



**ELUCIDATING THE MOLECULAR
BASIS OF COPPER STRESS IN
*Erwinia amylovora***

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ELUCIDATING THE MOLECULAR BASIS OF COPPER STRESS IN *Erwinia amylovora*

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***El talento, en buena medida, es una cuestión
de insistencia***
(For the most part, talent is a matter of persistence)

-Francisco Umbral (1932-2007); Spanish writer



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SUMMARY

Erwinia amylovora, a quarantine organism of the European Union (EU), is the causal agent of fire blight. This disease causes substantial economic losses in all countries where it is present and its control turns out difficult, due to the absence of effective chemical and biological treatments and the ability of persistence and dissemination of *E. amylovora*. Cupric treatments constitute the base of the integrated management of fire blight in the European Union countries, because the antibiotics, although have been proved useful against this disease, are forbidden in the EU for plant treatments.

This thesis, mostly performed in a P2 security lab, is aimed to dilucidate molecular mechanisms implicated in the response of *E. amylovora* to copper sulfate as a stress factor, considering that copper is a well known toxic element for bacterial cells over a certain threshold concentration. The global objective was first addressed with the study of a selection of genes that have been related in other bacterial models with copper stress or with stress in general. The quantification of the *rpoS* gene expression in presence of copper showed that, at least in long-term survival, this gene may be involved in the *E. amylovora* response to copper stress.

Second, a transcriptomic study was performed by microarray after subdue the bacteria to a copper shock treatment. The analysis of the microarray results showed that 44 genes were differentially expressed in presence of this metal. Each one of these genes was studied by gene ontology and, after comparing them with databases

published in NCBI, they were classified in functional categories. The gene expression of twenty-five out of forty-four differentially expressed genes was validated by real-time PCR. In the validation, **copA** gene was expressed more than 19-fold in presence than in absence of copper and, because of that, it was selected together with other seven genes (**soxS**, **yjcE**, **ygcF**, **yhhQ**, **galF**, **arcB**, **EAM_3469**), which also showed an increased expression, to generate mutants of *E. amylovora*. The responses of mutants to copper, and the fact that the wild phenotype was restored in the complemented mutants, has shown the role of **copA**, **soxS**, **yjcE**, **ygcF**, **arcB** and **yhhQ** genes in the *E. amylovora in vitro* survival against copper stress. Besides, the implication of **copA** gene has also been proved *in planta*, in copper treated shoots from pear trees. Finally, all the results obtained along this thesis have allowed to elaborate a putative model of the different genetic mechanisms that seem are involved in the interaction between *E. amylovora* and copper. The most important mechanism seems to be to face up reactive oxygen species (ROS) by the activation of the **soxS** and **yjcE** genes. The activity of these genes is supported by CopA protein, which pumps copper from inside the cell out to the periplasmic space. The activation of **arcB** gene, which allows the change from aerobic metabolism to anaerobic metabolism, would also help *E. amylovora* to reduce ROS.

Taking together, the results of this thesis have allowed an approximation to the genetic basis of *E. amylovora* response to copper stress and they constitute a start point to move forward in

the knowledge of the molecular mechanisms underlying that response.

RESUMEN

Erwinia amylovora, organismo de cuarentena en la Unión Europea (UE), es el agente causal del fuego bacteriano. Esta enfermedad produce grandes pérdidas económicas en todos los países en los que está presente y su control resulta muy difícil, debido a la carencia de tratamientos químicos y biológicos eficaces y a la persistencia y facilidad de diseminación de *E. amylovora*. Los tratamientos con compuestos cúpricos constituyen la base de la gestión integrada del fuego bacteriano en los países de la UE, puesto que el uso de antibióticos, aunque se ha demostrado útil contra esta enfermedad, está prohibido en la UE para el tratamiento de bacteriosis en plantas.

Esta tesis, realizada en su mayoría en un laboratorio de seguridad biológica P2, pretende dilucidar mecanismos moleculares implicados en la respuesta de *E. amylovora* al sulfato de cobre como factor de estrés, ya que este metal es un elemento tóxico para las células bacterianas por encima de una determinada concentración umbral. El objetivo global se abordó, en primer lugar, con el estudio de una selección de genes que se han relacionado en otros modelos bacterianos con el estrés que produce el cobre o con el estrés en general. La cuantificación de la expresión del gen *rpoS* en presencia de cobre mostró que este gen puede estar implicado en la supervivencia a largo plazo de *E. amylovora* para combatir el estrés que produce este metal.

En una segunda aproximación, se realizó un estudio transcriptómico mediante *microarray* tras someter a la bacteria a un breve tratamiento de cobre. El análisis de los resultados del *microarray* reveló que 44 genes se expresaban de forma diferencial en presencia del metal. Cada uno de ellos se estudió mediante *gene ontology* y por comparación con las bases de datos publicadas en el NCBI, y así se clasificaron en categorías funcionales. Las categorías de estrés y transporte fueron las más abundantes, tanto respecto a los genes que aumentaron su expresión tras la aplicación de cobre como a los que la disminuyeron. De los 44 genes que se expresaron de forma diferencial, se validó la expresión de 25 de ellos por PCR en tiempo real. En dicha validación, el gen ***copA*** se expresó 19 veces más en presencia que en ausencia de cobre, por lo que fue seleccionado, junto con siete genes más (***soxS***, ***yjcE***, ***ygcF***, ***yhhQ***, ***galF***, ***arcB***, ***EAM_3469***), en los que el incremento en la expresión fue menos pronunciado, para generar mutantes de *E. amylovora*. La respuesta de los mutantes a la presencia de cobre, y la restauración de fenotipos al complementar las mutaciones generadas, han revelado el papel de los genes ***copA***, ***soxS***, ***yjcE***, ***ygcF***, ***arcB*** y ***yhhQ*** en la supervivencia *in vitro* de *E. amylovora* frente al estrés por cobre. Además, la implicación del gen *copA* se ha demostrado también *in planta* en brotes de peral tratados con cobre. Finalmente, todos los resultados obtenidos han permitido elaborar un posible modelo de los diferentes mecanismos genéticos que parecen estar implicados en la interacción de *E. amylovora* con el cobre. El mecanismo más importante parece ser combatir las especies reactivas del oxígeno

(ERO), mediante la activación de la expresión de los genes *soxS* e *yjcE*. La actividad de estos genes está apoyada, además, por la proteína CopA, que bombea cobre desde el interior celular al espacio periplásmico. La activación del gen *arcB*, que permite el cambio de un metabolismo aerobio a uno anaerobio, también ayudaría a la reducción de las ERO. En definitiva, los resultados han permitido una aproximación al sustrato genético de la respuesta de *E. amylovora* al estrés por cobre, y constituyen un punto de partida para avanzar en el conocimiento de los mecanismos moleculares implicados en dicha respuesta.

RESUM

E. amylovora, organisme de quarantena a la Unió Europea (UE), és l'agent causal del foc bacterià. Aquesta malaltia produeix grans pèrdues econòmiques a tots els països on està present, i el seu control resulta molt difícil, a causa de l'absència de productes químics i biològics eficaços i també per la capacitat de persistència i disseminació d'*E. amylovora*. Els tractaments amb composts cúprics constitueixen la base de la gestió integrada del foc bacterià als països europeus, ja que l'ús d'antibiòtics, tot i que s'ha demostrat eficaç per a combatre aquesta malaltia, està prohibit a la UE per al tractament de bacteriosi en plantes.

