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TESIS DOCTORAL

**Función y origen de los plásmidos en especies de
Erwinia patógenas y epifitas de frutales de pepita**

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CERTIFICAN:

Que Dña. Silvia Barbé Martínez ha realizado bajo su dirección en el Departamento de Bacteriología del Centro de Protección Vegetal y Biotecnología del Instituto Valenciano de Investigaciones Agrarias, el trabajo que con el título “Función y origen de los plásmidos en especies de *Erwinia* patógenas y epifitas de frutales de pepita”, presenta para optar al grado de Doctor.

Para que así conste a los efectos oportunos, firman el presente certificado en

Valencia, a 12 de Junio de 2017.

Dr. María Milagros López González

Dr. Pablo Llop Pérez

"Cada persona que pasa por nuestra vida es única.
Siempre deja un poco de sí y se lleva un poco de nosotros.
Habrá los que se llevarán mucho, pero no habrá de los
que no nos dejarán nada. Esta es la prueba evidente de
que dos almas no se encuentran por casualidad"
El árbol de los amigos.

*A Sara.
Que la vida te devuelva toda la felicidad que nos estás dando.*

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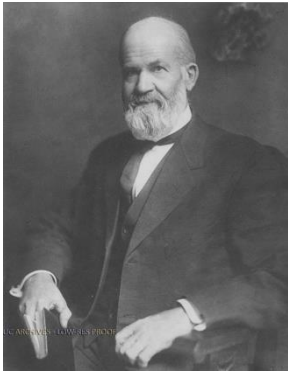
Quiero agradecer a mis padres por su apoyo, por su esfuerzo para que llegara hasta aquí, por darme todo lo que necesitaba y ayudarme en todo momento. A mi tete Javi, por ejercer de hermano y por estar siempre a mi lado. A María, por hacer feliz a mi tete y haber traído al mundo a mi chico, que es único y que nos puso un poco más fácil todo con su llegada. A los que no están pero siento cerca. Al Serio, al que tengo muy presente y que me acompaña en todo momento (hubiera sido muy bonito que la hubieses podido acunar). Agradecer a José y Rosario por cuidarme y mimarme como a una hija en todo momento.

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Revisión histórica

La existencia de microorganismos se presumió en el ámbito de la medicina en siglo XI, pero hasta el XIV estos no se relacionaron con las enfermedades infecciosas. No fue hasta 1674 cuando, gracias a los trabajos de microscopía de Anton van Leeuwenhoek, se descubrieron las bacterias, aunque este campo tuvo mayor relevancia en el siglo XIX por las investigaciones de científicos como Louis Pasteur, Robert Koch y Martinus W. Beijerinck.



Thomas J. Burrill. Fuente:
<http://archives.library.illinois.edu/index.php>,
consulta: 24/04/2017

En cuanto a la Fitopatología, ya en 1870, Thomas J. Burrill empezó su carrera científica en la Universidad de Illinois con el estudio de una devastadora enfermedad en los perales, llamada “*fire blight*” y por esa época asociada a un hongo como agente causal. Tras ocho años de investigación, Burrill, bajo el microscopio afirmó que, en lo que hoy conocemos como exudados y en los tejidos afectados, se podían observar “*como átomos en movimiento. Algo vivo, como las bacterias...*”. Su presencia era algo que aunque ya estaba demostrado por aquella fecha en animales, era impensable en plantas.

El interés por el fuego bacteriano siguió en aumento. En 1880 Burrill consiguió reproducir dicha enfermedad en perales sanos a partir de ejemplares sintomáticos y dos años más tarde describió el patógeno, hoy conocido con el nombre de *Erwinia amylovora*, denominándolo *Micrococcus amylovorus*, aún sin conseguir aislarlo en un medio de cultivo.

En 1885 Joseph C. Arthur defendió la primera tesis doctoral sobre el fuego bacteriano, afirmando que *M. amylovorus*, era el agente causal de la enfermedad, gracias a su trabajo hacia una aproximación para aislar la bacteria

en medio líquido. A esta tesis le siguieron numerosos estudios sobre los síntomas y el ciclo de la enfermedad, los métodos de control, la transmisión y diseminación, los hospedadores y su distribución.

En 1890 Merton B. Waite fue el primero en iniciar un proyecto de investigación sobre el fuego bacteriano de las rosáceas. Este científico hizo descubrimientos tan destacables como que el patógeno era capaz de penetrar en la planta por los nectarios de la flor sin necesidad de punciones o que las abejas propagaban fácilmente la enfermedad. Demostrando Waite estas teorías y junto con otros investigadores, se iniciaron los estudios de las medidas preventivas frente a la enfermedad.



Merton B. Waite. Fuente: <https://www.nal.usda.gov/illinois.edu/index.php>, consulta: 24/04/2017

En los primeros años del siglo XX investigadores como F. D. Chester, H. H. Whetzel, V. B. Stewart y W. G. Sackett entre otros estudiaron temas como la susceptibilidad en tejidos y la formación de chancros, haciendo recomendaciones para el control del fuego bacteriano en las que se incluía ya la desinfección de las herramientas.

Por esa época, en el Departamento de Agricultura de Estados Unidos, Erwin F. Smith empezaba a tener fama a nivel mundial, publicando en 1905 una serie de informes de sobre patógenos bacterianos de plantas en donde se incluía el agente causal del fuego bacteriano, llamado entonces *Bacillus amylovorus*. En su primer libro ya hacía una descripción detallada de este patógeno y tras un importante esfuerzo logró demostrar la capacidad de las bacterias para inducir la enfermedad en plantas. Este hito fue el inicio real y el establecimiento de la Fitopatología en ciencia.



Erwin F. Smith Fuente:
<https://www.nal.usda.gov/illinois.edu/index.php>,
consulta: 24/04/2017

Es importante destacar que Erwin F. Smith impulsó la contratación en sus estudios de más de una veintena de mujeres para realizar estos trabajos, excluidas por cuestión de género hasta dichas fechas en el Departamento de Agricultura de Estados Unidos, y abriendo paso a las mujeres al mundo de la investigación. Gracias a ello, se formaron grandes pioneras en el mundo de la fitopatología como Mary Katherine Bryan, Florence Hedges, Lucia McCulloch o Agnes J. Quirk, cuyas aportaciones han sido imprescindibles en los principales estudios de bacterias fitopatógenas como *Clavibacter michiganensis*, *Ralstonia solanacearum* o *Agrobacterium tumefaciens*.

Desde entonces, los trabajos relacionados con *E. amylovora* han ido aumentando exponencialmente año tras año, según el desarrollo de las nuevas técnicas para un mejor conocimiento del patógeno y comprensión de la enfermedad que causa, imprescindibles para la prevención y control de esta grave enfermedad.

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Funciones y orígenes de los plásmidos en las especies de *Erwinia* patógenas o epifitas asociadas a frutales de pepita

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Resumen

El fuego bacteriano está considerada la enfermedad más grave que afecta a frutales de pepita y rosáceas ornamentales y silvestres, por su difícil control químico, su fácil diseminación y por afectar a especies vegetales de gran importancia económica, como manzano, peral, níspero y membrillero. El agente causal de esta enfermedad es la bacteria *Erwinia amylovora*, perteneciente a la familia *Erwiniaceae* y considerada organismo de cuarentena en la Unión Europea.

Esta especie ha sido ampliamente estudiada, pero a nivel genómico todavía queda mucho por conocer, ya que en la actualidad existen únicamente dos genomas publicados, completamente secuenciados y anotados, y otros trece ensamblados en *scaffolds*, de cepas de *E. amylovora* de diferentes orígenes geográficos y huéspedes. Por ello su pangenoma se considera abierto, aunque con un *core* o genoma conservado con una alta identidad de secuencia en estas cepas. Con esos datos podemos decir que existe muy poca variabilidad intraespecífica, lo que se manifiesta en una escasa diversidad genotípica, advirtiéndose que los plásmidos son la mayor fuente de variabilidad genética. Esta variabilidad podría explicar las diferencias en virulencia en cepas portadoras de plásmidos, así como su mejor adaptación a diferentes condiciones ambientales.

Esta tesis consta de una introducción general, una revisión bibliográfica específica y publicada, sobre los plásmidos de las especies de *Erwinia* patógenas y epifitas asociadas a frutales de pepita y rosáceas ornamentales, y cuatro trabajos experimentales sobre *E. amylovora* y *E. piriflorinigrans*.

El origen de este trabajo surgió al observar que el plásmido pEA29 descrito en la mayoría de las cepas de *E. amylovora*, y con un efecto cuantitativo en virulencia, no se encontró en algunos aislados españoles de la bacteria. El estudio de estas cepas sin plásmido dio paso al descubrimiento de otro plásmido de unas 70 Kb

que se ha denominado pEI70, el cual está presente en cepas de *E. amylovora* de varios países europeos. Por ello, después del pEA29, el plásmido pEI70 es el de mayor presencia en las cepas de esta especie. Se ha estudiado la función, distribución y contenido genético de este plásmido, así como el efecto del pEA29 y del pEI70 sobre la expresión de los genes cromosómicos de la cepa que los porta, tras la infección en fruto inmaduro. Los experimentos de inoculación en fruto con las cepas a las que se les había introducido el plásmido pEI70 o el pEA29, en comparación con esa misma cepa sin plásmidos, mostraron un aumento de la virulencia, que se manifestaba en una reducción en el tiempo de la aparición de síntomas y en que éstos se presentaban de forma más agresiva.

Por todo ello, con el fin de estudiar si la presencia de cada uno de estos dos plásmidos podría producir efectos sobre determinados genes cromosómicos que explicarían esa variación en virulencia de la cepa portadora, se realizó un experimento de expresión génica diferencial usando un *microarray* con sondas del genoma completo de la bacteria, así como de los plásmidos pEA29 y pEI70. Los resultados obtenidos demostraron el papel de ambos plásmidos al afectar a la expresión de entre 120 y 180 genes cromosómicos según el plásmido que porte la cepa, enriqueciéndose en cada caso categorías funcionales diferentes, aunque 28 de ellos fueron coincidentes en los dos casos.

Por otro lado, *E. piriflorinigrens* es una especie patógena descrita recientemente que produce necrosis sólo en las flores de peral, pero no parece afectar a otros órganos. Además, ambas especies comparten características fenotípicas y moleculares, haciendo difícil su distinción. Su detección y correcta identificación era un reto debido a que los síntomas que provoca en flores son prácticamente indistinguibles a los causados por *E. amylovora*. En este trabajo se ha estudiado el contenido genético de un plásmido de 37 Kb, denominado pEPIR37, que se ha comprobado está presente en todas las cepas analizadas de la especie *E. piriflorinigrens*. Además, se ha observado que cuando este plásmido es

introducido en cepas de la especie *E. amylovora* curadas de plásmidos, y que por ello presentan una virulencia reducida, mostraron un nivel de virulencia mayor, comparable a la observada en las cepas portadoras del plásmido pEA29, lo que parece indicar que este plásmido produce un efecto similar. Por todo ello en este trabajo también se han desarrollado dos protocolos específicos y sensibles de PCR en tiempo real y convencional para identificar, detectar y diferenciar *E. piriflorinigra* de *E. amylovora* y de otras especies de este género, usando iniciadores diseñados a partir de secuencias específicas del plásmido pEPIR37. Ello ha permitido identificar esta nueva especie en otros huéspedes como *Pyracantha* sp., además de peral y en otras regiones españolas en donde anteriormente no se había detectado.

Asimismo, estos resultados han permitido conocer aspectos biológicos y epidemiológicos de *E. piriflorinigra* que aportan nueva información científica que resultaría clave para establecer estrategias para su control en frutales de pepita.

El estudio de los plásmidos y sus funciones en estas dos especies tan relacionadas filogenéticamente y su papel en la adaptación al medio en el que ambas habitan, así como en la virulencia de las cepas que los portan, podría dar nuevas pistas sobre el origen de estos patógenos, su evolución, su ciclo biológico y su interacción con las plantas hospedadoras.

Abstract

Fire blight is considered the most serious disease affecting pome fruit and ornamental and wild rosaceae, due to its difficult chemical control, its easy dissemination and to affect plant species of great economic importance, such as apple, pear, loquat and quince. The causal agent of this disease is the bacterium *Erwinia amylovora*, belonging to the family *Erwiniaceae* and considered a quarantine organism in the European Union.

This species has been extensively studied, but at the genomic level there is still much to know, as there are currently only two genomes published completely sequenced and annotated and another thirteen were assembled in scaffolds of strains of *E. amylovora* from different geographical origins and guests. Therefore its pangenoma is considered open, although with a core or conserved genome with a high sequence identity in these strains. With these data we can say that there is very little intraspecific variability, which is manifested in a low genotypic diversity, being noticed that the plasmids are the major source of genetic variability. This could explain the differences in virulence in strains that harbour plasmids, as well as their better adaptation to the different environmental conditions.

This thesis consists in a general introduction chapter, a specific and published bibliographical revision, based in the plasmids of the species of *Erwinia* pathogenic and epiphytes associated to pepita fruit trees and ornamental rosaceae, and other four experimental works of *E. amylovora* and *E. piriflorinigrans*.

On one hand, the origin of this work arose when observing that the plasmid pEA29 described in the majority of the strains of *E. amylovora*, and with a quantitative effect in virulence, was not found in some Spanish isolates of the bacterium. The study of these strains without plasmid gave way to the discovery of another plasmid of about 70 Kb called pEI70, which is present in strains of *E. amylovora* of several European countries. Therefore, after pEA29, the plasmid pEI70 is the one with the highest presence in the strains of this species. The

function, distribution and genetic content of this plasmid, as well as the effect of pEA29 and pEI70 on the expression of the chromosomal genes in strain bearing them, after infection in immature fruit, have been studied. The inoculation experiments on fruit with the strains to which the plasmid pEI70 or pEA29 had been introduced compared to that same strain without plasmids showed an increase in virulence, which was manifested in a reduction in the time of the emergence of symptoms and in which they appeared more aggressively.

Taking into account these results, an experiment was carried out using a microarray, in order to study if the presence of each one of these plasmids could affect the expression on certain chromosomal genes that would explain that variation in virulence of the carrier strain. To have this information a differential gene expression experiment was performed using a microarray with probes from the complete genome of the bacterium as well as the plasmids pEA29 and pEI70. The results obtained demonstrated the role of both plasmids to affect gene expression, between 120 and 180 chromosomal genes according to the plasmid carrying the strain, in each case enriching different functional categories, although 28 of them were coincident in the two cases.

On the other hand, *E. piriflorinigrans* is a newly described pathogenic species that produces necrosis only in pear blossoms but does not appear to affect other organs. In addition, both species share phenotypic and molecular characteristics, making their distinction difficult. Its detection and correct identification was a challenge because the symptoms it causes in flowers are practically indistinguishable from those caused by *E. amylovora*. In this work the genetic content of the new plasmid pEPIR37 found in this new species was studied also. This plasmid is present in all analyzed strains from *E. piriflorinigrans* and it has also been evaluated. When this plasmid was introduced into strains of the species *E. amylovora* cured of plasmids, and therefore with reduced virulence, they showed an increase in virulence comparable to that observed in the strains carrying the plasmid pEA29, suggesting that pEPIR37 plasmid produces a similar effect. Therefore, two

specific and sensitive real-time and conventional PCR protocols have also been developed to identify, detect and differentiate *E. piriflorinigrans* from *E. amylovora* and other species of this genus using primers designed from specific sequences, annotated in this same work, from plasmid pEPIR37. This has allowed to identify this new species in other hosts as *Pyracantha* sp., besides pear tree and in other regions where previously it had not been detected.

Likewise, these results have allowed to know biological and epidemiological aspects of *E. piriflorinigrans* that contribute to have new key scientific information to establish strategies for its control in pome fruit trees.

The study of the plasmids and their functions in these two phylogenetically related species and their role in the adaptation to the environment in which these species live, as well as in the virulence of the strains that carry them, could give new clues about the origin of both pathogens, their evolution, their biological cycle and interaction with the host plants.

Resum

El foc bacterià està considerada la malaltia més greu que afecta arbres fruiters de pinyol i rosàcies ornamentals i silvestres, pel seu difícil control químic, la seua fàcil disseminació i per afectar espècies vegetals de gran importància econòmica, com la pomera, la perera, el nesprer i el codonyer. L'agent causal d'aquesta malaltia és el bacteri *Erwinia amylovora*, pertanyent a la família *Erwiniaceae* i considerat organisme de quarantena a la Unió Europea.

Aquesta espècie ha estat àmpliament estudiada, però a nivell genòmic encara queda molt per conèixer, ja que en l'actualitat únicament existeixen dos genomes publicats, completament seqüenciats y anotats, i altres tretze assemblees *scaffolds*, de soques de diferents orígens geogràfics i hostes. Per això el seu pangenoma es considera obert, tot i que aquests soques posseeixen un *core* o genoma conservat amb una alta identitat de seqüència. Amb aquestes dades podem dir que hi ha molt poca variabilitat intraespecífica, el que es manifesta en una escassa diversitat genotípica, advertint-se que els plasmidis són la major font de variabilitat genètica. Aquesta variabilitat podria explicar les diferències en virulència a les soques portadores d'aquests plasmidis, així com la seua millor adaptació a les diferents condicions ambientals.

L'origen d'aquest treball va sorgir en observar que el plasmidi pEA29 descrit en la majoria de les soques d'*E. amylovora*, i amb un efecte quantitatiu en virulència, no es va trobar en alguns aïllats espanyols del bacteri. L'estudi d'aquests soques sense plasmidi va donar pas al descobriment d'un altre plasmidi d'unes 70 Kb anomenat pEI70, el qual està present en soques d'*E. amylovora* de diversos països europeus. Per això, després del pEA29, el plasmidi pEI70 és el de major presència a les soques d'aquesta espècie. S'ha estudiat la funció, distribució i contingut genètic d'aquest plasmidi, així com l'efecte del pEA29 i del pEI70 sobre l'expressió dels gens cromosòmics de la soca que els porta, després de la infecció en fruit immadur. Els experiments d'inoculació en fruit amb les soques a les que se'ls havia introduït el plasmidi pEI70 o el pEA29,

en comparació amb aquesta mateixa soca sense plasmidis, van mostrar un augment de la virulència, que es manifestava en una reducció en el temps de l'aparició de símptomes i en què aquests es presentaven de forma més agressiva. Per això, per tal d'estudiar si la presència de cada plasmidi podria produir efectes sobre determinats gens cromosòmics que explicarien aquesta variació en virulència de la soca portadora, es va realitzar un experiment d'expressió genètica diferencial mitjançant un *microarray* amb sondes del genoma complet i dels plasmidis pEA29 i pEI70. Els resultats obtinguts van demostrar el paper dels dos plasmidis en afectar l'expressió d'entre 120 i 180 gens cromosòmics segons el plasmidi que porta la soca, enriquint-se en cada cas categories funcionals diferents, tot i que 28 d'ells van ser coincidents en els dos casos.

D'altra banda, *E. piriflorinigrans* és una espècie patògena descrita recentment que produeix necrosi només en les flors de perera, però no sembla afectar altres òrgans. A més, les dues espècies comparteixen característiques fenotípiques i moleculars, fent difícil la seua distinció. La seua detecció i correcta identificació era un repte pel fet que els símptomes que provoca en flors són pràcticament indistingibles als causats per *E. amylovora*. En aquest treball també s'ha avaluat el contingut genètic d'un plasmidi de 37 Kb, anomenat pEPIR37, present en totes les soques analitzades de l'espècie *E. piriflorinigrans*, i a més s'ha observat que quan aquest plasmidi era introduït en soques de l'espècie *E. amylovora* curades de plasmidis, que per això tenen una virulència reduïda, van mostrar una virulència major, comparable amb l'observada en les soques portadores del plasmidi pEA29, el que indicaria que aquest plasmidi produeix un efecte similar. Per tot això en aquest treball també s'han desenvolupat dos protocols específics i sensibles de PCR en temps real i convencional per identificar, detectar i diferenciar *E. piriflorinigrans* d'*E. amylovora* i d'altres espècies d'aquest gènere, usant iniciadors dissenyats a partir de seqüències específiques,

anotades en aquest mateix treball, del plasmidi pEPIR37. Això ha permès identificar aquesta nova espècie a altres hostes com *Pyracantha* sp., a més de perera i en altres regions on anteriorment no s'havia detectat.

Així mateix, aquests resultats han permès conèixer aspectes biològics i epidemiològics d'*E. piriflorinigrans* que aporten nova informació científica clau per establir estratègies per al seu control en arbres fruiters de pinyol.

L'estudi dels plasmidis i les seues funcions en aquestes dues espècies tan relacionades filogenèticament i el seu paper en l'adaptació al medi on habiten, així com en la virulència de les soques que els porten, podria donar noves pistes sobre l'origen dels dos patògens, la seua evolució, el seu cicle biològic i la seua interacció amb les plantes hostes.

Capítulo 1

Introducción general



1. El género *Erwinia*.

El género *Erwinia* fue creado en 1920 en memoria del fitopatólogo Erwin F. Smith, para agrupar a los bacilos Gram negativos, fermentativos, no formadores de esporas y con flagelos peritricos, responsables de enfermedades de plantas (Winslow *et al.*, 1920). Este género incluyó enterobacterias patógenas y no patógenas asociadas a plantas, así como patógenos oportunistas de humanos y animales (Brenner, 1984). Se clasificaron todas las especies que forman el género en cuatro grupos (Dye, 1968, 1969 a, b, c):

- Grupo amylovora: engloba a las especies patógenas causantes de marchitez y necrosis.
- Grupo carotovora: incluye las especies patógenas causantes de podredumbres blandas.
- Grupo herbicola: compuesto por las especies consideradas saprofitas.
- Grupo de erwinias atípicas.

Esta clasificación agrupaba especies sin una relación filogenética estrecha y era muy heterogénea, por lo que el género sufrió varias reclasificaciones hasta el desarrollo de las técnicas moleculares. En 1998 Hauben *et al.*, en base a la comparación de las secuencias del ADN ribosómico 16S, propusieron la reorganización del género en cuatro grupos filogenéticos que se corresponden con cuatro géneros:

- *Erwinia*: formado por especies productoras de necrosis y/o marchitamiento en plantas y especies epifitas.
- *Pectobacterium*: formado por especies que producen podredumbres blandas debido a su actividad pectolítica en una gran variedad de plantas.

- *Brenneria*: formado por especies que provocan chancros y exudados en especies leñosas.
- *Pantoea*: formado por patógenos oportunistas de plantas, animales y humanos.

En 2016, Adeolu *et al.*, basándose en estudios filogenéticos, mediante la concatenación de 1548 proteínas del *core* bacteriano, 53 proteínas ribosomales y análisis de secuencias multilocus de los genes *gyrB*, *rpoB*, *atpD* e *infB*, describieron dentro del antiguo orden *Enterobacteriaceae*, ahora denominado *Enterobacterales*, siete nuevas familias: *Enterobacteriaceae*, *Erwiniaceae*, *Pectobacteriaceae*, *Yersiniaceae*, *Hafniaceae*, *Morganellaceae* y *Budviciaceae*.

Dentro de la nueva familia *Erwiniaceae* se incluye el género *Erwinia* (Hauben *et al.*, 1998) como género tipo, y los géneros *Buchnera* (Munson *et al.*, 1991), *Pantoea* (Brady *et al.*, 2010), *Phaseolibacter* (Halpern *et al.*, 2013), *Tatumella* (Hollis *et al.*, 1981) y *Wigglesworthia* (Aksoy, 1995).

En esta actual reclasificación, la familia *Erwiniaceae* incluye bacterias catalasa positivas, oxidasa negativa y no productoras de indol o disulfuro de hidrógeno. La mayoría de las especies son positivas para el test Voges-Proskauer, a excepción de *Erwinia toletana*, *Erwinia typographi* y algunas cepas de *Erwinia oleae*. Los miembros de la nueva familia *Erwiniaceae* forman un grupo monofilético distinto basado en el estudio de sus genomas, así pueden distinguirse de todas las otras bacterias por doce inserciones/delecciones conservadas en determinadas proteínas.

En esta tesis doctoral únicamente se tratará de algunas especies del género *Erwinia* asociadas a frutales de pepita y rosáceas ornamentales, principalmente de las especies patógenas *Erwinia amylovora* y *Erwinia piriflorinigrans*, que serán descritas con más detalle en apartados posteriores.

En esta primera parte de la introducción se realizará una descripción de cada especie del género basándose en su capacidad para producir o no patologías en las diferentes especies que comprenden los frutales de pepita y rosáceas ornamentales y silvestres.

1.1. Especies de *Erwinia* asociadas a frutales de pepita y rosáceas ornamentales

Varias especies del género *Erwinia* están asociadas de forma epifita o con carácter patógeno a frutales de pepita como el peral, manzano, níspero o membrillero.

En primer lugar, cabe resaltar que la primera enfermedad infecciosa de plantas en la que se demostró que el agente causal era una bacteria fue el fuego bacteriano de las rosáceas (Burril, 1883; Winslow *et al.*, 1920) y su agente causal, *E. amylovora* fue identificado como tal en el siglo XIX. Desde entonces, los estudios realizados sobre esta especie bacteriana son innumerables, pero en los últimos años se han descrito otras especies patógenas de este mismo género, como *Erwinia pyrifoliae*, *Erwinia* sp. de Japón que causa la enfermedad denominada “*bacterial shoot blight of pear*” (BSBP, por sus siglas en inglés), *E. piriflorinigrans* causante de necrosis de floral y *Erwinia uzenensis*, agente causal de la enfermedad denominada “*bacterial black shoot disease of European pear*” (BBSDP). Otras especies de este mismo género no causan sintomatología en el hospedador y son consideradas como especies epifitas, como *Erwinia billingiae*, *Erwinia tasmaniensis* y *Erwinia gerundensis*.

1.1.1. Características de las especies de *Erwinia* patógenas y de las enfermedades que causan

1.1.1.1. *E. amylovora*

1.1.1.1.1. Distribución geográfica

El fuego bacteriano se describió en pomáceas en Hudson Valley (Nueva York) en el año 1780 y se extendió rápidamente por distintos estados de Estados Unidos, llegando a infectar cultivos en Canadá en un breve periodo de tiempo. En 1919 fue detectado en Nueva Zelanda, traspasando las fronteras continentales. En Europa se detectó por primera vez en 1957, en el sur de Inglaterra, diseminándose rápidamente en el continente y siete años más tarde fue detectado en el continente africano, en el valle del Nilo (Egipto). En 1966 se identificó de nuevo en Europa, en los Países Bajos y desde entonces ha ido avanzando en sentido noroeste y suroeste, por la mayoría de países del norte y centro de Europa, en el Mediterráneo y también en Oriente medio, llegando a nuevas zonas del norte de África y del oeste de Asia.

En la actualidad, la enfermedad se ha descrito en todos los continentes en un total de 118 países (<https://gd.eppo.int/taxon/ERWIAM/distribution>, 02/05/2017) (Figura 1), pero según los datos de la OEPP/EPPO del año 2017 existen importantes zonas frutícolas en el hemisferio sur en donde actualmente no se ha detectado la bacteria y está ausente la enfermedad como Chile, Argentina o Brasil o como en Australia donde se considera erradicada tras un pequeño foco detectado en 1997. En el caso de Corea del Sur, en donde en 2015 se detectó la bacteria en un total de 768 perales y 324 manzanos, actualmente se encuentra en proceso de erradicación temporalmente, por lo que su estatus se define como transitorio.

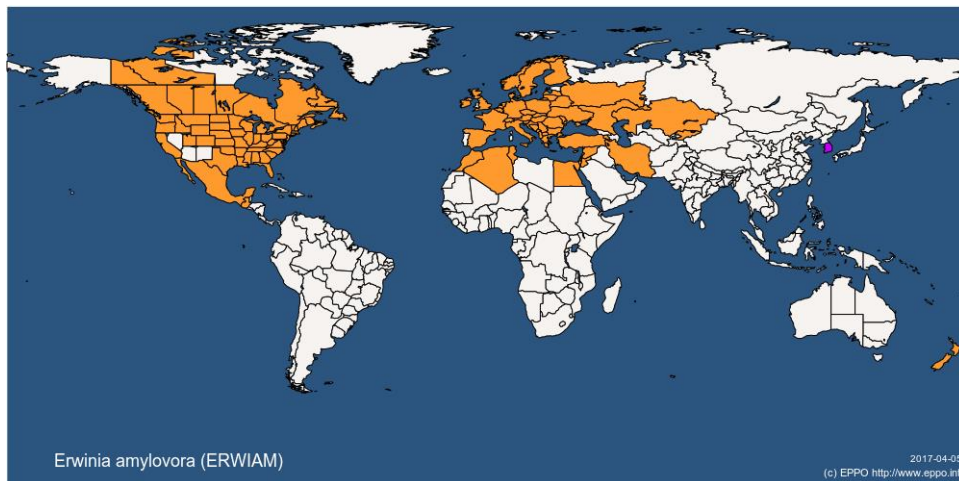


Figura 1. Distribución de *E. amylovora* basada en datos de la OEPP/EPPO (fecha de consulta: 05/04/2017). Las zonas en naranja corresponden a los países en donde la enfermedad está presente mientras que las zonas en blanco corresponden a los países en donde no hay confirmación de la enfermedad o esta ha sido erradicada. La zona en morado (Corea del Sur) representa un estatus transitorio, en donde actualmente la enfermedad está en proceso de erradicación.

1.1.1.1.2. Situación del fuego bacteriano en España

España fue considerada Zona protegida para la bacteria *E. amylovora* desde 1999 (Palacio-Bielsa y Cambra, 2010). Se intentó evitar la introducción del fuego bacteriano mediante una estricta regulación contenida en la legislación y con análisis del material vegetal importado, principalmente plantones de frutales y plantas ornamentales desde algunos países en donde la enfermedad estaba presente (López *et al.*, 1988; López *et al.*, 2002, Cambra y Díaz, 2010).

Pese a ello, la primera detección de *E. amylovora* en el país se produjo en agosto de 1995 en manzano de sidra, en Lezo (Guipúzcoa), a 10 Km del sur de Francia (Butrón, 1995), donde la enfermedad estaba establecida desde 1978. En esta provincia, en los dos años posteriores se declararon diversos focos y las prospecciones confirmaron que la enfermedad estaba extendida en la zona. En Vizcaya se encontraron pequeños focos en peral, entre 1999 y 2004, que fueron erradicados al igual que los detectados en Álava entre 2000 y 2005. En 1996 se confirmó la enfermedad en Navarra, en especies de frutales y plantas ornamentales, fundamentalmente *Pyracantha* spp. En esta provincia se han detectado focos hasta el año 2006 y se han tomado medidas de erradicación, encontrándose únicamente un foco en dicho año.

En Castilla y León se han ido detectando focos desde 1996 pero no en todos ellos se tomaron medidas de erradicación o no fueron suficientemente estrictas (López *et al.*, 1999; López *et al.*, 2002), por lo que en 2011 esta comunidad se salió de la Zona Protegida. En la Comunidad de Madrid se detectó y erradicó la enfermedad en 1999, en *Pyracantha* spp.

En Lleida (Cataluña) se detectó la enfermedad en cultivos de peral en 1998 y 1999 (Montesinos *et al.*, 1999) hasta que en 2003 se detectó un foco en Puigcerdá (Gerona), muy cerca de la frontera francesa y se erradicó en 2006. En 2015 se detectaron múltiples focos en los distritos de Segriá, Noguera, Pla d'Urgell, Garrigues y Urgell, de Lérida siendo entonces excluida esta área de la Zona Protegida.

En Aragón se han declarado focos de fuego bacteriano en plantas ornamentales en 1998 y 2000 y en este último año en frutales de la provincia de Zaragoza. En principio se tomaron en la zona estrictas medidas de erradicación (Cambra *et al.*, 2005), pero en 2011 la enfermedad se volvió a detectar y se estaba trabajando en la erradicación en la zona activamente, pero en 2013, por la extensión de la enfermedad Aragón solicitó salir de la Zona Protegida. En La Rioja en 2000 se detectó la enfermedad en peral y se aplicaron medidas contra

la misma (López *et al.*, 2002) aunque desde el año 2005 hasta 2013 se han ido encontrando focos en perales y plantas ornamentales de la zona y en este último año esta comunidad autónoma también salió de la Zona Protegida. En Cantabria, se detectó un único foco en 2004 en peral Nashi.

En Andalucía la enfermedad se detectó por primera vez en el año 2010 en Granada y se han ido detectando a lo largo de estos últimos años, nuevos brotes en Almería, Cádiz, Granada, Jaén, Málaga y Sevilla, por ellos, y junto con Murcia, solicitaron dejar de ser Zona Protegida.

En 2009 se detectan varios focos de la enfermedad en las zonas de Vegas Altas y Talayuela, en Extremadura. En consecuencia se tomaron medidas fitosanitarias de erradicación en diversas explotaciones agrícolas y viveros de frutales de pepita de los términos municipales pacenses de Acedera, Don Benito, Guareña, Navalvillar de Pela, Rena, Rucas, Villanueva de la Serena y Villar de Rena, así como en los términos cacereños de Campo Lugar, Madrigalejo, Majadas del Tiétar y Talayuela, pertenecientes a la provincia de Cáceres. A finales del año 2010 se volvieron a detectar nuevos focos en Acedera, Badajoz, Bienvenida, Don Benito, Guareña, La Garrovilla, Navalvillar de Pela, Rena, Valdeterres, Villanueva de la Serena y Villar de Rena, todos en Badajoz, y en Alagón del Río, Campo Lugar, Galisteo y Miajadas. En 2011, a pesar de las medidas fitosanitarias ejecutadas, se determina que la enfermedad en esta comunidad autónoma no se había logrado erradicar ni controlar los focos iniciales por lo que se estaba propagando en todo el ámbito territorial de Extremadura y por ello se declaró establecida en este territorio la enfermedad, saliendo de la Zona protegida.

En la Comunidad Valenciana, la enfermedad se identificó en peral en 2011 en varios puntos cerca de Villena (Alicante). Tras la destrucción de plantas infectadas y próximas, realizaron periódicamente prospecciones y análisis con el fin de erradicar la enfermedad. En 2013 hubo nuevos brotes en la zona, en un cultivo de membrilleros el cual fue arrancado casi en su totalidad. Pero desde la

fecha, han aparecido más brotes por el abandono de la erradicación, por cuestiones económicas, lo que supone una amenaza real de introducción de la bacteria en la zona de cultivo del níspero de Alicante. Poco tiempo después, se detectó la enfermedad en los municipios de Alborache y Turís, en la provincia de Valencia. Se tomaron medidas en 2013 con vistas a la erradicación de de la enfermedad en estas zonas, pero han resultado ineficaces, por lo que en 2014 salieron de las Zonas protegidas.

Por lo tanto, actualmente la situación de la enfermedad en España es preocupante ya que aunque el fuego bacteriano en sus inicios se localizó en el norte de la península, desde el año 2001 han ido apareciendo nuevos focos que en unos casos se han podido controlar, pero otros, en años posteriores se han mantenido y en algunos casos se han ido extendiendo, llegando a aparecer en regiones del sur de la península en la Comunidad autónoma de Andalucía, que confirman la extensión de la enfermedad en la península.

A pesar de los focos de la enfermedad detectados, España sigue siendo en algunas partes Zona Protegida de la enfermedad para la Unión Europea (Anónimo, 2000 b), ya que la enfermedad no está presente o han sido adoptadas las medidas de erradicación pertinentes en ciertas zonas, evitando numerosas pérdidas en la producción y económicas en cuanto a la reconversión varietal en las zonas afectadas (Palacio-Bielsa y Cambra, 2010). Además se han establecido medidas para evitar la introducción y propagación de este patógeno de cuarentena (Directiva Europea 2000/29 y su modificación 2003/116). Pero, actualmente la situación en el territorio nacional es grave ya que en 2011 algunas comunidades autónomas o parte de ellas han perdido este estatus de Zona Protegida, debido a que la enfermedad se ha establecido en parte o en la totalidad de su territorio (Castilla y León, Extremadura, Castilla la Mancha, la Rioja, Navarra, País Vasco (Guipúzcoa), Aragón, Murcia y Comunidad Valenciana (Comarcas de L'alt Vinalopó y El Vinalopó Mitjà en la provincia de Alicante y los

municipios de Turís y Alborache en Valencia)). El resto de Comunidades Autónomas, parcial o totalmente, continúan manteniendo el estatus de Zona Protegida ante el fuego bacteriano (Figura 2).

La distribución espacial y temporal de la enfermedad en España, así como el análisis de las cepas aisladas en esos focos, hacen pensar que ha habido varias introducciones de la enfermedad en el territorio mediante material vegetal contaminado y de diferentes orígenes (López *et al.*, 2002; Donat *et al.*, 2007).

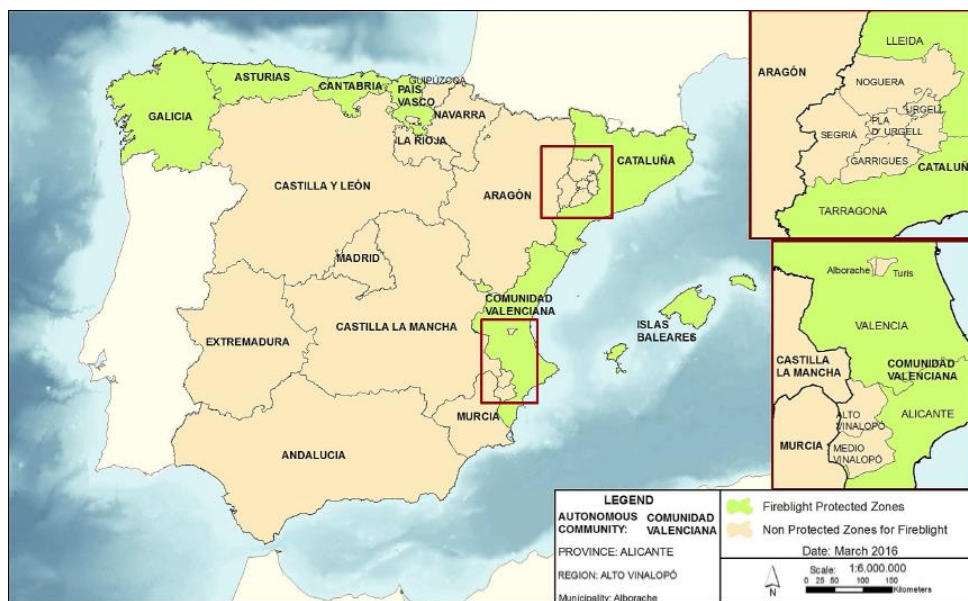


Figura 2. Mapa de España en donde se marcan en color marrón los territorios excluidos de la Zona Protegida de la enfermedad en 2016: Castilla y León, Aragón, Castilla la Mancha, Navarra, La Rioja, País Vasco (Guipúzcoa), Murcia, Extremadura, Andalucía y ciertas comarcas de la Comunidad Valenciana (en Alicante y Valencia) y de Cataluña (Lleida).

1.1.1.1.3. Características de la enfermedad

El agente causal de esta enfermedad, *Erwinia amylovora*, es una bacteria Gram-negativa con morfología bacilar, anaerobia facultativa, de flagelación peritrica y con material capsular perteneciente a la familia *Erwiniaceae* (Paulin, 2000; Adeolu *et al.*, 2016). Este patógeno es considerado como un organismo de cuarentena en la Unión Europea, produciendo una de las enfermedades más graves que afectan a diversas especies de plantas pertenecientes a la familia *Rosaceae*, en la que se incluyen especies de árboles frutales de gran importancia económica del género *Pyrus* (peral), *Malus* (manzano), *Eryobotrya* (níspero) y *Cydonia* (membrillero), así como plantas ornamentales y silvestres como *Amelanchier* spp., *Chaenomeles* spp., *Crataegus* spp. (espino albar), *Cotoneaster* spp. *Photinia davidiana*, *Pyracantha* spp. (espino de fuego), y *Sorbus* spp. (serbal), todas ellas pertenecientes a la subfamilia *Spiraeoideae* y contempladas en la legislación de la Unión Europea como los huéspedes más importantes y de mayor interés comercial y económico (Balduque *et al.*, 1996; van der Zwet y Beer, 1995). También se ha descrito en otras plantas ornamentales de la subfamilia *Rosoideae* como el frambueso y la zarzamora (*Rubus* spp.), además de en ciruelo japonés (*Prunus salicina*) de la subfamilia *Amigdaloidae* (Mohan y Thomsom, 1996; Starr *et al.*, 1951).

Desde su descubrimiento, esta enfermedad se consideró la más destructiva de los frutales de pepita, sobre todo en manzano y peral, siendo un factor limitante del desarrollo de estos cultivos. La enfermedad es devastadora en muchas zonas y de difícil control, afectando en particular a la producción de frutos y llegando a causar la muerte de los árboles afectados en un breve periodo de tiempo, debido a la rápida diseminación de la bacteria en la planta (Vanneste, 2000; Cambra *et al.*, 2002). Desde hace más de un siglo, todos los años se ha reportado un impacto económico significativo (Sobiczewski *et al.*, 1997; Vanneste, 2000). Las condiciones favorables para el desarrollo de la enfermedad y el uso de

antibióticos y la resistencia generada en cepas de la bacteria en algunas zonas de Estados Unidos e Israel (Norelli *et al.*, 2003), hace que se sigan dando pérdidas económicas muy graves, como en 1998, donde se estimaron unas pérdidas de 68 millones de dólares en EEUU, junto a 100 millones de pérdidas adicionales por las resistencias a estreptomycin. Otro ejemplo es el de la década de los 90 en Italia, en donde se destruyeron más de medio millón de árboles (Vanneste, 2000).

Su agente causal es capaz de dispersarse con gran facilidad por diferentes medios (viento, lluvia, insectos, herramientas y vestimenta relacionada con el laboreo) y sobrevive en las plantas hospedadoras, insectos, material vegetal comercializado e incluso en agua por largos periodos de tiempo (Biosca *et al.*, 2008). A todo ello se añade que actualmente no existen métodos de control eficaces, produciendo grandes pérdidas económicas cuando el patógeno se establece en una zona de cultivo con variedades sensibles y las condiciones medioambientales son favorables para su supervivencia y diseminación.

1.1.1.1.4. Sintomatología

El fuego bacteriano debe su nombre al síntoma más típico que provoca esta enfermedad en las especies sensibles, independientemente de la edad de la planta (van der Zwet y Walter, 1996; Montesinos y López, 2000). En las plantas afectadas, sus flores, hojas, brotes y frutos toman una coloración negra, como quemados por fuego, en el caso del peral. Este tono oscuro es más rojizo o pardo en otros huéspedes (Figuras 3, 4 y 5). Este síntoma tan característico se observa en los comienzos de la enfermedad, principalmente en los corimbos, durante la época de floración y brotación. En primavera y verano, dependiendo de diferentes factores bióticos y abióticos, la enfermedad avanza rápidamente pudiendo producir la muerte del árbol, como se detallará posteriormente.

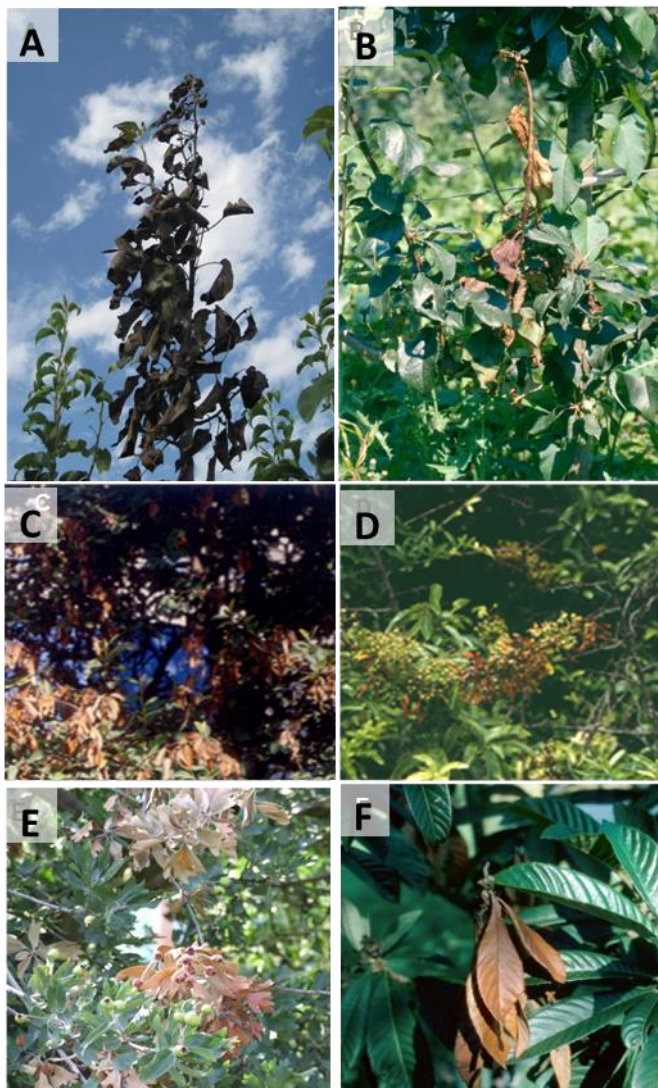


Figura 3. Aspecto de los diferentes huéspedes afectados por el fuego bacteriano. A) Peral, B) Manzano, C) *Cotoneaster* sp., D) *Pyracantha* sp., E) Acerolo y F) Níspero (Fotos: IVIA).

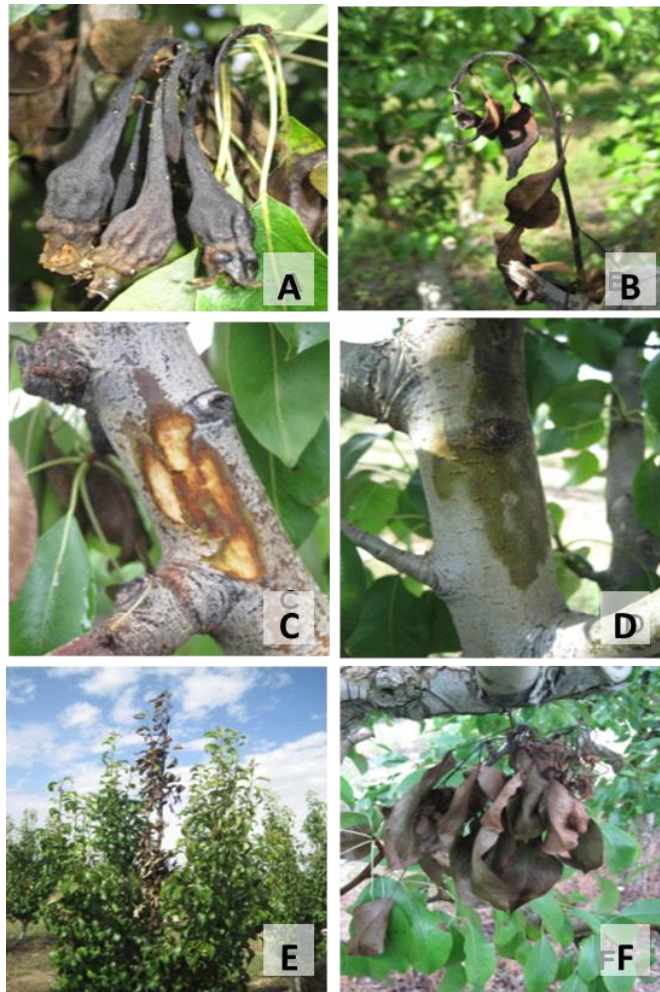


Figura 4. Distintos síntomas de fuego bacteriano en peral. A) frutos inmaduros necrosados con microexudados en su superficie; B) cayado de pastor; C) chancro descortezado en tronco; D) superficie de un chancro en tronco; E) aspecto “quemado” en ramas de un árbol infectado; F) hojas y corimbo floral necrosado; (fotos: IVIA).



Figura 5. Síntomas de fuego bacteriano en órganos de diferentes huéspedes. A) Ennegrecimiento de los estambres de flor de manzano al inicio de la infección (foto: EPPO Global Database); B, C, D y E) Aspecto pardo de ramas infectadas de manzano; F) Detalle del inicio de la infección en la base del peciolo de hojas de manzano (fotos: IVIA); G, H e I) Aspecto pardo de las ramas infectadas de *Pyracantha* sp. (fotos: IVIA); J) Aspecto rojizo que presentan las zonas infectadas en *Cotoneaster*; K) Frutos necrosados de *Cotoneaster* con el típico aspecto quemado (fotos: EPPO Global Database); L) Detalle de la infección en *Crataegus* sp., hojas necrosadas y chancros descortezados en tronco afectado (fotos: EPPO Global Database).

Los brotes herbáceos en formación o crecimiento activo, en el inicio del verano y otoño, pueden presentar síntomas como el aspecto húmedo, marchitez y ennegrecimiento. En ellos es característica la pérdida de rigidez y curvatura tomando una forma conocida como “cayado de pastor”.

Cuando la enfermedad llega a las hojas desde el brote afectado, lo hace a partir del peciolo en donde en fases iniciales se puede observar una zona húmeda y oscura cerca del limbo de la hoja. Seguidamente se produce una necrosis del nervio principal acompañada de otras manchas necróticas en los márgenes y la superficie de las hojas. Las lesiones se van extendiendo indicando la evolución sistémica de la enfermedad llegando también a la base de las ramas, donde se produce rápidamente la marchitez de todas sus hojas debido a que la bacteria impide su correcta nutrición. Al igual que ocurre con las flores afectadas, no se produce defoliación, y las hojas permanecen necrosadas en la rama, adquiriendo diferentes tonos rojizos o pardos en manzano, níspero, *Pyracantha*, *Crataegus* y *Cotoneaster* muy oscuros o negros en peral.

Los frutos pueden infectarse en todos sus estadios fisiológicos ya que *E. amylovora* es capaz de penetrar a través de las aberturas naturales (lenticelas) o por heridas provocadas en el cultivo o por factores climáticos. Estos frutos

presentan un aspecto húmedo, más tarde empiezan a oscurecerse a causa de la necrosis celular y finalmente se mantienen en el árbol momificados.

Cuando la enfermedad está avanzada, en cada ciclo de infección se puede observar la aparición de chancros en ramas y en el tronco. Al descortezar en estas zonas son visibles vetas de aspecto húmedo en el parénquima cortical y de color pardo o rojizo. Estas estrías pueden presentar un límite difuso, lo que indicaría la zona de avance de la infección y por lo tanto que el agente causal está vivo. Los síntomas en raíces y cuello del árbol son similares a los descritos en el tronco, y aunque estos no son habituales, cuando se dan, nos indica la muerte inminente del árbol.

Dependiendo principalmente de la humedad ambiental, se pueden observar en los distintos órganos afectados otro síntoma característico de esta enfermedad, que son los típicos exudados. Estos se pueden encontrar más frecuentemente en brotes y frutos infectados cuando la temperatura es templada y la humedad ambiental alta, y están formados por una mezcla de exopolisacáridos y células bacterianas en forma de gotas y/o filamentos mucilaginosos (van der Zwet y Beer, 1995; Montesinos *et al.*, 1999; Montesinos y López, 2000). Se ha demostrado que los exudados son una importante fuente de inóculo ya que ayudan a la bacteria en su diseminación, pudiendo causar nuevas infecciones o infecciones secundarias, así como también cumplen una función de mantener a las células viables y patogénicas durante largos periodos de tiempo (Beer, 1976; López *et al.*, 1988; Thomson, 2000; Slack *et al.*, 2016). Actualmente se ha descrito que en estas gotas de exudados la población celular es muy elevada (desde 10^6 hasta 10^{10} UFC/ μ l) y que estos, no sólo emergen las bacterias patógenas por las aberturas naturales de la planta, sino que en las zonas más afectadas lo hacen por heridas inducidas por el propio patógeno (Slack *et al.*, 2016).

1.1.1.1.5. Epidemiología

Los factores que condicionan el desarrollo de la enfermedad, y por tanto la manifestación, velocidad y magnitud de los síntomas, según Cambra *et al.* (2002) son:

- Cantidad de inóculo disponible: cuanto más material infectado existe en un cultivo, más se incrementa la intensidad de la infección así como la velocidad de desarrollo de la enfermedad. Cuando se producen exudados, aumenta la cantidad de inóculo, ya que grandes cantidades de bacteria emergen del interior de la planta en ellos, facilitando la diseminación de la enfermedad.
- Receptividad de la planta huésped: los periodos del ciclo vegetativo en los que la planta es más sensible son los de floración y crecimiento vegetativo, al igual que en la formación de los frutos. Dentro de la floración, las floraciones secundarias son más sensibles debido a las condiciones climáticas (temperatura suave y humedad ambiental alta) que favorecen la multiplicación de la bacteria. También el vigor de la planta se relaciona con su receptividad, ya que a más robustez en la planta, más sensible es. Un desequilibrio nutricional en el árbol o los ataques de diversas plagas a brotes en crecimiento, también puede hacerlos más sensibles (Schroth *et al.*, 1974).
- Factores edáficos: los suelos arcillosos, con mal drenaje y excesivamente fertilizados facilitan la predisposición de la enfermedad (van der Zwet y Beer, 1995).
- Sensibilidad de la planta huésped: En el caso de las Pomáceas, el peral es más sensible que el manzano y existen diferencias entre los cultivares de estas especies, al igual que ocurre en plantas ornamentales y silvestres entre géneros e incluso entre especies. El peral (*Pyrus communis*) es la especie que presenta una sintomatología más

característica cuando es atacado por *E. amylovora*, es de resaltar que es la que posee más cultivares altamente sensibles como Alexandrine Douillard, Decana del Comicio, Packam's Triumph y Passe Crassane (van der Zwet y Beer, 1995), todas ellas cultivadas en España. El manzano también tiene variedades de elevada sensibilidad (como Idared y Reina de Reinetas), pero en menor cantidad que el peral y se sabe que algunas de ellas cultivadas en España, como las de manzano de sidra, son sensibles a la enfermedad. Algunos de estos cultivares tienen una floración más tardía que las de peral y manzano de consumo en fresco, por lo que este periodo de floración coincide con los periodos con fuerte riesgo de infección (Palacio-Bielsa y Cambra, 2010). En las plantas ornamentales y silvestres también existen diferencias en cuanto a su sensibilidad a la enfermedad entre los diferentes géneros.

- Condiciones climáticas: Una temperatura de 18 a 29 °C, con una humedad ambiental superior al 70 % y lluvia o rocío, son las condiciones óptimas para el desarrollo de la enfermedad (van der Zwet y Beer, 1995). Algunos fenómenos meteorológicos como el granizo o el viento pueden producir daños físicos o lesiones en la planta que son fácilmente colonizables por la bacteria y por tanto, actuar como nuevos punto de infección. Todas estas condiciones son variables según el área geográfica y la climatología en ese año.

La infección por *E. amylovora* en una planta únicamente se producirá si esta pertenece a una especie sensible, si el inóculo de la bacteria es adecuado y si las condiciones ambientales son favorables para la supervivencia y crecimiento de la misma. La interacción planta-patógeno con las condiciones medioambientales ha sido descrita por diversos autores (Jones y Aldwinckle, 1990, van der Zwet y Beer, 1995, Beer, 2002) aunque aún sigue habiendo algunos aspectos poco estudiados en el ciclo.

Se considera que el ciclo de la enfermedad se inicia en primavera, cuando se produce el inóculo primario y la infección de las flores (Figura 6). El inóculo primario se localiza en los tejidos vivos de la corteza del árbol que rodean los chancros del ciclo anual anterior, en los márgenes difusos de los que antes se hablaba, donde *E. amylovora* ha sobrevivido a las condiciones invernales. Este inóculo primario también puede localizarse en diferentes tejidos de la planta, en donde las bacterias permanecen de forma epífita o endófito (van der Zwet *et al.* 1988). Cuando las condiciones ambientales son favorables, estos chancros del tronco o ramas viejas formados en otoño se activan, es decir, se produce una multiplicación de la bacteria, que es capaz de emerger a la superficie en forma de exudados mucosos (Brooks, 1926; Rosen, 1929; Pierstorff, 1931; van der Zwet, 1969). Los exudados superficiales en forma de gotas de mayor o menor tamaño, son la principal fuente de inóculo secundario por la gran cantidad de bacterias que contienen. Cuando estos exudados se encuentran en la superficie de la planta, la diseminación de la bacteria en la misma, y hacia otros hospedadores, mediante insectos, lluvia y aerosoles es muy propicia. En este punto, *E. amylovora* es capaz de llegar a los estigmas de las flores principalmente mediada por los insectos polinizadores, y de allí a los nectarios, donde se multiplica. Desde los exudados también puede llegar a aberturas naturales de la planta, como estomas, o lesiones causadas por otros factores externos bióticos y humanos, como por ejemplo las picaduras de insectos o la poda, o abióticos como el granizo o el viento. Todo ello produce las llamadas infecciones secundarias, asociadas a un crecimiento tardío de los brotes o una nueva brotación, en época de lluvia. Las infecciones secundarias son las que frecuentemente causan daños más graves en la planta y cuando se dan hay un mayor número de órganos sensibles (floraciones secundarias, brotes tiernos, frutos inmaduros), por lo que pueden ser más numerosas y por lo tanto haber más posibilidades de diseminación de la bacteria.

En otoño, cuando las condiciones ambientales son desfavorables para que la bacteria se multiplique, esta se instala en los tejidos lignificados. Allí, al bajar la temperatura, produce los chancros y detiene su multiplicación con la parada del ciclo vegetativo del árbol hospedador. En los tejidos vegetales vivos circundantes a los chancros, permanece una población de células de patógeno viables, que durante el invierno sobrevivirán a las adversas condiciones climáticas e iniciarán de nuevo el ciclo cuando las temperaturas sean más suaves y la humedad ambiental sea elevada.

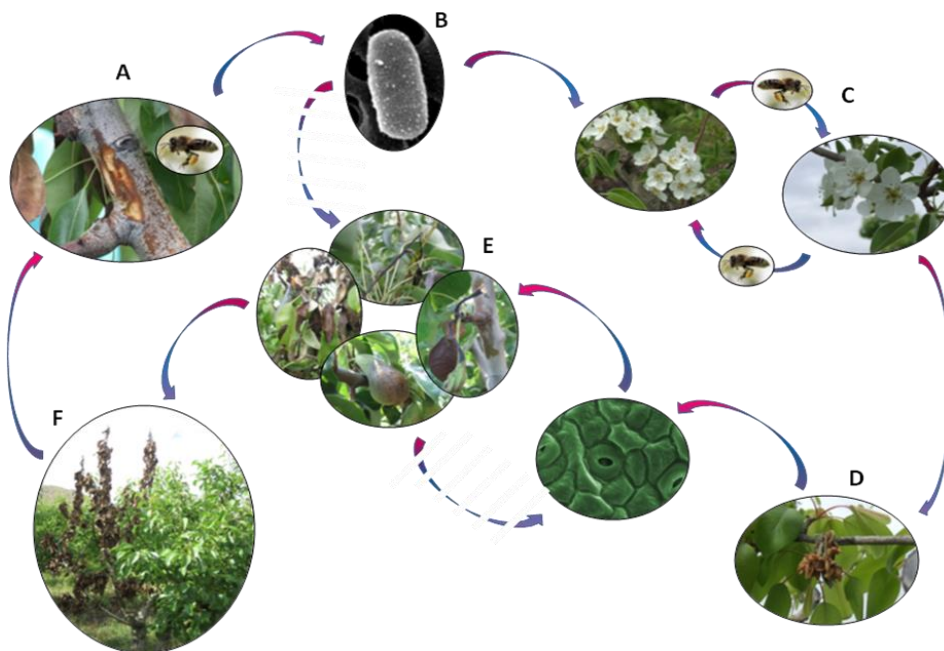


Figura 6. Ciclo biológico del fuego bacteriano. A y B) Inóculo primario (las bacterias de la superficie o de los chancros se dispersan hacia las flores mediante el viento o insectos); C) Fase epífita en las flores (la bacteria se multiplica en la superficie de las flores y se dispersa a otras flores mediante la lluvia o insectos); D) Infecciones florales (infecciones primarias); E) Infecciones secundarias en frutos,

brotos, ramas y producción del inóculo secundario, el exudado; F) Expansión de la enfermedad a otras partes de la planta y producción de nuevos chancros. (esquema modificado de: <http://www.apsnet.org/edcenter/intropp/lessons/prokaryotes/Pages/FireBlight.aspx>, 24/04/2017)

1.1.1.2. *E. pyrifoliae*

Esta especie patógena se aisló en Corea en los cultivares Shingo y Mansamgil de peral asiático (*P. pyrifolia*) y fue descrita por primera vez por Kim *et al.*, en 1999. Se caracteriza por causar síntomas muy similares a los provocados por *E. amylovora*, aunque en condiciones naturales únicamente se ha observado la enfermedad en estos dos cultivares de peral, por lo que se considera que su rango de huéspedes podría ser muy limitado (Figura 7). En inoculaciones controladas en laboratorio, es capaz de producir síntomas en algunos cultivares de peral europeo (*P. communis*) y en el cultivar Idared en manzano (*Malus domestica*) (Kim *et al.*, 2001b).

Los síntomas que causa son el oscurecimiento de la nervadura central, el peciolo y manchado de la hojas, pudiendo progresar a otras partes de la planta, ennegreciendo las flores y frutos inmaduros (Rhim *et al.*, 1999). Hasta su nuevo descubrimiento en cultivo de fresa en Holanda (NPPO, 2014) (Figura 7), esta enfermedad se restringía geográficamente a Asia (Corea del Sur) (Kim *et al.*, 1999; McGhee *et al.*, 2002) aunque estos datos podrían variar debido a los escasos estudios realizados sobre este patógeno en otras zonas (Smits *et al.*, 2010a).

Esta enfermedad, al igual que el fuego bacteriano, provoca pérdidas graves por la reducción de frutos, aunque es de menor impacto (Rhim *et al.*, 1999). El patógeno podría entrar a la planta de la misma manera que *E. amylovora*, por aberturas naturales y heridas, pero esto no se puede asegurar al ser todavía

escasa la información disponible en cuanto a su biología. También es probable que la propagación principal de este patógeno sea mediante material asintomático contaminado (Jock *et al.*, 2005).

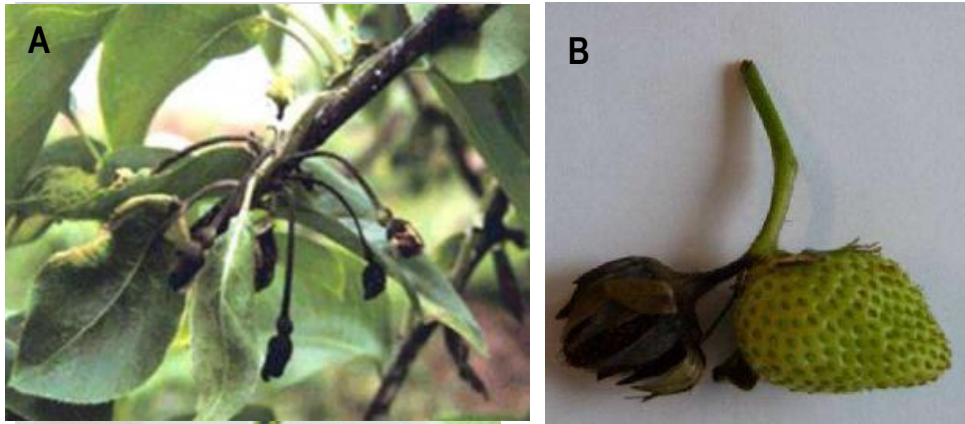


Figura 7. Sintomatología causada por *E. pyrifoliae* en peral (A) (Foto: Bacterial Genetics and Biotechnology Laboratory. <http://pbt.padil.gov.au/pbt/index.php?q=node/20andpbtID=114>, 24/04/2017) y (B) en frutos inmaduros de frambueso (Fuente: https://www.ippc.int/static/media/files/pestreport/2014/03/28/20140128_pest_report_findings_of_erwinia_assigned_to_the_e_pyrifoliae.pdf, 24/04/2017)

1.1.1.3. *Erwinia* sp. de Japón (BSBP)

La enfermedad denominada bacteriosis de los brotes de peral (BSBP: *Bacterial shoot blight of pear*), se ha descrito como una afección similar al fuego bacteriano que afecta a peral asiático (*P. ussuriensis*) cultivar Mishirazu y en principio, se creyó que también a peral europeo (*P. communis*). Esta

enfermedad, considerada erradicada desde 1999, se restringía a la isla de Hokkaido (Japón) y fue descrita por Tanii *et al.*, en 1981.

Los síntomas que produce son la aparición de necrosis en la base del fruto y hojas, y el marchitamiento y muerte de las flores infectadas. Los brotes en crecimiento activo también se ven afectados y se pueden producir exudados dependiendo de las condiciones climáticas, al igual que en el fuego bacteriano.

En 1983, Tanii *et al.*, describieron a las cepas aisladas de estos árboles enfermos como un patotipo de *E. amylovora* (*E. amylovora* pv. *pyri*), aunque no se dio como válido este nombre. Posteriormente 36 cepas de este patógeno se clasificaron taxonómicamente como *E. amylovora* biovar 4 basándose en las diferencias en algunos test fisiológicos y bioquímicos (Mizuno *et al.*, 2000). Aparentemente, sólo dos de esas cepas fueron investigadas de nuevo por Kim *et al.*, en 2001b, concluyéndose que estas eran filogenéticamente más próximas a *E. pyrifoliae* que a *E. amylovora*.

Geider *et al.*, en 2009 complementaron estos resultados con nuevos estudios, llegando a la conclusión que cuatro de esas cepas de las bacterias causantes de BSBP estaban estrechamente relacionadas con *E. pyrifoliae*. Sin embargo, este es un tema aún sin clarificar, ya que Mizuno *et al.*, en 2010, basándose en análisis moleculares, aunque admiten cierta variabilidad en las cepas, consideran que estas no son suficientes para determinar que *E. pyrifoliae* y el agente causal del BSBP sean especies diferentes.

Definitivamente, es complicado clasificar las cepas de BSBP que se conservan como *E. pyrifoliae* y se deberían hacer nuevas comparaciones con las cepas clasificadas como BSBP y las otras especies del género *Erwinia* ya descritos, así como comparaciones a nivel de los genomas de estas especies (Palacio-Bielsa *et al.*, 2012; Smits *et al.*, 2010b).

1.1.1.4. E. piriflorinigrans

Descrita en 2011 por López *et al.*, esta nueva especie fitopatógena se aisló por primera vez en 1999 de corimbos necróticos de peral cultivar Ercolini (Coscia) y Tendral (utilizado como polinizador), en una plantación situada en Turís (Valencia, España) en donde se observaron árboles que contenían un 10-80% de corimbos necrosados (Figura 8). El hecho de afectar a las flores, produce una reducción significativa de los frutos (Roselló *et al.*, 2006). Los síntomas que produce son muy similares a los provocados por el fuego bacteriano en flores, y la morfología colonial en su aislamiento en placa es también muy similar a la descrita para *E. amylovora*. Hasta ahora no se había descrito que esta bacteria progresara a otras partes de la planta y tampoco se habían visualizado síntomas en otras especies potencialmente sensibles (hospedadoras para el fuego bacteriano por proximidad entre las especies bacterianas) pese a que las condiciones de temperatura y humedad fueran idóneas para el desarrollo de la enfermedad (Roselló *et al.*, 2006).

Al igual que las otras especies del género *Erwinia*, *E. piriflorinigrans* es una especie Gram negativa, de forma bacilar, no formadora de esporas y con carácter móvil debido a sus flagelos peritricos (López *et al.*, 2011).

Los aislados de *E. piriflorinigrans* se multiplican en cultivo sin agitación en caldo nutritivo a 25 y 30 °C, pero no a 37 °C ni temperaturas superiores (Roselló *et al.*, 2006), además en condiciones de agitación a una temperatura de 26 °C es capaz de formar biopelículas en materiales como el vidrio y diferentes tipos de plásticos (datos no publicados).

En cuanto a las características metabólicas se ha descrito que los aislados de *E. piriflorinigrans* son quimiorganoheterótrofos, no utilizan el oxígeno en la cadena de transporte de electrones (oxidasa negativa) aunque son anaeróbicos

facultativos y son capaces de fermentar la glucosa en medio Hugh-Leifson sin producción de gas, tras 24 horas de incubación a 25 °C (Hugh y Leifson, 1953). La descripción fenotípica de López *et al.*, 2011, indica que en los test miniaturizados API 20E y API 50CH, los aislados muestran unas características bioquímicas y metabólicas homogéneas. Todos ellos muestran perfiles similares a los que se dan en las cepas de referencia de *E. pyrifoliae* y *E. amylovora*. (Roselló *et al.*, 2006; López *et al.*, 2011; Palacio-Bielsa *et al.*, 2012) (Figura 9).

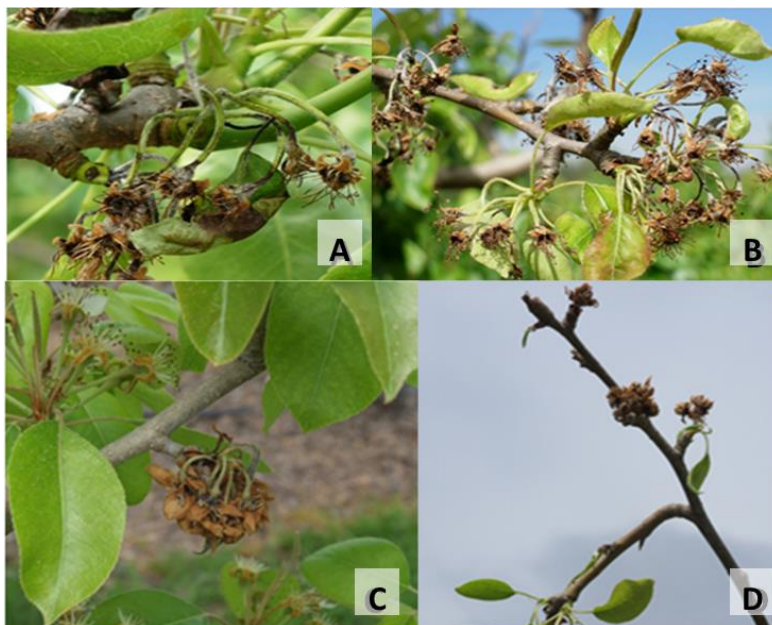


Figura 8. Sintomatología provocada por *E. piriflorinigrans* en corimbos florales de peral (Fotos: A y B: Montserrat Roselló; Fotos C y D: IVIA)

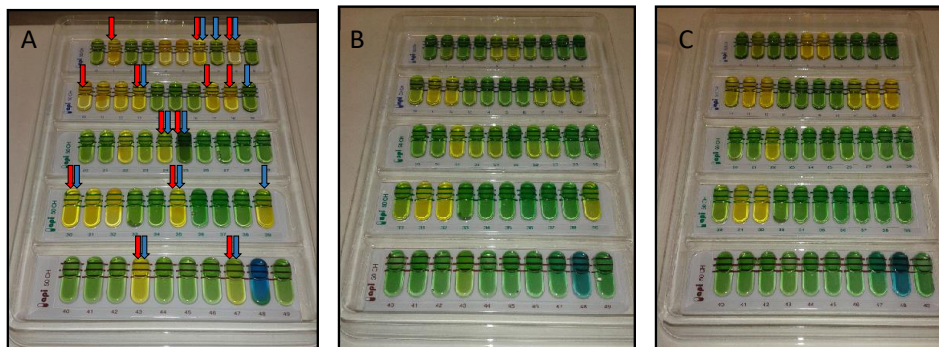


Figura 9. Diferencias metabólicas visibles en los test API 50CH a las 48 horas post-inoculación de *E. piriflorinigrans* CFBP 5888 (A), *E. amylovora* CFBP 1430 (B) y *E. pyrifoliae* CFBP 4172 (C). Las fechas rojas indican las diferencias en el metabolismo de los carbohidratos de *E. piriflorinigrans* y *E. amylovora*. Las fechas azules indican estas diferencias entre *E. piriflorinigrans* y *E. pyrifoliae*.

1.1.1.5. *E. uzenensis* (BBDSP)

E. uzenensis provoca en *P. communis* la denominada enfermedad bacteriana de los brotes negros (BBDSP: *bacterial black shoot disease pathogen*) (Matsuura *et al.*, 2012) y ha sido la penúltima especie descrita en este género. Esta nueva enfermedad se descubrió en 2007, en los brotes de peral europeo cv. La France en Yamagata (isla de Honshu, Japón) (Mizuno *et al.*, 2010). Los síntomas que produce son ligeramente diferentes a los causados por *E. amylovora*, *E. pyrifoliae* y la especie de *Erwinia* sp. de Japón que causa BSBP (Mizuno *et al.*, 2010). La necrosis únicamente afecta a los brotes jóvenes en crecimiento del peral y no llega a afectar a las ramas, aunque en algunos casos también afecta al peciolo de las hojas infectadas, pero nunca llega a progresar a las flores ni a los frutos (Figura 10). En los brotes en crecimiento activo afectados no se producen los cayados de pastor característicos de *E. amylovora* y de la especie de *Erwinia*

de Japón. Los síntomas se restringen a los brotes de la planta y un cultivar y la enfermedad se limita a esta área de Japón. *E. uzenensis* está descrita como un patógeno minoritario con baja virulencia (Mizuno *et al.*, 2010).

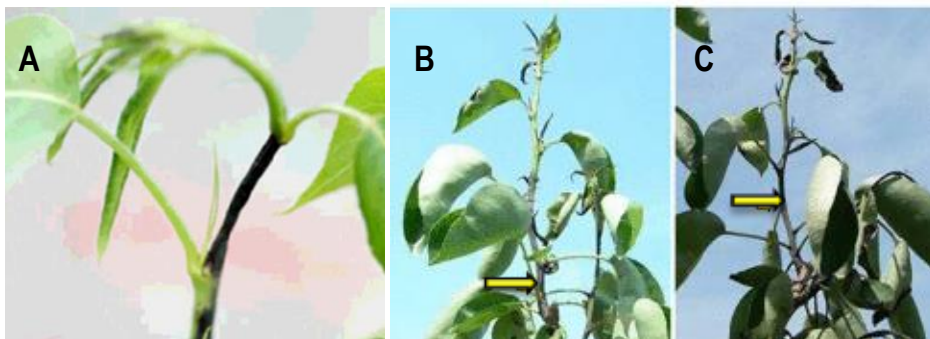


Figura 10. A) Síntomas producidos en brotes de peral cv. La France inoculado con un aislado de *E. uzenensis* (anteriormente este aislado se había clasificado como *Erwinia* sp. de Japón). B y C) las flechas amarillas señalan las zonas de los brotes con síntomas típicos de BBSDP (Foto: Mizuno *et al.*, 2010).

1.1.2. Especies de *Erwinia* no patógenas

Existen otras especies del género *Erwinia* y aisladas en frutales de pepita que son consideradas especies no patógenas, como *E. billingiae* y *E. tasmaniensis*. Estas dos especies se consideran parte de la microbiota epifita del peral y el manzano.

Debido a su carácter no patógeno, actualmente no se tiene suficiente información en cuanto a sus estilos de vida. Sin embargo, hoy en día se ha secuenciado una cepa de cada especie y sobre estos datos se han propuesto hipótesis sobre los mecanismos que ambas especies emplean para su

supervivencia en el medio y la colonización de la planta (Kube *et al.*, 2008 a y 2010).

Se ha observado que ambas especies comparten nicho con otras especies de *Erwinia* patógenas y un aumento de su biomasa en la planta podría interferir en el crecimiento de las especies patógenas, como *E. amylovora* (Jakovljevic *et al.*, 2008). Este antagonismo se ha demostrado sobre frutos inmaduros de peral y flores de manzano infectadas con *E. amylovora*, en los que la aplicación de un inóculo de diferentes cepas de *E. tasmaniensis* y *E. billingiae* dio lugar a una reducción en el crecimiento de la especie patógena (Geider *et al.*, 2006 y 2008; Jakovljevic *et al.*, 2008; Kube *et al.*, 2008 a y b y 2010; Mohammadi y Geider, 2007).

1.1.2.1. *E. billingiae*

Anteriormente esta especie se denominaba *E. herbicola* no pigmentada, y después pasó a denominarse *Pantoea agglomerans* (Billing y Baker, 1963; Vendonck *et al.*, 1987), hasta que con las hibridaciones ADN-ADN se reclasificó dentro del género *Erwinia* y pasó a denominarse como actualmente es conocida (Hauben *et al.*, 1998; Mergaert *et al.*, 1999).

Las cepas de esta especie se han aislado de chancros de ramas, flores necrosadas y frutos inmaduros de diferentes huéspedes como peral, manzano, cerezo, *Crataegus* sp. y olmo. Esta especie tiende a invadir tejidos necrosados de la planta y por ello se considera una especie invasora secundaria que actúa tras la infección de los patógenos primarios y que ayudaría a extender las lesiones provocadas por estos, aunque no a iniciarlas (Billing y Baker, 1963).

E. billingiae se aisló por primera vez de manzano en el Reino Unido y actualmente se ha detectado también en Alemania, Polonia y España (Geider *et al.*, 2008; López M. M., comunicación personal).

1.1.2.2. E. tasmaniensis

E. tasmaniensis se considera como una especie epifita, aunque en su genoma se han localizado algunos elementos genéticos implicados potencialmente en la virulencia del género *Erwinia* (Kube *et al.*, 2010). Esta especie se ha aislado de las flores y corteza de manzano y peral en Australia (Victoria, Tasmania y Queensland; Geider *et al.*, 2006), África (Cape-Suráfrica) y Europa (Alemania; Geider *et al.*, 2006; Jakovljevic *et al.*, 2008; Kube *et al.*, 2008a; España; López M.M., comunicación personal). Es muy posible que esta especie se encuentre distribuida a nivel mundial, al igual que otras especies de este mismo género que aún están poco estudiadas, pero que por la poca información de que se dispone actualmente no estén identificadas como tal.

1.1.2.3. E. gerundensis

E. gerundensis es la especie de *Erwinia* asociada a frutales de pepita más recientemente descrita (Rezzonico *et al.*, 2016). La cepa tipo de esta especie, EM595^T, ha sido aislada de hojas de peral común (*P. communis*) cultivar Winter Nellis en La Tallada d'Empordà, Gerona (España).

Actualmente y pese a su reciente descripción, esta nueva especie se ha localizado en otros orígenes geográficos como Australia y Kazajistán y en diferentes huéspedes como manzano, peral y en raíces de trigo. Con estos datos y su aparente amplia distribución, es de suponer que se podría localizar en otros países y en distintas especies de plantas.

Las características morfológicas y fenotípicas de esta nueva especie se corresponden con las del género, y sus pruebas de hibridación ADN-ADN, confirman que es una nueva especie. Su morfología colonial es similar a la de *Pantoea agglomerans*, así como su pigmentación, inusual en el género *Erwinia*.

2. Características fenotípicas, bioquímicas y fisiológicas comunes y diferenciales de las especies del género *Erwinia*

2.1. Características

Fenotípicamente las especies del género *Erwinia* se caracterizan por ser bacilos Gram-negativos con capacidad de movimiento mediante flagelos peritricos. Su crecimiento es aerobio y anaerobio facultativo, son oxidasa negativa y catalasa positiva y producen ácido a partir de glucosa, fructosa y galactosa (Hauben y Swings, 2005; Hauben, 1998). Las especies que componen este género difieren en algunas características fisiológicas y bioquímicas (Bereswill *et al.*, 1997; Geider *et al.*, 2006, 2009; Kim *et al.*, 1999; López *et al.*, 2011; Mergaert *et al.*, 1999; Mizuno *et al.*, 2010; Rhim *et al.*, 1999; Roselló *et al.*, 2006; Shrestha *et al.*, 2003). Actualmente debemos tener en cuenta el número de cepas utilizadas en cada estudio y de cada especie, ya que varía de forma notoria. Por ejemplo, el número de cepas de *E. amylovora*, estudiada por lo que definir las características genotípicas y fenotípicas generales para estas especies es complicado y no se deben generalizar a la especie en sí, si no a la cepa estudiada en concreto (Tabla 1).

Esta información se puede ampliar con los recientes datos descritos para *E. gerundensis*, ya que las dos cepas españolas de esta especie se caracterizan por su capacidad de fermentación de glicerol, L-arabinosa, D-manosa, L-ramnosa, inositol, D-manitol, maltosa, melibiosa, sacarosa, rafinosa, xilitol, D-arabitol, gluconato de potasio y potasio 2-cetoglutarato (Rezzonico *et al.*, 2016).

Tabla 1. Características fenotípicas, descritas por diversos autores, de las especies *E. amylovora* (*Eamy*), *E. pyrifoliae* (*Epyr*), *Erwinia* sp. de Japón (BSBP y BBSDP), *E. piriflorinigrans* (*Epfm*), *E. uzenensis* (*Euze*), *E. billingiae* (*Ebil*) y *E. tasmaniensis* (*Etas*).

Características	<i>Eamy</i> (a)	<i>Epyr</i> (b)	BSBP (c)	BBSDP (d)	<i>Epfm</i> (e)	<i>Euze</i> (f)	<i>Ebil</i> (g)	<i>Etas</i> (h)
Hidrólisis de Tween 20	-	-	Nd	Nd	+	Nd	+	-
Reducción de nitratos	-	-	-	-	-	Nd	+	-
β -galactosidasa	-	-	-	+	+	+	+	+
Producción de ácido a partir de:								
- α -metil-D-glucopiranosido	-	-	-	V (j)	+	+d	-	-
- β -glucosidasa (hidrólisis de esculina)	+d	-	-	+d	+	+d	+	-
- Adonitol	-	-	-	-	+	Nd	-	-
- D-arabitol	-	-	Nd	Nd	-	Nd	+	-
- D-fucosa	-	-	Nd	Nd	+	Nd	-	-
- D-maltosa	-	-	Nd	Nd	-	+d	+	-
- D-manosa	-	-	Nd	Nd	-	+	+	-
- D-rafinosa	-	-	Nd	Nd	+	Nd	-	-
- L-rhamnosa	-	-	Nd	Nd	-	Nd	+	-
- D-xylosa	+	-	Nd	Nd	+	+	+	-
- Glicerol	-	-	+	+	+	Nd	-	-
- Sacarosa	+	+	+	+	+	Nd	-	+
- Sorbitol	+	+	+	+	-	Nd	+	-
Producción de levano	+	+	+	+	-	Nd	+	-

+: Reacción positiva, -: Negativa, Nd: No determinada o datos no robustos, V: Variable, d: Reacción débil, según:

^a Geider *et al.*, (2006, 2009), López *et al.*, (2011), Mizuno *et al.*, (2010), Roselló *et al.*, (2006).

^b Geider *et al.*, (2006), Kim *et al.*, (1999), Rhim *et al.*, (1999), Roselló *et al.*, (2006), Shrestha *et al.*, (2003).

^c Mizuno *et al.*, (2000).

^d Mizuno *et al.*, (2010).

^e Roselló *et al.*, (2006), López *et al.*, (2011).

^f Matsuura *et al.*, (2012).

^g Geider *et al.*, (2006), López *et al.*, (2011), Mergaert *et al.*, (1999).

^h Geider *et al.*, (2006), López *et al.*, (2011).

ⁱ Existen cepas deficientes en levano descritas por Bereswill *et al.*, (1997).

^j Principalmente positivas según Mizuno *et al.*, (2010).

2.2. Morfología colonial

Los medios de cultivo utilizados en el aislamiento de estas especies son de tipo no selectivo como medio B de King (King *et al.*, 1954) y SNA o levano (Billing *et al.*, 1961; EPPO 2013) y el medio semiselectivo CCT (Ishimaru *et al.*, 1984).