Aquesta tesi, realitzada majoritàriament a un laboratori de seguretat biològica P2, pretén dilucidar mecanismes moleculars implicats en la resposta d'*E. amylovora* davant del coure com a factor d'estrés, ja que el coure és un element tòxic per la cèl·lula per damunt d'una determinada concentració umbral. L'objectiu global es va abordar, en primer lloc, amb l'estudi d'una selecció de gens relacionats en altres models bacterians amb l'estrés que produeix el coure, o amb l'estrés en general. La quantificació de l'expressió del gen *rpoS* en presència de coure va mostrar que aquest gen pot estar implicat en la supervivència a llarg termini d'*E. amylovora* per a combatre l'estrés que produeix aquest metall.

En una segona aproximació, es va realitzar un estudi transcriptòmic mitjançant *microarrays* després de sotmetre els bacteris a un breu tractament de coure. L'anàlisi dels resultats dels

microarrays va revelar que 44 gens s'expressen de forma diferencial en presència del metall. Cadascun d'ells es va estudiar mitjançant *gene ontology* i, per comparació amb les bases de dades publicades al NCBI, es van classificar en categories funcionals. Les categories d'estrés i transport van ser les més enriquides, tant en els gens que augmentaren la seua expressió després de l'aplicació de coure com en aquells que la van reduir. Dels 44 gens que s'expressaren de forma diferencial, es va validar l'expressió de 25 d'ells per PCR a temps real. En la validació, el gen ***copA*** es va expressar 19 vegades més en presència que en absència de coure, per aquesta raó va ser seleccionat junt amb set gens més (***soxS***, ***yjcE***, ***ygcF***, ***yhhQ***, ***galF***, ***arcB***, ***EAM_3469***), en els que l'increment de l'expressió va ser menys pronunciada, per a generar mutants d'*E. amylovora*. La resposta dels mutants a la presència de coure, i la restauració dels fenotips originals al complementar les mutacions generades, han revelat el paper dels gens ***copA***, ***soxS***, ***yjcE***, ***ygcF***, ***arcB*** i ***yhhQ*** en la supervivència *in vitro* d'*E. amylovora* davant a l'estrés per coure. A més a més, la implicació del gen *copA* s'ha demostrat també *in planta*, en brots de perera tractats amb coure. Finalment, tots els resultats obtinguts han permès elaborar un possible model dels diferents mecanismes genètics que semblen estar implicats en la interacció d'*E. amylovora* amb el coure. El mecanisme més important sembla ser combatre les espècies reactives de l'oxigen (ERO), mitjançant l'activació de l'expressió dels gens *soxS* i *yjcE*. L'activitat d'aquests gens és recolzada també per l'acció de la proteïna *copA*, que bombeja coure des de l'interior cel·lular a l'espai periplàsmic.

L'activació del gen *arcB*, que permet el canvi d'un metabolisme aerobi a un metabolisme anaerobi, també ajudaria a reduir la producció de les ERO. En conclusió, els resultats han suposat una aproximació al substrat genètic de la resposta d'*E. amylovora* a l'estrés per coure, i constitueixen un punt de partida per avançar en el coneixement dels mecanismes moleculars implicats en aquesta resposta.

1

INTRODUCTION

1.1. Fire blight of *Rosaceae*

1.1. Fire blight of *Rosaceae*

Fire blight is a destructive and highly infectious disease of apple, pear and other plants of the Spiraeoideae subfamily of the *Rosaceae*. It is apparently indigenous to North America, as the disease was first noticed in the late 18th century in New York State and was not reported in any other country until over a century later. Thus, it was detected in New Zealand in 1919, Europe in 1957 and Africa in 1964 (van der Zwet *et al.*, 2012). Today, the most important question is how to stop fire blight spreading all over the world. At the present time, chemicals against fire blight and the cultivation of fire blight resistant hosts seem to be the most helpful plant protection measurements against this disease (Fischer, 2012). Nevertheless it is very important not to forget the role that ornamental plants may play as disease reservoirs (Giayetto and Rossini, 2011).

1.1.1. Symptomatology of fire blight and host range of *Erwinia amylovora*

The symptoms of fire blight are easily recognized and, with few exceptions, are readily distinguished from those of other apple and pear diseases. According to van der Zwet *et al.* (2012), the name fire blight describes the most characteristic symptom of the disease, a blackening of twigs, flowers, and foliage looking like they had been burnt by fire (Fig. 1). The disease is also known by other names, depending on the plant part affected, such as blossom blight, twig blight, fruit blight, and trunk and collar blight (Eastgate, 2000). Often,

1.1. Fire blight of *Rosaceae*

when succulent shoots are affected, they bend forming the characteristic shepherd's crook (van der Zwet *et al.*, 2012) (Fig. 2).

Blossom blight is usually the first symptom of fire blight (van der Zwet *et al.*, 2012). Blossoms first appear water-soaked; they then wilt, shrivel, and turn brownish to black. According to van der Zwet *et al.* (2012), the blight progresses into the peduncle, which also may appear water-soaked and turns dark green and then black. In the most favorable weather conditions for fire blight development, that is, when it is warm and humid, ooze droplets sometimes exude from the peduncle. Tissues affected by fire blight turn black, appear dried and shriveled, but usually remain attached to the tree. This disease has a rapid spread using the midrib and main veins to invade adjacent tissues.

Figure 1. General view of a pear tree with a branch affected by fire blight (Photo by E. Marco-Noales, Spain)



1.1. Fire blight of *Rosaceae*

Figure 2. The characteristic fire blight symptom of shepherd's crook in a pear shoot (Photo by E. Marco-Noales, Spain)



Fruit also becomes blighted at any time but most commonly during the growing season following a severe hailstorm (van der Zwet and Keil, 1972; van der Zwet *et al.*, 1974). The affected part of the fruit is first appearing oily or water-soaked and later, as infection progress, becomes brown to black. Infection spreads directly through lenticels in the skin, through wounds, or from an infected spur into the fruit (Fig. 3) (van der Zwet *et al.*, 2012). Infected fruit, such as infected tissues, remains attached to the spur, with a mummified appearance (Fig. 4), and may even produce ooze favoured by hail damage and warm weather (van der Zwet *et al.*, 2012).

Leaves may become infected after blight bacteria enter directly through stomata, trichomes, and hydathodes, but more often through wounds caused by insects, hail, or wind whipping (van der Zwet *et al.*, 2012). When infection occurs in the leaf blade, a necrotic section usually appears within 48h. This part of the leaf may dry, but infection often spreads through the secondary veins into the midrib, then into the petiole and the supporting stem (Fig. 5) (van der Zwet *et al.*, 2012). There is often a characteristic blackening of the petiole and leaf midrib and ooze drops are usually present. Leaves can also show symptoms when the branch is infected and they can not receive enough nutritive substances, showing the typical necrotic aspect (van der Zwet *et al.*, 2012).

1.1. Fire blight of *Rosaceae*

Figure 3. Fire blight infected pear (Photo by E. Marco-Noales, Spain)



Figure 4. Blossom blight and ooze production (red arrow) in pear (Photo by E. Marco-Noales, Spain)



1.1. Fire blight of *Rosaceae*

Figure 5. Necrotic pear-shoot after fire blight infection (Photo by E. Marco-Noales, Spain)



In fire blight-susceptible hosts, the disease may advance downward from blossoms, shoots, or fruit through the larger scaffold limbs to older branches and eventually into the trunk (van der Zwet *et al.*, 2012), and the advance can also be observed in the subcortex tissue. The disease may cause small or large cankers in limb and trunk tissues (Fig. 6), which consist mainly of dead and collapsed bark cortex and phloem tissues. For the fire blight pathogen, the main overwintering sites are indeterminate cankers formed at the base of blighted shoots and fruit spurs, water sprouts, limbs, or branches or trunk, staying alive usually in the healthy tissue immediately adjacent to the edge of the visible canker (van der Zwet *et al.*, 2012). Often, when conditions are conducive, abundant ooze flows along the bark, accompanying the progress of infection. Flies, using the ooze to feed on, may be also an instrumental vehicle to spread the disease (van der Zwet *et al.*, 2012; Ordax *et al.*, 2015).

Fire blight was first described as a disease of apple and pear (van der Zwet *et al.*, 2012). Since that time knowledge of the disease has grown exponentially, and many more host plants have become known (Table 1). To determine the types and number of host plants susceptible to the disease, most natural blight observations and artificial inoculations of plants were made during the period 1925-1935 (Rosen and Groves, 1928; Rosen, 1929; Pierstorff, 1931; Thomas and Thomas, 1931; Thomas and Parker, 1933; Thomas and Ark, 1934; Parker *et al.*, 1956). Since that time, many more records have been collected. Besides genera *Malus* and *Pyrus*, 129 species in

1.1. Fire blight of *Rosaceae*

37 genera of the *Rosaceae* family have been reported being susceptible to fire blight. Of these genera, six are fruit crops: *Cydonia*, *Eriobotrya*, *Fragaria*, *Mespilus*, *Prunus* and *Rubus*. The remaining 21 genera, with 37 species, are nearly all ornamental host plants and trees (Thomas and Thomas, 1931; Shaw, 1934; Thomas and Ark, 1934; Jock *et al.*, 2000; Vogelsanger *et al.*, 2006; Bastas and Sahin, 2014). Among them, those that are most susceptible and are the cause of more economic losses, and usually exhibit the most severe blight, are species of *Cotoneaster*, *Crataegus*, *Pyracantha*, and *Sorbus* (van der Zwet *et al.*, 2012) (Table 1).

Figure 6. Canker caused by *E. amylovora* in a pear tree. (Photo by E. Marco-Noales, Spain)



1.1. Fire blight of Rosaceae

Table 1. Main genera and species susceptible to fire blight.

| Genus | Main representative fire blight host | Common name |
|--------------------------|--------------------------------------|-----------------|
| Fruit trees | | |
| <i>Malus</i> | <i>Malus communis</i> | Apple |
| <i>Pyrus</i> | <i>Pyrus communis</i> | Pear |
| <i>Cydonia</i> | <i>Cydonia oblonga</i> | Quince |
| <i>Eriobotrya</i> | <i>Eriobotrya japonica</i> | Japanese medlar |
| <i>Mespilus</i> | <i>Mespilus germanica</i> | Medlar |
| <i>Prunus</i> | <i>Prunus salicina</i> | Plum |
| Ornamental plants | | |
| <i>Crataegus</i> | | |
| <i>Chaenomeles</i> | | |
| <i>Cotoneaster</i> | | |
| <i>Photinia</i> | | |
| <i>Pyracantha</i> | | |
| <i>Sorbus</i> | | |
| <i>Stranvaesia</i> | | |
| <i>Rubus</i> | | |

1.1.2. Economic impact

A number of bacterial diseases are of major economic importance, with direct losses due to decreased agricultural production, both in quality and quantity, and indirect losses due to the implementation of expensive control measures (van der Zwet *et al.*, 2012). *E. amylovora* has proved to be extremely destructive to the apple and pear industries in many countries and also to the cultivation of various ornamentals as mentioned before (van der Zwet *et al.*, 2012). In apple and pears, the disease not only destroys the current season's crops but may also lead to loss of branches and whole trees, leading to long-term devastation of orchards and fruit tree nurseries (van der Zwet *et al.*, 2012). If during blooming there

have been favorable weather conditions for the pathogen, blossoms are affected, because of that yield is considerably reduced and in some cases nullified and also the next year's productivity is significantly affected because of the destruction of fruiting spurs (van der Zwet *et al.*, 2012). In susceptible hosts, the infection spreads so rapidly through the tree under favorable conditions. Once trees are infected, they cannot be saved even in spite of drastic and immediate surgery and usually die in a short time after the first visual sign of infection (van der Zwet *et al.*, 2012).

It is difficult to get information about the economic losses caused annually by fire blight but they are quite high (van der Zwet and Keil 1979; van der Zwet *et al.*, 2012). It is necessary to add to the cost of direct production losses, associated costs of the control measures (treatments, intensive vigilance, analysis, infected trees destruction, etc...) and the obligatory varietal structure modification of the fruit growing sector. The fast dissemination of fire blight and the progressive death of susceptible cultivars (especially pear trees) have made to quit its cultivation in some areas of United States of America (USA) and the European Union (EU).

To get an idea of the significance of losses, we can look at some numbers through years. In USA, detailed accounts of the early history of fire blight in California (USA) have been published, suggesting that two-thirds of the pear trees cultivar "Bartlett" were eliminated, at a cost of \$5 million, during the period 1903-1908 (Woods, 1909; Gardner and Ark, 1924; Baker, 1971). More recently, fire blight was

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particularly severe in 1991 in south-western Michigan, where the estimate of losses was \$3.8 million (van der Zwet and Beer, 1995). In Netherland, in 1982, a particularly severe year, the combined economic impact of the disease on nurseries and fruit orchards and the total cost of eradication and control were estimated at \$6 million (Vanneste, 2000). At the end of 1996, in Hungary more than 65,000 trees and shrubs were eradicated by the Ministry of Agriculture at a total cost of \$1.1 million (Vanneste, 2000). The more detailed recent data are those about the epidemy of Michigan (USA) in 2000, having an evaluation of crop costs and trees losses near to 80 million dollars (van der Zwet *et al.*, 2012). In Spain, only in Aragón and in a period between the years 2000 and 2004, costs of the disease (inspections and eradications) were estimated in more than one million euros (Palacio-Bielsa *et al.*, 2012). And these are only some examples of the economical losses caused by fire blight.

This disease also have negative consequences for the producers and the nursery sector (Lanthier, 2011), because the prohibition to export plants and in some cases fruits from countries with *E. amylovora* to countries that are free of the disease.

1.1.3. Global distribution

After its origin in the Hudson valley of New York in 1780, fire blight has moved into most states of the USA. This process took a period of more than hundred years at the same time as the movement of humans and the advance of industrialization (van der

Zwet and Keil, 1979; van der Zwet *et al.*, 2012). By 1925 fire blight had spread across USA border into Canada and Mexico and had moved overseas to New Zealand (van der Zwet *et al.*, 2012). From 1925, the disease spread to 42 additional countries, and up to date it has been reported in a total of 53 countries (Table 2, Fig. 7). According to van der Zwet *et al.* (2012), sometime during the 1950s the fire blight organism was most probably disseminated, via infested bud wood or trees, to two different areas from North America to north-western Europe and to the north-east corner of Africa. Without a doubt, human activity has been very influential in the spread of fire blight and another important factor for the fast spreading of the disease has been the increasing use of susceptible cultivars and rootstocks, as well as high tree densities in nurseries and young planting orchards (Vanneste, 2000).

In Europe, in 1958 the disease was first detected in England (Crosse *et al.*, 1958), suggesting, although never proved, that the introduction was due to contaminated fruit crates, which were recycled in those orchards in Kent where the initial blight symptoms were observed in 1956/57 (Lelliott, 1959). In 1966 it appeared on the mainland of the European continent, in The Netherlands (Netherlands Plant Protection, 1966) and the Baltic coast of Poland (Borecki *et al.*, 1967). It was also detected in Denmark in 1968, in Germany in 1971, and in Belgium and France in 1972 (Vanneste, 2000). Because the first reports of the disease in Denmark (1968) and the northern coast of former West Germany (1971), Belgium (1972)

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and France (1972) all appeared within six years, it has been suggested although not proved, that migratory birds may have been instrumental in the dissemination of the bacterium across the English Channel to the western and northern European coastlines (Meijneke, 1972; van der Zwet, 1994; Billing and Berrie, 2002).