En todas las especies se ha observado un cierto dimorfismo colonial en estos medios de cultivo. *E. amylovora* en el medio B de King forma colonias blanquecinas, circulares y mucosas de forma general. Habitualmente, en levano las colonias formadas por esta especie son convexas, de color crema, circulares y mucosas, al igual que en CCT, aunque en este medio toman un color violeta claro con el borde brillante. Esta morfología puede dar lugar a confusión con otra especie del género, *E. tasmaniensis*, capaz de multiplicarse en estos tres medios con una morfología similar a las 48 horas de la siembra (figura 11).

E. pyrifoliae también se multiplica en estos tres medios, aunque de forma más lenta. La morfología de esta especie en medio sólido se distingue más fácilmente de las anteriores, aunque en medio B de King las colonias también son circulares, algo mucosas y de color cremoso-blanquecino. Su crecimiento en levano es bastante mucoso y no abombado, y las colonias muestran un color cremoso opaco y de aspecto fluido. Al igual que en el anterior medio, en CCT se observa un crecimiento más lento a las 48 horas a 25°C, y las colonias, con un

diámetro menor al de las anteriores, presentan un color violáceo y de aspecto cremoso, no son abombadas y su diámetro es inferior a las demás especies descritas con anterioridad.

E. piriflorinigrans presenta una morfología colonial muy similar a la descrita en *E. amylovora* cuando es aislada o cultivada en estos tres medios (figura 12). En el medio B de King esta especie tampoco produce fluorescencia, formando colonias circulares de aproximadamente 2-3-mm de diámetro, con márgenes regulares, ligeramente convexas, mucosas y de color blanquecino muy similares a las colonias formadas por *E. amylovora* a las 48 horas tras una incubación a 25 °C, pero algo mayores. En levano las colonias que aparecen a las 48 horas de incubación son circulares y de diámetro mayor a las formadas por *E. amylovora*, son muy mucosas por la producción de levano, convexas y de color cremoso y más transparentes. En CCT a las 48 horas las colonias son circulares, de un diámetro superior en comparación con las colonias de *E. amylovora*, pasado este tiempo las colonias se esparcen y pierden esta forma circular, convexa y de bordes brillantes, aunque mantienen en común su color violeta claro.

E. billingiae también es capaz de multiplicarse en estos tres medios, aunque de forma más lenta respecto a todas las especies anteriores. Las colonias formadas por esta especie son muy similares a las formadas por *E. pyrifoliae*.

Aunque morfológicamente hay muy pocos datos de *E. uzenensis* en la actualidad, se ha observado que algunas cepas de esta especie producen dimorfismo en placa, formando la misma cepa colonias mucosas y secas al sembrarse en la misma placa y en iguales condiciones (Figura 11).

E. tasmaniensis presenta una morfología colonial circular, mucosa, aunque ligeramente más amarillenta que la presentada por *E. amylovora* en el medio B de King. Las colonias de este patógeno en levano y CCT aparentemente son como las formadas por *E. amylovora*, aunque de diámetro un poco mayor.

E. gerundensis, forma colonias similares a las especies del género, pero llama la atención su pigmentación amarillenta en los medios generales B de King, Luria-

Bertani (Bertani *et al.*, 1951) y Nutrient agar (Wright, 1934) por tratarse de un carácter atípico en el género, por lo que en un primer momento, de forma visual podría confundirse con la especie *Pantoea agglomerans*. En Nutrient agar forma colonias poco convexas con márgenes redondeados y no es capaz de producir levano en medio SNA (EPPO, 2013), aunque sí secreta abundantes exopolisacáridos, dándole un aspecto muy mucoso a la colonia.

3. Características genéticas del género *Erwinia* (estructura y composición del genoma)

En los últimos años se ha secuenciado el genoma completo de algunas cepas de las diferentes especies englobadas en el género *Erwinia*. Con estos genomas se han realizado estudios comparativos proporcionando diferentes aproximaciones de sus características comunes y diferenciales.

Las características generales de los genomas de las especies de *Erwinia* asociadas a frutales de pepita y rosáceas ornamentales se resumen en la Tabla

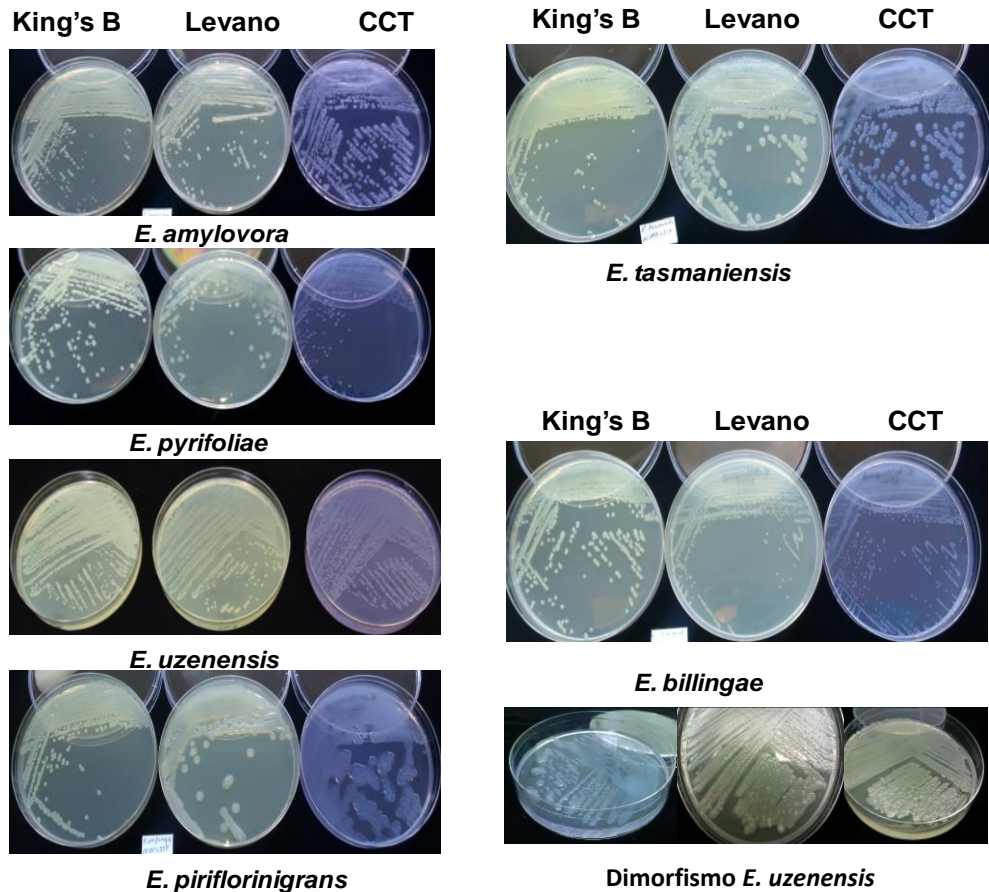


Figura 11. Morfología de las colonias de las distintas especies patógenas y epifitas de frutales de pepita en los tres medios principales que se emplean para su aislamiento. Se observa la gran similitud en la forma, tamaño y color de las colonias entre algunas de ellas, lo que dificulta su diagnóstico correcto *de visu*.

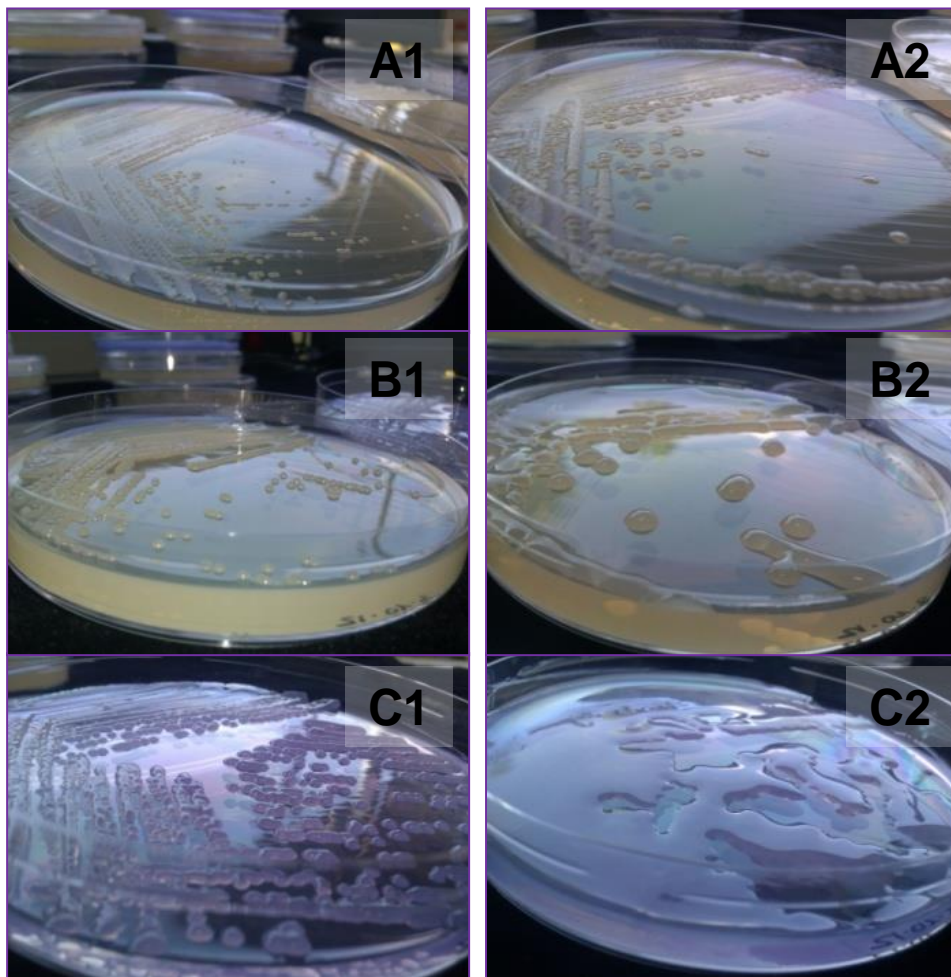


Figura 12. Comparación de la morfología colonial de la cepa CFBP 1430 de *E. amylovora* (A1, B1 y C1) y la cepa CFBP 5888 de *E. piriflorinigrans* (A2, B2 y C2) en los medios de cultivo B de King (A), Levano (B) y CCT (C) a las 48 horas tras una incubación a 25 °C.

Tabla 2. Características generales de los genomas de las cepas secuenciadas de las diferentes especies del género *Erwinia* que afectan a los frutales de pepita y rosáceas ornamentales.

Especie y cepa	Origen	Huésped	Composición	Tamaño (pb)	% G+C	CDS	Ref.
<i>E. amylovora</i> (°)							
ATCC 49946	EEUU	<i>M. domestica</i>	Cromosoma pEA29 pEA72	3805874 28243 71487	53.6	3712	Sebahia <i>et al.</i> , 2010 a
CFBP 1430	Francia	<i>Crataegus</i>	Cromosoma pEA29	3805573 28259	53.6	3706	Smits <i>et al.</i> , 2010a
CFBP 1232 [†]	Reino Unido	<i>P. communis</i>	Cromosoma pEA29	3767276 29586	53.6	3734	Mann <i>et al.</i> , 2013
Ea266	Canadá	<i>Malus</i> sp.	Cromosoma pEA29	3758663 28261	53.6	3804	Mann <i>et al.</i> , 2013
UPN 527	España	<i>Malus</i> sp.	Cromosoma	3766971	53.6	3746	Mann <i>et al.</i> , 2013
ACW 56400	Suiza	<i>P. communis</i>	Cromosoma pEA29 pEI70	- - 65840	-	-	Mann <i>et al.</i> , 2013; Llop <i>et al.</i> , 2011.
<i>E. tasmaniensis</i>							
Et1/99 [†]	Australia	<i>M. domestica</i>	Cromosoma pET09 pET35 pET45 pET46 pET49	3883467 9299 35494 44694 46159 48751	53.4	3804	Kube <i>et al.</i> , 2008 a

Introducción general

E. billingiae

Eb661 ^T	Reino Unido	<i>P. communis</i>	Cromosoma pEB102 pEB170	5100167 102323 169778	55	5029	Kube <i>et al.</i> , 2010
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Erwinia sp. (BSBP)

Epi617	Japón	<i>P. pyrifolia</i>	Cromosoma pEJ01 pEJ02 pEJ03 pEJ04 pEJ05	3909168 30866 5296 6417 3237 2691	53.6	3769	Park <i>et al.</i> , 2011
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E. piriflorinigrans

CFBP 5888 ^T	Valencia, España	<i>P. communis</i>	Cromosoma pEPIR37	3968031 37376	52.9	3857	Smits <i>et al.</i> , 2013; Barbé <i>et al.</i> , 2012
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E. uzenensis

NCPBP 4475 ^T	Kaminoyama, Japón	<i>P. communis</i>	Nd	Nd	Nd	Nd	Nd
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E. gerundensis

EM595 ^T	Gerona, España	<i>P. communis</i>	Cromosoma pEM01 pEM02	3775020 571293 134943	55.4	Nd	Rezzonico <i>et al.</i> , 2016
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Nd: datos no disponibles en la actualidad.

(a): Se citan únicamente los genomas de *E. amylovora* publicados y los más completos de los trece disponibles en la base de datos de NCBI (consulta el 29/04/2017).

3.1. *E. amylovora*

En la actualidad, y excluyendo tres cepas que infectan a *Rubus* sp., existen secuenciados nueve genomas completos de esta especie, aunque en siete de ellos el estado de su anotación es preliminar (Mann *et al.*, 2013). Estas cepas han sido aisladas de diferentes huéspedes como *Crataegus* (cepa: CFBP 1430), *M. domestica* (ATCC 49946 o Ea273) y *Malus* sp. (UPN 527; Ea 266 ó E4001A), *P. communis* (CFBP 1232^T ó NCPPB 683^T; ACW 56400), *Sorbus* sp. (CFBP 2585 o Ea495; 01SFR-BO) y *Cotoneaster* sp. (Ea356 o Ea1/79) y de diferentes orígenes geográficos (Tabla 2). Todas estas cepas poseen un cromosoma circular de aproximadamente 3.8 Mb con un porcentaje de G+C del 53.6% y el contenido en plásmidos de las mismas es variable.

E. amylovora es la especie del género de la que más información genética poseemos actualmente debido a la importancia de la enfermedad que produce y a su distribución mundial. Por ello sabemos que el 86% de su genoma pertenece a CDS (*Coding DNA Sequence* o Secuencias de ADN Codificantes). La densidad de estas secuencias es de una por cada Kb y oscila entre 3706 y 3804 CDS por genoma, dependiendo de la cepa (Mann *et al.*, 2013). Hoy en día, su pangenoma (conjunto de todos los genes de la especie) se considera abierto ya que el número de cepas secuenciadas y anotadas es aún pequeño para poder definir correctamente la especie.

Se ha descrito que el 88-92% del total de sus CDS pertenecen al llamado *core* o genoma conservado, es decir, que en este alto porcentaje de CDS la información genética básica que existe en todas las cepas de la especie y que tan sólo un 8-12% del genoma es variable y por lo tanto podría ser considerado como información supletoria específica de cada cepa. En dicho *core* se da una identidad de secuencia de aminoácidos del 99%, por lo que podemos afirmar que hay escasa variabilidad intraespecie y prácticamente nula diversidad

genética en este patógeno (Mann *et al.*, 2013). Probablemente esta escasa variabilidad tenga que ver con el grado de especialización en cuanto al nicho ecológico de las cepas que afectan a frutales de pepita (Smits *et al.*, 2011).

En cuanto a las características moleculares básicas de este patógeno y en base a sus factores de virulencia, se ha descrito que *E. amylovora* no tiene la capacidad de biosíntesis de metabolitos fitotóxicos o de secretar enzimas proteolíticos que degraden la pared celular de los tejidos vegetales a los que afecta (Seemüller y Beer, 1976). Sin embargo, actualmente se han identificado cuatro factores esenciales para la patogénesis de esta especie:

- Genes *hrp* (*hypersensitive response and pathogenicity*): estos genes intervienen en el desarrollo de la reacción de hipersensibilidad (HR) en plantas no hospedadoras y/o en la patogénesis en plantas huésped. El *cluster*, de 40 Kb, constituye una isla de patogenicidad incluida en el cromosoma (Kim y Beer, 2000). Los genes que la forman codifican proteínas reguladoras, secretoras y secretadas. Las proteínas reguladoras, como el factor de transcripción HrpL, controlan la expresión de otros genes del *cluster*, en respuesta a factores ambientales (Wei y Beer, 1995; Wei *et al.*, 2000). Las proteínas secretoras liberan proteínas al exterior de la célula bacteriana y las inyectan a las células vegetales mediante el sistema de secreción de tipo III. Estas proteínas forman los componentes estructurales de dicho sistema de secreción en forma de pilus. Los genes encargados de codificar las proteínas secretoras son *hrcV*, *hrcC*, *hrcJ* y *hrcT* (Alfano y Collmer, 1997). Las proteínas efectoras más estudiadas son las harpinas (HrpN), aisladas por primera vez en *E. amylovora* aunque presentes también en otras bacterias fitopatógenas. Estas intervienen en la reacción de hipersensibilidad o en la patogénesis en plantas no

hospedadoras y hospedadoras respectivamente, e inducen resistencia sistémica frente a patógenos (Kim y Beer, 2000; Wei *et al.*, 1992).

- Genes *dps* (*disease specific*): son un conjunto de genes co-regulados por el factor de transcripción HrpL, que se encuentran en la región contigua anterior a los genes *hrp*. Estos genes son imprescindibles para el desarrollo de los síntomas de la enfermedad en plantas sensibles, pero no para la reacción de hipersensibilidad en plantas no hospedadoras (Bogdanove *et al.*, 2000).
- Exopolisacáridos: estos polisacáridos extracelulares forman una cápsula que envuelve a la célula bacteriana y la protege de los mecanismos de defensa de la planta. Los principales en esta especie son el amyovorano y el levano (Geider, 2000). Ambos con un importante papel en la patogénesis de *E. amylovora*.
- Sideróforos o desferrioxiaminas: por su forma de vida, *E. amylovora* tiene limitado el hierro disponible en el medio. El escaso hierro existente se encuentra en los tejidos de la planta hospedadora, por lo que la bacteria sintetiza agentes quelantes y transportadores de hierro para poder asimilarlo (Dellagi *et al.*, 1998). Estos sideróforos también actúan como defensa del patógeno ante el efecto tóxico de algunos compuestos químicos que sintetiza la planta en las fases iniciales de la infección (Expert *et al.*, 2000).

En los estudios moleculares realizados sobre esta especie se ha visto que algunas cepas poseen resistencia a antibióticos, como la estreptomina, adquiridas por mutaciones cromosómicas o por la adquisición de plásmidos que codifican para enzimas capaces de inactivar estas sustancias (Jones y Schnabel, 2000). Esto ocurre en zonas en donde son frecuentes los tratamientos en campo con productos que contienen este antibiótico, pero de forma general, la mayoría de cepas de esta especie son sensibles a los antibióticos más utilizados.

3.2. *E. pyrifoliae*

Actualmente sólo se han secuenciado dos cepas de *E. pyrifoliae*, ambas aisladas en Corea del Sur de *P. pyrifolia*. El genoma de ambas es algo mayor al tamaño medio descrito para *E. amylovora*. Ambas cepas poseen aproximadamente 4 Mb y su contenido en G+C es prácticamente igual al de *E. amylovora*, 53.4%. La anotación de ambos genomas muestra que la cepa DSM 12163^T posee 4114 CDS (Smits *et al.*, 2010 b), mientras que la cepa Ep1/96 posee 3852 CDS (Kube *et al.*, 2010) debido probablemente a los diferentes sistemas de anotación empleados. En ambas cepas se han secuenciado cuatro plásmidos.

Los análisis filogenéticos basados en genes *housekeeping* (16S rRNA, *gpd* y *recA*) muestran que esta especie está más cercana a *E. amylovora* que a *E. tasmaniensis* y está bastante más alejada de *E. billingiae* (Geider *et al.*, 2009).

3.3. *Erwinia* sp. de Japón

El genoma de la cepa Epj617 de la especie *Erwinia* sp. de Japón y agente causal de la enfermedad llamada BSBP también ha sido anotado (Park *et al.*, 2011). La secuencia de esta cepa se compone de 3.9 Mb con un contenido en G+C idéntico al de *E. amylovora* (53.6%). En esta anotación se incluyen los cinco plásmidos que contiene y que son prácticamente iguales a los descritos en las cepas DSM12163^T y Ep1/96 de *E. pyrifoliae*.

Su genoma contiene 3769 CDS, que representan el 84.68% de su secuencia (Park *et al.*, 2011).

En comparación con los genomas de *E. pyrifoliae* se observa una baja diversidad genética entre estas cepas, exceptuando la variabilidad en el contenido genético

plasmídico (Shrestha *et al.*, 2007), y por ello Geider *et al.*, (2009) engloban esta cepa en la especie *E. pyriformis*.

3.4. *E. uzenensis*

Actualmente se tiene poco conocimiento a nivel genético del agente causal de la enfermedad BBSDP, *E. uzenensis*. Ningún genoma de esta especie se ha secuenciado hasta hoy, pero sí que se han secuenciado algunos de sus genes *housekeeping* (16S rRNA, *gyrB* y *rpoD*).

Se estima que el contenido en G+C de la cepa tipo YPPS 95113^T es del 53.4%. Los análisis de estas secuencias junto a las hibridaciones ADN-ADN de los 18 aislados descritos para esta especie con otras especies del mismo género, han demostrado que están próximos filogenéticamente a *E. amylovora* y *E. pyriformis* (Mizuno *et al.*, 2010; Matsuura *et al.*, 2012).

3.5. *E. piriflorinigra*

El genoma de la cepa tipo de *E. piriflorinigra* (CFBP5888^T) ha sido recientemente secuenciado y anotado (Smits *et al.*, 2013) revelando su cercanía con *E. amylovora* y *E. pyriformis* pero sobre todo con *E. tasmaniensis*, aunque con pequeñas pero importantes diferencias, como la capacidad de producir amylovorano, lo que podría caracterizar la patogenicidad de esta especie frente a *E. tasmaniensis*.

Su genoma consta de 3.9 Mb con un contenido en G+C del 52.9% en donde se han localizado 3815 CDS cromosómicos y 42 CDS plasmídicos de las cuales, casi un tercio (750) codifican pequeñas proteínas hipotéticas y el 65,34% del total

(2520) están presentes en otras especies de *Erwinia* cercanas, coincidiendo muchas de ellas con la cepa de *E. tasmaniensis* Et1/99.

En el genoma de esta cepa también se han descrito algunas islas genómicas que contienen genes con funciones desconocidas por ahora.

Esta cepa solo posee un plásmido de los dos descritos hasta ahora en esta especie, de los que se hablará en el sexto capítulo de esta tesis.

3.6. *E. billingiae*

Al igual que en la especie anterior, sólo hay una cepa secuenciada y anotada de *E. billingiae*, Eb661^T, por ello la información disponible a nivel genético actualmente es muy limitada (Kube *et al.*, 2010).

Esta cepa posee un cromosoma circular algo mayor del presente en las demás especies (5.1 Mb). La anotación de esta cepa presenta 5029 CDS y un contenido en G+C del 55%. En esta cepa también se han secuenciado sus dos plásmidos, que en comparación con los presentes en las otras especies del género descritas con anterioridad, son de mayor tamaño.

3.7. *E. tasmaniensis*

Únicamente se ha secuenciado la cepa tipo de esta especie, Et1/99^T (Kube *et al.*, 2008a). Su genoma, formado por un cromosoma circular y cinco plásmidos, tiene un tamaño aproximado de 3.9 Mb con un contenido en G+C del 53.4%.

Los análisis de hibridación ADN-ADN y de los genes *housekeeping* muestran que es una especie no patógena muy próxima filogenéticamente a las anteriores especies, especialmente a las patógenas.

3.8. *E. gerundensis*

El genoma de la cepa tipo de esta nueva especie se ha secuenciado, mostrando un cromosoma circular de alrededor de 3.8 Mb con un contenido en G+C del 55.4%. Contiene dos plásmidos circulares de gran tamaño: pEM01 de alrededor de 571 Kb, con un contenido en G+C de 55.1 %, y el plásmido pEM02 de alrededor de 135 Kb, con un contenido en G+C del 52.3 %, este último muy parecido al pEI70 de *E. amylovora* y al pEB102 de *E. billingiae* por poseer un elemento integrativo y conjugativo (ICE) de gran tamaño (Rezzonico *et al.*, 2016).

Su perfil de ácidos grasos (Geider *et al.*, 2006; Matsuura *et al.*, 2012; Skrodenytė-Arbačiauskienė *et al.*, 2012) y la secuenciación del gen16S rRNA junto con el análisis de las secuencias parciales de los genes concatenados *atpD*, *gyrB*, *infB* y *rpoB* (Brady *et al.*, 2008, 2009; Moretti *et al.*, 2011; Wayne *et al.*, 1987) y los estudios de hibridación ADN-ADN de las cepas analizadas para la descripción de esta nueva especie, EM595^T y EM486, mostraron su alejamiento del género *Pantoea*, sugiriendo así el descubrimiento de esta nueva especie del género.

E. gerundensis se considera una especie no patógena ya que los ensayos de hipersensibilidad en tabaco y geranio muestran resultados negativos a las 24 y 72 horas postinoculación (Rezzonico *et al.*, 2016).

Actualmente y gracias a la secuenciación y anotación de todos estos genomas, se han podido hacer comparaciones preliminares a nivel genético intra e interespecíficos, relacionando sus diferencias y semejanzas fenotípicas. Con ellas podemos decir que las cepas secuenciadas de *E. amylovora*, *E. pyrifoliae*, *E. tasmaniensis* y *E. piriflorinigrans*, pese a sus reorganizaciones

cromosómicas, muestran una alta colinearidad. En cambio, el genoma de la cepa secuenciada de *E. billingiae* muestra más diferencias en cuanto a su contenido y disposición de los genes. En base al pangenoma de *E. amylovora*, *E. tasmaniensis* tiene un *core* genético con una identidad en el número de aminoácidos del 92.66%, 95.44% con *E. pyrifoliae* y 92.77% con *E. piriflorinigrans* (Smits *et al.*, 2013).

En cuanto a la anotación del genoma de la especie *E. piriflorinigrans*, que se estudia en varios capítulos de esta tesis, se ha descrito su similitud genética con la cepa secuenciada de *E. tasmaniensis* aunque presenta pequeñas pero importantes diferencias, como por ejemplo la capacidad de producir amylovorano, lo que podría caracterizar la patogenicidad de esta especie frente a *E. tasmaniensis*. Cabe resaltar que las especies patógenas de frutales de pepita y rosáceas ornamentales de este género presentan una alta concordancia en sus genes, donde se incluyen los genes implicados en virulencia, al igual que sucede con los genes que condicionan el estilo de vida epifito en las especies no patógenas (Kube *et al.*, 2010). Pero las diferencias en cuanto al contenido genético interespecífico, así como la expresión de los genes relacionados con la virulencia, nos puede esclarecer los factores implicados en la capacidad de patogénesis de una especie u otra, y por ello en el siguiente apartado se hace un análisis de dichos factores en las diferentes especies de este género.

4. Genes comunes y diferenciales relacionados con patogenicidad, virulencia y eficacia biológica

Actualmente se han realizado múltiples estudios basados en los genes implicados en el desarrollo de los síntomas, la colonización y otras interacciones con el hospedador, para conocer y entender los mecanismos y factores implicados en la patogenicidad y virulencia de este género. En ellos la especie usada como referencia es *E. amylovora*. Hoy en día, la producción de

exopolisacáridos (EPS), los sistemas de secreción, los genes de patogenicidad, colonización y supervivencia en el nicho, así como otros genes que le otorgan unas ventajas metabólicas, son los más conocidos (Bugert y Geider, 1995; Eastgate, 2000; Oh y Beer, 2005; Oh *et al.*, 2005; Zhao *et al.*, 2009 a).

La caracterización genética de estos aspectos a nivel biológico y fenotípico da paso al entendimiento del diferente rango de hospedadores en estas especies y su grado de virulencia, así como a las hipótesis de la evolución y especialización dentro del género. En este sentido Smits *et al.* (2011) han planteado que el ancestro original del género poseía diversos mecanismos de virulencia como la biosíntesis de levano, el metabolismo del sorbitol, los sistemas de secreción tipo III y los de tipo IV, mientras que otros factores, como los genes para la biosíntesis de amylovorano, o los genes que sintetizan un segundo flagelo o dos glicosiltransferasas, se han ido adquiriendo o perdiendo. También se ha demostrado la existencia de ADN exógeno que habría contribuido a la evolución del género mediante transferencia horizontal de genes.

En los siguientes puntos se describen los mecanismos moleculares más importantes implicados en la patogénesis de estas especies hasta ahora conocidos, al igual que la comparación de los mismos (Tabla 3).

Tabla 3. Características comunes y diferenciales relacionados con patogenicidad, virulencia y eficacia biológica de las especies *E. amylovora* (*Eamy*), *E. pyrifoliae* (*Epyr*), *E. tasmaniensis* (*Etas*), *E. piriflorinigrans* (*Epfm*) y *E. billingiae* (*Ebil*) y localizados en el genoma de cada especie.

Características	<i>Eamy</i>	<i>Epyr</i>	<i>Etas</i>	<i>Epfm</i>	<i>Ebil</i>
SST3	PAI-1 PAI-2 PAI-3	PAI-1 PAI-3	PAI-1 incompleta PAI-2 incompleta PAI-3	PAI-1 incompleta PAI-2 PAI-3	PAI-2 parcial
CRISPR	Cas tipo Ecoli	Cas tipo Ecoli e Ypest	Cas tipo Ypest	Cas tipo Ypest	Estructuras cuestionables
Sistemas flagelares:	Si	Si	Si	Si	Si
Fimbrias	K88 (F4) Tipo V	K88 (F4) Tipo V	K88 (F4) Tipo I Tipo V Tipo IV (pET49) Otros	Nd	Nd
Biopelículas	Si, en superficies abióticas y bióticas	Probable pero no demostrado	Probable pero no demostrado	Demostrado en superficies abióticas (no publicado)	Probable pero no demostrado
EPS	Amylovorano Levano Glucano	Pyrifolano (amylovorano)	Levano No cantidades detectables de EPS capsulares	Levano (información limitada en síntesis EPS)	Otros
SST1	Sistema <i>prtADEF</i>	Ausente	Ausente	Ausente	Ausente
SST2	Presente	Presente	Presente	Presente	Ausente
SST4	Presente en pEU30 y pEL60	Ausente	Incompleto en pET49	Nd	Nd
SST5	Ausente	Ausente	Ausente	Nd	Presente

SST6	Cluster 1, 2 y 3	Cluster 1 y 2	Cluster 1 y 2	Cluster 1 y 2	Cluster 1 y 2
Quorum sensing	Ausencia de enzimas para generar señales AI-1 (No síntesis de AHL). Ausencia de genes accesorios para producir respuesta a la señal AI-2.	Ausencia de enzimas para generar señales AI-1 (No síntesis de AHL). Ausencia de genes accesorios para producir respuesta a la señal AI-2.	Ausencia de enzimas para generar señales AI-1 (No síntesis de AHL). Ausencia de genes accesorios para producir respuesta a la señal AI-2. Sistema Q-S ExpRI.	Ausencia de enzimas para generar señales AI-1 (No síntesis de AHL). Ausencia de genes accesorios para producir respuesta a la señal AI-2. Sistema Q-S ExpRI.	Síntesis de AHL. (k) Ausencia de genes accesorios para producir respuesta a la señal AI-2.
Adquisición de hierro:	Regulación de la síntesis y captación por Fur. Sideróforos: Desferrioxamina E. Receptores TonB	Regulación de la síntesis y captación por Fur. Sideróforos: Desferrioxamina E. Receptores TonB	Regulación de la síntesis y captación por Fur. Sideróforos: Desferrioxamina E. Receptores TonB y TonB adicionales con transportadores tipo ABC necesarios.	Regulación de la síntesis y captación por Fur. Sideróforos: Desferrioxamina E. Receptores TonB y TonB adicionales con transportadores tipo ABC necesarios. <i>Cluster</i> para la síntesis de chrysobactina para la captación de hierro.	Regulación de la síntesis y captación por Fur.
Metabolismo de azúcares:					
- Sorbitol	Si	No	No	No	No en todas las cepas
- Sacarosa	Si	Si	Si	Si	No
- Xilitol	No	No	Si (algunas cepas)	Nd	Si

Introducción general

Factores extracelulares:

- De la necrosis	<i>cnf 1 y cnf 2</i>	<i>cnf 1 y cnf 2</i>	<i>cnf 1 y cnf 2</i>	<i>cnf 1 y cnf 2</i> (parcial)	No
- Proteasas	PrtA, PrtB	PrtB	PrtB	PrtB	PrtB

Nd: datos no disponibles en la actualidad.

T(1-6)SS: sistemas de secreción tipo (1-6)

EPS: exopolisacárido.

PAI: isla de patogenicidad

AHL: N-acil homoserina lactona

AI: autoinductor

CRISPR: repeticiones palindrómicas cortas agrupadas y regularmente interespaciadas

4.1. Sistemas de secreción tipo III (SST3): Hrp (PAI-1) e *inv/spa* (PAI-2 y PAI-3)

Se ha demostrado que estos sistemas constituyen un determinante de virulencia de estos patógenos ya que sintetizan proteínas que actúan como factores de patogenicidad en el espacio extracelular o en el citoplasma de las células vegetales. En otras bacterias no patógenas, estos componentes son fundamentales en las interacciones bacteria-planta y otras interacciones como las que se dan entre las bacterias e insectos (Kube *et al.*, 2010; Smits *et al.*, 2010a).

En el pan-genoma de *E. amylovora* se encuentran tres tipos de SST3. El más importante es la isla de patogenicidad 1 (PAI-1), que contiene el grupo de genes o *cluster* llamado Hrp y codifica la reacción de hipersensibilidad (HR) en las plantas no hospedadoras y/o patogénesis en las que sí lo son, mediante la liberación en la célula eucariota de proteínas efectoras (He *et al.*, 2004; Oh *et al.*, 2005; Mann *et al.*, 2013). La isla de patogenicidad Hrp de *E. amylovora*, se delimita por genes que sugieren su transferencia genética horizontal y se compone de cuatro regiones diferentes, la región *hrp/hrc*, la región HEE (efectores y elicitores Hrp), la región HAE (enzimas asociados a Hrp), curiosamente incompleta en la especie no patógena *E. tasmaniensis*, y la región IT (isla de transferencia) (Oh *et al.*, 2005).

En el genoma de la cepa DSM12162T de *E. pyrifoliae* se han descrito el *cluster* de genes *hrp/dsp* y las regiones HAE y HEE, análogas a las presentes en *E. amylovora*. En *E. tasmaniensis* y *E. piriflorinigrans*, también se han localizado estas regiones, a excepción de la HAE, mientras que en *E. billingiae* no se ha localizado ninguna región homóloga al sistema SST3 (Smits *et al.*, 2013). La presencia de estas regiones en la especie *E. tasmaniensis*, considerada no patógena, podría explicarse por el papel de estos genes en la vida epifita de la

bacteria y en la respuesta de hipersensibilidad que sus cepas inducen en plantas no hospedadoras.

En *E. amylovora*, los genes *hrp* (*hypersensitive response and pathogenicity*) se agrupan en la región cromosómica que forma la isla de patogenicidad PAI-1 de 40 Kb (Oh *et al.*, 2005, Smits *et al.*, 2010 b). Estos genes codifican tres tipos de proteínas diferentes en cuanto a su función: reguladoras, secretoras y secretadas.

Entre las proteínas reguladoras, cabe destacar el factor de transcripción HrpL, que en respuesta a factores ambientales, controla la expresión de los otros genes *hrp* (Wei y Beer, 1995; Wei *et al.*, 2000). Las proteínas secretoras son componentes estructurales del SST3, con estructura en forma de *pilus*, que libera proteínas fuera de la célula bacteriana. Los principales genes que codifican las proteínas secretoras de este sistema en *E. amylovora* son: *hrcV*, *hrcC*, *hrcJ* y *hrcT* (Alfano y Collmer, 1997). Las proteínas que secreta este aparato de secreción al exterior de la célula bacteriana son la harpina (HrpN) que es el principal efector de la hipersensibilidad y se ha demostrado su implicación en la patogenicidad de *E. amylovora* (Wei *et al.*, 1992), y otras proteínas potencialmente efectoras, como DspA (Venisse *et al.*, 2003), HrpW (Kim y Beer, 1998) y otras, que contribuyen de alguna manera a la proliferación de *E. amylovora* en la planta, aunque sus funciones biológicas no se conocen del todo bien (Kim y Beer, 2000).

Otro grupo de genes asociado a los *hrp* son los *dsp* (*disease specific*), adyacentes a la región anterior en la isla de patogenicidad PAI-1. Los genes *dsp* se requieren para el desarrollo de los síntomas de la enfermedad, pero no para la respuesta de hipersensibilidad, y están co-regulados por la proteína HrpL (Bogdanove *et al.*, 2000). La proteína DspA/E codificada por este grupo de genes, está implicada en la generación del estrés oxidativo durante la enfermedad y la

supresión de la formación de los depósitos de calosa (Boureau *et al.*, 2006). Esta proteína está descrita como factor de virulencia en *E. amylovora*, ya que la mutación de los genes *dspA/E* en la bacteria la hacen no patógena (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998).

En el pan-genoma de *E. amylovora* también se encuentran las islas PAI-2 y PAI-3, que contienen genes relacionados con dos tipos de SST3 en donde se encuentran genes *inv/spa*, de los cuales se tienen diferentes teorías en cuanto a su función, como la formación de un complejo que codifica proteínas fundamentales para la entrada del patógeno en el hospedador (Boyd *et al.*, 1997) o según Smits *et al.*, (2010a) podrían no estar relacionados con la virulencia en planta, sino con la interacciones con insectos vectores. Este complejo *inv/spa* situado en la PAI-3 también ha sido descrito en *E. tasmaniensis* y en *E. pyrifoliae*, pero no en *E. billingiae*. La PAI-2 se perdió íntegramente en *E. pyrifoliae* pero de forma parcial en las especies consideradas no patógenas, como *E. tasmaniensis* y *E. billingiae* (Smits *et al.*, 2010a) (Tabla 3).

4.2. Secuencias CRISPR

Las repeticiones de secuencias palindrómicas cortas dispuestas regularmente (CRISPR por sus siglas en inglés; *Clustered Regularly Interspaced Short Palindromic Repeats*), son secuencias nucleotídicas presentes en el 60% de las bacterias conocidas actualmente, con un tamaño que oscila entre 21 y 47 pb. En las bacterias, estas secuencias actúan como mecanismo de defensa ante un material genético exógeno a su propio genoma proveniente de bacteriófagos, plásmidos conjugativos u otras secuencias de ADN foráneo relacionadas con la transferencia horizontal de genes. Así, las secuencias CRISPR se considerarían un mecanismo de defensa análogo al RNA de interferencia de los eucariotas y formaría, por así decirlo, una especie de sistema inmune en las bacterias

(Palacio-Bielsa *et al.*, 2012; McGhee y Sundin, 2012). Estas secuencias codifican proteínas putativas denominadas Cas (*CRISPR-associated*) y Cse (*CRISPR-Cascade complex*), imprescindibles y exclusivas de este mecanismo.

Actualmente se han observado secuencias CRISPR en el genoma de *E. amylovora* al igual que en el de *E. pyrifoliae* y en la cepa Epj617 de la especie de Japón, formadas por ocho genes ortólogos a los Cas descritos en *Escherichia coli*. En *E. tasmaniensis*, *E. piriflorinigrans* y también en *E. pyrifoliae* se han encontrado secuencias Cas ortólogas a las localizadas en el genoma de *Yersinia pestis* (Smits *et al.*, 2013; McGhee y Sundin, 2012).

4.3. Genes flagelares

Como la mayoría de las bacterias fitopatógenas, especialmente del género *Erwinia*, *E. amylovora* es móvil por su flagelación peritrica, lo que favorece la virulencia de esta especie (Wang *et al.*, 2010). Esta movilidad se ha asociado con una quimiotaxis específica, dependiente de la temperatura, el pH y otros factores ambientales (Raymundo y Ries, 1980a, b; Palacio-Bielsa *et al.*, 2012). Su movimiento en superficie es tipo *swarming*, el cual requiere de un conjunto de células hiperflageladas y de componentes extracelulares, como EPS y surfactantes (Zhao *et al.*, 2009 a). Se ha descrito que en la regulación de la síntesis de los flagelos también se encuentran implicados los genes de patogenicidad de *E. amylovora* (Cesbron *et al.*, 2004).

En el genoma de la cepa CFBP1430 de *E. amylovora* se localizan dos conjuntos de genes relacionados con la síntesis de flagelos y la quimiotaxis. Uno de estos *clusters* está formado por cuatro genes y es similar a los localizados en las especies *E. pyrifoliae* y *E. tasmaniensis*. El segundo *cluster* está formado por un solo gen (*Flg-2*) que a su vez es análogo al localizado en la cepa tipo de *E.*

pyrifoliae. Este segundo *cluster* no se ha localizado en *E. tasmaniensis* ni en *E. piriflorinigrans*.

4.4. Fimbrias

Todas las cepas del género *Erwinia* secuenciadas en la actualidad poseen un *cluster* de genes que codifica para adhesinas fimbriales de tipo F4 (K88), similar al descrito en las cepas de *E. coli* enterotoxigénicas, excepto la cepa secuenciada de *E. billingiae*. Este *cluster* de genes está incompleto en el género *Erwinia* y no está implicado en la patogenicidad, aunque sí está considerado un factor de virulencia (Kube *et al.*, 2010).

Otro sistema fimbrial de tipo I está presente únicamente en *E. tasmaniensis*. Este está relacionado con la adhesión de las células a superficies, y hace posible la agregación bacteriana y la formación de biopelículas supliendo en esta especie su carencia de exopolisácaridos capsulares, lo que contribuiría a la colonización bacteriana en la planta, así como a su eficacia biológica en el medio donde se encuentran (Kube *et al.*, 2008 a y 2010; Blumer *et al.*, 2005). En *E. tasmaniensis* también se ha descrito un sistema de *pilus* tipo IV, no presente en las otras especies, que es codificado en el plásmido pET49. Este sistema tiene un papel importante en la colonización del huésped, ya que dota a la especie de un movimiento en las biopelículas, independiente de los flagelos (Kube *et al.*, 2008 a y b).

En todas las especies del género *Erwinia* se ha descrito la presencia de genes que codifican para proteínas fimbriales tipo V, también descritas en las cepas de *E. coli* enterotoxigénicas. Estas tienen un efecto acumulativo sobre las de tipo F4, en cuanto a la adherencia a receptores de diferentes células hospedadoras (Kube *et al.*, 2010). Por el hecho de estar presentes en especies patógenas y

epifitas, actualmente es difícil relacionar la función de estos sistemas con la patogenicidad.

Los genes *hns* (*heat-stable nucleoid-structuring*) para la regulación global de la expresión fimbrial, están presentes en todas estas especies del género *Erwinia* (Edwards *et al.*, 1998). Esta regulación afecta a la síntesis de exopolisacáridos (EPS) ya que la proteína que sintetiza se une a la región promotora de los genes *Isc* de *E. amylovora* (Hildebrand *et al.*, 2010).

4.5. Biopelículas

La función principal de las biopelículas es la protección de las bacterias ante las fluctuaciones rápidas que se pueden producir en el medio en donde se encuentran, protegiéndolas ante cualquier estrés de tipo externo como puede ser la exposición a antibióticos o ante las defensas del hospedador (Koczan *et al.*, 2009). En estas biopelículas se crea un microcosmos en donde se favorece la transferencia horizontal de genes. Actualmente se ha demostrado que *E. amylovora* puede formar biopelículas en superficies inertes, así como en superficies bióticas (como en pedúnculos de manzanas y en la mosca de la fruta); para ello juega un papel imprescindible la biosíntesis de amylovorano, y aunque prescindible, es importante para este fin la biosíntesis de levano (Koczan *et al.*, 2009; Ordax *et al.*, 2009).

Aunque no se ha estudiado si las otras especies del género forman biopelículas, gracias a la secuenciación de la cepa Eb661^T de *E. billingiae* se sabe que su genoma contiene genes que codifican para la formación de esta matriz. Recientemente también se ha observado que *E. piriflorinigra* es capaz de formar biopelículas en superficies inertes, aunque estos ensayos son muy preliminares (datos no publicados) (Figuras 13 y 14).

Por su modo de vida en planta y por lo comentado en el apartado anterior en relación con las fimbrias, es muy probable que *E. pirifoliae* y *E. tasmaniensis* también tengan la capacidad de producir biopelículas.

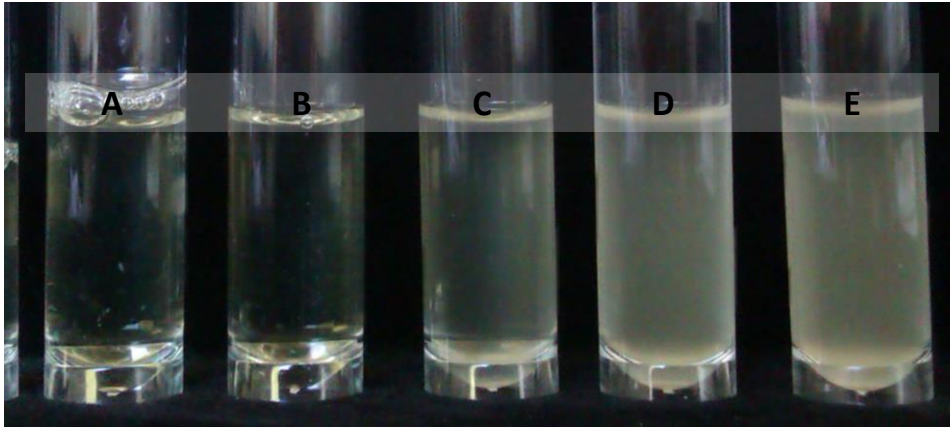


Figura 13. Crecimiento de la cepa tipo de *E. piriflorinigans*, CFBP 5888, en medio de cultivo B de King a diferentes temperaturas: A) 42 °C, B) 37 °C, C) 30 °C, D) 25 °C, E) 22 °C en donde es capaz de formar biopelículas.

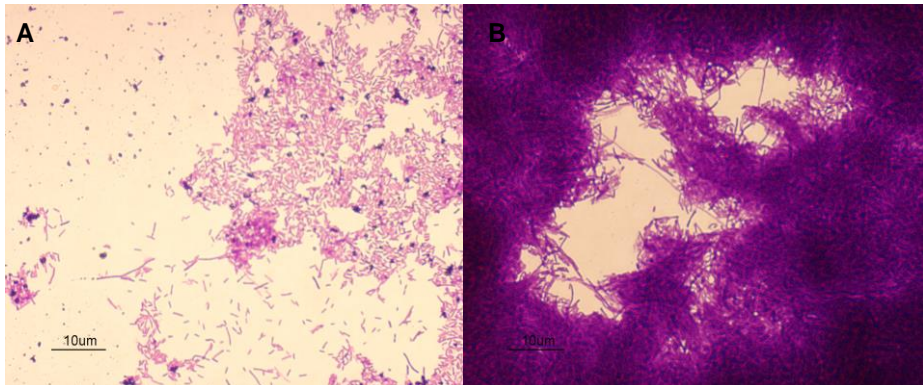


Figura 14. Microscopía de células teñidas por tinción de Gram (Gram, 1884) de la cepa CFBP 5888 de *E. piriflorinigans* en medio líquido (A) y células de esta misma especie formando biopelícula (B).

4.6. Biosíntesis de exopolisacáridos

La biosíntesis de exopolisacáridos (EPS) juega un papel importante en la formación de síntomas en las plantas huéspedes. *E. amylovora* produce tres tipos de EPS: amylovorano, levano y glucano, aunque este último es minoritario en la especie y está muy poco estudiado (Bugert y Geider, 1995).

4.6.1. Amylovorano

El amylovorano es un heteropolímero complejo, de elevado peso molecular (Jumel *et al.*, 1997), capaz de colapsar el tejido infectado produciendo de esta forma el marchitamiento de los brotes (Sjulin y Beer, 1978). Está considerado un factor de patogenicidad en *E. amylovora*, ya que los mutantes con déficit en la síntesis del mismo pierden la capacidad de producir síntomas. Además dota a la bacteria que lo sintetiza de una protección frente a las defensas del hospedador (Bellemann y Geider 1992; Bugert y Geider, 1995). Su biosíntesis está codificada por el operón *ams* (*amylovoran synthesis*) constituido por 12 genes que codifican proteínas transportadoras de azúcares (*amsB*, *D* y *E*), transferasas de lípidos (*amsG*), componentes de un transportador de membrana (*amsA* y *amsL*), una polisacárido polimerasa (*amsF*), proteínas de transporte del EPS a través de la membrana celular externa (*amsH*), una fosfatasa ácida (*amsL*), una UDP-glucosa fosforilasa (*amsM*) y una epimerasa (*galE*) (Geider *et al.*, 2006). Este operón está regulado por los genes *rsc* (*regulation of capsular synthesis*). La mutación de estos genes y la reducción en la síntesis de EPS, provoca una disminución en la virulencia de estas bacterias (Bernhard *et al.*, 1990; Bereswill y Geider, 1997; Geider *et al.*, 2000).

En el genoma de *E. piriflorinigrans* también se han localizado los genes *ams* para la producción de amylovorano, con una alta homología a los localizados en *E.*

amylovora, sobre todo los ortólogos *amsD* y *amsE*, los cuales están ausentes en la especie no patógena *E. tasmaniensis* y sería uno de los factores por los que en esta especie no se producen cantidades detectables de este heteropolímero (Kube *et al.*, 2010).

4.6.2. Levano

El levano es un homopolímero de fructosa de bajo peso molecular que se polimeriza extracelularmente mediante el enzima levanosacarasa (Gross *et al.*, 1992). Este enzima está codificado por el gen *lsc* (Geier y Geider, 1993) y en las bacterias juega un papel importante en la protección contra el reconocimiento de las defensas del hospedador (principalmente en la colonización a la planta), aparte de su actividad hidrolizando la sacarosa y polimerizando la fructosa. El levano no es estrictamente necesario para la patogénesis de esta especie (Kube *et al.*, 2010) y de hecho se han encontrado cepas patógenas en la naturaleza deficientes en su producción (Bereswill *et al.*, 1997). *E. tasmaniensis* y *E. piriflorinigrans* poseen los genes *lsc* para la síntesis de levanosacarasa y por tanto son capaces de producir levano, reduciendo con esta enzima los niveles de sacarosa en el medio donde se encuentran, como en el néctar de las flores (Jakovljevic *et al.*, 2008; Smits *et al.*, 2013).

En *E. amylovora*, la regulación global de ambos EPS está mediada por una proteína de unión al ADN denominada H-NS, que se une a la región *lsc*, también presente en el genoma de *E. piriflorinigrans*, y se ha sugerido que está relacionada con la percepción por esta especie de las condiciones ambientales que las rodean, así como el ajuste de su metabolismo para la adaptación a dichas condiciones (Geider *et al.*, 2006; Hildebrand *et al.*, 2006; Smits *et al.*, 2013). *E. pyrifoliae*, las especies de *Erwinia* sp. de Japón y *E. billingiae* no

sintetizan levano ya que no poseen los genes necesarios para su biosíntesis (Park *et al.*, 2011; Kube *et al.*, 2010).

E. tasmaniensis es capaz de sintetizar polifrufructano, proteína similar al levano, que actúa ante las defensas de la planta, y la glucosa que libera podría ser una fuente de carbono adecuada para su supervivencia (Jakovljevic *et al.*, 2008, Kube *et al.*, 2008 b).

4.6.3. Glucano y otros exopolisacáridos

Aparte del amylovorano y levano, *E. amylovora* produce glucano, un EPS de bajo peso molecular con la función de estabilizar la estructura celular de la bacteria cuando ésta está en situación de cambios osmóticos, como se ha descrito para otras bacterias, y de ayuda en la protección del amylovorano frente a la pérdida de agua en condiciones medioambientales desfavorables (Weissborn *et al.*, 1992).

Otro EPS descrito en el género es el pyrifulano, el único producido en *E. pyrifuliae*, tiene la misma composición en azúcares que el amylovorano en *E. amylovora* aunque se diferencian en la falta de la segunda cadena lateral de glucosa, de hecho, los genes que sintetizan ambos EPS tienen una similitud genética superior al 85% (Kube *et al.*, 2010). Se ha descrito que este EPS se sintetiza a partir de un *cluster* de 14 genes y que está relacionado con la virulencia de esta especie.

Los EPS de *E. amylovora*, en su condición de factores esenciales para la patogenicidad, realizan una serie de funciones clave tanto para el inicio como para el avance, desarrollo y diseminación de la enfermedad. Estos EPS principales forman un complejo necesario para realizar diversas funciones en la

colonización del patógeno en las plantas huéspedes, una de ellas es la defensa física ante las condiciones de sequía o pérdida de agua, a la vez que la protección ante la defensa de la planta y el estrés al cobre (Barney *et al.*, 1990; Bellemann y Geider, 1992; Ordax *et al.*, 2010; Steinberger y Beer, 1988; Vanneste *et al.*, 1990).

4.7. Sistema de secreción tipo I (SST1)

El SST1 está presente en la cepa secuenciada CFBP 1430 de *E. amylovora*. Este sistema codifica una proteasa que se excreta (PtrA) y su sistema de secreción (PtrDEF) (Smits *et al.*, 2010 a). La ausencia de este sistema en la especie no patógena *E. tasmaniensis* se relaciona con su pérdida de virulencia en los frutales de pepita (Kube *et al.*, 2008 b). Sin embargo, también está ausente en las especies patógenas *E. pyrifoliae* y *E. piriflorinigrans*. Por ello se sugiere que este tipo de sistema de secreción puede contribuir a la virulencia en un amplio rango de huéspedes (como ocurre en el caso de *E. amylovora*) más que ser un determinante en la virulencia de la especie (Smits *et al.*, 2010a; Smits *et al.*, 2013).

4.8. Sistema de secreción tipo II (SST2)

El SST2 está presente en el genoma de las cepas CFBP 1430 de *E. amylovora*, DSM 12163^T de *E. pyrifoliae*, CFBP 5888 de *E. piriflorinigrans* y en la especie no patógena *E. tasmaniensis* (cepa Et1/99), lo que hace pensar que aunque se induzca en los ensayos de inoculación en pera inmadura, no es un factor implicado en la virulencia (Zhao *et al.*, 2005; Smits *et al.*, 2010b, Smits *et al.*, 2013). Este *cluster* posee el gen *chiV* cuya proteína tiene un dominio de unión a quitina, indicando que este sistema de secreción podría contribuir a las interacciones de la bacteria con insectos vectores (Smits *et al.*, 2010b).

4.9. Sistema de secreción tipo IV (SST4)

El SST4 es un sistema de secreción que media el movimiento de plásmidos entre bacterias mediante conjugación. Este sistema de secreción de sustratos está presente en algunas especies del género *Erwinia*, y se ha descrito que puede contribuir a la patogénesis en otros géneros bacterianos (Sundin, 2007).

En *E. amylovora* los genes que lo codifican se encuentran en plásmidos como en el pEU30 y pEL60, aunque no están presentes en el plásmido conjugativo pEI70, comprometiendo el papel de este tipo de sistema en la transferencia de genes mediante conjugación (Llop *et al.*, 2011).

En el plásmido pET49 de *E. tasmaniensis* este sistema de secreción también está presente aunque de forma incompleta (Kube *et al.*, 2010).

4.10. Sistema de secreción tipo V (SST5)

El SST5 es un sistema de secreción únicamente observado en la especie *E. billingiae* (Kube *et al.*, 2010). Funciona como un mecanismo de exportación de proteínas muy sencillo. Básicamente es un autotransportador ya que él mismo lleva a cabo su ensamblaje y la secreción de proteínas efectoras que tienen función en la adhesión, invasión y degradación (Henderson *et al.*, 2004).

4.11. Sistema de secreción tipo VI (SST6)

El SST6 es un sistema de secreción que juega un papel en procesos relacionados con la patogénesis y en otros procesos como la adhesión, citotoxicidad, crecimiento intracelular, invasión, supervivencia y persistencia en el huésped y otras interacciones simbióticas con células eucariotas (Cascales, 2008; Palacio-

Bielsa *et al.*, 2012). Este sistema se ha descrito tanto en especies patógenas como en epifitas del género *Erwinia* (Smits *et al.*, 2010 b).

En *E. amylovora* han sido identificados tres grupos de genes que codifican para este sistema (Smits *et al.*, 2010b). Los dos primeros grupos de genes son muy similares a los descritos en *E. pyrifoliae*, *E. tasmaniensis*, *E. piriflorinigrans* y *E. billingiae*, a excepción de algunos genes que codifican para proteínas hipotéticas, mientras que el tercer conjunto está ausente en estas especies (Bingle *et al.*, 2008, Smits *et al.*, 2010b; Smits *et al.*, 2013). En *E. amylovora* este tercer grupo se encuentra dentro de una isla genómica que posiblemente haya sido adquirida mediante transferencia horizontal de genes (Smits *et al.*, 2010b).

4.12. Elementos de autoinducción (*quorum sensing*): LuxI y LuxS

La autoinducción o *quorum sensing* (QS) es un mecanismo de control de la expresión génica o mecanismo de control dependiente de la densidad celular. Es un sistema químico ya que se basa en la existencia de moléculas de señalización extracelular, que regulan diferentes aspectos de las bacterias tales como la producción de factores de virulencia, motilidad y simbiosis.

En bacterias Gram-negativas se conocen dos sistemas QS principales, ambos con señales autoinductoras (AI) de naturaleza química (Miller y Bassler, 2001). El sistema AI-1 utiliza N-acil-homoserina lactonas (AHLs) como moléculas señal, producidas por las proteínas de la familia LuxI.

Se han descrito una amplia variedad de AHLs en bacterias Gram-negativas patógenas y no patógenas, y está controlada por el par de genes ortólogos *luxRI* (Smits *et al.*, 2010 a).

El segundo sistema se basa en la producción de furanosil borato, que es la molécula señal AI-2, controlada por la familia de proteínas LuxS y extendida

tanto en bacterias Gram-negativas como en Gram-positivas. Se ha escrito que este sistema está probablemente implicado en la comunicación entre especies (Smits *et al.*, 2010a).

Los sistemas AI-1 y 2 se han descrito en *E. amylovora*, *E. pyrifoliae* y *E. tasmaniensis*, a pesar que la síntesis de AHL ha sido detectada en *E. billingiae* pero no en las otras tres especies (Smits *et al.*, 2010b; Jakovljevic *et al.*, 2008). Aunque sí existen genes similares a los implicados en la síntesis de AI-2 en el genoma de *E. amylovora* (Mohammadi y Geider, 2007), pero sin embargo la señal de síntesis enzimática para los autoinductores AI-1 no se localiza en el genoma de *E. amylovora* ni de *E. pyrifoliae*, por lo que carecen de los genes accesorios esenciales en la respuesta a la señal producida por AI-2 (Smits *et al.*, 2010 a, b). Sin estos genes, la función de LuxS en *E. amylovora* se debe restringir a un papel metabólico de la enzima implicada en la activación del ciclo del metilo en donde se recicla la metionina y se forma la principal fuente de metilos, S-adenosil-L-metionina (SAM) en procariotas (Winzer *et al.*, 2003; Rezzonico y Duffy, 2008).

Recientemente se ha descrito que en el genoma de *E. piriflorinigra* se codifica un sistema QS que muestra una sintenia con respecto a los localizados en el cromosoma de *E. tasmaniensis*, no presente en *E. amylovora* ni en *E. pyrifoliae* (Smits *et al.*, 2013).

4.13. Factores determinantes en la absorción de hierro

El hierro es un factor nutricional esencial para la vida de los organismos, ya que actúa como cofactor en numerosas proteínas. La absorción de este hierro en las bacterias se regula de forma global por el receptor de la captación Fur, que regula la biosíntesis de sideróforos y su afinidad hacia el hierro (Hantke, 1981;

de Lorenzo *et al.*, 1987; Dellagi *et al.*, 1998). Los genes *fur* se han encontrado en todas estas especies del género *Erwinia* (Smits *et al.*, 2010 a). Bajo condiciones limitantes de hierro, *E. amylovora* produce y secreta estos sideróforos pertenecientes a desferroxaminas E (DFOs) (Feistner *et al.*, 1993; Kachadourian *et al.*, 1996). No solo funcionan como sistemas de adquisición de hierro, sino también como agentes protectores y con función en virulencia, formando complejos con este metal e interrumpiendo la reacción de estrés oxidativo generada por la planta en fases iniciales de la infección (Expert *et al.*, 2000; Oh y Beer, 2005).

Los genes para la desferroxamina E han sido recientemente identificados en *E. pyrifoliae* (Smits *et al.*, 2010a) y sus ortólogos también se han encontrado en el genoma de *E. amylovora* y *E. tasmaniensis*.

La reabsorción de Fe^{3+} unido a sideróforos requiere un receptor dependiente TonB que transporta el sideróforo al espacio periplasmático. El genoma de *E. amylovora* presenta cuatro receptores dependientes de Ton (Kachadourian *et al.*, 1996; Dellagi *et al.*, 1998). A excepción de uno de ellos, que es inactivo en *E. pyrifoliae*, todos ellos están descritos en *E. pyrifoliae* y *E. tasmaniensis* (Smits *et al.*, 2010b). En *E. amylovora*, se encuentran vestigios de otros receptores dependientes de TonB, que se encuentran intactos en los genomas de *E. pyrifoliae* y *E. tasmaniensis*, y otros ausentes en las otras dos especies (Smits *et al.*, 2010a).

En el genoma de *E. amylovora* se encuentran únicamente tres sistemas de captación de hierro tipo ABC y todos ellos están también presentes en los genomas de *E. tasmaniensis* y *E. pyrifoliae* (Smits *et al.*, 2010b).

E. pyrifoliae y *E. tasmaniensis* tienen en común un grupo de genes relacionados con la producción de sideróforos: un receptor de ferrioxamina (codificada por el gen *foxR*) y también presente en el genoma de *E. billingiae* (Kube *et al.*, 2010); el enzima L-lisina 6-monooxygenasa (NADPH) para su biosíntesis (codificada por el gen *dfoA*) y la probable síntesis de alcaligina (codificada por el gen *alcA*).

La base genética para la absorción limitada de hierro en *E. amylovora*, se refleja en una utilización de los sideróforos de este microorganismo (Kachadourian *et al.*, 1996). El hecho de que el dicitrato férrico no se utilice, se confirma con la ausencia completa del sistema *fec*, mientras que la inactivación de *fhuA* aboliría la captación de ferricromo (Smits *et al.*, 2010b).

El *cluster* para la desferrioxamina, *dfoJAC*, también ha sido identificado en *E. piriflorinigrans* con el gen del receptor asociado a ferrioxamina *foxR*, en la misma configuración genética que la cepa CFBP 1430 de *E. amylovora* (Smits *et al.*, 2013).

En el genoma de *E. piriflorinigrans* también se ha detectado un *cluster* putativo de biosíntesis de crisobactina similar al descrito en la cepa 3937 de *Dickeya dadantii* (Franza y Expert, 2010). En este *cluster* se localiza un gen que sintetiza un receptor de ferrocrisobactina que podría ser utilizado para la captación de hierro. También se encuentra un precursor de 2,3-dihidroxibenzoato para la biosíntesis de crisobactina en el *cluster* de genes *cbsCEBA*. Ambos *clusters* están ausentes en los genomas de las otras especies de *Erwinia* secuenciadas. En este patógeno también se encuentra un sistema de captación de citrato férrico, situado adyacentemente al receptor de ferrioxamina E, *foxA* (Smits *et al.*, 2011), un sideróforo receptor de hierro putativo y un sistema de captación del tipo ABC igual al observado en *E. tasmaniensis* Et1/99 (Smits *et al.*, 2010b).

4.14. Metabolismo de los azúcares

Un importante factor relacionado con la virulencia en *E. amylovora* es el metabolismo de los azúcares. La distribución de estos en la planta es dependiente de las condiciones ambientales, de la especie vegetal y del tejido en cuestión (Palacio-Bielsa *et al.*, 2012; Blachinsky *et al.*, 2006; Zhang y Geider, 1999).

4.14.1 Sorbitol

En las rosáceas, es el polialcohol de azúcares dominante y se utiliza para el transporte de carbohidratos en lugar de la sacarosa, como en muchas otras plantas. En *E. amylovora*, su metabolismo se considera un factor de virulencia (Oh y Beer, 2005) y se ha descrito como fuente de carbono para la síntesis de amylovorano (Belleman *et al.*, 1994).

La interrupción de los genes implicados en la captación de sorbitol de la planta, que forman parte del operón *slr* en *E. amylovora*, causan la pérdida de patogenicidad de la bacteria en plántulas de manzano (Aldridge *et al.*, 1997) y una disminución en la virulencia en pera (Qazi *et al.*, 2004).

Las proteínas para el metabolismo del sorbitol también se han descrito en *E. billingiae*, *E. pyrifoliae* y en las especies causales del BSBP y BBSDP, (Megaert *et al.*, 1999; Mizuno *et al.*, 2010; Rhim *et al.*, 1999; Smits *et al.*, 2010a), pero no en la especie epifita *E. tasmaniensis*, ni en especie patógena *E. piriflorinigrans*, (Kube *et al.*, 2010; Geider *et al.*, 2006; Kube *et al.*, 2008 a y b; López *et al.*, 2011, Roselló *et al.*, 2006; Smits *et al.*, 2013).

4.14.2 Sacarosa

La sacarosa es otro azúcar que también actúa en el transporte de carbohidratos en la planta y su metabolismo juega un importante papel en la colonización de las plantas por las bacterias. La mayor concentración de este azúcar se encuentra en los nectarios de las plantas hospedadoras, que se asume que son el principal sitio de entrada de *E. amylovora* (Pusey *et al.*, 2008; Wilson *et al.*, 1990).

Como se ha comentado con anterioridad, este patógeno puede metabolizar la sacarosa a través de la secreción de levanosacarasa, la cual polimeriza el homopolisacárido levano y libera glucosa de la sacarosa (Geier y Geider, 1993; Gross *et al.*, 1992). El metabolismo de este azúcar se lleva a cabo por la expresión de un operón compuesto por cinco genes (*scrK*, *scrY*, *scrA*, *scrB* y el represor *scrR*) que tienen analogía con otros regulones *scr* (Bogs y Geider, 2000) y que también están presentes en el genoma de *E. pyrifoliae* y *E. tasmaniensis* (en esta última especie se han descrito dos copias de los genes *scrA* y *scrB*) y no se ha localizado en la cepa secuenciada de *E. billingiae* (Kube *et al.*, 2010). Los mutantes de *E. amylovora* en el operón *scr* se muestran avirulentos confirmándose que este azúcar es estrictamente necesario para la patogenicidad (Kube *et al.*, 2010).

4.14.3 Xilitol

E. billingiae puede metabolizar xilitol, ampliamente distribuido en la naturaleza (Kube *et al.*, 2010). Esta especie epífita utiliza el xilitol como fuente de carbono, a pesar de su bajo valor nutricional, para evitar la competencia en su adquisición con otras bacterias presentes en el medio y que no lo pueden metabolizar, como *E. amylovora*, *E. pyrifoliae* y algunas cepas de *E. tasmaniensis* (López *et al.*, 2011; Kube *et al.*, 2010).

4.15. Factores citotóxicos en la necrosis y proteasas

En *E. amylovora*, *E. pyrifoliae* y *E. tasmaniensis* se ha descrito la presencia de los genes *cnf1* y *cnf2* que codifican para factores citotóxicos necrotizantes. Dichos factores denominados Cnf1 y Cnf2, son proteínas que debilitan la respuesta de defensa de la planta hospedadora y facilitan la propagación de la bacteria en ella. Ninguno de estos factores se han identificado en la especie no patógena *E. billingiae* (Kube *et al.*, 2010).

Se ha demostrado que Cnf1 inhibe la apoptosis, lo que ayudaría a las bacterias a sobrevivir tras la invasión celular y así multiplicarse en un entorno adaptado (Fiorentini *et al.*, 1998).

Las peptidasas (también conocidas como proteasas) también son consideradas factores citotóxicos relacionados con la necrosis celular. Al contrario que otras especies del género *Erwinia*, *E. amylovora* carece de la capacidad de degradar los componentes de la pared celular de las plantas por la acción de enzimas que degradan los carbohidratos. Sin embargo, esta especie produce y secreta PrtA, una metaloproteasa secretada mediante SST1 que está relacionada con la virulencia de la especie, ya que facilita la colonización de la bacteria en las hojas de manzano (Oh y Beer, 2005). Este operón no está presente en el genoma de *E. pyrifoliae*, *E. tasmaniensis*, ni en el de *E. billingiae*.

4.16. Expresión de genes en *Erwinia amylovora*

Aunque el conocimiento de los genes de cada especie es algo fundamental para estos estudios, hoy en día podemos utilizar las nuevas tecnologías para evaluar los genes que se expresan en ciertas condiciones, como en la infección de la

planta. Esto se puede evaluar con diferentes técnicas, una de las cuales es mediante *microarrays* personalizados específicamente para *E. amylovora*.

Para el estudio de la expresión genética de esta especie, se ha diseñado un *microarray* de ADN complementario (ADNc) que está compuesto de un cristal con 8 *arrays* que contienen 4860 sondas por triplicado (aproximadamente 15000 *spots*). Las sondas de este *array* están basadas en el genoma de la cepa tipo de *E. amylovora* ATCC 49946, y contiene también sondas de otros plásmidos descritos en otras cepas de esta especie (McNally *et al.*, 2011; Águila-Clares *et al.*, 2016). Esta técnica ha sido empleada para demostrar que el sistema Rsc de *E. amylovora* regula de forma diferencial la síntesis de polisacárido capsular y algunos fenotipos tales como el movimiento por *swarming*, la resistencia a determinados antibióticos, la formación de biopelículas y la virulencia en la especie (Wang *et al.*, 2012).

Con la misma metodología se ha demostrado que el regulón HrpL del que se conocía su expresión durante la patogénesis, aparte de regular el sistema de secreción tipo III, regula directa o indirectamente otras señales de las que hasta ahora no se tenía constancia de su implicación en el proceso (McNally *et al.*, 2011).

Esta técnica también ha sido utilizada en el capítulo quinto de esta tesis con el objetivo de conocer el papel que ejercen diferentes plásmidos sobre la expresión de genes del cromosoma de una cepa de *E. amylovora* durante la formación de síntomas *in planta*.

En un trabajo anterior (Triplett *et al.*, 2006) se empleó la técnica de la hibridación sustractiva para estudiar la expresión de genes en *E. amylovora*, en donde por primera vez se pudo observar la expresión de los genes relacionados con la patogenicidad y virulencia de esta especie a gran escala. En otro estudio posterior se demostró que alrededor de 400 genes se inducían en condiciones de infección en pera inmadura y estos fueron clasificados en nueve categorías

funcionales que corresponden a: interacción huésped-patógeno (3.8%), respuesta al estrés (5.3%), regulación (11.8%); superficie celular (8.9%), transporte (13.5%), elementos móviles (1%), metabolismo (20.3%), adquisición y síntesis de nutrientes (15.5%) y proteínas hipotéticas o desconocidas (19.8%) (Zhao *et al.*, 2005). En el mismo estudio se demostró la sobreexpresión de genes ya conocidos implicados en virulencia entre los que se incluyen los componentes del sistema de secreción tipo III (*hrp/hrc*), el gen efector *dspE*, el sistema de secreción tipo II, el enzima levanosacarasa sintetizado por el gen *lsc*, y reguladores de la biosíntesis de levanosacarasa y amylovorano (Águila-Clares *et al.*, 2016).

4.17. Aspectos evolutivos del género *Erwinia*

Se ha propuesto que los géneros *Erwinia* y *Pantoea* provienen de un antecesor enterobacteriano común (Rezzonico *et al.*, 2009) y que de éste derivan las especies productoras de podredumbres blandas (*Pectobacterium* sp. y *Dickeya* sp.), así como las productoras de chancros en leñosas (*Brenneria* sp.) y otras enterobacteriales más lejanas genéticamente.

Esta genealogía del género *Erwinia* se corresponde en gran medida con la filogenia, pero introduce niveles adicionales de diferenciación, ya que se supone que el ancestro del género podría haber evolucionado escalonadamente, en primer lugar con la separación de las especies no productoras de necrosis (*E. billingiae*) o no relacionadas con las rosáceas (*E. rhapontici*, *E. persicina*). Las demás especies en base a su *core* genético, estarían agrupadas con un ancestro común, del que derivaría el grupo de las especies patógenas de rosáceas y el grupo no patógeno formado por *E. tasmaniensis* (Smits *et al.*, 2010b; Smits *et al.*, 2011).

El grupo patógeno se diferenciaría del ancestro por la inserción de una isla genómica que incluye un nuevo sistema flagelar, excepto en el caso de *E. piriflorinigrans*, mientras que el grupo no patógeno sufre unas variaciones que llevan a una pérdida de patogenicidad en la especie en la que deriva, *E. tasmaniensis*.

El ancestro patogénico se diferencia del antecesor común del género *Erwinia* en la incorporación de un segundo sistema flagelar y un cambio en el exopolisacárido que producen, pasando de stewartano a amylovorano.

De este ancestro también derivaría *E. pyrifoliae*, en donde se perdería uno de los dos SST3 de tipo Inv/Spa, así como uno de los dos SST6 de forma parcial. En esta especie también se perdieron los genes para la biosíntesis de levanosacarosa, pero a diferencia del ancestro contiene en su genoma fagos y transposones no encontrados en las otras especies patógenas secuenciadas en la actualidad.

En este sentido *E. piriflorinigrans* derivaría del antecesor común patógeno, teniendo en común los sistemas de secreción tipo III y VI, así como los genes para la biosíntesis de amylovorano y levanosacarosa, pero esta especie ha perdido uno de los dos sistemas flagelares y únicamente posee proteínas asociadas a CRISPRS de tipo Ypest. Curiosamente esta especie se asemeja mucho genéticamente a *E. tasmaniensis*, tanto a nivel del gen 16S rRNA, como por ejemplo en la posesión del gen *expRI* que codifica la síntesis de un autoinductor del QS hasta ahora sólo conocido en esta especie (Figura 15)

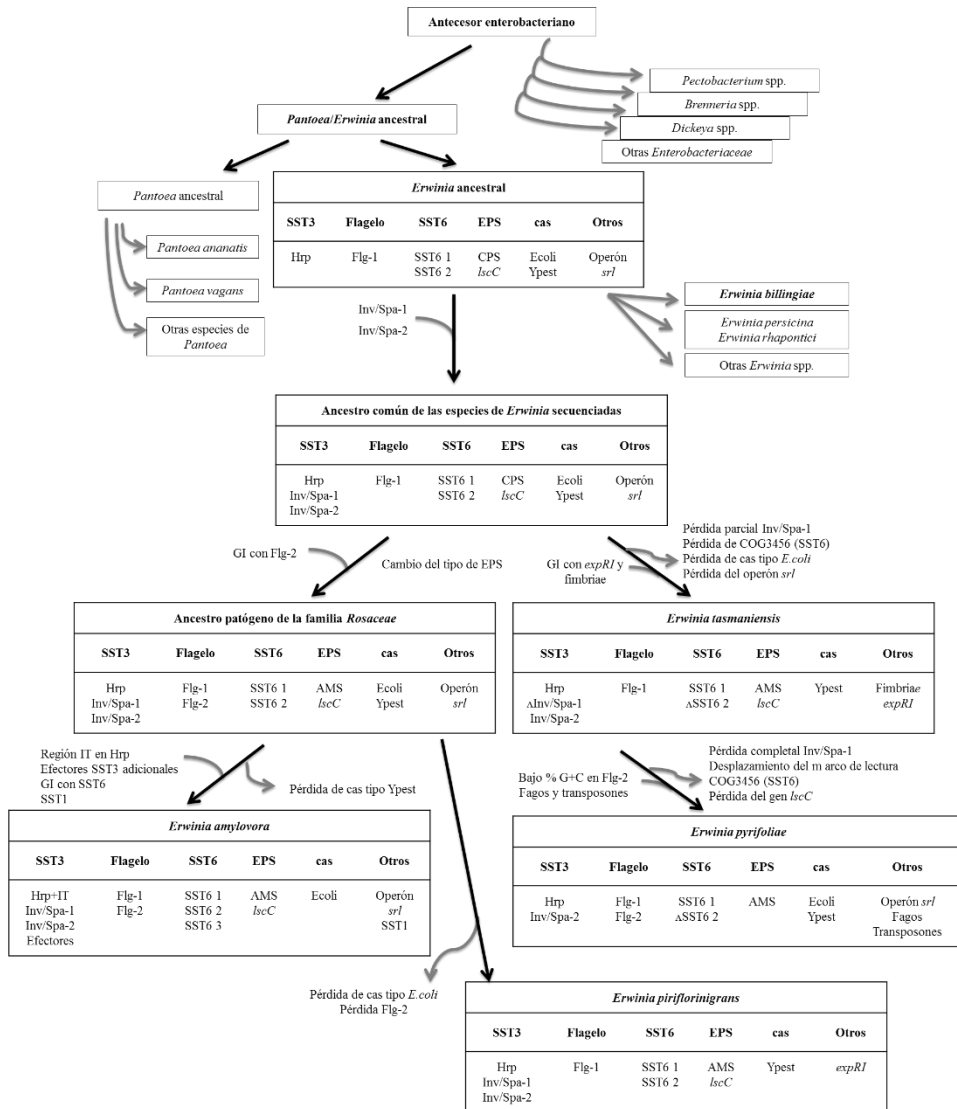


Figura 15. Genealogía del género *Erwinia* basado en los esquemas de Smits *et al.*, 2011, y modificado al incluir los factores presentes en *E. piriflorinigra*ns. Se representan los principales factores de virulencia basados en la secuenciación de los diferentes genomas de las especies que conforman el género. Abreviaturas: SST3: sistema de secreción de tipo III; Inv / Spa: SST3 relacionados con *Salmonella enterica*; Flg: flagelo; SST6: sistema de secreción tipo VI; EPS: exopolisacárido; CPS: stewartano; AMS: amyovorano; *lscC*: gen levanosacarasa; *cas*: subtipo de genes *cas* según Haft *et al.*, 2005; operón *srl*: metabolismo de sorbitol; *expRI*: genes que codifican la biosíntesis de AI-2; GI: isla genómica.

5. Diagnóstico de *E. amylovora* y *E. piriflorinigra*ns

5.1. Métodos de diagnóstico, detección e identificación de *E. amylovora* (EPPO, 2013)

La sintomatología del fuego bacteriano es fácilmente reconocible en las principales plantas hospedadoras, aunque también pueden confundirse con los síntomas producidos por otros patógenos o por otras causas abióticas o bióticas. Por ello, es imprescindible un protocolo de diagnóstico en donde se aísle y se identifique el agente causal basándose en sus características bioquímicas, serológicas, moleculares y patogénicas (López *et al.*, 1988; López y Cambra, 1996), que lo diferencie frente a especies filogenéticamente próximas. Un diagnóstico erróneo podría suponer una gravísima amenaza para los cultivos e incluso perjudicar al comercio internacional del país al ser esta bacteria un patógeno de cuarentena en la Unión Europea (López *et al.*, 2003, 2006a, b; Álvarez, 2004; Palacio-Bielsa *et al.*, 2010).

Del mismo modo, estas técnicas deben ser rápidas, sensibles, específicas y fiables, que permitan confirmar la presencia de la bacteria en material vegetal sintomático o asintomático, con el fin de tomar rápidamente las medidas

fitosanitarias legisladas para evitar su propagación (Anónimo 2000 a, 2003; López *et al.*, 2003; 2006 a, b).

Para un buen diagnóstico en el laboratorio, un factor importante es la toma de muestra y la rapidez con la que se transporte al laboratorio, así como tener en cuenta si las plantas han recibido algún tratamiento previo a la toma de la muestra con bactericidas o cobre.

La muestra de material vegetal, sin tratamiento fitosanitario previo, debería ser tomada en primavera, verano o principios de otoño, cuando las condiciones climáticas son más favorables para la multiplicación de la bacteria. Si hay disponibilidad, el material vegetal preferente para el análisis debe estar formado por flores, brotes sintomáticos (pero no totalmente necrosados) y/o chancros activos en donde el patógeno se encuentra en mayor concentración.

5.1.1. Prospecciones y toma de muestras

Las prospecciones sistemáticas para descubrir la presencia de *E. amylovora* están reguladas por la Dirección General competente en materia de Sanidad Vegetal a nivel territorial de las C.C.A.A. (RD 1201/1999 de 19 de julio - BOE núm. 184, de 3 de agosto de 1999 y RD 1512/2005 de 22 de diciembre - BOE núm. 312, de 30 de diciembre de 2005) (MAPA, 1999 y 2005). Estas prospecciones se realizan en plantas hospedadoras del patógeno, ya sean ornamentales, frutales de pepita o silvestres.

Las prospecciones consisten en inspecciones visuales de estas plantas en una red permanente de puntos geográficos específicos o itinerarios definidos, incluyendo los viveros que producen o comercialicen las especies vegetales hospedadoras. Esta vigilancia consiste también en la toma de muestras para el análisis en laboratorio y en el control del cumplimiento de la legislación

respecto a los documentos del material vegetal en los viveros, y también del pasaporte fitosanitario en su modalidad de zona protegida.

En las zonas con plantas cultivadas o silvestres, cuando se detecten síntomas de la enfermedad, inmediatamente ha de ser comunicado a la Dirección General competente en materia de Sanidad Vegetal.

Para el correcto diagnóstico en el laboratorio, idealmente el material vegetal debe tener síntomas iniciales con lesiones y con frente de avance, aunque la muestra a analizar debería también contener toda la gama de síntomas, desde material vegetal aparentemente sano hasta el más afectado, sin estar muerto o descompuesto. La muestra debe de ser abundante y su transporte preferentemente ha de ser refrigerado, en condiciones estancas y de forma individual para prevenir contaminaciones, especificando la fecha de su toma, el tipo de cultivo, la variedad y patrón, los datos del propietario, la ubicación de la parcela y el porcentaje de plantas afectadas en la misma así como su distribución.

5.1.2. Preparación de muestras sintomáticas

Las muestras sintomáticas se pueden procesar de manera individual o en pequeños grupos, combinando material vegetal de varias muestras. Estas se procesan en tampón de maceración antioxidante (Gorris *et al.*, 1996 a y b) en caso de enriquecimiento posterior de la muestra, o simplemente tampón fosfato salino (PBS) o agua cuando se realicen inmediatamente después el aislamiento directo, pruebas de inmunofluorescencia o PCR.

Para el análisis se seleccionan las partes más frescas del material sintomático y los exudados.

5.1.2.1. Aislamiento

En muestras sintomáticas es relativamente sencillo el aislamiento de *E. amylovora*, ya que en ellas, el número de células cultivables es habitualmente alto. En estos casos, se realiza el cultivo del macerado vegetal y sus diluciones en los tres medios de cultivo empleados para su aislamiento: B de King, levano y CCT, incubándose a 25 oC y realizándose lecturas a las 48, 72 y 96 horas. Las colonias presuntivas se han de conservar en cultivo puro para poder hacer los análisis de confirmación posteriores con otros métodos.

Cuando las muestras presentan síntomas muy avanzados o las condiciones ambientales no son favorables para el crecimiento del patógeno, el número de células cultivables se ve reducido considerablemente. En este caso y cuando en la muestra se encuentren antagonistas del patógeno, se han de realizar enriquecimientos en dos medios líquidos con el macerado con el fin de multiplicar la población inicial de bacterias en la muestra. Este paso previo también es necesario en los análisis por ELISA por su bajo nivel de sensibilidad cuando se usan anticuerpos monoclonales y en algunos análisis por PCR para incrementar su sensibilidad. Como la composición y la cantidad de microbiota es desconocida en la muestra, estos enriquecimientos se realizan en el medio no selectivo B de King y en el medio semiselectivo CCT.

Los métodos de aislamiento y la obtención de cultivos puros de la bacteria requieren varios días y éstos, junto con las pruebas de patogenicidad de los aislados, son los que confirmarán la presencia del patógeno en el material analizado.

5.1.2.2. Pruebas de detección rápida

Este tipo de pruebas se realizan en muestras sintomáticas en donde la concentración mínima de bacteria suele estar entre 10^5 y 10^6 cél/ml. Al menos

dos de estos test, con diferentes principios biológicos, se deben de realizar con cada muestra. Es recomendable realizar un test serológico, a poder ser utilizando un anticuerpo monoclonal específico, y otro molecular basado en la PCR. Las características generales de estas técnicas se muestran en la Tabla 4.

Los protocolos aconsejados para la extracción de ADN para los test moleculares son el descrito por Llop *et al.*, en 1999, el descrito por Taylor *et al.*, 2001 y el kit comercial RED-Extract N-Amp T Plant de Sigma-Aldrich.

5.1.3. Preparación de las muestras asintomáticas

La detección de *E. amylovora* en muestras asintomáticas es complicada. Las muestras se han de tomar cuando la temperatura media sea superior a 18 °C, para aumentar la probabilidad de detectar al patógeno (van der Zwet y Beer, 1995) y deben estar formadas por flores, brotes y frutos jóvenes o partes de tallo. Cuando las muestras se tengan que tomar en invierno, estas estarán formadas por yemas (5-10 por planta).

Este tipo de muestras debe ser enriquecida en medio líquido durante 72 horas a 25 °C, ya que la población bacteriana en ellas suele ser baja y el análisis directo suele ser negativo (Gorris *et al.*, 1996b). Tras ello debe ser procesada para los aislamientos y al menos por dos técnicas moleculares y/o serológicas.

Tabla 4. Comparación de las técnicas serológicas y moleculares utilizadas en el diagnóstico de *E. amylovora*.

Test	Base	Sensibilidad	Observaciones
Serológicos			
Inmunofluorescencia indirecta (IF)	Antisueros policlonales o anticuerpos monoclonales	$10^3 - 10^4$ cél/ml	- Reacciones cruzadas al usar anticuerpos policlonales
ELISA-DASI	Anticuerpos monoclonales	- En material vegetal: $10 - 10^2$ cél/ml - Tras etapa de enriquecimiento: $10^3 - 10^4$ cél/ml	- Evita reacciones cruzadas - Comercializado en kit
Tiras de flujo lateral	Anticuerpos fijados en una membrana.	$- 10^5 - 10^6$ cél/ml	- Indicado en el análisis de material vegetal sintomático - Uso de colonias aisladas de la muestra - Reacciones cruzadas con otras especies del género <i>Erwinia</i> - Comercializado en kit únicamente
Moleculares			
LAMP (Loop-mediated isothermal amplification)	Amplificación isotérmica de secuencias del plásmido pEA29	10^3 cél/ml	- Indicado en el análisis de material vegetal sintomático - Temple <i>et al.</i> , 2008; Temple y Johnson, 2011. - Uso en laboratorios de diagnóstico sin termocicladores.
PCR convencional			
Bereswill <i>et al.</i> , 1992	Amplificación de secuencias del plásmido pEA29	- En material vegetal: $10^5 - 10^6$ cél/ml - Tras enriquecimiento: $10^2 - 10^3$ cél/ml (López <i>et al.</i> , 2006 b)	- Amplicón: 900-1100 pb debido al número de repeticiones de una secuencia de 8 pb en esa zona (Lecomte <i>et al.</i> , 1997; Jones y Geider, 2001)

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Taylor <i>et al.</i> , 2001	Amplificación de secuencias del cromosoma	10^3 - 10^4 cél/ml	- Ampliación: 187 pb - Recomendado el protocolo de extracción de ADN del artículo o el descrito por Llop <i>et al.</i> , en 1999
Stöger <i>et al.</i> , 2006	Amplificación de secuencias del plásmido pEA29 (iniciadores descritos por Llop <i>et al.</i> , 2000)	10^4 - 10^6 cél/ml	- Ampliación: 391 pb - Recomendado el kit comercial RED-Extract N-Amp T Plant de Sigma-Aldrich
Gottsberger, 2010	Amplificación de secuencias del cromosoma	10^3 - 10^4 cél/ml	- Ampliación: 458 pb - Optimización del protocolo de Obradovic <i>et al.</i> , 2007 - Recomendado el protocolo de extracción de ADN descrito por Llop <i>et al.</i> , en 1999
Nested PCR (Llop <i>et al.</i> , 2000)	Amplificación de secuencias del plásmido pEA29 - Iniciadores externos: McManus y Jones en 1995 e internos: Llop <i>et al.</i> , 2000	10^3 - 10^4 cél/ml	- Ampliación: 391 pb - Dos reacciones de amplificación consecutivamente en el mismo tubo - Recomendado el protocolo de extracción de ADN descrito por Llop <i>et al.</i> , en 1999
PCR en tiempo real			
Pirc <i>et al.</i> , 2009	Amplifica una región del gen cromosómico <i>amsC</i>	10^3 - 10^4 cél/ml	- Ampliación: 74 pb - Se usa una modificación del protocolo original para el diagnóstico - Recomendado el protocolo de extracción de ADN descrito por Llop <i>et al.</i> , en 1999
Gottsberger, 2010	Amplifica una región de un gen cromosómico que codifica para una proteína hipotética	10^3 - 10^4 cél/ml	- Amplificación: 138 pb - Recomendado el protocolo de extracción de ADN descrito por Llop <i>et al.</i> , en 1999

5.1.4. Identificación

Los cultivos puros de aislados, presuntamente de *E. amylovora*, deben ser confirmados como tales con al menos dos pruebas combinadas que caractericen a esta especie según sus particularidades moleculares (mediante PCR convencional y/o en tiempo real y LAMP), serológicas (inmunofluorescencia indirecta, ELISA-DASI o tiras de flujo lateral) y/o bioquímicas (tiras API). Posteriormente también se debe evaluar la capacidad del aislado de producir una reacción de hipersensibilidad (habitualmente en tabaco) y la patogenicidad del aislado en frutos inmaduros de peral, manzano o níspero.

5.2. Métodos de diagnóstico, detección e identificación de *E. piriflorinigra*s

Para un correcto diagnóstico de esta bacteria, tanto las prospecciones como la toma de muestras deben ser idénticas a las realizadas para el diagnóstico del fuego bacteriano. A su vez, se deben realizar aislamientos para poder observar las típicas colonias de este patógeno en los mismos medios de cultivo y condiciones a los descritos en los protocolos EPPO con *E. amylovora* (EPPO, 2013). Las colonias tienen la morfología descrita en anteriores apartados de esta introducción, estas deben ser purificadas, porque eso es necesario para las pruebas de hipersensibilidad y la determinación de su poder patógeno.

5.2.1. Pruebas de hipersensibilidad y patogenicidad

Las cepas de *E. piriflorinigra*s provocan una respuesta de hipersensibilidad en hojas de tabaco y tomate a las 24-48h tras su infiltración, independientemente de su origen geográfico, del hospedador y del tejido de donde se haya aislado, al igual que las otras especies patógenas del género (Figura 16).

Las inoculaciones en frutos inmaduros y en hojas de níspero y plantones y brotes de peral y manzano con esta nueva especie no mostraron ningún

síntoma, pero tras su inoculación en flores individualizadas y en corimbos de peral cv. Ercolini se desarrollaron los típicos síntomas de necrosis en parte o totalidad de la flor a los 3-5 días (Roselló *et al.*, 2006) (Figura 17), esto también se ha observado en experimentos preliminares en flor de manzano (datos no publicados).



Figura 16. Respuesta de hipersensibilidad en hojas de tabaco infiltradas con *E. amylovora* (A), *E. piriflorinigrens* (B) y la especie no patógena *E. billingiae* a las 48 horas de la infiltración.

5.2.2. Pruebas serológicas

Se ha descrito que las cepas de *E. piriflorinigrens* reaccionan en los ensayos de inmunofluorescencia con los anticuerpos policlonales: PAb Loewe, PAb Adgen, PAb IVIA EPS 1430 y PAb IVIA 1430 y con el anticuerpo monoclonal: MAb IVIA 7A, empleados para la detección de *E. amylovora*. En cambio, con los anticuerpos monoclonales MAb IVIA 3B e IVIA 5H y con la mezcla de los dos en la técnica ELISA-DASI, los resultados fueron negativos en las cepas de *E. piriflorinigrens* y positivos con las cepas de *E. amylovora* (Roselló *et al.*, 2006). Esta técnica podría servir para descartar que el patógeno de la muestra fuera *E. amylovora*.



Figura 17. Síntomas causados por inoculación de *E. piriflorinigrans* en flores de peral cv. 'Ercolini' (Foto: M. Roselló).

5.2.3. Pruebas moleculares

En cuanto a las pruebas moleculares realizadas con esta nueva especie, se ha descrito que las cepas de *E. piriflorinigrans* tienen una alta similitud de secuencia con algunas regiones del genoma de *E. amylovora*. Esta afirmación se basa en la capacidad de producir amplificadores mediante PCR con los iniciadores diseñados por Maes *et al.* (1996) y Bereswill *et al.*, (1995) dirigidas a las regiones cromosómicas del 23S ADNr y la región de los genes que codifican para la biosíntesis de amylovorano (*ams*). Otros iniciadores empleados en la detección de *E. amylovora*, como los diseñados por Guilford *et al.*, en 1996, dirigidos a amplificar secuencias cromosómicas y Bereswill *et al.* en 1992, McManus y Jones en 1995 y Llop *et al.*, 2000, para secuencias del pEA29, no producen ningún amplicón (Roselló *et al.*, 2006). Estos datos sugieren el interés del diseño de unos iniciadores para la detección, identificación y diferenciación de *E. amylovora*, específicos de esta nueva especie y este tema será desarrollado en el capítulo séptimo de esta tesis.

Sólo con una correcta toma de muestra y empleando varias pruebas para la identificación basadas en diferentes principios biológicos y por último la secuenciación, se podrá realizar un preciso y correcto diagnóstico diferencial entre las especies de este género.



Capítulo 2

Justificación y objetivos

La extensión del fuego bacteriano en España y otros países europeos es actualmente alarmante, amenazando los cultivos de variedades sensibles de frutales de pepita en las grandes zonas productoras, al mismo tiempo que a especies de rosáceas ornamentales y silvestres. El conocimiento del agente causal de la enfermedad y concretamente del genoma de distintas cepas es esencial para desarrollar estrategias de prevención.

Pese a los numerosos avances en los estudios genéticos sobre *E. amylovora*, en este patógeno todavía hay aspectos esenciales que hoy en día se desconocen, como por ejemplo los relativos a sus plásmidos. En muchos de ellos no se ha podido determinar su papel en la cepa que los porta, ni su implicación en aspectos del ciclo de vida de la bacteria o su interacción con el huésped, que pueden ser abordados mediante análisis transcriptómico.

La información de los plásmidos encontrados en *E. amylovora*, aunque limitada, es más abundante que la referente a bacterias de este mismo género pero de descubrimiento mucho más reciente, como es el caso de la nueva especie *E. piriflorinigrans*, en la que hasta hoy no se habían hecho estudios previos de su material extracromosómico.

A nivel más aplicado e inmediato, estos resultados moleculares facilitarían el diseño de nuevas metodologías para el correcto diagnóstico de cada una de las enfermedades causadas por bacterias patógenas del género *Erwinia* con la inequívoca y precisa identificación de cada agente causal.

Por todo lo anterior, en la presente tesis doctoral se plantean los siguientes objetivos:

- 1- Estudiar comparativamente los plásmidos de las diferentes especies del género *Erwinia* descritas en base a la bibliografía disponible, ya que constituyen la mayor fuente de variabilidad genética, realizando una revisión bibliográfica de los plásmidos descritos en las especies patógenas y

- epifitas del género asociadas a frutales de pepita, que sirviera de base a los trabajos experimentales posteriores.
- 2- Establecer las posibles funciones del plásmido pEI70 de *E. amylovora* mediante su secuenciación, anotación y ensayos de virulencia *in planta* y analizar la distribución geográfica del mismo.
 - 3- Determinar el papel de los plásmidos pEA29 y pEI70 en la expresión de los genes cromosómicos en *E. amylovora, in planta*, mediante análisis con *microarray*.
 - 4- Establecer las posibles funciones de los plásmidos presentes en *E. piriflorinigrans* mediante secuenciación, anotación y estudio de su posible función biológica en las cepas de *E. amylovora*. Realizar una comparación de los mismos con otros plásmidos de tamaño similar presentes en otras especies de *Erwinia*.
 - 5- Diseñar un método de diagnóstico rápido, sensible y específico para detectar *E. piriflorinigrans* y diferenciarla del agente causal del fuego bacteriano, así como de otras especies de *Erwinia* patógenas.



Capítulo 3

Funciones y orígenes de los plásmidos en las especies de *Erwinia* patógenas o epifitas asociadas a frutales de pepita

Functions and origin of plasmids in *Erwinia* species that are pathogenic to or epiphytically associated with pome fruit trees

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Abstract

The genus *Erwinia* includes plant-associated pathogenic and non-pathogenic species. Among them, all species pathogenic to pome fruit trees (*E. amylovora*, *E. pyrifoliae*, *E. piriflorinigrans*, *Erwinia* sp. from Japan) cause similar symptoms, but differ in their degrees of aggressiveness, i.e. in symptoms, host range or both. The presence of plasmids of similar size, in the range of 30 kb, is a common characteristic that they possess. Besides, they share some genetic content with high homology in several genes associated with exopolysaccharide production and hence, with virulence, as well as in some other genes. Knowledge of the content of these plasmids and comparative genetic analyses may provide interesting new clues to understanding the origin and evolution of these pathogens and the level of symptoms they produce. Furthermore, genetic similarities observed among some of the plasmids (and genomes) from the above indicated pathogenic species and *E. tasmaniensis* or *E. billingiae*, which are epiphytic on the same hosts, may reveal associations that could expose the mechanisms of origin of pathogens. A summary of the current information on their plasmids and the relationships among them is presented here.

Genetic material in bacteria: chromosomes and plasmids

Bacterial genomes are formed by chromosomal and plasmid genetic material. Their size is directly proportional to the number of genes they contain and correlates with the bacterial lifestyle. Larger genomes are common in bacteria that face changing environmental conditions and occupy more complex and variable niches, whereas bacteria that are very specialised tend to have smaller genomes.

Bacterial chromosomes are circular and/or linear, covalently closed loops of double-stranded DNA, supercoiled and with a single origin of replication, and their numbers vary from one to several per cell. In prokaryotes, the frequency of repetitive sequences is low compared to other organisms, but on the contrary the content in mobile elements such as plasmids, phages, transposable elements or genomic islands is high (Russell, 1998).

Plasmids are molecules of extrachromosomal DNA, capable of being replicated and transcribed autonomously (independently of the chromosomal DNA) that are very common in prokaryotic cells. They can also be circular or, less commonly, linear. Their size and copy number is very variable, although generally it decreases with increasing size. The number can vary from one copy to hundreds in high-copy number plasmids. Plasmid sizes range from a few kb to a few Mb and when they are close to the size of chromosomes are called megaplasmids (Rosenberg *et al.*, 1982). Although there is still no general consensus as to the minimum size at which a plasmid is considered a megaplasmid, some authors suggest that it should be around 100 kb (Antón *et al.*, 1995; Barton *et al.*, 1995). Megaplasmids may be related to the survival strategies of the bacteria in their special surroundings, as reported for halophilic bacteria (Argandoña *et al.*, 2003). In plant-associated bacteria, plasmids with sizes ranging from 150 kb to 1.5 Mb are found in many species, such as *Rhizobium meliloti*, *Agrobacterium tumefaciens* and *Ralstonia solanacearum* (Burkardt and Burkardt, 1984; Unger *et al.*, 1985; Negishi *et al.*, 1993).

Some authors hypothesise that recent events, thought to have occurred since the development of agriculture, led to the conversion of a non-pathogenic or a weak pathogen into a virulent pathogen after acquiring the genes needed for pathogenicity from a different bacterial species (Stukenbrock and McDonald, 2008). Plant pathogens could emerge in agricultural ecosystems through several mechanisms, including host-tracking, host jumps, hybridisation and horizontal

gene transfer. Some pathogens may have been domesticated along with their hosts during the development of agriculture, but some others appear to have emerged very recently and developed almost instantaneously following horizontal gene transfer or hybridisation (Stukenbrock and McDonald, 2008). Currently, pathogen populations in modern agroecosystems are regularly challenged by plant resistance genes, pesticides, crop rotations, and a variety of other cultural practices aimed at reducing plant infections. Consequently, selected genotypes that can adapt to these management practices, including the ability to overcome host resistance genes and chemical treatments, may increase rapidly in frequency and be dispersed to neighbouring fields. Plasmids can play a role in these adaptations by providing bacterial cells with specific genes enabling them to survive or multiply under certain unfavourable conditions (Stukenbrock and McDonald, 2008).

Plasmids are very common in phytopathogenic bacteria, conferring the host bacteria with different traits that enhance their fitness, adaptability to a specific niche, virulence or metabolic features, symbiotic abilities, resistance to certain antibiotics or other characteristics, which can improve their environmental adaptability and contribute significantly to their genetic evolution. An important characteristic is that they can be transferable genetic elements, moving from one organism to another (Sundin, 2007). Usually they are not essential for the host bacterium, but in most cases they confer on the bacteria decisive features for their survival in the environment that provide them with selective advantages.

Horizontal gene transfer, also known as lateral gene transfer, is the exchange of specific genes or genomic regions between species that are normally reproductively isolated (de la Cruz and Davies, 2000). Horizontal gene transfer can also result in unexpected gene homologies among organisms and, in the case of pathogens, in a patchy distribution of virulence genes among different

phylogenetic lineages. Plasmids and conjugative transposons are very frequently transferred horizontally among species of bacterial strains (Panopoulos and Peet, 1985).

The ability of bacteria to adapt to new environments most frequently results from the acquisition of new genes through horizontal gene transfer rather than by the alteration of gene functions through mutations. Most plasmids characterised in phytopathogenic bacteria are self-transmissible and can include insertion sequences and other transposable elements that contribute to the movement of sequences within and between genomes. These features convert plasmids into dynamic instruments of change for bacterial genomes and confer them with genetic plasticity and different advantages in host–pathogen interactions and environmental traits (Sundin, 2007).

The roles of plasmids described in plant pathogenic bacteria are considered in terms of the phenotypes they confer to their host cells, although most of them are cryptic. Many of these genes are acquired from other bacteria via horizontal transfer, in which the plasmids play an important role as tools of genetic exchange that enable rapid evolution of the genome of the microorganisms (Sundin, 2007). The genes they carry can be integrated into the host chromosome. Through integration events, the genetic content of plasmids can be stabilised within the genome and further disseminated.

Functions encoded by plasmids in plant pathogenic bacteria

Plasmids are not restricted to the bacterial species from which they originated due to the horizontal transfer of genes. Their genetic content is variable and they represent the flexible part of the bacterial genome and contribute to the ecological and pathogenic differences among strains of the same species, since they usually code for genomic islands (Sundin, 2007). Plasmids, with their

potential for inter-strain mobility, play an important role in bacterial interaction with plants and a substantial number of genes involved in pathogenicity and host specificity have been described in plasmids in some species or particular strains. Other characteristics frequently encoded in plasmids are toxin and hormone production, resistance to bactericides such as copper or antibiotics, and to UV irradiation (Vivian *et al.*, 2001). Plasmids can also acquire a number of different antibiotic-resistance genes by means of integrons. Integrons are transposons that can carry multiple gene clusters, called gene cassettes that move as a unit from one piece of DNA to another. In this way, a number of different antibiotic-resistance genes can be transferred from one bacterium to another.

Some of the most relevant genes and phenotypes described in plant pathogenic bacteria that can be encoded in plasmids are:

- Pathogenicity and host-specificity genes. The most notable are avirulence (*avr*) and virulence (*vir*) genes and the *hrp* genes involved in a type III protein secretion system. The *hrp* genes are mainly coded by the chromosome, and only in *Pantoea agglomerans* (formerly *Erwinia herbicola*) pv. *gypsophylae* and *Ralstonia solanacearum* are the *hrp* genes coded in plasmids (Lichter *et al.*, 1995; Nizan *et al.*, 1997; Boucher *et al.*, 1986). In contrast, the *avr* genes that have been described are evenly divided between plasmid and chromosomal locations (Vivian *et al.*, 1997). A totally different system encoding for pathogenicity is the Ti plasmid of *Agrobacterium tumefaciens* which contains the T-DNA and *vir* regions that play a direct role in tumour induction and oncogenicity (Watson *et al.*, 1975).

- Toxins One example is coronatine, whose genetic determinants in the species *Pseudomonas syringae* are generally located on plasmids (Cuppels and Ainsworth, 1995).
- Plant hormones. Genes for indoleacetic acid (IAA) production are located on the Ti plasmid in *A. tumefaciens* and also harboured by a plasmid in *P. agglomerans* pv. *gypsophilae* (Manulis *et al.*, 1991; Clark *et al.*, 1993). In *Pseudomonas savastanoi* pv. *savastanoi*, the IAA genes are located in plasmids in oleander strains, but they are chromosomally located in ash and most olive strains (Caponero *et al.*, 1995).
- Resistance to copper. Appears to be plasmid-borne in several phytopathogenic bacteria (*Xanthomonas* spp. or *X. campestris* pv. *vesicatoria*), *Pseudomonas syringae* pv. *syringae*; and *P. syringae* pv. *tomato*) (Stall *et al.*, 1986; Bender and Cooksey, 1986, 1987; Mellano and Cooksey, 1988; Kidambi *et al.*, 1995). It is generally located on large plasmids, with the exception of *X. arboricola* pv. *juglandis*, in which the genes are chromosomally located (Lee *et al.*, 1994).
- Resistance to antibiotics and other compounds. Plasmid borne resistance to streptomycin has been detected in *P. syringae* pv. *papulans* and other Gram-negative bacteria (Norelli *et al.*, 1991). Copper resistance is often linked to streptomycin resistance and dual resistance to both bactericides has been detected in conjugative plasmids of *P. syringae* pv. *syringae* (Sundin and Bender, 1993).
- Resistance to UV irradiation. Resistance determinants to UV light, *rulAB*, have been identified and characterised in plasmids in *P. syringae* (Sundin and Murillo, 1999). They are homologous to the DNA repair operon, *umuDC*, in *E. coli* and they are harboured on pPT23A-like

plasmids. These replicons are indigenous residents of the species *P. syringae* and also tend to encode determinants of importance in host–pathogen interactions. The precise role of the *ruAB* genes in protection against UV needs cautious assessment since it is clear from the work of Arnold *et al.* (2000) that these genes may no longer be functional. Consequently, their role among *Ps. syringae* strains, whether in protection against UV irradiation or as mobile regions of potential homology for integration of virulence genes, is still to be determined.

- Insertion sequences (IS) and transposons. They have a role in the transfer and mobility of genes among bacteria. In some cases they can inactivate genes, such as the effect of IS51 and IS52 on the *iaaM* gene in *P. savastanoi* pv. *savastanoi*, leading an inability to induce gall formation (Comai and Kosuge, 1983; Yamada *et al.*, 1986) and IS476, which inactivates the avirulence gene *avrBs1* in *Xanthomonas* spp. (*X.campestris* pv. *vesicatoria*) (Kearney and Staskawicz, 1990). Others may serve as sites of potential mobile homology, such as IS1327 in *P. agglomerans* pv. *gypsophila*, which is probably also involved in the horizontal acquisition of the IAA gene cluster in *P. savastanoi* pv. *phaseolicola* (Szabo and Mills 1984; Lichter *et al.*, 1996; Vivian *et al.*, 2001).

Few other phenotypes have been shown to be plasmid derived among phytopathogenic bacteria, but given the size of several plasmids already described, the genes they harbour may play a role in many other characteristics that remain still unknown.

Brief description of phytopathogenic *Erwinia* sp. and their relationship with non-pathogenic *Erwinia* sp.

The genus *Erwinia* essentially comprises plant-associated bacteria and includes several species that are pathogenic to pome fruit trees (Palacio-Bielsa *et al.*, 2012). The most important pathogenic species is *Erwinia amylovora*, causal agent of fire blight on a wide range of rosaceous hosts (pear, apple, quince, loquat, ornamental plants), and which is capable of producing symptoms in different organs of these plants. Once established in a tree, *E. amylovora* invades old tissues through the current season's growth. Death of infected branches can be so rapid that the leaves do not fall off the tree. Young trees of susceptible cultivars can be easily killed by the infection while mature trees can survive, but the economic losses are very high (van der Zwet and Keil, 1979; Bonn and van der Zwet, 2000). *Erwinia amylovora* is present worldwide, including in North America, Europe, the Eastern Mediterranean, and some countries in Pacific Asia and North Africa (Bonn and van der Zwet, 2000; Shrestha *et al.*, 2003).

Related species producing symptoms similar to those of fire blight in pome fruit trees described in the last decades include:

- *Erwinia pyrifoliae*. A pathogen described in Asian pear and, thus far, only isolated in South Korea. Affected plants show symptoms very similar to fire blight in Nashi cultivars (Kim *et al.*, 1999; Rhim *et al.*, 1999). Some strains of the previously described bacterial shoot blight of pear (BSBP) in Japan were found to be closely related to the Korean pathogen *E. pyrifoliae* (Kim *et al.*, 2001a). Recently a polyphasic characterisation has assigned some strains of these *Erwinia* from Japan to *E. pyrifoliae* (Geider *et al.*, 2009).

- *Erwinia piriflorinigrans*. In 1999, necrotic pear blossoms were observed in pear trees of cvs. *Ercolini* (Coscia) and Tendral in Valencia, Spain, and the disease was also observed in subsequent years (López *et al.*, 2011). The affected trees only presented infected blossoms similar to fire blight in spring, but these were the only symptomatic parts of the plant, in contrast to the disease caused by *E. amylovora* (Roselló *et al.*, 2002, 2006). A new *Erwinia* species was described based on the characteristics of the strains isolated from these symptoms, and called *E. piriflorinigrans*. In 2011, the disease was reported only from some orchards in Valencia, Spain.
- A new pathogenic *Erwinia* sp. that produces bacterial black shoot disease (BBSDP) on European pear has also been recently reported (Mizuno *et al.*, 2010). So far, the disease is restricted to one island in Japan. Isolates of this new pathogen are closely related, but different to, *E. amylovora* and *E. pyrifoliae*. Their taxonomic classification is not yet defined. No information about the possible spread of this new pathogenic *Erwinia* is available.

The symptoms that all these pathogenic erwinias produce on pear are shown in Fig. 1. Other species of this genus isolated from pome fruit are *Erwinia billingiae* and *Erwinia tasmaniensis*. Due to the epiphytic occurrence of these latter species, they can grow competitively with *E. amylovora* on flowers and may be applied as antagonists for control of fire blight (Kube *et al.*, 2010). All these *Erwinia* species are genetically and phenotypically closely related, although they can be distinguished by taxonomic criteria (Palacio-Bielsa *et al.*, 2012).



Fig. 1. Symptoms produced on pear trees by the different pathogenic *Erwinia* species. a) Symptoms caused by *E. amylovora* on a branch. Photo: IVIA, Spain. b) Symptoms of *E. amylovora* on a shoot, producing the typical “shepherd’s crook”. Photo: IVIA, Spain. c) Necrotic symptoms caused by *E. pyrifoliae*. Photo: Bacterial Genetics and Biotechnology Laboratory. d) Symptoms caused by *E. piriflorinigrans*. Necrosis on flowers of cv. *Ercolini*. Photo: IVIA, Spain. e) Symptoms on young shoots of cv. *La France* inoculated with isolates of the new pathogenic *Erwinia* sp. from Japan. Photo from Mizuno *et al.*, (2010).

Plasmids in *E. amylovora*

Genomic studies performed on strains of *E. amylovora* have revealed that although the species is very homogeneous at the biochemical and serological levels (Paulin, 2000; Smits *et al.*, 2010a), there are differences among strains found in different analyses, including host range and virulence (Norelli *et al.*, 1984; Cabrefiga and Montesinos, 2005; Wang *et al.*, 2009). The diversity that could explain such differences may primarily be attributed to the flexible genome that comprised plasmids. Several works have reported finding different plasmids in strains of this pathogen (Laurent *et al.*, 1989; Chiou and Jones 1991; McGhee *et al.*, 2002; Maxson-Stein *et al.*, 2003; Foster *et al.*, 2004), but such studies were performed using a low number of isolates from few geographic areas or countries. Consequently, there is a lack of information on the plasmid content of large collections of isolates of *E. amylovora* (Foster *et al.*, 2004; Llop *et al.*, 2008a). This topic has acquired more attention in recent years since the discovery of strains lacking plasmid pEA29, which was thought to be universal (Llop *et al.*, 2006, 2008a, 2011; Mohammadi *et al.*, 2009; Mohammadi, 2010). The search for isolates lacking the pEA29 plasmid led to the discovery of new isolates with different plasmid contents (Llop *et al.*, 2006, 2011). Thus, the genetic information encompassed by this extrachromosomal material could be more important in the life cycle, aggressiveness or fitness of *E. amylovora* than expected. Here, we describe current knowledge about the different plasmids found in *E. amylovora* strains, their possible functions and their comparison to other plasmids found in related pathogenic and non-pathogenic *Erwinia* species. A summary of the plasmids found in *E. amylovora* and the other pome fruit *Erwinia* species is shown in Table 1.

Table 1. Species of *Erwinia* from pome fruits, the plasmids they harbour and other characteristics.

Plasmid	Strain of origin	Size (bp)	Bacterial host plant	Country of origin	Accession numbers	References
<i>Erwinia amylovora</i>						
pEA72	Ea273	71,487	Apple	USA	FN666577	Sebahia <i>et al.</i> , 2010
pEI70	IVIA1614-2a	65,84	<i>Crataegus</i>	11 European countries	CP002951	Llop <i>et al.</i> , 2008b; 2011
pEL60	LebB66	60,145	Apple, pear	Lebanon	NC_005246	Foster <i>et al.</i> , 2004
pCPP60*	Ea322	≈56kb	<i>Crataegus</i>	France	Na	Steinberger <i>et al.</i> , 1990
pEA34	CA11	≈34kb	Pear, apple	Michigan, USA	Na	Chiou and Jones, 1991; 1993
pEU30	UTRJ2	30,314	Pear, apple	USA	NC_005247	Foster <i>et al.</i> , 2004
pEA29	Ea88	28,185	Pear	Washington, USA	AF264948	McGhee and Jones, 2000
	Ea273	28,243	Apple	New York, USA	FN666576	Sebahia <i>et al.</i> , 2010
	CFBP1430	28,259	<i>Crataegus</i>	France	FN43411	Smits <i>et al.</i> , 2010a
pEA8.7	CAR3	near 9 kb	Apple	California, USA	Na	Palmer <i>et al.</i> , 1997
pEAR5.2	ATCCBAA2158	5,251	<i>Rubus</i> sp	Illinois, USA	FR719211	Powney <i>et al.</i> , 2010
pEAR4.3	ATCCBAA2158	4,369	<i>Rubus</i> sp	Illinois, USA	FR719210	Powney <i>et al.</i> , 2010
pEA2.8	IL-5	2,825	<i>Rubus</i> sp	Illinois, USA	AY123047	McGhee <i>et al.</i> , 2002
pEA1.7	IH3-1	1,711	<i>Crataegus</i>	Louisiana, USA	AY123046	McGhee <i>et al.</i> , 2002

E. pyrifoliae

pEP36	Ep1/96	35,904	Asian pear	Korea	AY123045	McGhee et al., 2002
	Ep1/96	35,909	Asian pear	Korea	FP236829	Kube et al., 2010
	DSM12163T	35,901	Asian pear	Korea	FN392238	Smits et al., 2010a
pEJ01	Ejp617	30,866	Asian pear	Japan	CP002125	Park et al., 2011
pEJ30	Ejp556	29,593	Asian pear	Japan	AY255829	Maxson-Stein et al., 2003
pJE03	Ejp617	6,417	Asian pear	Japan	CP002127	Park et al., 2011
pJE02	Ejp617	5,296	Asian pear	Japan	CP002126	Park et al., 2011
pEP05	Ep1/96	4,955	Asian pear	Korea	FP236828	Kube et al., 2010
pJE04	Ejp617	3,237	Asian pear	Japan	CP002128	Park et al., 2011
pEP03	Ep1/96	3,07	Asian pear	Korea	FP236827	Kube et al., 2010
pJE05	Ejp617	2,691	Asian pear	Japan	CP002129	Park et al., 2011
pEP2.6	Ep1/96	2,59	Asian pear	Korea	FN392236	Kube et al., 2010

E. tasmaniensis

pET49	Et1/99	48,751	Apple	Tasmania	CU468131	Kube et al., 2008b
pET46	Et1/99	46,159	Apple	Tasmania	CU468133	Kube et al., 2008b
pET45	Et1/99	44,694	Apple	Tasmania	CU468132	Kube et al., 2008b
pET35	Et1/99	35,494	Apple	Tasmania	CU468130	Kube et al., 2008b
pET09	Et1/99	9,299	Apple	Tasmania	CU468128	Kube et al., 2008b

E. billingiae

pEB170	Eb661	169,778	Pear	UK	FP236830	Kube et al., 2010
pEB102	Eb661	102,323	Pear	UK	FP236826	Kube et al., 2010

E. piriflorinigrans

pEPIR37	CFBP5888	37,376	Pear	Spain	NC_022963	Barbé et al., 2013
pEPIR5	CFBP5887	5,529	Pear	Spain	Pending	Barbé et al., 2013

* Recent data obtained in the sequencing of several *E. amylovora* strains indicate that this plasmid, thought to be present in several strains, corresponds in reality to two different ones: it has been renamed pEA72 in strain Ea273 from USA (Sebahia et al., 2010) and it has been reported as pEI70 in some strains from Europe (Llop et al., 2011). See text for details.

- pEA72. This plasmid has been described in strain Ea273 (ATCC 49946), which has been sequenced recently (Sebahia *et al.*, 2010). The strain was isolated from an infected apple tree growing in an orchard in New York State (USA) in the 1970s. In a previous work on strains Ea273 and Ea322, a plasmid thought to have a size of 56 kb was reported and named pCPP60 (Steinberger *et al.*, 1990). However, recent sequencing of strain Ea273 has revealed a plasmid with a size of 71,487 kb (GenBank accession number FN666577) and the designation of pEA72 was given instead. No specific genes relating to important characteristics that could be provided to the host bacterium are present and as its function is unknown it is considered cryptic.
- pEI70. A plasmid of nearly 66 kb in size was found during screening of the plasmid content of Spanish strains of *E. amylovora* (Llop *et al.*, 2006). This plasmid has important characteristics that make it unusual among the other plasmids found in this pathogen: (1) it is conjugative (Llop *et al.*, 2011); (2) it is widespread in 11 European countries, being present in 5–92% of strains analysed, depending on the country; and (3) more importantly, it has been observed that when introduced into low virulence strains of *E. amylovora*, it was able to induce faster development of symptoms than without it (Llop *et al.*, 2008 a, 2011). It has been sequenced, but the annotation has not revealed known virulence genes. The sequencing yielded a consensus sequence of 65,840 base pairs, with an overall G+C content of 52.2% (GenBank accession number CP002951). A total of 70 open reading frames (ORFs) have been identified and annotated. It presents a high sequence identity to sequences of plasmid pEB102 from *E. billingiae* strain Eb661, and the protein RepA has 99% sequence identity to the RepA of the same plasmid, indicating a potential common origin. It is thought to

provide a fitness advantage to the host bacterium, but its specific role has not yet been determined (Llop *et al.*, 2011).

- pCPP60. A plasmid from *E. amylovora* strain Ea322 (CFBP1368), isolated from *Crataegus* in France was described with a size of approximately 56 kb, and called pCPP60 (Steinberger *et al.*, 1990). Other strains including Ea208, Ea220 and Ea273 from the USA and strain Ea321 from France (CFBP1367), were reported to also contain pCPP60. However, as explained above, the plasmid in strain Ea273 was renamed pEA72, while sequences of strain Ea321 now demonstrate that it harbours pEI70 (T. Smits, personal communication). As strains Ea321 and Ea322 were isolated from the same place and at the same time, they could be clonal, and the plasmid in strain Ea322 may also prove to be pEI70. The presence of pCPP60 in other strains, claimed to be in 25% of 40 isolates analysed from the USA and France (Steinberger *et al.*, 1990), is misleading because pEI70 has not yet been found in the USA (Llop *et al.*, 2011).
- pEL60. This plasmid, described by Foster *et al.*, (2004) has been reported in strains from Lebanon and was present in 47% of the 49 *E. amylovora* strains analysed from three plant hosts (apple, pear and quince). This plasmid has 60,145 bp with a G+C content of 51.5% (GenBank accession number NC_005246). Annotation of its sequence revealed 68 ORFs, but putative functions could only be assigned to 46 (68.7%) of them, encoding a variety of genes thought to may be of environmental significance to *E. amylovora*, because mutagenic DNA repair determinants confer UV radiation tolerance that may enhance bacterial fitness on plant surfaces.

- pEA34. This plasmid was reported from some strains of *E. amylovora* with resistance to streptomycin (SmR), isolated during a study on resistance to this antibiotic in Michigan (USA) (Chiou and Jones 1991, 1993) and seems to be present only in strains from there. It has a size of ca. 34 kb and seems that SmR is due to the presence of a transposon (Tn5393) that contains the genes encoding this resistance. pEA34 likely originated from the insertion of the transposon into a 28 kb plasmid present in some strains of *E. amylovora*. Hybridisation studies indicated that pEA34 and this plasmid were not related to the pEA29 plasmid (Chiou and Jones 1993). Although this transposon appears to be restricted to plant-associated bacteria, the SmR genes of Tn5393 are identical to the genes found in at least 14 genera of Gram-negative animal and human pathogens worldwide (Sundin and Bender 1996). The plasmid pEA34 has not been fully sequenced.
- pEU30. This plasmid was reported from several strains from Washington, Utah and Oregon, in the USA (Foster *et al.*, 2004) where it was present in 41.0% of the analysed strains. Plasmid pEU30 contains 30,314 bp with a G+C content of 48.2%; its annotation revealed 25 putative ORFs, and 23 of these (92.0%) had homology with existing gene sequences (GenBank accession number NC_005247). The genetic content of pEU30 is similar to that of other plasmids inhabiting plant pathogenic bacteria, but there are no specific genes related to virulence or fitness reported (Foster *et al.*, 2004). In Fig. 2 the maps of this plasmid and pEL60 are shown in the way normally employed to observe the gene composition they harbour.

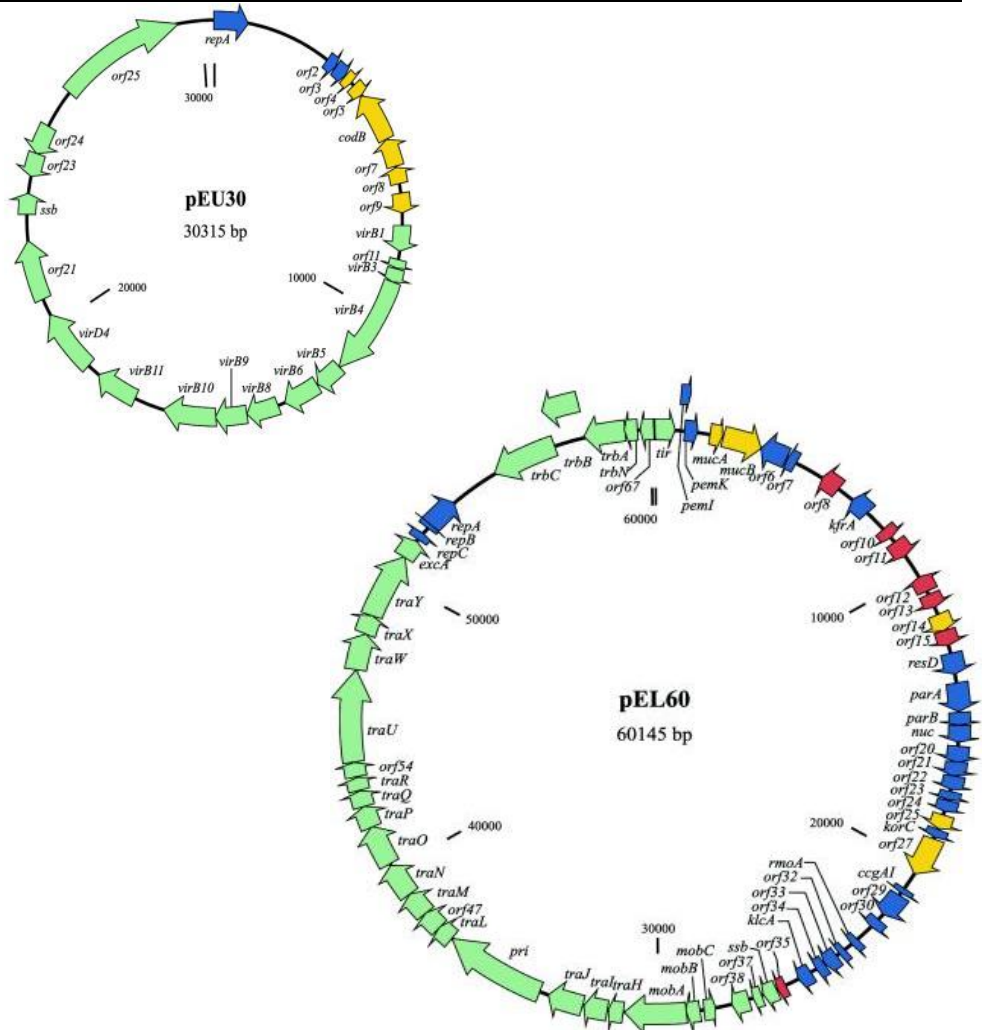


Fig. 2. Circular genetic maps of the 30,314-bp plasmid pEU30 (a) and 60,145-bp plasmid pEL60 (b) and their 25 and 68 identified ORFs, respectively. Genes are color coded on the basis of function as follows: replication and stability, blue; ecological fitness, yellow; transcriptional regulators, red; and conjugation or plasmid-specific functions, green. The number or identity of each ORF is located inside the circle. From Foster *et al.*, (2004).

- pEA29. This plasmid was first described by Merckaert *et al.* (1982) and since then the information about its presence and functions is abundant. For many years it was assumed that all pathogenic *E. amylovora* wild strains should carry plasmid pEA29, due to its high stability, role in virulence and non-transmissibility (Laurent *et al.*, 1989; Falkenstein *et al.*, 1989; McGhee and Jones 2000). Nevertheless, in Spain, wild strains lacking this plasmid were found in symptomatic hosts (Llop *et al.*, 2006) and these strains showed similar virulence levels to strains harbouring this plasmid. The complete sequence of plasmid pEA29 from *E. amylovora* strain Ea88 showed it has 28,185 bp with a 50.2% G+C content [the GenBank accession number from strain Ea88 is AF264948 (McGhee and Jones 2000), whereas the EMBL accession number for pEA29 from ATCC49946 is FN666576 (Sebahia *et al.*, 2010), and from strain CFBP1430 it is FN43411 (Smits *et al.*, 2010a)]. There are differences in the sizes of plasmids in strains from different origins due to a variable number of the short sequence repeats (SSRs) harboured. Thus, its size can actually vary from 27.6 to 34.9 kb (Schnabel and Jones 1998; Kim and Geider 1999; McGhee and Jones 2000). Thirteen ORFs that encoded predicted proteins with similarities to known proteins of other bacteria were identified, along with two ORFs related to hypothetical proteins found in GenBank and six ORFs with no similarities to the existing GenBank entries. Annotation revealed the following: predicted products of ORF with similarity to the thiamine biosynthetic genes *thiO*, *thiG*, and *thiF*; a *betT* gene encoding for choline transport; an *msrA* gene encoding for the enzyme methionine sulfoxide reductase; a putative methyl-accepting chemotaxis gene; an aldehyde dehydrogenase gene; a *hns* DNA-binding gene; a LysR-type transcriptional regulator; and *parA* and *parB* partitioning genes. The plasmid encodes a thiamine biosynthesis operon and several candidate

genes that could affect virulence and survival in plants (McGhee and Jones 2000). Thiamine prototrophy has also been associated with pEA29 (Chiou and Jones 1993), which could play a role in the physiology or metabolism of extracellular polysaccharide production, and these traits are associated with full virulence of the pathogen (McGhee and Sundin 2008). Strains cured of this plasmid exhibited reduced virulence, thiamine auxotrophy, and altered exopolysaccharide production on minimal medium without thiamine (Falkenstein *et al.*, 1989; Laurent *et al.*, 1989; McGhee and Jones 2000). *Pst*I and *Kpn*I restriction patterns for pEA29 isolated from fruit tree strains of *E. amylovora* were homogenous, but different from those for pEA29 isolated from *Rubus* (raspberry) strains (McGhee and Jones 2000), although the number of strains analysed was too small to generalise. Sequencing data have shown that pEA29 plasmids from genotypically diverse strains of *E. amylovora* share 99% sequence identity (100% coverage) (Ea88, McGhee and Jones 2000; Ea273, Sebahia *et al.*, 2010; CFBP1430, Smits *et al.*, 2010a).

- pEA8.7. This plasmid was first reported and analysed after a survey performed in California (USA) looking for strains showing SmR (Palmer *et al.*, 1997). It was initially thought that resistance was due to the plasmid pEA34, as was the case with strains from Michigan described above, but in fact it was observed that the same genes of resistance (*strA-strB*) present in pEA34 were also harboured in a new plasmid, which was approximately 9 kb in size and was named pEA8.7. Analyses by PCR, sequencing, hybridisation, and restriction data indicate that pEA8.7 from strain CA3R is closely related, if not identical, to the broad host-range plasmid RSF1010. RSF1010-like plasmids have been reported in a wide variety of clinical bacteria, and have also been detected in *P.*

agglomerans isolates from apple orchards in New Zealand (Palmer *et al.*, 1997; Vanneste, personal communication). The plasmid has not yet been fully sequenced.

- Other small plasmids. Two small plasmids were observed in *E. amylovora* strain IH3-1, isolated from *Crataegus* (McGhee *et al.*, 2002); one of which has been sequenced and named pEA1.7, because it has a size of 1,711 bp (GenBank accession number AY123046). It has an ORF with 74% nucleotide similarity to the *rom* gene of the pEP2.6 plasmid from *E. pyrifoliae*, but no origin of replication similar to that of pEP2.6 was detected. Three additional ORFs do not exhibit homology to known genes. No information is available for the other plasmid (McGhee *et al.*, 2002).
- Plasmids in *E. amylovora* strains from *Rubus*. Three small plasmids (pEA2.8, and two others of unknown size and function) have been found in *E. amylovora* strain IL-5 isolated from *Rubus* (McGhee *et al.*, 2002). Plasmid pEA2.8 is 2,825 bp in size (GenBank accession number AY123047). Its *oriV* sequence presented 84% nucleotide similarity to the origin of replication found in the pEP2.6 plasmid from *E. pyrifoliae*. It also contained an ORF coding for the ampicillin resistance protein β -lactamase. Two additional ORFs do not exhibit sequence homology to existing entries in GenBank. The other two plasmids from this strain hybridise with the *ori* region of plasmid pEP2.6, but no further information is available.

Recent sequencing of the genome of strain ATCC BAA2158, restricted to *Rubus* spp., has shown the presence of three circular plasmids: pEA29 (28,138 bp with 50% G+C), pEAR5.2 (5,251 bp with 52.2% G+C), and pEAR4.3 (4,369 bp with 51.5% G+C) (Powney *et al.*, 2011). Plasmid

pEAR5.2 (EMBL accession number FR719211) encodes for six ORFs and pEAR4.3 (EMBL accession number FR719210) encodes for four ORFs. Both seem to be unique to strain ATCC BAA-2158 at the moment, and share 88 and 89% sequence identity (57 and 53% coverage) with pEP5 of *E. pyrifoliae* DSM 12163T (Smits *et al.*, 2010a), respectively.

Latest screenings of plasmid content in *E. amylovora* isolates from different collections are bringing to light strains harbouring novel plasmids, such as that of ca. 60 kb found in isolates from Poland (now called pEA68: Ismail *et al.*, 2014). These screenings will increase our knowledge about the extrachromosomal material present in *E. amylovora* and the genetic information they provide to the host bacteria (*).

Plasmids in *E. pyrifoliae*

The plasmid content of this pathogen of Asian pear (Rhim *et al.*, 1999) has been studied quite extensively, despite its recent discovery and the availability of only a few strains (Smits *et al.*, 2010b). Since the first reports of this new pathogen, plasmids have been found in different isolates, and have been compared to the plasmids described in *E. amylovora* (Rhim *et al.*, 1999; McGhee *et al.*, 2002; Shrestha *et al.*, 2007). The plasmid profiles of 25 strains of *E. pyrifoliae* were investigated by Shrestha *et al.* (2007) and four groups were observed, suggesting intraspecific plasmid diversity. The *E. pyrifoliae* strains showed diverse plasmid profiles and restriction patterns typical of larger plasmids.

Variability in plasmid content was also observed in strains of *Erwinia* from Japan causing BSBP, some of which are now classified as *E. pyrifoliae* (Maxson-Stein *et al.*, 2003).

(*) After publication of this review a new plasmid called pEA78, was sequenced by Smits *et al.*, in 2013. It was found in the strain LA636 isolated from a Golden Delicious apple tree in Mexico.

To date, the following six plasmids and their possible functions have been reported from *E. pyrifoliae*.

- pEP36. Plasmid pEP36 (EMBL accession number FN392238) was the most commonly encountered among the 25 isolates analysed from this species (Shrestha *et al.*, 2007). A larger plasmid has been found in only two strains, but so far no information is available regarding its relatedness to pEP36. Only a part of Tn5394 (*tnpA* gene), which is specific to the pEP36 plasmid, was detected by hybridisation, indicating that this transposon was also present in the larger plasmid (Shrestha *et al.*, 2007). The number of nucleotides of the 36 kb plasmid from strain Ep1/96 varies from 35,904 to 35,909 bp (GenBank accession numbers AY123045 and FP236829, respectively), depending on the sequencing project (McGhee *et al.*, 2002; Kube *et al.*, 2010) with a G+C content of 49.6%, and 32 ORF. Strain DSM 12163T possesses a plasmid of 35,901 bp (GenBank accession number FN392238; Smits *et al.*, 2010b). Plasmid digestion with *Bam*HI enzyme generates several DNA fragments, which clearly differentiate it from the *E. amylovora* pEA29 plasmid (Rhim *et al.*, 1999). One of the nine *Bam*HI restriction sites detected in pEP36 corresponds to the unique *Bam*HI site in pEA29. However, pEP36 was found to be incompatible with clones containing the reciprocal origin regions of pEA29. The sequencing of pEP36 has shown that the same RepA protein was present in both, which explains the incompatibility observed. Sequence analysis also showed that pEP36 carried the transposon Tn5394 of approximately 6.4 kb in size. Genes similar to thiamine biosynthesis operon genes of pEA29 were found in this plasmid, but the possibility that pEP36 contributes to the virulence of *E. pyrifoliae* remains to be determined.

- pEJ30. This plasmid was reported from strain Ejp556 (MaxsonStein *et al.*, 2003; Geider *et al.*, 2009) and its complete nucleotide sequence was determined to be 29,593 bp with a G+C content of 49.7% (GenBank accession number AY255829). The sequencing revealed that this plasmid was nearly identical to plasmid pEP36 and was closely related to the non-transferable plasmid pEA29 from *E. amylovora*. The annotated sequence of pEJ30 was very similar to that of pEP36 (in terms of conservation of ORFs and gene order). Major differences of pEJ30 compared to pEP36 include the presence of an additional ORF in pEJ30 (ORF EJ8), the absence of transposon Tn5394, which accounts for the difference in size of pEP36 (6 kb), and a slightly different insertion site and reversed orientation of the insertion sequence IS285.

Recently, the entire genome of another *E. pyrifoliae* strain was sequenced (Ejp617) along with the plasmids it contains (Park *et al.*, 2011). Five plasmids were sequenced and the largest, pJE01 (30,866 bp; GenBank accession number CP002125), which contains 34 predicted ORFs was compared to pEJ30, and it was concluded that they were almost identical (Park *et al.*, 2011).

- Small plasmids. Several small plasmids have been found and sequenced by different authors (Rhim *et al.*, 1999; McGhee *et al.*, 2002; Park *et al.*, 2011) from strain Ep1/96 (pEP2.6, pEP05 and pEP03) and strain Ejp617 (pJE02, pJE03, pJE04, and pJE05). The sequence of plasmid pEP2.6 indicated that it was a 2,590 bp ColE1-like plasmid, but in the strain DSM 12163T this plasmid was slightly larger (2,610 bp). It also contained an ORF with 45% similarity to a rom gene found in *E. coli* plasmid pEC157 (GenBank accession number AF432497). Three additional hypothetical ORFs were located on pEP2.6, but none had similarity to the existing GenBank entries. The ColE1-related origin of replication

probe of the pEP2.6 plasmid hybridised with plasmid DNA in some *E. pyrifoliae* strains, but failed to do so with the DNA from others, indicating that the presence of these plasmids is limited to certain isolates.

Plasmids pEP05 and pEP03 are 4,955 and 3,070 bp in size, respectively. They were also classified as ColE1-type plasmids based on the presence of an *oriV* origin of replication, as were plasmids pJE02 (5,296 bp) pJE03 (6,417 bp), pEJ04 (3,237 bp) and pJE05 (2,691 bp). Plasmid pJE02 contains six ORFs with one mobilisation protein A (MobA), whereas no GenBank BLAST hits were found in the cases of pJE03, pJE04, and pJE05. Plasmid pEP03 shares a region with plasmid pEP2.6. The rest of the plasmid contains a large region that is similar (78% identity) to a portion of plasmid pEP05 that encodes three ORF, two of which are hypothetical proteins and one encodes Hcp, a putative type VI secretion system (T6SS) effector protein (Smits *et al.*, 2010b).

The annotated sequences of *E. pyrifoliae* strain Ep1/96 plasmids are deposited in Genbank/EMBL/DDBJ under accession numbers FP928999 (pEp2.6), FP236827 (pEP03), FP236828 (pEP05), FP236829 and AY123045 (both pEP36) and the sequences from strain DSM 12163 plasmids in EMBL are: FN392239 (pEP5), FN392237 (pEP3) and FN392236 (pEP2.6).

The sequences of the plasmids pJE01, pJE02, pJE03, pJE04, and pJE05 from strain Ejp617 have been deposited in GenBank under accession numbers CP002125, CP002126, CP002127, CP002128, and CP002129, respectively.

Plasmids in *E. piriflorinigrans*

The information about the plasmid content in this species, first reported in 2002 (Roselló *et al.*, 2002), is limited because only a plasmid screening of eight strains from this species has so far been performed. The plasmid pattern of these isolates generated only one band, larger than the 29 kb plasmid of *E. amylovora*, but similar in size to pEP36 of *E. pyrifoliae*. The plasmid was named pEPIR37 (Barbé *et al.*, 2013). One *E. piriflorinigrans* strain (CFBP5887) harboured two plasmids, one of ca. 36 kb and another of ca. 5 kb, named pEPIR5 (López *et al.*, 2011). Restriction analyses were performed using the *Bam*HI restriction enzyme and the plasmid profiles obtained showed the same pattern for all the *E. piriflorinigrans* strains, but were different to those of pEA29 and pEP36. Sequencing and genetic analyses of these plasmids are detailed in Barbé *et al.*, 2013.

Plasmids in *E. tasmaniensis*

Only strain Et1/99 of *E. tasmaniensis* has been sequenced and annotated. Besides the 4 Mb chromosomal sequence, five plasmids were characterised, which encode gene functions for autonomous plasmid transfer, bacteriocin-like proteins and synthesis of antibiotic precursors (Kube *et al.*, 2010). The plasmids were named pET09 (9,299 bp), pET35 (35,494 bp), pET45 (44,694 bp), pET46 (46,159 bp) and pET49 (48,751 bp) and they may play a role in fitness of the bacteria on plant surfaces. Plasmid pET45 harbours an *hns* gene in addition to the two chromosomal copies, while plasmid pET46 encodes klebicin-like proteins and a type IV pilus system. Several of these plasmids show the potential for conjugal transfer (pET35, pET45, pET46 and pET49). The GenBank accession numbers are: pET09 (CU468128), pET35 (CU468130), pET45 (CU468132), pET46 (CU468133), pET49 (CU468131). No data on screening of plasmid content in other strains of this species are available.

Plasmids in *E. billingiae*

Strain Eb661 of *E. billingiae* has been sequenced and annotated (Kube *et al.*, 2010). The authors found two plasmids in this strain: pEB170 (169,778 bp, a G+C content of 52.3% and 220 ORF) and pEB102 (102,323 bp, 51.7% G+C and 114 ORF). Plasmid pEB170 shows the potential for conjugal transfer, whereas no mobilisable or transfer genes are harboured by pEB102. The GenBank accession numbers are FP236826 (pEB102) and FP236830 (pEB170). No additional information on plasmid content in other isolates is available.

Comparative analyses of plasmids of similar size

As observed in this brief description of the plasmid content in different *Erwinia* species, all pathogenic and non-pathogenic strains harbour plasmids of different sizes, present in variable numbers. Among the pathogenic species, one striking characteristic is the presence of a plasmid with a similar size of around 30 kb (plasmids pEA29 in *E. amylovora*, pEJ30 and pEP36 in *E. pyriformis*, and pEPIR37 in *E. piriflorinigra*). All have been sequenced entirely (McGhee and Jones 2000; Maxson-Stein *et al.*, 2003; Smits *et al.*, 2010b; Barbé *et al.*, 2013) and comparative sequence analyses found that similar numbers of common ORFs were present in approximately the same order and orientation (Fig. 3). Among the 21 putative ORFs conserved between pEJ30 and pEP36, 12 of these were also found in pEA29 from *E. amylovora* Ea88. The gene content of these plasmids is highly conserved, sharing *thiO* (glycine oxidase), *thiS* (sulphur carrier protein), *thiG* (thiazole synthase) and *thiF* (adenylyl transferase) in conserved order. These genes are also present in the pEPIR37 plasmid from *E. piriflorinigra*, and were also found in the chromosome of *E. tasmaniensis*.

The following specific features shared by these plasmids of ca. 30 kb in *Erwinia* spp. have been reported (data from pEPIR37 plasmid will be shown in following chapters of this thesis):

- Short-sequence DNA repeats (SSR) conserved between pEJ30 and pEP36. The sequences of these SSR were different to those found in pEA29 (Jock *et al.*, 2003) but the repeat ATTACAGA present in all *E. amylovora* strains was found to be reiterated 3–15 times in pEA29. The *E. pyrifoliae* strains from Japan contained the major repeat sequence GGATTCTG, which was reiterated 16–24 times in the pEJ30 plasmid. The sequence ATTACAGG, which resembles the SSR of *E. amylovora*, was reiterated two or three times in *E. pyrifoliae* strains from Japan, differing only in the terminal nucleotide (G instead of A) from the *E. amylovora* repeat sequence.
- A common *Bam*HI restriction site in plasmids pEJ30, pEP36 and pEA29 located at the beginning of the *repA* gene was highly similar in all three plasmids.
- The order and organisation of 21 presumptive genes in pEP36 and pEJ30 was also very similar.
- Plasmids pEJ30 and pEP36 showed several IS elements and transposons not found in pEA29. The transposon Tn5394 was present in pEP36, but not in pEJ30.

Plasmids in *Erwinia* species

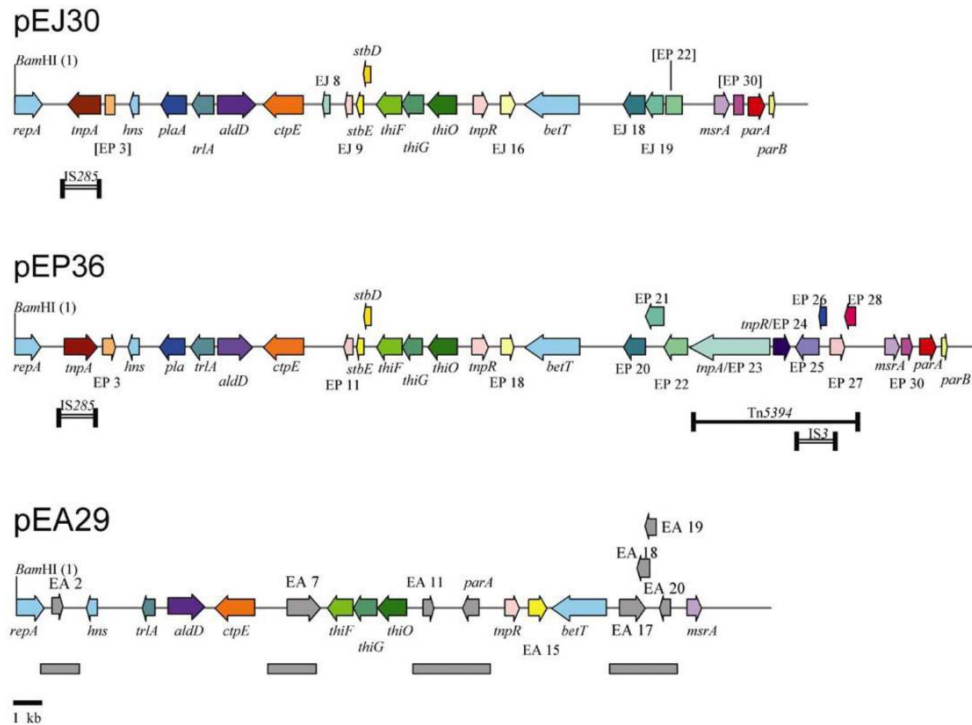


Fig. 3. Linear genetic maps for plasmid pEA29 from *Erwinia amylovora* Ea88 (accession no. AF264948), pEP36 from *E. pyrifoliae* Ep1/96 (AY123045), and pEJ30 from *E. pyrifoliae* strain Ejp556 (AY255829). A common *Bam*HI site was used as the origin for each map. Putative genes were inferred from sequence data in databases and arrows with similar color indicate similar genes and their order. Brackets indicate pseudogenes. The bottom row of boxes indicate regions of pEA29 that were not detected in the other plasmids. Reprinted by permission from Maxson-Stein *et al.*, (2003). Genetic analysis of a pathogenic *Erwinia* sp. isolated from pear in Japan. *Phytopathology*. Vol. 93, 1393–1399

The similarity between these ca. 30 kb plasmids from pathogenic pome fruit *Erwinia* raises the question as to whether these plasmids play the same function as pEA29 in *E. amylovora*. Thiamine genes are related to extracellular polysaccharide production and are involved in exacerbating the symptoms that pEA29 causes in *E. amylovora* (McGhee and Sundin 2008) and are also present in plasmids pEP36, pEJ30 and pEPIR37. Consequently, we could ask: do other plasmids elicit the same increase in symptoms development? To address this question, plasmids pEP36, pEJ30 and pEPIR37 should be introduced into *E. amylovora* pEA29 cured strains and inoculation assays performed to determine whether the *E. amylovora* strains with these plasmids show similar symptom development and incidence of disease as in the reference strains, and whether these are more extensive than the strains lacking pEA29. The results would demonstrate if these plasmids of ca. 30 kb from different species of *Erwinia* can have the same function in pathogenicity as pEA29, as suggested by their high genetic similarities.

Apart from the high similarity of plasmid genetic content and the possible similar functioning that these 30 kb plasmids could provide, the relationship observed among other medium and small plasmids in *Erwinia* species gives the impression of a common background shared among pear-associated *Erwinia* spp., both pathogenic and nonpathogenic. The high genetic identity between plasmids pEI70, from the pathogen *E. amylovora* and pEB102, from the epiphytic species *E. billingiae* indicates that lateral transfer of near entire extrachromosomal material could take place between species sharing host and niche (Llop *et al.*, 2011). This genetic similarity among plasmids of other species may also be observed, supporting the mosaic nature and mobility of plasmid DNA. Analyses of *E. amylovora* plasmid pEA8.7 have shown that it was closely related or identical to a broad host-range plasmid RSF1010 found in many species of bacteria, but this was the first report of its presence in plant

pathogenic bacteria (Palmer *et al.*, 1997). Plasmid pEP2.6 from *E. pyrifoliae* strain Ep1/ 96 contained sequences found in small plasmids of *E. amylovora* strains IL-5 and IH3-1 (McGhee *et al.*, 2002).

Plasmid pEA34 may have arisen from the insertion of a transposable element into an existing plasmid or by some *E. amylovora* strains acquiring a new plasmid (Chiou and Jones 1991). The possible origin and distribution of pEA34 has been studied (Chiou and Jones 1993) and a 34 kb plasmid resembling it was not reported among strains of *E. amylovora* examined for cryptic plasmids (Falkenstein *et al.*, 1988; Steinberger *et al.*, 1990). Finally, it was concluded that plasmid pEA34 could have originated from the insertion of Tn5393 into a 28 kb plasmid (not described here), because such plasmid with DNA homologous to pEA34 was detected in a streptomycin-sensitive strain of *E. amylovora* (EL01). Hybridisation analyses indicated that the 28 kb plasmid and plasmid pEA34 were not related to pEA29 (Chiou and Jones 1993).

The SmR provided by pEA34 to some *E. amylovora* isolates is an example of how a particular trait can originate from and be extended by horizontal gene transfer to different plasmids. Thus, the first SmR strains reported contained both pEA34 and the almost ubiquitous nonconjugative plasmid, pEA29 (Chiou and Jones 1991). More recently, SmR strains in which Tn5393 is present in pEA29 were observed, suggesting that the transposon could have moved via transposition from pEA34 to pEA29. However, almost all of the strains containing Tn5393 in pEA29 had lost pEA34.

This research on the possible origin of genes for SmR, an important characteristic for the survival of the bacterium, and their exchange by several transfer systems is a perfect example of the ways in which genes disseminate through horizontal transfer. The donor strain could be a non-pathogenic species that has the same ecologic niche as the pathogenic one, as may be the case

between plasmids pEB102 and pEI70, as explained above, or there can be a transfer between non-pathogenic and pathogenic isolates of the same species. As an example, the Tn5393 transposon, involved in conferring SmR on *E. amylovora*, has been shown to be widely distributed among other SmR plant pathogens and is also carried by other bacteria from the phyllosphere (Chiou and Jones 1993; Sundin and Bender 1995). In addition, a number of enterobacterial species harbouring this transposon were isolated from apple flowers and leaves, with *P. agglomerans* being the most prevalent species. It has been hypothesised that *P. agglomerans* could be the source of the SmR plasmid pEA34, which was later acquired by *E. amylovora* (Chiou and Jones 1993).

Several of the plasmids reported above show the potential for conjugal transfer, such as pEI70 from *E. amylovora*, pEB170 of *E. billingiae*, pET35, pET45, pET46, pET49 of *E. tasmaniensis* and pEL60 of *E. amylovora* from Lebanon, and others carry *mob* genes and may contain an *oriT* to be mobilised by Tra proteins of other plasmids (plasmids pEP05 and pEt46 from *E. pyrifoliae* and *E. tasmaniensis*) (Kube *et al.*, 2010). This indicates the wide variability of genetic interchange available in this genus, but it could also include genetic material from other genera sharing the same niches.

On the other hand, the importance of stable plasmids as epidemiological markers has only been explored in the case of pEI70 (Llop *et al.*, 2011), but knowledge of stable and non-conjugative plasmids present in strains of one area could provide information about possible sources of inoculum.

Plasmid and evolution: hypotheses

Horizontal transfer is an important mechanism for genetic exchange. In this sense, the existence of the SSR array in the genome of *E. amylovora* and *E. pyrifoliae* could intervene in DNA replication (Bramhill and Kornberg 1988), DNA

repair (Strand *et al.*, 1993), and recombination, favouring genomic evolution, although this remains to be proved. The presence of near identical plasmids (pEI70 and pEB102) between pathogenic and epiphytic erwinias, or the close relationship of pEL60 with other enterobacterial IncL/M plasmids suggests that the plant pathogen *E. amylovora* can access the gene pools of other enteric bacteria through horizontal transfer. IncL/M plasmids are widespread among enteric pathogens, they typically encode resistance to multiple antibiotics, and they harbour mobile elements including integrons and IS elements associated with antibiotic-resistance genes (Proding *et al.*, 1996; Villa *et al.*, 2000).

Other plasmids, such as pEU30 and pEL60, may represent DNA that can spread efficiently with limited effects on the reproductive fitness of their bacterial host. Coexistence of pEU30 or pEL60 with their *E. amylovora* hosts could be a system for acquisition of novel genetic determinants that would improve the ecological fitness of bacteria (Foster *et al.*, 2004).

The presence of IS elements in some derivatives of pEA29 and of transposon Tn5394 in pEP36, also suggests that horizontal transfer has played a role in the evolution of these plasmids. The presence of such similar plasmids in different species from distant geographic origins having common hosts, which cause relatively similar symptoms, raises the following question: how did pEP36, pEJ30, pEPIR37 and pEA29 become established in their respective hosts? Although there is evidence of horizontal transfer of plasmids with genes for SmR in *E. amylovora* (Chiou and Jones 1995), horizontal transfer of pEA29 has not been proven, and no genes involved in conjugative transfer were found in either plasmid. A helper plasmid may have taken part in transfer of non-transferable plasmids, but until spontaneous horizontal transfer of pEPIR37, pEP36, pEJ30 and pEA29 can be demonstrated, the origin of pEA29-related plasmids in their hosts through horizontal transfer remains a conjecture (McGhee *et al.*, 2002). As non-transferable plasmids, inherited vertically within a bacterial cell line, the

evolution of this family of pEA29 related plasmids is suggestive of the evolution of these pathogens (Maxson-Stein *et al.*, 2003).

Do pEA29 and other similar plasmids confer a fitness advantage on their host bacteria? The answer to this question is not yet known. Although plasmids may carry genes beneficial to their bacterial hosts, they may also reduce fitness. The introduction of pEA29 to several *E. amylovora* strains naturally lacking it appears to enhance the fitness of some, but not all, strains. In general, strains without pEA29 are substantially less aggressive than strains containing it (Mohammadi *et al.*, 2009). However, some strains lacking pEA29 and having instead other plasmids, such as pEI70, or even strains without any plasmids can show similar virulence levels to isolates harbouring pEA29 (Llop *et al.*, 2011). Since it has been reported in *A. tumefaciens* that strains with several plasmids were more virulent than strains with a single plasmid (Ogawa and Mii 2001), it is tempting to wonder whether plasmids found in different strains of pathogenic *Erwinia* might also have an additive role in virulence.

It would also be interesting to determine if ancestral strains of *E. amylovora* harbour the pEA29 plasmid. It is intriguing to speculate that *E. amylovora* evolved by acquiring plasmids from other bacteria through horizontal gene transfer with subsequent selection-based deletion of the gene(s) for conjugal transfer. It is possible that the ancestral lines were plasmid-free and less aggressive to their host plants, and became more pathogenic by acquiring plasmid DNA from other microbial sources. Thus, plasmid free strains may represent ancestral strains rather than a more recent lineage as suggested by Ochman *et al.* (2000).

The examination of other pEA29-related plasmids in strains of *E. pyrifoliae*, *E. piriflorinigrans* and in other *Erwinia* species, such as those recently described from Japan (Mizuno *et al.*, 2010), and in the non-pathogenic species, will

provide further insight into the evolution of these plasmids and their bacterial hosts.

Conclusions

The genus *Erwinia* includes plant-associated pathogenic and non-pathogenic species of *Enterobacteriaceae*, some of which have been reported in pome fruit trees. Important pathogens such as *E. amylovora*, the causative agent of fire blight of *Rosaceae*, *E. pyrifoliae* that causes bacterial shoot blight of Asian pear, and *E. piriflorinigrans* that causes necrosis on pear blossoms, belong to this genus. Other species such as *E. tasmaniensis* and *E. billingiae* are epiphytic bacteria and may be used as antagonists for fire blight biocontrol (Kube *et al.*, 2010). All these species harbour plasmids of similar sizes in variable numbers, but although the role of the genetic information they contain is known for some of them, for many others it remains to be revealed. Plasmids in *Erwinia* species from pome fruits are very common, and further screenings of worldwide isolates will probably augment those so far described. Such screenings and the sequencing of more plasmids may reveal other possible features that they provide to the host bacteria, facilitating a better understanding of the epidemiology and life cycle of these pathogens, and possibly the genetic relationship between pathogenic and non-pathogenic species. Although there are still many cryptic plasmids, the data available suggest that the common background in the genes harboured by different plasmids and their genetic exchanges could explain the evolution and the presence of several new characteristics in some *Erwinia* strains.

Plasmids inherited vertically, and maintained as residents in the long-term should co-evolve with their bacterial hosts (Sundin, 2007). Although the

different species of pome fruit pathogenic erwinias seem to have distant geographic origins, *E. amylovora* being widespread but other species showing a restricted habitat until now, they elicit more or less similar symptoms in these pome fruit hosts and have a pool of plasmids that share high genetic similarity. The origin of these plant pathogenic bacteria, and the way they evolve from a possible epiphytic life to an increasingly aggressive pathogen is a very interesting subject. The studies on pome fruit pathogenic and non-pathogenic *Erwinia* spp., including their plasmids, could provide an insight into how an important pathogen such as *E. amylovora* evolves through its interactions with other bacteria. Whole genome sequence analyses of more strains, together with the use of advanced molecular tools, will elucidate the means of exchange of genetic material among different but related species.

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Capítulo 4

**Un nuevo plásmido de *Erwinia amylovora*,
pEI70: secuencia completa, biogeografía y papel
en la agresividad del agente causal del fuego
bacteriano**

***Erwinia amylovora* novel plasmid pEI70: complete sequence, biogeography, and role in aggressiveness in the fire blight phytopathogen**

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* S. Barbé has contributed in performing partially sequence analysis and cloning of pEI70, analyses of plasmid content by PCR and extraction analyses to detect the presence of plasmid in strains from geographically different localizations, analysis of aggressiveness using *ex vivo* plant assays and finally wrote part of the manuscript.

Abstract

Comparative genomics of several strains of *Erwinia amylovora*, a plant pathogenic bacterium causal agent of fire blight disease, revealed that its diversity is primarily attributable to the flexible genome comprised of plasmids. We recently identified and sequenced in full a novel 65.8 Kb plasmid, called pEI70. Annotation revealed a lack of known virulence-related genes, but found evidence for a unique integrative conjugative element related to that of other plant and human pathogens. Comparative analyses using BLASTN showed that pEI70 is almost entirely included in plasmid pEB102 from *E. billingiae*, an epiphytic *Erwinia* of pome fruits, with sequence identities superior to 98%. A duplex PCR assay was developed to survey the prevalence of plasmid pEI70 and also that of pEA29, which had previously been described in several *E. amylovora* strains. Plasmid pEI70 was found widely dispersed across Europe with frequencies of 5-92%, but it was absent in *E. amylovora* analyzed populations from outside of Europe. Restriction analysis and hybridization demonstrated that this plasmid was identical in at least 13 strains. Curing *E. amylovora* strains of pEI70 reduced their aggressiveness on pear, and introducing pEI70 into low-aggressiveness strains lacking this plasmid increased symptoms development in this host. Discovery of this novel plasmid offers new insights into the biogeography, evolution and virulence determinants in *E. amylovora*.

Introduction

Erwinia amylovora is the causal agent of fire blight, the most serious disease that affects pome fruit trees worldwide (Vanneste, 2000). This bacterium has been studied for several decades, and it has been found that *E. amylovora* can infect a wide range of hosts within the family *Rosaceae* (apple, pear, quince,

loquat, and many ornamentals) (van der Zwet and Keil, 1979) compared to other *Erwinia* species pathogenic to pome fruit trees (e.g., *E. pyrifoliae*, *E. piriflorinigrans*) (Kim *et al.*, 1999; López *et al.*, 2011). Although phenotypic traits can be different in *E. amylovora* isolates (e.g., metabolic activity, virulence) (Norelli *et al.*, 1984; Aldridge *et al.*, 1997; Sholberg *et al.*, 2001; Cabrefiga and Montesinos, 2005), several genetic analyses have demonstrated that it is a very homogeneous species (Kim *et al.*, 1995; Zhang and Geider, 1997; Oh and Beer, 2005; Triplett *et al.*, 2006). The genes involved in pathogenicity, virulence and related behavior are highly conserved and rarely show significant differences in sequence or structure. Whole-genome sequence analysis has demonstrated that the two available genome sequences have over 99.99 % nucleotide sequence identity (Smits *et al.*, 2010a), which is in accordance with the high homogeneity that this species presents. This is supported by the preliminary analysis of four further genome sequences from European isolates that showed that, apart from plasmid content, the pan-genome of *E. amylovora* is closed (Smits *et al.*, 2010b).

The most obvious difference between the *E. amylovora* genomes of the sequenced strains CFBP 1430 and Ea 273 is the presence of plasmid pEA72 in the latter strain (Smits *et al.*, 2010a; Sebahia *et al.*, 2010). Plasmids also appear to be the main elements contributing to diversity within the pan-genome of *E. amylovora* (Smits *et al.*, 2011). However, although a total of 14 plasmids have been detected in isolates of this species (Chiaou and Jones, 1991; Laurent *et al.*, 1989; Steinberger *et al.*, 1990; Foster *et al.*, 2004) (Table 1), in practice, knowledge of the existence of this extra-chromosomal material is limited to some strains and few plasmids. A reason for the lack of information on the presence of plasmids could be that no intensive screening analyses have been performed worldwide to determine the plasmid content in *E. amylovora* strain collections from a large number of countries. Apart from the near-ubiquitous

plasmid pEA29, whose role in *E. amylovora* is still unclear but includes biosynthesis of thiamine (Sundin *et al.*, 2004), plasmids of 8.7 Kb and 34 Kb size have been assigned to confer resistance to streptomycin, and a plasmid of 2.8 Kb contains a gene with high sequence identity to a β -lactamase that could confer resistance to ampicillin (Chiaou and Jones, 1991; Palmer *et al.*, 1997; McGhee *et al.*, 2002b). All other plasmids reported in Table 1 are cryptic. Despite studies having elucidated the effect of plasmid pEA29 (Laurent *et al.*, 1989; Steinberger *et al.*, 1990; Sundin *et al.*, 2004), the presence/absence of this plasmid alone does not explain the differences in aggressiveness observed among *E. amylovora* isolates. Recently, a strain obtained from a fire blight outbreak in Spain was shown to lack pEA29 but it harbors a different plasmid of approximately 70 Kb (Llop *et al.*, 2006). This plasmid, designated pEI70, was sequenced and annotated in the present study. We analyzed its presence in a wide collection of isolates from different geographic origins, hosts and years. It was shown that pEI70 was present at different percentages in isolates from European countries, but not in isolates from elsewhere. Furthermore, its influence on aggressiveness was examined.

Materials and Methods

Bacterial strains, plasmids and growth media

The *E. amylovora* isolates analyzed for plasmid content, the vectors employed for cloning pEI70 and the bacteria used for virulence analyses and genetic comparisons are described in Table 2. Bacteria were cultured on LB agar, supplemented with antibiotics as appropriate for mutants, and incubated at 26°C (*E. amylovora*) or 37°C (*Escherichia coli*). Throughout, the following antibiotic concentrations were used for: *E. coli*, kanamycin 50 μ g/ml; and for *E.*

amylovora, streptomycin 25 µg/ml and kanamycin 50 µg/ml. When required, nicotinic acid (1 mg/ml) and thiamine (0.2%) were added to M9 minimal broth medium (mmT) (Maniatis *et al.*, 1982)

Characteristics of plasmid pEI70

Stability analyses were performed following the protocol described in Foster *et al.*, (2004), consisting of a 200-generation serial transfer experiment in mmT medium. Briefly, 10 ml of a bacterial suspension were grown up for 24 h at 26°C, and 10 µl were transferred to a new 10 ml tube of the same medium and incubated again. These transfers were performed over 20 consecutive days. We employed one strain harboring only pEI70 (IVIA 1614-2a), and another strain that carries pEA29 in addition to pEI70 (IVIA 1614-1), to observe the possible influence of pEA29 on the stability of pEI70. One hundred colonies were taken after 20 days and checked for the presence of pEI70. Additionally, 25 colonies were analyzed for plasmid content by plasmid extraction and by restriction analysis to confirm that the plasmid had not integrated into the chromosome.

Plasmid pEI70 was marked with a transposon with resistance to kanamycin using the EZ-Tn5 Insertion kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions, yielding plasmid pEI70::Tn5. Conjugative analysis was performed using two derivatives of plasmid free *E amylovora* strain CGJ2. One carried the plasmid pEA29::Tn5393 from strain MI11-1, a wild-type strain harboring pEA29 with a natural transposon which confers resistance to streptomycin, isolated from apple (McGhee *et al.*, 2002b). The other CGJ2 derivative contained plasmid pEI70::Tn5. Suspensions were adjusted to 10⁷ cfu/ml of each strain and 250 µl of each suspension were placed together on a 0.22 µm filter placed on a LB plate containing both antibiotics. On similar filters, one suspension of each strain was plated separately as controls. After 48 h

incubation at 26°C, colonies appeared only in the mixed suspensions, and were analyzed for plasmid content by PCR using the duplex system.

Sequence analysis of pEI70

Plasmid pEI70 from Spanish strain IVIA 1614-2a was sequenced using shotgun cloning and Sanger sequencing. The coverage was 13.0x with 929 reads (average read length 922 bp) in a single contig. Genes on pEI70 were predicted using a combined strategy (McHardy *et al.*, 2004) based on the CDS prediction programs Glimmer (Salzberg *et al.*, 1998) and Critica (Badger *et al.*, 1999). Subsequently, the potential function of each predicted gene was automatically assigned using the GenDB annotation pipeline (Meyer *et al.*, 2003). The resulting plasmid annotation was manually curated. Routine sequence manipulations were completed using several subroutines of the LASERGENE package (DNASTAR, Madison, WI, USA). In the framework of a larger genome sequencing project (Powney *et al.*, unpublished), the genome of a recent Swiss isolate *E. amylovora* strain ACW 56400 (Rezzonico *et al.*, 2011), was sequenced using Illumina sequencing. This genome contained an identical copy of pEI70 next to plasmid pEA29. The sequence of pEI70 from *E. amylovora* strain ACW 56400, identical in nucleotide sequence to strain IVIA 1614-2a, is available at NCBI under accession number CP002951.

Table 1. Other plasmids different from pEI70 that have been found and studied in different *E. amylovora* strains. Three plasmids (one from strain IH3-1 and two other from *Rubus* strain IL5) have been reported, but names and sequence information are unavailable and they are not included.