In the early 1960s, fire blight also appeared in Egypt in north-east corner of the African continent (El-Helaly *et al.*, 1964), whereas the first report of fire blight in Israel dates from 1985 (Zutra and Shabi, 1985), and on the island of Cyprus from 1986 (Psallidas and Dimova, 1986). In 1985, fire blight was reported from Turkey and the following year from Crete (Greece) (EPPO, 1987). Once fire blight became established in the Egypt-Cyprus-Israel triangle, it was only a matter of time before the disease appeared in neighbouring countries (Vanneste, 2000). By 1988, the disease was reported in Lebanon (EPPO, 1988), in 1990 in Jordan (Tehabsim *et al.*, 1992), and in 1995 in Iran (Afunian and Rahimian, 1996). After Crete and Turkey, fire blight was found into the mainland areas of Greece and then into Bulgaria (Bobev, 1990), Romania (Baicu *et al.*, 1994), Macedonia (Mitrev, 1996), and Hungary (Hevesi, 1996). Once fire blight was established throughout the southern Balkans, it came as no surprise the observation of symptoms in the southern part of Italy (Cariddi and Piglionica, 1992). In 1995, the first outbreak of fire blight was identified in northern Spain (de la Cruz Blanco, 1996). Since then, the disease has been reported in new countries and now *E. amylovora* has been identified in all the EU members including Finland. In 2000,

host plants of *E. amylovora* in the Royal Botanic Gardens of Melbourne (Australia) were positive for the presence of the pathogen, although it was not isolated from wood samples (Jock *et al.*, 2000). In 2008, fire blight was first reported in Morocco on pear, apple and quince (Fatmi *et al.*, 2008) and in 2013 a report showed that the characterization of a selection of strains from Middle Atlas Mountains of Morocco had notable similarities with a Spanish strain obtained from plants imported from Belgium (Hannou *et al.*, 2013). Recently, in 2012, *E. amylovora* was reported from different host plants and locations in Serbia and Montenegro (Ivanovic *et al.*, 2012) and it is also present in other countries like Tunisia, first reported in 2014 in pear (Rhouma *et al.*, 2014) or Algeria, that had its first characterization of isolates in 2012 (Laala *et al.*, 2012).

The last first report of *E. amylovora* has been on pear trees in Finland (Soukainen *et al.*, 2015), that have a Protected Zone status against fire blight in the EU, and in Kyrgyzstan, Kazakhstan and South Korea (Myung *et al.*, 2016; Zhao and Sundin, 2017).

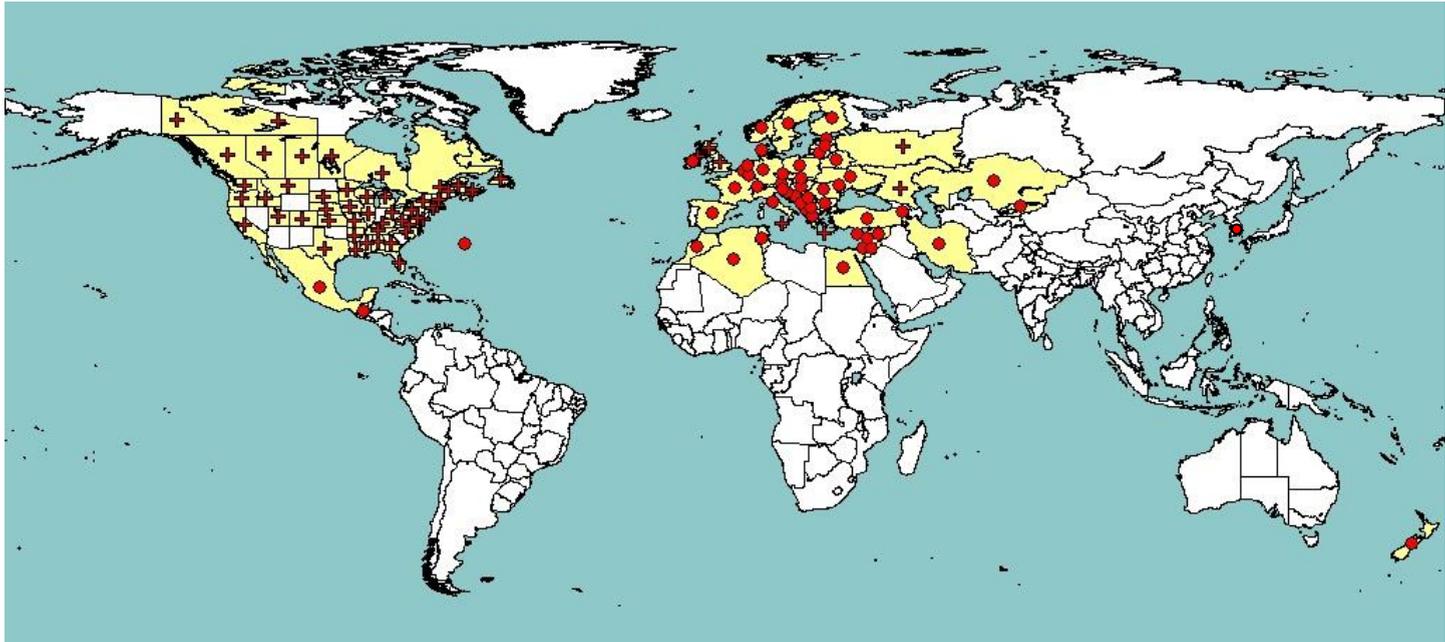
1.1. Fire blight of *Rosaceae*

Table 2. Countries where fire blight is present (EPPO, 2017).

| Area | Countries | |
|----------------|--------------------|---------------|
| Africa | Tunisia | Algeria |
| | Morocco | Egypt |
| America | Bermuda | Mexico |
| | Canada | United States |
| | Guatemala | |
| Asia | Iran | Kazakhstan |
| | Israel | Lebanon |
| | Jordan | Syria |
| | Kyrgyzstan | South Korea |
| Europe | Albania | Lithuania |
| | Armenia | Luxemburg |
| | Austria | FYROM* |
| | Belarus | Moldova |
| | Belgium | Montenegro |
| | Bosnia-Herzegovina | Netherlands |
| | Bulgaria | Norway |
| | Croatia | Poland |
| | Cyprus | Romania |
| | Czech Republic | Rusia |
| | Denmark | Serbia |
| | Estonia | Slovakia |
| | Finland | Slovenia |
| | France | Spain |
| | Germany | Sweden |
| | Greece | Switzerland |
| Hungary | Turkey | |
| Ireland | Ukraine | |
| Italy | United Kingdom | |
| Latvia | | |
| Oceania | New Zealand | |

*Former Yugoslav Republic of Macedonia, mentioned as Macedonia in the EPPO database.

Figure 7. Global distribution of fire blight (EPPO 2017). Red circle indicates countries. Red cross indicates regions or states inside some countries.