Name	Strain(s)	Host	Origin	Features ^b	Accession Number	References
pEA72	Ea273	Apple	NY, USA	Cryptic	FN666577	(Sebahia <i>et al.</i> , 2010)
pEL60	LebB66	Apple, pear	Lebanon	Cryptic	NC_005246	(Foster <i>et al.</i> , 2004)
pEA34	CA11	Apple/pear	Michigan, USA	Str ^R	-	(Chiou and Jones, 1991)
pEU30	UTRJ2	Apple/pear	Utha, USA	Cryptic	NC_005247	(Foster <i>et al.</i> , 2004)
pEA29 ^a	Ea88	Pear	Washington, USA	Thiamine	AF264948	(McGhee and Jones, 2000b)
pEA29 ^a	Ea273	Apple	NY, USA	Thiamine	FN666576	(Sebahia <i>et al.</i> , 2010)
pEA29 ^a	CFBP 1430	<i>Crataegus</i> sp.	France	Thiamine	FN43411	(Smits <i>et al.</i> , 2010a)
pEA29 ^a	ATCC BAA-2158	<i>Rubus</i> sp.	Illinois, USA	Thiamine	FR719212	(Powney <i>et al.</i> , 2011)
pEA8.7	CA3R	Apple	California, USA	Str ^R	-	(Palmer <i>et al.</i> , 1997)
pEAR5.2	ATCC BAA-2158	<i>Rubus</i> sp.	Illinois, USA	Cryptic	FR719211	(Powney <i>et al.</i> , 2011)
pEAR4.3	ATCC BAA-2158	<i>Rubus</i> sp.	Illinois, USA	Cryptic	FR719210	(Powney <i>et al.</i> , 2011)
pEA2.8	IL-5	<i>Rubus</i> sp.	Illinois, USA	Amp ^R	AY123047	(McGhee <i>et al.</i> , 2002b)
pEA1.7	IH3-1	<i>Crataegus</i> sp.	Louisiana, USA	Cryptic	AY123046	(McGhee <i>et al.</i> , 2002b)

^a plasmid pEA29 is present in almost all *E. amylovora* strains. Here, only the four plasmids that have been sequenced and their hosts are shown.

^b Str^R: resistance to streptomycin; Thiamine: thiamine biosynthesis; Amp^R: possible resistance to ampicillin. Not functionally demonstrated; Cryptic: plasmids that have no known function, with no apparent effect on the phenotype of its host cell.

Table 2. *Erwinia amylovora* strains analyzed for plasmid content and for genetic comparison, and plasmids employed in the different experiments.

Bacterial strains	Host	Plasmid content	Origin/year of isolation	References
CFBP 1430	<i>Crataegus</i> sp.	pEA29	France/1972	(Paulin and Samson, 1973)
PMV 6014	-	-	France	(Laurent <i>et al.</i> , 1989)
Ea 273	Apple	pEA29, pEA72	USA/1971	(Beer <i>et al.</i> , 1991)
IVIA 1614-1	<i>Pyracantha</i> sp.	pEA29, pEI70	Spain/1996	This work
IVIA 1614-2a	<i>Crataegus</i> sp.	pEI70	Spain/1996	(Llop <i>et al.</i> , 2006)
IVIA 1596	Pear	pEI70	Spain/1996	This work
BC3	Apple	-	Serbia /2003	(Llop <i>et al.</i> , 2008a)
CGJ2	Apple	-	Serbia/2003	(Llop <i>et al.</i> , 2008a)
E 70	<i>Cotoneaster</i> sp.	pEA29, pEI70, 30Kb plasmid	Ireland/1997	(Brennan <i>et al.</i> , 2002)
NCPPB 3299	<i>Pernettya</i> sp.	pEI70	UK/1983	(Mohd-Zain <i>et al.</i> , 2004)
IVIA 1614-2a-pEI70	-	-	Spain/2005	This work
IVIA 1596-pEI70	-	-	Spain/2005	This work
ACW 56400	Pear	PEA29, pEI70	Switzerland/2007	(Rezzonico <i>et al.</i> , 2011)
Plasmids	Characteristics		Origin	References
pBBRMCS2	Kan ^R , cloning vector	-	USA	(Kovach <i>et al.</i> , 1995)
pEA29::Tn5393	Str ^R	-	USA	(McGhee <i>et al.</i> , 2001)
pEI70::Tn5	Kan ^R , labelled	-	Spain	This work

Analyses of plasmid content by PCR and extraction analyses

E. amylovora strains were analyzed for the presence of pEA29 by PCR using primers AJ75 and AJ76 of McManus and Jones (1995). To screen plasmid pEI70, a conventional PCR protocol was designed, using primers 1.7F (5'-CCCCGTGAACAACAGACCACC-3') and 1.7R (5'-AATCTGACAGCCGCAACCCG-3') derived from the sequence of a 1.7 Kb *Bam*HI fragment of plasmid pEI70. Briefly, PCR reaction mix was as follows: 1X buffer (Tris-HCl 75 mM; KCl 50 mM; (NH₄)₂SO₄ 20mM); 0.1 mM dNTPs; 1.5 mM MgCl₂; 5 μM primer 1.7F; 5 μM primer 1.7R; 1 U DNA polymerase (Biotools, Madrid, Spain). The PCR amplification conditions were 3 min at 94°C initial denaturation followed by 40 cycles of 45 s at 94°C, 40 s at 60°C and 1 min at 72°C, with a final extension at 72°C for 10 min.

Additionally, a duplex PCR using both pairs of primers was developed to allow detection of pEA29 and pEI70 in a single assay. The reaction mix for the duplex PCR was: 1X buffer; 0.1 mM dNTPs; 1.5 mM MgCl₂; 5 μM primers AJ75-AJ76; 2.5 μM primers 1.7F-1.7R; 1 U DNA polymerase. The PCR conditions were identical to those of the single PCR. PCR products were 800 bp for pEA29 and 539 bp for pEI70 amplicons, analyzed on 1.2 % agarose gels. Isolates providing deviant results by PCR (strains negative for pEA29 plasmid, positive for pEI70, or negative for both plasmids) were subjected to plasmid extraction using the protocol of Zhou *et al.* (1990) to verify the lack and/or presence of the corresponding plasmids. With 13 isolates positive for pEI70 from different origins chosen at random, restriction analysis was performed using the enzyme *Bam*HI which generates only one band for pEA29 and 14 bands for pEI70, to confirm that the plasmid was identical in all isolates. Plasmid restrictions were analyzed on 0.8 % agarose gels.

Presence of plasmid pEI70 in *E. amylovora* strains from geographically different locations

Using the duplex PCR system, 1,480 *E. amylovora* strains from different collections from 21 countries in Europe, North America and Asia were analyzed to examine the distribution of strains that harbor pEI70 and whether strains without pEA29 are widespread in nature. Several isolates were obtained from international and laboratory collections and others were analyzed in laboratories in Belgium, Poland, Slovenia, Spain and Switzerland (Table 3).

Transformation of *E. amylovora* strains

Plasmids pEI70::Tn5 and pEA29::Tn5393 were introduced into *E. amylovora* strains by electroporation. Plasmids were introduced into the low aggressive strains BC3 and CGJ2 (Table 2), and also into strain PMV 6014 (strain CFBP 1430 cured of plasmid pEA29) that was employed as a control in the inoculation experiments (Table 4). Strains CGJ2+pEI70::Tn5 and CGJ2+pEA29::Tn5393 were also employed for the conjugation assays.

Construction of plasmid-less strains

Plasmid pEI70 was eliminated from strains IVIA 1614-2a and IVIA 1596 using a subclone containing a 1 Kb *AsuI-EcoRI* fragment that includes the *repA* gene in vector pBBR-MCS2. After introduction of the vector into the strains, the resulting kanamycin-resistant transformants were subcultured several times on LB with kanamycin. Individual colonies were screened for the loss of pEI70 by plasmid extraction and visualization on a 0.8 % agarose gel. The cured strains were analyzed for aggressiveness compared to the same strains harboring the plasmid.

Erwinia amylovora plasmid pEI70

Table 3. Screening of presence of plasmids pEA29 and pEI70 in strains of different international and collections from Institutes and laboratories using the duplex PCR system. Additional information is provided in Tables S2 and S3.

European countries	Strains analyzed	Strains with pEI70	% of strains with pEI70	Strains without pEA29	Strains without any plasmid
Austria	25	0	-	0	0
Belgium	70	65	92.8	2	1
Bulgaria	4	0	-	0	0
Czech Republic	2	1	50.0	0	0
France	43	3	6.9	0	0
Germany	12	0	-	0	0
Greece	15	0	-	0	0
Hungary	10	0	-	0	0
Ireland	14	12	85.7	0	0
Italy	5	3	60.0	0	0
The Netherlands	4	2	50.0	0	0
Poland	120	7	5.8	0	0
Serbia	2	0	-	2	2
Slovenia	526	331	62.9	0	0
Spain	142	20	14.0	4	3
Switzerland	247	13	5.2	0	0
UK	8	2	25.0	1	0
Total	1249	458	34.6	9 (0.7%)	6 (0.48%)
Non European countries	Strains analyzed	Strains with pEI70	% of strains with pEI70	Strains without pEA29	Strains without plasmids
Canada	36	0	-	0	0
Lebanon	6	0	-	0	0
USA	117	0	-	0	0
Turkey	72	0	-	0	0
Total	231	0	-	0	0

Table 4. Strains analyzed for aggressiveness on basis of severity of infections (0-100%) produced in immature pear fruits before and after introducing tagged plasmids pEA29::Tn5393 and/or pEI70::Tn5.

Strains analyzed	Aggressiveness	Plasmid content	Plasmids introduced	Aggressiveness after plasmid introduction
CFBP 1430 ^a	92.1	pEA29	-	-
PMV 6014	47.5	-	pEI70	85.3
IVIA 1614-2a	70.6	pEI70	pEA29	79.8
IVIA 1596	83.1	pEI70	pEA29	82.8
BC3	22.2	-	pEA29	54.7
BC3	13.2	-	pEI70	75.0
CGJ2	20.0	-	pEA29	57.2
CGJ2	15.6	-	pEI70	68.6

^a positive control.

Analysis of aggressiveness using *ex vivo* plant assays

Several analyses were performed to evaluate the aggressiveness of the different types of strains under study and to observe how the introduction of plasmids pEI70 and pEA29 affected this feature. The consequence of curing pEI70 from some strains harboring only this plasmid was also assessed. The material used in the different assays consisted of: i) strains that do not harbor any plasmid as confirmed by PCR and plasmid extraction, ii) the above strains with plasmid pEA29 and/or pEI70 introduced by electroporation, and iii) wild strains with only pEI70 (Table 4).

The first assay was performed to test the effect of plasmid pEA29 (pEA29::Tn5393), (McGhee *et al.*, 2001) when introduced into strains that do not harbor this plasmid and also in strains harboring only plasmid pEI70, and, further, to test the effect of curing pEI70 from strains harboring it alone (Table 4). The assay was performed using ‘Passe Crassane’ immature pear fruits. Briefly, pear fruits of 2-3 cm diameter, collected at 6-weeks after fruit set were washed in running water and soaked in a 1% sodium hypochlorite solution for

20 min. Subsequently, they were washed with sterile water and left to remove excessive water on a sterile bench. Bacterial suspensions were adjusted to 0.1 OD₆₀₀ (10⁸ cfu/ml) and diluted to the corresponding concentration. Two concentrations were tested, 10⁵ cfu/ml and 10⁶ cfu/ml. Four wounds were performed with a pipette tip on each pear, and 10 µl of the diluted suspension placed on each wound. Then, fruits were placed in polystyrene trays, covered with plastic bags to maintain conditions of high humidity, and incubated at 25 °C. The experimental design consisted of three repetitions of three pears (nine fruits) per strain and dose.

The incidence and severity of infected wounds (%) for each repetition (3 pears x 4 wounds each) was assessed after 5 days of incubation. Wounds were considered as infected when either drops of bacterial exudates or necrosis appeared in and around the inoculation site. Severity of symptoms was evaluated by means of a visual scale (from 0 to 3). The scale was based on necrosis progression as follows: 0 = no symptoms, 1 = exudates located at the inoculation point, 2 = necrosis affecting area around the wound, 3 = necrosis expanding through the fruit. Disease severity (S) was calculated according to the following formula:

$$S = \sum_{i=1}^i \left(\frac{SI_i}{n \cdot 3} \right) \cdot 100$$

where *S* is the disease severity, *SI* is the corresponding severity index in an inoculated wound, *i* is the wound number, *n* the total wounds inoculated and 3 the maximum severity index.

A second assay was also performed in immature ‘*Passé Crassane*’ pear fruits to test the effect of the introduction of plasmid pEI70::Tn5 in strains lacking pEA29. The inoculation methodology and the experimental design were the same as

described above for the first assay. The assay was performed at 10^5 cfu/ml and 5×10^5 cfu/ml pathogen concentrations. The incidence and severity of infected wounds (%) for each repetition (3 pears x 4 wounds each) was assessed after 7 days incubation.

Statistical analysis

ANOVA was performed to analyze the effect of each strain treatment on infection incidence and means were separated by the Tukey's test at $P \leq 0.05$. The analysis was done with the GLM procedure of the PC-Statistical Analysis System version 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Identification and sequencing of pEI70 in *E. amylovora* strain IVIA 1614-2a

E. amylovora strain IVIA 1614-2a was previously shown to contain an approximately 70 Kb plasmid and to lack the ubiquitous plasmid pEA29 (Llop *et al.*, 2006). The sequencing of the plasmid yielded a final consensus sequence of 65,840 base pairs, with an overall G+C content of 52.2%, close to the G+C level of the host (Smits *et al.*, 2010a; McManus and Jones, 1995). A total of 70 CDS were identified by GenDB and manually annotated (Fig. 1).

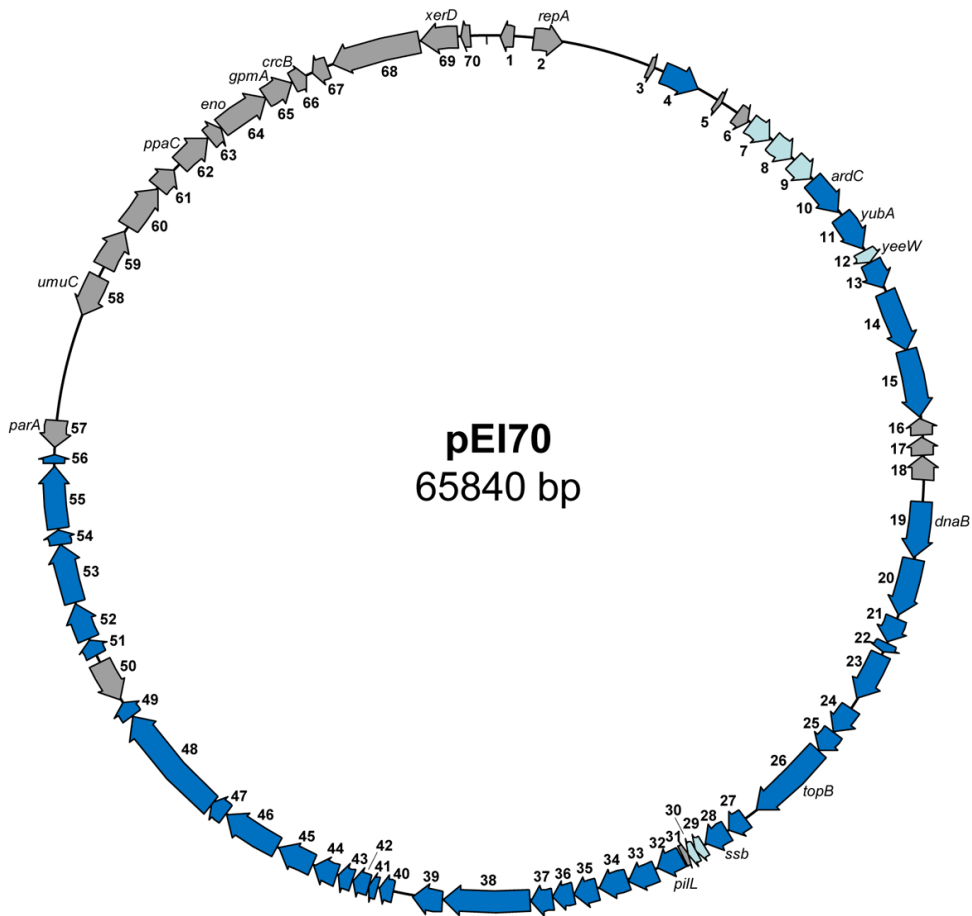


Fig. 1. Circular representation of plasmid pEI70. Locus tags (EaACW_pEI700xx) are indicated in the graph. Genes in dark blue are genes with orthologs in ICE elements. Genes that were also identified in the ICE element of *E. pyrifoliae*, but which are not related to a function in the ICE element are highlighted in light blue. Genes in grey are unrelated to the ICE element.

Sequence analysis of pEI70

Of the 70 CDS identified, 61 have more than 98% sequence identity to sequences of plasmid pEB102 from *E. billingiae* strain Eb661 (see Table 4-S1 in supplementary information at the end of this memory). The initiator replication protein RepA has 99% sequence identity to the RepA of plasmid pEB102 and a 98% identity to plasmid pPATH from *Pantoea agglomerans* pv. *gypsophilae* 824-1 (Weinthal *et al.*, 2007), indicating a potential common origin of these three plasmids. The nucleotide sequences of genes encoded on pEI70 have more than 98% identity to their counterparts on plasmid pEB102, as observed by BLASTN analysis. The organization of the CDS in plasmids pEI70 and pEB102 are identical as well, but a 36-Kb region in pEB102 is absent in pEI70 (Fig. 2). In this region, genes encoding proteins putatively involved in LPS biosynthesis (EbC_pEb10200120-130) and resistance to arsenate (EbC_pEb10200180-210) are present among many others with no or only a general function prediction (Kube *et al.*, 2010). A major feature of pEI70 is the presence of an Integrating Conjugative Element (ICE) that shares similarities to regions of PFGI-01 of *Pseudomonas fluorescens* Pf-5 (Mavrodi *et al.*, 2009) and HAI2 of *Pectobacterium atrosepticum* SCRI 1043 (Toth *et al.*, 2006), containing a fragment of the *pill* gene and lacks all the *tra* genes (Fig. 2C). We were not able to find potential attenuation sites (Williams, 2002) in the sequence of pEI70 that would allow an insertion into or close to known insertion sites in the genome of *E. amylovora* CFBP 1430 (Smits *et al.*, 2010a; Williams, 2002). This would indicate that the ICE might be unable to integrate into the chromosome and therefore, remains only present as a plasmid in *E. amylovora* strains.

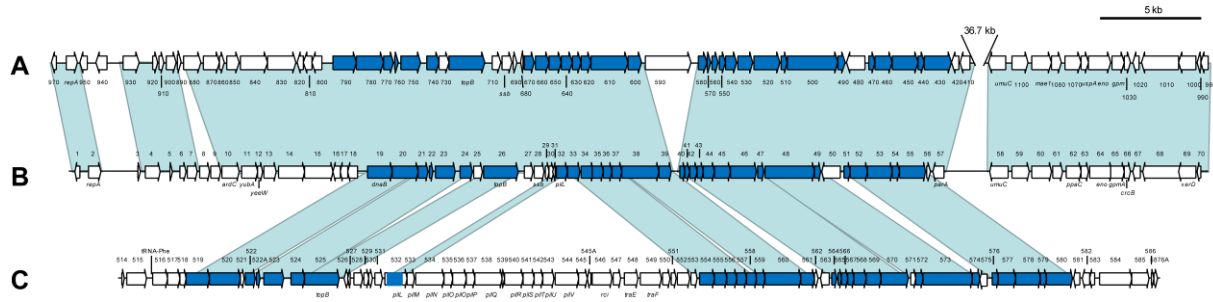


Fig. 2. Comparison of plasmid pEB102 of *E. billingiae* Eb661 (A) with *E. amylovora* ACW 56400 plasmid pEI70 (B), and the conserved region of GAI-2 of *Pectobacterium atrosepticum* SCRI 1043 (C). Orthologous genes are indicated by blue shading (conserved ICE element genes) and shading. Genes in white do not have orthologs in these regions.(Available at <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0028651>, April 24, 2017:)

Plasmid stability and conjugability of pEI70

We evaluated the stability of pEI70 in two native host strains: one with only this plasmid (strain IVIA 1614-2a) and another harboring pEA29 and pEI70 (strain IVIA 1614-1) in a 200-generation analysis. A total of 100 colonies per strain were positive by PCR for the presence of the plasmid after the 200-generations transfer in each of the two analyses performed. All the plasmid extractions performed (25 colonies) demonstrated that the plasmid was not integrated into the chromosome of the bacterium and that the plasmid did not change over time. Plasmid maintenance of pEI70 was 100% after 200 generations, both in the presence and absence of pEA29, and this latter plasmid was also found in 100% of the colonies analyzed. These experiments indicated that both plasmids are highly stable in their native *E. amylovora* host strains, and also the high stability of the pEI70 even in the presence of pEA29.

Conjugation experiments showed that pEI70 can be transferred between *E. amylovora* strains. A Tn5-tagged plasmid pEI70::Tn5 was conjugatively transferred into the strain CGJ2 that already contained the transposon-tagged plasmid pEA29::Tn5393, resulting in a trans-conjugant with both plasmids and two antibiotic resistances. The possibility that the trans-conjugants containing both plasmids arose from the mobilization of pEA29::Tn5393 into the strain containing pEI70 is quite unlikely, as the former plasmid was not shown to be conjugative (McGhee and *and* Jones, 2000) and the analysis of plasmid content showed that no plasmids were present in strain CGJ2.

Distribution of pEI70 within isolates from geographically different regions

The results obtained from the duplex PCR that allows the simultaneous detection of plasmids pEA29 and pEI70 in the different strains analyzed are

shown in Table 3 and Fig. 3. Additional information on the strains positive for pEI70 analyzed, is shown in Table 4-S2 in supplementary information at the end of this memory. Plasmid pEI70 was found to be widespread throughout European countries. In total, this new plasmid was found in 458 strains out of 1,249 isolates analyzed from Europe (36.6 %). The proportion of strains containing pEI70 plasmid was variable, ranging from 5% to 92% of the European strains analyzed, depending on the country (Table 3). Prevalence was as high as 62.9%, 85% and 92% in Slovenia, Ireland, and Belgium, respectively. In some countries (i.e., Austria, Bulgaria, Germany, Greece and Hungary), no strains containing pEI70 were detected, but in several of these countries, the number of isolates analyzed was quite low. Additional data on the strains negative for pEI70 analyzed is shown in Table 4-S3 in supplementary information at the end of this memory. In other countries (i.e., Czech Republic, Italy and The Netherlands), at least one strain was found with plasmid pEI70, even when only a few strains were analyzed. In Spain, from 142 strains obtained in several outbreaks from 1995 to 2009, 20 strains (14%) were found carrying pEI70, and of these, three were also devoid of pEA29. The information from supplementary Table 4-S2 demonstrated that at least since 1972 the plasmid was present in a French strain and since 1979 in another from Belgium. It is interesting to remark that more than twenty years later, strains with this plasmid were still isolated in Belgium confirming the natural stability of this plasmid in wild *E. amylovora* strains, which has been observed in our *in vitro* assays. Of the 231 strains analyzed from countries of other continents (e.g., Canada, Lebanon, Turkey, USA), pEI70 has thus far not been detected even in the USA, where fire blight originated.

The presence of pEI70 was confirmed by plasmid extraction and restriction with the *Bam*HI enzyme in 13 strains (eight from Poland, three from Spain, one from UK and one from Ireland), all providing the same restriction pattern. The strain from Ireland (E70) harbored another plasmid of around 30 Kb, different to

pEA29, as observed after restriction analyses and hybridization with pEA29 as a probe (Fig. 4).

Only six strains (three from Spain, two from Serbia and one from Belgium) were found not to carry plasmids

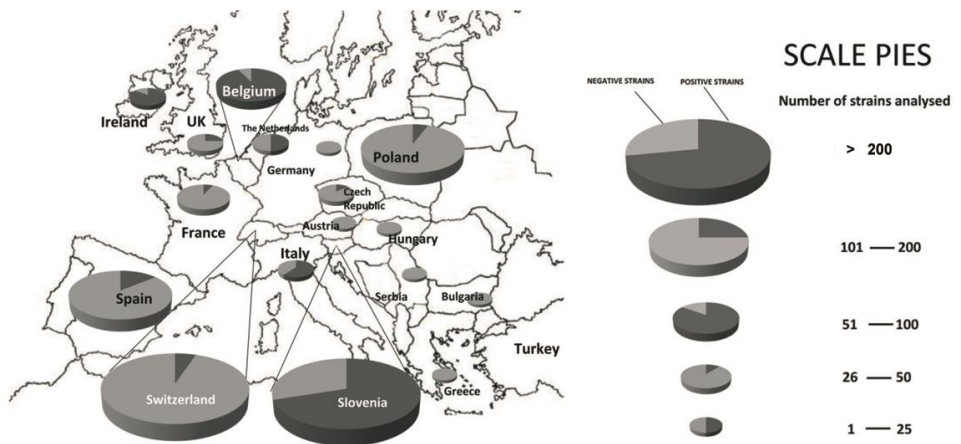


Figure 3. Biogeographic distribution map of strains harboring pEI70 in different European countries obtained by duplex-PCR. (0.5%).

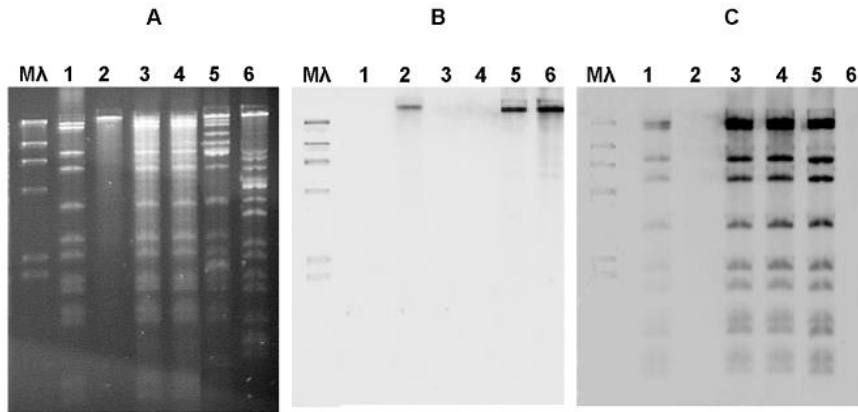


Fig. 4. Analysis of several strains by restriction digestion with *Bam*HI (A) and hybridisation analyses using pEA29 as probe (B) or pEI70 as probe (C). Isolate E 70 (lane 5) from Ireland shows a slightly different profile, with two extra bands that do not belong either to pEA29 or pEI70. Lane 1: strain IVIA 1614-2a; lane 2: CFBP 1430; lane 3: IVIA 1596; lane 4: NCPPB 3299; lane 5: E70; lane 6: Ea 273. M λ: marker Lambda (Invitrogen).

Analyses of aggressiveness using *ex vivo* plant assays

The role of pEA29 and pEI70 on the aggressiveness of *E. amylovora* strains was tested in immature pear fruits. In the first assay, large differences in aggressiveness between strains with different plasmid contents were observed (Table 4; Fig. 5). Plasmid-less strains CGJ2 and BC3 showed a very low level of aggressiveness, similar to the strain PMV 6014 (strain CFBP 1430 cured of pEA29); whereas the most aggressive strains contained at least one of the plasmids. When comparing the strains before and after introducing pEA29 it was observed that the presence of this plasmid significantly increased the incidence and severity of infections in the least aggressiveness strains (i.e., BC3, CGJ-2 and PMV 6014), independently of the inoculation dose. However,

differences were more evident when strains were inoculated at a lower dose (10^5 cfu/ml). The strains cured of pEI70 (i.e., IVIA 1596-pEI70 and IVIA 1614-2a-pEI70) caused significantly less infection incidence and lower severity compared to the wild type, and again this was more evident with a lower dose of inoculation (Fig. 5).

In the second assay, an increase in aggressiveness was also observed when pEI70 was introduced in strains that did not contain this plasmid (Fig. 6). This increase was observed at both doses assessed and was similar to the increase observed after the introduction of pEA29 in the strains that showed lower levels of aggressiveness.

Therefore, we can conclude that pEI70 has a clear effect on the aggressiveness of these strains (Table 4, Fig. 5, Fig. 6).

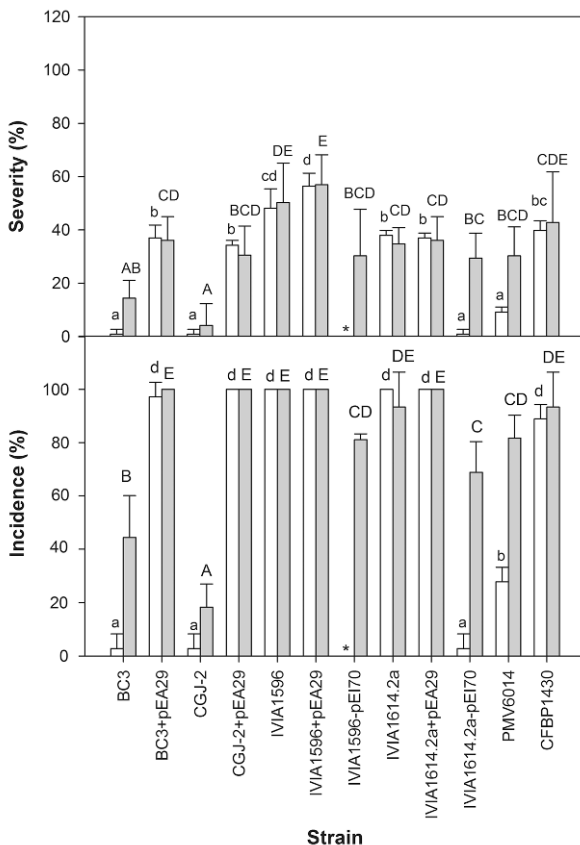


Fig. 5. Severity and infection incidence in immature pear fruits by inoculation of strains of *E. amylovora* before and after receiving plasmid pEA29 or after curing of pEI70. The severity and incidence of infection were measured 5 days after inoculation. The experiment was performed at 1×10^5 cfu/ml (white columns) and 1×10^6 cfu/ml (black columns). Means with the same letters (lower-case letters for low pathogen dosages, upper-case letters for high pathogen dosages) do not differ significantly according to Tukey's test ($P \leq 0.05$). Asterisks '*' indicate assays that were not performed.

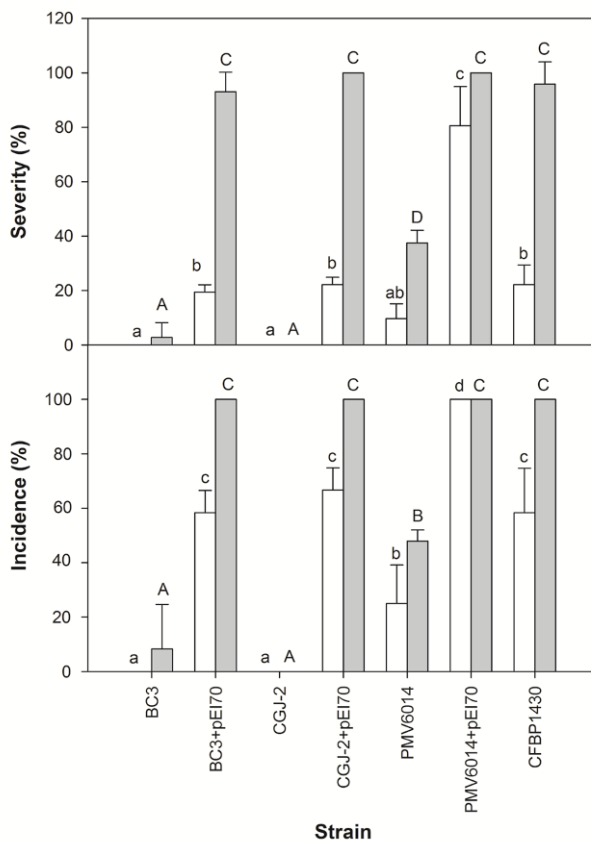


Fig. 6. Severity and infection incidence in immature pear fruits by inoculation of strains of *E. amylovora* with or without pEI70. The severity and incidence of infection were measured 7 days after inoculation. The experiment was performed at 1×10^5 cfu/ml (white columns) and 5×10^5 cfu/ml (black columns). Means with the same letters (lower-case letters for low dosages, upper-case letters for high dosages) do not differ significantly according to Tukey's test ($P \leq 0.05$).

Discussion

Several studies on the aggressiveness and phenotypic characteristics of *E. amylovora* strains have shown different levels of variability among them, regardless of the homogeneous genetic content observed in this species (Oh and Beer, 2005; Triplet *et al.*, 2006; Smits *et al.*, 2010a). An important source of genetic variability comes from gaining new genes through plasmid acquisition, as observed in other bacterial models (Arnol *et al.*, 2003; Stavrinides and Guttman, 2004; Zhao *et al.*, 2005; Sundin, 2007). In *E. amylovora*, some studies have been performed on the plasmid content of the strains and the functions they could provide to the bacterial genome, but these have been very limited in terms of the number of strains analyzed. Until now, apart from the plasmid pEA29 and its functionality in virulence, no recent data are available (Chiou and Jones, 1991; Laurent *et al.*, 1989; McGhee and Sundin, 2007). The detection of wild isolates lacking pEA29, and the presence of plasmid pEI70 that some of these strains instead carry (Llop *et al.*, 2006; Llop *et al.*, 2008b) has prompted a detailed analysis of this new plasmid and the elucidation of its role in the interaction between bacterium and plant host.

Analysis of the pEI70 sequence showed a very high sequence identity (more than 98% identity) to plasmid pEB102 from *E. billingiae* Eb661, which suggests a common origin of both plasmids. Plasmid pEI70 presumably encodes an ICE, but lacks the Type IV Secretion System (T4SS) *tra* and *pil* genes. Their gene products are commonly known to be involved in conjugative transfer, but they are not present in all known ICEs (Mohd-Zain *et al.*, 2004; Gaillard *et al.*, 2006). Nevertheless, plasmid pEI70 is conjugatively transferred to other *E. amylovora* strains, disputing the role of T4SS in the conjugative transfer of this kind of mobile element.

The genes in the latter quarter of the plasmid sequence do not belong to the core of the ICE, and may represent the “cargo” genes. The annotation predicts a metabolic function for several of these genes, which would suggest that these genes optimize the performance of the pathways they are involved in. The *eno* and *gpmA* genes (enolase and phosphoglyceromutase) present in the plasmid are enzymes of the glycolysis pathway. In preliminary experiments, it was observed that the growth rates of several strains assayed in minimal medium with sucrose were significantly faster in strains with pEI70, compared to the same strains lacking the plasmid. This effect was not observed when these strains were grown in rich medium like LB, which would indicate that the glycolysis genes may play a role (data not shown). *In planta*, this may improve competence for degrading these compounds, increasing pathogen aggressiveness. We hypothesize that the glycolysis in *E. amylovora* may be one of the bottlenecks in the bacterial growth and subsequent infection *in planta*. The fact that these genes are almost identical in plasmid pEB102 suggests a fitness effect rather than a role in virulence because the effect it provides in the epiphytic *E. billingiae* would likely confer a metabolic advantage. The relatedness of plasmids among closely related *Erwinia* species suggests occurrence of horizontal genetic transfer.

An interesting attribute of pEI70 is that it seems to be widespread in Europe because it has been detected in strains from 11 countries. The screening of 1,249 strains from 17 countries for the presence of this new plasmid has shown that it can be very abundant in some Western, Central and North-eastern European countries, but it is absent in isolates from the South-eastern European countries analyzed, or in countries outside the European continent. In Belgium, the percentage of strains positive for pEI70 was very high (more than 90% of isolates analyzed) but some distant countries also show high percentages (e.g., Slovenia, 62.9%). For instance, in Spain, pEI70 was present in 14% of the strains

analyzed and in 6 out of the 9 regions where the disease was reported up to 2009. In other European countries, plasmid pEI70 was not detected (e.g., Austria and Germany), but the low number of strains analyzed and proximity to countries with high prevalence of plasmid pEI70 (Fig. 3) suggest that further sampling will probably reveal its presence. More complete analyses could provide some useful information about the dispersal of the disease in Europe, although it is necessary to analyze more strains from the remaining countries and increase the number of isolates in some others to obtain solid conclusions (Tables S2 and S3).

Although a large number of strains from North America were examined, plasmid pEI70 was not detected, even though the disease originated in the USA (van der Zwet *et al.*, 1979), suggesting that this plasmid maybe was introduced once *E. amylovora* spread to Europe. Other regions where fire blight occurs (e.g., Northern Africa, New Zealand and Mexico) remain to be surveyed using the primer sets described in this paper.

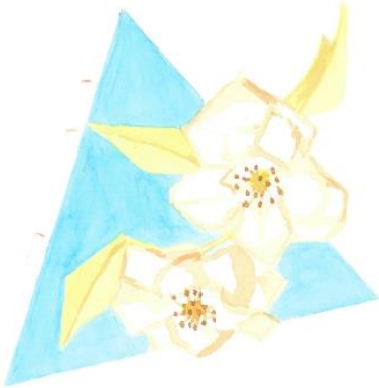
The analyses on immature pear fruit have shown that, when pEI70 was introduced into strains with low levels of aggressiveness, the intensity of symptoms increased. Moreover, after curing this plasmid from two strains (i.e., IVIA 1596 and IVIA 1614-2a) that show a standard level of aggressiveness, the intensity of symptoms decreased to levels similar to the strains without plasmids or to the strain PMV 6014, cured of pEA29. However, all strains tested containing only pEI70 had a standard to high level of aggressiveness. In preliminary experiments, the analyses of strains devoid of plasmids showed the same variability in aggressiveness as typical *E. amylovora* strains harboring pEA29: we found strains with high aggressiveness (e.g., UPN527 from Spain) and also strains with low aggressiveness (e.g., BC3 and CGJ2 from Serbia). Wild-type strains lacking pEA29, but containing plasmid pEI70, presented similar levels of aggressiveness compared to the reference strain CFBP 1430, that harbors only

pEA29 (Fig. 5). In contrast, no significant effect of the introduction of pEA29 was observed on the incidence of infection in two strains that naturally only harbor plasmid pEI70 (i.e., IVIA 1596 and IVIA 1614-2a) (Table 4). In other studies, introduction of pEA29 in strains without this plasmid provided different levels of aggressiveness, depending on the strain studied USA (Mohammadi, 2010). These results suggest the possible influence of other factors such as genomic background, host interactions and environmental conditions on the variability in aggressiveness that remains unexplained. In recent years, several *E. amylovora* strains with differing plasmid contents, including strains without pEA29 or strains with new plasmids, have been discovered (Chiou and Jones, 1991; Laurent *et al.*, 1989; Steinberger *et al.*, 1990; McGhee *et al.*, 2002a; Foster *et al.*, 2004; Sundin *et al.*, 2004; Palmer *et al.*, 1997; McGhee *et al.*, 2002b; *et al.*, 1989; Llop *et al.*, 2006). The results obtained on symptom development after their inoculation suggest that variable genomic backgrounds could be behind the differences in aggressiveness of *E. amylovora* strains, and that still unknown mechanisms may play a role in its aggressiveness, including a possible effect of the plasmid genes on the expression of chromosomal genes. Bacterial plasmids can contain so-called “fitness island” sequences (FIs), which consist of genes responsible for the epiphytic fitness of the bacterium that could enhance their pathogenicity and virulence as an additional advantage (Stavrínides and Guttman 2004; Zhao *et al.*, 2005; McGhee and Sundin, 2008). Plasmid pEI70 seems to provide some features that compensate for the lack of pEA29 and could explain the standard aggressiveness levels observed in the strains harboring it. Recently, in *E. amylovora* strains from Poland a new plasmid has been found (Isamil *et al.*, 2014), which indicates the need for additional detailed studies on the only factor currently known to influence the pan-genome in this important plant pathogen (Smits *et al.*, 2011). Most likely, different plasmids are still waiting to be discovered in *E. amylovora* strains, and further studies at genomic, proteomic and metabolomic levels could contribute to fully

understanding the role of extra-genetic elements in the *E. amylovora*-plant interaction.

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Capítulo 5

Análisis del efecto de los plásmido pEA29 y pEI70 de *Erwinia amylovora* en la expresión de los genes cromosómicos durante el desarrollo de los síntomas en ensayos en pera inmadura

Analysing the effect on chromosomal gene expression of plasmids pEA29 and pEI70 in symptoms development on pear in *Erwinia amylovora*

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Para ser enviado a publicación.

* S. Barbé has contributed in the design of experiments, performing pear assays, RNA isolation and purification and partially microarray hibridization, analyzing data of differential expression from normalization and processing data and interpretation of functional profiling, and writing the first version of the manuscript.

Abstract

Erwinia amylovora, the causal agent of fire blight, is considered a genetically homogeneous species, and the major genetic differences among strains are based on extrachromosomal material, being the plasmids the factor providing genetic differences. Plasmid pEA29, almost universal in this species, has been studied about its effect in symptoms development and several of its genes were identified as important for the virulence of *E. amylovora*. Another plasmid recently discovered called pEI70, widespread in isolates all over Europe, provides a similar effect on the increase of symptoms, despite the different gene content compared to pEA29. Some studies on the virulence of this pathogen have focused on specific genes involved in pathogenicity, whereas other looked at the expression of the genes induced in *E. amylovora* during symptoms formation, providing very useful information on the pathogenesis. Despite these advances, nothing is known about how plasmids that play important roles in virulence influence the expression of the genes of this bacterium. Here we describe the use of a microarray designed with probes from the *E. amylovora* strain ATTC 99446. Using this technology, we have studied the expression of chromosomal genes in presence and absence of plasmids pEA29 and pEI70 in immature pear assays in independent experiments. The results show that the presence of pEA29 up regulates 38 genes from the chromosome whereas 142 are down regulated, with fold-change expression ratios greater than 1.6. In the case of pEI70, its presence increases the expression of 60 genes and 60 other genes are down regulated. The functional analysis of these genes will provide additional and important clues about pathogenicity factors in correlation to this extrachromosomal material, filling an important gap in the information about how the virulence of this bacterium is regulated.

Introduction

Erwinia amylovora is the causative agent of fire blight, the most serious disease of pomaceous plants worldwide, like apple and pear, and other members of the family Rosaceae (Vanneste, 2000). This bacterium has been studied for long time, and several genetic analyses have demonstrated that it is a very homogeneous species (Zhang and Geider, 1997; Oh and Beer, 2005; Triplet *et al.*, 2006; Smits *et al.*, 2010) although *E. amylovora* isolates can have different phenotypic traits (Aldridge *et al.*, 1997; Sholberg *et al.*, 2001; Kim *et al.*, 1995; Vanneste, 1995).

Currently, it is known that the genomes of the European strain CFBP 1430 and the American strain Ea 273 have high genetic-homogeneity (>99.99% nucleotide sequence identity) (Smits *et al.*, 2010 a). Genome sequences from four European isolates and comparative genomic analysis of 12 strains representing distinct populations (e.g., geographic, temporal, host origin) of *E. amylovora*, support that the pan-genome of *E. amylovora* is closed except for its plasmid content. (Smits *et al.*, 2011; Mann *et al.*, 2013). The genes involved in virulence and pathogenicity are highly conserved and rarely show significant differences in sequence and structure (Llop *et al.*, 2011), and the only differential trait is the presence of an extra plasmid (pEa72) in the U.S. strain (Sebahia *et al.*, 2010; Smits *et al.*, 2010 a). Thus, the diversity that could explain the phenotypic differences between strains may be attributed primary to the flexible genome comprised by them. Currently, there are several plasmids described in *E. amylovora* strains, but the most important is pEA29 (AF264948, McGhee and Jones, 2000; FN666576, Sebahia *et al.*, 2010; FN43411, Smits *et al.*, 2010a) due to the role it shows in the aggressiveness of the bacterium. In the last years, new isolates with different plasmid content have been discovered (Foster *et al.*, 2004; Chiou and Jones, 1991, 1993; McGhee and Jones, 2000; Sebahia *et al.*, 2010; Smits *et al.*, 2010 a and 2014; Llop *et al.*, 2008 and 2011, Isamil *et al.*,

2014) but only for pEI70 the demonstration of its role in virulence is available. Probably provides a better fitness to the bacterium because its sequencing did not reveal known virulence genes described previously (Llop *et al.*, 2008, 2011).

Plasmid pEA29 encodes a thiamine biosynthesis operon and several candidate genes that could affect virulence and survival in plants (McGhee and Jones, 2000) and which could play a role in the physiology or metabolism of extracellular polysaccharide production, and these traits are associated with the full virulence of the pathogen (McGhee and Sundin, 2008). Strains cured of this plasmid, as mutant PMV 6014, exhibit reduced virulence, thiamine auxotrophy, and altered exopolisaccharide production on minimal medium without thiamine (Falkenstein *et al.*, 1989; Laurent *et al.*, 1989; McGhee and Jones 2000; Llop *et al.*, 2011). Since the discovery of strains lacking plasmid pEA29, more efforts have been put on the study of plasmid content in *E. amylovora* strains and their possible role they play in the pathogen. Among the new plasmids described in *E. amylovora*, plasmid pEI70 has unusual characteristics among the other plasmids found in *E. amylovora* because it is conjugative (Llop *et al.*, 2011), it is widespread in 11 European countries, being present in 5–92% of strains analysed, depending on the country; and more importantly, it has been observed that, when introduced into plasmidless strains of *E. amylovora*, it was able to induce faster development of symptoms.

In *E. amylovora* the genes involved in pathogenicity and virulence have also been studied in many works (Kim and Beer, 2000; Wei and Beer, 1995; Wei *et al.*, 2000; Alfano and Collmer, 1997; Wei *et al.*, 1992; Bogdanove *et al.*, 2000; Geider, 2000; Dellagi *et al.*, 1998; Expert *et al.*, 2000) however, all the genes described involved in virulence are situated in the chromosome. Furthermore, several studies on the effect of specific genes on the expression of different pathogenic features have been performed in *E. amylovora*. In many cases the role of these plasmidic genes has been confirmed/established related to

pathogenicity, and several gene expression studies of the effect of specific plasmid genes have been achieved (Wang *et al.*, 2012; McNally *et al.*, 2011) but the influence of the presence of plasmids on the expression of chromosomal genes during symptoms development has never been attempted.

Microarray-based gene expression profiling has been recognized as a powerful tool to observe global gene expression and thus, study the physiological processes involved in response to a particular stimulus (Maleck *et al.*, 2000; Schenk *et al.*, 2000). Many research has been conducted in the last years to elucidate the virulence factors that intervene in the disease produced by *E. amylovora*, and among them, the T3SS, the amylovoran, levan and protease related genes, and genes involved in sorbitol and sucrose metabolism have been described (Oh and Beer, 2005; Oh *et al.*, 2005; Zhao *et al.*, 2011; Zhao *et al.*, 2009a, b; Bogs and Geider, 2000; Smits and Duffy, 2011; Águila-Clares *et al.*, 2016). Other works have focused on the expression of these genes in the interaction with the plant, but still many aspects of the disease are unknown.

In this work, the expression profile of the chromosomal genes in one *E. amylovora* strain, under the influence of two plasmids, one almost universal and known to have a role in virulence (pEA29, Falkenstein *et al.*, 1989) and another recently described which has also a role in symptoms development, pEI70 (Llop *et al.*, 2011) in immature pear fruit infection conditions has been performed through the use of a customized array of the chromosome and several plasmid sequences of *E. amylovora* and the preliminary functional analyses are shown.

This is the first attempt to obtain a global view of the effect that these plasmids have on the expression of chromosomal genes during symptoms development, giving a new insight on the molecular knowledge of disease production.

Material and methods

Bacterial strain and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in King's B medium (King *et al.*, 1954) at 26°C for 24 hours. *E. amylovora* CFBP 1430 was used as the strain harbouring the pEA29 plasmid; strain PMV 6014 which is strain CFBP 1430 cured of pEA29 and shows low aggressiveness, was used as control strain. Plasmid pEI70::Tn5 was introduced into strain PMV 6014 by electroporation (Llop *et al.*, 2011) and was used as the sample harbouring pEI70 plasmid. All these strains contain the same chromosomal background (strain CFBP 1430) and different plasmid content: no plasmids (PMV 6014); plasmid pEA29 (CFBP 1430); and plasmid pEI70 (PMV 6014 + pEI70::Tn5).

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains	Host	Plasmid content	Origin/Year	Ac. number chromosome	Ac. number plasmid	References
CFBP 1430	<i>Crataegus</i> sp.	pEA29	France/1972	FN434113	FN434114	Paulin and Samson, 1973. Smith <i>et al.</i> , 2010a.
PMV 6014	-	-	France	-	-	Laurent <i>et al.</i> , 1989.
PMV 6014 + pEI70::Tn5	-	pEI70::Tn5	Spain	-	CP002951	Llop <i>et al.</i> , 2011.

Pear assays

Strains were grown for 24 hour in King's B medium at 26°C. Cell suspensions were prepared and concentrations adjusted to 10^3 colony-forming units (cfu/mL) at 600 nm in 0.5x phosphate-buffered saline (PBS; 0.36% NaCl, 0.018% $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$, 0.12% $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$) as described in Barbé *et al.* (2012). These suspensions were used for inoculation of immature pear fruits cv. Williams.

Plant material was prepared according to the procedure used by Cabrefiga and Montesinos (2005). 15 to 20 wounds of one cm long and 0.5 cm deep were performed on each fruit with 3-4 cm diameter, using a scalpel blade, and 20 to 24 fruits were inoculated with 10 μl suspension introduced into each wound. Fruits were incubated at 26°C under high humidity conditions. Symptoms were evaluated daily until the fourth day. Exudates were collected on the third day post inoculation and stored with *RNAlater*[®] (Life Technologies) following the instructions of manufacturer. The experiment was performed two times with each strain (Fig. 1).



Fig. 1. A: Inoculation on immature pear fruits var. Williams in trays with high humidity and incubation at 26°C during 4 days. 10 μ l of 10^3 CFU/ml per wound and 20-24 wounds per fruit. B: close up of exudates taken as starting material.

RNA isolation

Exudates were defrosted on ice and harvested by centrifugation for 5 minutes at 19000 G-force. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hidden, Germany) as recommended by the manufacturer. DNase digestion was performed using Ambion® TURBO DNA-free™ (Invitrogen) following the instructions for “*Rigorous DNase treatment*” recommended by the manufacturer.

RNA was quantified using Nano-Drop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and RNA quality was checked using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Microarray hybridization

The Agilent-021826 UIUC Erwinia 15K.V1.0, is a cDNA microarray designed at the James Hutton Institute (Scottish Crop Research Institute), and developed by Agilent Technologies, Inc. (Palo Alto, CA). Each slide comprises eight arrays and each array has nearly 4,860 probes of 60-mer length oligonucleotide (nearly 15,000 spots) in triplicate (Wang *et al.*, 2012; McNally *et al.*, 2011). This custom microarray was made using *E. amylovora* ATCC 49946 (Ea273) genome information, and also sequences from several plasmids described in other strains were included (Table 2).

Table 2. Genome information of Agilent-021826 UIUC Erwinia 15K.V1.0 custom microarray.

Strain	Locus tags site	Probes	Plasmid name	Size (pb)	CDS	Acc. number	Ref.
ATCC 49946 (Ea273)	Chromosome	3483		3805874	3483	NC_013971	Sebaihia <i>et al.</i> , 2010
ATCC 49946 (Ea273)	Plasmid	149	Plasmid 1 (pEA29)	28243	21*	NC_013972	Sebaihia <i>et al.</i> , 2010 McGhee and Jones, 2000
ATCC 49946 (Ea273)	Plasmid	466	Plasmid 2 (pEA72)	71487	87	NC_013973	Sebaihia <i>et al.</i> , 2010
LebB66	Plasmid	278	pEL60	60145	68	NC_005246	Foster <i>et al.</i> , 2004
LebB66	Plasmid	110	pEU30	30314	25	NC_005247	Foster <i>et al.</i> , 2004
IVIA 1614-2a (ACW 56400)	Plasmid	379	pEI70	65840	70	CP002951	Llop <i>et al.</i> , 2011

The cRNAs of the exudates from the three strain were obtained using Agilent's Low Input Quick Amp Labeling WT Kit and Spike-In Kit (Agilent). Cy3 labeled samples were added as recommended for 100 ng input RNA per reaction through this linear amplification protocol. cRNA yields and specific activities were measured using a NanoDrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Labeling and hybridizations were performed using a protocol based in One-Color Microarray-Based Exon Analysis-LIQA WT Labeling Protocol (Agilent) as recommended by the manufacturer (application note). One slide of 8 arrays was used with the following repeats: CFBP1430, 3 replicates; PMV6014, 2 replicates; PMV 6014 + pEI70::Tn5, 3 replicates. Slides were scanned using an Agilent DNA Microarray Scanner G2565C.

Data normalization and preprocessing

Microarray images were imported into Agilent Feature Extraction Software and raw data were logarithmically transformed and normalized using Babelomics v4.3 platform (Medina *et al.*, 2010). Agilent Processed Signal (Agilent Feature Extraction Software) was standardized across arrays using quantile normalization algorithm (Bolstad *et al.*, 2003).

Differential expression

Differential gene expression assessment of all comparisons (strain CFBP 1430 with pEA29 plasmid vs. strain PMV 6014 without plasmids; strain PMV 6014 with plasmid pEI70::Tn5 vs. strain PMV 6014 without plasmids) was carried out using limma moderated t-statistics. We evaluated only the chromosomal genes. Standard microarray analysis techniques perform one test for each gene (or probe-set) in the microarray and in the comparison. Thus, for each gene a statistical test statistic is reported together with its corresponding p-value (also

termed raw). This raw p-value was corrected for multiple testing in order to minimize the amount of false positives.

Functional profiling

Enrichment analysis and gene set analysis were carried out for the Gene Ontology terms using FatiGO (Al-Shahrour *et al.*, 2004) and Logistic Regression Model (Sartor *et al.*, 2008) integrated in the Babelomics suite.

Gene Ontology annotation for the genes in the microarray were generated from Blast2GO (Conesa *et al.*, 2005).

Conventional multiple testing p-value correction procedure proposed by Benjamini and Hochberg (1995) was used to derive adjusted p-values.

Results

Data normalization and preprocessing

All arrays could be read and pre-processed appropriately. The Box plots show the intensity distribution for each of the arrays in the study. Data distributions are shown after normalization (Fig. 2). Normalization reduced technical artefacts enhancing the biological signal (Fig. 3).

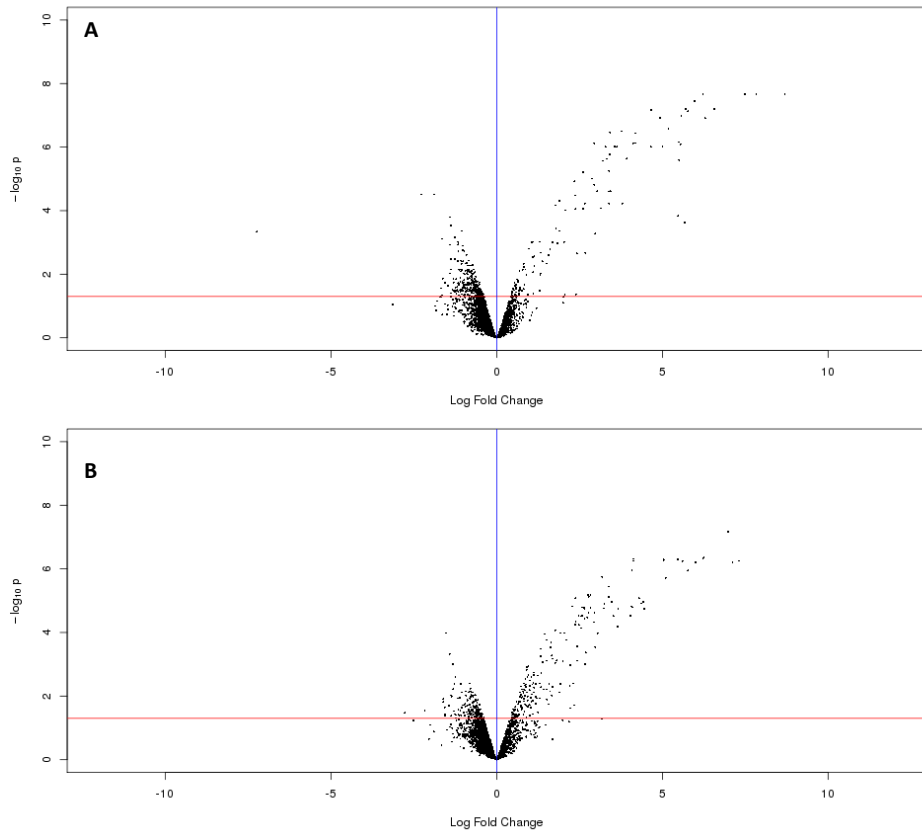


Fig. 2. Volcano plots arrange genes along dimensions of biological and statistical significance. A) comparison of strain CFBP 1430 with pEA29 plasmid vs. strain PMV 6014 without plasmids; B) comparison of strain PMV 6014 with plasmid pEI70::Tn5 vs. strain PMV 6014 without plasmids), using in both cases limma moderated t-statistics. The first (horizontal) dimension is the fold change between the two groups (on a log scale, so that up and down regulation appear symmetric), and the second (vertical) axis represents the p-value for a t-test of differences between samples (most conveniently on a negative log scale – so smaller p-values appear higher up). The first axis indicates biological impact of the change; the second indicates the statistical evidence, or reliability of the change. Red line indicates fold change = 0 and blue line means adjusted p-value = 0.05.

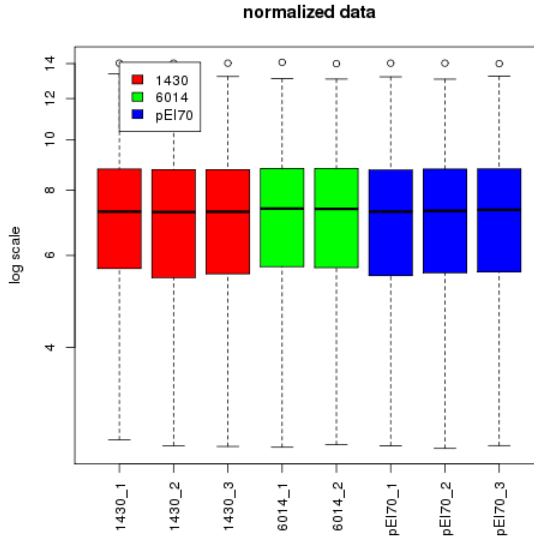


Fig. 3. The Box plots show intensity data distribution for each of the arrays after normalization. In red arrays hybridized with strain CFBP 1430, in green arrays hybridized with strain PMV 6014 and in blue arrays hybridized with strain PMV 6014 - pEI70::Tn5.

Differential expression

Table 3 shows the list of differentially expressed genes (Affymetrix probe-sets) in each of the comparisons or contrasts evaluating only chromosomal genes (3477 genes). The differentially expressed genes were classified as (a) Over-expressed in the presence of a plasmid: those genes with a fold change ≥ 1.6 and a p-value ≤ 0.05 ; (b) Under-expressed: genes with a fold change ≤ -1.6 with a p-value ≤ 0.05 ; (c) non differentially expressed, genes with a fold change between 1.6 and -1.6 with a p-value ≤ 0.05 . Of 3915 probes covering the genome of strain ATCC 49946 in which the array is based, 3477 probes are chromosomal and 438 are plasmidic (from plasmids pEA72, pEI70, pEL60, pEA29

and pEU30). A total of 5.17% of the chromosomal genes were differentially expressed when comparing strain CFBP 1430 (carrying plasmid pEA29) and PMV 6014 (with no plasmids). This percentage includes 38 over-expressed genes in the strain with pEA29 plasmid (CFBP 1430), representing 1.09% of chromosome genes, and 142 under-expressed genes, representing 4.08%. When comparing the strain to which it has been introduced the plasmid pEI70 (PMV 6014 + pEI70::tn5) against the strain without plasmids (PMV 6014), in presence of pEI70, 3.44% of chromosomal genes were differentially expressed. This percentage includes 60 genes overexpressed, representing 1.72% of the genes of the chromosome, and 60 genes (1.72%) are under-expressed. Statistically, 96.56% of the genes in the chromosome were non-differentially expressed (Table 3).

Table 3. Differentially expressed chromosomal genes.

Biological condition	Under-expressed	Non differentially expressed	Over-expressed
CFBP 1430 vs. PMV 6014	142 (4.08%)	3131 (94.83%)	38 (1.09%)
PMV 6014 + pEI70::Tn5 vs. PMV 6014	60 (1.72%)	3252 (96.56%)	60 (1.72%)

The genes were grouped into eleven categories according to the functions described for each gene (Tables 4 and 5). A large percentage of these differentially expressed genes in the strain with pEA29 were listed in the functional category of metabolism of fatty acids, carbohydrates, amino acids, nucleosides and glucose (Fig. 4), as observed also for the expressed genes when plasmid pEI70 was present (Fig. 5). The following categories with high number of genes differentially expressed are: hypothetical proteins, transport of different substances (as ions, sugars, amino acids and nucleotides), and genes listed as “other” which include metiltransferases, DNA repair, degradation of RNA and is in this category where more genes are coincident between pEA29 and pEI70

(4 genes) (Table 6, Fig. 6). The analyses of the complete set of genes differentially expressed in presence of one and the other plasmid have revealed a number of genes grouped in the different categories, and before a confirmation by means of q-PCR analyses of the expression values of selected genes is performed, the main aspects to be highlighted of the results obtained with the microarray are described below.

The genes differentially regulated with pEA29 in the functional categories were: In the category of mobile elements and phages showed one gene upregulated (EAM_3026) which corresponds to a putative phage-regulator protein and three putative transposases and a putative phage holin were repressed (EAM_1525, EAM_2457, EAM_3461, EAM_1407).

In the Interaction (host, microorganism and environment) category, an outer membrane protein gene related to the transport of hydrophobic molecules showed a high increase in expression (*ompW*), and also one gene which encodes a CRISPR-associated protein (EAM_0767). Seven genes repressed are related to growth in anaerobiosis, biofilm formation, growth in CO₂ and production of toxic protein colicin (EAM_2412, *dgkA*, *cvpA*, *suhB*, *can*, EAM_1297, EAM_1731).

In the Metabolism category, of 19 genes upregulated, at least seven are mainly involved in energy production (glycolysis and sugar metabolism) and nitrogen regulation (EAM_1711, *adhP*, *nrdD*, *pall*, *nrdG*, *fumA*, EAM_1539, *pyrD*, *tpiA*, EAM_2054, EAM_2268, *wabM*, EAM_0404, *yafA*, *astD*, *astB*, *holC*, *glmS*, *glgP*). 36 genes downregulated are involved in the biosynthesis of purine and pyrimidine compounds and fatty acids (*smtA*, *purT*, *rob*, EAM_1860, *srmB*, *ribF*, EAM_2309, *pyrF*, *rpsT*, *atpI*, *hutG*, *yfbR*, *rsuA*, *mqa*, *carA*, *gtrA*, *holE*, *purM*, *pyrE*, *accB*, *hutI*, *rluC*, *idi*, *gntK*, EAM_2254, *aroQ*, *rep*, *pyrB*, *hutH* EAM_3339, *folA*, *ndh*, *puuE*), and include three of the regulon genes of thiamine metabolism

(*thiE*, *thiC* and *thiM*) held at the chromosome that were repressed in ooze material.

In Cellular motility, two flagellar genes were repressed (*flhA* and *flgM*), and none was upregulated;

In the Gene expression regulation group, only one gene belonging to the LuxR family presented an intense increase (fold change 4,16). This gene regulates the cell division. The 14 genes repressed are involved in the catabolism and transport of arabinose and other sugars, synthesis of pyrimidines and asparagine, DNA binding in stress response, block replication and regulation of biofilms, among other (*hns*, EAM_0871, *mlrA*, EAM_1756, EAM_0771, *pyrI*, *tus*, *soxR*, EAM_3348, *ascG*, *phdR*, EAM_2923, EAM_0942, *rcsV*).

One gene related to stress response showed down regulation of protection against heat shock (*dnaJ*).

Only one gene which encodes a putative membrane protein showed upregulation in the Cellular surface and transmembrane area category (EAM_2958), while 14 genes are repressed (*gmhB*, EAM_1885, *waaQ*, *murB*, EAM_0959, EAM_0083, EAM_3371, *gcvH*, EAM_0235, EAM0275, EAM_0322, EAM_0921, *aaeX*, *ampH*). The majority of them are related to membrane protein genes and synthesis of LPS and peptidoglycan.

The transport category showed three genes upregulated (*ttdT*, EAM_0347, EAM_1712), one with high increase, *ttdT*, related to tartrate/succinate transport, and 20 genes repressed in the transport of different amino acids and sugars as glucose and ribose (*mgIB*, EAM_1333, EAM_2601, EAM_2847, EAM_2385, *ptsG*, *kup*, EAM_1965, *ansP*, *gntU*, *sdaC*, EAM_0040, EAM_0117, EAM_0858, EAM_1276, EAM_2308, EAM_2307, *rbsD*, *aaeA*, EAM_2306).

Only downregulated genes appeared in the category of TTSS, the majority putative exported proteins (EAM_2924, EAM_2864, *prtF*, EAM_0518, EAM_3363, EAM_0538), and the gene *hrpW*, a harpin protein that induces rapid tissue collapse (Kim and Beer, 1998).

In the hypothetical proteins category, the second most abundant with pEA29, many were conserved while other are just hypothetical, with nine upregulated (EAM_2194, EAM_0488, EAM_2242, EAM_1111, EAM_1540, EAM_2269, EAM_0402, EAM_0914, EAM_3020) and 25 repressed (EAM_2767, EAM_3347, EAM_0999, EAM_3436, EAM_1743, EAM_1792, EAM_3370, EAM_3209, EAM_1121, EAM_0732, *syIX*, EAM_0705, EAM_1978, EAM_0470, EAM_1969, EAM_0884, EAM_1528, EAM_0956, EAM_1472, EAM_0149, EAM_1791, EAM_0110, EAM_0781, EAM_1502, EAM_1068).

In the last category of “Other”, two genes are upregulated, a oxidase (*hemN*) and a putative methyltransferase (EAM_0403), are related to energy and electron transport, and the down regulated (12) have different functions (EAM_3439, EAM_0855, *mioC*, EAM_0852, *exoX*, EAM_2991, *rph*, EAM_0230, EAM_0941, EAM_3438, EAM_1257, EAM_3340) (Table 4).

The genes differentially regulated with pEI70 in the microarray experiment were:

Mobile elements showed two genes that appear downregulated also with pEA29 (EAM_1525 and EAM_2457).

In the Interaction category, a putative anhydrase that has been shown to function in the survival of intracellular pathogens within their host (EAM_0510) (Smith and Ferry, 2000) was upregulated, as well as another putative cytotoxic factor (EAM_0738), which has been described as a virulence factor in *Erwinia* (Kube *et al*, 2010) and a non-ribosomal peptide synthetase (EAM_2971). The

genes repressed were also three (EAM_2425, *mdtA*, EAM_2412) and included an Rhs-family protein described as a possible virulence protein (EAM_2425) (Kung *et al.*, 2012). It is also related to the T6SS in *P. aeruginosa*, involved in different functions, including virulence as well (Jones *et al.*, 2014) and is defined as a self-proteolysis protein.

In Metabolism category, the genes upregulated (24) were mainly involved in energy uptake and production, as with pEA29, although they are different (*fadH*, *fadE*, EAM_2048, *fadB*, *acs*, *metE*, *metF*, *fadA*, EAM_2126, *treA*, EAM_2268, EAM_2125, *argI*, *nrdF*, *argH*, *argG*, *ndrB*, EAM_1242, *pckA*, *metB*, *argD*, *nrdE*, *atpH*, *gltA*). Among the genes downregulated (14) several are involved in the catabolism of galactose (*galK*, *galM* and *galT*) as well as genes for galactose metabolism in glycolysis and gluconeogenesis, and genes related in carbohydrates metabolism in anaerobiosis (*rsuA*, EAM_0881, *hutG*, *gntK*, *yfbR*, EAM_2254, *fruK*, *hutI*, EAM_3340, EAM_3439, *glpD*). In this category, eight genes downregulated in presence of pEI70 were also repressed in presence of pEA29 (*rsuA*, *hutG*, *gntK*, *yfbR*, EAM_2254, *hutI*, EAM_3340, EAM_3439).

In Cellular motility, three flagellar genes were repressed (*fliT*, *flgI*, *flgA*), and none is upregulated; they are different from the ones downregulated in presence of pEA29, but probably the effect is similar, because the bacterium does not need flagella in the EPS matrix forming the ooze.

In the Gene expression regulation group a putative transcriptional regulator was upregulated (EAM_2487) and two genes are downregulated (EAM_2961 and EAM_3307) involved in the catabolism and transport of arabinose and in catalyzed the phosphorylation of ribose.

Genes upregulated from the Stress response (*cspD* and *spy*) were involved in heat and cold shock, along with a putative exported protein with unknown

function. The genes repressed (*umuD*, EAM_1297, *asr*) are related to protection against UV radiation, oxidative stress and acid stress.

Transport category showed high number of genes upregulated (20), involved in transport of amino acids, sugars and other compounds (*rbsC*, *actP*, EAM_2047, *rbsB*, EAM_0511, *rbsA*, *pstB*, *mgIC*, *fadL*, *gltI*, *gltK*, *mtIA*, *dppD*, *livG*, *mtID*, *dppF*, *dctA*, *livJ*, EAM_2130) and interestingly, gene *rbsD*, involved in ribose transport as a source of energy is one of the upregulated genes. Ten genes were repressed involved in the transport of different compounds (*gntU*, EAM_1577, *fruA*, EAM_0058, EAM_2601, *scrY*, *aaeB*, EAM_1708, EAM_0873, *aaeA*).

In the Cellular surface category, three putative membrane proteins were increased (EAM_0326, EAM_1241, EAM_1390, EAM_0565) while four other genes of putative membrane proteins are repressed (EAM_1808, EAM_0945, *aaeX*).

Genes in the category of TTSS were all downregulated, involved in harpin injection and secretion proteins (*hrcT*, *prrF*, EAM_1853).

In the Hypothetical proteins category, two were upregulated (EAM_2269, EAM_1243) and 14 repressed (EAM_3400, EAM_0867, EAM_0924, EAM_0705, EAM_1978, EAM_1791, EAM_2888, EAM_1725, EAM_1578, EAM_2767, EAM_2764, EAM_0601, EAM_0884, EAM_1806) with six genes coincident with pEA29, one upregulated (EAM_2269) and five down (EAM_0705, EAM_1978, EAM_1791, EAM_2767, EAM_0884).

In the last category of “Other”, one of the four of the upregulated genes (EAM_3477) is related to synthesis of ATP (EAM_2488, EAM_2486, EAM_0403), and the three genes downregulated (*sbcD*, EAM_1257, EAM_3438) have different functions (Table 5).

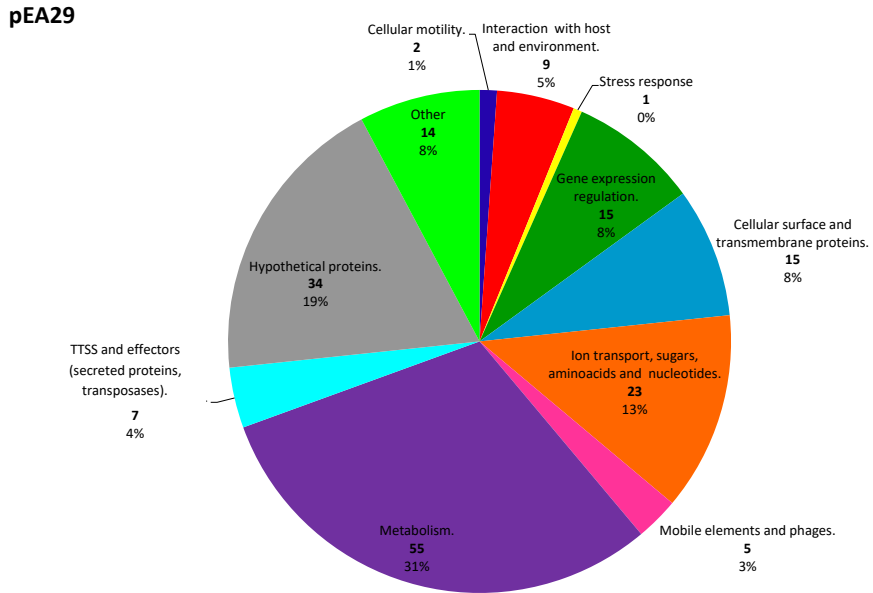


Fig. 4. Genes differentially expressed in presence of pEA29 in the ooze obtained after inoculation of immature pear, grouped using the functional categories.

pEI70

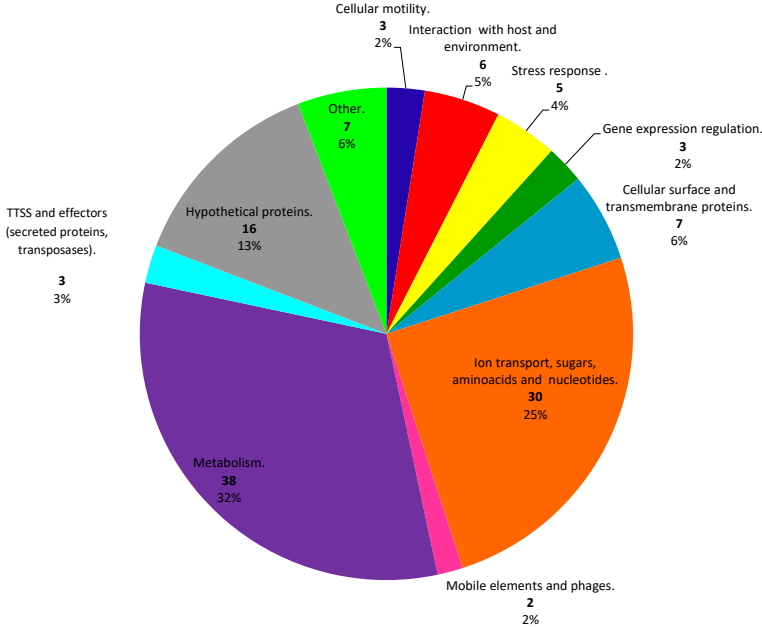


Fig. 5. Genes differentially expressed in presence of pEI70 in the ooze obtained after inoculation of immature pear, grouped using the functional categories.

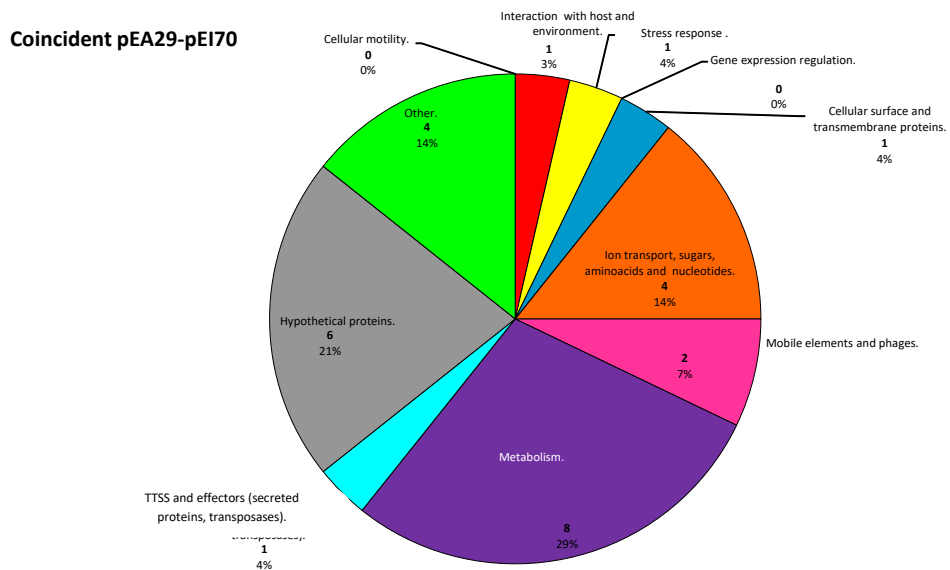


Fig. 6. Genes differentially expressed in presence of pEA29 or pEI70 in the ooze obtained after inoculation of immature pear, grouped using the functional categories that are coincident.

Table 4. Genes grouped into eleven categories according to the functions described for each gene in presence of plasmid pEA29.

Functional category	Locus tag	Gene symbol	Protein description and gene position in the chromosome	Fold change
1. Mobile elements and phages				
Up-regulated	EAM_3026		putative phage-regulatory protein 3307405:3307602 forward	1,68
Down-regulated	EAM_1525		putative phage holin 1646185:1646454 forward	-1,72
	EAM_2457		putative transposase 2684184:2684882 reverse	-1,78
	EAM_3461		putative transposase 3781391:3781669 reverse	-1,78
	EAM_1407		putative transposase 1533169:1534080 forward	-1,95
2. Interaction: host, microorganism and environment				
Up-regulated	EAM_1884	<i>ompW</i>	outer membrane protein W 2026228:2026860 reverse	10,64
	EAM_0767		CRISPR-associated protein 865814:866416 forward	1,93
Down-regulated	EAM_2412		putative nitrate/nitrite responsive two-component sensor kinase 2611461:2612453 forward	-1,70
	EAM_0263	<i>dggA</i>	diacylglycerol kinase 333971:334336 forward	-1,71
	EAM_2319	<i>cvpA</i>	colicin V production protein 2513815:2514300 reverse	-1,72
	EAM_2495	<i>suhB</i>	inositol-1-monophosphatase 2725995:2726798 forward	-1,75
	EAM_0784	<i>can</i>	carbonic anhydrase 2 883432:884094 reverse	-1,84
	EAM_1297		glutaredoxin 1410620:1410883 reverse	-1,88
	EAM_1731		putative 1-phosphatidylinositol phosphodiesterase 1863865:1865253 reverse	-2,28
3. Metabolism				
Up-regulated	EAM_1711		putative dethiobiotin synthetase 1842752:1843414 forward	18,04

	EAM_1064	<i>adhP</i>	alcohol dehydrogenase, propanol-preferring 1167941:1168957 reverse	13,85
	EAM_3059	<i>nrdD</i>	anaerobic ribonucleoside-triphosphate reductase 3351789:3353926 forward	10,42
	EAM_1823	<i>pall</i>	sucrose isomerase 1962167:1963966 reverse	8,16
	EAM_3060	<i>nrdG</i>	anaerobic ribonucleoside-triphosphate reductase activating protein 3353923:3354387 forward	7,39
	EAM_1537	<i>fumA</i>	fumarate hydratase class I, aerobic 1656229:1657875 reverse	4,20
	EAM_1539		putative NADH:flavin oxidoreductase 1660144:1662921 forward	3,70
	EAM_1366	<i>pyrD</i>	dihydroorotate dehydrogenase 1489345:1490355 forward	3,23
	EAM_0111	<i>tpiA</i>	triosephosphate isomerase 139323:140090 reverse	2,85
	EAM_2054		putative nitroreductase (pseudogene) 2204436:2205189 forward	2,49
	EAM_2268		putative ferredoxin 2453569:2453829 forward	2,18
	EAM_0085	<i>wabM</i>	glycosyl transferase 114401:115381 reverse	2,15
	EAM_0404		putative histidyl-tRNA Synthetase 485742:487007 forward	2,14
	EAM_0897	<i>yafA</i>	esterase 1009328:1010575 forward	2,06
	EAM_1608	<i>astD</i>	N-succinylglutamate 5-semialdehyde dehydrogenase 1734773:1736242 forward	1,86
	EAM_1609	<i>astB</i>	N-succinylarginine dihydrolase 1736239:1737564 forward	1,77
	EAM_3045	<i>holC</i>	DNA polymerase III, Chi subunit 3334138:3334593 forward	1,76
	EAM_3471	<i>glmS</i>	glucosamine-fructose-6-phosphate aminotransferase [isomerizing] 3791363:3793192 reverse	1,72
	EAM_3268	<i>glgP</i>	glycogen phosphorylase 3558196:3560643 reverse	1,71
Down-regulated	EAM_1349	<i>smtA</i>	putative S-adenosylmethionine-dependent methyltransferase 1465505:1466293 forward	-1,69
	EAM_1994	<i>purT</i>	phosphoribosylglycinamide formyltransferase 2 2139459:2140637 forward	-1,70
	EAM_0638	<i>rob</i>	right origin-binding protein 714915:715799 reverse	-1,72
	EAM_1860		putative translation initiation factor 2000735:2001061 reverse	-1,75
	EAM_2525	<i>srmB</i>	ATP-dependent RNA helicase 2758936:2760264 forward	-1,81

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EAM_0654	<i>ribF</i>	riboflavin biosynthesis protein [includes: riboflavin kinase; FMN adenyltransferase] 733385:734323 forward	-1,83
EAM_2309		putative NUDIX-family hydrolase 2506142:2506672 reverse	-1,83
EAM_1861	<i>pyrF</i>	orotidine 5'-phosphate decarboxylase 2001061:2001777 reverse	-1,86
EAM_0653	<i>rpsT</i>	30S ribosomal protein S20 732806:733069 reverse	-1,88
EAM_3481	<i>atpI</i>	ATP synthase protein I 3801770:3802150 reverse	-1,91
EAM_2193	<i>thiM</i>	hydroxyethylthiazole kinase 2360346:2361137 reverse	-1,94
EAM_1259	<i>hutG</i>	N-formylglutamate amidohydrolase 1370377:1371165 forward	-1,95
EAM_2295	<i>yfbR</i>	putative nucleoside 5'-monophosphate phosphohydrolase 2485611:2486207 forward	-1,95
EAM_2235	<i>rsuA</i>	ribosomal small subunit pseudouridine synthase A 2408522:2409226 reverse	-1,98
EAM_0109	<i>mgo</i>	malate:quinone oxidoreductase 137364:138926 reverse	-1,98
EAM_0660	<i>carA</i>	carbamoyl-phosphate synthase small chain 740642:741790 forward	-2,00
EAM_0960	<i>gtrA</i>	bactoprenol-linked glucose translocase 1069084:1069449 reverse	-2,05
EAM_1989	<i>holE</i>	DNA polymerase III, theta subunit 2134658:2134888 forward	-2,05
EAM_2441	<i>purM</i>	phosphoribosylformylglycinamide cyclo-ligase 2664109:2665149 forward	-2,09
EAM_0068	<i>pyrE</i>	orotate phosphoribosyltransferase 99381:100022 forward	-2,10
EAM_3141	<i>accB</i>	biotin carboxyl carrier protein of acetyl-CoA carboxylase 3440869:3441339 forward	-2,11
EAM_1258	<i>hutI</i>	imidazolonepropionase 1369163:1370380 forward	-2,15
EAM_1451	<i>rIuC</i>	ribosomal large subunit pseudouridine synthase C 1576340:1577302 forward	-2,17
EAM_1238	<i>idi</i>	putative isopentenyl-diphosphate delta-isomerase 1349046:1349591 forward	-2,23
EAM_3275	<i>gntK</i>	thermo-resistant gluconokinase 3570673:3571221 reverse	-2,24
EAM_2254		putative 4'-phosphopantetheinyl transferase 2431436:2432164 forward	-2,25
EAM_0239	<i>thiE</i>	thiamine-phosphate pyrophosphorylase 299897:300541 reverse	-2,28
EAM_3140	<i>aroQ</i>	3-dehydroquinate dehydratase 3440395:3440847 forward	-2,33

EAM_0159	<i>rep</i>	ATP-dependent DNA helicase 196638:198656 forward	-2,36
EAM_3054	<i>pyrB</i>	aspartate carbamoyltransferase catalytic subunit 3344444:3345379 forward	-2,40
EAM_1254	<i>hutH</i>	histidine ammonia-lyase 1364602:1366149 reverse	-2,40
EAM_3339		putative FAD-binding oxidoreductase 3640833:3641795 reverse	-2,43
EAM_0240	<i>thiC</i>	thiamine biosynthesis protein 300538:302472 reverse	-2,46
EAM_0663	<i>folA</i>	dihydrofolate reductase 745970:746449 forward	-2,61
EAM_1473	<i>ndh</i>	NADH dehydrogenase 1595884:1597188 forward	-3,69
EAM_2305	<i>puuE</i>	4-aminobutyrate aminotransferase 2501548:2502810 forward	-4,85

4. Motion

Up-regulated: 0

Down-regulated	EAM_2587	<i>flhA</i>	flagellar biosynthesis protein 2818272:2820377 forward	-1,77
	EAM_2557	<i>flgM</i>	negative regulator of flagellin synthesis (anti-sigma-28 factor) 2790859:2791158 forward	-1,83

5. Regulation

Up-regulated	EAM_3314		LuxR-family transcriptional regulator 3615272:3615871 reverse	4,16
Down-regulated	EAM_1915	<i>hns</i>	DNA-binding protein (histone-like protein Hlp-II) 2058982:2059389 forward	-1,70
	EAM_0871		putative transcriptional regulator 984838:985677 forward	-1,80
	EAM_1247	<i>mlrA</i>	MerR-family transcriptional regulator 1356353:1357084 reverse	-1,81
	EAM_1756		AraC-family transcriptional regulator 1894757:1895098 forward	-1,84
	EAM_0771		transcriptional regulator 870365:871114 reverse	-2,02
	EAM_3055	<i>pyrI</i>	aspartate carbamoyltransferase regulatory subunit 3345392:3345856 forward	-2,07
	EAM_1706	<i>tus</i>	DNA replication terminus site-binding protein 1836836:1837765 reverse	-2,08
	EAM_0317	<i>soxR</i>	redox-sensitive transcriptional activator (MerR-family transcriptional regulator) 377056:377523	-2,10

Effect of plasmids in *E. amylovora*

			forward	
	EAM_3348		AraC-family transcriptional regulator 3648728:3649060 reverse	-2,24
	EAM_2929	<i>ascG</i>	LacI-family transcriptional regulator 3192132:3193157 reverse	-2,33
	EAM_0746	<i>pdhR</i>	pyruvate dehydrogenase complex repressor (GntR- family transcriptional regulator) 839648:840412 forward	-2,62
	EAM_2923		AsnC-family transcriptional regulator 3188075:3188560 forward	-3,08
	EAM_0942		AraC-family transcriptional regulator 1047597:1048664 forward	-3,13
	EAM_0780	<i>rcsV</i>	LuxR-family transcriptional regulator 880258:880935 forward	-150,80

6. Stress response

Up-regulated: 0

Down-regulated	EAM_0650	<i>dnaJ</i>	chaperone protein (heat shock protein J) 729768:730913 forward	-1,69
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7. Cell surface and transmembrane area

Up-regulated	EAM_2958		putative membrane protein 3223293:3223949 forward	3,42
Down-regulated	EAM_0850	<i>gmhB</i>	D,D-heptose 1,7-bisphosphate phosphatase 956438:956998 forward	-1,70
	EAM_1885		putative membrane protein 2027219:2027956 forward	-1,70
	EAM_0079	<i>waaQ</i>	putative heptosyl transferase 107859:108872 forward	-1,75
	EAM_0218	<i>murB</i>	UDP-N-acetylenolpyruvoylglucosamine reductase 271427:272464 forward	-1,75
	EAM_0959		bactoprenol glucosyl transferase 1068158:1069087 reverse	-1,83
	EAM_0083		putative lipopolysaccharide biosynthesis protein 112327:113283 forward	-1,85
	EAM_3371		putative membrane protein 3675954:3676526 reverse	-1,86
	EAM_2790	<i>gcvH</i>	glycine cleavage system H protein 3055238:3055624 reverse	-1,86
	EAM_0235		fimbrial protein 295451:296227 forward	-1,89

EAM_0275		putative membrane protein 342232:342621 reverse	-2,05
EAM_0322		putative membrane protein 382921:383349 forward	-2,19
EAM_0921		putative membrane protein 1027870:1028526 reverse	-2,21
EAM_3127	<i>aaeX</i>	putative membrane protein 3423440:3423643 reverse	-2,47
EAM_3009	<i>ampH</i>	penicillin-binding protein 3294268:3295407 forward	-2,60

8. Transport of ions, sugars, amino acids y nucleotides

Up-regulated	EAM_1538	<i>ttdT</i>	putative tartrate transporter (tartrate/succinate antiporter) 1658056:1659567 reverse	6,10
	EAM_0347		putative amino acid efflux protein 415122:415748 reverse	2,20
	EAM_1712		major facilitator superfamily protein 1843839:1845284 forward	1,75
Down-regulated	EAM_2207	<i>mglB</i>	galactoside ABC transporter, substrate-binding protein 2374600:2375595 reverse	-1,72
	EAM_1333		major facilitator superfamily protein 1447873:1449024 forward	-1,74
	EAM_2601		major facilitator superfamily protein 2832086:2833270 forward	-1,76
	EAM_2847		probable Fe(2+)-trafficking protein 3110254:3110526 forward	-1,79
	EAM_2385		sodium bile acid symporter family protein 2584209:2585234 forward	-1,79
	EAM_1466	<i>ptsG</i>	glucose-specific PTS system, IIBC component 1588960:1590393 forward	-1,80
	EAM_0006	<i>kup</i>	low affinity potassium transport system protein 6718:8586 forward	-1,83
	EAM_1965		putative transporter 2110783:2112336 reverse	-1,92
	EAM_2410	<i>ansP</i>	L-asparagine permease 2609060:2610508 reverse	-1,94
	EAM_3274	<i>gntU</i>	low-affinity gluconate transporter 3569336:3570676 reverse	-1,99
	EAM_1294	<i>sdaC</i>	serine transporter 1405569:1406852 reverse	-2,02
	EAM_0040		putative purine permease 68539:69927 reverse	-2,03
	EAM_0117		putative transport protein 144825:145826 forward	-2,15
	EAM_0858		putative cytosine/purines permease 969460:970956 reverse	-2,36

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EAM_1276		putative efflux pump HlyD-family protein 1386833:1387966 forward	-2,52
EAM_2308		putative ABC transporter, permease protein 2505251:2506078 forward	-2,61
EAM_2307		putative ABC transporter, permease protein 2503972:2505240 forward	-2,64
EAM_0015	<i>rbsD</i>	high affinity ribose transport protein 33153:33572 forward	-2,68
EAM_3126	<i>aaeA</i>	p-hydroxybenzoic acid efflux pump subunit 3422500:3423432 reverse	-2,76
EAM_2306		putative ABC transporter, substrate-binding protein 2502887:2503912 forward	-3,15

9. TTSS and effectors (secreted protein, general secretory pathways)

Up-regulated: 0

Down-regulated	EAM_2924	putative exported protein 3188657:3188989 forward	-1,70
	EAM_2864	putative exported protein 3128342:3129058 reverse	-1,85
	EAM_3364	<i>prtF</i> type I secretion system protein 3667942:3669330 reverse	-1,85
	EAM_0518	putative exported protein 603553:603786 forward	-2,02
	EAM_3363	putative exported protein 3667597:3667818 forward	-2,04
	EAM_0538	putative exported endonuclease 613308:613832 reverse	-2,07
	EAM_2873	<i>hrpW</i> putative pectate lyase 3142192:3143535 forward	-2,14

10. Hypothetical proteins

Up-regulated	EAM_2194	conserved hypothetical protein 2361533:2361742 reverse	10,70
	EAM_0488	hypothetical protein 576497:576706 reverse	7,69
	EAM_2242	hypothetical protein 2416570:2416698 reverse	5,25
	EAM_1111	hypothetical protein 1210519:1210626 forward	3,69
	EAM_1540	conserved hypothetical protein 1662994:1663962 forward	2,50
	EAM_2269	conserved hypothetical protein 2453903:2454160 forward	2,13
	EAM_0402	hypothetical protein 483968:484549 forward	2,11
	EAM_0914	conserved hypothetical protein 1021480:1021683	1,96

		reverse	
	EAM_3020	conserved hypothetical protein 3303920:3304240 reverse	1,79
Down-regulated	EAM_2767	hypothetical protein 3027440:3027562 reverse	-1,69
	EAM_3347	hypothetical protein 3648434:3648628 reverse	-1,70
	EAM_0999	conserved hypothetical protein 1108352:1109047 reverse	-1,72
	EAM_3436	hypothetical protein 3754307:3754564 reverse	-1,72
	EAM_1743	conserved hypothetical protein 1877732:1878181 reverse	-1,73
	EAM_1792	hypothetical protein 1936074:1936223 reverse	-1,76
	EAM_3370	hypothetical protein 3675763:3675927 forward	-1,77
	EAM_3209	<i>slyX</i> conserved hypothetical protein 3494648:3494866 forward	-1,84
	EAM_1121	conserved hypothetical protein 1215870:1216133 reverse	-1,85
	EAM_0732	hypothetical protein 820394:820567 reverse	-1,86
	EAM_2908	<i>hsvC</i> conserved hypothetical protein 3170343:3171587 reverse	-1,90
	EAM_0705	conserved hypothetical protein 795545:796060 reverse	-1,98
	EAM_1978	conserved hypothetical protein 2125051:2126487 forward	-1,98
	EAM_0470	conserved hypothetical protein 561129:561488 forward	-2,00
	EAM_1969	conserved hypothetical protein 2114483:2115493 reverse	-2,09
	EAM_0884	hypothetical protein 996297:996449 forward	-2,13
	EAM_1528	conserved hypothetical protein 1647524:1648342 reverse	-2,15
	EAM_0956	conserved hypothetical protein 1064849:1065430 reverse	-2,20
	EAM_1472	conserved hypothetical protein 1594250:1595338 forward	-2,21
	EAM_0149	conserved hypothetical protein 184912:185250 forward	-2,24
	EAM_1791	hypothetical protein 1935846:1936058 reverse	-2,27
	EAM_0110	hypothetical protein 139070:139354 forward	-2,30

Effect of plasmids in *E. amylovora*

EAM_0781	hypothetical protein 880999:881187 forward	-2,40
EAM_1502	hypothetical protein 1621979:1622128 forward	-2,95
EAM_1068	hypothetical protein 1171936:1172094 forward	-3,22

11. Others

Up-regulated	EAM_0029	<i>hemN</i>	oxygen-independent coproporphyrinogen III oxidase 54899:56272 forward	9,16
	EAM_0403		putative methyltransferase 484646:485701 forward	3,05
Down-regulated	EAM_3439		putative carbohydrate kinase 3757692:3758645 forward	-1,71
	EAM_0855		putative methyltransferase 967194:967895 forward	-1,72
	EAM_0001	<i>mioC</i>	putative flavoprotein 32:472 reverse	-1,77
	EAM_0852		putative methyltransferase (pseudogene) 964158:964886 forward	-1,83
	EAM_1990	<i>exoX</i>	exodeoxyribonuclease X 2135034:2135699 forward	-1,86
	EAM_2991		putative aromatic ring-opening dioxygenase 3273365:3274189 reverse	-1,87
	EAM_0067	<i>rph</i>	ribonuclease PH 98609:99325 forward	-2,00
	EAM_0230		pentapeptide repeat protein 289423:290022 reverse	-2,00
	EAM_0941		putative phosphotransferase/kinase 1046438:1047442 forward	-2,08
	EAM_3438		putative sugar isomerase 3756939:3757688 forward	-2,14
	EAM_1257		putative chlorohydrolase 1367726:1369081 reverse	-2,20
	EAM_3340		putative (2Fe-2S) protein 3641804:3642838 reverse	-2,67

Table 5. Genes grouped into eleven categories according to the functions described for each gene in presence of plasmid pE170.

Functional category	Locus tag	Gene symbol	Protein description and gene position in the chromosome	Fold change
1. Mobile elements and phages				
Up-regulated: 0				
Down-regulated	EAM_1525		putative phage holin 1646185:1646454 forward	-1,79
	EAM_2457		putative transposase 2684184:2684882 reverse	-1,90
2. Interaction: host, microorganism and environment				
Up-regulated	EAM_0510		putative carbonic anhydrase 594172:594798 forward	1,87
	EAM_0738		putative cytotoxic necrotizing factor 826310:828832 reverse	1,83
	EAM_2971		non-ribosomal peptide synthetase 3238785:3240053 forward	1,82
Down-regulated	EAM_2425		Rhs-family protein 2647129:2648514 forward	-1,75
	EAM_2183	<i>mdtA</i>	multidrug resistance protein 2345788:2347005 forward	-1,89
	EAM_2412		putative nitrate/nitrite responsive two-component sensor kinase 2611461:2612453 forward.	-2,42
3. Metabolism				
Up-regulated	EAM_0507	<i>fadH</i>	2,4-dienoyl-CoA reductase [NADPH] (2,4-dienoyl coenzyme A reductase) 591030:593039 reverse	3,18
	EAM_0890	<i>fadE</i>	acyl-CoA dehydrogenase 1000788:1003232 reverse	3,09
	EAM_2048		putative aldehyde dehydrogenase 2197402:2198922 reverse	2,82
	EAM_0213	<i>fadB</i>	fatty oxidation complex subunit alpha [includes: enoyl-CoA hydratase; delta(3)-cis-delta(2)-trans-enoyl-CoA isomerase; 3-hydroxyacyl-CoA dehydrogenase; 3 hydroxybutyryl-CoA epimerase] 258586:260772 reverse	2,73
	EAM_0327	<i>acs</i>	acetyl-coenzyme A synthetase 386917:388872 reverse	2,54
	EAM_0198	<i>metE</i>	5-methyltetrahydropteroyltrimethylglutamate--homocysteine methyltransferase 242743:245016 forward	2,52
	EAM_0135	<i>metF</i>	5,10 methylenetetrahydrofolate reductase 162385:163290 forward	2,50

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EAM_0212	<i>fadA</i>	3-ketoacyl-CoA thiolase (fatty oxidation complex beta subunit) 257412:258575 reverse	2,19	
EAM_2126		putative aminotransferase 2279510:2280478 forward	2,04	
EAM_1533	<i>treA</i>	periplasmic trehalase 1651353:1653029 reverse	2,04	
EAM_2268		putative ferredoxin 2453569:2453829 forward	2,00	
EAM_2125		putative phenylacetate-coenzyme A ligase 2278101:2279510 forward	2,00	
EAM_3053	<i>argI</i>	ornithine carbamoyltransferase chain I 3343140:3344150 forward	1,94	
EAM_2597	<i>nrdF</i>	ribonucleoside-diphosphate reductase 2 beta chain 2827123:2828082 forward	1,91	
EAM_0140	<i>argH</i>	argininosuccinate lyase 169902:171275 forward	1,91	
EAM_0139	<i>argG</i>	argininosuccinate synthase 168595:169809 forward	1,88	
EAM_2267	<i>nrdB</i>	ribonucleoside-diphosphate reductase 1 beta subunit 2452436:2453566 forward	1,88	
EAM_1242		putative cyclopropane-fatty-acyl-phospholipid synthase 1351970:1353214 reverse	1,84	
EAM_3251	<i>pckA</i>	phosphoenolpyruvate carboxykinase 3536473:3538092 forward	1,82	
EAM_0133	<i>metB</i>	cystathionine gamma-synthase 158533:159693 forward	1,76	
EAM_3227	<i>argD</i>	acetylornithine/succinyldiaminopimelate aminotransferase 3513123:3514340 reverse	1,76	
EAM_2596	<i>nrdE</i>	ribonucleoside-diphosphate reductase 2 alpha chain 2824921:2827071 forward	1,73	
EAM_3477	<i>atpH</i>	ATP synthase delta subunit 3799528:3800061 reverse	1,72	
EAM_3111	<i>gltA</i>	putative glutamate synthase [NADPH] large subunit 3402866:3408400 forward	1,69	
Down-regulated	EAM_2235	<i>rsuA</i>	ribosomal small subunit pseudouridine synthase A 2408522:2409226 reverse	-1,69
	EAM_0881		putative carbon-nitrogen hydrolase 993462:994229 reverse	-1,72
	EAM_1195	<i>galM</i>	aldose 1-epimerase 1303157:1304191 reverse	-1,72
	EAM_1196	<i>galk</i>	galactokinase 1304185:1305333 reverse	-1,75
	EAM_1259	<i>hutG</i>	N-formylglutamate amidohydrolase 1370377:1371165 forward	-1,77
	EAM_3275	<i>gntK</i>	thermoresistant gluconokinase 3570673:3571221 reverse	-1,78
	EAM_2295	<i>yfbR</i>	putative nucleoside 5'-monophosphate phosphohydrolase 2485611:2486207 forward	-1,93
	EAM_2254		putative 4'-phosphopantetheinyl transferase 2431436:2432164 forward	-2,00

	EAM_2222	<i>fruK</i>	1-phosphofructokinase 2393746:2394687 reverse	-2,03
	EAM_1258	<i>hutI</i>	imidazolonepropionase 1369163:1370380 forward	-2,16
	EAM_1197	<i>galT</i>	galactose-1-phosphate uridylyltransferase 1305330:1306382 reverse	-2,37
	EAM_3340		putative (2Fe-2S) protein 3641804:3642838 reverse	-2,62
	EAM_3439		putative carbohydrate kinase 3757692:3758645 forward	-2,68
	EAM_3265	<i>glpD</i>	aerobic glycerol-3-phosphate dehydrogenase 3554700:3556205 forward	-2,91

4. Motion

Up-regulated: 0

Down-regulated	EAM_2559	<i>fliT</i>	flagellar protein 2791822:2792160 reverse	-1,78
	EAM_2548	<i>flgI</i>	flagellar P-ring protein 2783558:2784679 reverse	-1,85
	EAM_2556	<i>flgA</i>	flagella basal body P-ring formation protein 2790082:2790786 forward	-2,23

5. Regulation

Up-regulated	EAM_2487		putative transcriptional regulator 2716872:2717564 reverse	3,16
Down-regulated	EAM_2961		AraC-family transcriptional regulator 3227136:3228053 reverse	-1,71
	EAM_3307		putative phosphosugar-binding RpiR-family transcriptional regulator 3605184:3606035 forward	-1,91

6. Stress response

Up-regulated	EAM_1318	<i>cspD</i>	cold shock-like protein 1429606:1429833 reverse	1,90
	EAM_1612	<i>spy</i>	putative exported protein (spheroplast protein Y) 1738950:1739438 forward	1,68
Down-regulated	EAM_2039	<i>umuD</i>	protein UmuD 2187688:2188107 forward	-1,71
	EAM_1297		glutaredoxin 1410620:1410883 reverse	-1,75
	EAM_1805	<i>asr</i>	acid shock protein 1944204:1944632 forward	-4,50

7. Cell surface and transmembrane area

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Up-regulated	EAM_0326		putative membrane protein 386443:386754 reverse	3,23
	EAM_1241		putative lipoprotein 1351358:1351891 reverse	2,15
	EAM_1390		putative membrane protein 1515578:1516234 reverse	2,12
	EAM_0565		putative membrane protein 631136:631369 forward	1,75
Down-regulated	EAM_1808		putative N-acetylmuramoyl-L-alanine amidase 1945815:1946684 forward	-1,75
	EAM_0945		putative lipoprotein 1050058:1050408 forward	-2,39
	EAM_3127	<i>aaeX</i>	putative membrane protein 3423440:3423643 reverse	-2,56

8. Transport of ions, sugars, amino acids y nucleotides

Up-regulated	EAM_0017	<i>rbsC</i>	ribose transport system, permease protein 35088:36056 forward	3,78
	EAM_0325	<i>actP</i>	cation/acetate symporter 384788:386446 reverse	3,39
	EAM_2047		putative permease 2195797:2197212 reverse	2,97
	EAM_0018	<i>rbsB</i>	D-ribose-binding periplasmic protein 36206:37087 forward	2,83
	EAM_0511		putative sulfate transporter 594795:596273 forward	2,62
	EAM_0016	<i>rbsA</i>	ribose transport system, ATP-binding protein 33580:35085 forward	2,52
	EAM_3464	<i>pstB</i>	phosphate ABC transporter, ATP-binding protein 3783746:3784516 reverse	2,27
	EAM_2205	<i>mgIC</i>	galactoside ABC transporter, permease protein 2371995:2373005 reverse	2,26
	EAM_2348	<i>fadL</i>	long-chain fatty acid transport protein 2546763:2548097 forward	2,17
	EAM_1136	<i>gltI</i>	glutamate/aspartate ABC transporter, substrate-binding protein 1230897:1231826 reverse	2,13
	EAM_1134	<i>gltK</i>	glutamate/aspartate ABC transporter, permease protein 1229189:1229866 reverse	2,11
	EAM_3416	<i>mtIA</i>	mannitol-specific PTS system, EIICBA components 3734654:3736585 forward	2,09
	EAM_3391	<i>dppD</i>	dipeptide ABC transporter, ATP-binding protein 3708151:3709137 reverse	1,94
	EAM_3287	<i>livG</i>	high-affinity branched-chain amino acid ABC transporter, ATP-binding protein 3582565:3583332 reverse	1,91
	EAM_3417	<i>mtID</i>	mannitol-1-phosphate dehydrogenase 3736683:3737831 forward	1,81
	EAM_3390	<i>dppF</i>	dipeptide ABC transporter, ATP-binding protein 3707160:3708161 reverse	1,79

	EAM_3380	<i>dctA</i>	C4-dicarboxylate transport protein 3689794:3691068 reverse	1,78
	EAM_3290	<i>livJ</i>	high-affinity branched-chain amino acid ABC transporter, substrate-binding protein 3585611:3586726 reverse	1,77
	EAM_2130		putative LysE-type translocator 2282672:2283298 forward	1,75
	EAM_0015	<i>rbsD</i>	high affinity ribose transport protein 33153:33572 forward	1,70
Down-regulated	EAM_3274	<i>gntU</i>	low-affinity gluconate transporter 3569336:3570676 reverse	-1,73
	EAM_1577		putative sulphate transporter 1701451:1702935 forward	-1,98
	EAM_2221	<i>fruA</i>	fructose-specific PTS system, EIIBC component 2392038:2393729 reverse	-2,13
	EAM_0058		putative mobilization protein 88526:89119 reverse	-2,15
	EAM_2601		major facilitator superfamily protein 2832086:2833270 forward	-2,27
	EAM_1597	<i>scrY</i>	sucrose-specific outer membrane porin 1719514:1721058 reverse	-2,46
	EAM_3125	<i>aaeB</i>	p-hydroxybenzoic acid efflux pump subunit 3420528:3422486 reverse	-2,71
	EAM_1708		major facilitator superfamily protein 1838964:1840232 reverse	-2,94
	EAM_0873		ABC transporter, permease protein 986725:987390 forward	-2,96
	EAM_3126	<i>aaeA</i>	p-hydroxybenzoic acid efflux pump subunit 3422500:3423432 reverse	-3,06

9. TTSS and effectors (secreted protein, general secretory pathways)

Up-regulated: 0

Down-regulated	EAM_2904	<i>hrcT</i>	type III secretion system protein 3167467:3168264 forward	-1,71
	EAM_3364	<i>prtF</i>	type I secretion system protein 3667942:3669330 reverse	-2,09
	EAM_1853		putative exported protein 1993462:1994118 forward	-2,48

10. Hypothetical proteins

Up-regulated	EAM_2269		conserved hypothetical protein 2453903:2454160 forward	2,18
	EAM_1243		conserved hypothetical protein 1353218:1353931 reverse	1,85
Down-regulated	EAM_3400		hypothetical protein 3718052:3718282 reverse	-1,70
	EAM_0867		conserved hypothetical protein 981030:981218 reverse	-1,72

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	EAM_0924	hypothetical protein 1029526:1029705 forward	-1,72
	EAM_0705	conserved hypothetical protein 795545:796060 reverse	-1,79
	EAM_1978	conserved hypothetical protein 2125051:2126487 forward	-1,85
	EAM_1791	hypothetical protein 1935846:1936058 reverse	-1,85
	EAM_2888	hypothetical protein 3153170:3153346 reverse	-1,87
	EAM_1725	conserved hypothetical protein 1857801:1858553 forward	-1,94
	EAM_1578	hypothetical protein 1702932:1703072 forward	-1,99
	EAM_2767	hypothetical protein 3027440:3027562 reverse	-2,00
	EAM_2764	conserved hypothetical protein (partial) 3026266:3026421 reverse	-2,08
	EAM_0601	hypothetical protein 673711:674190 forward	-2,23
	EAM_0884	hypothetical protein 996297:996449 forward	-2,50
	EAM_1806	hypothetical protein 1944647:1944802 forward	-6,83
<hr/>			
11. Others			
<hr/>			
Up-regulated	EAM_2488	putative lyase 2717637:2718557 reverse	4,62
	EAM_2486	putative proline racemase 2715938:2716867 reverse	2,27
	EAM_0403	putative methyltransferase 484646:485701 forward	1,95
	EAM_3477	ATP synthase delta subunit 3799528:3800061 reverse	1,71
Down-regulated	EAM_0948	<i>sbcD</i> exonuclease subunit D 1054467:1055690 reverse	-1,70
	EAM_1257	putative chlorohydrolase 1367726:1369081 reverse	-1,85
	EAM_3438	putative sugar isomerase 3756939:3757688 forward	-3,05
<hr/>			

Table 6. Differentially expressed chromosomal genes in presence of pEA29 or pEI70 in exudates of immature pear of *E. amylovora* strain CFBP 1430 with fold change $\geq 1,6$ / $\leq -1,6$ of genes showing a P value $< 0,05$.

Locus tag	Fold change pEA29	Fold change pEI70	Gene	Protein description
EAM_0015	-2,6	1,6	<i>rbsD</i>	ribose pyranase
EAM_0403	3,1	1,9		putative methyltransferase
EAM_2268	2,2	1,8		putative ferredoxin
EAM_2269	2,1	2,2		conserved hypothetical protein
EAM_0705	-2	-1,8		conserved hypothetical protein
EAM_0884	-2,1	-2,5		hypothetical protein
EAM_1257	-2,2	-1,9		putative chlorohydrolase
EAM_1258	-2,1	-2,2	<i>hutI</i>	3-(5-oxo-4,5-dihydro-3H-imidazol-4-yl)propanoate amidohydrolase
EAM_1259	-2	-1,8	<i>hutG</i>	N-formyl-L-glutamate amidohydrolase
EAM_1297	-1,9	-1,8		glutaredoxin
EAM_1525	-1,7	-1,8		putative phage holin
EAM_1791	-2,3	-1,8		hypothetical protein
EAM_1978	-2	-1,8		conserved hypothetical protein
EAM_2235	-2	-1,7	<i>rsuA</i>	pseudidine synthase
EAM_2254	-2	-2,2		putative 4'-phosphopantetheinyl transferase
EAM_2295	-2	-2	<i>yfbR</i>	5'-deoxynucleotidase
EAM_2412	-1,7	-2,4		putative nitrate/nitrite responsive two-component sensor kinase
EAM_2457	-1,8	-1,9	<i>flgM</i>	flagellar synthesis regulation
EAM_2601	-1,8	-2,3		major facilitator superfamily protein
EAM_2767	-1,7	-2		hypothetical protein
EAM_3126	-2,8	-3,1	<i>aaeA</i>	component of hydroxylated aromatic carboxylic acid efflux transporter
EAM_3127	-2,5	-2,6	<i>aaeX</i>	putative membrane protein of efflux system
EAM_3274	-2	-1,7	<i>gntU</i>	transporter for gluconate
EAM_3275	-2	-1,8	<i>gntK</i>	thermoresistant gluconokinase
EAM_3340	-2,7	-2,6		putative (2Fe-2S) protein
EAM_3364	-1,9	-2,1	<i>prtF</i>	type I secretion outer membrane protein
EAM_3438	-2,1	-3,1		putative sugar isomerase
EAM_3439	-1,7	-2,7		putative carbohydrate kinase

Functional analyses

From the results of differentially expressed genes a functional analysis based on the terms of the Gene Ontology (GO) to elucidate the influence of the presence of each plasmid in the bacterium was conducted. In this analysis 3915 mapped sequences (between chromosome and plasmid sequences) were included, 808 of which could not be assigned to any GO term.

The ontology covers three domains: a) cellular component, b) molecular function and c) biological process.

In the cellular component domain five GO terms were upregulated in CFBP 1430 and none was downregulated. 15 GO terms were upregulated and 2 downregulated in presence of plasmid pEI70 (see GOgraphs and detailed information in Fig. 5-S1 and Table 5-S1 in supplementary information at the end of this memory).

In molecular function domain 17 GO terms were upregulated and other 17 were downregulated in presence of plasmid pEA29. In this domain and in presence of pEI70 89 GO terms were upregulated and 3 downregulated (see GOgraphs and detailed information in Fig. 5-S2 and Table 5-S2 in supplementary information at the end of this memory).

In biological process domain 35 GO term were upregulated and 45 downregulated, and in presence of pEI70 135 GO term were upregulated and 10 downregulated (Fig. 7) (see GOgraphs and detailed information in Fig. 5-S1, Fig. 5-S2 and Fig. 5-S3 in supplementary information at the end of this memory).

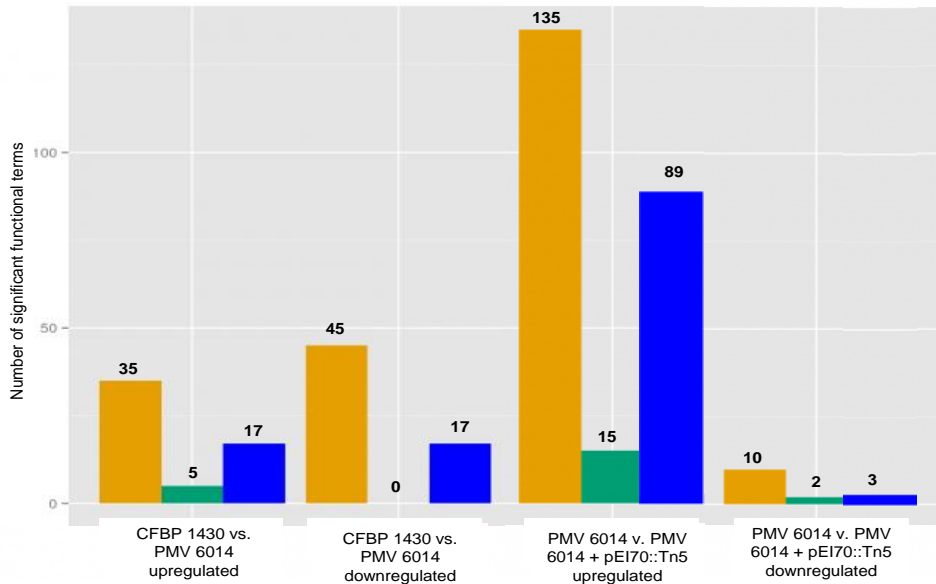


Fig. 7. Functional analysis based on GO terms. In ochre cellular component domain; in green molecular function domain and in blue biological process domain.

Discussion

Microarray-based gene expression profiling has been recognized as a powerful tool to observe global gene expression and the physiological processes involved in response to a particular stimulus (Maleck *et al.*, 2000; Schenk *et al.*, 2000). Many investigations has been conducted in the last years to elucidate the

virulence factors that intervene in the disease produced by *E. amylovora*, and among them, the T3SS, the amylovoran, levan and protease genes, and genes involved in sorbitol and sucrose metabolism have been described (Oh and Beer, 2005; Oh *et al.*, 2005; Zhao *et al.*, 2011; Zhao *et al.*, 2009a, b; Bogs and Geider, 2000; Smits and Duffy, 2011). Other works have focused on the expression of these genes in the interaction with the plant, but still many aspects of the disease are unknown. All the pathogenicity determinants are situated in the chromosome, and only plasmids pEA29 and pEI70 have been reported to play a role in symptoms development in pear assays (McGhee and Jones, 2000; Llop *et al.*, 2011). In the case of pEA29, the thiamine genes, related to amylovoran and cell wall synthesis, have been described as a possible explanation of its effect in virulence. Other genes in pEA29 with a possible effect on disease have been proposed (*msrA*, *betT*), but they have not demonstrated their effect in virulence (McGhee and Jones, 2000). Plasmid pEI70 has a similar effect in virulence increase in pear assays, but its sequence has not shown any gene related to pathogenicity (Llop *et al.*, 2011). There is a lack of knowledge of the way these plasmids play a role in pathogenicity, and in this work, a research to address the effect they produce on the host bacterium has been performed. Differential expression analyses of the chromosomal genes during plant-pathogen interaction have been performed using strain CFBP 1430 that harbors pEA29, and the same strain cured of the plasmid. This cured strain was used to introduce plasmid pEI70, and the same differential expression analysis was performed compared to the plasmidless strain. To perform these studies, a microarray that has been already employed in previous works on the effect of specific genes in the regulation of pathogenicity genes of *E. amylovora* (McNally *et al.*, 2011; Wang *et al.*, 2012; Águila-Clares *et al.*, 2016) was used.

Although the results obtained in both comparison experiments need the confirmation via qRT-PCR analysis, the data provided is giving a first glimpse on

the possible influence that each plasmid has on the virulence of *E. amylovora*. To validate the gene expression data from the microarrays, qRT-PCRs will be carried out to confirm the values of differential expression some of the of CFBP 1430 chromosomal genes of interest. Total RNA will be reversed-transcribed to cDNA. Vector NTI would be used to obtain the optimal oligo sequences for qRT-PCR. Cycle threshold (Ct) will be determined using an endogenous control. With these data, a relative quantification value will be calculated for each differential expressed gene and compared with the microarray.

The analysis of strain harboring plasmid pEA29 showed it increases the expression of 38 genes while down regulates 142. From all them, metabolism category showed the highest number of genes differentially expressed (48, 27% of total genes), followed by hypothetical proteins (34, 19%) and transport of several compounds (23, 13%). Is also in the metabolism category where more genes are upregulated (17) and nine in hypothetical proteins, while few other genes appear upregulated in the other functional categories.

The presence of plasmid pEI70 showed the same number of genes up and down regulated in the experiments (60 each). Similarly to pEA29, the majority of genes differentially expressed belong to metabolism (33, 27%) and transport (30, 25%), followed by hypothetical proteins (16, 13%), while the other categories are less common. Again, due to the high number of genes involved, is the metabolism category where more genes appear up and down regulated (22 and 11 respectively), and only 2 up and 14 down in hypothetical proteins. The genes differentially regulated with pEA29 or pEI70 are in accordance with the results obtained in symptoms development in experiments *in planta*. These data showed that strains harboring any of these plasmids overexpress genes related to metabolism, including the metabolic pathway of glycolysis, in order to obtain energy, which may be advantageous for the strains carrying them during the plant infection process. The annotation of pEI70 (Llop et al., 2011) predicted a

metabolic function for several of these genes, and suggested a function in optimization of the glycolysis pathway. This was observed in growth experiments, where the growth rates of several strains assayed in minimal medium with sucrose were significantly faster in strains with pEI70, compared to the same strains lacking the plasmid. This effect was not observed when these strains were grown in rich medium like LB, which would indicate that the glycolysis genes may play a role (data not shown). *In planta*, this may improve competence for degrading these compounds, increasing pathogen aggressiveness. The glycolysis in *E. amylovora* may be one of the bottlenecks in the bacterial growth and subsequent infection *in planta*. These genes are present also in plasmid pEB102 from the epiphytic *E. billingiae* (Kube *et al.*, 2010) and this suggests that the effect they provide are related to fitness more than a role in virulence.

There are three genes commonly overexpressed in the strain carrying the plasmid pEA29 or pEI70 (EAM_2268, EAM_2269, EAM_0403). One of these is a gene encoding a ferredoxin, which are iron-sulfur proteins involved in electron transport in certain metabolic reactions (Valentine, 1964). The electron transport chain consists of a series of proteins found in the plasma membrane of bacteria by biochemical reactions and they produce adenosine triphosphate (ATP), which is the energy compound used by living beings, therefore it can be deduced that the metabolism of the strain harbouring one of both plasmids is more intense. The other two genes overexpressed in both conditions correspond to a gene encoding a methyltransferase, which probably play a role in the regulation of gene expression and a gene encoding a highly conserved hypothetical protein.

The genes underexpressed with pEA29 are mainly related to synthesis of different amino acids, whereas the presence of pEI70 underexpress genes mostly related to galactose metabolism. The number of genes underexpressed

in presence of pEA29 was much higher (4.1% of the 5% of the differentially expressed genes) than with pEI70 (1.72% of the 3.44% of the differentially expressed genes), and therefore, the functional categories that were grouped were more diverse. The functional category termed "others" include genes involved in common cellular processes, followed by the category of regulation of gene expression and cell surface transmembrane proteins and systems secretion and effectors. The categories where more underexpressed genes were shown in presence of pEI70 were hypothetical proteins, metabolism, mobile elements and functional phages and the category "other".

Within the chromosomal genes differentially expressed with pEA29 in common with pEI70 compared to the strain without plasmids, there were 23 genes underexpressed in both cases, five of which correspond to hypothetical proteins, three encode proteins with very general functions (hydrolysis of substrates, isomerases and a protein of the electron transport chain), one of these related to the protection against oxidative stress, six of these genes were classified into the functional category of metabolism. In addition, four of these genes were classified into the category of transport of sugars, ions, amino acids and nucleotides (*gntU*, involved in transport of glucan; *aaeA*, related to the transport of aromatic carboxylic acids is part of the Krebs cycle, and another gene involved in transmembrane facilitated diffusion). Two of the genes fall into the category of mobile elements and phage and coding for holin (involved in cell wall degradation host) similar to those described in the phage and a transposase (Smits *et al.*, 2010). Another gene encodes a membrane protein (AAEX) and finally classified in the interaction with the host and/or medium (which is part of a system sensor kinase). Within the chromosomal genes differentially expressed in common is the gene *rhsD*, overexpressed with pEI70 and repressed in strain with pEA29. This gene encodes the protein RBS, a carrier protein with high affinity for ribose, used as source of carbon and energy.

The genes repressed with pEA29 in Gene expression category operate in the catabolism and transport of sugar arabinose, synthesis of pyrimidines and regulation of biofilms. As a special feature, gene *rcsV*, belonging to the LuxR family transcriptional regulator, similar to the only gene upregulated in this category, suffers an important decrease (150 fold change decrease). This gene is involved in the coordination expression of virulence factors, synthesis of antibacterial substances, transfer of plasmids and biofilm formation. Taken together, the genes up and downregulated in presence of pEA29 in the interaction with the plant follow the results obtained in other works although they were not studying the role of the plasmids (Kim and Beer, 1998; Zhao *et al.*, 2005, 2006; Wang *et al.*, 2012; McNally *et al.*, 2011; Pester *et al.*, 2012).

These results could provide a possible explanation of the advantages the plasmid genes produce on the bacterial host during the interaction with plant material (increase in energy production, repression of biofilm formation, lack of flagellar and stress response gene expression and EPS synthesis diminished regulated, sugar metabolism increased). Comparing to other works employing this technology looking at gene expression *in planta* and *in vitro* conditions with strains harboring pEA29, the number of genes expressed are similar (around 50-100 genes) (Wang *et al.*, 2012; McNally *et al.*, 2011). Only in the work of Zhao *et al.* (2005) where they studied the genes expressed in the interaction with immature pear near 400 genes (394) differentially expressed were observed. The majority of them (20%) were related to metabolism and another 19% to hypothetical or unknown genes. Other functional groups with genes expressed were transport (13%) and regulation (12%). These data are similar to the ones obtained in our results, but interestingly the genes involved are not the same as the ones we found in our experiment in the majority of cases, only six are coincident. This is logical because the expression of genes due to the interaction with the plant cannot be observed in our study, but only the genes expressed by

the presence of the plasmid. It has to be taken into account that in their study the technique employed was not a microarray but an IVET system that expands all over the genome but has some limitations over the microarray technology. The other works that were using the microarray performed studies limited to specific pathogenicity genes that were later mutated to see the effect in other genes (Wang *et al.*, 2012; McNally *et al.*, 2011). In another work using the same microarray to study the effect of a copper shock, the authors did not find a strict correlation between the microarray and the qPCR results, but they consider that the microarray supplied a global panorama (Águila-Clares *et al.*, 2016)

Our results indicate that plasmids pEA29 and with pEI70 could have an influence on the interaction with the plant and the effect is very probably more profound than only the expression of thiamine genes or other genes that have been proposed to have influence in virulence (McGhee and Jones, 2000).

In general, the effect that plasmid pEI70 produces on the expression of different genes of the bacterium during plant interaction agrees with a final phenotypic outcome of an increase in symptoms development. This is reflected in the different categories the GO groups the genes, and although many genes need further research to confirm the role they play, some of them are in accordance with previous works.

Although the results with the presence of pEI70 cannot be compared with these researches, we can observe that the number and category of genes differentially expressed with this new plasmid are similar to the ones appearing in presence of pEA29. In contrast, only 28 genes are coincident between the two plasmids, 3 upregulated and 24 down regulated, and only one gene showed different expression level, up with pEI70 and down with pEA29.


Collectively, our data show that: (i) in presence of pEA29 the chromosomal genes of *E. amylovora* related to the outer membrane and capsule polysaccharide involved in pathogenicity are activated. Also in metabolism and glycolysis, (ii) in presence of pEI70 groups related to transmembrane active transport, metabolism and glycolysis are activated and (iii) in both cases, genes related to the synthesis of flagella are repressed.

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Capítulo 6



Secuencia completa de los plásmidos pEPIR37 y pEPIR5 de *Erwinia piriflorinigrans* y papel del pEPIR37 en la virulencia del patógeno

Complete sequence of *Erwinia piriflorinigrans* plasmids pEPIR37 and pEPIR5 and role of pEPIR37 in pathogen virulence

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* S. Barbé has contributed in designing of experiments, performing plasmids isolation and labelling, sequence analyses and annotation of plasmids pEPIR37 and pEPIR5, analyses of plasmid content by extraction analyses, comparison with others plasmids, aggressiveness assays in pear fruits, cloning pEPIR37 in different strains of *Erwinia amylovora*, performing phylogenetics analyses with pEPIR37 sequences and writing the first version of the manuscript.

Abstract

Erwinia piriflorinigrans is a newly described pathogen causing necrosis of pear blossoms. Complete sequencing of the 37 Kb plasmid pEPIR37 common to 27 *E. piriflorinigrans* strains revealed homology to sequences of the ubiquitous plasmids pEA29 of the fire blight pathogen *E. amylovora*, plasmid pEP36 of *E. pyrifoliae*, plasmid pEJ30 of *Erwinia* sp. from Japan, and to genomic regions of the related *Rosaceae* epiphytic *Erwinia* species *E. tasmaniensis* and *E. billingiae*. A second 5.5 Kb cryptic plasmid pEPIR5, found in 12 *E. piriflorinigrans* strains, was also sequenced revealing mobilization and replication proteins with similarities to many small ColE1-type plasmids in *Erwinia* spp. and other enterobacteria. Functional analyses of pEPIR37 introduced into a strain of *E. amylovora* cured of pEA29 plasmid, which has a reduced virulence, showed a role in increasing symptoms development similar to that observed in *E. amylovora* carrying plasmid pEA29.

Introduction

Several *Erwinia* species have recently been described that have restricted geographic distribution, host-range, and/or virulence expression on European pear (*Pyrus communis*) and/or Asian pear (syn. Nashi pear; *P. pyrifolia*), relative to the broader host-range fire blight pathogen of pear and other *Rosaceae*, *Erwinia amylovora* (Palacio-Bielsa *et al.* 2012). Among them, *E. pyrifoliae* causes blight of Asian pear and has thus far been reported only from Korea and Japan (Kim *et al.* 1999; Geider *et al.* 2009), *Erwinia* sp. strain Ejp617 causes pear shoot blight (Park *et al.* 2011), and *E. uzenensis* causes bacterial black shoot disease of pear (Matsuura *et al.* 2011). *E. tasmaniensis* is a *Rosaceae* epiphyte, not known to be pathogenic to pear (Geider *et al.* 2006), but having genomic indications of

potential phytopathogenicity to unknown hosts (Kube *et al.* 2008 b), and *E. billingiae* (formerly *Pantoea agglomerans*, *Enterobacter agglomerans*) is a cosmopolitan epiphyte with no indications of phytopathogenicity (Mergaert *et al.* 1999; Kube *et al.* 2010). *E. piriflorinigrans* is one of the newest members of this cast, causing necrosis of pear flowers but no advancing symptoms into shoots, it does not affect other *Rosaceae* host plants and thus far it has only been reported in Spain (López *et al.* 2011).

Comparative genomic analysis of *E. amylovora*, *E. pyrifoliae*, *E. tasmaniensis*, and *E. billingiae* has shown a high similarity in gene content, organization and virulence and/or ecological fitness factors (Smits *et al.* 2011; Smits *et al.* 2010b). Pathogenic species have hallmark genetic determinants for virulence and fitness on pome fruit hosts, including type IV, type III, and type VI secretion systems and effectors, exopolysaccharides, sucrose and sorbitol metabolism, and desferroxamine siderophores (Smits *et al.* 2010a; De Maayer *et al.* 2011; Kamber *et al.* 2012; Powney *et al.* 2011; Smits and Duffy 2011). Variations of this repertoire, however, occur in pome fruit non-pathogenic and epiphytic species indicating that some auxiliary elements may contribute to full manifestation of specific pathogenic phenotypes (Sundin, 2007).

All these *Erwinia* species harbor a variety of plasmids and the presence of one plasmid in the range of 30 Kb is a common characteristic they have. Some of these plasmids have been studied, showing a role in host-pathogen interactions (Maxson-Stein *et al.* 2003). The best characterized is plasmid pEA29, which is essentially ubiquitous in *E. amylovora* (Laurent *et al.* 1989; McGhee and Jones 2000) and it has been implicated in symptom development (Laurent *et al.* 1989; McGhee and Sundin 2008; Llop *et al.* 2012). The presence of comparably sized plasmids (approximately 30-40 Kb), homology of plasmid-encoded genes (e.g., thiamine biosynthetic genes, *msrA*) (Llop *et al.* 2012), and low virulence retention in pEA29 devoid strains (Laurent *et al.* 1989), highlights the genetic

versatility in *Erwinia* spp., but also the importance of characterizing novel plasmids.

The objective of this study was to investigate the plasmids present in strains of the recently described species *E. piriflorinigrens*: plasmid pEPIR37, common to all analyzed strains, and pEPIR5 found in some strains. Both plasmids were completely sequenced and a comparative genomics analysis of pEPIR37 identified similarities and differences with other characterized *Erwinia* plasmids and chromosomes. Besides, the contribution of pEPIR37 to virulence was investigated using heterologous transfer to *E. amylovora*.

Materials and methods

Bacterial strains and growth media

Bacterial strains used in this study are listed in Table 1. Plasmid extractions on 27 strains of *E. piriflorinigrens* were performed using the Real Miniprep Turbo Kit (Durviz, Valencia, Spain). *Escherichia coli* strain JM109 was used for cloning experiments. All strains were grown on LB medium, with kanamycin (50 µg/l) added when appropriate.

Table 1. Bacterial strains and plasmids used.

Bacterial strains	Host or characteristics	Plasmid content	Origin/year isolation	Reference
<i>E. piriflorinigrans</i>				
CFBP 5881	Pear	pEPIR37	Spain/1999	López <i>et al.</i> (2011)
CFBP 5882	Pear	pEPIR37	Spain/1999	López <i>et al.</i> (2011)
CFBP 5883	Pear	pEPIR37	Spain/2000	López <i>et al.</i> (2011)
CFBP 5884	Pear	pEPIR37	Spain/2000	López <i>et al.</i> (2011)
CFBP 5885	Pear	pEPIR37	Spain/2000	López <i>et al.</i> (2011)
CFBP 5886	Pear	pEPIR37	Spain/2000	López <i>et al.</i> (2011)
CFBP 5887	Pear	pEPIR37, pEPIR5	Spain/2000	López <i>et al.</i> (2011)
CFBP 5888 ^T	Pear	pEPIR37,	Spain/1999	López <i>et al.</i> (2011)
IVIA 3926-1.1	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3926-1.2	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3926-2.1	Pear	pEPIR37	Spain/2011	This work
IVIA 3926-2.2	Pear	pEPIR37	Spain/2011	This work
IVIA 3928-5.1	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3928-5.2	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3929-8.2	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3929-11.1	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3930-18.1	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3930-20.1	Pear	pEPIR37	Spain/2011	This work
IVIA 3930-22.1	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3930-23.1	Pear	pEPIR37	Spain/2011	This work
IVIA 3930-23.2	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3930-29.1	Pear	pEPIR37	Spain/2011	This work
IVIA 3930-29.2	Pear	pEPIR37	Spain/2011	This work
IVIA 3930-32.1	Pear	pEPIR37	Spain/2011	This work
IVIA 3930-32.2	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3930-33.1	Pear	pEPIR37, pEPIR5	Spain/2011	This work
IVIA 3930-33.2	Pear	pEPIR37	Spain/2011	This work
<i>E. amylovora</i>				
CFBP 1430	<i>Crataegus</i>	pEA29	France/1972	Paulin <i>et al.</i> (1973)
PMV 6014 ^a	-	-	France	Laurent <i>et al.</i> (1989)
<i>E. tasmaniensis</i>				
Et 1/99	Apple	pET46	Australia/1999	Kube <i>et al.</i> (2008b)
<i>E. billingiae</i>				
Eb661	Pear	pEB102	UK/1959	Kube <i>et al.</i> (2010)
<i>E. pyrifoliae</i>				
Ep 1/96	Asian pear	pEP36	South Korea/1999	Kube <i>et al.</i> (2010)
DMS 12163	Asian pear	pEP36	South Korea/1999	Smits <i>et al.</i> (2010b)

***Erwinia* sp.**

Ejp 617 ^a	Asian pear	pJE01	Japan/1992	Park <i>et al.</i> (2011)
Ejp 556 ^b	Asian pear	pEJ30	Japan/1992	Maxson-Stein <i>et al.</i> (2003)

E. coli

JM109	<i>LacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)U169 <i>gyrA96</i>		Invitrogen
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Plasmids

pBBRMCS2	Kan ^R , cloning vector	Kovach <i>et al.</i> (1995)
pEPIR37::Tn5	Kan ^R , labeled plasmid	This work
pEPIR5::Tn5	Kan ^R , labeled plasmid	This work

^a Strain CFBP 1430 cured of plasmid pEA29.

^b According to Geider *et al.*, 2009 these strains are now *E. pyrifoliae*.

Plasmid DNA isolation and labeling

A plasmid, named pEPIR37, was isolated from the type strain *E. piriflorinigra*ns (CFBP 5888^T) and one of smaller size named pEPIR5 from strain CFBP 5887, from an overnight suspension in LB at 26 °C (Table 1). The extraction was analyzed by electrophoresis in 0.8% agarose. Plasmids were extracted from gel using the QIAEX II kit (QIAGEN Inc., CA, USA) and purified with a phenol-chloroform step and resuspended in distilled water. Restriction analyses with *Bam*HI and *Eco*RI were performed to confirm the identity of the plasmids. To carry out the *in vitro* transposition labeling we used the EZ-Tn5TM <KAN-2> Insertion Kit with kanamycin resistance (Epicenter, Madison, WI, USA), following the manufacturer's instructions. Briefly, 0.2 μg of plasmid DNA was added in 10 μl reaction with 1x reaction buffer, 1 μl transposase and a volume molar equivalent of transposon EZ-Tn5 <KAN-2>, (corresponding to 0.08 pmoles), followed by incubating at 37°C for 2 h, and transforming into *E. coli* JM109 strains, obtaining plasmids pEPIR37::Tn5 and pEPIR5::Tn5, respectively.

Plasmid sequencing

Labeled plasmids were transformed separately into *E. coli* strains JM109 by electroporation. Transformation was performed at 2.5Kv 200 ohms in a Gene Pulser X Cell™ (BIO-RAD, Hercules, CA. USA) using 0.2 cm electroporation cuvettes. After incubation for 1 h at 37 °C in LB medium, suspensions were plated on LB amended with kanamycin (50 µg/ml) and incubated for 24 h at 37 °C. Transformants were checked by plasmid extraction, digested with *EcoRI* and *BamHI* and analyzed by electrophoresis in 0.8% agarose to confirm the labeling of plasmids. Labeled plasmid pEPIR37 was purified and sequenced following a shotgun cloning and Sanger sequencing procedure (Macrogen, Seoul, Korea). The coverage was 11.8x with 440 reads (average read length 702 bp) in a single contig. Plasmid pEPIR5 was sequenced through direct sequencing from Tn5 transposon with overlapping primers at the sequencing facility of the Instituto de Biología Molecular y Celular de Plantas (IBMCP) of Valencia, Spain.

Sequence analyses

Genes on pEPIR37 were predicted using a combined strategy as described in Llop *et al.*, (2011). The resulting plasmid annotation was manually curated. Routine sequence manipulations were completed using several subroutines of the LASERGENE package (DNASTAR, Madison, WI, USA). Plasmid pEPIR5 reads were assembled into one contig using VectorNTI v. 8 (Invitrogen, Life Technologies, CA, USA), and annotated using the approach described above for pEPIR37.

Comparison of pEPIR37 with plasmids pEA29, pEP36, and pEJ30

The common features among plasmids pEA29 of *E. amylovora*, pEP36 of *E. pyrifoliae*, and pEJ30 from Japanese *Erwinia* strain Ejp556 (now identified as *E. pyrifoliae* by Geider *et al.*, 2009), reported by Maxson-Stein *et al.*, (2003) were

investigated in case they were also present in pEPIR37 plasmid. CDS found in pEPIR37 were analyzed and gene content compared against the other plasmids.

Phylogenetic analyses with the amino acid sequences of thiamine biosynthetic genes (*thiF*, *thiG*, *thiS* and *thiO*) of related pathogenic and non-pathogenic *Erwinia* spp. (*E. piriflorinigrans*, *E. amylovora*, *E. pyrifoliae*, and *E. tasmaniensis*) and with some *Pantoea* species were performed using Neighbor-Joining. *Citricella* sp. SE45 was chosen as outgroup. Significance of nodes was evaluated by Bootstrap with 1000 replicates. Analyses of amino acid distance were based on the Dayhoff matrix based model (Schwarz *et al.*, 1979) and all positions containing gaps and missing data were eliminated. The analyses were performed using MEGA version 5 (Tamura *et al.*, 2011). *Erwinia billingiae* proteins were not included in the analyses because this species harbors a different thiamine biosynthesis pathway.

Aggressiveness assays with pEPIR37 introduced into *E. amylovora* strains

Plasmid pEPIR37::Tn5, introduced into *E. coli* strain JM109, was extracted for transforming *E. amylovora* strain PMV 6014 that is the strain CFBP 1430 of *E. amylovora* cured of pEA29 and shows low aggressiveness level. Transformation was performed by electroporation as described previously, but the strains were incubated for 2 h at 26 °C in LB medium before plating. The transformants were incubated on LB agar with 50 µg/ml kanamycin for 48 h at 26 °C.

Pear assays

The possible function of plasmid pEPIR37 in symptom development was analyzed in *E. amylovora* strain PMV6014. This strain was tested formerly with the immature pear assay described previously (Llop *et al.*, 2011). Strains were grown overnight at 26 °C on LB agar with kanamycin. Cell suspensions were prepared and concentrations adjusted at 600 nm by serial dilutions in 0.5x PBS

(0,36% NaCl, 0.018% NaH₂PO₄·2 H₂O, 0,12% Na₂HPO₄·12 H₂O). Suspensions of 10³ cfu/ml were used for inoculation of pear var. Williams fruits as described in Llop *et al.*, (2011) to observe if there was an increase in aggressiveness when plasmid pEPIR37::Tn5 was introduced. Plant material was prepared according to the procedure used by Cabrefiga and Montesinos (2005). Four wounds were made on pear fruits of 3-4 cm diameter using a pipette tip, and 3 µl of each suspension placed per wound. Then, fruits were incubated at 26 °C under controlled relative humidity conditions. Aggressiveness was evaluated daily until 10th day. Wounds were considered as infected when either drops of bacterial exudates or necrosis appeared in and around the inoculation site of the pathogen. The experimental design consisted of three repetitions consisting of three pears (total of nine pears) per each strain and concentration. The incidence of infected wounds (%) for each repetition was assessed and severity was evaluated by means of a visual scale (from 0 to 3). The scale was based on necrosis progression or exudates production where 0 = no symptoms, 1 = exudates located at the inoculation point, 2 = necrosis affecting area around the wound, and 3 = necrosis expanding through the fruit. Disease severity (S) was calculated using the formula:

$$S = \sum_{i=1}^i \left(\frac{SI_i}{n \cdot 3} \right) \cdot 100$$

where *SI* is the corresponding severity index in an inoculated wound, *i* is the wound number, *n* the total wounds inoculated and 3 the maximum severity index.

E. amylovora strain CFBP 1430 was employed as positive control, and PBS buffer and *E. piriflorinigrens* CFBP 5888^T were utilized as negative controls in all assays. The inoculation experiments were performed twice.

Statistical analysis

ANOVA was performed to analyze the effect of each strain treatment on infection incidence and means were separated by the Tukey's test at $P \leq 0.05$. The analyses were done with the GLM procedure of the PC-Statistical Analysis System version 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Sequence analyses and plasmid annotation of pEPIR37

We analyzed 27 *E. piriflorinigrens* strains isolated from 1999 to 2011 in Valencia, Spain (Table 1) by plasmid extraction followed by digestion with *Bam*HI and *Eco*RI (data not shown), we observed that all harbored the same plasmid pEPIR37, and 12 of them also contained the plasmid pEPIR5 (Fig. 1). Plasmid pEPIR37 was determined to be 37,376 bp with a G+C content of 49.8%. The annotation of the sequence identified 42 CDS (Fig. 2, Table 2), for which the best BLAST hits were one CDS common to CDS present in plasmid pEA29, two CDS common to pEJ01, seven CDS common to pEP36, four CDS to pET46 and one CDS to pEB102. Another 12 CDS showed homology to putative proteins from the chromosome of *E. pyrifoliae* (two CDS), *E. tasmaniensis* (six CDS), and *E. billingiae* (four CDS). Another six CDS were homologs of those observed in different *Enterobacteriaceae* species (i.e., *Serratia odorifera*, *Edwardsiella ictaluri*, *Klebsiella pneumonia*, *Yersinia enterocolitica*, *Dickeya dadantii*, *E. coli*, *Pantoea* spp., and *Pectobacterium wasabiae*) and one *Clostridiaceae* (*Clostridium* sp.).

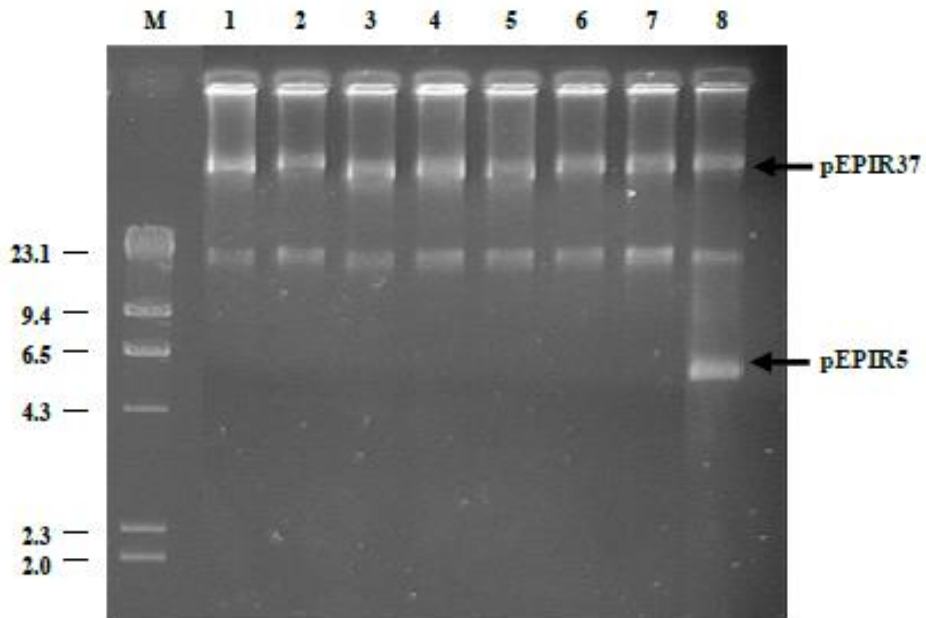


Fig. 1. Plasmid analysis of 8 isolates of *E. piriflorinigrans* obtained in pear orchards in 1999 and 2011 in Turis, Valencia (Spain) showing the plasmids of 37 and 5 Kb. Lanes 1 to 8, different strains of *E. piriflorinigrans*. Lane M, ladder λ *Hind*III (New England-biolabs).

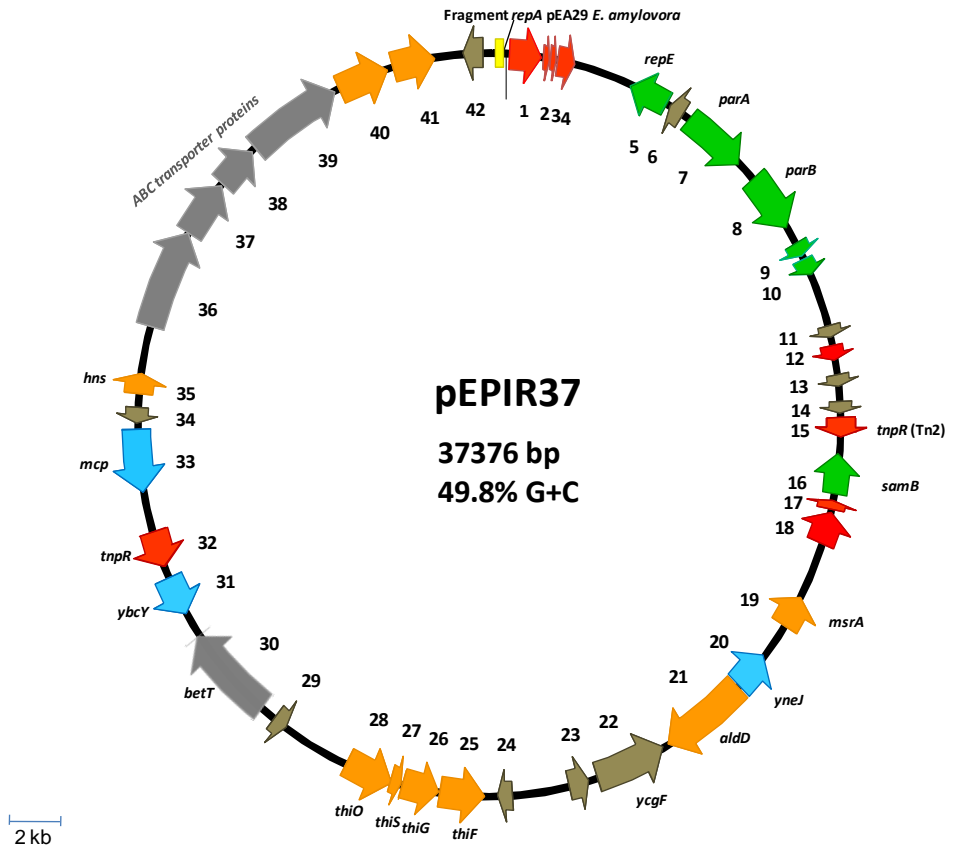


Fig. 2. Circular representation of plasmid pEPIR37. Color chart: Transposon and Insertion Sequences, red; replication and stability, green; ecological fitness, orange; transport, gray; transcriptional regulators, blue; hypothetical proteins, brown.

The replication origin was found to have high homology with RepE proteins and two partitioning proteins (ParA and ParB) in related bacteria (e.g., *E. tasmaniensis* pET46 (Kube *et al.*, 2008 b) or *P. vagans* pPag3 (Smits *et al.*, 2010 c) (Table 2). A 120 bp fragment of RepA protein (94% sequence identity), similar to the replication protein present in pEA29, pEJ30, and pEP36 plasmids is also present in pEPIR37 (Fig. 3). Remnants of an insertion sequence (IS), (CDS 18) and one Tn3-like and one Tn2-like-transposon (CDS 14 and 15), similar to other present in related enterobacteria (*Y. enterocolitica* and *E. coli*) were also found in pEPIR37, but they were different from those found in pEP36 and pEJ30. Analyses of pEPIR37 also showed that the genes for thiamine biosynthesis (*thiO*, *thiS*, *thiG*, and *thiF*; CDS 25 to 28) were similar to genes present in *E. amylovora*, *Erwinia* sp., *E. pyrifoliae*, and *E. tasmaniensis*. These genes have a protein identity of 90-94% with the proteins observed in pEA29, pEP36, and pEJ01, and also in the genome of *E. tasmaniensis*. (Table 2). The phylogenetic analysis of the concatenated ThiOSGF proteins in the different *Erwinia* species studied is shown in Fig. 4 and for separate proteins in Supplemental Fig. 6-1 in supplementary information at the end of this memory. The dendrogram shows that the *E. piriflorinigrans* plasmidic proteins for thiamine biosynthesis are more closely related to the other compared *Erwinia* spp.

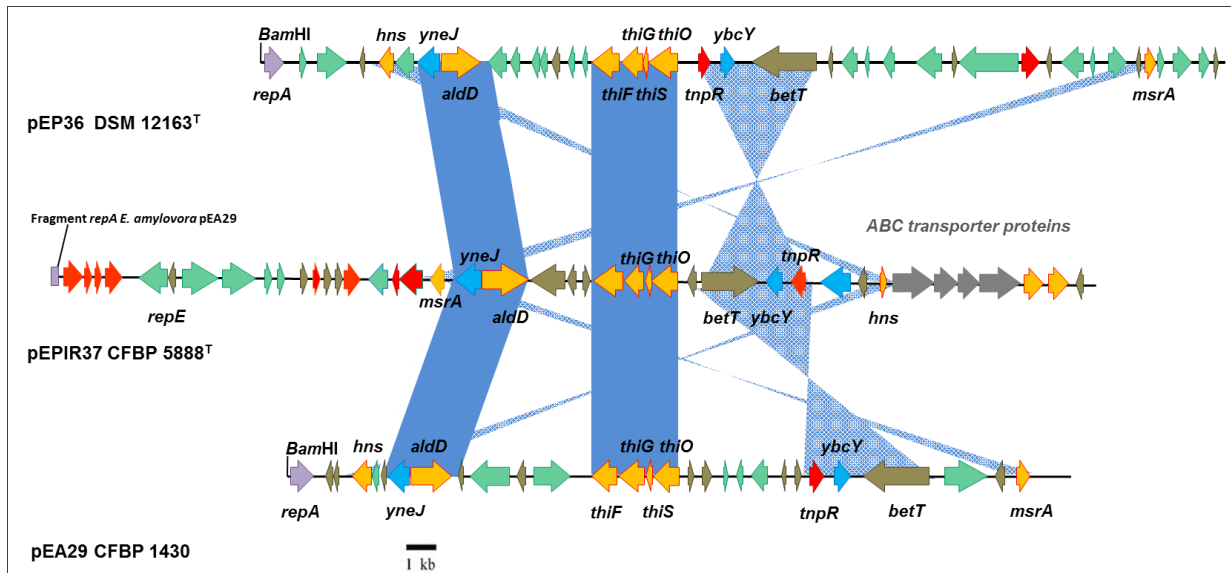


Fig. 3. Linear genetic maps for plasmid pEPIR37 from *E. piriflorinigra*ns CECT 5888^T, plasmid pEA29 from *Erwinia amylovora* CFBP 1430 (Smits *et al.*, 2010a), and pEP36 from *E. pirifoliae* DSM 12163 (Smits *et al.*, 2010b). The *Bam*HI restriction site common to pEA29 and pEP36 was used as the origin for each map. Homologous genes are connected by solid shading in genes that are in the same orientation and broken shadings when in opposite. Plasmid pEJ30 is not employed in the comparison due to its similarity to pEP36 without transposon Tn5394.

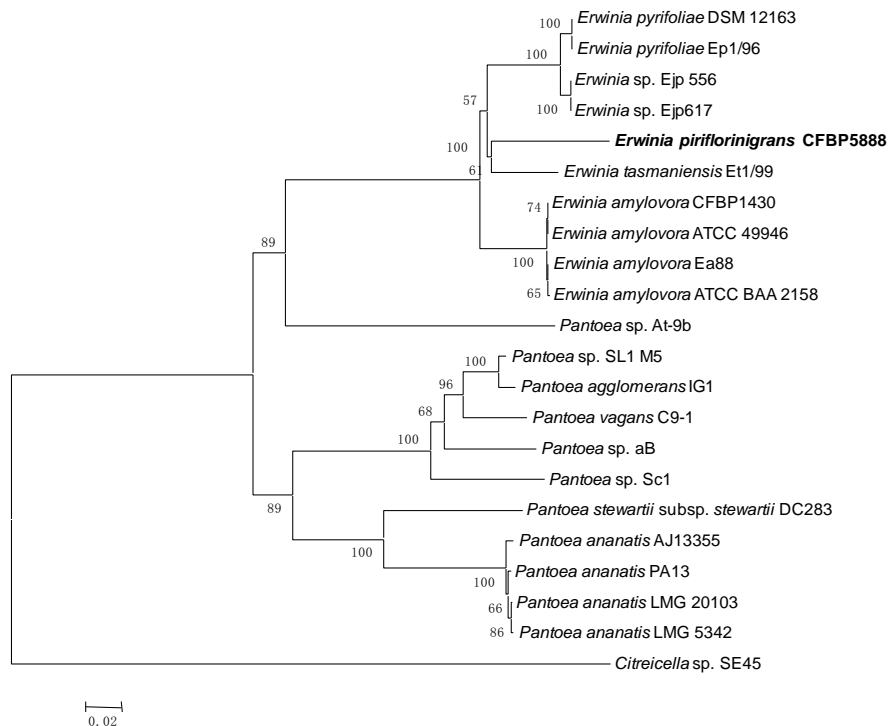


Fig. 4. Phylogenetic tree of concatenated amino acid sequences of ThiOSGF, constructed using Neighbor-Joining method. Bootstrap percentages (1000 replications) are indicated only for branches that show a value >50%. *Citreicella* sp. SE45 was used as an outgroup. Branch lengths are proportional to the amino acid distances. All analyses were performed using program MEGA version 5.

Table 2. Predicted CDS to proteins of pEIPR37 in the GenBank nonredundant database. In bold are indicated the genes that show a highest sequence identity to genes in pEIPR37 due to maximum percentage of identity, query coverage, E-value and/or biological significance.

Locus tag	Gene name	Description GenDB	from	Locus tags of orthologs in*:					Genome	% sequence identity	Best hit description	Acc. Nº
				pEA29 (<i>E.a.</i> CFBP 1430)	pJE01 (<i>E. sp.</i> Ejp 617)	pEP36 (<i>E. py.</i> DSM1 2163)	pET46 (<i>E.tas.</i> Et1/99)	Cromos. <i>E.tas.</i> Et1/99				
1	IS2A	Insertion element IS2A uncharacterized 48.2 kDa protein								98	HMPREF07 58_4959	ZP_0664 1623
2		Putative transposase								91	-	YP_0019 08788
3	IS407	Insertion element IS407 uncharacterized 31.7 kDa protein;									-	
4	<i>int</i>	Site-specific recombinase					ETA_pET46 0350			63	-	YP_0019 05944
5	<i>231epe</i>	Replication initiation protein					ETA_pET46 0360			93	-	YP_0019 05945
6		Hypothetical protein								42	CSBG_013 56	EEH9773 0
7	<i>parA</i>	Putative replication protein A					ETA_pET46 0370	<i>EbC_pEb17</i> 201400		93	-	YP_0019 05946
8	<i>parB</i>	Plasmid partition protein B					ETA_pET46 0380			92	-	YP_0019 05947
9	<i>stbD</i>	putative protein StbD	stability		EJP617_ A250	EPYR_ 04001				68	-	CAY7638 6
10	<i>stbE</i>	Addiction toxin StbE	module		EJP617_ A240					80	NT01EI_20 09	YP_0029 33420
11		Hypothetical protein									-	

Erwinia piriflorinigrans plasmids

12		Transposase for insertion sequence element IS1328					93	Dd586_0818	YP_003332411	
13		Hypothetical protein						-		
14		Hypothetical protein						-		
15	<i>tnpR</i>	R46 site-specific recombinase					80	-	DQ406736.1	
16	<i>samB</i>	Protein SamB				EbC_pEb10201110	68	-	YP_003739167	
17		Insertion element IS630 uncharacterized 39 kDa protein					87	EPYR_00476	CAY72822.1	
18	IS630	Insertion element IS630 uncharacterized 39 kDa protein					82	EPYR_03693	CAY76073	
19	<i>msrA</i>	Peptide methionine sulfoxide reductase	EAMY_3740	EJP617_ A080	EPYR_04022	ETA_07790	EbC_19820	89	-	YP_003208092.1
20	<i>yneJ</i>	HTH-type transcriptional regulator	EAMY_3718	EJP617_ A180	EPYR_03993	ETA_07780	EbC_22570	95	-	YP_001906722
21	<i>aldD</i>	Aldehyde dehydrogenase AldD	EAMY_3719	EJP617_ A190	EPYR_03994	ETA_07770	EbC_22560	94	-	CAY76379.1
22	<i>ycgF</i>	Uncharacterized protein ycgF				ETA_07760	EbC_22990	92	-	YP_001906720
23		Protein of unknown function DUF2132	EAMY_3722	EJP617_ A230	EPYR_03999	ETA_07750	EbC_21020	80	-	YP_003522543
24		Hypothetical protein							-	
25	<i>thiF</i>	Thiamine biosynthesis protein ThiF	EAMY_3724	EJP617_ A260	EPYR_04002	ETA_07740		94	-	YP_001906718
26	<i>thiG</i>	Thiazole synthase protein ThiG	EAMY_3725	EJP617_ A270	EPYR_04003	ETA_07730		94	-	CAY76388
27	<i>thiS</i>	Thiamine	EAMY_3726	EJP617_	EPYR_	ETA_07720		92	-	CAY7638

		biosynthesis protein ThiS	A280	04004					9.1	
28	<i>thiO</i>	Glycine oxidase ThiO	EAMY_3727	EJP617_	EPYR_	ETA_07710	90	-	YP_0019 06715	
			A290	04005						
29		Hypothetical protein						-		
30	<i>betT</i>	High-affinity choline transport protein	EAMY_3737	EJP617_	EPYR_	ETA_07690	EbC_19780	98	-	YP_0019 06713
				A320	04008					
31	<i>ybcY</i>	Uncharacterized protein ybcY	EAMY_3736	EJP617_	EPYR_	ETA_07680		86	-	ADP1331 2
				A310	04007					
32	<i>tnpR</i>	Resolvase-domain containing protein	EAMY_3735	EJP617_	EPYR_			89	-	CAY7639 1.1
				A300	04006					
33	<i>mcp</i>	Putative methyl- accepting chemotaxis protein				ETA_07660		85	-	YP_0019 06710
34		Hypothetical protein							-	
35	<i>hns</i>	DNA-binding protein H-NS	EAMY_3715	EJP617_	EPYR_	ETA_07650	EbC_19760	98	-	CAY7637 6.1
				A160	03991					
36	<i>fnrA</i>	ABC transporter, periplasmic component					EbC_20610	78	-	YP_0037 41442
37	<i>fnrB</i>	ABC transport system permease protein					EbC_20600	74	-	YP_0037 41441.1
38	<i>fnrC</i>	ABC transport system permease protein					EbC_20590	77	Pecwa_44 58	YP_0032 61757
39	<i>fnrD</i>	ABC transport system, ATP-binding protein					EbC_20580	66	-	YP_0037 41439
40		Monoxygenase, luciferase family					EbC_20570	70	-	YP_0037 41438
41		peroxidase-like protein					EbC_20560	62	Pat9b_570 8	YP_0041 18420
42		Hypothetical protein							-	

*E.a.: *Erwinia amylovora*; E. sp.: *Erwinia* sp. from Japan; E. py.: *Erwinia pyrifoliae*; E.tas.: *Erwinia tasmaniensis*; E. bil.: *Erwinia billingiae*

Common and different features observed in plasmids pEA29, pEP36, pEJ01 and pEPIR37

The different plasmids in the range of 30-40 Kb from pathogenic pome fruit *Erwinia* species showed several similarities as well as some differences between them (Fig. 3). Plasmids pEP36 and pEJ30 are almost identical, except for the presence of a transposon (Tn5394) of approximately 6 Kb in pEP36 that explains the difference in size they show (Maxson-Stein *et al.*, 2003). The analysis of the plasmid sequence also shows that pEPIR37 lacks short sequence repeats (SSR) and a *Bam*HI restriction site position, common features in pEA29, pEP36, and pEJ01 (Table 2). It shows IS elements and transposons different to pEP36, but similar to six other genes observed in related enterobacteria as indicated above. Twelve CDS were common to pEP36, pEJ30 and pEA29 whereas 12 CDS were similar to genes present in the genomes of *E. pyrifoliae*, *E. tasmaniensis* and *E. billingiae*. Comparisons of the four plasmids showed that plasmids pEPIR37 and pEA29 have 12 CDS in common while plasmids pEP36 and pEJ01 showed 14 common CDS with pEPIR37 (Table 3). Besides, the organization of genes *hns* and *msrA* is identical to the gene cluster located on the chromosome of *E. tasmaniensis*.

Sequence analyses and plasmid annotation of pEPIR5

The cryptic ColE1-type plasmid pEPIR5 has 5.5 Kb, with a G+C content of 54.3%, and nine CDS (Fig. 5 and Table 4). This plasmid presents mobilization proteins similar to the ones present in the plasmid pRK10 of *Serratia marcescens* as well as in the chromosome of *E. coli* strains MS78-1 and E482, and also to a hypothetical protein present in plasmid pPAT9B02 of *Pantoea* sp. AT-9b. Four CDS showed no homology with sequences registered in the NCBI database.

Table 3. Common features observed in the 30 Kb plasmids from pathogenic *Erwinia* spp. compared to pEPIR37.

Features	Plasmid pEPIR37	Plasmid pEA29	Plasmid pEP36	Plasmid pEJ01
Presence of short-sequence repeats (SSR) ^a	no	(GAATTACA) 3-7-8	(ATTCTGGG) 9-16	(ATTCTGGG) 21-22
Common <i>Bam</i> HI site	no	yes	yes	yes
Transposons	no	no	yes (Tn5394)	no
IS elements	no	no	yes (IS3, IS285)	yes (IS285)
Thiamine metabolism genes (<i>thiF</i> , <i>thiG</i> , <i>thiS</i> , <i>thiO</i>)	yes	yes	yes	yes
Replication protein	RepE	RepA	RepA	RepA
Common CDS to pEPIR37 ^b	-	12	13	14

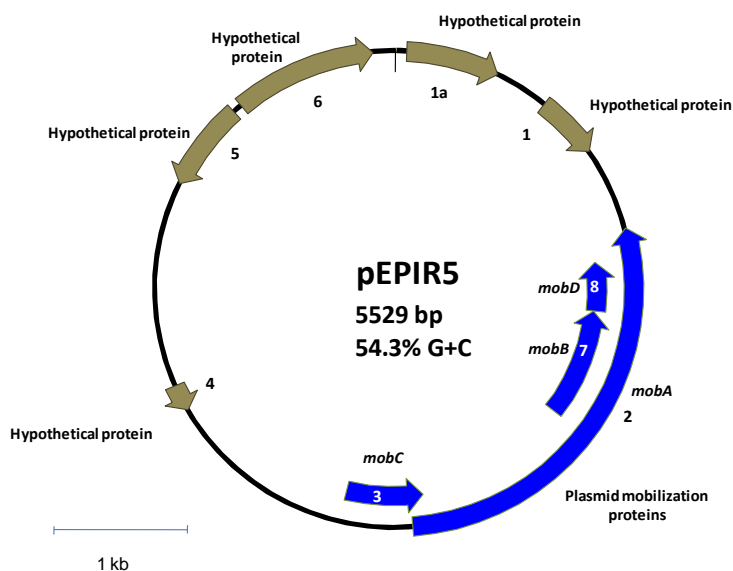


Fig. 5. Circular representation of plasmid pEPIR5. Color chart: mobilization, dark blue; hypothetical proteins, brown.

Table 4. Predicted CDS to proteins of pEPIR5 in the GenBank nonredundant data base.

Locus tag	Gene name	Description from GenBank	% sequence identity	Best hit	Accession number
01		Hypothetical protein			
02		Hypothetical protein Pat9b_5020 [<i>Pantoea</i> sp. At-9b]	49	Pat9b_5020	YP_004118867.1
03	<i>mobD</i>	Mbed/mobD like protein [<i>Escherichia coli</i> MS 78-1]	97	HMPREF9535_04353	ZP_07222674.1
04	<i>mobB</i>	Mobilization protein B [<i>Serratia marcescens</i>]	96	pRK10_p4	YP_001941151.1
05	<i>mobA</i>	Mobilization protein A [<i>Serratia marcescens</i>]	95	pRK10_p3	YP_001941150.1
06	<i>mobC</i>	Mobilization protein [<i>Escherichia coli</i> E482]	97	ERDG_04527	EGB35077.1
07		Hypothetical protein predicted by Glimmer/Critica			
08		Hypothetical protein predicted by Glimmer/Critica			
09		Hypothetical protein			

Virulence assays

To test whether the plasmid is involved in virulence, the effect on incidence and severity of symptoms of the introduction of pEPIR37::Tn5 into the low virulence level *E. amylovora* strain PMV 6014 without plasmid pEA29 in immature pear fruits was studied (Fig. 6). As expected, the type strain of *E. piriflorinigrans* showed no symptoms in pear fruitlets and strain PMV 6014 showed significantly lower severity (19.1 ± 1.2) than strain CFBP 1430 (73.5 ± 1.2). However, severity increased significantly in strain PMV 6014 with pEPIR37::Tn5 introduced when it was inoculated, in the two sets of assays (54.6 ± 5.4) even though the Tn5 was inserted in gene *thiF*, affecting the ability to synthesize thiamine.

Regarding the symptoms observed after 6 days in immature pear assays, 30.5% of wounds inoculated with strain PMV 6014 showed symptoms of necrosis, in

contrast to 69.4% and 80.5% of wounds inoculated with strains PMV 6014 with pEPIR37::Tn5 and CFBP 1430, respectively (Fig. 7).