1.1. Fire blight of *Rosaceae*

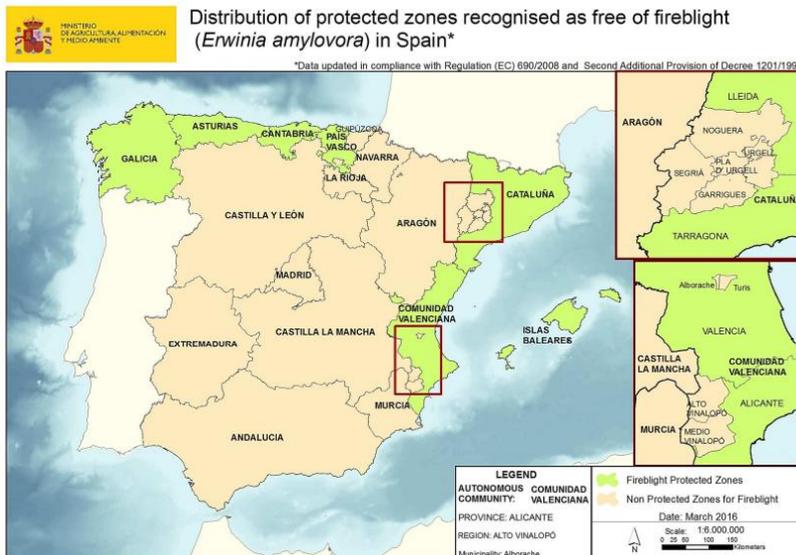
1.1.4. Situation in Spain

In 1984, while fire blight was spreading across north-western Europe, Sampayo and Palazón (1984) published a detailed list of preventive measures to try to keep the disease out of Spain. In spite of this, the first report of fire blight in Spain dates from 1995 (de la Cruz Blanco, 1996), as mentioned before. The disease was observed in August 1995, mainly on cider apple trees, a few kilometers south of the French border, in Guipúzcoa, near the Atlantic coast. In the following two years, fire blight was observed in pear, apple, quince, loquat, and several ornamental hosts (López *et al.*, 1999). The spatial and temporal distribution of the disease foci strongly suggest that infested plant material was responsible for the introduction of the disease (López *et al.*, 2002). Moreover, molecular analysis of a large number of Spanish strains of *E. amylovora* support the hypothesis of several introductions in Spain of infected plant material from different European countries (Donat *et al.*, 2007).

Spain was recognized as “protected zone” (PZ) for fire blight in 2000 (DOCE 2000). This is the most important protection considered in the European Union Phytosanitary legislation. Countries or places where a pathogen is not established and perform surveys to try to find it may obtain the status of PZ, and introduction of vegetal material and its movement are subjected to high severe quarantine requirements (Real Decreto 58/2005).

In 2011 and the following years, there were new fire blight outbreaks in many of the pome fruit production zones of the national territory. Even though measures to eradicate the disease or delay its establishment have been adopted in some cases, the situation today in 2017 is not encouraging. It is considered that the disease is established in the following areas that have lost, therefore, the PZ status: Castilla y León, Extremadura, La Rioja, Castilla La Mancha, Murcia, Navarra, Guipúzcoa (País Vasco), Aragón, Murcia and Valencia (green spots in Fig. 8). Recently, several regions of a city of Catalonia named Lleida lost its PZ status as well (official communication).

Figure 8. Current distribution of fire blight in Spain (www.magrama.gob.es 2017).



1.1. Fire blight of *Rosaceae*

1.1.5. Fire blight epidemiology and control

1.1.5.1. Fire blight cycle

Fire blight bacteria hibernate in the bark at the edge of cankers formed during previous growing seasons. As weather becomes warm in the spring and temperature reach 18-30°C the bacteria multiply and ooze to the surface in sticky droplets (Palacio-Bielsa and Cambra, 2009). Under low relative humidity the bacteria can survive in the dry exudates for over a year (Rosen, 1938). Relatively few cankers survive winter, becoming active and producing bacteria in the spring. However, a single active canker will produce millions of bacteria, enough to infect an entire orchard. Cankers produce bacteria in droplets of ooze that are transferred to flowers by splashing rain or by insects, mostly bees, flies and ants (Fig. 9).

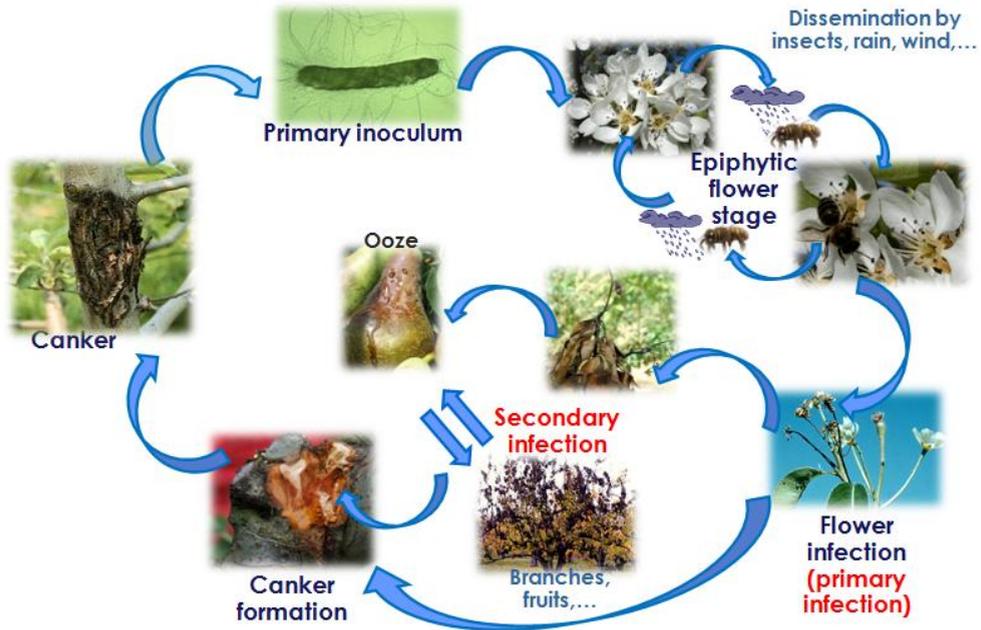
According to van der Zwet *et al.* (2012), once on the flower stigmas, the bacteria can grow epiphytically under favorable circumstances and reaching 10^6 - 10^7 colony-forming units (CFU) per healthy flower. The presence of high densities of epiphytic bacteria on healthy flowers facilitates an efficient movement of the bacteria from flower to flower by rain or by any insect that visits the flowers (Fig. 9). Blossom infection occurs when the bacteria are washed by rain to natural openings at the flower base. These openings located in the hypanthium are specialized stomata, termed nectar glands. Blossoms wilt and die in about 1-2 weeks after infection occurs, and the bacteria that ooze from them provide inoculum for secondary

spread to other flowers and to young succulent shoots (Fig. 9). The bacteria are moved to shoots by insects and rain. Bacteria may enter the leaves through stomata and water pores (hydathodes) but usually they enter through wounds made by insects, hailstorms and other mechanical damage. As the season advances, shoots become progressively less susceptible to new infections as their growth slows and stops. Bacterial progression through woody tissues also slows and cankers are formed (Fig. 9), where some bacteria overwinter and renew the disease cycle the following spring.

Hibernating bacteria occasionally move internally from canker margins to nearby shoots, which they infect in a systemic way. If bacteria reach the phloem, they are carried upward to the tip of the twig and to the leaves. Invasion of large twigs and branches is restricted primarily to the cortex.

1.1. Fire blight of Rosaceae

Figure 9. Fire blight cycle.