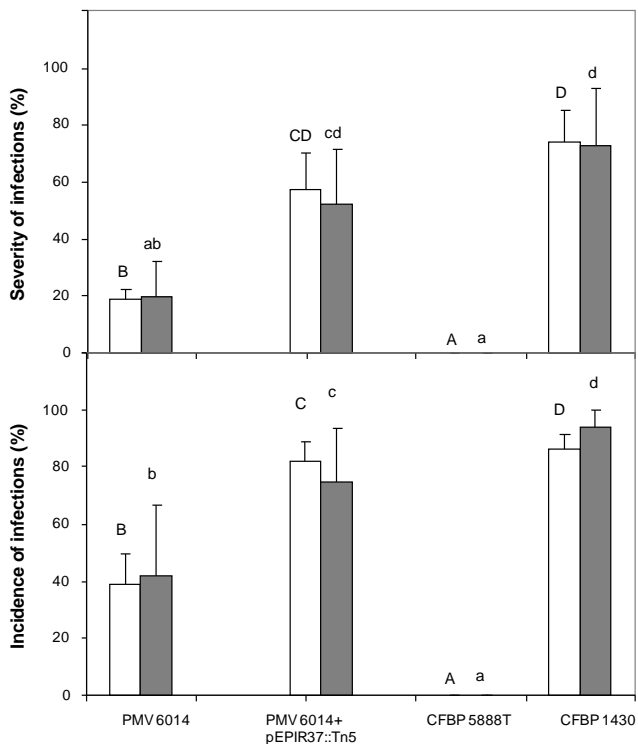


Fig. 6. Severity and incidence of infections in immature pear by inoculation of strain PMV 6014 of *E. amylovora*, before and after receiving plasmid pEPIR37::Tn5. The experiment was performed at 1×10^3 cfu/ml (white columns, 6 days post inoculation, black columns, 5 days post inoculation). Means with the same letters (upper-case letters for assay 1, lower-case letters for assay 2) do not differ significantly according to Waller-Duncan's test ($P \leq 0.05$). Strain *E. piriflorinigra*s CFBP 5888^T was included as negative control, whereas strain *E. amylovora* CFBP 1430 was included as positive control.

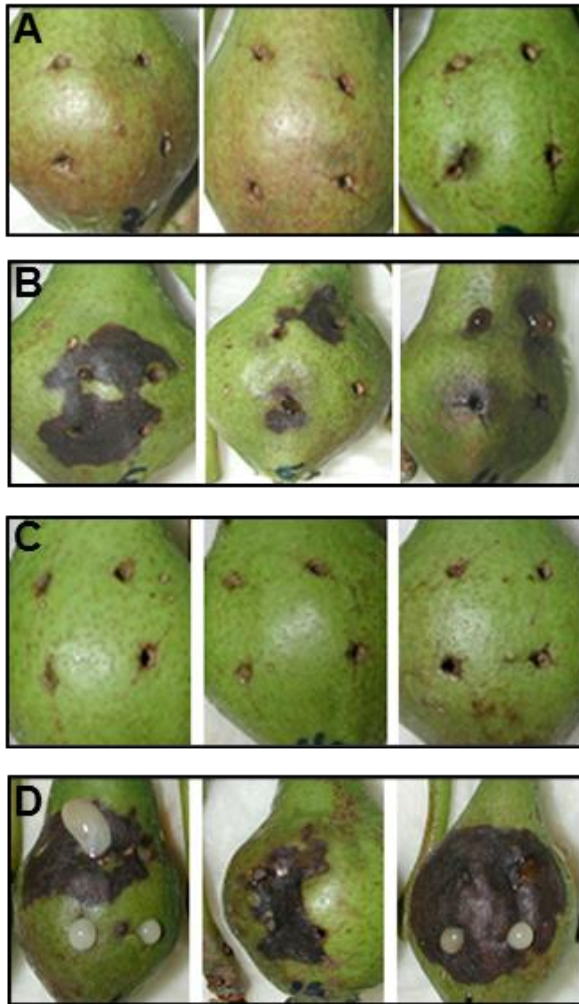


Fig. 7. Symptoms observed after 6 days of inoculation of immature pears when inoculated with 10^3 cfu/ml suspensions. A) strain *E. amylovora* PMV 6014 , B) strain PMV 6014 + pEPIR37::Tn5, C) *E. piriflorinigrans* strain CFBP 5888^T (negative control), D) strain *E. amylovora* CFBP 1430 (positive control).

Discussion

This is the first report on the complete sequencing of two plasmids of the recently described species *E. piriflorinigrans*. Our data show that all analyzed strains harbor a plasmid of 37 Kb and many of them another of 5 Kb. Relatedness among pathogenic bacteria from genus *Erwinia* can be due to several common characteristics they share (Smits *et al.*, 2011) because they have the same hosts and they harbor plasmids of either similar sizes (in the range of 30 - 40 Kb) and/or small cryptic plasmids of similar size content (Llop *et al.*, 2011). These species cause more or less similar symptomatology on pear but appear to have decreasing disease severity *E. amylovora* > *E. pyrifoliae* > Japanese *Erwinia* > *E. piriflorinigrans*. The information about virulence of *E. piriflorinigrans* strains is scarce because the bacterium has only been reported in pear orchards in Valencia (Spain), and the damage caused by the disease seem to be limited to necrosis of pear blossoms (Roselló *et al.*, 2006). However, observations of differential virulence should consider appropriateness of host and or pathogen intraspecies genotype (e.g., cultivars; Norelli *et al.*, 1984) for drawing such comparisons, and also pooling observations of different studies where experimental conditions that can influence disease severity typically vary (e.g., pathogen inoculum concentration, inoculation conditions; Cabrefiga and Montesinos 2005; Billing, 2011).

Recent genome comparison studies have shown that *E. amylovora* strains share more than 99.99% identity at the nucleotide level, indicating only minimal evolution since its geographical dispersal (Smits *et al.*, 2010a; Kamber *et al.*, 2012). Besides, the genomes of two *E. pyrifoliae* strains from Korea (Ep1/96 and DSM 12163^T) have shown they are almost identical. Chromosomal colinearity of *E. amylovora* to the closely related *E. pyrifoliae* strains, *Erwinia* sp. Ejp617, and *E. tasmaniensis* was observed despite that chromosomal large-scale rearrangements were detected (Kamber *et al.*, 2012).

The main difference among *E. amylovora* strains is reported to be their plasmid content, being pEA29 the plasmid most frequently reported. This plasmid pEA29 has been described as playing a role in the increment of symptoms, due to the presence of genes involved in the biosynthesis of thiamine (*thiO*, *thiS*, *thiG*, and *thiF*) (Laurent *et al.*, 1989; McGhee and Jones 2000; McGhee and Sundin 2008). Thiamine genes are also located on plasmids pEP36 and pEJ30 in *E. pyrifoliae* and on the chromosome in *E. tasmaniensis*. The gene content of plasmids pEA29, pEP36 and pEJ30 is highly conserved, and along with the *thi* genes, other genes are also present in the same order and orientation (e.g., *msrA*, *betT*) and we have shown that all these genes are also present on *E. piriflorinigrans* pEPIR37. Phylogenetic analyses performed on the concatenated ThiOSGF proteins of the related pathogenic and non-pathogenic *Erwinia* spp. and *Pantoea* spp. revealed a closer relationship between the proteins encoded on pEPIR37 and those from other *Erwinia* spp. For as far as known, the *thiOSGF* clusters in *Pantoea* spp. are present on plasmids as well (Smits *et al.*, 2010 c; De Maayer *et al.*, 2012).

The fact that pEPIR37 has a RepE replication protein related to that of *E. tasmaniensis* plasmid pET46 and ABC transporter proteins similar to *E. billingiae* and *Pantoea* spp., also possibly links it with these two species of non-pathogenic *Erwinia* and *Rosaceae* epiphytes.

This can be supported also by the fact that *E. piriflorinigrans* is able to cause necrosis of pear blossoms, but the symptoms do not progress to other parts of the plant and it does not affect immature pear fruits. However, heterologous introduction of pEPIR37 into plasmidless *E. amylovora* strain increased its virulence on immature fruits to a similar degree as pEA29 does, despite the fact that gene *thiF* is mutated in the labeled plasmid, affecting the biosynthesis of thiamine.

This study also annotated a cryptic plasmid pEPIR5, with no clear evidence of what type of role it provides to its host. Together with genes from pEPIR37 that showed no homology to previously sequenced genes they can be called orphan genes (Mira *et al.*, 2002).

The sequencing of more plasmids from other strains of the *Erwinia* species may confirm their genome flexibility. Saprophytic and non-pathogenic *Erwinia* have similar rosaceous plants as hosts as several pathogenic species, and their geographic distribution could be much more spread than currently reported. They could serve as a reservoir for transmission of plasmids or horizontal gene transfer and a step in evolutionary adaptation to different environments or host genotypes. Whole genome analyses of more strains of the species *Erwinia* that share fruit trees as habitat, like the most recent described *Erwinia* species, *E. uzenensis* (Matsuura *et al.*, 2011), or other uncharacterized *Erwinia* from Japan (Palacio *et al.*, 2012) from which no information about plasmid content and genome composition is yet known, could provide additional information about their plasmids and their gene content, composition and evolution, giving new clues on the origin of these plant pathogens and a better understanding of their life cycle.

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Capítulo 7

**PCR convencional y en tiempo real para la
detección de *Erwinia piriflorinigrans* y su
distinción del agente causal del fuego
bacteriano, *Erwinia amylovora***

Conventional and real-time PCR to detect *Erwinia piriflorinigrans* allow its distinction from the fire blight pathogen *Erwinia amylovora*

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* S. Barbé has contributed in designing of experiments, performing new isolation of *Erwinia piriflorinigrans* strains, stability analyses of pEPIR37, primers and probe design and design of PCRs conditions, specificity and sensitivity assays, analyses of plant material samples and writing the first version of the manuscript.

Abstract

Erwinia piriflorinigrans is a new pathogenic bacterial species of the genus *Erwinia*, recently described in Spain. Accurate detection and identification of *E. piriflorinigrans* is challenging because its symptoms on pear blossoms are similar to those caused by *Erwinia amylovora*, the causal agent of fire blight. Moreover, both species share phenotypic and molecular characteristics. Two specific and sensitive conventional and real-time PCR protocols were developed to identify, detect and differentiate *E. piriflorinigrans* from *E. amylovora* or other species of this genus. These protocols were based on sequences from plasmid pEPIR37, which is present in all analyzed strains of *E. piriflorinigrans*. After demonstrating plasmid stability, the specificity of the protocols was confirmed by the amplification of all *E. piriflorinigrans* strains tested whereas, 304 closely related pathogenic and non-pathogenic *Erwinia* strains and microbiota from pear trees were not amplified. In sensitivity assays, 10^3 cells/ml extract were detected in spiked plant material using conventional PCR, 10^2 cells/ml using real-time PCR after DNA extraction and 10^3 cells/ml without previous DNA extraction. The developed protocols herein succeeded in detecting *E. piriflorinigrans* in 102 out of 564 symptomatic and asymptomatic naturally infected pear samples (flowers, cortex stem tissue, leaves, shoots and fruitlets), in necrotic *Pyracantha* sp. blossoms and in mixed infections with *E. amylovora* in pear and apple necrotic tissues. Therefore, these new tools can be used in epidemiological studies that will contribute to improving the understanding of the life cycle of *E. piriflorinigrans* in different hosts and plant tissues, and its interaction with *E. amylovora*.

Introduction

Erwinia piriflorinigrans is a Gram-negative plant pathogenic bacterium causing pear blossom necrosis (López *et al.*, 2011). The disease affects pear production and can reduce fruit yield. It was first observed in Valencia (Spain) in 1999 (Roselló *et al.*, 2002; Roselló *et al.*, 2006). After this first identification, the disease was observed at the same place in later years (López *et al.*, 2011). Due to its recent discovery, the biology, ecology and life cycle of this new species is still poorly understood.

Unlike *E. amylovora*, the causal agent of fire blight, *E. piriflorinigrans* affects blossoms and not other parts of plants (Roselló *et al.*, 2006). Nevertheless, in culture media, colonies of *E. piriflorinigrans* can be easily mistaken for *E. amylovora* e.g. using CCT (Ishimaru and Klos, 1984), King's medium B (King *et al.*, 1954) and SNA or Levan medium which are usually utilized and recommended to isolate *E. amylovora* (EPPO, 2013; Billing *et al.*, 1961). Both pathogens show similar metabolic profiles based on the commercial strips API 20E, API 20NE, API ZYM and API 50CH (bioMérieux, France), as well as close fatty acid profiles. *E. piriflorinigrans* can react with several antisera and even with one of the monoclonal antibodies, used to detect *E. amylovora* (Roselló *et al.*, 2006). Moreover, *E. piriflorinigrans* reacts positively in PCR using primers based on the 23S rDNA sequence designed by Maes *et al.* (1996) to detect *E. amylovora*. As a result, similarities between *E. piriflorinigrans* and *E. amylovora* could lead to false negatives in the detection of this new pathogenic species (Mergaert *et al.*, 1999) or its misidentification with *E. amylovora*.

The genus *Erwinia* includes pear pathogens such as *E. pyrifoliae* reported in South Korea (Kim *et al.*, 1999), *Erwinia* spp. causing bacterial shoot blight of pear (BSBP) (Tanii *et al.*, 1981; Beer *et al.*, 1996), *E. uzenensis* causal agent of bacterial black shoot disease of European pear in Japan (BBSDP) (Mizuno *et al.*, 2010; Matsuura *et al.*, 2012) and the rosaceous epiphytes *E. tasmaniensis*

(Geider *et al.*, 2006) and *E. billingiae* (Roselló *et al.*, 2006). All these species share some biochemical, metabolic and genetic characteristics with *E. piriflorinigrans*. Thus, a polyphasic approach is required to identify each pathogen accurately. Recent genomic analyses have provided information on the taxonomic position of *Erwinia* species and an insight into their evolution (Smits *et al.*, 2011; Palacio-Bielsa *et al.*, 2012). These data strongly support the need for unambiguous identification methods.

Barbé *et al.* (2012) reported that *E. piriflorinigrans* isolates harbor a plasmid of 37 Kb (pEPIR37), which plays a similar role in virulence to the plasmid pEA29 when introduced into *E. amylovora*. As these authors indicated that pEPIR37 was non-conjugative based in the analysis of its sequence, the first goal of this study was to design a conventional and a real-time PCR protocol targeting plasmid pEPIR37 present in all known strains of *E. piriflorinigrans*. Primers and probe for both PCR protocols were developed to fulfill accurate detection and identification of this pathogen in rosaceous samples and to distinguish it from other pathogenic and non-pathogenic *Erwinia* species. Specificity and sensitivity of the developed PCR protocols were also assayed. As a second goal, the real-time PCR protocol was used to test two hypotheses: (i) the presence of *E. piriflorinigrans* in organs other than blossoms and in hosts other than pear and (ii) its presence in different Spanish areas alone or with *E. amylovora*.

Materials and methods

Bacterial strains

Bacterial strains used are listed in Tables 1 and 2. All strains were routinely grown on King's medium B (King *et al.*, 1954) for 48 h at 26°C. For sensitivity evaluation, cell suspensions were adjusted to 10⁸ cells/ml by optical density at 600 nm (OD₆₀₀ of 0.2) and serial dilutions were performed in sterile water.

Table 1. *Erwinia piriflorinigrans* strains used in this study.

Strains	Host	Year of isolation	Reference
CFBP 5881	<i>Pyrus</i> sp.	1999	(López <i>et al.</i> , 2011)
CFBP 5882	<i>Pyrus</i> sp.	1999	(López <i>et al.</i> , 2011)
CFBP 5883	<i>Pyrus</i> sp.	2000	(López <i>et al.</i> , 2011)
CFBP 5884	<i>Pyrus</i> sp.	2000	(López <i>et al.</i> , 2011)
CFBP 5885	<i>Pyrus</i> sp.	2000	(López <i>et al.</i> , 2011)
CFBP 5886	<i>Pyrus</i> sp.	2000	(López <i>et al.</i> , 2011)
CFBP 5887	<i>Pyrus</i> sp.	2000	(López <i>et al.</i> , 2011)
CFBP 5888 ^T	<i>Pyrus</i> sp.	1999	(López <i>et al.</i> , 2011)
IVIA 3926-1.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3926-1.2	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3926-2.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3926-2.2	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3928-5.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3928-5.2	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3929-8.2	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3929-11.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-18.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-20.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-22.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-23.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-23.2	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-29.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-29.2	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-32.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-32.2	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-33.1	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)
IVIA 3930-33.2	<i>Pyrus</i> sp.	2011	(Barbé <i>et al.</i> , 2012)

CFBP: Collection Française des Bactéries Phytopathogènes. INRA, Angers, France.

IVIA. Instituto Valenciano de Investigaciones Agrarias Collection. Moncada, Spain.

Table 2. Bacterial strains used in specificity assays.

Bacterial strains	Host	Origin	Reference
<i>Agrobacterium vitis</i>			
IVIA 339-26	Grapevine	Spain	(Albiach <i>et al.</i> , 1992)
<i>Agrobacterium tumefaciens</i>			
C58	<i>Prunus cerasus</i>	USA	(Watson <i>et al.</i> , 1975)
<i>Agrobacterium rhizogenes</i>			
K84	Soil	Australia	(Kerr <i>et al.</i> , 1974)
<i>Dickeya chrysanthemi</i>			
CFBP 2048	<i>Chrysanthemum morifolium</i>	USA	(Samson <i>et al.</i> , 2005)
<i>Dickeya dadantii</i>			
CFBP 1269	<i>Pelargonium capitatum</i>	Comoro Is.	(Samson <i>et al.</i> , 2005)
<i>Dickeya</i> sp.			
3937	<i>Saintpaulia ionantha</i>	France	(Kotoujansky <i>et al.</i> , 1982)
<i>Erwinia amylovora</i>			
BC3	<i>Pyrus</i> sp.	Serbia	(Llop <i>et al.</i> , 2011)
BPic 909	<i>Pyrus</i> sp.	Greece	(Llop <i>et al.</i> , 2011)
BPic 913	<i>Pyrus</i> sp.	Greece	(Llop <i>et al.</i> , 2011)
BPic 917	<i>Cydonia</i> sp.	Greece	(Llop <i>et al.</i> , 2011)
BPic 1041	<i>Cydonia</i> sp.	Bulgaria	(Llop <i>et al.</i> , 2011)
BPic 1614	<i>Pyrus</i> sp.	Greece	(Llop <i>et al.</i> , 2011)
BPic 1624	<i>Pyrus</i> sp.	Greece	(Llop <i>et al.</i> , 2011)
CFBP 1430	<i>Crataegus</i> sp.	France	(Paulin <i>et al.</i> , 1973)
CFBP 2584	<i>Cotoneaster</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
CFBP 3041	<i>Pyrus</i> sp.	United Kingdom	(Llop <i>et al.</i> , 2011)
CFBP 3042	<i>Malus</i> sp.	United Kingdom	(Llop <i>et al.</i> , 2011)
CFBP 3049	<i>Malus</i> sp.	Canada	-
CFBP 3050	<i>Malus</i> sp.	Canada	-
CFBP 3051	<i>Malus</i> sp.	USA	-
CFBP 3053	<i>Malus</i> sp.	Germany	(Llop <i>et al.</i> , 2011)
CFBP 3054	<i>Pyrus</i> sp.	Germany	(Llop <i>et al.</i> , 2011)
CFBP 3056	<i>Cotoneaster</i> sp.	Germany	(Llop <i>et al.</i> , 2011)
CFBP 3063	<i>Pyrus</i> sp.	Greece	(Llop <i>et al.</i> , 2011)
CFBP 3064	<i>Pyrus</i> sp.	Greece	(Llop <i>et al.</i> , 2011)
CFBP 3065	<i>Pyrus</i> sp.	Greece	(Llop <i>et al.</i> , 2011)
CFBP 3093	<i>Pyrus</i> sp.	Greece	(Llop <i>et al.</i> , 2011)
CFBP 7528	<i>Pyrus</i> sp.	Morocco	(Hannou <i>et al.</i> , 2013 a)
CFBP 7522	<i>Pyrus</i> sp.	Morocco	(Hannou <i>et al.</i> , 2013 a)
CFBP 7509	<i>Pyrus</i> sp.	Morocco	(Hannou <i>et al.</i> , 2013 a)
CFBP 7543	<i>Cydonia</i> sp.	Morocco	(Hannou <i>et al.</i> , 2013 a)
CFBP 7426	<i>Pyrus</i> sp.	Morocco	(Hannou <i>et al.</i> , 2013 a)
CFBP 7546	<i>Pyrus</i> sp.	Morocco	(Hannou <i>et al.</i> , 2013 a)

CFBP 7532	<i>Pyrus</i> sp.	Morocco	(Hannou <i>et al.</i> , 2013 a)
CFBP 7514	<i>Pyrus</i> sp.	Morocco	(Hannou <i>et al.</i> , 2013 a)
CGJ-2	<i>Pyrus</i> sp.	Serbia	(Llop <i>et al.</i> , 2008 a)
E-70	<i>Cotoneaster</i> sp.	Ireland	(Brennan <i>et al.</i> , 2002)
Ea 3-2	-	Canada	-
Ea 29-7	-	Canada	-
Ea 31-B	-	Canada	-
Ea 34-A	-	Canada	-
Ea 273	<i>Malus</i> sp.	USA	(Norelli <i>et al.</i> , 1987)
Ea D-7	-	Canada	-
Ea G-5	-	Canada	-
Ea G-7	-	Canada	-
Ea PO 394	-	Poland	-
Ea PO 632	-	Poland	-
Ea PO 653	-	Poland	-
Ea PO 659	-	Poland	-
Ea PO 663	-	Poland	-
EAR 3	-	Turkey	-
EAR 4	-	Turkey	-
EAR 5	-	Turkey	-
EAR 6	-	Turkey	-
EAR 8	-	Turkey	-
EAR 101	-	Turkey	-
EAY 123	-	Turkey	-
EMU 1	-	Turkey	-
FG 2	<i>Pyrus</i> sp.	Bulgary	-
INRA 2279	-	Egypt	-
INRA 2582	<i>Malus</i> sp.	France	(Llop <i>et al.</i> , 2011)
INRA 3012	<i>Crataegus</i> sp.	Belgium	-
INRA 3098	<i>Malus</i> sp.	Israel	-
IVIA 1525-6	<i>Cotoneaster</i> sp.	Spain	(Llop <i>et al.</i> , 2011)
IVIA 1554	<i>Crataegus</i> sp.	Spain	(Llop <i>et al.</i> , 2011)
IVIA 1596	<i>Pyrus</i> sp.	Spain	(Llop <i>et al.</i> , 2011)
IVIA 1614-1	<i>Pyracantha</i> sp.	Spain	(Llop <i>et al.</i> , 2011)
IVIA 1614-2	<i>Crataegus</i> sp.	Spain	(Llop <i>et al.</i> , 2011)
IVIA 1614-2a	<i>Crataegus</i> sp.	Spain	(Llop <i>et al.</i> , 2006)
IVIA 1626-6	<i>Malus</i> sp.	Spain	(Llop <i>et al.</i> , 2011)
IVIA 1739-1	<i>Malus</i> sp.	Spain	(Llop <i>et al.</i> , 2011)
IVIA 1951-8	<i>Cotoneaster</i> sp.	Spain	(Llop <i>et al.</i> , 2011)
KG 6-45	<i>Cydonia</i> sp.	Germany	(Llop <i>et al.</i> , 2011)
KG 9-7	<i>Pyrus</i> sp.	Germany	(Llop <i>et al.</i> , 2011)
KG 9-43	<i>Cotoneaster</i> sp.	Germany	(Llop <i>et al.</i> , 2011)
KG 9-75	<i>Cotoneaster</i> sp.	Germany	(Llop <i>et al.</i> , 2011)
KG 250	<i>Malus</i> sp.	Germany	(Llop <i>et al.</i> , 2011)
KG 285	-	Germany	-
KG 1179	-	Germany	-

LNPV 1585	<i>Cotoneaster</i> sp.	France	(Llop <i>et al.</i> , 2011)
LNPV 1594	<i>Cotoneaster</i> sp.	France	(Llop <i>et al.</i> , 2011)
LNPV 1601	<i>Pyrus</i> sp.	France	(Llop <i>et al.</i> , 2011)
LNPV 1613	<i>Pyracantha</i> sp.	France	(Llop <i>et al.</i> , 2011)
LNPV 1626	<i>Pyrus</i> sp.	France	(Llop <i>et al.</i> , 2011)
LNPV 1709	<i>Cotoneaster</i> sp.	France	(Llop <i>et al.</i> , 2011)
LNPV 1710	<i>Cotoneaster</i> sp.	France	(Llop <i>et al.</i> , 2011)
LNPV 1775	<i>Cotoneaster</i> sp.	France	(Llop <i>et al.</i> , 2011)
LNPV 1778	<i>Cotoneaster</i> sp.	France	(Llop <i>et al.</i> , 2011)
LNPV 1879	<i>Cotoneaster</i> sp.	France	-
MK 1	<i>Malus</i> sp.	Hungary	(Llop <i>et al.</i> , 2011)
MK 17	<i>Cydonia</i> sp.	Hungary	(Llop <i>et al.</i> , 2011)
MK 23	<i>Pyrus</i> sp.	Hungary	(Llop <i>et al.</i> , 2011)
MK 26	<i>Pyrus</i> sp.	Hungary	(Llop <i>et al.</i> , 2011)
MK 28	<i>Crataegus</i> sp.	Hungary	(Llop <i>et al.</i> , 2011)
MK 295/93	<i>Cotoneaster</i> sp.	Austria	(Llop <i>et al.</i> , 2011)
MK 483/98	<i>Cotoneaster</i> sp.	Austria	(Llop <i>et al.</i> , 2011)
MK 1082/00	<i>Pyrus</i> sp.	Austria	(Llop <i>et al.</i> , 2011)
MK 1180/00	<i>Malus</i> sp.	Austria	(Llop <i>et al.</i> , 2011)
MK 1186/00	<i>Sorbus</i> sp.	Austria	(Llop <i>et al.</i> , 2011)
MK 2447/01	<i>Sorbus</i> sp.	Austria	(Llop <i>et al.</i> , 2011)
NCPBP 595	<i>Pyrus</i> sp.	United Kingdom	(Llop <i>et al.</i> , 2000)
NIB 311	<i>Pyrus</i> sp.	Slovenia	(Llop <i>et al.</i> , 1989)
PMV 6014	<i>Pyrus</i> sp.	France	(Laurent <i>et al.</i> , 2011)
PMV 6084	-	France	(Llop <i>et al.</i> , 1999)
SL 2156	<i>Cotoneaster</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
SL 2157	<i>Cotoneaster</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
SL 2158	<i>Sorbus</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
SL 2159	<i>Sorbus</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
SL 2160	<i>Sorbus</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
SL 2161	<i>Sorbus</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
SL 2162	<i>Sorbus</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
SL 2163	<i>Cotoneaster</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
SL 2164	<i>Sorbus</i> sp.	Ireland	(Llop <i>et al.</i> , 2011)
UPN 527	<i>Malus</i> sp.	Spain	(Llop <i>et al.</i> , 2011)
<i>Erwinia tasmaniensis</i>			
NCPBP 4357	<i>Malus</i> sp.	Australia	(Geider <i>et al.</i> , 2006)
NCPBP 4358	<i>Pyrus</i> sp.	Australia	(Geider <i>et al.</i> , 2006)
<i>Erwinia billingiae</i>			
IVIA 3571-1	<i>Malus</i> sp.	Spain	-
IVIA 3571-3	<i>Malus</i> sp.	Spain	-
IVIA 3571-6	<i>Malus</i> sp.	Spain	-
IVIA 3579-2	<i>Malus</i> sp.	Spain	-
IVIA 3579-3	<i>Malus</i> sp.	Spain	-
LMG 2624	<i>Crataegus</i> sp.	France	(Mergaert <i>et al.</i> , 1999)
LMG 2641	<i>Malus</i> sp.	France	(Mergaert <i>et al.</i> , 1999)

Erwinia piriflorinigrans PCR

NCPPB 1261	<i>Malus</i> sp.	United Kingdom	-
<i>Erwinia pyrifoliae</i>			
CFBP 4172	<i>Pyrus pyrifolia</i>	South Korea	(Kim <i>et al.</i> , 1999)
CFBP 4243	<i>Pyrus pyrifolia</i>	South Korea	(Kim <i>et al.</i> , 1999)
LMG 25885	<i>Pyrus pyrifolia</i>	Japan	(Brady <i>et al.</i> , 2012)
LMG 25886	<i>Pyrus pyrifolia</i>	Japan	(Brady <i>et al.</i> , 2012)
<i>Erwinia uzenensis</i>			
LMG 25843	<i>Pyrus communis</i>	Japan	(Matsuura <i>et al.</i> , 2012)
YPPS 950	<i>Pyrus communis</i>	Japan	(Matsuura <i>et al.</i> , 2012)
YPPS 952	<i>Pyrus communis</i>	Japan	(Matsuura <i>et al.</i> , 2012)
YPPS 956	<i>Pyrus communis</i>	Japan	(Matsuura <i>et al.</i> , 2012)
<i>Pantoea agglomerans</i>			
IVIA 2019-2a	<i>Olea europaea</i>	Spain	-
<i>Pectobacterium carotovorum</i>			
NCPPB 312	<i>Solanum tuberosum</i>	Denmark	(Gardan <i>et al.</i> , 2003)
<i>Pectobacterium atrosepticum</i>			
7	<i>Solanum tuberosum</i>	Spain	-
13	<i>Solanum tuberosum</i>	Spain	-
14	<i>Solanum tuberosum</i>	Spain	-
<i>Pseudomonas fluorescens</i>			
1499	-	Spain	-
<i>Pseudomonas syringae</i> pv. <i>syringae</i>			
IVIA 773-1	<i>Pyrus</i> sp.	Spain	-
<i>Pseudomonas viridiflava</i>			
IVIA 3684-7	<i>Prunus cerasus</i>	Spain	-

137 strains of unidentified epiphytic bacteria of pear trees from France and Spain.

BPIC: Benaki Phytopathological Institute Collection, Athens, Greece.

CFBP: Collection Française de Bactéries Phytopathogènes, INRA, Angers, France.

INRA : Institut National de la Recherche Agronomique, UMR de Pathologie Végétale, Beaucauzé, France.

IVIA: Instituto Valenciano de Investigaciones Agrarias Collection, Moncada, Spain.

LMG: Collection of the Laboratory of Microbiology. Rijksuniversiteit, Gent, Belgium.

LNPV: Laboratoire National de la Protection des Végétaux, Beaucauzé, France.

NCPPB: National Collection of Plant Pathogenic Bacteria, York, United Kingdom.

NIB: National Institute of Biology, Ljubljana, Slovenia. PMV: Laboratoire de Pathologie Moléculaire et Végétale INRA/INA-PG, Paris, France.

SL: State Laboratory, Dublin, Ireland.

UPN: Universidad Pública de Navarra. Pamplona, Spain.

Stability analyses of pEPIR37

E. piriflorinigrans strains CFBP 5888^T and CFBP 5887 (Table 1) were used to conduct four independent stability assays according to the procedure of Foster et al. (Foster *et al.*, 2004). A single colony of each bacterial strain was incubated in 4.5 ml of Luria-Bertani (LB) broth medium (Bertani *et al.*, 1951) at 26° C for 24 h under shaking at 220 rpm (Barnstead Lab-Line MaxQ 4000, USA). Then, each suspension was adjusted to 10⁸ cells/ml and 45 µl were added to fresh medium. This process was repeated daily during 100 days (about 1000-generations). Every 72 h, 3 µl of each culture were plated on LB and every 20 days 25 colonies from the LB plates were analyzed by real-time PCR targeting plasmid pEPIR37 (described below). Besides, at the end of the stability experiment (100 days), 125 colonies from each strain were analyzed using the Real Miniprep Turbo Kit (Durviz, Spain) to confirm plasmid presence according to Barbé *et al.* (2012). Restriction analyses with *Bam*HI and *Eco*RI enzymes were performed to confirm that plasmid pEPIR37 had not suffered rearrangements.

Primers and probe design

A nucleotide specific sequence from pEPIR37 was selected to design appropriate primers and probe. Alignment of nucleotide sequences was conducted using several databases (GenBank, EMBL and DDBJ). Vector NTI v.8 (Life Technologies, USA) and ABI PRISM Primer Express software (Life Technologies, USA) were used to obtain the optimal oligonucleotide primers and probe sequences. To evaluate specificity, primers, probe and PCR product sequences were compared with available sequences in NCBI databases using BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST>) (21). For conventional PCR, primer sequences were: P37-F (5'-TGG ATA GCG TGG ACG GTA A-3') and P37-R (5'-GGC AGT CAC TCC CAG CAG A-3'). For real-time PCR, primer sequences

were: P37rt-F (5'-TGC GCC CCT GGA ATA CCT-3') and P37rt-R (5'-GTG ATC CTG TGG GCT TTG GT-3'). The TaqMan probe sequence was the following: P37-Epfn (5'-FAM-CGG TAC TGC /ZEN/ TCC AGT CG-IBFQ-3') (Integrated DNA Technologies, Inc., USA).

PCR conditions

Conventional PCR was performed in a GeneAmp 9700 thermal cycler (Life Technologies, USA). With culture suspensions and dilutions 1:10 of plant material extract we used a final volume of 50 μ l (45 μ l of mix and 5 μ l of sample). For plant material after DNA extraction, we used 47 μ l of mix and 3 μ l of sample. The reaction mixture contained 1 U *Taq* DNA polymerase (Biotools, Spain) when culture suspensions were analyzed, and 2 U *Taq* DNA polymerase for plant material after DNA extraction (see below), 1 μ M of each primer, 0.1 mM of each dNTP, 3 mM MgCl₂. The samples with the mix was subjected to an initial denaturation step of 3 min at 94°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 45 seconds at 72°C and a final extension step of 10 min at 72°C. The amplification products obtained by conventional PCR were visualized in 2% (w/v) agarose gel electrophoresis in 0.5X TAE buffer (40 mM Tris, 20 mM NaOAc, 1 mM EDTA, pH 8/HOAc), after ethidium bromide staining. A 100 bp molecular weight marker (New England Biolabs, USA) was used. In preliminary experiments, different concentrations of primers (0.5 μ M, 0.75 μ M, and 1 μ M) were tested to optimize reaction conditions.

Real-time PCR assays were performed in a LightCycler 480 thermocycler (Roche, USA) and in a SmartCycler (Cepheid, USA). With culture suspensions and plant samples after DNA extraction we used a final volume of 12 μ l (9 μ l of mix and 3 μ l of sample). For dilutions 1:10 of plant material extract (without DNA extraction), we used 20 μ l of mix and 5 μ l of sample. The reaction mixture containing 2X reaction MasterMix (Biotools, Spain), 0.8 μ M of each primer and

0.1 μM of TaqMan probe. The amplification conditions were: an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 70°C.

Specificity assays

To test the specificity of conventional and real-time PCR protocols, we used 137 strains of epiphytic bacteria of pear trees from France and Spain (Table 2), 153 strains from different *Erwinia* species: *E. piriflorinigrans* (27) (Table 1), *E. amylovora* (108), *E. pyrifoliae* (4), *E. tasmaniensis* (2), *E. billingiae* (8) and *E. uzenensis* (4) (Table 2), and 14 strains from plant pathogenic bacteria species of the genera *Agrobacterium* (3), *Dickeya* (3), *Pantoea* (1), *Pectobacterium* (4) and *Pseudomonas* (3) (Tables 2). It was evaluated on bacterial suspensions treated at 100°C for 10 min. All PCR reactions included *E. piriflorinigrans* type strain CFBP 5888^T as positive control. Negative controls were sterile water and *E. amylovora* strain CFBP 1430. Each bacterial strain was analyzed in three different PCR assays.

Sensitivity assays and comparison of DNA extraction protocols

A suspension of *E. piriflorinigrans* CFBP 5888^T was adjusted to 10⁸ cells/ml at 600 nm (OD₆₀₀ of 0.2) to determine the sensitivity of each PCR protocol. Serial dilutions from 10⁸ to 10 cells/ml were performed. Plate count on King's medium B and flow cytometry, using the nucleic acid stain SybrGreen (Marie *et al.*, 1997), were used to check bacterial concentration in each suspension. A BD FACS Canto™ II flow cytometry system (Deckton Dickinson, USA) was used with pure cultures. For spiked samples, one gram of healthy pear flowers was slightly crushed with 50 ml of antioxidant maceration buffer as described in the protocol of the European and Mediterranean Plant Protection Organization for

E. amylovora (EPPO, 2013). Then it was macerated with the serial dilution suspensions indicated above to reach final concentrations of 10^7 to 1 cells/ml. These spiked macerates were analyzed after DNA extraction or diluted 1:10 in sterile distilled water and used directly in the PCR protocols without DNA extraction.

DNA extractions were performed using four protocols: (i) DNeasy Plant Mini kit (QIAGEN Inc., Germany), (ii) DNA extraction protocol modified from Taylor *et al.*, 2001, (iii) DNA extraction protocol described in Llop *et al.*, 1999 and (iv) cetyl trimethyl ammonium bromide (CTAB) extraction protocol described in Murray and Thompson (1980). Extracted DNA was immediately used for PCR or stored at -20°C . Finally, samples from bacterial suspensions, spiked samples after DNA extractions, and 1:10 dilutions from spiked plant material without DNA extraction were assayed in the PCR analyses. Four technical replicates were analysed. All assays included positive and negative controls.

Comparative analyses of pear material for *E. piriflorinigra*s by isolation and PCR

PCR detection of *E. piriflorinigra*s and isolation were compared in pear blossoms and in stems, leaves and fruitlets. One g of symptomatic or asymptomatic pear blossoms was collected from pear trees in Valencia (Spain) according to the procedure described in EPPO for *E. amylovora* (EPPO, 2013). Each sample was placed in a plastic bag, and slightly crushed and macerated for 5 min at room temperature in 50 ml of antioxidant maceration buffer. Each macerate was plated in three media: King's medium B, Levan and CCT as indicated in the EPPO protocol (EPPO, 2013) and 1 ml was transferred into two sterile Eppendorf tubes: one for PCR analyses and another stored with 30% glycerol at -20°C for further confirmation.

Besides, in order to detect the possible epiphytic presence of *E. piriflorinigrans* in other organs of pear trees, healthy leaves, fruitlets and stems from orchards where *E. piriflorinigrans* had been detected since 1999 were processed according to the protocol described in Quesada *et al.* (2007). Twenty g of stems or fruitlets or 10 g of leaves in 200 ml of Ringer ¼ solution (38.5 mM NaCl, 1.5 mM KCl, 1.1 mM CaCl₂, 0.6 mM NaHCO₃, pH 7) were used. A 0.05% of Tween 20 was added to this solution and shaken for 1 h at 200 rpm at room temperature. After shaking, 50 µl of washing solutions from shoots, fruitlets and leaves and their 1:10 and 1:100 dilutions were plated on CCT, King's medium B and Levan medium and incubated at 26°C for 72 h and also analyzed by PCR. Presumptive *E. piriflorinigrans* colonies were purified on King's medium B and characterized by phenotypic and molecular tests.

In 2011, 34 symptomatic and asymptomatic pear blossom samples were collected. In 2012, 94 samples of secondary pear blossoms, leaves and stems were collected from pear orchards of cv. Ercolini and Tendral from Valencia (Spain). In these orchards, *E. piriflorinigrans* was the only pathogenic *Erwinia* species detected since 1999 until 2012. When typical *Erwinia* colonies were not recovered from the plates, 1 ml of each plant extract was added to the same volume of King's medium B and CCT liquid media for enrichment, following the same protocol described for *E. amylovora* (EPPO, 2013). Presumptive *Erwinia* colonies were then purified on King's medium B and analyzed by PCR. Plant extracts were diluted 1:10 and DNA was extracted from these samples for PCR analysis. Positive and negative controls were as indicated above, including healthy plant samples.

Analyses of naturally infected samples for *E. piriflorinigrans* and *E. amylovora*

Compulsory surveys according to the European Union legislation are performed each year in Spain in nurseries and orchards in all the regions. After visual

inspection, following a established field itinerary and in nurseries, the inspectors take officially suspicious samples that are analyzed (or confirmed) in the National Reference Laboratory of Spain. Fire blight symptomatic and asymptomatic samples of flowers, cortex stem tissue, leaves, shoots and fruitlets from these samples were analyzed. No presence of ooze was observed in the samples analyzed. Plant extracts were screened for *E. amylovora* according to the procedure of EPPO (EPPO, 2013) and strict procedures were followed to analyze the samples to avoid contaminations, including the use of several controls.

For *E. piriflorinigrans*, the developed real-time PCR assay and the isolation procedure described herein were used. Only the analyzed samples that were positive for *E. amylovora*, *E. piriflorinigrans* or both are shown. A total of 564 samples from several Spanish locations were analyzed for both pathogens in 2011, 2012 and 2013 from different hosts (*Cotoneaster* sp., *Cydonia* sp., *Eriobotrya japonica*, *Malus* sp., *Pyracantha* sp., *Pyrus* sp. and *Sorbus* sp.) (Tables 5 and 6). Negative and positive controls were included. Presumptive colonies of *E. piriflorinigrans* and *E. amylovora* were purified on King's medium B and characterized by phenotypic, molecular tests and 16S rRNA partial sequence analysis.

Results

Stability analyses of pEPIR37

Plasmid pEPIR37 was stable during 1000 generations. All analyzed colonies from *E. piriflorinigrans* strains CFBP 5888^T and CFBP 5887 were positive using real-time PCR for plasmid pEPIR37 amplification. Moreover, results from plasmid

extraction and restriction analysis confirmed that the plasmid had not suffered rearrangements (data not shown).

Primers, probe and PCR conditions

Primers and probe were selected from the *fncC* gene sequence from plasmid pEPIR37 of *E. piriflorinigrans* (GenBank accession number: HE792893.1 and the locus tag of the target sequence: EPIR_pEPIR37038). The bioinformatic analysis showed that this gene codifies for an ABC-type dipeptide/oligopeptide/nickel transport system membrane, with protein coding accession number: CCG55415. The region where these target sequences were employed for the design of primers and probe was classified as a “nickel ABC transporter, permease subunit NikC”. Using conventional PCR, the designed primers specifically amplified all tested *E. piriflorinigrans* strains and produced a DNA fragment of 255 bp. Using real-time PCR, primers and probe specifically amplified *E. piriflorinigrans* and produced the expected product of 71 bp. Further amplification reactions were performed at the optimized concentration using 1 μ M of each primer in conventional PCR and 0.8 μ M of each primer and 100 nM TaqMan probe in real-time PCR.

Specificity of the PCRs

The 137 strains of epiphytic bacteria isolated from pear trees, 153 strains of pathogenic and non-pathogenic *Erwinia* species and 14 strains of plant pathogenic and epiphytic bacterial species were all negative by the conventional and real-time PCRs developed. Amplifications were only positive for *E. piriflorinigrans* strains. These analyses confirmed previous *in silico* specificity results obtained with sequences available in NCBI database.

Sensitivity assays and comparison of DNA extraction protocols

Cell concentration in bacterial suspensions was confirmed by plate counting and by flow cytometry. Amplification was obtained for concentrations of *E. piriflorinigrans* as low as 10 cells/ml to 10^3 cells/ml, depending on the extraction protocol used. For bacterial suspensions, the obtained sensitivity was 10^2 cells/ml (conventional PCR) and 10 cells/ml (real-time PCR) (Cycle threshold, Ct: 34.04 ± 0.91) (Table 3, Fig. 1). For spiked samples after DNA extraction, sensitivity was 10^3 cells/ml (conventional PCR) and 10^2 cells/ml (real-time PCR) (Ct: 35.05 ± 0.51). For 1:10 dilutions from plant material without DNA extraction, sensitivity was 10^3 cells/ml (conventional PCR and real-time PCR) (Ct: 33.46 ± 1.10). These results were confirmed in both the LightCycler 480 thermocycler (Roche, USA) and the SmartCycler (Cepheid, USA).

Among the DNA extraction methods tested, DNeasy Plant Mini kit (QIAGEN Inc., Germany) showed the best detection limit (10^2 or 10^3 cells/ml) using real-time PCR or conventional protocols. The DNA extraction protocol modified from Taylor *et al.* (2001), the method described in Llop *et al.* (1999) and the CTAB extraction protocol modified by Murray and Thompson (1980), showed less efficiency.

Table 3. Sensitivity of detection of *Erwinia piriflorinigrans* strain CFBP 5888^T in serial dilutions (10^7 to 1 cells/ml); in (1:10) extract of spiked flowers and after a DNA extraction using DNeasy Plant Mini Kit (Qiagen Inc., Germany) from the same plant extract, using conventional and real-time PCR protocols. In real-time PCR, Ct values and standard deviation values were calculated for four PCR reactions.

		Cells/ml of strain CFBP 5888 ^T								
Conventional PCR:	1	10	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	Negative control	
Bacterial suspension	-	-	+	+	+	+	+	+	-	
Flower extract (1:10)	-	-	-	+	+	+	+	+	-	
Flower extract (DNA extraction) ^a	-	-	-	+	+	+	+	+	-	
Real-time PCR	1	10	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	Negative control	
Bacterial suspension	-	34.04±0.91	32.38±1.69	30.63±1.26	27.61±1.47	24.93±1.17	22.86±1.34	19.49±1.37	-	
Flower extract (1:10)	-	-	-	33.46±1.10	31.25±0.72	27.40±0.39	24.72±1.63	19.83±0.29	-	
Flower extract (DNA extraction) ^a	-	-	35.05±0.51	33.11±3.04	32.89±0.97	29.57±0.50	28.80±1.04	22.13±1.33	-	

^a DNA was extracted by using the DNeasy Plant Mini kit (Qiagen Inc., Germany).

+, detection; -, no detection.

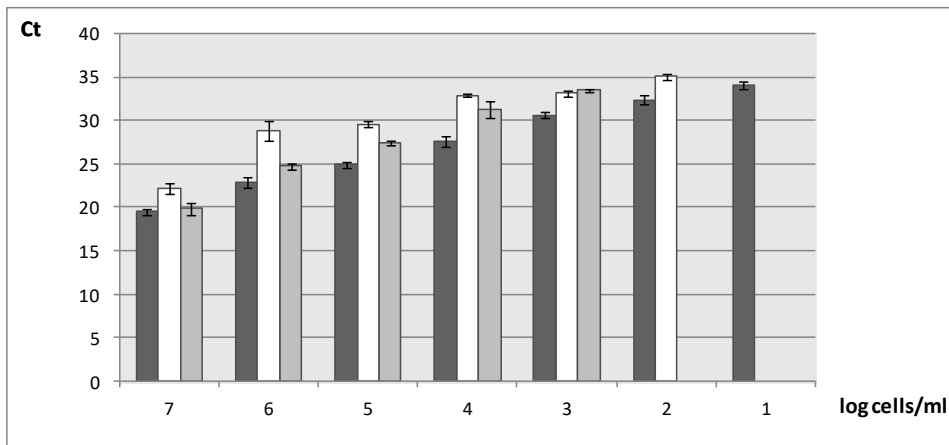


Fig. 1. Threshold cycles (Ct) obtained in real-time PCR with *Erwinia piriflorinigrans* strain CFBP 5888^T in a serial dilution (1 to 10⁷ CFU/ml) in sterile distilled water, in spiked flowers (1:10) and after DNA extraction from these plant materials. Serial dilutions are shown in dark grey (linear correlation (R^2) of the standard curve = 0.9905), DNA extraction from spiked flowers in white (R^2 = 0.9729) and spiked flowers (1:10) in light grey (R^2 = 0.9842). X-axis represents the logarithm of cells/ml in the sample and Y-axis represents the cycle threshold value (Ct).

Comparative analyses of pear material for *E. piriflorinigrans* by isolation and PCR

A total of 22.2% of necrotic and asymptomatic flower samples from cv. Ercolini and Tendral were positive using conventional PCR protocol; whereas 26.9% were positive with real-time PCR protocol (Table 4). All positive samples were flowers collected in April 2011 and were further confirmed by bacterial isolation. Samples collected in August 2012 were all negative by real time PCR, isolation and enrichment-isolation. No other species of the genus *Erwinia* were detected in samples collected in 2011 and 2012. A representative number (16

colonies) obtained by isolation from positive samples were identified by phenotypic and molecular test and checked for their plasmid content and showed the presence of the expected 37 kb plasmid for *E. piriflorinigrans*.

Analyses of naturally infected samples for *E. piriflorinigrans* and *E. amylovora*

A total of 270 samples of *Eriobotrya japonica*, *Cotoneaster* sp., *Cydonia* sp., *Malus* sp., *Pyracantha* sp., *Pyrus* sp. and *Sorbus* sp., were collected in 2011 and in 2012 from Spanish nurseries and orchards. From them, 132 were positive for *E. amylovora* by real-time PCR, but were negative using the real-time PCR designed to detect *E. piriflorinigrans* (Table 5). Isolation and enrichment-isolation were positive for *E. amylovora* but not for *E. piriflorinigrans*. However, 43 pear samples that were negative for *E. amylovora* were positive for *E. piriflorinigrans* by isolation and PCR. In addition, one sample of *Pyracantha* with necrotic blossoms was also negative for *E. amylovora* but positive for *E. piriflorinigrans*.

Mixed infection of *E. amylovora* and *E. piriflorinigrans* was found in 38 out of 265 samples from pear trees collected from Spanish orchards and analyzed in 2013. These samples were positive for *E. amylovora* using the EPPO protocol (EPPO, 2013) and also for *E. piriflorinigrans* using the real-time PCR designed in this study (Table 6). Furthermore, both species were jointly isolated not only from flowers, but also from shoots, leaves, fruitlets and cortex stem tissues. In the same way, 20 out of 29 samples of flowers, shoots and leaves of apple from one Spanish orchard were also positive for *E. amylovora* and *E. piriflorinigrans* (Table 6). Representative colonies of both bacterial species were identified by phenotypic and molecular tests as above indicated. *E. piriflorinigrans* was confirmed by sequencing the 16S rRNA gene of 13 colonies.

Table 4. Detection of *Erwinia piriflorinigrans* in pear tree samples by isolation and/or enrichment, conventional and real-time PCR protocols using DNA extraction.

Organ	Month/year	Samples (No.)	Isolation and or enrichment	Conventional PCR	Real-time PCR
Flowers	April/2011	34	17	14	17
Flowers	August/2012	29	0	0	0
Stems	August/2012	20	0	Nd ^a	0
Leaves	August/2012	20	0	Nd ^a	0
Fruitlets	August/2012	25	0	0	0

^a Nd: not determined.

Table 5. Detection of *E. amylovora* and *E. piriflorinigrans* in symptomatic and asymptomatic samples from Spanish nurseries and orchards.

Host	Tissue	No. samples	Only <i>E. amylovora</i> positive	Only <i>E. piriflorinigrans</i> positive	<i>E. amylovora</i> and <i>E. piriflorinigrans</i> negative	<i>E. amylovora</i> and <i>E. piriflorinigrans</i> positive
<i>Pyrus</i> sp.	Flowers	102	37	43	22	0
	Shoots	36	15	0	21	0
	Leaves	38	14	0	24	0
	Fruitlets	35	10	0	25	0
	Cortex stem tissues	16	16	0	0	0
<i>Malus</i> sp.	Flowers	4	4	0	0	0
	Shoots	1	1	0	0	0
	Leaves	4	4	0	0	0
	Fruitlets	2	2	0	0	0
	Cortex stem tissues	3	3	0	0	0
<i>Eriobotrya japonica</i>	Flowers	3	3	0	0	0
	Cortex stem tissues	1	0	0	1	0
<i>Cydonia</i> sp.	Flowers	1	1	0	0	0
	Shoots	3	3	0	0	0
	Leaves	3	3	0	0	0
<i>Cotoneaster</i> sp.	Leaves	4	4	0	0	0
	Cortex stem tissues	1	1	0	0	0
<i>Sorbus</i> sp.	Flowers	6	6	0	0	0
	Leaves	4	4	0	0	0
<i>Pyracantha</i> sp.	Flowers	1	0	1	0	0
	Leaves	2	1	0	1	0
TOTAL		270	132	44	94	0

Table 6. Detection of mixed infection of *E. amylovora* and *E. piriflorinigrens* from pear and apple orchards from Spain.

Host	Tissue	No. samples	Only <i>E. amylovora</i> positive	Only <i>E. piriflorinigrens</i> positive	<i>E. amylovora</i> and <i>E. piriflorinigrens</i> negative	<i>E. amylovora</i> and <i>E. piriflorinigrens</i> positive
<i>Pyrus</i> sp. ^a	Flowers	117	102	0	0	15
	Shoots	40	36	0	0	4
	Leaves	43	38	0	0	5
	Fruitlets	46	35	0	0	11
	Cortex stem tissue	19	16	0	0	3
<i>Malus</i> sp. ^b	Flowers	12	4	0	0	8
	Shoots	6	1	0	0	5
	Leaves	11	4	0	0	7
TOTAL		294	236	0	0	58

^a From three Spanish origins, located in three different regions.

^b From one origin.

Discussion

Two PCR protocols based on the recently described plasmid pEPIR37 were designed to detect, identify and differentiate *E. piriflorinigrens* from *E. amylovora* and other *Erwinia* species. Plasmid pEPIR37 was considered as non-transmissible because its sequence was devoid of *tra*-like genes involved in conjugal transfer (Barbé *et al.*, 2012). The analyses performed by plasmid extraction and by restriction analysis confirmed that the plasmid had not integrated into the chromosome or suffered rearrangements under the experimental conditions and demonstrate its stability.

Although most *Erwinia* species from pome fruits have plasmids of similar sizes in variable numbers (Llop *et al.*, 2012), specific *E. piriflorinigrens* sequences were selected for the new PCR protocols. The designed primers and probe did not amplify *E. pyrifoliae* or *E. amylovora* as templates, as well as other closely related species that harbor similar sized plasmids. Specificity was even observed

using *E. tasmaniensis* as template, the closest species to *E. piriflorinigrans* at phylogenetic level (Barbé *et al.*, 2012; Smits *et al.*, 2013). Moreover, using the developed PCR protocols, only *E. piriflorinigrans* strains showed amplification and none of the strains of other bacterial genus or epiphytic *Erwinia* and non *Erwinia* species were amplified. These results indicate the selected sequences from pEPIR37 were only present in strains of the target species.

The conventional and real-time PCR protocols could detect the target bacterium directly from plant material without DNA extraction with an acceptable efficiency (10^3 cells/ml) and the use of this direct PCR without DNA extraction reduces the risk of contamination, saves time and decreases the cost per reaction. Thus, it is useful for processing large numbers of samples. As regards DNA extraction method, DNeasy Plant Mini kit (QIAGEN Inc., Germany) was the recommended method for both PCR protocols for the most sensitive analyses of plant material.

Real-time PCR is considered the most accurate and reliable method for the detection and diagnosis of plant pathogens, mainly bacteria (López *et al.*, 2009; Palacio-Bielsa *et al.*, 2009; De Boer and López, 2012) and our results support these findings because 10^2 cells/ml were routinely detected with DNA extraction. In contrast to published real-time PCR protocols designed for other plant pathogenic bacteria, we used a double-quencher probe with an internal quencher named ZEN™ (Integrated DNA Technologies, Inc., USA). This kind of probe has been reported to provide a better amplification efficiency, to reduce background noise and to increase the fluorescence level of the probe, improving PCR sensitivity in plant pathogenic fungi (Arif *et al.*, 2013). Besides, using this type of probes in other bacteria as '*Candidatus Liberibacter asiaticus*' and '*Candidatus Liberibacter solanacearum*' or some plant viruses such as *Plum pox virus*, it is possible to reduce some cycles the amplification curves (unpublished data).

For diagnostic purposes and for processing large number of samples, we recommend using a 1:10 dilution in sterile distilled water of plant extract suspected of being infected with this pathogen as template for real-time PCR in a first screening test.

As regards the biology of *E. piriflorinigrans*, the target could only be detected and isolated from pear samples in spring but not in secondary blooming. High concentrations of *E. piriflorinigrans* were isolated from asymptomatic as well as from symptomatic flowers and confirmed by real-time PCR, in primary blooming. Our findings show differences between *E. piriflorinigrans* and *E. amylovora* because fire blight pathogen can also be detected in secondary blooming (Thomson *et al.*, 2000). A possible explanation for this might be related to the high temperatures (maximum 41.5°C) recorded in the sampled orchard in summer 2012 compared with spring. However, with a one-year sampling period, caution must be applied, as the findings might not be transferable to a broader time period. These results together with those previously reported in Roselló *et al.* (2006) and López *et al.* (2011), suggest that the most favorable conditions to detect and isolate *E. piriflorinigrans* are encountered during pear blooming in spring.

In this study *E. piriflorinigrans* was detected not only in pear flowers but also in shoots, leaves, fruitlets and cortex stem tissue of samples infected also with *E. amylovora*. These results support the need for research on basic life cycle of this pathogen. Until now, *E. piriflorinigrans* had only been reported in necrotic pear blossoms. Our study provides further support for the hypothesis that *E. piriflorinigrans* can be present alone in different tissues in pear and apple trees, and also in organs which can be affected by *E. amylovora*. These results, together with the detection in *Pyracantha* sp. necrotic flowers indicate that *E. piriflorinigrans* could share other host plants, besides pear trees, with the fire blight pathogen. However, further research should be done to investigate the interaction of both pathogens in the same niche. In our isolations, *E. amylovora*

was present more frequently than *E. piriflorinigrans* and when interacting, it might be possible that *E. amylovora* became dominant. In some cases, the relative amount of *E. amylovora* and *E. piriflorinigrans* found in mixed infection was approximately 10:1 in CCT medium in some samples. Moreover, *E. amylovora* has shown a good competitive fitness against other pathogens of the same genus, like *E. pyrifoliae* (Lehman *et al.*, 2008).

The present study was first designed to obtain accurate, rapid and simple protocols to detect *E. piriflorinigrans* and to distinguishing it from the causal agent of fire blight, *E. amylovora*, the most destructive disease of pome fruits. The new real-time PCR protocol succeeded in accurately identifying this new pathogen in plant material samples.

Related to the second goal of improving the knowledge on the geographical distribution and the habitat of *E. piriflorinigrans*, we have found this target in new areas in Spain and in apple and *Pyracantha* in addition to pear. Necrotic symptoms of pear flowers can be due both to *E. amylovora* and *E. piriflorinigrans*, as well as to other bacterial species, such as *Pseudomonas* sp., fungal infections or plant physiological disorders. Consequently, it is inadvisable to make diagnosis based solely on visual inspections. Furthermore, the identification of *E. piriflorinigrans* can be problematic because it shares phenotypic characteristics with some epiphytes and pathogenic *Erwinia* species. This probably explains why *E. piriflorinigrans* has not been reported yet outside of Spain. Based on our recent findings, it is expected that this bacterium could be also found in other countries, and has been mistakenly identified as *E. amylovora* or other *Erwinia* species, as it occurred in Japan with some other *Erwinia* (Palacio-Bielsa *et al.*, 2012). The developed techniques could also be used in surveys that would shed light on the current distribution of *E. piriflorinigrans* inside and outside Spain, epidemiological studies that will help in providing information about the life cycle of *E. piriflorinigrans* in different hosts and plant tissues, and in exploring its interaction with *E. amylovora*.

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Capítulo 8

Discusión general

El género *Erwinia* incluye especies de la familia *Erwiniaceae* patógenas y no patógenas asociadas a especies cultivadas y silvestres de la familia de las rosáceas (Adeolu *et al.*, 2016). Dentro de los patógenos más importantes de este género destaca *E. amylovora*, agente causal del fuego bacteriano de numerosas especies de rosáceas, mientras que otras especies del mismo únicamente son capaces de infectar a un número de especies vegetales más limitado, como *E. pyrifoliae* que afecta a determinados cultivares de *P. pyrifolia* (Kim *et al.*, 1999). Las otras especies del género *Erwinia* patógenas de frutales de pepita, provocan síntomas limitados a ciertos órganos de la planta, como se ha descrito en *E. piriflorinigrans* (López *et al.*, 2011) o *Erwinia* sp. de Japón y *E. uzenesis*, agentes causales de las enfermedades “*bacterial shoot blight of pear*” (BSBP) y “*bacterial black shoot disease of European pear*” (BBSDP), respectivamente (Tanii *et al.*, 1981; Mizuno *et al.*, 2010). Otras especies como *E. tasmaniensis*, *E. billingiae* y *E. gerundensis* están consideradas como epifitas (Kube *et al.*, 2010, Rezzonico *et al.*, 2016).

Todas estas especies patógenas y epifitas de frutales de pepita, son portadoras de un número variable de plásmidos de tamaño similar, la mayoría de los cuales se han secuenciado, y aunque de muchos de ellos aún no se ha podido determinar su papel en la cepa que los porta, en otros casos sí se ha asociado su contenido genético con aspectos fenotípicos, así como con el grado de virulencia de la misma en la planta hospedadora (Llop *et al.*, 2012).

Por ello, el primer objetivo de esta tesis fue realizar una revisión bibliográfica de los plásmidos descritos en las especies patógenas y epifitas del género asociadas a frutales de pepita, que sirviera de base a los trabajos experimentales posteriores.

La secuenciación y el estudio del contenido genético de estos plásmidos pueden revelar aspectos de la interacción de la bacteria portadora con el huésped, así como proporcionar información del ciclo de vida de la bacteria que los porta y

su epidemiología. Del mismo modo, también nos pueden aportar pistas sobre la relación genética de estas especies de *Erwinia* patógenas y no patógenas, ya que los plásmidos que portan estas especies poseen regiones con alta similitud de secuencia y cierta sintenia (Maxson-Stein *et al.*, 2003).

Aunque muchos de los plásmidos descritos en estas especies actualmente se consideran crípticos, los datos disponibles revelan también la existencia de genes comunes también en ellos, por lo que su intercambio genético podría explicar la evolución de estas especies a partir de una posible vida epífita y la pérdida o ganancia de nuevas características en algunas cepas portadoras de los mismos. Estos plásmidos se transfieren verticalmente y tras mantenerse en la especie co-evolucionan con sus huéspedes bacterianos (Sundin, 2007, Smits *et al.*, 2011), pero también interactúan con el medio que les rodea e incluso con los genes cromosómicos propios de la especie portadora, pudiendo así explicarse desde diferentes puntos esta visión evolutiva.

Las diferentes especies de *Erwinia* asociadas de frutales de pepita parecen tener orígenes geográficos distantes, siendo *E. amylovora* la única especie distribuida a nivel mundial (Van der Zwet, 2001). Esta observación está basada en las respectivas detecciones en distintos países, pero ello podría reflejar solo una parte de la realidad, ya que al producir las otras especies síntomas similares a los de *E. amylovora* o ser epifitas, podrían pasar desapercibidas en países en donde el fuego bacteriano está presente, o bien directamente atribuirse sus síntomas a los provocados por el fuego bacteriano. Resulta interesante señalar, que tanto *E. pyrifoliae*, como *E. piriflorinigrans*, *E. tasmaniensis* o *E. uzenensis*, y las especies de *Erwinia* de Japón, se han descrito en países como Corea, España, Australia o Japón, donde *E. amylovora* no estaba presente o se tomaban medidas de erradicación frente a ella, lo que hacía que cualquier nuevo aislado sospechoso fuera estudiado en profundidad y con diversas técnicas basadas en

diferentes principios biológicos, caso muy diferente al de los países en los que *E. amylovora* está muy extendida, en los que prácticamente su diagnóstico se realiza de forma visual. Todo ello apoyaría nuestra recomendación de la necesidad de un estudio en profundidad de los aislados para poder aportar más información en cuanto a la epidemiología de las diferentes especies del género, así como su ciclo de vida e interacciones.

Del mismo modo, el rango real de plantas hospedadoras y órganos a los que pueden afectar las distintas especies de *Erwinia* es discutible en la actualidad, ya que muchas de estas especies están poco estudiadas, por el hecho del bajo impacto económico de las enfermedades que provocan en comparación con los daños ocasionados por el fuego bacteriano, o por ser epifitas (Palacio-Bielsa *et al.*, 2012). En este sentido es prioritario el estudio de aspectos moleculares, que podrían utilizarse con el fin de crear nuevas herramientas para su correcta identificación, a la vez que aportar una gran información en cuanto a las características de cada una de las especies.

Actualmente diversos estudios sobre la agresividad y otras características fenotípicas de *E. amylovora* han demostrado la homogeneidad cromosómica entre cepas de esta especie patógena, y una importante fuente de diversidad genética es la adquisición de nuevos genes debido a la incorporación de nuevos plásmidos, como se ha demostrado en otros modelos bacterianos y lo que explicaría la gran variabilidad entre cepas (Arnold *et al.*, 2003; Sundin, 2007; Oh y Beer, 2005; Cabrefiga y Montesinos, 2005; Smits *et al.*, 2010 a). Hasta ahora en *E. amylovora* se habían realizado pocos estudios sobre el contenido plasmídico de las cepas y sus funciones en la bacteria portadora, aunque actualmente estos estudios se han incrementado en cuanto al número de cepas analizadas y plásmidos descritos gracias a alguno de nuestros trabajos previos y

a esta tesis doctoral (Llop *et al.*, 2006, 2011, 2012; Hannou *et al.*, 2013 b; Ismail *et al.*, 2014; Smits *et al.*, 2014).

Por ello, el segundo objetivo de este trabajo fue la identificación de nuevos factores de virulencia, con el estudio de uno de sus plásmidos recientemente descrito pEI70, y el plásmido anteriormente considerado ubicuo en la especie pEA29.

Los datos más relevantes obtenidos con el estudio de este nuevo plásmido, después de analizar 1249 cepas de 17 países, muestran que está ampliamente distribuido en Europa occidental, central y noreste, pero ausente en los aislados de los países del suroeste así como en países de fuera del continente europeo, excepto en el norte de África (Marruecos y Argelia) (Hannou *et al.*, 2013 b). En dichos países probablemente los aislados encontrados tendrían un origen europeo, por frecuentes importaciones de plantas de países de la Unión Europea. En América del norte, donde la enfermedad se detectó por primera vez (van der Zwet y Keil, 1979), y pese a que el número de aislados analizados es amplio, no se han encontrado cepas con este plásmido, lo que sugiere que el pEI70 se pudo introducir en *E. amylovora a posteriori*, una vez que la especie se propagó por Europa.

En España, el pEI70 se detectó en el 14% de las cepas analizadas y en 6 de las 9 regiones en donde la enfermedad se detectó hasta 2009. Además, conociendo el origen de la introducción de *E. amylovora* con material vegetal importado de Bélgica, en viveros de Segovia y Guadalajara en 1996 y 1997 (Donat *et al.*, 2007), resulta lógico pensar que esa amplia distribución podría estar directamente relacionada con la comercialización de plantas de dichos viveros en otras comunidades autónomas. En otros países europeos el número de cepas analizadas es menor y en casos como Austria y Alemania no se ha logrado detectar pese a su proximidad a países donde se encuentra ampliamente distribuido. Ello sugiere que se han de realizar más prospecciones y disponer de

más cepas para confirmar la ausencia de dicho plásmido para tener una aproximación más real.

El análisis genético del nuevo plásmido pEI70, con una alta identidad de secuencia con el plásmido pEB102 de *E. billingiae* (Kube *et al.*, 2010) y algunos genes relacionados con la conjugación bacteriana, reafirma la teoría de la existencia de una transferencia horizontal de genes en especies de *Erwinia* próximas y su posterior adquisición por las cepas aeuropneas de *E. amylovora*. A su vez, vemos que algunos de los genes anotados en este plásmido sintetizan enzimas implicados en la glucólisis, para la obtención de energía celular y por lo tanto como una ventaja metabólica, y no relacionados directamente con la patogenicidad o virulencia, lo que podría dar sentido al encontrarlos en especies no patógenas como *E. billingiae*.

En *E. amylovora*, esta ventaja adaptativa, por su mayor capacidad metabólica y energética, presumiblemente se podría reflejar en la optimización de las cepas de la bacteria para degradar ciertos compuestos vegetales, otorgándoles el aumento de agresividad que hemos observado en todos nuestros ensayos.

En este trabajo, se ha demostrado que cuando el pEI70 era introducido en cepas de *E. amylovora* con un bajo nivel de virulencia, la intensidad de los síntomas se incrementaba y por otra parte, tras curar del plásmido a dos cepas que originalmente lo portaban con un nivel estándar en cuanto a su agresividad, la intensidad de los síntomas que provocaban disminuía a niveles similares a la cepa sin plásmidos. Las cepas que no contienen el plásmido pEA29, pero que por el contrario si contenían el pEI70, mostraban altos niveles en cuanto a su agresividad en la planta, siendo estos comparables a los de la cepa de referencia CFBP 1430, portadora del pEA29. En cambio, no se observó ningún efecto significativo de la introducción del pEA29 sobre la incidencia de infección de dos cepas que originariamente solo contenían el plásmido pEI70, es decir, no se

observó ningún efecto acumulativo en cuanto a la agresividad en estas cepas con ambos plásmidos. Se ha observado también que al introducir el plásmido pEA29 en algunas cepas, su nivel de agresividad variaba dependiendo de esta misma cepa, sugiriendo con ello la influencia de otros factores del fondo genómico, de las interacciones con el huésped y de las condiciones ambientales. Del mismo modo, el plásmido pEI70 parece proveer algunas características que compensan a la cepa de la pérdida del pEA29 y podría explicar los niveles de agresividad observados en las cepas que lo portan. Con estos resultados podemos afirmar que el pEI70 juega un papel importante en la virulencia de las cepas que lo portan como único plásmido, produciendo un efecto similar en la planta al que se observa en infecciones con las cepas portadoras del pEA29.

Por otro lado hay que señalar que existen cepas de *E. amylovora* no portadoras de ningún plásmido con un nivel de agresividad variable, como por ejemplo la cepa UPN527 aislada en España, que tiene un nivel alto de agresividad, o las cepas BC3 y CGJ2 aisladas de Serbia, que tienen un nivel de agresividad bajo, indicando la necesidad de estudios para conocer la interacción entre los plásmidos y el cromosoma, y las diferencias en la expresión de los genes cromosómicos durante la infección *in planta*, ya que todas estas ventajas no serían únicamente atribuibles a estos elementos extracromosómicos. Todo esto hace resaltar la complejidad de la biología de este patógeno en cuanto a su fondo genético y a su interacción con el medio que le rodea.

Por ello, como tercer objetivo de esta tesis, se decidió analizar el efecto de la presencia de los dos plásmidos que se sabe producen un incremento en la formación de síntomas, sobre la expresión de los genes cromosómicos de *E. amylovora* durante la interacción con la planta. Este análisis podría esclarecer estas diferencias en la agresividad de las cepas de esta especie, ya que todavía

hay mecanismos desconocidos que pueden desempeñar un papel en la virulencia ya sea directa o indirectamente, en este importante patógeno.

Para ello, se realizó mediante la hibridación y el análisis de datos de un *microarray*, un estudio comparativo entre cepas con el mismo fondo genético portadoras y no de plásmidos relacionados con la virulencia (pEA29 y pEI70). En este marco, se estudió la expresión diferencial de los genes cromosómicos durante el desarrollo de síntomas en pera inmadura y se realizó unos posteriores análisis funcionales de los mismos para complementar con una visión biológica.

Los resultados obtenidos indicaron que las cepas portadoras del pEA29, cuando están afectando a la planta tienen la capacidad de sobreexpresar genes que codifican para componentes celulares implicados de una forma directa con la patogenicidad de la especie, como los genes relacionados con la membrana externa y cápsula celular. En cambio, en cepas con el pEI70 se activan genes relacionados con la formación de complejos proteicos para el transporte activo transmembrana de sustancias. Así, este aumento de la virulencia en las cepas portadoras del pEI70, a diferencia de las que portan el pEA29, se vería determinada por un mayor metabolismo y movimiento de sustancias que dotaría a las mismas de una ventaja en cuanto a su modo de vida *in planta*, pudiendo producir mayores efectos dañinos sobre el huésped, sin relacionarse estos grupos de genes directamente con la virulencia de la especie.

Los procesos biológicos de los genes sobreexpresados de ambas cepas se engloban dentro de generación de energía mediante glucólisis y metabolismo de aminoácidos, confirmando la hipótesis general mostrada en los estudios anteriores con el pEI70, de que las cepas portadoras de plásmidos activan determinados mecanismos para la síntesis de energía celular en mayor proporción que las cepas de *E. amylovora*, que no son portadoras de ninguno de estos dos plásmidos (Llop *et al.*, 2006, 2008 a, 2008 b).

En este estudio también fue interesante observar que en cepas que portan alguno de estos plásmidos, existe una represión de los genes cromosómicos relacionados con la síntesis de flagelos. Estos resultados confirman los datos de Cesbron *et al.*, (2004) en donde se demuestra que los sistemas flagelares y los *hrp* están regulados de forma inversa en *E. amylovora*. Actualmente es conocido que la motilidad es un factor determinante para la virulencia en *E. amylovora*. Esta es necesaria en las primeras fases de la infección cuando la bacteria se desplaza hacia las aberturas de la planta, seguida de la adherencia y penetración que iniciaría la producción de la sintomatología. Llegados a este punto, una vez colonizada la planta hospedadora, los genes que codifican para el movimiento bacteriano no serían necesarios, sin embargo sí que se empezarían a expresar genes relacionados directamente con la patogénesis de la especie (Cesbron *et al.*, 2004). En nuestro caso, la diferencia de expresión en la represión de determinados genes relacionados con la movilidad estaría determinada por los diferentes genes que se reprimen en el caso del pEA29 (*flhA* y *flgM*) en comparación con los reprimidos en el caso del pEI70 (*fliT*, *flgL* y *flgA*) frente a la cepa sin plásmidos. De manera general, la estrategia para la inhibición de la motilidad en esta fase de la infección en las cepas portadoras del plásmido de 29 Kb sería la represión de la síntesis de flagelina que impide por otro lado la expresión de determinados genes relacionados con la formación del filamento y la quimiotaxis. Además, la estrategia que seguirían para el mismo fin las cepas portadoras del pEI70, sería la represión de algunos genes implicados en la formación del sistema flagelar, ya que expresan para proteínas flagelares y proteínas formadoras del anillo flagelar.

Con estos datos podemos decir que este es un primer estudio global a nivel de expresión cromosómica que demuestra que hay un efecto de la presencia de los plásmidos completos (pEA29 y pEI70), que después de su confirmación por PCR cuantitativa, podría proporcionar información novedosa sobre la correlación

entre el nivel de virulencia de la cepa portadora frente a la cepa sin plásmidos, con el mismo fondo cromosómico. Para confirmar todas estas hipótesis haría falta en primer lugar realizar una validación de los datos obtenidos con este análisis con *array*, de la expresión diferencial de los genes seleccionados (como por ejemplo los genes *flgM*, *rcsV*, EAM_1806, EAM_2488, *ompW* o *adhP*) mediante PCR cuantitativa. Para ello habría que hacer una transcripción reversa del ARN con el que se hibridó el *array* y con iniciadores diseñados específica e individualmente para estos genes para poder determinarlos cuantitativamente, en comparación con un control endógeno como el gen *gyrA* (de expresión no variable) (McNally *et al.*, 2011; Águila-Clares *et al.*, 2016) . Esta validación daría paso a ensayos de mutagénesis de los genes seleccionados por su nivel de expresión diferencial, para posteriormente analizar el efecto de estos mutantes en ensayos en plantas hospedadoras. Del mismo modo en futuros estudios se deberían analizar los genes plasmídicos, su diferente nivel de expresión debido a la interacción de la bacteria portadora con la planta.

Las prospecciones llevada a cabo en 1999 en Valencia, en una comunidad autónoma libre en esos años de *E. amylovora*, se descubrió una nueva especie de *Erwinia* patógena de peral, *E. piriflorinigrans*, que en cuanto a los síntomas que produce y sus características bioquímicas y fenotípicas es afín al agente causal del fuego bacteriano (Roselló *et al.*, 2002, 2006; López *et al.*, 2011). En estos trabajos se observó que esta especie es portadora de un plásmido de alrededor de 30 Kb que podía hibridar con sondas del pEA29 de *E. amylovora*, al igual que sucedía con otros plásmidos de un tamaño similar descritos en otras especies del género, como por ejemplo el pEP36 y pEJ30 de *E. pyrifoliae* (López *et al.*, 2011; (Maxon-Stein *et al.*, 2003).

Por ello, como cuarto objetivo de esta tesis y en el contexto del estudio de estos plásmidos de similar tamaño en especies de *Erwinia* patógenas de frutales de pepita, analizamos los de *E. piriflorinigrans*. Siguiendo los estudios previos de

este patógeno, en primer lugar se analizó el contenido genético de los dos plásmidos de esta nueva especie, el de 37 Kb (pEPIR37), considerado actualmente ubicuo en los aislados analizados, y el plásmido de 5 Kb (pEPIR5), presente casi en la mitad de los mismos y actualmente considerado críptico. Estos estudios permitieron conocer la existencia *in silico* de una cierta semejanza a nivel de la secuencia del plásmido pEPIR37 de esta nueva especie con el plásmido pEA29 de *E. amylovora*.

Es sabido que la característica más notable en el pEA29, presente en la mayoría de cepas de *E. amylovora*, es la presencia de los genes de biosíntesis de tiamina (*thiO*, *thiS*, *thiG* y *thiF*) (Laurent *et al.*, 1989; McGhee y Jones, 2000; McGhee y Sundin, 2008). Diversos trabajos han postulado que estos genes son los principales responsables del aumento de la agresividad de las cepas portadoras del plásmido de 29 Kb (Geier *et al.*, 1993; Zhao *et al.*, 2005; Smits *et al.*, 2010 a). El *cluster thiOSGF* también se ha encontrado en otros plásmidos de un tamaño cercano a los 30 Kb descritos en las otras especies de *Erwinia* patógenas de frutales de pepita (como por ejemplo en los plásmidos pEP36 y pEJ30 en *E. pyrifoliae*) (Smits *et al.*, 2010 b), así como en el cromosoma de *E. tasmaniensis* (Kube *et al.*, 2010; Kube *et al.*, 2008 b).

En este trabajo se ha observado que en el plásmido pEPIR37 de *E. piriflorinigra*s existe este *cluster (thiOSGF)* y su secuencia muestra una relación estrecha filogenéticamente entre las especies de *Erwinia* patógenas pero curiosamente está más cercano a la especie considerada no patógena *E. tasmaniensis*, que además es la especie de la que está más próxima desde el punto de vista taxonómico, en base al análisis del 16S rRNA.

Con esta información nos preguntamos si estos plásmidos desarrollan la misma función que la del plásmido pEA29 de *E. amylovora* y por lo tanto favorecen el incremento de los síntomas en la planta hospedadora (McGhee y Sundin, 2008).

Con la introducción del pEPIR37 en cepas de *E. amylovora* sin plásmidos se comprobó el incremento de la virulencia en fruto inmaduro al mismo nivel que lo hace el pEA29, haciendo patente que este plásmido, al igual que sucede con el pEA29 (y probablemente ocurra de forma general con los plásmidos con las mismas características), puede contribuir al aumento de los síntomas provocados por las cepas que los poseen.

El hecho de que los genes de la biosíntesis de tiamina se localicen a nivel de los plásmidos de alrededor de 30 kb en estas especies nos hace pensar que en un ancestro universal, estos genes ya existían y se localizaban en el cromosoma bacteriano, como sigue pasando en *E. tasmaniensis*, pero en su evolución estos se desplazarían a este tipo de plásmidos altamente estables y conservados, creándole una dependencia a la bacteria de los mismos para llevar esta importante función relacionada con el tipo de vida patógeno. Del mismo modo, estos plásmidos también podrían evolucionar mediante recombinación con otros elementos extracromosómicos (o con el cromosoma del portador), especializándose pero de forma limitada, ya que su transferencia sería vertical y no horizontal al no considerarse plásmidos conjugativos por no poseer genes *tra*.

Estos genes se considerarían ortólogos por su posible origen común, y su limitación para evolucionar podría explicar la semejanza entre todos ellos y su similar papel en las especies patógenas (Smits *et al.*, 2011). El hecho de que el *cluster* se encuentre en la especie *E. tasmaniensis*, junto con otros genes implicados en la virulencia del género, hace pensar que aunque está presente a) no es del todo funcional, b) no está regulado o c) está reprimido, entre otros factores, ya que no se han detectado niveles altos de exopolisacáridos en dicha especie. Por tanto sería una de las razones por la que esta especie no tuviera la capacidad de producir daños graves en las especies vegetales con las que se ha trabajado o en las que se ha aislado.

Estos plásmidos de alrededor de 30 Kb descritos en las especies de *Erwinia* patógenas y epifitas asociadas a frutales de pepita, aunque les confieran ventajas a la cepa portadora, no son necesarios para la supervivencia de la bacteria. Todo ello explicaría la existencia de por ejemplo cepas *E. amylovora* sin el plásmido de 29 Kb, aunque parecen ser casos excepcionales en comparación con las que lo portan, estas cepas perderían en algún momento el plásmido pudiendo sobrevivir y persistir en el medio, pese su desventaja por la auxotrofia de la vitamina B1 y por el déficit en la biosíntesis de amylovorano.

Por otro lado y desde el punto de vista epidemiológico, hasta la fecha, la información sobre la biología de las cepas de *E. piriflorinigrans* era escasa ya que únicamente se había detectado en cultivos de peral en Valencia y el daño causado por la enfermedad parecía limitado (Roselló *et al.*, 2006). La semejanza en la sintomatología con la del fuego bacteriano, el hecho de compartir los huéspedes y la similar morfología colonial en los medios de aislamiento empleados para la detección e identificación de ambos patógenos hacen que sea difícil distinguir las dos especies si no se buscan exhaustivamente o no se tiene experiencia en las mismas (López *et al.*, 2011). Por ello se realizaron en este trabajo diversos estudios epidemiológicos sobre esta nueva especie en distintos momentos del año para intentar conocer su forma de diseminación y la cantidad de inóculo presente en muestras con y sin síntomas en estados fenológicos del peral presumiblemente más favorables. Para ello fue necesario como quinto objetivo de esta tesis, desarrollar un sistema de detección altamente específico y sensible que nos permitiera distinguir *E. piriflorinigrans* de otras especies de *Erwinia* que pudieran estar presentes en ese mismo nicho, principalmente *E. amylovora*. Para ello, se aprovechó la información molecular y fisiológica disponible, es decir, la secuencia previamente estudiada del pEPIR37 y su estabilidad y universalidad en esta especie.

En este trabajo se diseñaron dos protocolos de PCR basados en secuencias específicas de este plásmido con el fin de detectar, identificar y diferenciar a *E. piriflorinigra* de otras especies. El diseño de ambas PCRs se basa en la amplificación de un fragmento del gen *frnC*, que codifica para un transportador de membrana de níquel tipo ABC. Una vez demostrada la alta especificidad de ambas técnicas se analizó la sensibilidad mediante diferentes protocolos de extracción de ADN bacteriano, e incluso sin extracción previa de ADN, mostrando una buena eficiencia. Este resultado reduce el riesgo de contaminación durante la manipulación de las muestras, ahorra tiempo en el procesado de las mismas y minimiza los costes por reacción, resultando por tanto muy útil para procesar grandes cantidades de muestras. Del mismo modo, el protocolo de PCR en tiempo real se considera el protocolo más preciso y seguro para la detección y el diagnóstico de este patógeno, como sucede frecuentemente en la detección de otros agentes bacterianos fitopatógenos (Smits *et al.*, 2013; López *et al.*, 2009; Palacio-Bielsa *et al.*, 2009).

El uso de estos protocolos dio paso a nuevas prospecciones en las zonas donde este patógeno se descubrió por primera vez, y en otras comunidades autónomas en las que se había detectado fuego bacteriano. Ello permitió conocer mejor su biología, así como estimar su concentración en muestras asintomáticas y sintomáticas. Con esta metodología se ha podido localizar a *E. piriflorinigra* en muestras coinfectadas con *E. amylovora*, en donde en un primer momento podría haberse confundido o pasado por alto su identificación de forma visual en los aislamientos. Estas muestras coinfectadas pertenecen a especies vegetales diferentes a los cultivares de peral en donde se había encontrado a *E. piriflorinigra* de forma exclusiva, hallándose en este trabajo en manzano y *Pyracantha*, especies hospedadoras del fuego bacteriano, así como en diferentes órganos aparte de los corimbos florales. Por todo ello se hace patente la necesidad de investigar el ciclo de vida de *E. piriflorinigra*, así como su distribución geográfica, hallándose en un breve lapso de tiempo, a raíz

de la puesta a punto de estos nuevo métodos de diagnóstico, esta especie en otras áreas de España alejadas del lugar en donde se detectó por primera vez (como por ejemplo Almería o Navarra), así como en diferentes países (comunicación personal B. Duffy). Algo similar ha ocurrido en otras especies del género como *E. pyrifoliae* en Corea, Japón y en los Países Bajos (Shrestha *et al.*, 2007; <https://gd.eppo.int/taxon/ERWIPY/distribution>, consultada el 25/04/2017). En base a estos hallazgos creemos que esta especie será probablemente detectada en muchos más países, y que probablemente en alguno de ellos pudiera haber sido identificada erróneamente como *E. amylovora*, como ocurrió al describir por primera vez las cepas de *Erwinia* sp. en Hokkaido (Tanii *et al.*, en 1981; Palacio-Bielsa *et al.*, 2012).