(Modified from APS (www.aspn.net.org) by Ordax, 2008)

1.1.5.2. Preventive and cultural measures

To prevent fire blight, it is basic not to illegally introduce vegetal material of *Rosaceae* from infected areas or countries. The plant purchase must be made exclusively from authorized nurseries, requiring the phytosanitary passport which guarantees the requirement compliance established in the European legislation (BOE 2005; DOCE 2000). The elimination of symptomatic or infected plants laid down in the Real Decreto 1201/99 (BOE 1999) as eradication measures has been very effective (Palacio-Bielsa *et al.*, 2012) and it is necessary to carry out the eradication measures as faster as possible to reduce inoculum and avoid *E. amylovora* dissemination.

Control of fire blight will be only possible if disease symptoms are detected prematurely in the orchard. It has been observed that fire blight progresses faster when more symptomatic plant material is present in an orchard or closer to it. Therefore, it is very important to examine the orchard during and after bloom, rain, storms and above all after hail. Moreover, June, July and September are in general the most critical months for symptom detection since trees have an active vegetative growth and more susceptible material is available.

All factors that favor plant susceptibility and/or pathogen spreading should be controlled. Pruning is recommended only when trees are hibernating to remove all suspicious cankers, favoring the maximum ventilation of the orchard, combined with disinfecting frequently all the tools used, and burning the plant debris. Other recommended practices include the removing of secondary bloom, a

1.1. Fire blight of *Rosaceae*

limited use of nitrogen fertilizer to prevent vegetative overgrowth, and avoidance of spraying watering.

1.1.5.3. Chemical control

Chemicals are applied to reduce the number of *E. amylovora* cells or, at least, inhibit their multiplication. To achieve these effects, it is necessary to eliminate sources of inoculum, such as overwintering cankers on fruit trees or alternate hosts, or to protect potential invasion sites, such as blossoms, shoots, leaves, or fruit, especially after wounding (Palacio-Bielsa and Cambra, 2009). In this sense, to be effective against this pathogen, any bactericide should be applied during the three distinct periods of the host: when the tree or plant is dormant, in bloom, and during the postbloom period. To prevent the development of new blossom infections, chemical agents are used during the dormant period and before budbreak, since *E. amylovora* may overwinter in cankers. During bloom the objective is to decrease pathogen populations and blossom necrosis and in the postbloom to avoid shoot infections (van der Zwet *et al.*, 2012).

Two groups of chemical agents, antibiotics and copper compounds, have played the most important role in controlling fire blight of apples and pears since the 1930s. In the early 1950s, discovery and application of antibiotics was the most important development in fire blight control, due to their successful use in treating human diseases (van der Zwet *et al.*, 2012). Moreover, for

fire blight control, in general, streptomycin gave better result than copper, and normally it caused no fruit russet. This lack of injury probably appealed to many growers even though several treatments are necessary and the antibiotic cost is considerably higher than that of copper (van der Zwet *et al.*, 2012). In early antibiotic research, a widely used formulation contained 15% streptomycin sulfate and 1.5% oxytetracycline (Terramycin) (Dunegan *et al.*, 1954; Higdon, 1954; Ark, 1958; Keil, 1963). Laboratory studies showed that *E. amylovora* developed resistance far more slowly with this combination than with streptomycin alone (English and van Halsema, 1954). Then, it was used commercially for many years, until oxytetracycline was removed during the 1960s, apparently because the combination showed no advantage when used in the field (van der Zwet *et al.*, 2012). According to Stockwell and Duffy (2013), streptomycin is applied in several countries of the United States to control fire blight before infection have place, in springtime, because after infection antibiotics are ineffective. Antibiotics have been indispensable for crop protection in USA for more than 50 years without reports of adverse effects on human health or persistent impacts on the environment, since antibiotics are active on plants for less than a week (Stockwell and Duffy, 2013). A study with streptomycin to control fire blight in experimental orchards of Europe was recently carried out by Walsh *et al.* (2014). They demonstrated that there was not abundance of streptomycin or tetracycline resistant genes in the bacteria neither a negative impact on the bacterial community after three streptomycin treatments.

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Numerous copper compounds have given variable results in the control of fire blight, ranging from poor to excellent (Rosen, 1932, 1934; Pinckard *et al.*, 1936; Sherbakof and Andes, 1939; Veerkamp, 1945; Gardiner, 1951-1957; Agrios, 1968; Hickey *et al.*, 1998; Bastas *et al.*, 2010). Copper sulfate mixed with lime (Bordeaux mixture) has been used more often than any other form of copper. It is applied only during the dormant period and the bloom period, to avoid fruit russet that is directly proportionate to the copper content of the formulation. Probably because of this fruit injury, copper is not used more often.

A wide and renew overview about the use of copper in the management of bacterial diseases of fruit trees will be explained in section 1.2.

1.1.5.4. Biological control

The possibility of biological control of fire blight, using different microorganisms, has been investigated, discussed, and reviewed for more than four decades (Schroth *et al.*, 1974; Aldwinckle and Beer, 1979; Pusey, 2002; Stockwell *et al.*, 2002; Ozaktah and Bora, 2004; Cabrefiga *et al.*, 2014). At first, mainly antagonistic bacteria were tested as potential biological control agents, but since then several natural compounds (plant extracts and etheric oils) have also been assayed against the fire blight pathogen (Briffaerts *et al.*, 1996; Zeller and Laux, 2006; Farkas *et al.*, 2012). Increased research on the biocontrol of this disease has been motivated by the development of

resistance in *E. amylovora* to the antibiotic streptomycin, and the restrictions to the use of several chemicals and antibiotics in countries of the EU and other states (Zeller and Laux, 2001, 2002a, 2002b; Montesinos *et al.*, 2009; BOE 2012).

The surface of the stigma, located on top of the floral pistil, is the site where bacterial bio-control agents must interact with and successfully antagonize *E. amylovora* (Hattingh *et al.*, 1986; Thomson, 1986; Wilson *et al.*, 1989; Vanneste, 1995; Farkas *et al.*, 2012). In fact, biological control of fire blight is successful when a bacterial antagonist establishes and develops a large population on the stigmatic surface prior to the establishment of *E. amylovora* (Wilson *et al.*, 1992; Johnson *et al.*, 1993; Wilson and Lindow, 1993). These populations, through a combination of mechanisms, can suppress the establishment and epiphytic growth of the pathogen (Farkas *et al.*, 2012). Decrease of *E. amylovora* population on stigmatic surfaces reduces the probability of floral infection and spread of the pathogen to other blossoms. Effective biological control requires colonization of the most stigmas of the flowers in the orchard by the bacterial antagonists (Johnson *et al.*, 1993; Lindow *et al.*, 1996), and requires a larger population of them on these surfaces (Farkas *et al.*, 2012). Fire blight is a good candidate for biological control because the bacterial antagonists need to persist on the nutrient-rich, stigmatic surfaces for only about one week to suppress blossom infection effectively (Cabrefiga *et al.*, 2007; Cabrefiga *et al.*, 2011; Farkas *et al.*, 2012; Cabrefiga and Montesinos, 2017).

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1.1.5.5. Cultivar susceptibility and genetic control

A very limited number of apple and pear cultivars are responsible for a large proportion of annual world production (Vanneste, 2000). To retain a cultivar with fruiting desirable characteristics and to introduce disease-resistance genes by conventional breeding methods is virtually impossible, because of apple and pear heterozygosity, long generation time and self-incompatibility. All this make back-cross programs of several generation prohibitively long term and expensive (Vanneste, 2000). The use of biotechnology can now overcome these handicaps by introducing resistance genes directly into current valuable commercial cultivars and thereby transforming them into resistant forms of the same cultivars (van der Zwet *et al.*, 2012).