Es conveniente resaltar que en los aislamientos iniciales para confirmar el diagnóstico, en las muestras coinfectadas por *E. amylovora* y *E. piriflorinigra*s, la primera estaba presente en mayor proporción que *E. piriflorinigra*s. Sin embargo, se necesita más investigación para conocer con precisión la interacción de ambos patógenos en el mismo nicho.

Los síntomas provocados en flor de peral descritos hasta la fecha para *E. piriflorinigra*s pueden ser debidos a infecciones provocadas tanto por *E. amylovora* como *E. piriflorinigra*s, así como por otras especies bacterianas del mismo género o de otros géneros como *Pseudomonas* sp., infecciones por hongos o por trastornos fisiológicos en la planta. En consecuencia, es importante comprender que no es aconsejable hacer un diagnóstico de *visu* únicamente en las inspecciones en campo, pero tampoco limitarse a un diagnóstico en placa o serológico, ya que la identificación correcta de *E. piriflorinigra*s se debería complementar con alguna de estas dos nuevas técnicas moleculares, y por tanto con un diferente fundamento biológico, como se recomienda de forma general, en los protocolos de la EPPO para el diagnóstico,

detección e identificación bacteriana. En todos los casos en los que se ha detectado, las pérdidas ocasionadas por esta especie, no parecen ser comparables a las causadas por *E. amylovora*, por lo que se podría considerar como una especie de reducida patogenicidad o incluso como un patógeno oportunista, pero todo ello deberá ser evaluado en futuros estudios.

El análisis total del genoma de un mayor número de cepas de las diferentes especies de *Erwinia* relacionados con los frutales de pepita, como *E. uzenensis* (Matsuura *et al.*, 2012), la especie recientemente descrita *E. gerundensis* (Rezzonico *et al.*, 2016) u otras especies de *Erwinia* aún sin caracterizar procedentes de Japón (Palacio *et al.*, 2012) de las que aún no hay apenas información disponible ni sobre su contenido en plásmidos, ni su composición genómica, podría proporcionar información adicional sobre la evolución de estas especies a la vez que dar nuevas pistas sobre el origen de las mismas y ayudar a una mejor comprensión de su ciclo de biológico.

En conclusión, los resultados recogidos en esta memoria han permitido avanzar y profundizar en el conocimiento del genoma del agente causal del fuego bacteriano de las rosáceas *E. amylovora*, con el estudio de un nuevo plásmido, se ha demostrado el efecto de los plásmidos de esta especie implicados en la virulencia en la expresión de genes cromosómicos durante la interacción con la planta. A su vez se han esclarecido aspectos del ciclo de vida de *E. piriflorinigrans* no conocidos hasta ahora, así como desarrollado métodos para su correcta detección e identificación que nos permitirán seguir avanzando en el conocimiento de la biología y distribución de esta nueva especie bacteriana.

Capítulo 9

Conclusiones

- Las conclusiones de esta memoria, se refieren a dos bacterias fitopatógenas del género *Erwinia*. Respecto a *E. amylovora*, el exhaustivo análisis del nuevo plásmido pEI70 de *E. amylovora* mostró, una alta identidad de secuencia nucleotídica (más del 98%) con el plásmido pEB102 de *E. billingiae* Eb661, lo que sugiere un origen común de ambos plásmidos. Este plásmido se transfiere de forma conjugativa a otras cepas de *E. amylovora*, y posiblemente por ello está ampliamente distribuido en 11 países de Europa occidental, nororiental y central, y en algunos países colindantes como Marruecos y Argelia.
- Su papel en la virulencia de *E. amylovora* ha sido comprobado ya que la introducción del pEI70 en cepas de *E. amylovora* con bajo nivel de virulencia produce un incremento en los síntomas de la enfermedad en en pera inmadura, del mismo modo que tras curar el plásmido de diferentes cepas con un nivel estándar en cuanto a su agresividad, la gravedad e incidencia de la infección se ve reducida. La anotación de los genes del plásmido pEI70 no predijo genes directamente relacionados con la virulencia.
- El análisis funcional de la cepa de *E. amylovora* portadora del plásmido pEA29 en un *microarray* ha mostrado una expresión diferencial de genes cromosómicos, al igual que ocurrió con la cepa de *E. amylovora* portadora del plásmido pEI70, con respecto a la misma cepa de *E. amylovora* curada de plásmidos, durante la interacción del patógeno con el fruto. Algunos de los genes expresados diferencialmente en las cepas portadoras de alguno de los plásmidos eran coincidentes.
- En todos los casos, los genes expresados diferencialmente en las cepas con alguno de los dos plásmidos, se agrupan en un mayor número

dentro de la categoría funcional del metabolismo con respecto a la cepa curada de ellos. La diferencia en cuanto a las funciones metabólicas implica un aumento indirecto de la agresividad de estas cepas que ya se había observado en el incremento en los síntomas de la enfermedad en un menor tiempo.

- Todas las cepas analizadas de la especie *Erwinia piriflorinigra* son portadoras del plásmido de 37 Kb (pEPIR37) y muchas de las mismas también son portadoras de otro plásmido de 5 Kb (pEPIR5). Se ha analizado la secuencia completa de los dos plásmidos (pEPIR37 y pEPIR5), y se ha comparado con plásmidos de tamaño similar de otras especies de *Erwinia*, observándose que su contenido genético está altamente conservado en todas las especies de *Erwinia* filogenéticamente próximas a *E. piriflorinigra*.
- La introducción del plásmido pEPIR37 en una cepa de *E. amylovora* con nivel de virulencia reducido, provoca un aumento de su agresividad similar al provocado en las cepas portadoras del pEA29, en los experimentos de inoculación en pera inmadura.
- Se han diseñado dos protocolos de PCR (convencional y en tiempo real) basados en el plásmido pEPIR37 recientemente descrito para detectar, identificar y diferenciar la especie *E. piriflorinigra* de *E. amylovora* y de otras especies de *Erwinia*. Ambos protocolos son capaces de detectar de forma específica y con una buena sensibilidad la especie *E. piriflorinigra* directamente en material vegetal, sin previa extracción de ADN bacteriano. Con su empleo se ha conseguido detectar al nuevo patógeno en cuatro nuevos tejidos vegetales y dos nuevos huéspedes, al mismo tiempo que se han hallado muestras coinfectadas con el

agente causal del fuego bacteriano. Con ellos también se ha demostrado que la distribución geográfica del patógeno es más amplia, encontrándose en áreas nuevas de España (por ejemplo en Almería y Navarra) y de fuera de sus fronteras.

- El conjunto de resultados presentados en esta memoria, abre la puerta a nuevos estudios funcionales sobre el papel de los plásmidos en las distintas especies de *Erwinia* y sobre la biología y el ciclo de vida de *E. piriflorinigra*s.

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Información suplementaria

Información suplementaria capítulo 4

Erwinia amylovora novel plasmid pEI70: complete sequence, biogeography, and role in aggressiveness in the fire blight phytopathogen

Table 4-S1. Predicted CDS to proteins of pEI70 in the GenBank nonredundant database.

Table 4-S2. Additional information available from *E. amylovora* strains positive for pEI70 analyzed in European countries.

Table 4-S3. Additional information available from *E. amylovora* strains negative for pEI70 analyzed in European countries.

Table 4-S1. Predicted CDS to proteins of pEI70 in the GenBank nonredundant database.

Locus tag	Gene name	Best BlastP hit Sequence identity	% amino acid identity	Accession number
01		ArdK protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739154.1
02	<i>repA</i>	RepA replication initiation protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739153.1
03		No significant similarity found	-	-
04		Integrase <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739150.1
05		No significant similarity found	-	-
06		Hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739148
07		Hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739147.1
08		Hypothetical protein <i>Escherichia coli</i> UMNK88	33	AEE59532
09		Hypothetical protein <i>Serratia</i> sp. AS12	31	YP_004498793.1
10	<i>ardC</i>	Antirestriction protein ArdC <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739145.1
11	<i>yubA</i>	Protein of unknown function (DUF1281) <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739144.1
12	<i>yeeW</i>	Hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739143.1
13		Hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739142.1
14		DNA helicase <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739141.1
15		Putative helicase/relaxase <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739140.1
16		Predicted thioesterase <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739139.1
17		Hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739138.1
18		Hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739137.1
19	<i>dnab</i>	Replicative DNA helicase <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739136.1
20		ICE-PFGI_1_parB, ParB family protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739135.1
21		DUF2857 superfamily protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739134.1
22		Conserved hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739133.1
23		Conserved hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739132.1
24		Integrating conjugative element protein, PFL_4669 family <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739131.1
25		Conserved hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739130.1
26	<i>topB</i>	DNA topoisomerase III <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739129.1
27		Conserved hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739128.1
28	<i>ssb</i>	Single-stranded DNA-binding protein <i>E. billingiae</i> Eb661	99	YP_003739127.1

	plasmid pEB102		
29	Conserved hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739126.1
30	Hypothetical protein <i>Serratia symbiotica</i> str. Tucson	76	ZP_08039662.1
31	Hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739125.1
32	<i>pill</i> Similar to type IV B pilus protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739124.1
33	Hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739123.1
34	ICE protein, PFL_4693 family <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739122.1
35	Putative lytic transglycosylase, catalytic <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739121.1
36	ICE protein, PFL_4695 family <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739120.1
37	Restriction endonuclease, type IV-like <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739119.1
38	Conjugative coupling factor TraD, SXT/TOL subfamily <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739118.1
39	ICE membrane protein, PFL_4697 family <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739117.1
40	Plasmid conserved hypothetical protein, RAQPRD family <i>E. billingiae</i> Eb661 plasmid pEB102	98	YP_003739115.1
41	ICE protein, PFL_4701 family <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739114.1
42	ICE membrane protein, PFL_4702 family <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739113.1
43	Conjugative transfer region protein, TIGR03750 family <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739112.1
44	ICE protein, PFL_4703 family <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739111.1
45	ICE protein, PFL_4704 family <i>E. billingiae</i> Eb661 plasmid pEB102	98	YP_003739110.1
46	ICE protein, PFL_4705 family <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739109.1
47	Conjugative transfer region lipoprotein, TIGR03751 family <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739108.1
48	Putative plasmid-related outer membrane ATPase <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739107.1
49	Conjugative transfer ATPase, PFL_4706 family <i>E. billingiae</i> Eb661 plasmid pEB102	98	YP_003739106.1
50	Hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739105.1
51	Conserved hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739104.1
52	ICE protein, PFL_4709 family <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739103.1
53	ICE protein, PFL_4710 family <i>E. billingiae</i> Eb661 plasmid	100	YP_003739102.1

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		pEB102		
54		ICE protein, PFL_4711 family <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739101.1
55		Conserved hypothetical protein <i>E. billingiae</i> Eb661 plasmid pEB102	99	YP_003739100.1
56		TraG_N superfamily protein <i>Burkholderia gladioli</i> BSR3 chromosome 2	26	YP_004349864.1
57	<i>parA</i>	Putative site-specific recombinase, resolvase family <i>E. billingiae</i> Eb661 plasmid pEB102	98	YP_003739098.1
58	<i>umuC</i>	DNA-repair protein, UmuC-like <i>Pantoea</i> sp. At-9b plasmid pPAT9B05	83	YP_004119580.1
59		WD40 superfamily protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739166.1
60	<i>mae1</i>	C4-dicarboxylate transporter/malic acid transport protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739165.1
61		Uracil-DNA glycosylase family protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739164.1
62	<i>ppaC</i>	Inorganic pyrophosphatase <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739163.1
63		Universal stress protein A <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739162.1
64	<i>eno</i>	Enolase <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739161.1
65	<i>gpmA</i>	3-bisphosphoglycerate-dependent phosphoglycerate mutase <i>Pantoea ananatis</i> AJ13355	92	BAK12605.1
66	<i>crcB</i>	Camphor resistance CrcB family protein <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739159.1
67		Glyoxalase/bleomycin resistance protein/dioxygenase <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739158.1
68		Site-specific recombinase, phage integrase family <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739157.1
69	<i>xerD</i>	Tyrosine recombinase <i>xerC</i> <i>E. billingiae</i> Eb661 plasmid pEB102	100	YP_003739156.1
70	<i>samB</i>	DNA polymerase IV.DNA-directed DNA polymerase <i>Pantoea</i> sp. At-9b plasmid pPAT9B05	79	YP_004119580.1

Table 4-S2. Additional information available from *E. amylovora* strains positive for pEI70 analyzed in European countries.

Country	Collection	Isolates positive for pEI70	Host	Year of isolation
Belgium	LMG	LMG 1880	Pear	1979
Belgium	LMG	LMG 1881	<i>Crataegus</i> sp.	1980
Belgium	LMG	LMG 1882	Pear	1977
Belgium	LMG	LMG 1883	Pear	1979
Belgium	LMG	LMG 1885	<i>Crataegus</i> sp.	1980
Belgium	LMG	LMG 1889	<i>Crataegus</i> sp.	1980
Belgium	LMG	LMG 1892	<i>Cotoneaster</i> sp.	1980
Belgium	LMG	LMG 1893	Pear	1980
Belgium	LMG	LMG 1895	<i>Crataegus</i> sp.	1980
Belgium	LMG	LMG 1899	Pear	1980
Belgium	LMG	LMG 1906	<i>Crataegus</i> sp.	1980
Belgium	LMG	LMG 1907	Pear	1980
Belgium	LMG	LMG 1910	Pear	1980
Belgium	LMG	LMG 1911	<i>Cotoneaster</i> sp.	1980
Belgium	LMG	LMG 1931	Pear	1980
Belgium	LMG	LMG 1932	<i>Cotoneaster</i> sp.	1980
Belgium	LMG	LMG 1933	<i>Cotoneaster</i> sp.	1980
Belgium	LMG	LMG 1934	Pear	1980
Belgium	LMG	LMG 1936	<i>Crataegus</i> sp.	1980
Belgium	LMG	LMG 1937	<i>Cotoneaster</i> sp.	1980
Belgium	LMG	LMG 1946	Pear	1980
Belgium	LMG	LMG 1947	<i>Crataegus</i> sp.	1980
Belgium	LMG	LMG 1955	<i>Crataegus</i> sp.	1980
Belgium	LMG	LMG 1961	<i>Crataegus</i> sp.	1980
Belgium	CRA-W	EaP1	Pear	2001
Belgium	CRA-W	EaP2	Pear	2001
Belgium	CRA-W	EaP5	Pear	2001
Belgium	CRA-W	EaP18	Pear	2001
Belgium	CRA-W	EaP24	Pear	2001
Belgium	CRA-W	EaP25	Pear	2001
Belgium	CRA-W	EaP26	Pear	2001
Belgium	CRA-W	EaP27	Pear	2001
Belgium	CRA-W	EaP28	Pear	2001
Belgium	CRA-W	EaP29	Pear	2002
Belgium	CRA-W	EaP30	Pear	2002
Belgium	CRA-W	EaP31	Pear	2002
Belgium	CRA-W	EaP32	Pear	2002
Belgium	CRA-W	EaP33	Pear	2002

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Belgium	CRA-W	EaP34	Pear	2002
Belgium	CRA-W	EaP35	Pear	2002
Belgium	CRA-W	EaP36	Pear	2002
Belgium	CRA-W	EaP38	Pear	2002
Belgium	CRA-W	EaP39	Pear	2002
Belgium	CRA-W	EaP40	Pear	2002
Belgium	CRA-W	EaP41	Pear	2002
Belgium	CRA-W	EaP42	Pear	2002
Belgium	CRA-W	EaP43	Pear	2002
Belgium	CRA-W	EaP44	Pear	2002
Belgium	CRA-W	EaP46	Pear	2002
Belgium	CRA-W	EaP47	Pear	2002
Belgium	CRA-W	EaP48	Pear	2002
Belgium	CRA-W	EaP49	Pear	2002
Belgium	CRA-W	EaP50	Pear	2002
Belgium	CRA-W	EaP51	Pear	2002
Belgium	CRA-W	EaP52	Pear	2002
Belgium	CRA-W	EaP53	Pear	2004
Belgium	CRA-W	EaA9	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaA10	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaA11	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaA12	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaA14	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaA15	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaA17	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaA20	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaA54	<i>Crataegus</i> sp.	2001
Czech Republic	BPIC	BPIC 1634	<i>Sorbus</i> sp.	1990
France	CFBP	CFBP1367	<i>Crataegus</i> sp.	1972
France	CFBP	CFBP1368	Pear	1972
France	LNPV	LNPV 1585	<i>Cotoneaster</i> sp.	na
Ireland	SL	SL 1014	<i>Pyracantha</i> sp.	1986
Ireland	SL	SL 2156	<i>Cotoneaster</i> sp.	na
Ireland	SL	SL 2157	<i>Cotoneaster</i> sp.	na
Ireland	SL	SL 2158	<i>Sorbus</i> sp.	na
Ireland	SL	SL 2159	<i>Sorbus</i> sp.	na
Ireland	SL	SL 2160	<i>Sorbus</i> sp.	na
Ireland	SL	SL 2161	<i>Sorbus</i> sp.	na
Ireland	SL	SL 2162	<i>Sorbus</i> sp.	na
Ireland	SL	SL 2163	<i>Cotoneaster</i> sp.	na
Ireland	SL	SL 2164	<i>Sorbus</i> sp.	na
Ireland	CFBP	CFBP 2584	<i>Cotoneaster</i> sp.	1986
Ireland	DERM	E-70	<i>Cotoneaster</i> sp.	1997

Italy	OMP	OMP-BO 1185	Apple	1997
Italy	SFR	SFR-BO 685	<i>Crataegus</i> sp.	1999
Italy	SFR	SFR- BO 984	<i>Crataegus</i> sp.	1999
Poland	DPP	2	<i>Crataegus</i> sp.	2000
Poland	DPP	600	<i>Crataegus</i> sp.	1993
Poland	DPP	601	Pear	1993
Poland	DPP	603	<i>Crataegus</i> sp.	1994
Poland	DPP	606	<i>Crataegus</i> sp.	1994
Poland	DPP	665	Pear	1993
Poland	DPP	666	<i>Crataegus</i> sp.	1996
Slovenia	NIB	109	<i>Cydonia</i> sp.	2002
Slovenia	NIB	110	<i>Cydonia</i> sp.	2002
Slovenia	NIB	114	<i>Cydonia</i> sp.	2002
Slovenia	NIB	115	<i>Cydonia</i> sp.	2002
Slovenia	NIB	170	<i>Cydonia</i> sp.	2003
Slovenia	NIB	171	Pear	2003
Slovenia	NIB	172	<i>Cydonia</i> sp.	2003
Slovenia	NIB	173	Apple	2003
Slovenia	NIB	175	Apple	2003
Slovenia	NIB	176	<i>Cydonia</i> sp.	2003
Slovenia	NIB	177	Apple	2003
Slovenia	NIB	178	<i>Pyracantha</i> sp.	2003
Slovenia	NIB	180	<i>Pyracantha</i> sp.	2003
Slovenia	NIB	181	Apple	2003
Slovenia	NIB	182	Pear	2003
Slovenia	NIB	183	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	185	Apple	2003
Slovenia	NIB	189	<i>Crataegus</i> sp.	2003
Slovenia	NIB	190	Apple	2003
Slovenia	NIB	191	<i>Cydonia</i> sp.	2003
Slovenia	NIB	192	Pear	2003
Slovenia	NIB	193	Apple	2003
Slovenia	NIB	195	Apple	2003
Slovenia	NIB	199	Pear	2003
Slovenia	NIB	202	Pear	2003
Slovenia	NIB	203	Apple	2003
Slovenia	NIB	204	Pear	2003
Slovenia	NIB	205	Apple	2003
Slovenia	NIB	206	Apple	2003
Slovenia	NIB	207	Apple	2003
Slovenia	NIB	208	Apple	2003
Slovenia	NIB	210	Apple	2003
Slovenia	NIB	211	Apple	2003
Slovenia	NIB	212	Apple	2003
Slovenia	NIB	213	Apple	2003
Slovenia	NIB	215	Apple	2003

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Slovenia	NIB	216	Apple	2003
Slovenia	NIB	217	Pear	2003
Slovenia	NIB	218	Apple	2003
Slovenia	NIB	219	Apple	2003
Slovenia	NIB	224	Apple	2003
Slovenia	NIB	225	Apple	2003
Slovenia	NIB	226	Apple	2003
Slovenia	NIB	228	<i>Pyracantha</i> sp.	2003
Slovenia	NIB	231	Apple	2003
Slovenia	NIB	235	Pear	2003
Slovenia	NIB	236	Apple	2003
Slovenia	NIB	237	Apple	2003
Slovenia	NIB	238	Pear	2003
Slovenia	NIB	239	Apple	2003
Slovenia	NIB	241	Pear	2003
Slovenia	NIB	242	Apple	2003
Slovenia	NIB	244	Pear	2003
Slovenia	NIB	245	<i>Cydonia</i> sp.	2003
Slovenia	NIB	246	Apple	2003
Slovenia	NIB	251	Pear	2003
Slovenia	NIB	252	Pear	2003
Slovenia	NIB	253	<i>Crataegus</i> sp.	2003
Slovenia	NIB	254	Pear	2003
Slovenia	NIB	257	Apple	2003
Slovenia	NIB	258	Apple	2003
Slovenia	NIB	259	Pear	2003
Slovenia	NIB	261	Apple	2003
Slovenia	NIB	262	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	264	Pear	2003
Slovenia	NIB	265	Apple	2003
Slovenia	NIB	271	Pear	2003
Slovenia	NIB	273	Apple	2003
Slovenia	NIB	274	Pear	2003
Slovenia	NIB	275	Apple	2003
Slovenia	NIB	278	Pear	2003
Slovenia	NIB	283	Pear	2003
Slovenia	NIB	284	Apple	2003
Slovenia	NIB	285	Pear	2003
Slovenia	NIB	286	Pear	2003
Slovenia	NIB	290	Apple	2003
Slovenia	NIB	291	Apple	2003
Slovenia	NIB	293	Apple	2003
Slovenia	NIB	298	Apple	2003
Slovenia	NIB	300	Apple	2003
Slovenia	NIB	301	Apple	2003
Slovenia	NIB	302	<i>Cotoneaster</i> sp.	2003

Slovenia	NIB	303	Apple	2003
Slovenia	NIB	311	Pear	2003
Slovenia	NIB	312	Apple	2003
Slovenia	NIB	313	Apple	2003
Slovenia	NIB	314	Pear	2003
Slovenia	NIB	315	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	316	Pear	2003
Slovenia	NIB	317	Pear	2003
Slovenia	NIB	318	<i>Cydonia</i> sp.	2003
Slovenia	NIB	321	<i>Cydonia</i> sp.	2003
Slovenia	NIB	323	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	324	<i>Cydonia</i> sp.	2003
Slovenia	NIB	325	Apple	2003
Slovenia	NIB	331	<i>Cydonia</i> sp.	2003
Slovenia	NIB	332	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	333	<i>Cydonia</i> sp.	2003
Slovenia	NIB	343	<i>Cydonia</i> sp.	2003
Slovenia	NIB	345	Pear	2003
Slovenia	NIB	346	Pear	2003
Slovenia	NIB	348	<i>Cydonia</i> sp.	2003
Slovenia	NIB	349	Pear	2003
Slovenia	NIB	350	Apple	2003
Slovenia	NIB	352	Pear	2003
Slovenia	NIB	355	Apple	2003
Slovenia	NIB	356	Apple	2003
Slovenia	NIB	357	<i>Cydonia</i> sp.	2003
Slovenia	NIB	359	<i>Cydonia</i> sp.	2003
Slovenia	NIB	360	<i>Cydonia</i> sp.	2003
Slovenia	NIB	363	<i>Cydonia</i> sp.	2003
Slovenia	NIB	364	<i>Pyracantha</i> sp.	2003
Slovenia	NIB	367	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	368	<i>Cydonia</i> sp.	2003
Slovenia	NIB	376	Pear	2003
Slovenia	NIB	378	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	379	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	380	<i>Cydonia</i> sp.	2003
Slovenia	NIB	382	<i>Cydonia</i> sp.	2003
Slovenia	NIB	383	Pear	2003
Slovenia	NIB	384	Pear	2003
Slovenia	NIB	385	Apple	2003
Slovenia	NIB	386	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	387	Pear	2003
Slovenia	NIB	388	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	389	<i>Cydonia</i> sp.	2003
Slovenia	NIB	391	<i>Cydonia</i> sp.	2003
Slovenia	NIB	392	<i>Cydonia</i> sp.	2003

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Slovenia	NIB	394	Apple	2003
Slovenia	NIB	398	<i>Cydonia</i> sp.	2003
Slovenia	NIB	400	<i>Cydonia</i> sp.	2003
Slovenia	NIB	407	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	411	Apple	2003
Slovenia	NIB	412	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	413	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	424	Pear	2003
Slovenia	NIB	426	Apple	2003
Slovenia	NIB	430	Pear	2003
Slovenia	NIB	457	<i>Cydonia</i> sp.	2003
Slovenia	NIB	458	<i>Cydonia</i> sp.	2003
Slovenia	NIB	612	<i>Cotoneaster</i> sp.	2004
Slovenia	NIB	613	<i>Cotoneaster</i> sp.	2004
Slovenia	NIB	637	<i>Cotoneaster</i> sp.	2004
Slovenia	NIB	640	Pear	2004
Slovenia	NIB	641	Pear	2004
Slovenia	NIB	642	Pear	2004
Slovenia	NIB	649	Pear	2004
Slovenia	NIB	652	<i>Cotoneaster</i> sp.	2004
Slovenia	NIB	653	Pear	2004
Slovenia	NIB	736	Pear	2005
Slovenia	NIB	737	Pear	2005
Slovenia	NIB	739	Pear	2005
Slovenia	NIB	740	<i>Cotoneaster</i> sp.	2005
Slovenia	NIB	754	<i>Cotoneaster</i> sp.	2005
Slovenia	NIB	763	<i>Cotoneaster</i> sp.	2005
Slovenia	NIB	811	Apple	2006
Slovenia	NIB	883	Apple	2007
Slovenia	NIB	884	Apple	2007
Slovenia	NIB	885	Apple	2007
Slovenia	NIB	886	Apple	2007
Slovenia	NIB	887	<i>Cydonia</i> sp.	2007
Slovenia	NIB	888	<i>Cydonia</i> sp.	2007
Slovenia	NIB	889	<i>Cydonia</i> sp.	2007
Slovenia	NIB	890	<i>Cydonia</i> sp.	2007
Slovenia	NIB	893	Apple	2007
Slovenia	NIB	895	<i>Cydonia</i> sp.	2007
Slovenia	NIB	896	Pear	2007
Slovenia	NIB	898	Pear	2007
Slovenia	NIB	899	Pear	2007
Slovenia	NIB	900	Pear	2007
Slovenia	NIB	904	Apple	2007
Slovenia	NIB	905	Pear	2007
Slovenia	NIB	907	Apple	2007
Slovenia	NIB	914	<i>Crataegus</i> sp.	2007

Slovenia	NIB	915	Pear	2007
Slovenia	NIB	916	Apple	2007
Slovenia	NIB	921	<i>Crataegus</i> sp.	2007
Slovenia	NIB	922	<i>Cydonia</i> sp.	2007
Slovenia	NIB	923	Pear	2007
Slovenia	NIB	924	Pear	2007
Slovenia	NIB	928	Apple	2007
Slovenia	NIB	929	Apple	2007
Slovenia	NIB	930	Pear	2007
Slovenia	NIB	931	Pear	2007
Slovenia	NIB	932	Pear	2007
Slovenia	NIB	933	Apple	2007
Slovenia	NIB	934	Apple	2007
Slovenia	NIB	953	Apple	2007
Slovenia	NIB	955	Apple	2007
Slovenia	NIB	956	Apple	2007
Slovenia	NIB	957	Apple	2007
Slovenia	NIB	961	<i>Cydonia</i> sp.	2007
Slovenia	NIB	962	Pear	2007
Slovenia	NIB	963	<i>Cotoneaster</i> sp.	2007
Slovenia	NIB	964	Pear	2007
Slovenia	NIB	965	Pear	2007
Slovenia	NIB	967	Apple	2007
Slovenia	NIB	968	Apple	2007
Slovenia	NIB	970	Pear	2007
Slovenia	NIB	971	Apple	2007
Slovenia	NIB	973	Apple	2007
Slovenia	NIB	974	Pear	2007
Slovenia	NIB	975	Apple	2007
Slovenia	NIB	976	Apple	2007
Slovenia	NIB	977	Apple	2007
Slovenia	NIB	978	Apple	2007
Slovenia	NIB	979	Pear	2007
Slovenia	NIB	982	Pear	2007
Slovenia	NIB	983	Pear	2007
Slovenia	NIB	984	<i>Cydonia</i> sp.	2007
Slovenia	NIB	985	<i>Cotoneaster</i> sp.	2007
Slovenia	NIB	986	Apple	2007
Slovenia	NIB	987	Pear	2007
Slovenia	NIB	988	Pear	2007
Slovenia	NIB	989	Apple	2007
Slovenia	NIB	990	Pear	2007
Slovenia	NIB	991	Pear	2007
Slovenia	NIB	1000	Pear	2007
Slovenia	NIB	1001	Pear	2007
Slovenia	NIB	1002	Apple	2007

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Slovenia	NIB	1003	Apple	2007
Slovenia	NIB	1004	Apple	2007
Slovenia	NIB	1006	Pear	2007
Slovenia	NIB	1007	Pear	2007
Slovenia	NIB	1010	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1012	Pear	2007
Slovenia	NIB	1013	Pear	2007
Slovenia	NIB	1014	Pear	2007
Slovenia	NIB	1015	Pear	2007
Slovenia	NIB	1016	Pear	2007
Slovenia	NIB	1017	Pear	2007
Slovenia	NIB	1018	Pear	2007
Slovenia	NIB	1019	Pear	2007
Slovenia	NIB	1020	Pear	2007
Slovenia	NIB	1021	Pear	2007
Slovenia	NIB	1022	Pear	2007
Slovenia	NIB	1023	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1025	Pear	2007
Slovenia	NIB	1026	Pear	2007
Slovenia	NIB	1027	Apple	2007
Slovenia	NIB	1030	Pear	2007
Slovenia	NIB	1031	Pear	2007
Slovenia	NIB	1032	Pear	2007
Slovenia	NIB	1034	Pear	2007
Slovenia	NIB	1035	Pear	2007
Slovenia	NIB	1036	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1038	Pear	2007
Slovenia	NIB	1039	Pear	2007
Slovenia	NIB	1040	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1042	Pear	2007
Slovenia	NIB	1043	Pear	2007
Slovenia	NIB	1044	<i>Cotoneaster</i> sp.	2007
Slovenia	NIB	1045	Pear	2007
Slovenia	NIB	1047	Pear	2007
Slovenia	NIB	1054	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1057	Pear	2007
Slovenia	NIB	1058	Pear	2007
Slovenia	NIB	1060	Pear	2007
Slovenia	NIB	1061	Apple	2007
Slovenia	NIB	1062	Pear	2007
Slovenia	NIB	1063	Pear	2007
Slovenia	NIB	1064	Pear	2007
Slovenia	NIB	1065	Pear	2007
Slovenia	NIB	1069	Pear	2007
Slovenia	NIB	1070	Pear	2007
Slovenia	NIB	1071	Pear	2007

Slovenia	NIB	1072	Pear	2007
Slovenia	NIB	1076	Pear	2007
Slovenia	NIB	1077	Pear	2007
Slovenia	NIB	1083	Pear	2007
Slovenia	NIB	1084	Pear	2007
Slovenia	NIB	1085	Pear	2007
Slovenia	NIB	1088	Pear	2007
Slovenia	NIB	1092	Pear	2007
Slovenia	NIB	1094	Pear	2007
Slovenia	NIB	1095	Pear	2007
Slovenia	NIB	1096	Pear	2007
Slovenia	NIB	1097	Pear	2007
Slovenia	NIB	1098	Pear	2007
Slovenia	NIB	1099	Pear	2007
Slovenia	NIB	1101	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1112	<i>Photinia</i> sp.	2007
Slovenia	NIB	1113	Pear	2007
Slovenia	NIB	1114	Pear	2007
Slovenia	NIB	1115	Pear	2007
Slovenia	NIB	1116	Pear	2007
Slovenia	NIB	1117	Apple	2007
Slovenia	NIB	1118	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1119	Apple	2007
Slovenia	NIB	1120	Apple	2007
Slovenia	NIB	1121	Apple	2007
Slovenia	NIB	1122	Apple	2007
Slovenia	NIB	1127	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1128	<i>Cotoneaster</i> sp.	2007
Slovenia	NIB	1129	Pear	2007
Slovenia	NIB	1136	Pear	2007
Slovenia	NIB	1137	Pear	2007
Slovenia	NIB	1138	Pear	2007
Slovenia	NIB	1139	Pear	2007
Slovenia	NIB	1201	Apple	2008
Slovenia	NIB	1203	Pear	2008
Slovenia	NIB	1204	Apple	2008
Slovenia	NIB	1205	Apple	2008
Slovenia	NIB	1206	Pear	2008
Slovenia	NIB	1207	Apple	2008
Slovenia	NIB	1212	Apple	2008
Slovenia	NIB	1213	Apple	2008
Slovenia	NIB	1215	Pear	2008
Slovenia	NIB	1218	Pear	2008
Slovenia	NIB	1219	Pear	2008
Slovenia	NIB	1220	Pear	2008
Slovenia	NIB	1221	Apple	2008

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Slovenia	NIB	1222	Pear	2008
Slovenia	NIB	1225	Pear	2008
Slovenia	NIB	1226	<i>Cydonia</i> sp.	2008
Slovenia	NIB	1227	Apple	2008
Slovenia	NIB	1228	Apple	2008
Slovenia	NIB	1229	<i>Cydonia</i> sp.	2008
Slovenia	NIB	1230	Pear	2008
Slovenia	NIB	1261	Apple	2008
Slovenia	NIB	1262	Apple	2008
Slovenia	NIB	1264	Pear	2008
Slovenia	NIB	1265	<i>Cydonia</i> sp.	2008
Slovenia	NIB	1276	Pear	2008
Slovenia	NIB	1277	Pear	2008
Slovenia	NIB	1278	Apple	2008
Slovenia	NIB	1279	Pear	2008
Slovenia	NIB	1321	Pear	2009
Slovenia	NIB	1327	Pear	2009
Slovenia	NIB	1340	Pear	2009
Slovenia	NIB	1345	Pear	2009
Spain	IVIA	IVIA1554-1	<i>Crataegus</i> sp.	1996
Spain	IVIA	IVIA1554-2	<i>Crataegus</i> sp.	1996
Spain	IVIA	IVIA 1578-3	<i>Crataegus</i> sp.	1996
Spain	IVIA	IVIA 1596	Pear	1996
Spain	IVIA	IVIA 1603	<i>Cotoneaster</i> sp.	1996
Spain	IVIA	IVIA 1614-1	<i>Pyracantha</i> sp.	1996
Spain	IVIA	IVIA 1614-2a	<i>Crataegus</i> sp.	1996
Spain	IVIA	IVIA 1892-1	Pear	1998
Spain	IVIA	IVIA 1898-11	Apple	1998
Spain	IVIA	IVIA 1898-18	Apple	1998
Spain	IVIA	IVIA 1899-19	Pear	1998
Spain	IVIA	IVIA 1985	<i>Pyracantha</i> sp.	1998
Spain	IVIA	IVIA 2397	Pear	2000
Spain	IVIA	IVIA 3169	Pear	2006
Spain	IVIA	IVIA 3171	<i>Crataegus</i> sp.	2006
Spain	IVIA	IVIA 3172	Pear	2006
Spain	IVIA	IVIA 3173	Apple	2006
Spain	IVIA	IVIA 3174	<i>Crataegus</i> sp.	2006
Spain	IVIA	IVIA 3199-4	<i>Crataegus</i> sp.	2006
Spain	UPN	UPN 538	Pear	1998
Switzerland	ACW	Rac 3075	na	na
Switzerland	ACW	35697	Pear	2003
Switzerland	ACW	37678	Pear	2003
Switzerland	ACW	37809	Pear	2003
Switzerland	ACW	38262	<i>Crataegus</i> sp.	2003
Switzerland	ACW	38899	<i>Cydonia</i> sp.	2003
Switzerland	ACW	39005	<i>Pyracantha</i> sp.	2003

Switzerland	ACW	44081	Apple	2005
Switzerland	ACW	44274	Pear	2005
Switzerland	ACW	45072	Pear	2005
Switzerland	ACW	45534	<i>Crataegus</i> sp.	2005
Switzerland	ACW	56400	Pear	2007
Switzerland	ACW	56437	Pear	2007
The Netherlands	CFBP	CFBP 3017	<i>Crataegus</i> sp.	1973
The Netherlands	CFBP	CFBP 3021	Pear	1981
UK	NCPFB	NCPFB 3299	<i>Pernettya</i>	1983
UK	BBA	P-1570	<i>Cotoneaster</i> sp.	na

na: not available

ACW: Agroscope-Changins Wädenswil, Switzerland.

BBA: Federal Biological Institute for Agriculture and Forestry. Dossenheim, Germany.

BPIC: Benaki Phytopathological Institute Collection, Athens, Greece.

CFBP: Collection Française de Bactéries Phytopathogènes, INRA, Angers, France.

CRA-W : Centre Wallon de Recherches Agronomiques. Gembloux, Belgium.

DERM: Department of Environmental Resource Management, University College Dublin, Belfield, Ireland.

DPP: Department of Plant Protection collection, Institute of Horticulture, Pomology Division, Skierniewice, Poland.

IVIA: Instituto Valenciano de Investigaciones Agrarias collection, Moncada, Spain.

LMG: Collection of the Laboratory of Microbiology. Rijksuniversiteit, Gent, Belgium.

LNPV: Laboratoire National de la Protection des Végétaux, Beaucauzé, France.

NCPFB: National Collection of Plant Pathogenic Bacteria, York, UK.

NIB: National Institute of Biology, Ljubljana, Slovenia.

OMP: Osservatorio per le Malattie delle Piante, Bologna, Italy.

SFR: Servizio Fitosanitario Regionale, Bologna, Italy.

SL: State Laboratory, Dublin, Ireland.

UPN: Universidad Publica de Navarra. Pamplona, Spain.

Table 4-S3. Additional information available from *E. amylovora* strains negative for pEI70 analyzed in European countries.

Country	Collection	Isolates negative for pEI70	Host	Year of isolation
Austria	AGES	MK 295/3	<i>Cotoneaster</i> sp.	1993
Austria	AGES	MK 483/98	<i>Cotoneaster</i> sp.	1998
Austria	AGES	MK 1082/00	Pear	2000
Austria	AGES	MK 1180/00	Apple	2000
Austria	AGES	MK 1186/00	<i>Sorbus</i> sp.	2000
Austria	AGES	MK 2447/01	<i>Sorbus</i> sp.	2001
Austria	AGES	MK 295/83 (4)	<i>Cotoneaster</i> sp.	1993
Austria	AGES	MK 295/83 (54)	<i>Cotoneaster</i> sp.	1993
Austria	AGES	MK 295/93 (127)	<i>Cotoneaster</i> sp.	1993
Austria	AGES	MK 2694/01	Apple	2001
Austria	AGES	MK 2677/01	Apple	2001
Austria	AGES	MK 273/98	Apple	1998
Austria	AGES	MK 296/93	<i>Cotoneaster</i> sp.	1993
Austria	AGES	MK 324/98	Apple	1998
Austria	AGES	MK 328/98	<i>Cotoneaster</i> sp.	1998
Austria	AGES	MK 482/98	<i>Cotoneaster</i> sp.	1998
Austria	AGES	MK 640/99	Apple	1999
Austria	AGES	MK 668/00	Apple	2000
Austria	AGES	MK674/94	Pear	1994
Austria	AGES	MK 881/98	na	1998
Austria	AGES	MK 955/99	Pear	1999
Austria	AGES	MK 1059/03	Pear	2003
Austria	AGES	MK 1057/03	Apple	2003
Austria	AGES	MK 2057/02	<i>Prunus</i> sp.	2002
Austria	AGES	MK 2614/01	Apple	2001
Belgium	LMG	LMG 1930	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaP3	<i>Crataegus</i> sp.	1980
Belgium	CRA-W	EaA22	Pear	2001
Belgium	CRA-W	EaA23	<i>Crataegus</i> sp.	2001
Belgium	CRA-W	EaP45	Pear	2002
Bulgaria	CRA-W	KG 115.22a	<i>Cydonia</i> sp.	na
Bulgaria	CRA-W	KG 115.22b	<i>Cydonia</i> sp.	na
Bulgaria	BPIC	BPIC 1041	<i>Cydonia</i> sp.	1991
Czech Republic	BPIC	BPIC 1634	<i>Crataegus</i> sp.	1989
France	CFBP	CFBP1430	<i>Crataegus</i> sp.	1972
France	CFBP	CFBP 2314	Apple	1982
France	CFBP	CFBP 2597(1)	Apple	na
France	INRA	INRA 2543	Apple	1992
France	INRA	INRA 2546 (p)	Apple	1992
France	INRA	INRA 2550 (p)	Apple	1992
France	INRA	INRA 2552 (p)	Apple	1992
France	INRA	INRA 2553 (p)	na	na
France	INRA	INRA 2561 (p)	na	na
France	INRA	INRA 2576 (p)	Pear	na
France	INRA	INRA 2579 (p)	Apple	na
France	INRA	INRA 2580 (p)	Apple	na
France	INRA	INRA 2581	Apple	1992

France	INRA	INRA 2582	Apple	1992
France	INRA	INRA 2595	<i>Cotoneaster</i> sp.	na
France	BBA	KG 6-45	<i>Cydonia</i> sp.	na
France	BBA	KG 9-43	<i>Cotoneaster</i> sp.	na
France	BBA	KG 9-7	Pear	na
France	BBA	KG 9-75	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 1099	<i>Pyracantha</i> sp.	na
France	LNPV	LNPV 1594	<i>Cotoneaster</i> sp.	1993
France	LNPV	LNPV 1601	Pear	1993
France	LNPV	LNPV 1613	<i>Pyracantha</i> sp.	1993
France	LNPV	LNPV 1626	Pear	1993
France	LNPV	LNPV 1709	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 1710	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 1775	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 1778	<i>Cotoneaster</i> sp.	1993
France	LNPV	LNPV 1887	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 1888	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV1890	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 1892	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 1893	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 1894	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 1899	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 2018	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 2026	<i>Cotoneaster</i> sp.	na
France	LNPV	LNPV 2030	<i>Pyracantha</i> sp.	na
France	LNPV	LNPV 1257-B	<i>Photinia</i> sp.	na
France	LNPV	LNPV 1096	<i>Cotoneaster</i> sp.	na
Germany	BBA	Ea 115	Apple	1989
Germany	CFBP	CFBP 3053	Apple	1981
Germany	CFBP	CFBP 3054	Pear	1974
Germany	CFBP	CFBP 3057	<i>Cotoneaster</i> sp.	1974
Germany	BBA	KG 237	Apple	na
Germany	BBA	KG 250	Apple	na
Germany	BBA	KG 282	Pear	na
Germany	BBA	KG 286	Pear	na
Germany	BBA	KG 1/79	<i>Cotoneaster</i> sp.	na
Germany	BBA	KG FR-3/98	<i>Cotoneaster</i> sp.	na
Germany	AGES	MK DL 1	Apple	1993
Germany	AGES	MK 7/74	<i>Cotoneaster</i> sp.	1974
Greece	BPIC	BPIC 909	Pear	1985
Greece	BPIC	BPIC 917	<i>Cydonia</i> sp.	1985
Greece	BPIC	BPIC 913	Pear	1985
Greece	BPIC	BPIC 1624	Pear	1990
Greece	BPIC	BPIC 1614	Pear	1990
Greece	BPIC	BPIC 943	Pear	1984
Greece	BPIC	BPIC 932	Apple	1986
Greece	BPIC	BPIC 928	Pear	1986
Greece	BPIC	BPIC 939	Pear	1986
Greece	BPIC	BPIC 847	Pear	1984
Greece	BPIC	BPIC 1056	<i>Cydonia</i> sp.	1987
Greece	CFBP	CFBP 3063	Pear	1985
Greece	CFBP	CFBP 3064	Pear	1985
Greece	CFBP	CFBP 3065	Pear	1986

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Greece	CFBP	CFBP 3093	Pear	1986
Hungary	AGES	MK 1	Apple	1996
Hungary	AGES	MK 10	Pear	1997
Hungary	AGES	MK 15	<i>Cotoneaster</i> sp.	1997
Hungary	AGES	MK 16	<i>Cotoneaster</i> sp.	1997
Hungary	AGES	MK 17	<i>Cydonia</i> sp.	1997
Hungary	AGES	MK 22	<i>Crataegus</i> sp.	1998
Hungary	AGES	MK 23	Pear	1998
Hungary	AGES	MK 26	Pear	1998
Hungary	AGES	MK 28	<i>Crataegus</i> sp.	1998
Hungary	AGES	MK 31	<i>Pyracantha</i> sp.	1998
Hungary	AGES	MK 1	Apple	1996
Ireland	SL	AE 91	Pear	na
Ireland	SL	E-31	<i>Cotoneaster</i> sp.	1996
Italy	OMP	OMP-BO 787-1	<i>Crataegus</i> sp.	1992
Italy	OMP	OMP-BO 1077-7	Pear	1994
Poland	DPP	123	<i>Crataegus</i> sp.	2000
Poland	DPP	462	Apple	2002
Poland	DPP	464	Pear	2002
Poland	DPP	518	Pear	2002
Poland	DPP	538	Apple	2002
Poland	DPP	576	<i>Crataegus</i> sp.	2002
Poland	DPP	604	<i>Crataegus</i> sp.	1994
Poland	DPP	607	Apple	1994
Poland	DPP	608	Apple	1994
Poland	DPP	611	Apple	1995
Poland	DPP	612	Pear	1995
Poland	DPP	613	<i>Crataegus</i> sp.	1995
Poland	DPP	614	Apple	1995
Poland	DPP	615	<i>Crataegus</i> sp.	1995
Poland	DPP	616	Apple	1995
Poland	DPP	617	<i>Crataegus</i> sp.	1995
Poland	DPP	618	Pear	1995
Poland	DPP	619	Pear	1995
Poland	DPP	620	<i>Cotoneaster</i> sp.	1995
Poland	DPP	621	Pear	1995
Poland	DPP	622	<i>Cydonia</i> sp.	1995
Poland	DPP	623	<i>Crataegus</i> sp.	1995
Poland	DPP	624	Pear	1995
Poland	DPP	625	Pear	1995
Poland	DPP	627	Apple	1985
Poland	DPP	629	Apple	1985
Poland	DPP	632	Pear	1985
Poland	DPP	633	Pear	1985
Poland	DPP	634	<i>Crataegus</i> sp.	1985
Poland	DPP	635	<i>Crataegus</i> sp.	1985
Poland	DPP	636	<i>Crataegus</i> sp.	1985
Poland	DPP	637	Pear	1985
Poland	DPP	645	<i>Crataegus</i> sp.	1985
Poland	DPP	646	<i>Crataegus</i> sp.	1985
Poland	DPP	648	<i>Crataegus</i> sp.	1983
Poland	DPP	650	<i>Crataegus</i> sp.	1983
Poland	DPP	651	<i>Crataegus</i> sp.	1983

Poland	DPP	659	Apple	1986
Poland	DPP	660	Pear	1993
Poland	DPP	661	<i>Sorbus</i> sp.	1993
Poland	DPP	663	Apple	1993
Poland	DPP	664	Apple	1993
Poland	DPP	684	<i>Crataegus</i> sp.	1997
Poland	DPP	691	Apple	1998
Poland	DPP	692	<i>Sorbus</i> sp.	1998
Poland	DPP	694	Apple	2000
Poland	DPP	698	Apple	na
Poland	DPP	39/675	<i>Crataegus</i> sp.	2000
Poland	DPP	133/95	Quince	1995
Poland	DPP	16/96	Apple	1996
Poland	DPP	21/96	Pear	1996
Poland	DPP	2112/00	Pear	2000
Poland	DPP	2119/00	<i>Crataegus</i> sp.	2000
Poland	DPP	244/96	<i>Cotoneaster</i> sp.	1996
Poland	DPP	367/96	<i>Pyracantha</i> sp.	1996
Poland	DPP	74/95	<i>Sorbus</i> sp.	1995
Poland	DPP	96/96	<i>Sorbus</i> sp.	1996
Poland	DPP	E 26	Apple	2001
Poland	DPP	E1/I	Apple	1999
Poland	DPP	E1/II	Apple	1999
Poland	DPP	E10	Apple	2000
Poland	DPP	E11	Apple	2000
Poland	DPP	E12	Apple	2000
Poland	DPP	E13	Apple	2000
Poland	DPP	E14	Pear	2000
Poland	DPP	E15	Pear	2000
Poland	DPP	E16	Apple	2000
Poland	DPP	E17	Apple	2000
Poland	DPP	E18	Apple	2000
Poland	DPP	E19	Apple	2000
Poland	DPP	E2/I	Apple	2000
Poland	DPP	E2/II	Apple	2000
Poland	DPP	E20	Apple	2000
Poland	DPP	E21	Apple	2000
Poland	DPP	E22	na	2000
Poland	DPP	E23	Apple	2001
Poland	DPP	E24	Apple	2002
Poland	DPP	E25	Pear	2002
Poland	DPP	E27	Apple	2002
Poland	DPP	E28	Apple	2002
Poland	DPP	E29	Apple	2002
Poland	DPP	E3	Apple	2000
Poland	DPP	E4	Pear	2000
Poland	DPP	E5	Pear	2000
Poland	DPP	E6	Pear	2000
Poland	DPP	E7	Pear	2000
Poland	DPP	E8	Pear	2000
Poland	DPP	E9	Apple	2000
Poland	DPP	Pr 591	<i>Crataegus</i> sp.	2000
Poland	DPP	Pr 82	<i>Crataegus</i> sp.	2000

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Poland	DPP	TL 81	Apple	2000
Poland	DPP	TL 85	Apple	2000
Poland	DPP	TL 86	Apple	2000
Poland	DPP	TN44	<i>Crataegus</i> sp.	2000
Poland	DPP	604a	Apple	2001
Poland	DPP	608a	Apple	2002
Poland	DPP	609a	Pear	2003
Poland	DPP	610a	Apple	2003
Poland	DPP	611a	Pear	2003
Poland	DPP	613a	Pear	2004
Poland	DPP	614a	Pear	2004
Poland	DPP	615a	Pear	2006
Poland	DPP	616a	Pear	2006
Poland	DPP	617a	Pear	2005
Poland	DPP	618a	Apple	2007
Poland	DPP	620a	Apple	2007
Poland	DPP	621a	Apple	2007
Poland	DPP	622a	Apple	2007
Poland	DPP	623a	Apple	2007
Poland	DPP	624a	Pear	2007
Poland	DPP	626a	Apple	2007
Poland	DPP	627a	Apple	2007
Poland	DPP	629a	Apple	2007
Serbia	ARI	BC-3	Apple	2003
Serbia	ARI	CGJ-2	Apple	2003
Slovenia	NIB	174	Apple	2003
Slovenia	NIB	179	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	186	Apple	2003
Slovenia	NIB	187	Apple	2003
Slovenia	NIB	188	Apple	2003
Slovenia	NIB	194	Apple	2003
Slovenia	NIB	196	Pear	2003
Slovenia	NIB	197	Apple	2003
Slovenia	NIB	198	Apple	2003
Slovenia	NIB	200	Pear	2003
Slovenia	NIB	201	Apple	2003
Slovenia	NIB	209	Apple	2003
Slovenia	NIB	214	Apple	2003
Slovenia	NIB	227	Apple	2003
Slovenia	NIB	232	Apple	2003
Slovenia	NIB	233	Pear	2003
Slovenia	NIB	234	Apple	2003
Slovenia	NIB	240	Apple	2003
Slovenia	NIB	243	Apple	2003
Slovenia	NIB	256	Apple	2003
Slovenia	NIB	260	Pear	2003
Slovenia	NIB	263	Apple	2003
Slovenia	NIB	266	Apple	2003
Slovenia	NIB	267	Apple	2003
Slovenia	NIB	268	Apple	2003
Slovenia	NIB	269	Apple	2003
Slovenia	NIB	270	Apple	2003
Slovenia	NIB	276	Apple	2003

Slovenia	NIB	277	Apple	2003
Slovenia	NIB	279	Pear	2003
Slovenia	NIB	280	Apple	2003
Slovenia	NIB	281	Apple	2003
Slovenia	NIB	282	Apple	2003
Slovenia	NIB	292	Apple	2003
Slovenia	NIB	294	<i>Chaenomeles</i> sp.	2003
Slovenia	NIB	295	Apple	2003
Slovenia	NIB	299	Apple	2003
Slovenia	NIB	319	Apple	2003
Slovenia	NIB	320	Apple	2003
Slovenia	NIB	327	<i>Cydonia</i> sp.	2003
Slovenia	NIB	329	Apple	2003
Slovenia	NIB	334	Apple	2003
Slovenia	NIB	335	Apple	2003
Slovenia	NIB	336	Apple	2003
Slovenia	NIB	337	Apple	2003
Slovenia	NIB	338	Pear	2003
Slovenia	NIB	339	Apple	2003
Slovenia	NIB	340	Apple	2003
Slovenia	NIB	341	Pear	2003
Slovenia	NIB	342	Pear	2003
Slovenia	NIB	350	Apple	2003
Slovenia	NIB	353	<i>Cydonia</i> sp.	2003
Slovenia	NIB	354	Apple	2003
Slovenia	NIB	355	Apple	2003
Slovenia	NIB	356	Apple	2003
Slovenia	NIB	357	<i>Cydonia</i> sp.	2003
Slovenia	NIB	359	<i>Cydonia</i> sp.	2003
Slovenia	NIB	360	<i>Cydonia</i> sp.	2003
Slovenia	NIB	363	<i>Cydonia</i> sp.	2003
Slovenia	NIB	365	Apple	2003
Slovenia	NIB	366	Apple	2003
Slovenia	NIB	370	Apple	2003
Slovenia	NIB	371	<i>Cydonia</i> sp.	2003
Slovenia	NIB	374	Apple	2003
Slovenia	NIB	375	Apple	2003
Slovenia	NIB	401	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	402	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	403	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	404	Apple	2003
Slovenia	NIB	405	Apple	2003
Slovenia	NIB	414	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	415	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	416	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	417	<i>Cotoneaster</i> sp.	2003
Slovenia	NIB	425	Apple	2003
Slovenia	NIB	429	Pear	2003
Slovenia	NIB	609	Apple	2004
Slovenia	NIB	610	Apple	2004
Slovenia	NIB	611	Apple	2004
Slovenia	NIB	614	Apple	2004
Slovenia	NIB	615	Apple	2004

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Slovenia	NIB	616	Apple	2004
Slovenia	NIB	617	Pear	2004
Slovenia	NIB	618	<i>Cotoneaster</i> sp.	2004
Slovenia	NIB	633	Apple	2004
Slovenia	NIB	634	Apple	2004
Slovenia	NIB	636	<i>Cotoneaster</i> sp.	2004
Slovenia	NIB	638	Apple	2004
Slovenia	NIB	639	na	2004
Slovenia	NIB	643	na	2004
Slovenia	NIB	647	Apple	2004
Slovenia	NIB	648	Apple	2004
Slovenia	NIB	651	Apple	2004
Slovenia	NIB	654	Pear	2004
Slovenia	NIB	655	Pear	2004
Slovenia	NIB	656	Pear	2004
Slovenia	NIB	687	<i>Cotoneaster</i> sp.	2004
Slovenia	NIB	731	Apple	2005
Slovenia	NIB	733	Apple	2005
Slovenia	NIB	734	Apple	2005
Slovenia	NIB	735	Pear	2005
Slovenia	NIB	738	Pear	2005
Slovenia	NIB	743	Pear	2005
Slovenia	NIB	747	Pear	2005
Slovenia	NIB	812	Apple	2006
Slovenia	NIB	813	Apple	2006
Slovenia	NIB	814	Apple	2006
Slovenia	NIB	815	Pear	2006
Slovenia	NIB	816	Pear	2006
Slovenia	NIB	882	Apple	2007
Slovenia	NIB	891	Pear	2007
Slovenia	NIB	892	Pear	2007
Slovenia	NIB	894	na	2007
Slovenia	NIB	897	Apple	2007
Slovenia	NIB	899	Pear	2007
Slovenia	NIB	901	Apple	2007
Slovenia	NIB	902	Pear	2007
Slovenia	NIB	903	Apple	2007
Slovenia	NIB	906	Apple	2007
Slovenia	NIB	908	Apple	2007
Slovenia	NIB	909	Apple	2007
Slovenia	NIB	910	Apple	2007
Slovenia	NIB	911	Apple	2007
Slovenia	NIB	912	Pear	2007
Slovenia	NIB	913	Pear	2007
Slovenia	NIB	917	Apple	2007
Slovenia	NIB	918	Apple	2007
Slovenia	NIB	919	Apple	2007
Slovenia	NIB	920	Apple	2007
Slovenia	NIB	925	Apple	2007
Slovenia	NIB	926	Apple	2007
Slovenia	NIB	927	Apple	2007
Slovenia	NIB	935	Apple	2007
Slovenia	NIB	954	Pear	2007

Slovenia	NIB	958	Apple	2007
Slovenia	NIB	959	Apple	2007
Slovenia	NIB	960	Apple	2007
Slovenia	NIB	966	Pear	2007
Slovenia	NIB	969	Pear	2007
Slovenia	NIB	972	Apple	2007
Slovenia	NIB	980	Apple	2007
Slovenia	NIB	981	Apple	2007
Slovenia	NIB	992	Pear	2007
Slovenia	NIB	993	Apple	2007
Slovenia	NIB	994	<i>Cydonia</i> sp.	2007
Slovenia	NIB	995	<i>Crataegeus</i> sp.	2007
Slovenia	NIB	996	Apple	2007
Slovenia	NIB	997	Apple	2007
Slovenia	NIB	998	Pear	2007
Slovenia	NIB	999	Apple	2007
Slovenia	NIB	1005	Apple	2007
Slovenia	NIB	1008	Pear	2007
Slovenia	NIB	1009	Apple	2007
Slovenia	NIB	1028	Apple	2007
Slovenia	NIB	1029	Pear	2007
Slovenia	NIB	1033	<i>Crataegus</i> sp.	2007
Slovenia	NIB	1037	Pear	2007
Slovenia	NIB	1046	Pear	2007
Slovenia	NIB	1048	Apple	2007
Slovenia	NIB	1049	Pear	2007
Slovenia	NIB	1050	Pear	2007
Slovenia	NIB	1051	Pear	2007
Slovenia	NIB	1053	Apple	2007
Slovenia	NIB	1055	Pear	2007
Slovenia	NIB	1059	Pear	2007
Slovenia	NIB	1066	Pear	2007
Slovenia	NIB	1067	Pear	2007
Slovenia	NIB	1075	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1078	Pear	2007
Slovenia	NIB	1081	Pear	2007
Slovenia	NIB	1082	Pear	2007
Slovenia	NIB	1090	Pear	2007
Slovenia	NIB	1093	Pear	2007
Slovenia	NIB	1100	<i>Cydonia</i> sp.	2007
Slovenia	NIB	1123	Apple	2007
Slovenia	NIB	1124	Pear	2007
Slovenia	NIB	1126	<i>Cotoneaster</i> sp.	2007
Slovenia	NIB	1130	Apple	2007
Slovenia	NIB	1184	Apple	2007
Slovenia	NIB	1200	Pear	2008
Slovenia	NIB	1208	Apple	2008
Slovenia	NIB	1211	Apple	2008
Slovenia	NIB	1214	Apple	2008
Slovenia	NIB	1216	<i>Cydonia</i> sp.	2008
Slovenia	NIB	1217	<i>Mespilus</i> sp.	2008
Slovenia	NIB	1223	Apple	2008
Slovenia	NIB	1224	Pear	2008

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Slovenia	NIB	1259	Pear	2008
Slovenia	NIB	1260	Apple	2008
Slovenia	NIB	1263	Apple	2008
Slovenia	NIB	1334	Apple	2009
Slovenia	NIB	1335	Apple	2009
Slovenia	NIB	1339	Pear	2009
Spain	IVIA	IVIA 2887-21b	Pear	2004
Spain	IVIA	IVIA 2887-26	Pear	2004
Spain	IVIA	IVIA 2921-2	<i>Pyrus pyrifolia</i> (Nashi)	2004
Spain	IVIA	IVIA 2921-10	<i>Pyrus pyrifolia</i> (Nashi)	2004
Spain	IVIA	IVIA 3149	<i>Crataegus azarolus</i>	2006
Spain	IVIA	IVIA 3175	<i>Pyracantha</i> sp.	2006
Spain	IVIA	IVIA 3156 col. 12	Pear	2006
Spain	IVIA	IVIA 3156 col. 19	Pear	2006
Spain	IVIA	IVIA 3156 col. 20	Pear	2006
Spain	IVIA	IVIA 3156 col. 22	Pear	2006
Spain	IVIA	IVIA 2070-1	Pear	1999
Spain	IVIA	IVIA 2072-1	Pear	1999
Spain	IVIA	IVIA 2092	<i>Pyracantha</i> sp.	1999
Spain	IVIA	IVIA 2112-1	<i>Pyracantha</i> sp.	1999
Spain	IVIA	IVIA 2134-1	Pear	1999
Spain	IVIA	IVIA 2278-1	Pear	2000
Spain	IVIA	IVIA 2303-1	Apple	2000
Spain	IVIA	IVIA 2303-3	Pear	2000
Spain	IVIA	IVIA 2303-6	Pear	2000
Spain	IVIA	IVIA 2311-6	Pear	2000
Spain	IVIA	IVIA 2311-8	Pear	2000
Spain	IVIA	IVIA 2311-19	Pear	2000
Spain	IVIA	IVIA 2500	Apple	2001
Spain	IVIA	IVIA 2501	Pear	2001
Spain	IVIA	IVIA 1509-B	Apple	1995
Spain	IVIA	IVIA 1525-1	<i>Cotoneaster</i> sp.	1996
Spain	IVIA	IVIA 1525-6	<i>Cotoneaster</i> sp.	1996
Spain	IVIA	IVIA 1626	Apple	1996
Spain	IVIA	IVIA 1626-6	Apple	1996
Spain	IVIA	IVIA 1767-3	Apple	1997
Spain	IVIA	IVIA 1731-1	Pear	1997
Spain	IVIA	IVIA 1739-1	Apple	1997
Spain	IVIA	IVIA 1777-1	<i>Pyracantha</i> sp.	1997
Spain	IVIA	IVIA 1898-4	<i>Cotoneaster</i> sp.	1998
Spain	IVIA	IVIA 1899-21	<i>Cydonia oblonga</i>	1998
Spain	IVIA	IVIA1961-5	Pear	1998
Spain	IVIA	IVIA 1961-16	Pear	1998
Spain	IVIA	IVIA 1924-4	<i>Pyracantha</i> sp.	1998
Spain	IVIA	IVIA 1951-5	<i>Cotoneaster</i> sp.	1998
Spain	IVIA	IVIA 1951-8	<i>Pyracantha</i> sp.	1998
Spain	IVIA	IVIA 1951-6	<i>Sorbus</i> sp.	1998
Spain	IVIA	IVIA 1951-2	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 529	<i>Pyracantha</i> sp.	1997
Spain	UPN	UPN 525	<i>Pyracantha</i> sp.	1997
Spain	UPN	UPN 526	Pear	1997
Spain	UPN	UPN 527	Apple	1997
Spain	UPN	UPN 528	Apple	1997

Spain	UPN	UPN 530	Pear	1997
Spain	UPN	UPN 500	Pear	1998
Spain	UPN	UPN 511	Apple	1998
Spain	UPN	UPN 537	<i>Cotoneaster</i> sp.	1998
Spain	UPN	UPN 540	Pear	1998
Spain	UPN	UPN 545	Apple	1998
Spain	UPN	UPN 546	<i>Cydonia oblonga</i>	1998
Spain	UPN	UPN 547	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 564	<i>Cotoneaster</i> sp.	1998
Spain	UPN	UPN 582	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 583	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 584	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 585	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 586	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 587	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 588	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 589	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 590	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 591	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 592	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 593	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 594	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 609	<i>Sorbus</i> sp.	1998
Spain	UPN	UPN 611	<i>Cotoneaster</i> sp.	1998
Spain	UPN	UPN 612	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 613	na	Na
Spain	UPN	UPN 535	Apple	1998
Spain	UPN	UPN 536	Apple	1998
Spain	UPN	UPN 539	Pear	1998
Spain	UPN	UPN 541	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 542	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 543	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 544	<i>Cotoneaster</i> sp.	1998
Spain	UPN	UPN 548	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 549	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 550	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 551	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 552	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 553	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 554	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 555	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 556	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 557	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 558	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 559	Apple	1998
Spain	UPN	UPN 560	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 561	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 562	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 563	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 565	<i>Cotoneaster</i> sp.	1998
Spain	UPN	UPN 566	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 567	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 568	<i>Crataegus</i> sp.	1998

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Spain	UPN	UPN 569	<i>Cotoneaster</i> sp.	1998
Spain	UPN	UPN 570	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 571	Apple	1998
Spain	UPN	UPN 572	Apple	1998
Spain	UPN	UPN 573	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 574	Apple	1998
Spain	UPN	UPN 575	Pear	1998
Spain	UPN	UPN 576	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 577	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 578	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 579	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 580	<i>Pyracantha</i> sp.	1998
Spain	UPN	UPN 581	<i>Pyracantha</i> sp.	1998
Spain	EPS	EPS 101	Pear	na
Spain	SV	SV 4576	Pear	na
Switzerland	ACW	57218	na	2007
Switzerland	ACW	57065	na	2007
Switzerland	ACW	57210	na	2007
Switzerland	ACW	57112	na	2007
Switzerland	ACW	59998	na	2007
Switzerland	ACW	60039	na	2007
Switzerland	ACW	59999	na	2007
Switzerland	ACW	59868	na	2007
Switzerland	ACW	59969	na	2007
Switzerland	ACW	59912	na	2007
Switzerland	ACW	59973	na	2007
Switzerland	ACW	59956	na	2007
Switzerland	ACW	58459	na	2007
Switzerland	ACW	56965	na	2007
Switzerland	ACW	56961	na	2007
Switzerland	ACW	56968	na	2007
Switzerland	ACW	57114	na	2007
Switzerland	ACW	57467	na	2007
Switzerland	ACW	56929	na	2007
Switzerland	ACW	56963	na	2007
Switzerland	ACW	60019	na	2007
Switzerland	ACW	57045	na	2007
Switzerland	ACW	59677	na	2007
Switzerland	ACW	59959	na	2007
Switzerland	ACW	56934	na	2007
Switzerland	ACW	57143	na	2007
Switzerland	ACW	57113	na	2007
Switzerland	ACW	59889	na	2007
Switzerland	ACW	59888	na	2007
Switzerland	ACW	59557	na	2007
Switzerland	ACW	56928	na	2007
Switzerland	ACW	57488	na	2007
Switzerland	ACW	57111	na	2007
Switzerland	ACW	57078	na	2007
Switzerland	ACW	56932	na	2007
Switzerland	ACW	60021	na	2007
Switzerland	ACW	59717	na	2007
Switzerland	ACW	57575	na	2007

Switzerland	ACW	59987	na	2007
Switzerland	ACW	56839	na	2007
Switzerland	ACW	56508	na	2007
Switzerland	ACW	56867	na	2007
Switzerland	ACW	56823	na	2007
Switzerland	ACW	56310	na	2007
Switzerland	ACW	56216	na	2007
Switzerland	ACW	56657	na	2007
Switzerland	ACW	56363	na	2007
Switzerland	ACW	57117	na	2007
Switzerland	ACW	57560	na	2007
Switzerland	ACW	59690	na	2007
Switzerland	ACW	56933	na	2007
Switzerland	ACW	56098	na	2007
Switzerland	ACW	57546	na	2007
Switzerland	ACW	56847	na	2007
Switzerland	ACW	56283	na	2007
Switzerland	ACW	56486	na	2007
Switzerland	ACW	56842	na	2007
Switzerland	ACW	56511	na	2007
Switzerland	ACW	56959	na	2007
Switzerland	ACW	56948	na	2007
Switzerland	ACW	56930	na	2007
Switzerland	ACW	57132	na	2007
Switzerland	ACW	59517	na	2007
Switzerland	ACW	56605	na	2007
Switzerland	ACW	56848	na	2007
Switzerland	ACW	56121	na	2007
Switzerland	ACW	56120	na	2007
Switzerland	ACW	56387	na	2007
Switzerland	ACW	59910	na	2007
Switzerland	ACW	57506	na	2007
Switzerland	ACW	57561	na	2007
Switzerland	ACW	56902	na	2007
Switzerland	ACW	56933	Pear	2007
Switzerland	ACW	56232	na	2007
Switzerland	ACW	56482	na	2007
Switzerland	ACW	56601	na	2007
Switzerland	ACW	55792	na	2007
Switzerland	ACW	56199	na	2007
Switzerland	ACW	57116	na	2007
Switzerland	ACW	59718	na	2007
Switzerland	ACW	57530	na	2007
Switzerland	ACW	56934	na	2007
Switzerland	ACW	57479	na	2007
Switzerland	ACW	56437	na	2007
Switzerland	ACW	56499	na	2007
Switzerland	ACW	56298	Pear	2007
Switzerland	ACW	56200	na	2007
Switzerland	ACW	56785	na	2007
Switzerland	ACW	56762	na	2007
Switzerland	ACW	56099	na	2007
Switzerland	ACW	56177	na	2007

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Switzerland	ACW	56187	na	2007
Switzerland	ACW	38158	na	2007
Switzerland	ACW	39011	na	2007
Switzerland	ACW	45021	na	2007
Switzerland	ACW	RAC 3077	na	2007
Switzerland	ACW	38291	na	2007
Switzerland	ACW	39010	na	2007
Switzerland	ACW	37803+	na	2007
Switzerland	ACW	37602	na	2007
Switzerland	ACW	43577	na	2007
Switzerland	ACW	43579	na	2007
Switzerland	ACW	43802	na	2007
Switzerland	ACW	35734	na	2007
Switzerland	ACW	43590	na	2007
Switzerland	ACW	35696	na	2007
Switzerland	ACW	35697	na	2007
Switzerland	ACW	35698	na	2007
Switzerland	ACW	35731	na	2007
Switzerland	ACW	35683	na	2007
Switzerland	ACW	35684	na	2007
Switzerland	ACW	35686	na	2007
Switzerland	ACW	35689	na	2007
Switzerland	ACW	39005	na	2007
Switzerland	ACW	38900	na	2007
Switzerland	ACW	37915+	na	2007
Switzerland	ACW	37783+	na	2007
Switzerland	ACW	37686	na	2007
Switzerland	ACW	39002	na	2007
Switzerland	ACW	38249	na	2007
Switzerland	ACW	38878	na	2007
Switzerland	ACW	44567	na	2007
Switzerland	ACW	45015	na	2007
Switzerland	ACW	44432	na	2007
Switzerland	ACW	44736	na	2007
Switzerland	ACW	45534	na	2007
Switzerland	ACW	45009	na	2007
Switzerland	ACW	45034	na	2007
Switzerland	ACW	44193	na	2007
Switzerland	ACW	45069	na	2007
Switzerland	ACW	45962	na	2007
Switzerland	ACW	37607	na	2007
Switzerland	ACW	37812	na	2007
Switzerland	ACW	37825	na	2007
Switzerland	ACW	37920+	na	2007
Switzerland	ACW	RAC 2875	na	2007
Switzerland	ACW	RAC 99/85	na	2007
Switzerland	ACW	RAC 3080	na	2007
Switzerland	ACW	RAC 2235	na	2007
Switzerland	ACW	RAC 3072	na	2007
Switzerland	ACW	RAC 3074 B	na	2007
Switzerland	ACW	36915	na	2007
Switzerland	ACW	36916	na	2007
Switzerland	ACW	36670	na	2007

Switzerland	ACW	37600+	na	2007
Switzerland	ACW	37905+	na	2007
Switzerland	ACW	38019	na	2007
Switzerland	ACW	38925	na	2007
Switzerland	ACW	42919	na	2007
Switzerland	ACW	37706	na	2007
Switzerland	ACW	36076	na	2007
Switzerland	ACW	36694	na	2007
Switzerland	ACW	36670	na	2007
Switzerland	ACW	RAC 2414	na	2007
Switzerland	ACW	42918	na	2007
Switzerland	ACW	39012	na	2007
Switzerland	ACW	39018	na	2007
Switzerland	ACW	37811	na	2007
Switzerland	ACW	37623	na	2007
Switzerland	ACW	36097	na	2007
Switzerland	ACW	36064	na	2007
Switzerland	ACW	35978	na	2007
Switzerland	ACW	42306	na	2007
Switzerland	ACW	42915	na	2007
Switzerland	ACW	45008	na	2007
Switzerland	ACW	42906	na	2007
Switzerland	ACW	42951	na	2007
Switzerland	ACW	36056	na	2007
Switzerland	ACW	37830+	na	2007
Switzerland	ACW	37838	na	2007
Switzerland	ACW	39000	na	2007
Switzerland	ACW	39015	na	2007
Switzerland	ACW	42907	na	2007
Switzerland	ACW	42913	na	2007
Switzerland	ACW	37884	na	2007
Switzerland	ACW	39004	na	2007
Switzerland	ACW	35506	na	2007
Switzerland	ACW	28357	na	2007
Switzerland	ACW	32953	na	2007
Switzerland	ACW	35065	na	2007
Switzerland	ACW	na	na	2007
Switzerland	ACW	na	na	2007
Switzerland	ACW	35301	na	2007
Switzerland	ACW	35294	na	2007
Switzerland	ACW	35291	na	2007
Switzerland	ACW	35295	na	2007
Switzerland	ACW	35408	na	2007
Switzerland	ACW	35367	na	2007
Switzerland	ACW	35368	na	2007
Switzerland	ACW	35391	na	2007
Switzerland	ACW	35409	na	2007
Switzerland	ACW	35300	na	2007
Switzerland	ACW	35303	na	2007
Switzerland	ACW	35302	na	2007
Switzerland	ACW	35299	na	2007
Switzerland	ACW	36629	na	2007
Switzerland	ACW	35396	na	2007

Información suplementaria

Switzerland	ACW	35402	na	2007
Switzerland	ACW	35389	na	2007
Switzerland	ACW	35484	na	2007
Switzerland	ACW	35522	na	2007
Switzerland	ACW	35524	na	2007
Switzerland	ACW	35529	na	2007
Switzerland	ACW	35366	na	2007
Switzerland	ACW	35357	na	2007
Switzerland	ACW	35356	na	2007
Switzerland	ACW	35418	na	2007
Switzerland	ACW	35393	na	2007
Switzerland	ACW	35377	na	2007
Switzerland	ACW	35407	na	2007
Switzerland	ACW	35297	na	2007
Switzerland	ACW	35462	na	2007
Switzerland	ACW	35289	na	2007
Switzerland	ACW	35306	na	2007
Switzerland	ACW	35307	na	2007
Switzerland	ACW	35298	na	2007
Switzerland	ACW	35312	na	2007
Switzerland	ACW	35299	na	2007
Switzerland	ACW	35388	na	2007
Switzerland	ACW	35385	na	2007
Switzerland	ACW	35476	na	2007
Switzerland	ACW	32920	na	2007
Switzerland	ACW	32953	na	2007
Switzerland	ACW	32945	na	2007
Switzerland	ACW	34978	na	2007
Switzerland	ACW	34998	na	2007
Switzerland	ACW	34945	na	2007
Switzerland	ACW	32953	na	2007
Switzerland	ACW	34962	na	2007
Switzerland	ACW	35145	na	2007
Switzerland	ACW	35091	na	2007
Switzerland	ACW	35063	na	2007
Switzerland	ACW	35065	na	2007
Switzerland	ACW	35075	na	2007
Switzerland	ACW	35270	na	2007
Switzerland	ACW	35266	na	2007
Switzerland	ACW	35250	na	2007
Switzerland	ACW	35264	na	2007
Switzerland	ACW	35263	na	2007
Switzerland	ACW	35060	na	2007
Switzerland	ACW	35260	na	2007
Switzerland	ACW	35205	na	2007
Switzerland	ACW	35281	na	2007
Switzerland	ACW	39203	na	2007
The Netherlands	CFBP	CFBP 3020	Pear	1981
The Netherlands	NCPFB	NCPFB 3159	Apple	1980
UK	CFBP	CFBP 3041	Pear	na
UK	CFBP	CFBP 3042	Apple	na
UK	BBA	KG P-1573	<i>Cotoneaster</i> sp.	na
UK	BBA	KG 98 3042	Apple	na

UK	NCPFB	NCPFB 595	Pear	1958
UK	NCPFB	NCPFB 683	Pear	1959

na: not available.

ACW: Agroscope-Changins Wädenswil, Switzerland.

AGES: Austrian Agency for Health and Food Safety. Wien, Austria.

ARI: Agricultural Research Institute SERBIA - Pesticide and Environmental Research Centre, Belgrade-Zemun. Serbia.

BBA: Federal Biological Institute for Agriculture and Forestry. Dossenheim, Germany.

BPIC: Benaki Phytopathological Institute Collection, Athens, Greece.

CFBP: Collection Française de Bactéries Phytopathogènes, INRA, Angers, France.

CRA-W : Centre Wallon de Recherches agronomiques. Gembloux, Belgium.

EPS: Escola Politècnica Superior-Universitat de Girona, Spain.

INRA : Institut National de la Recherche Agronomique, UMR de Pathologie Végétale, Beaucauzé, France

IVIA: Instituto Valenciano de Investigaciones Agrarias collection, Moncada, Spain

LMG: Collection of the Laboratory of Microbiology. Rijksuniversiteit, Gent, Belgium

LNPV: Laboratoire National de la Protection des Végétaux, Beaucauzé, France.

NCPFB: National Collection of Plant Pathogenic Bacteria, York, UK.

NIB: National Institute of Biology, Ljubljana, Slovenia.

OMP: Osservatorio per le Malattie delle Piante, Bologna, Italy.

SL: State Laboratory, Dublin, Ireland.

SV: Servei de Protecció dels Vegetals-Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya, Barcelona, Spain.

UPN: Universidad Publica de Navarra. Pamplona, Spain.

Información suplementaria capítulo 5

Analysing the effect on chromosomal gene expression of plasmids pEA29 and pEI70 in symptoms development on pear in *Erwinia amylovora*.

Supplemental Figure 5-1. GOgraphs and detailed information of a) cellular components domains upregulated in presence of pEA29; b) cellular components domains downregulated in presence of pEA29; c) cellular components domains downregulated in presence of pEI70;

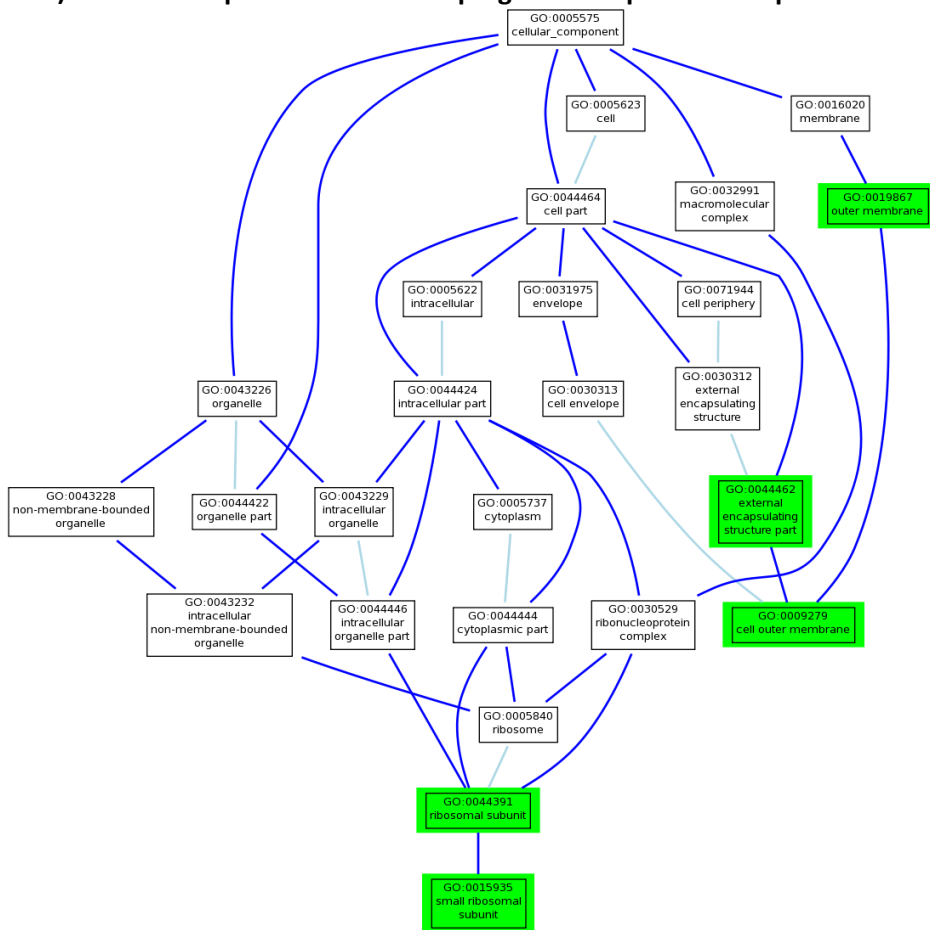
Supplemental Figure 5-2. GOgraphs and detailed information of a) molecular function domains upregulated in presence of pEA29; b) molecular function domains downregulated in presence of pEA29; c) molecular function domains upregulated in presence of pEI70; d) molecular function domains downregulated in presence of pEI70.

Supplemental Figure 5-3. GOgraphs and detailed information of a) biological process domains upregulated in presence of pEA29; b) biological process domains downregulated in presence of pEA29; c) biological process domains upregulated in presence of pEI70; d) biological process domains downregulated in presence of pEI70.

In the GO graph, the squares marked in green represent the terms up or down-represented in the functional analysis.