Targeting gene expression to fire blight-susceptible tissues or during specific developmental stages could be advantageous in providing resistance where and when needed (Vanneste, 2000). Exogenous application of plant resistance inducers (PRIs) able to activate plant defenses is the most novel approach for new integrated pest management practices (de Bernonville *et al.*, 2014).

1.1.5.6. Integrated control in Spain and the European Union (EU)

Optimal control of fire blight seems to be only achieved by eliminating all diseased plant material and by reducing host susceptibility with available cultural measures (Deckers and Porreya, 1987; Deckers *et al.*, 1987). The disease control must be considered

as an integrated strategy, selecting the lower sensitivity varieties and applying the prophylactic measures, the cultural techniques and necessary treatments to reduce the inoculum amount (Johnson and Stockwell, 1998; Palacio-Bielsa and Cambra, 2009; Johnson and Temple, 2013; Smith, 2014).

Genetic improvement of apple and pear trees to obtain resistant varieties with commercial interest did not provide the expected results despite that it has been obtained significant advances (van der Zwet *et al.*, 2012). Also, transgenic and cisgenic varieties of apple and pear trees with significant levels of fire blight resistance have been obtained (Litz and Padilla, 2012), but not authorized and commercialized in EU yet.

A combination of all available control measures (sanitation, selection of resistant varieties, and cultural practices) is the preferred way to keep the disease to a minimum.

1.1.5.7. Legislation

In Spain, the regulation against fire blight started with a Ministry Order in 1975 about the prohibition of host plants import from contaminated countries. In 1999, it was published the Real Decreto 1201/1999 (BOE 1999) on the National Program for eradication and control of fire blight, that was based on the European legislation but adapted to the Spanish situation. This law was modified in 2005 (Real Decreto 1512/2005), 2010 (Real Decreto 246/2010) and 2011 (Real Decreto 1786/2011). The following rules were determined as

1.1. Fire blight of *Rosaceae*

obligatory in disease free zones (PZ): a) official declaration of the disease, b) destruction of the affected vegetal material by particular people and public entities, and c) systematic research by the Autonomous Communities. Obligatory phytosanitary measures were established in this Real Decreto in zones where the disease was present, to slow down its propagation, as well as the prohibition to plant host ornamental species in public road and gardens in the hazard zones decided by each Autonomous Community (Montesinos *et al.*, 1999).

The European Economic Community (EEC) published in 1977 the Directive 77/93/CEE about the circulation of damaging organisms of plants where *E. amylovora* appeared as a quarantine organism (present in some countries of the EEC but not in all of them). Restrictions were imposed to the distribution of vegetal material coming from epidemic zones, which were considered as Protected Zones (PZ) for this disease, being Spain among them. The EEC member countries not yet affected by fire blight established a supervision network for maintaining these areas free of the disease. According to that, all the vegetal material susceptible to the disease must be commercialized under the phytosanitary passport with the initials PZ (Montesinos *et al.*, 1999).

In 2000, the Directive 2000/29/CE was published in the Official Diary of the European Economic Communities. The Directive is relative to the protection measures against the introduction in the Community of damaging organisms for the vegetables or vegetal

products and against their dissemination inside the Community. In Spain, the Plant Health law (Law 43/2002, of November 20th of 2002) regulates thoroughly general aspects relative to the prevention and fight against the different pests and diseases. The Autonomous Communities, basing on the Real Decreto 1201/1999 and 1512/2005, have adopted in Spain other complementary measures to reinforce the effects that are being pursued (Palacio-Bielsa and Cambra, 2009).

1.2. Copper and field application

1.2. Copper and field application

Bacterial infections of plants are some of the most difficult diseases to control because there is still little effective chemistry available (Civerolo, 1982; López *et al.* 2003; Janse, 2004, 2005). Antimicrobials for prophylactic treatment of bacterial diseases of plants are limited in availability, use, and efficacy, and therapeutic use is largely ineffective (Vidaver, 2002). One type of the products more frequently used are copper formulations that have been extensively used in agriculture since more than 200 years ago, with a significant track history of relative success, and nowadays the utilisation of different copper-based bactericides is a piece in the whole of controlling bacterial diseases.

1.2.1. The history of the use of copper

Copper is considered a unique metal known for its antimicrobial properties throughout millennia. It was the first metal used by humans, probably because of its metallic native form (Elguindi *et al.*, 2011). Thus, use of copper has been reported as far as in ancient Chinese civilization around 2500 B.C (Yu *et al.*, 1995); in ancient Egypt to sterilize drinking water and chest wounds (Dollwet and Sorenson 1985); by Greeks in 400 B.C., for treating pulmonary diseases and purifying drinking water (Dollwet and Sorenson, 1985); and by ancient Aztecs, in Mexico, for treating skin conditions. And during the circa 1850 cholera epidemic in Paris, copper workers were found to be immune to it (Michels *et al.*, 2005). Throughout history, men have exploited the antimicrobial attributes of copper.

As early as the beginning of 18th century, Prevost (1807) had demonstrated that bunt of wheat, caused by *Tilletia caries*, could be controlled to some degree by copper sulfate. He provided the first scientific evidence that this compound would kill fungal structures. Previously, Schulthess (1761) had observed that copper sulfate could provide some control of bunt but did not know the mode of action (van Zweiten *et al.*, 2007). It was in the latter half of the 19th century that chemical disease control really started to develop. Because of its high phytotoxicity, copper was not used as a foliar pesticide until 1885 (Millardet, 1885), when French scientist Millardet accidentally observed that a mixture of copper sulfate, lime and water, that had been applied to grapevines near roadways in order to discourage thieves from stealing the grapes, was not phytotoxic but exhibited a fungicidal action against *Plasmopara viticola* (Russell, 2005). His winter's spray formulation was then the fungicide of choice in USA and was named "Bordeaux mixture" (Borkow and Gabbay, 2005). It had represented the first large scale use of a fungicide in Europe and America (Dunegan and Doolittle, 1953; Floyd, 1991). It later proved also to be a good bactericide; in fact, it has been used widely against bacterial diseases on different crops (Jiang *et al.*, 2008). Already in one of the first publications on bacterial diseases of plants (Smith, 1920), it is reported that the number of infections in walnut blight in California was reduced by 50% using Bordeaux mixture, and that in Italy this mixture was recommended for olive trees following hailstorms to protect them against tuberculose.

1.2. Copper and field application

1.2.2. Copper formulations and mode of action

Copper bactericides work by coating the leaf surface with minute particles of copper which then react with acid and moisture on the surface to release copper ions that kill bacteria and prevent fungal spores from germinating. Over time the protective coating provided by copper bactericides is diluted by the action of rain, wind and the growth of the crop. Interestingly, despite the long-term use of copper as antimicrobial, its precise mode of action has not been fully elucidated (Dupont *et al.*, 2011; Fones and Preston, 2012).

The antimicrobial activity of copper is due to the soluble fraction of Cu^{++} or Cu^+ metal. Copper compounds used in agriculture are generally composed of the active ingredient Cu^{++} combined with an anionic component to form inorganic or organic salts, or quelates. The other ingredients improve water miscibility, adherence and spreadability to plant surfaces, and other additional properties.