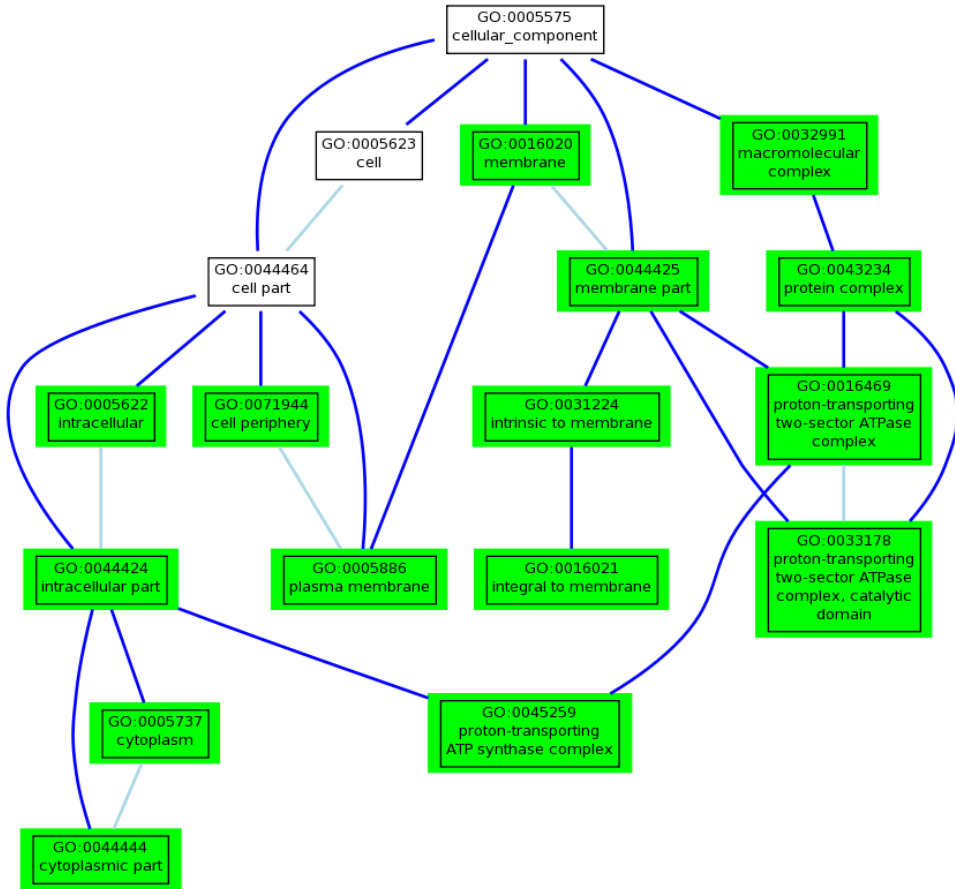
In detailed information a table with GO terms up or down-represented is showed, and only cells marked in pink colour show GO terms where genes differentially expressed were included (See tables 4 and 5 in “Capítulo 5”).

5-1. a) Cellular components domains upregulated in presence of pEA29



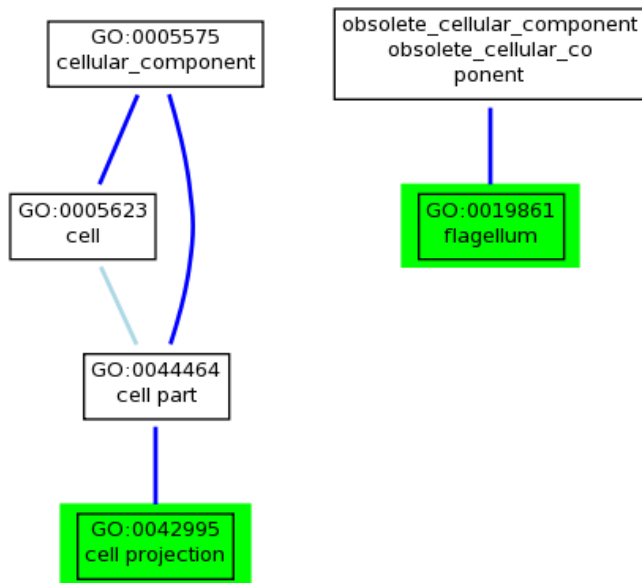
GO_id	GO_description
GO:0009279	cell outer membrane
GO:0015935	small ribosomal subunit
GO:0019867	outer membrane
GO:0044391	ribosomal subunit
GO:0044462	external encapsulating structure part

5-1. b) Cellular components domains downregulated in presence of pEA29



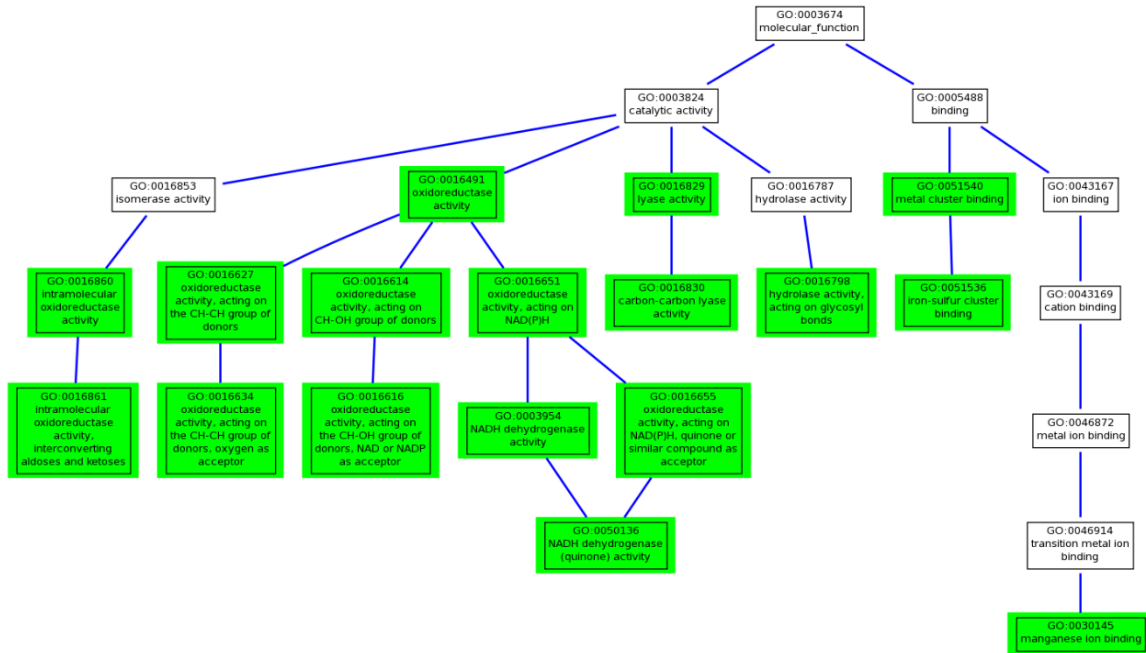
GO_id	GO_description
GO:0005622	intracellular
GO:0005737	cytoplasm
GO:0005886	plasma membrane
GO:0016020	membrane
GO:0016021	integral to membrane
GO:0016469	proton-transporting two-sector ATPase complex
GO:0031224	intrinsic to membrane
GO:0032991	macromolecular complex
GO:0033178	proton-transporting two-sector ATPase complex, catalytic domain
GO:0043234	protein complex
GO:0044424	intracellular part
GO:0044425	membrane part
GO:0044444	cytoplasmic part
GO:0045259	proton-transporting ATP synthase complex
GO:0071944	cell periphery

5-1. c) Cellular components domains downregulated in presence of pEI70



GO_id	GO_description
GO:0019861	flagellum
GO:0042995	cell projection

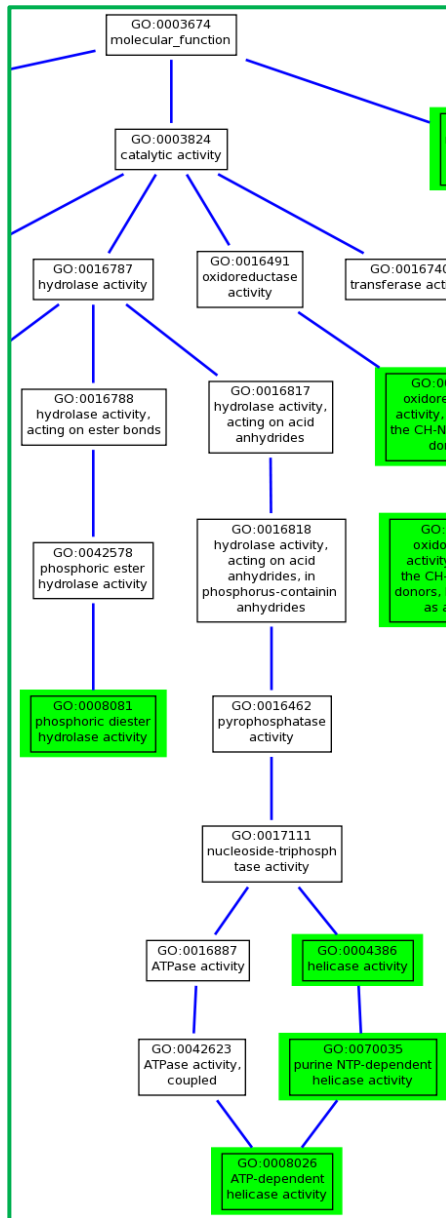
5-2. a) Molecular function domains upregulated in presence of pEA29

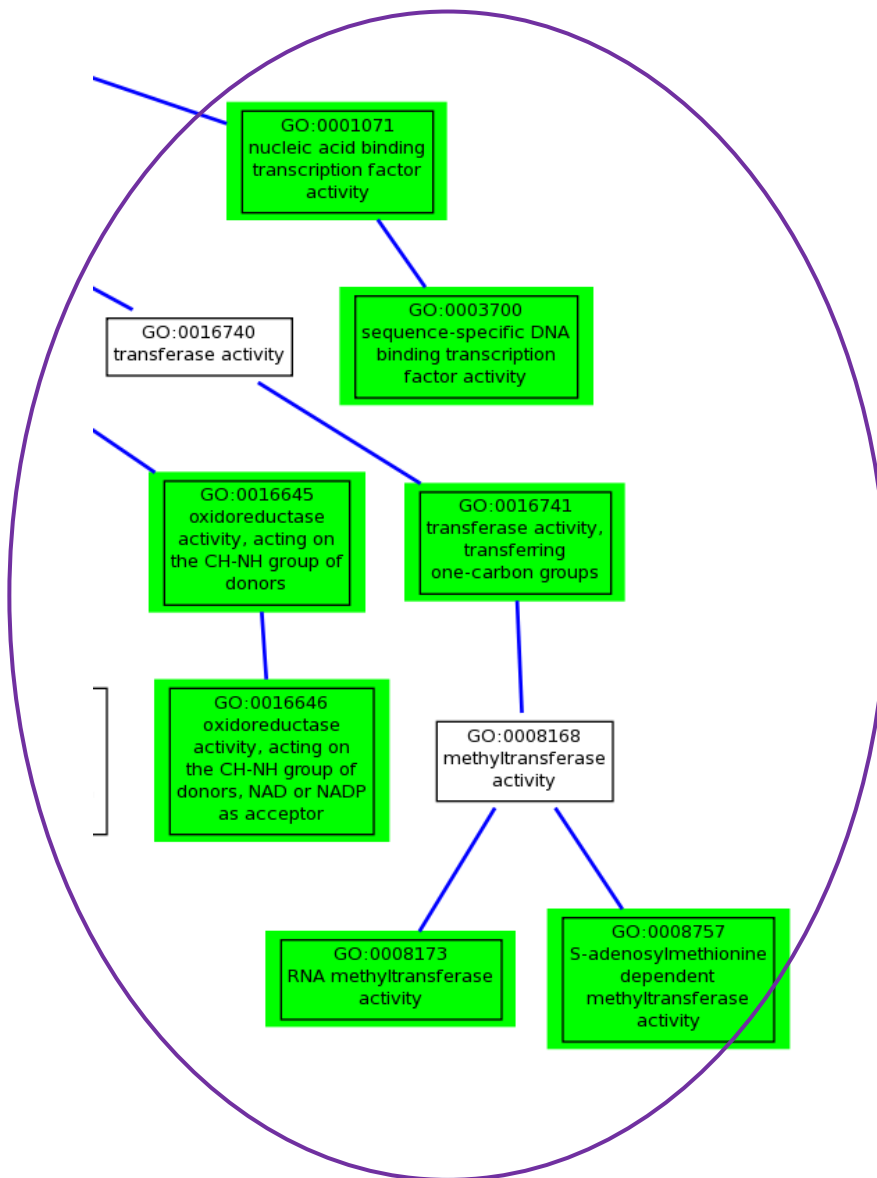


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GO_id	GO_description
GO:0003954	NADH dehydrogenase activity
GO:0016491	oxidoreductase activity
GO:0016614	oxidoreductase activity, acting on CH-OH group of donors
GO:0016616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors
GO:0016634	oxidoreductase activity, acting on the CH-CH group of donors, oxygen as acceptor
GO:0016651	oxidoreductase activity, acting on NADH or NADPH
GO:0016655	oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor
GO:0016798	hydrolase activity, acting on glycosyl bonds
GO:0016829	lyase activity
GO:0016830	carbon-carbon lyase activity
GO:0016860	intramolecular oxidoreductase activity
GO:0016861	intramolecular oxidoreductase activity, interconverting aldoses and ketoses
GO:0030145	manganese ion binding
GO:0050136	NADH dehydrogenase (quinone) activity
GO:0051536	iron-sulfur cluster binding
GO:0051540	metal cluster binding

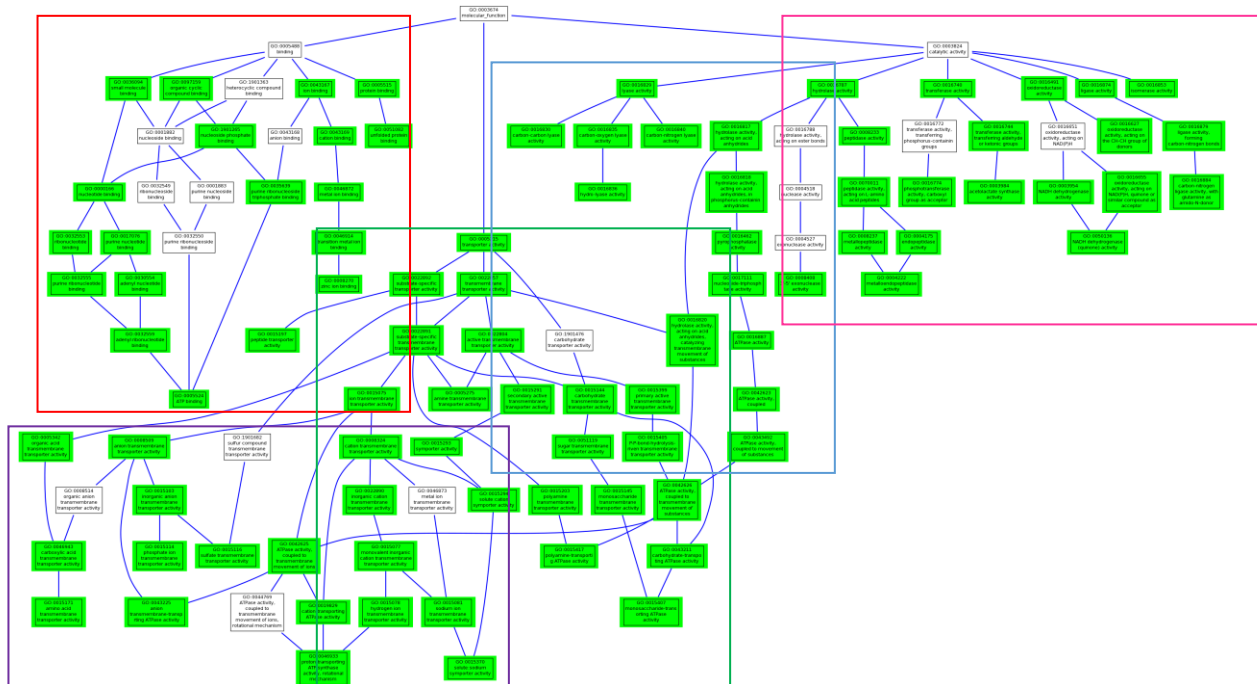


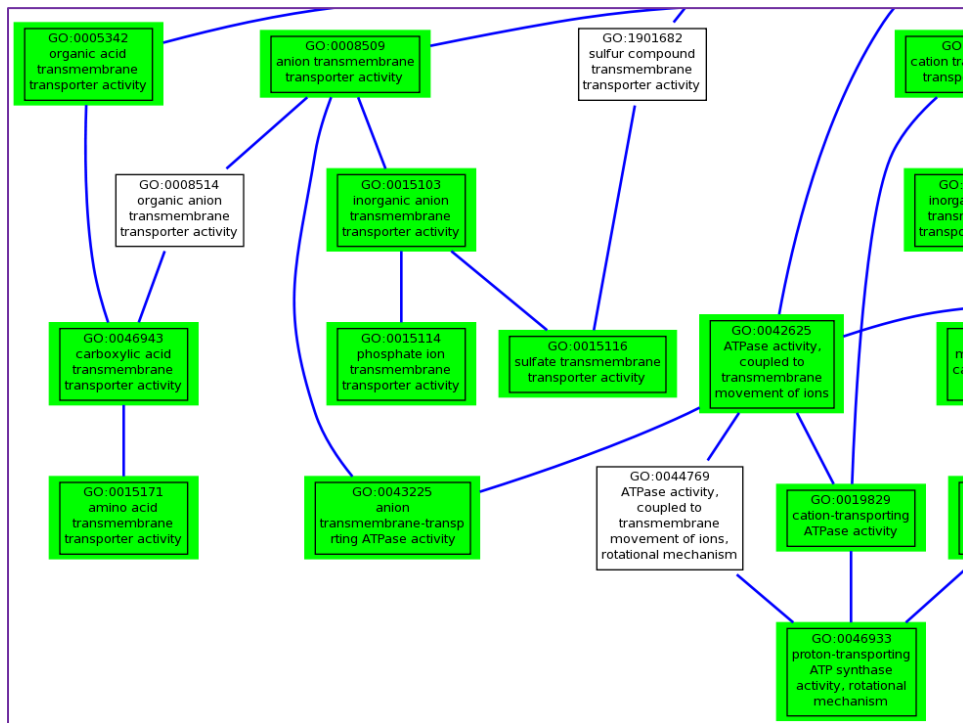


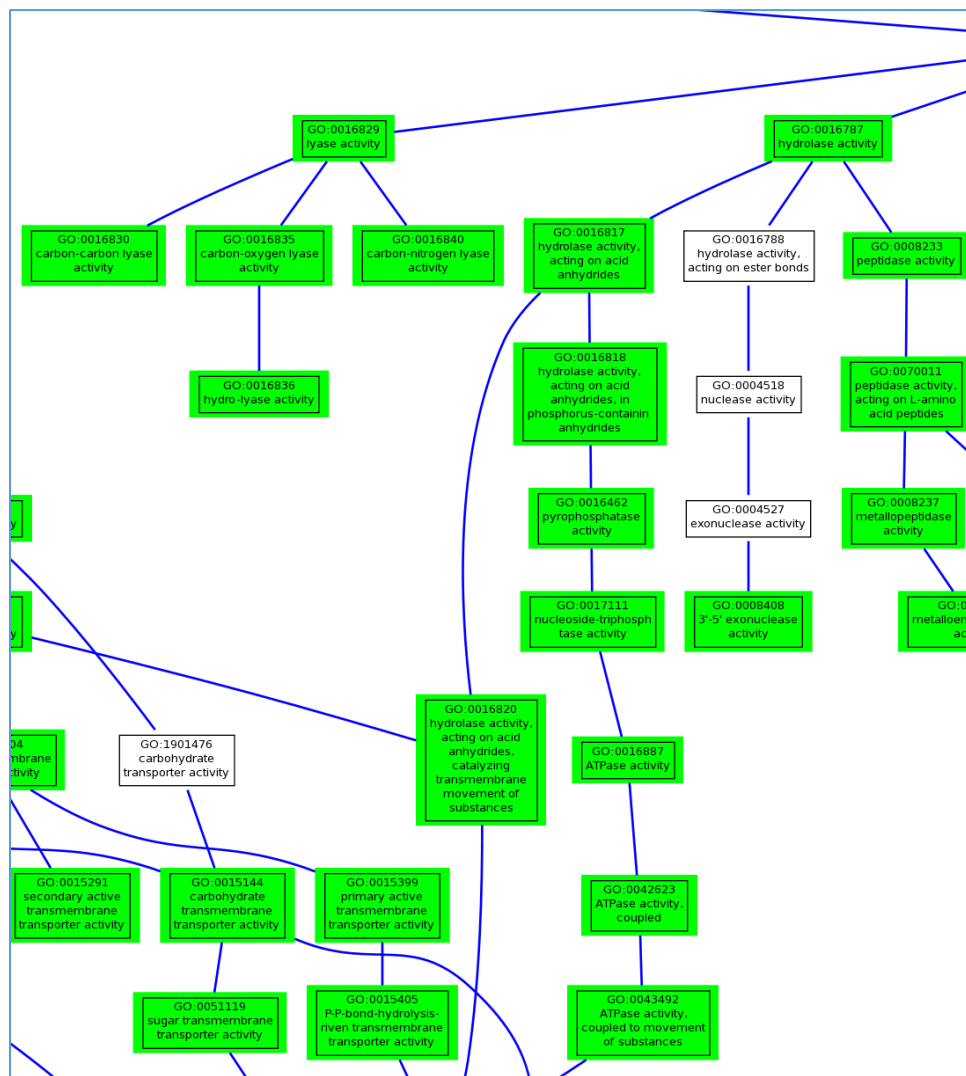


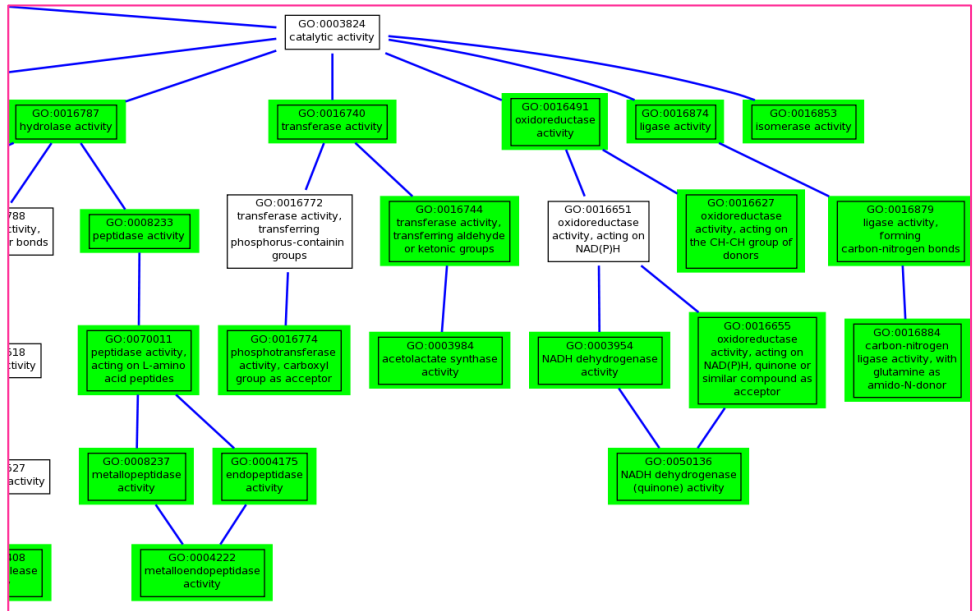
GO_id	GO_description
GO:0001071	nucleic acid binding transcription factor activity
GO:0003676	nucleic acid binding
GO:0003677	DNA binding
GO:0003700	sequence-specific DNA binding transcription factor activity
GO:0004386	helicase activity
GO:0008026	ATP-dependent helicase activity
GO:0008081	phosphoric diester hydrolase activity
GO:0008173	RNA methyltransferase activity
GO:0008757	S-adenosylmethionine-dependent methyltransferase activity
GO:0009982	pseudouridine synthase activity
GO:0016645	oxidoreductase activity, acting on the CH-NH group of donors
GO:0016646	oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor
GO:0016741	transferase activity, transferring one-carbon groups
GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
GO:0016812	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amides
GO:0016814	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amidines
GO:0070035	purine NTP-dependent helicase activity

5-2. c) Molecular function domains upregulated in presence of pEI70









Información suplementaria

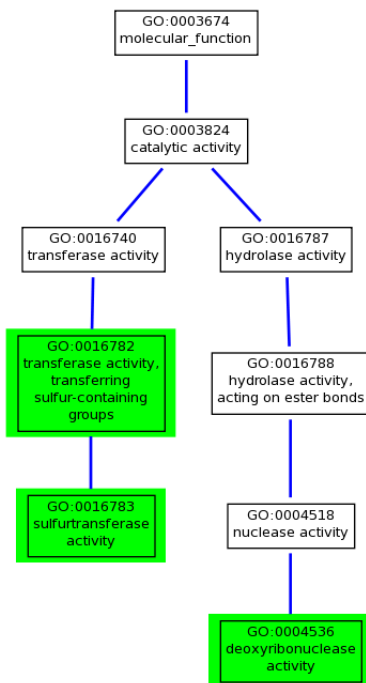
GO_id	GO_description
GO:0000166	nucleotide binding
GO:0003954	NADH dehydrogenase activity
GO:0003984	acetolactate synthase activity
GO:0004175	endopeptidase activity
GO:0004222	metalloendopeptidase activity
GO:0005215	transporter activity
GO:0005275	amine transmembrane transporter activity
GO:0005342	organic acid transmembrane transporter activity
GO:0005515	protein binding
GO:0005524	ATP binding
GO:0008233	peptidase activity
GO:0008237	metallopeptidase activity
GO:0008270	zinc ion binding
GO:0008324	cation transmembrane transporter activity
GO:0008408	3'-5' exonuclease activity
GO:0008509	anion transmembrane transporter activity
GO:0015075	ion transmembrane transporter activity
GO:0015077	monovalent inorganic cation transmembrane transporter activity
GO:0015078	hydrogen ion transmembrane transporter activity
GO:0015081	sodium ion transmembrane transporter activity
GO:0015103	inorganic anion transmembrane transporter activity
GO:0015114	phosphate ion transmembrane transporter activity
GO:0015116	sulfate transmembrane transporter activity
GO:0015144	carbohydrate transmembrane transporter activity
GO:0015145	monosaccharide transmembrane transporter activity
GO:0015171	amino acid transmembrane transporter activity
GO:0015197	peptide transporter activity
GO:0015203	polyamine transmembrane transporter activity
GO:0015291	secondary active transmembrane transporter activity
GO:0015293	symporter activity
GO:0015294	solute:cation symporter activity
GO:0015370	solute:sodium symporter activity
GO:0015399	primary active transmembrane transporter activity

GO:0015405	P-P-bond-hydrolysis-driven transmembrane transporter activity
GO:0015407	monosaccharide-transporting ATPase activity
GO:0015417	polyamine-transporting ATPase activity
GO:0016462	pyrophosphatase activity
GO:0016491	oxidoreductase activity
GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors
GO:0016655	oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor
GO:0016740	transferase activity
GO:0016744	transferase activity, transferring aldehyde or ketonic groups
GO:0016774	phosphotransferase activity, carboxyl group as acceptor
GO:0016787	hydrolase activity
GO:0016817	hydrolase activity, acting on acid anhydrides
GO:0016818	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides
GO:0016820	hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
GO:0016829	lyase activity
GO:0016830	carbon-carbon lyase activity
GO:0016835	carbon-oxygen lyase activity
GO:0016836	hydro-lyase activity
GO:0016840	carbon-nitrogen lyase activity
GO:0016853	isomerase activity
GO:0016874	ligase activity
GO:0016879	ligase activity, forming carbon-nitrogen bonds
GO:0016884	carbon-nitrogen ligase activity, with glutamine as amido-N-donor
GO:0016887	ATPase activity
GO:0017076	purine nucleotide binding
GO:0017111	nucleoside-triphosphatase activity
GO:0019829	cation-transporting ATPase activity
GO:0022804	active transmembrane transporter activity
GO:0022857	transmembrane transporter activity
GO:0022890	inorganic cation transmembrane transporter activity
GO:0022891	substrate-specific transmembrane transporter activity
GO:0022892	substrate-specific transporter activity
GO:0030554	adenyl nucleotide binding
GO:0032553	ribonucleotide binding

Información suplementaria

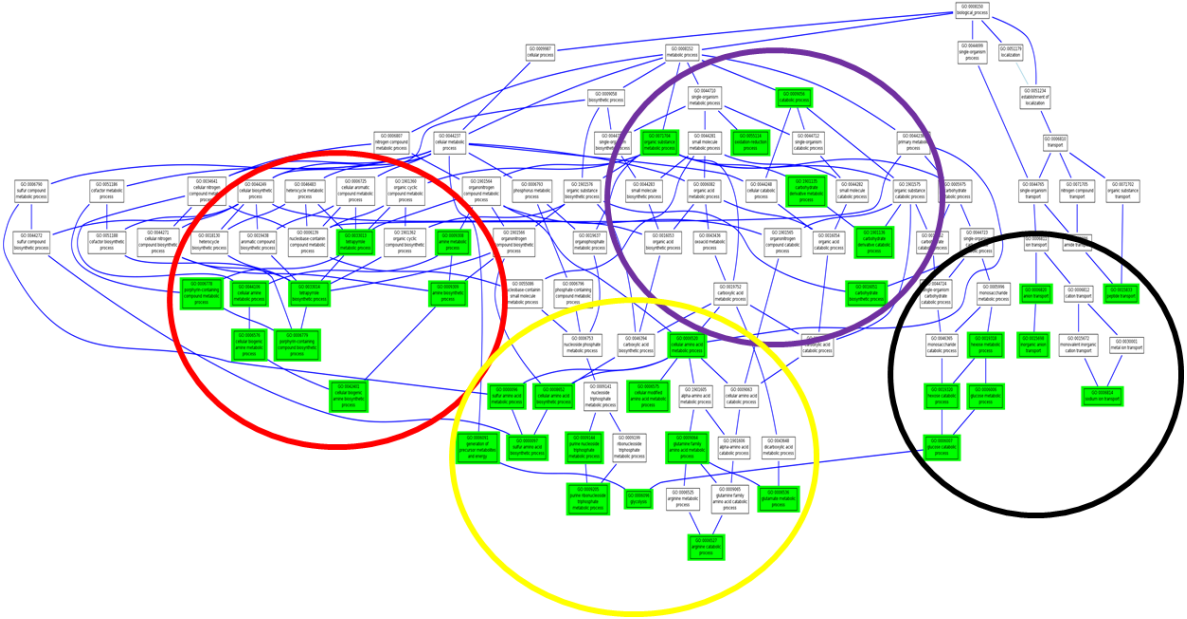
GO:0032555 purine ribonucleotide binding
GO:0032559 adenylyl ribonucleotide binding
GO:0035639 purine ribonucleoside triphosphate binding
GO:0036094 small molecule binding
GO:0042623 ATPase activity, coupled
GO:0042625 ATPase activity, coupled to transmembrane movement of ions
GO:0042626 ATPase activity, coupled to transmembrane movement of substances
GO:0043167 ion binding
GO:0043169 cation binding
GO:0043211 carbohydrate-transporting ATPase activity
GO:0043225 anion transmembrane-transporting ATPase activity
GO:0043492 ATPase activity, coupled to movement of substances
GO:0046872 metal ion binding
GO:0046914 transition metal ion binding
GO:0046933 hydrogen ion transporting ATP synthase activity, rotational mechanism
GO:0046943 carboxylic acid transmembrane transporter activity
GO:0050136 **NADH dehydrogenase (quinone) activity**
GO:0051082 **unfolded protein binding**
GO:0051119 sugar transmembrane transporter activity
GO:0070011 peptidase activity, acting on L-amino acid peptides
GO:0097159 organic cyclic compound binding
GO:1901265 nucleoside phosphate binding

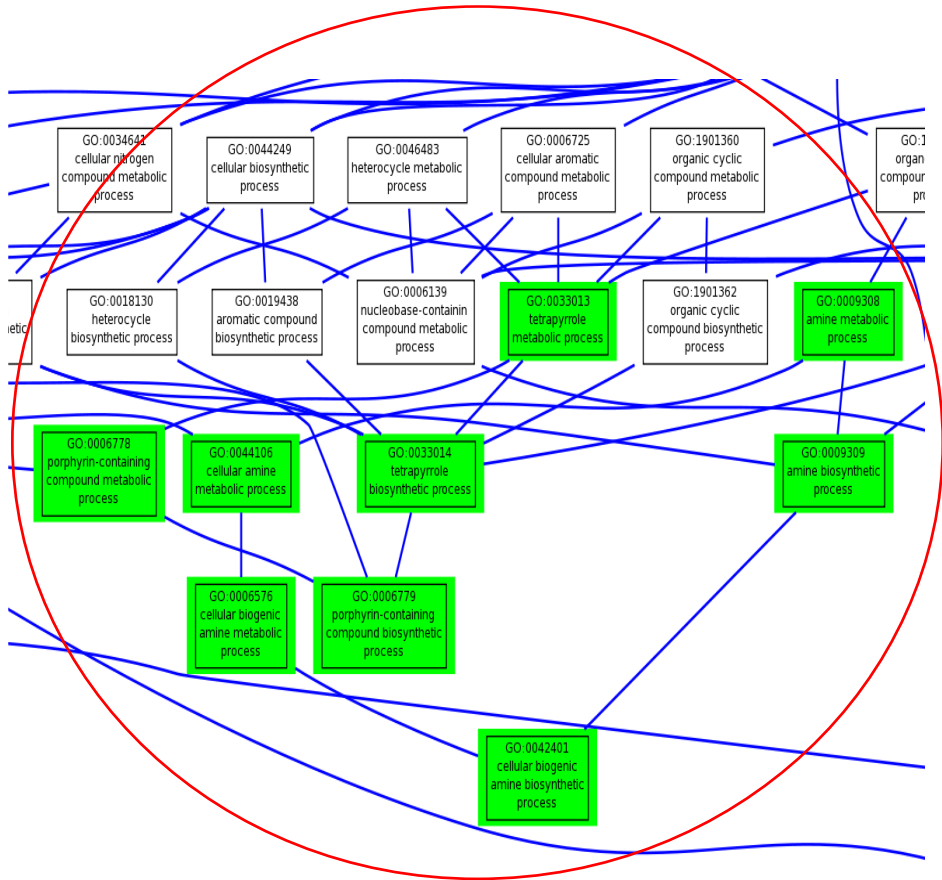
5-2. d) Molecular function domains downregulated in presence of pE170

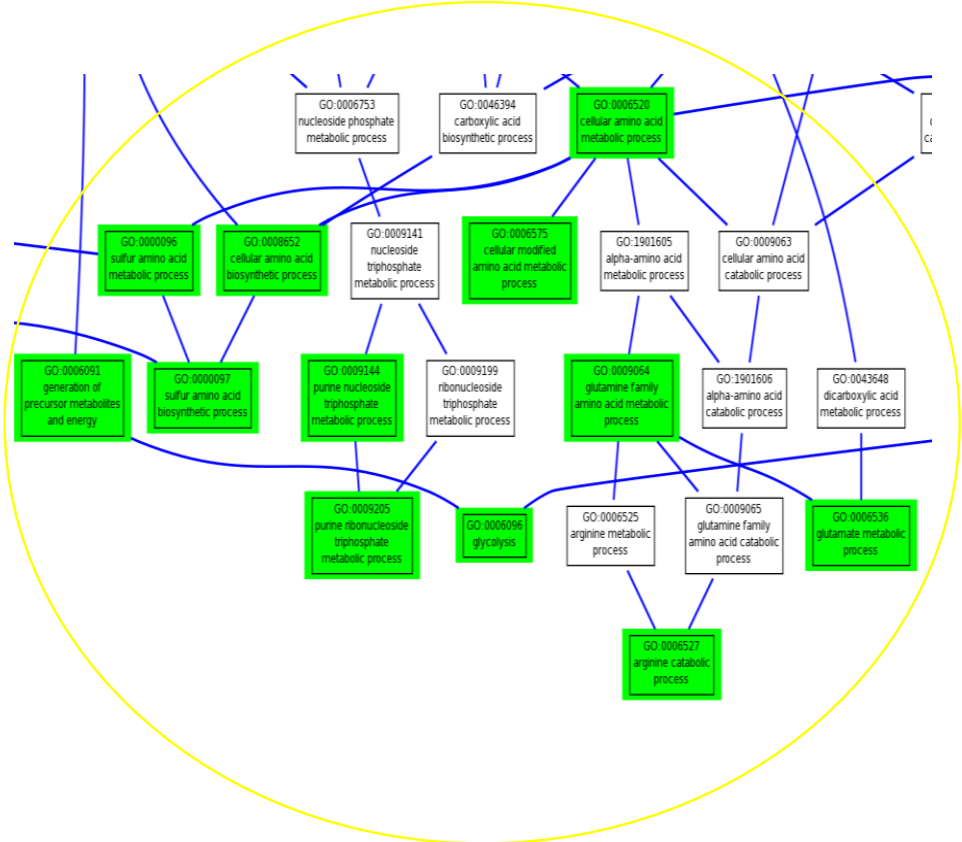


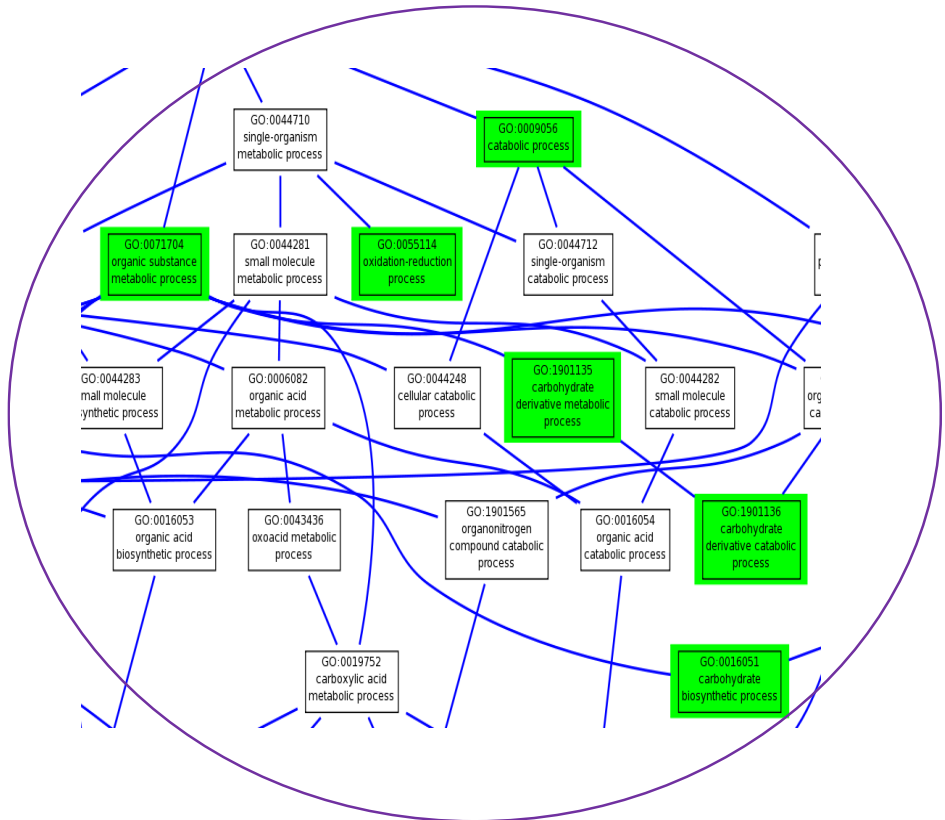
GO_id	GO_description
GO:0004536	deoxyribonuclease activity
GO:0016782	transferase activity, transferring sulfur-containing groups
GO:0016783	sulfurtransferase activity

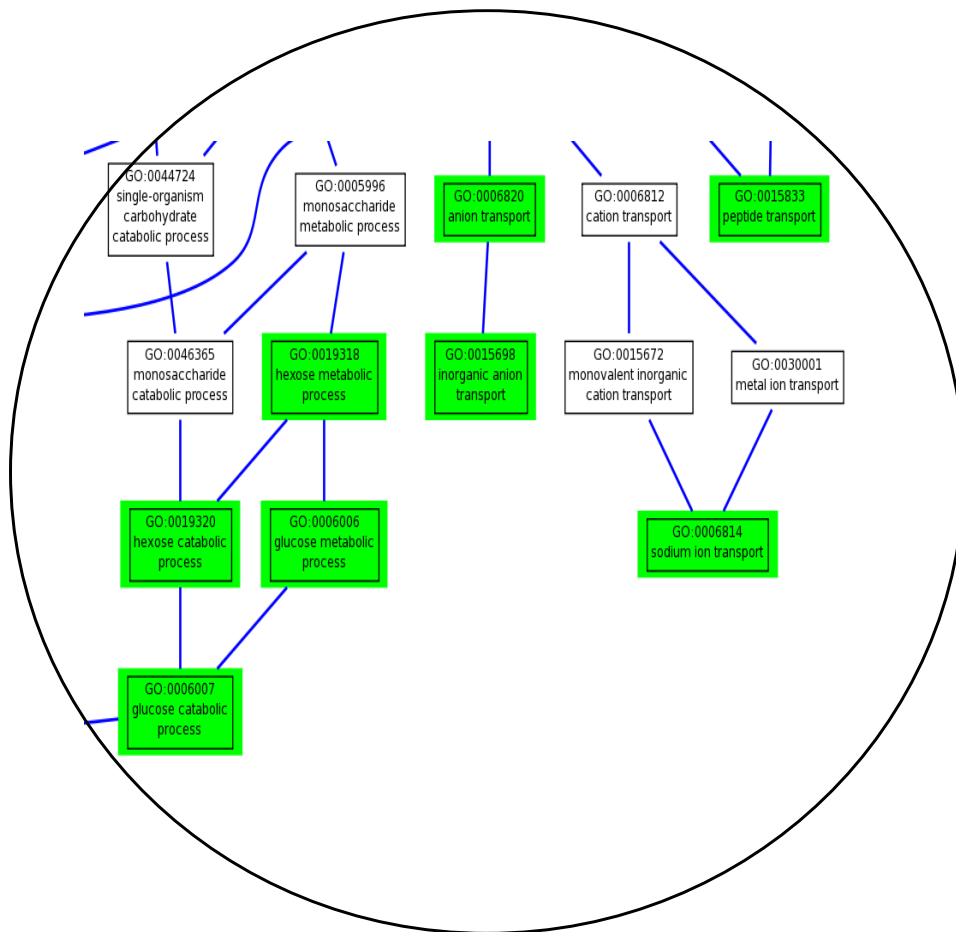
5-3. a) Biological process domains upregulated in presence of pEA29









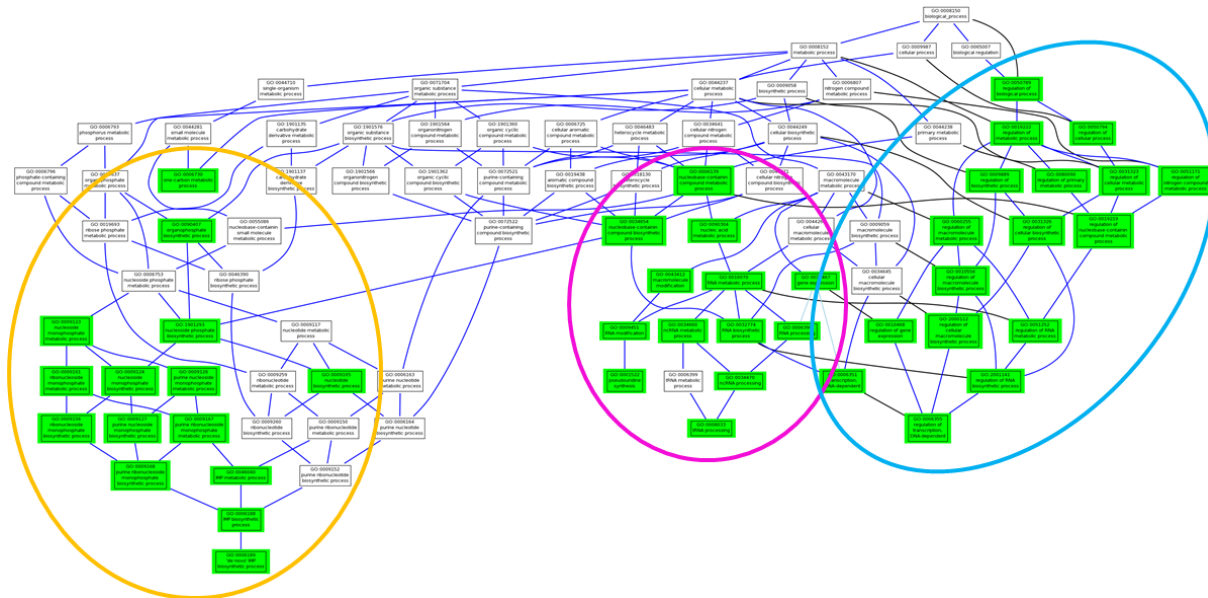


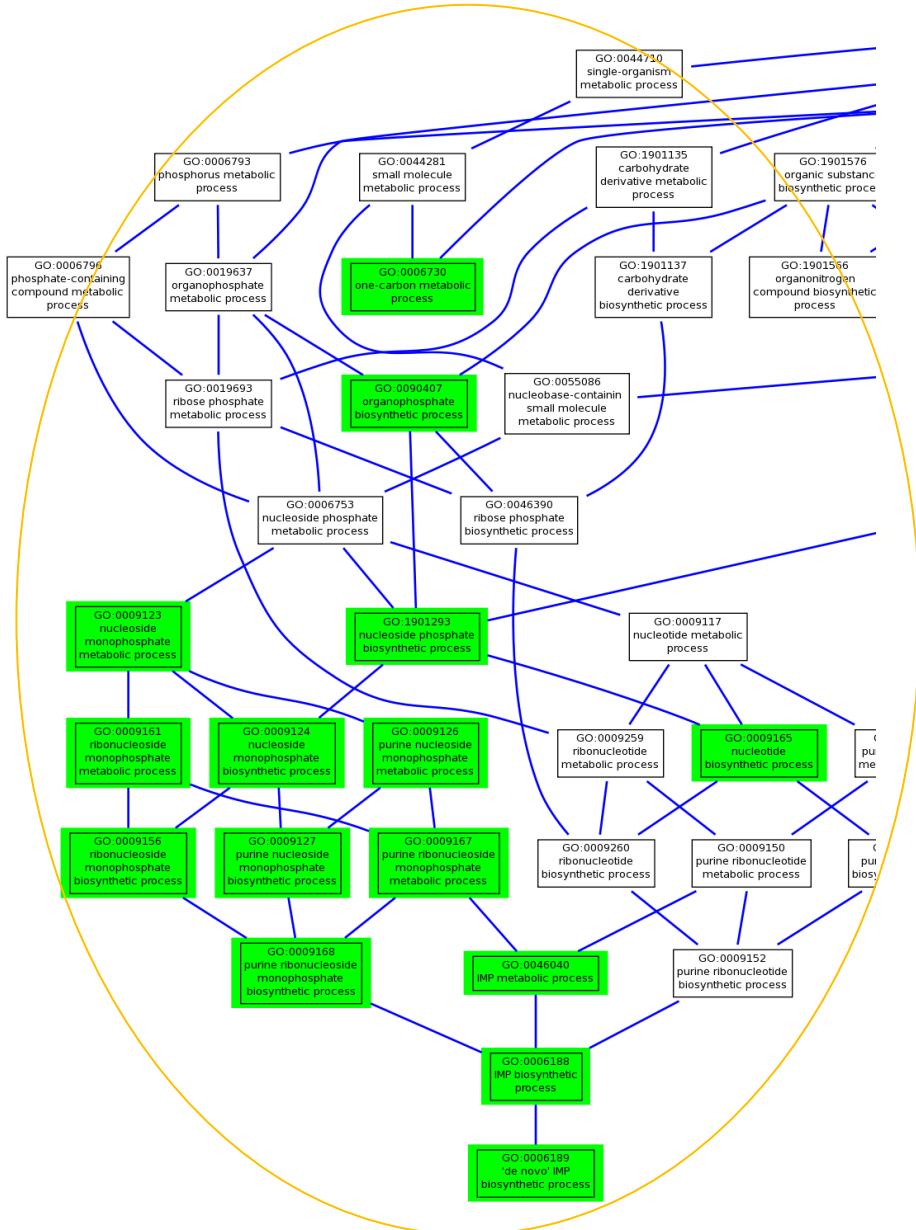
GO_id	GO_description
GO:0000096	sulfur amino acid metabolic process
GO:0000097	sulfur amino acid biosynthetic process
GO:0006006	glucose metabolic process
GO:0006007	glucose catabolic process
GO:0006091	generation of precursor metabolites and energy
GO:0006096	glycolysis
GO:0006520	cellular amino acid metabolic process
GO:0006527	arginine catabolic process
GO:0006536	glutamate metabolic process
GO:0006575	cellular modified amino acid metabolic process
GO:0006576	cellular biogenic amine metabolic process
GO:0006778	porphyrin-containing compound metabolic process
GO:0006779	porphyrin-containing compound biosynthetic process
GO:0006814	sodium ion transport
GO:0006820	anion transport
GO:0008652	cellular amino acid biosynthetic process
GO:0009056	catabolic process
GO:0009064	glutamine family amino acid metabolic process
GO:0009144	purine nucleoside triphosphate metabolic process
GO:0009205	purine ribonucleoside triphosphate metabolic process
GO:0009308	amine metabolic process
GO:0009309	amine biosynthetic process
GO:0015698	inorganic anion transport
GO:0015833	peptide transport
GO:0016051	carbohydrate biosynthetic process
GO:0019318	hexose metabolic process
GO:0019320	hexose catabolic process
GO:0033013	tetrapyrrole metabolic process
GO:0033014	tetrapyrrole biosynthetic process
GO:0042401	cellular biogenic amine biosynthetic process
GO:0044106	cellular amine metabolic process
GO:0055114	oxidation-reduction process

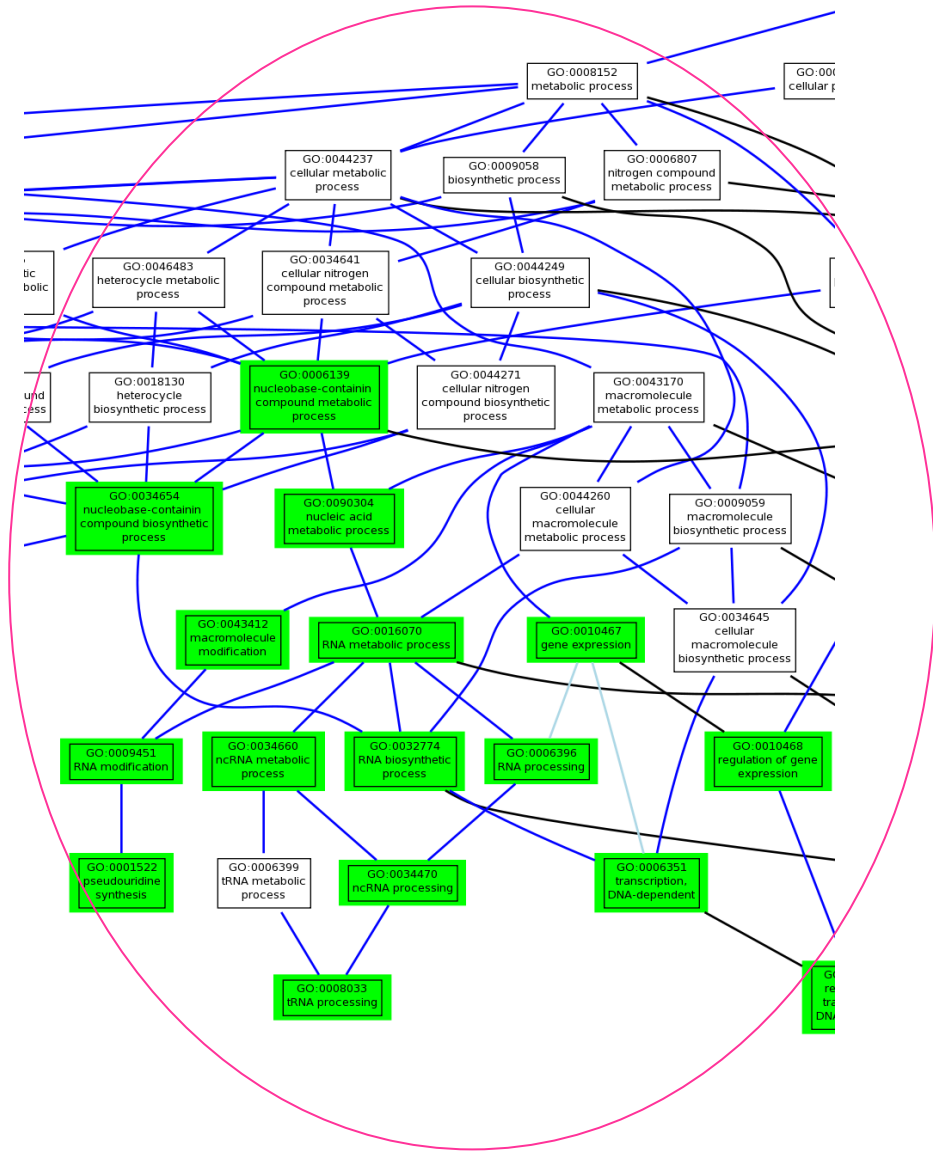
Información suplementaria

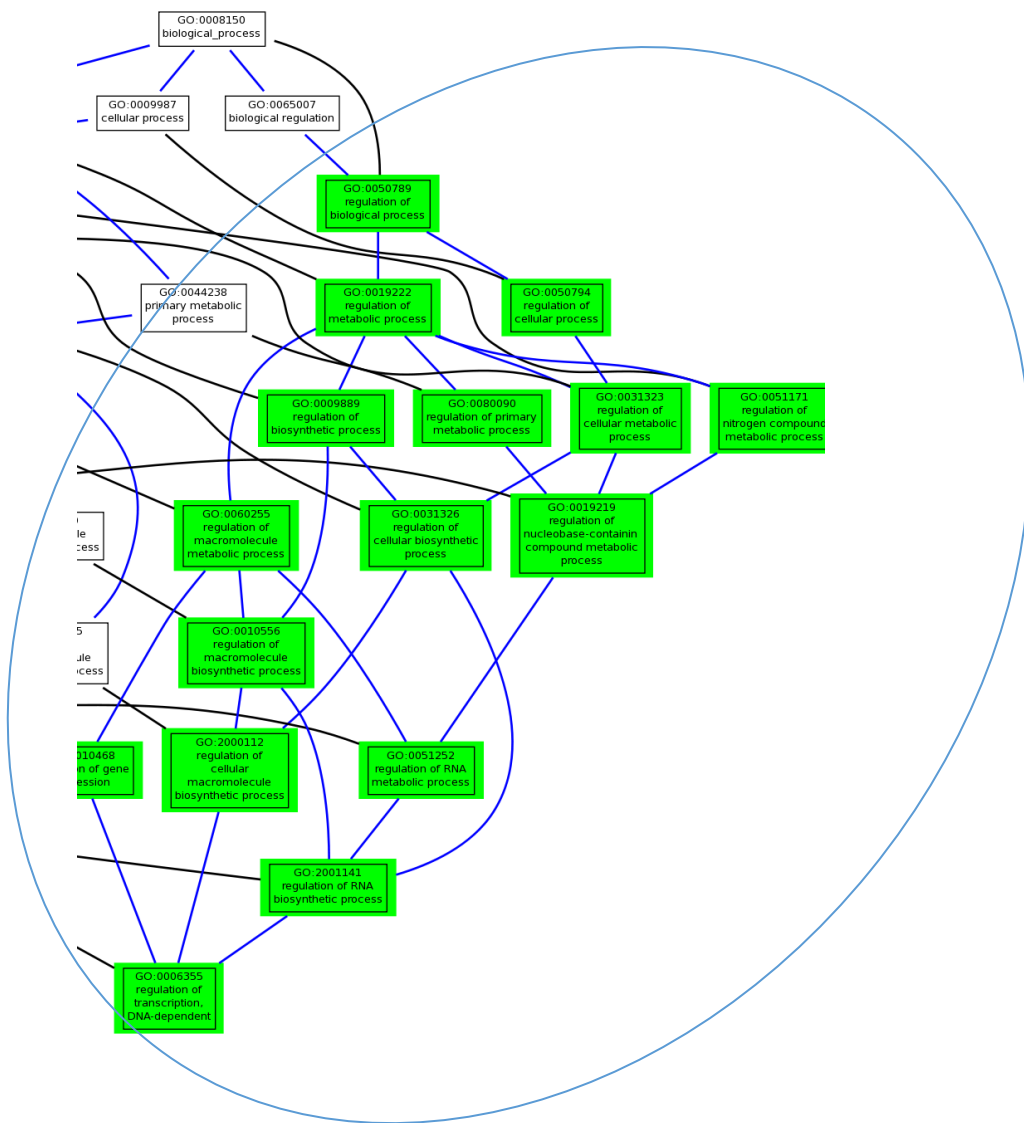
GO:0071704	organic substance metabolic process
GO:1901135	carbohydrate derivative metabolic process
GO:1901136	carbohydrate derivative catabolic process

5-3. b) Biological process domains downregulated in presence of pEA29







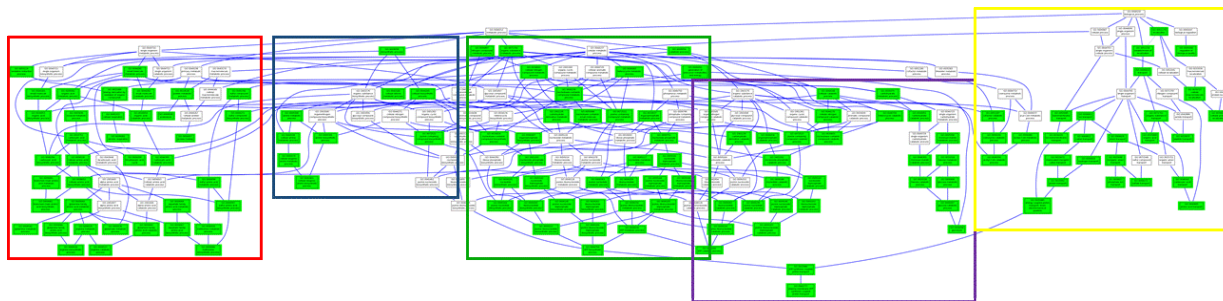


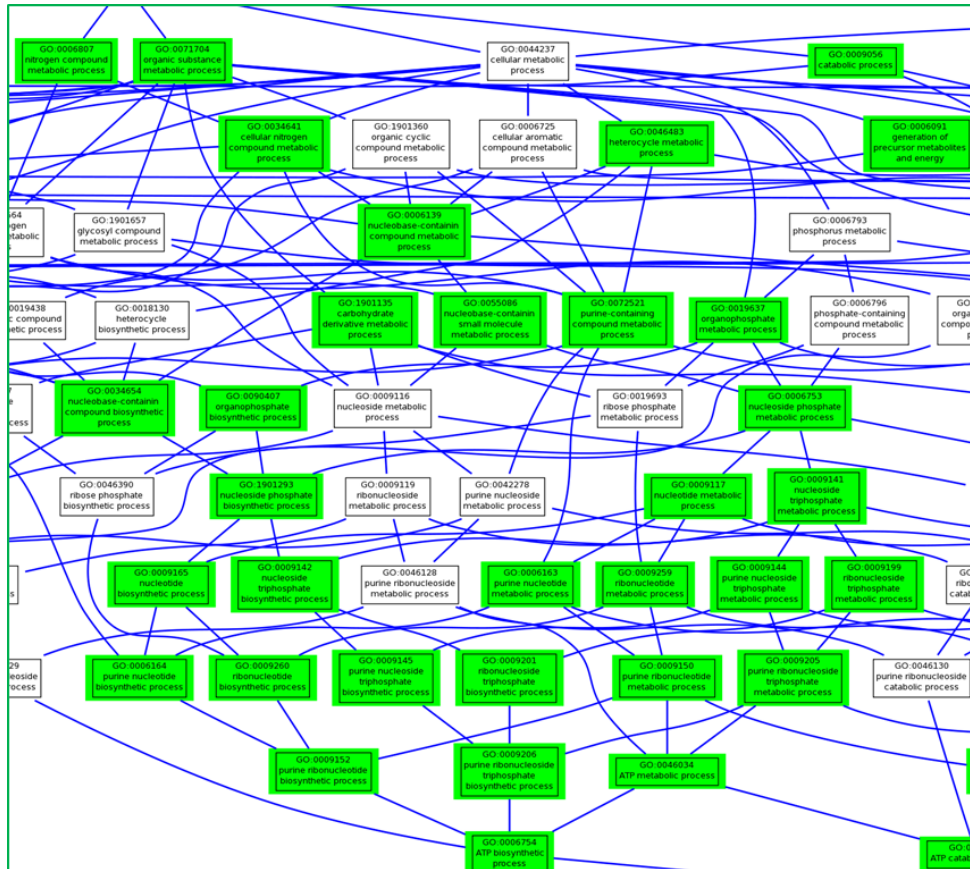
GO_id	GO_description
GO:0001522	pseudouridine synthesis
GO:0006139	nucleobase-containing compound metabolic process
GO:0006188	IMP biosynthetic process
GO:0006189	'de novo' IMP biosynthetic process
GO:0006351	transcription, DNA-dependent
GO:0006355	regulation of transcription, DNA-dependent
GO:0006396	RNA processing
GO:0006730	one-carbon metabolic process
GO:0008033	tRNA processing
GO:0009123	nucleoside monophosphate metabolic process
GO:0009124	nucleoside monophosphate biosynthetic process
GO:0009126	purine nucleoside monophosphate metabolic process
GO:0009127	purine nucleoside monophosphate biosynthetic process
GO:0009156	ribonucleoside monophosphate biosynthetic process
GO:0009161	ribonucleoside monophosphate metabolic process
GO:0009165	nucleotide biosynthetic process
GO:0009167	purine ribonucleoside monophosphate metabolic process
GO:0009168	purine ribonucleoside monophosphate biosynthetic process
GO:0009451	RNA modification
GO:0009889	regulation of biosynthetic process
GO:0010467	gene expression
GO:0010468	regulation of gene expression
GO:0010556	regulation of macromolecule biosynthetic process
GO:0016070	RNA metabolic process
GO:0019219	regulation of nucleobase-containing compound metabolic process
GO:0019222	regulation of metabolic process
GO:0031323	regulation of cellular metabolic process
GO:0031326	regulation of cellular biosynthetic process
GO:0032774	RNA biosynthetic process
GO:0034470	ncRNA processing
GO:0034654	nucleobase-containing compound biosynthetic process

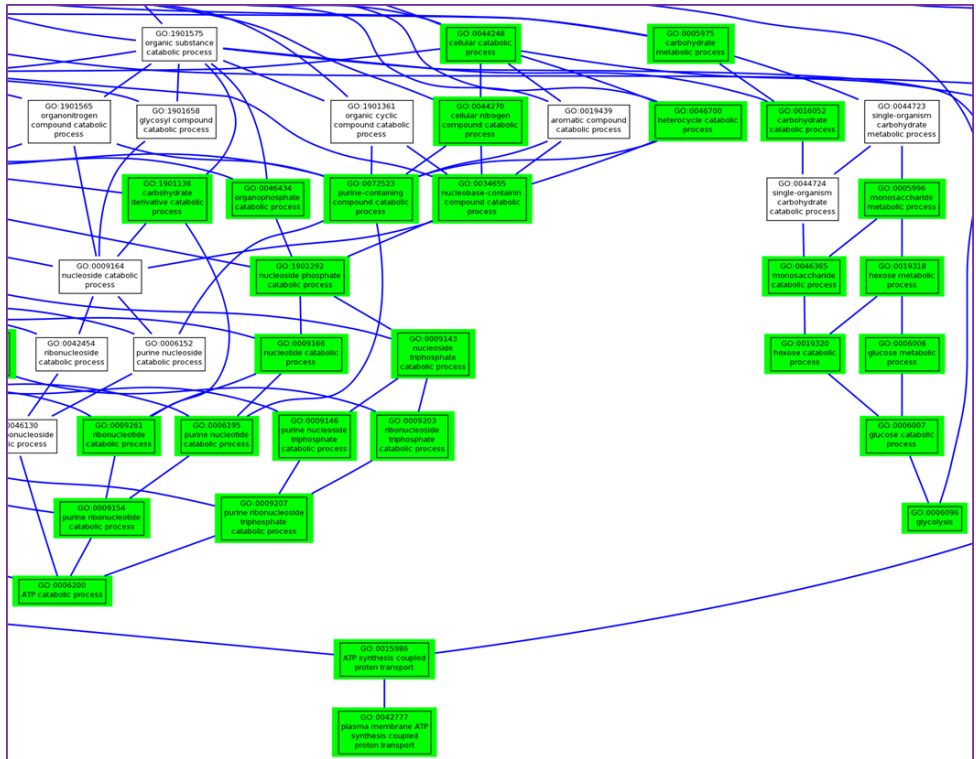
Información suplementaria

GO:0034660	ncRNA metabolic process
GO:0043412	macromolecule modification
GO:0046040	IMP metabolic process
GO:0050789	regulation of biological process
GO:0050794	regulation of cellular process
GO:0051171	regulation of nitrogen compound metabolic process
GO:0051252	regulation of RNA metabolic process
GO:0060255	regulation of macromolecule metabolic process
GO:0080090	regulation of primary metabolic process
GO:0090304	nucleic acid metabolic process
GO:0090407	organophosphate biosynthetic process
GO:1901293	nucleoside phosphate biosynthetic process
GO:2000112	regulation of cellular macromolecule biosynthetic process
GO:2001141	regulation of RNA biosynthetic process

5-3. c) biological process domains upregulated in presence of pEI70







GO_id	GO_description
GO:0000096	sulfur amino acid metabolic process
GO:0000097	sulfur amino acid biosynthetic process
GO:0005975	carbohydrate metabolic process
GO:0005996	monosaccharide metabolic process
GO:0006006	glucose metabolic process
GO:0006007	glucose catabolic process
GO:0006082	organic acid metabolic process
GO:0006084	acetyl-CoA metabolic process
GO:0006091	generation of precursor metabolites and energy
GO:0006096	glycolysis
GO:0006099	tricarboxylic acid cycle
GO:0006139	nucleobase-containing compound metabolic process
GO:0006163	purine nucleotide metabolic process
GO:0006164	purine nucleotide biosynthetic process
GO:0006195	purine nucleotide catabolic process
GO:0006200	ATP catabolic process
GO:0006457	protein folding
GO:0006508	proteolysis
GO:0006520	cellular amino acid metabolic process
GO:0006525	arginine metabolic process
GO:0006526	arginine biosynthetic process
GO:0006527	arginine catabolic process
GO:0006536	glutamate metabolic process
GO:0006541	glutamine metabolic process
GO:0006555	methionine metabolic process
GO:0006576	cellular biogenic amine metabolic process
GO:0006753	nucleoside phosphate metabolic process
GO:0006754	ATP biosynthetic process
GO:0006790	sulfur compound metabolic process
GO:0006807	nitrogen compound metabolic process
GO:0006810	transport
GO:0006811	ion transport

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GO:0006812	cation transport
GO:0006817	phosphate ion transport
GO:0006818	hydrogen transport
GO:0006820	anion transport
GO:0006865	amino acid transport
GO:0008272	sulfate transport
GO:0008652	cellular amino acid biosynthetic process
GO:0009056	catabolic process
GO:0009058	biosynthetic process
GO:0009060	aerobic respiration
GO:0009064	glutamine family amino acid metabolic process
GO:0009065	glutamine family amino acid catabolic process
GO:0009066	aspartate family amino acid metabolic process
GO:0009067	aspartate family amino acid biosynthetic process
GO:0009081	branched-chain amino acid metabolic process
GO:0009082	branched-chain amino acid biosynthetic process
GO:0009084	glutamine family amino acid biosynthetic process
GO:0009086	methionine biosynthetic process
GO:0009109	coenzyme catabolic process
GO:0009117	nucleotide metabolic process
GO:0009141	nucleoside triphosphate metabolic process
GO:0009142	nucleoside triphosphate biosynthetic process
GO:0009143	nucleoside triphosphate catabolic process
GO:0009144	purine nucleoside triphosphate metabolic process
GO:0009145	purine nucleoside triphosphate biosynthetic process
GO:0009146	purine nucleoside triphosphate catabolic process
GO:0009150	purine ribonucleotide metabolic process
GO:0009152	purine ribonucleotide biosynthetic process
GO:0009154	purine ribonucleotide catabolic process
GO:0009165	nucleotide biosynthetic process
GO:0009166	nucleotide catabolic process
GO:0009199	ribonucleoside triphosphate metabolic process
GO:0009201	ribonucleoside triphosphate biosynthetic process
GO:0009203	ribonucleoside triphosphate catabolic process

GO:0009205	purine ribonucleoside triphosphate metabolic process
GO:0009206	purine ribonucleoside triphosphate biosynthetic process
GO:0009207	purine ribonucleoside triphosphate catabolic process
GO:0009259	ribonucleotide metabolic process
GO:0009260	ribonucleotide biosynthetic process
GO:0009261	ribonucleotide catabolic process
GO:0009308	amine metabolic process
GO:0009309	amine biosynthetic process
GO:0015672	monovalent inorganic cation transport
GO:0015698	inorganic anion transport
GO:0015833	peptide transport
GO:0015849	organic acid transport
GO:0015980	energy derivation by oxidation of organic compounds
GO:0015985	energy coupled proton transport, down electrochemical gradient
GO:0015986	ATP synthesis coupled proton transport
GO:0015992	proton transport
GO:0016052	carbohydrate catabolic process
GO:0016053	organic acid biosynthetic process
GO:0016054	organic acid catabolic process
GO:0019318	hexose metabolic process
GO:0019320	hexose catabolic process
GO:0019538	protein metabolic process
GO:0019637	organophosphate metabolic process
GO:0019752	carboxylic acid metabolic process
GO:0034220	ion transmembrane transport
GO:0034613	cellular protein localization
GO:0034641	cellular nitrogen compound metabolic process
GO:0034654	nucleobase-containing compound biosynthetic process
GO:0034655	nucleobase-containing compound catabolic process
GO:0042180	cellular ketone metabolic process
GO:0042401	cellular biogenic amine biosynthetic process
GO:0042777	plasma membrane ATP synthesis coupled proton transport
GO:0043436	oxoacid metabolic process
GO:0044106	cellular amine metabolic process

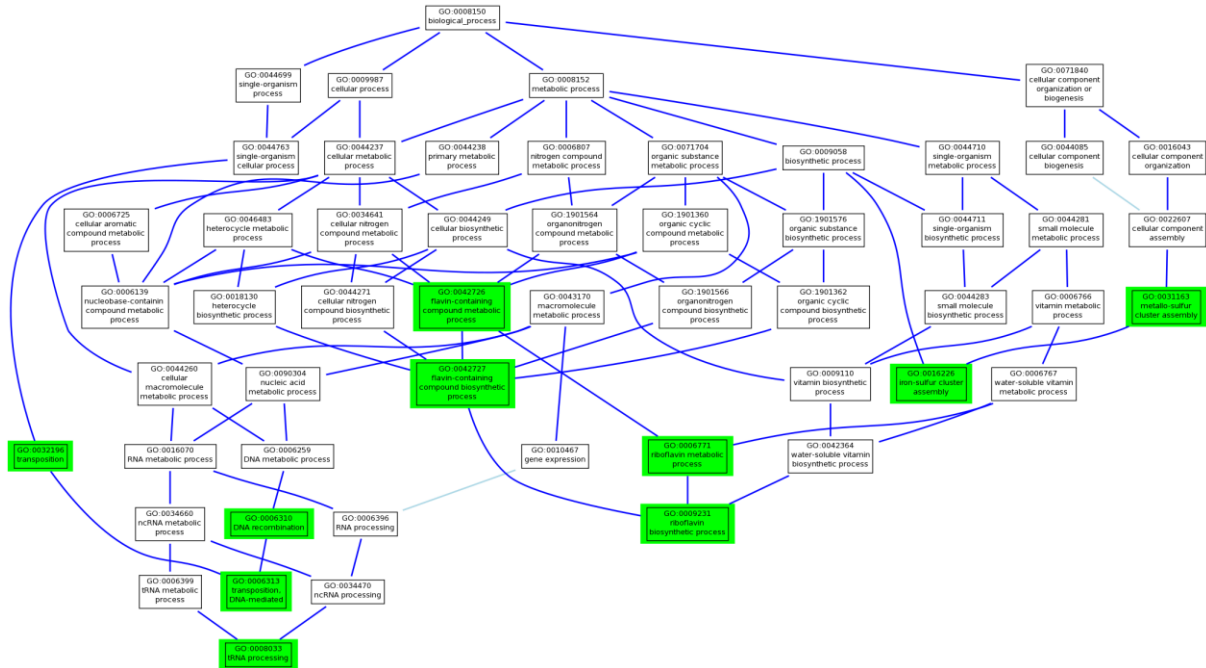
Información suplementaria

GO:0044248	cellular catabolic process
GO:0044249	cellular biosynthetic process
GO:0044270	cellular nitrogen compound catabolic process
GO:0044272	sulfur compound biosynthetic process
GO:0044281	small molecule metabolic process
GO:0044282	small molecule catabolic process
GO:0044283	small molecule biosynthetic process
GO:0045333	cellular respiration
GO:0046034	ATP metabolic process
GO:0046356	acetyl-CoA catabolic process
GO:0046365	monosaccharide catabolic process
GO:0046394	carboxylic acid biosynthetic process
GO:0046395	carboxylic acid catabolic process
GO:0046434	organophosphate catabolic process
GO:0046483	heterocycle metabolic process
GO:0046700	heterocycle catabolic process
GO:0046942	carboxylic acid transport
GO:0051179	localization
GO:0051187	cofactor catabolic process
GO:0051234	establishment of localization
GO:0055085	transmembrane transport
GO:0055086	nucleobase-containing small molecule metabolic process
GO:0055114	oxidation-reduction process
GO:0065008	regulation of biological quality
GO:0070727	cellular macromolecule localization
GO:0071702	organic substance transport
GO:0071704	organic substance metabolic process
GO:0072521	purine-containing compound metabolic process
GO:0072522	purine-containing compound biosynthetic process
GO:0072523	purine-containing compound catabolic process
GO:0090407	organophosphate biosynthetic process
GO:1901135	carbohydrate derivative metabolic process
GO:1901136	carbohydrate derivative catabolic process
GO:1901292	nucleoside phosphate catabolic process

GO:1901293

nucleoside phosphate biosynthetic process

5-3. d) biological process domains downregulated in presence of pE170



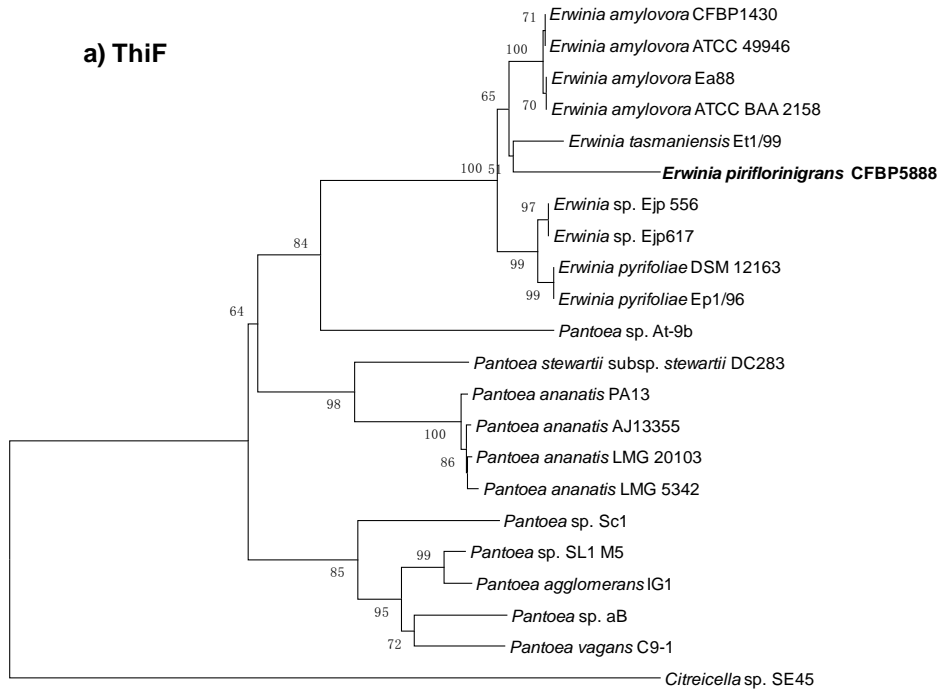
GO_id	GO_description
GO:0006310	DNA recombination
GO:0006313	transposition, DNA-mediated
GO:0006771	riboflavin metabolic process
GO:0008033	tRNA processing
GO:0009231	riboflavin biosynthetic process
GO:0016226	iron-sulfur cluster assembly
GO:0031163	metallo-sulfur cluster assembly
GO:0032196	transposition
GO:0042726	flavin-containing compound metabolic process
GO:0042727	flavin-containing compound biosynthetic process

Información suplementaria capítulo 6

Complete sequence of *Erwinia piriflorinigra*ns plasmids pEPIR37 and pEPIR5 and role of pEPIR37 in pathogen virulence

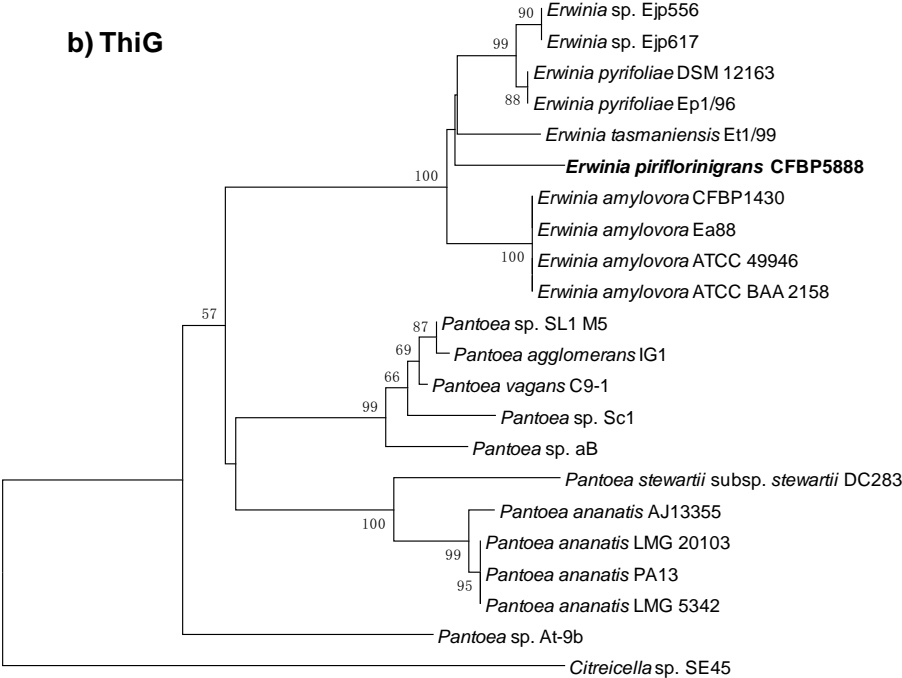
Supplemental Figure 6-1. Phylogenetic trees of amino acid sequences of a) ThiF; b) ThiG; c) ThiS; d) ThiO, constructed using Neighbor-Joining method. Bootstrap percentages (1000 replications) are indicated only for branches that show a value > 50 %. *Citricella* sp. SE45 was used as an outgroup. Branch lengths are proportional to the amino acid distances. All analyses were performed using program MEGA version 5.

a) ThiF



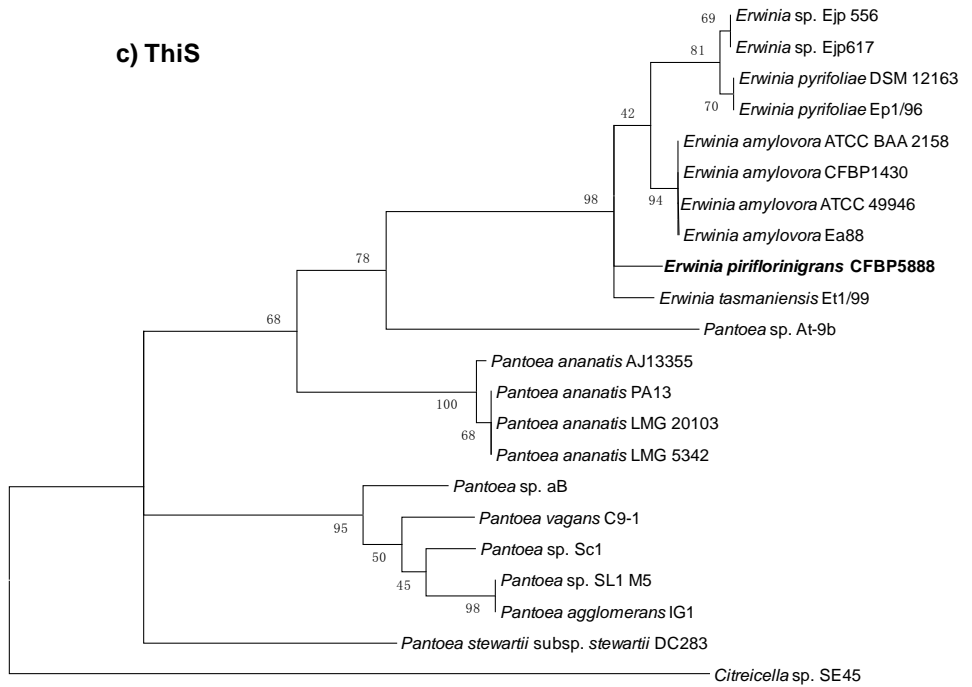
0.02

b) ThiG



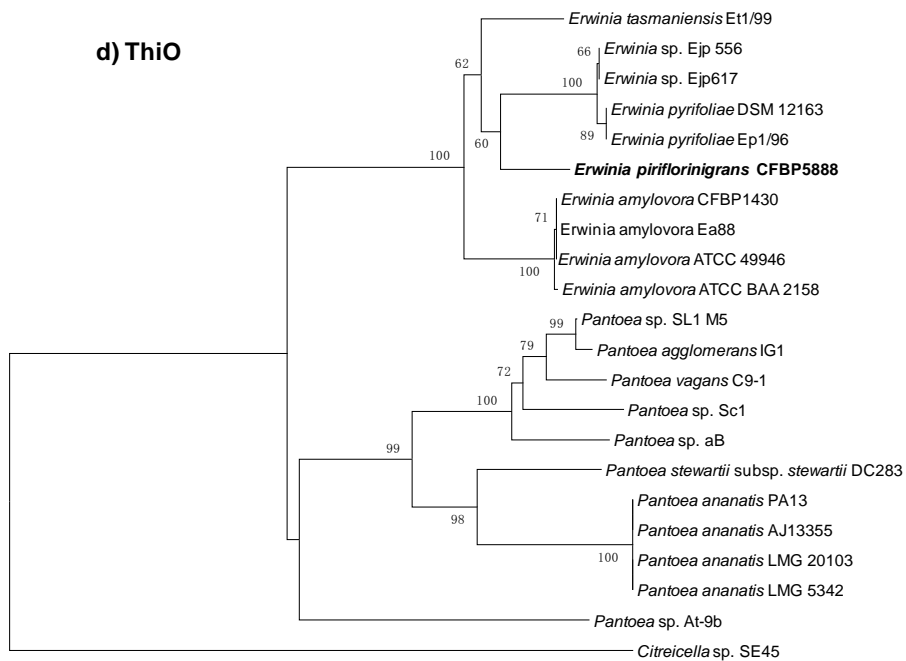
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c) ThiS



0.02

d) ThiO



0.02