Copper, although not target specific, kills all living cells. However, plants are less susceptible to copper toxicity than microorganisms, due to tissue barriers and structures, such as leaf cuticle, fruit wax or trunk suber, which avoid the entry of copper. The role of copper for control of bacterial plant diseases is primarily based on its direct action on the pathogenic cells. Copper can interact with many vital cell structures due to the reactive nature of Cu^{++} with anionic cell components. Moreover, increased intracellular copper levels may induce the synthesis of reactive oxygen species (ROS), causing an additional oxidative damage to lipids, proteins and DNA in

the internal structures of the bacterial cell. The result of these actions is a loss of the functionality of the bacterial cell envelope by membrane disruption, a protein-enzyme denaturation, inhibition of DNA replication, transcription, and protein synthesis. Therefore, copper exhibits a bactericidal activity. However, there is a second mechanism of action of copper, which is mediated by the host. It consists on a stress response induced by copper in the plant, with overproduction of ROS and increased levels of antioxidant enzymes (SOD, GPX, APX) (Ros-Barceló, 2006; Yadav *et al.*, 2010).

1.2.3. Field applications in the EU

As described before, copper compounds through agriculture history have been used for vegetable and fruit crops to restrict the spread of plant pathogens, both bacteria and fungi. They have constituted for decades the main protection instrument against plant diseases. However, today there is a strong tendency to reduce the use of copper and copper-based products to minimize their environmental impact. Development of copper resistant bacterial strains and accumulation of copper in soil are detrimental effects derived from use of this chemical.

In the EU, plant protection products containing copper must fulfil the safety requirements laid down in BOE (2012). According to it, several active substances were evaluated, and the following products were included in the Annex I of the Directive: copper hydroxide, copper oxychloride, copper oxide, Bordeaux mixture, and tribasic copper sulfate. The phytosanitary products that contain these

1.2. Copper and field application

substances can be therefore authorized in the Member States. Nevertheless, due to the fact that the risk assessment of copper compounds revealed eco-toxicological concerns, a restriction on the inclusion period is deemed necessary to allow Member States to review after a shorter period copper containing plant protection products already on the market.

Chemical treatments are very useful and contribute to reduce losses in fruit production due to bacterial infections. However, no complete protection can be expected by chemical treatments alone, but they should be considered as a part of an integrated control system aiming to decrease disease incidence. The objective for a sustainable disease management is to improve the efficiency of disease control while reducing the amount of copper application to an environmentally acceptable level. An attempt to adequately control diseases and limit unnecessary chemical applications is to select the copper compounds to be used, applying them in a strategic way, timed according to pathogen activity, and test alternative products and farming practices.

1.3. Copper, friend and foe for bacterial cells: targets for copper action and strategies

Copper is a transition metal utilized by bacteria in many cellular processes. However, while copper plays critical roles it can be toxic when levels are beyond cellular needs. Bacterial cells may prevent copper toxicity in part by keeping copper compartmentalized in the cell periphery. Trace copper is sufficient for survival as there are few Cu-dependent enzymes and they are most often localized within the cell periphery. The most widespread Cu-dependent enzyme in bacteria is cytochrome *c* oxidase (Cox), located in the cell membrane (Festa and Thiele, 2012).

Our current understanding of the molecular mode of action of copper ions against bacteria is still limited and the microorganism more studied is *Escherichia coli*. Rensing and Grass (2003) demonstrated that the copper translocating P-type ATPase, CopA, was the central component in *E. coli* for copper homeostasis, responsible for removing excess copper from the cytoplasm. Macomber and Imlay (2009) showed that copper toxicity involved the action of ROS and that the primary target of copper in *E. coli* was the iron-sulfur cluster of proteins. A connection between copper and cell integrity was discovered when Espirito Santo *et al.* (2011) challenged *E. coli* to dry copper surfaces. Cells suffered extensive membrane damage within minutes of exposure to dry copper.

1.3. Copper friend and foe for bacterial cells

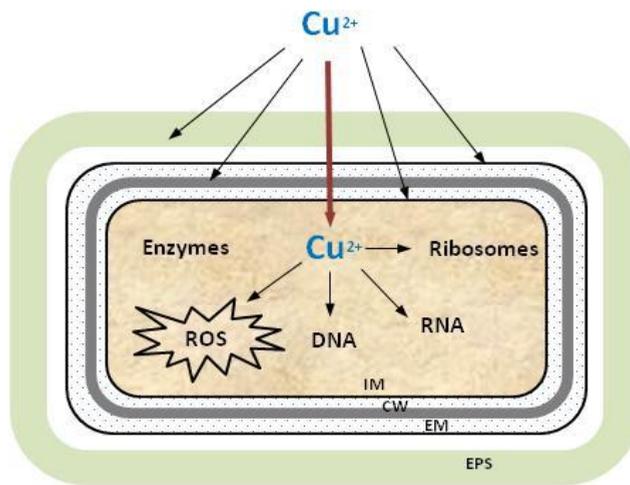
1.3.1. Copper and generation of reactive oxygen species

Metals can act directly as antimicrobial toxins, and, consequently, they can be used directly to limit pathogen growth (Fones and Preston, 2012). Besides that, the ability of copper to undergo redox changes between Cu^+ and Cu^{++} , although makes it essential for life by its indispensable role in various enzymatic processes, also makes it dangerous since it is able to elicit the production of ROS. Aerobically, copper readily catalyzes reactions that result in the production of hydroxyl radicals through the Fenton-like and Haber-Weiss reactions ($\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{HO}^\bullet + \text{HO}^- + \text{Cu}^{++}$; $\text{O}_2^{\bullet-} + \text{Cu}^{++} \rightarrow \text{Cu}^+ + \text{O}_2$) (Halliwell and Gutteridge, 1984, 1990; Rensing and Franke-McDevitt, 2013). The highly reactive oxygen intermediates are responsible for lipid peroxidation, oxidation of proteins and damage to nucleic acids (Halliwell and Gutteridge, 1984; Imlay and Linn, 1988; Stadtman, 1992). The effect of Cu^+ on generation of ROS can also be indirect, since free copper ions are able to oxidize sulfhydryl-groups, such as cysteine in proteins or the cellular redox-buffer glutathione (Stojs and Bagchi, 1995; Helbig *et al.*, 2008). Moreover, Cu^+ can attack and destroy iron-sulfur clusters releasing iron which can in turn cause oxidative damage through iron-based Fenton chemistry (Keyser and Imlay, 1996; Rensing and Franke-McDevitt, 2013).

It was believed that copper ion toxicity in bacteria was only mediated by oxidative DNA damage. However, under anaerobic conditions copper ions reduce the growth rate of *E. coli* even more strongly than under aerobic conditions (Outten *et al.*, 2001).

Figure 10 shows a summary of the multitarget action of copper in a Gram-negative bacterial cell.

Figure 10. The multitarget action of copper in a Gram-negative bacterial cell. IM: inner membrane, CW: cell wall, EM: external membrane, EPS: exopolysaccharide (Courtesy of Prof. E. Montesinos, University of Girona, Spain).



1.3.2. Copper uptake and homeostasis

Copper compounds have been used to control bacterial diseases since long time ago and consequently pathogens have been under copper pressure all that time. They have developed detoxification strategies and copper-resistance mechanisms to face up to the bactericide properties of high level copper concentrations (Rensing and Grass, 2003; Teitzel and Parsek, 2003; Waldron and Robinson, 2009; Fones and Preston, 2012).

1.3. Copper friend and foe for bacterial cells

Copper homeostasis must be carefully regulated in pathogens to allow enough metal to be available for protein assembly but below damage induction threshold. Within the cytoplasm, copper is sequestered by various proteins and molecules, including specific copper chaperones, to prevent cellular damage (Rae *et al.*, 1999). Hernández-Montes *et al.* (2012) reported that the bacteria of the plant pathogenic genera such as *Xanthomonas*, *Pantoea*, *Erwinia*, and *Pseudomonas*, have proteins involved in copper homeostasis (Table 3). They found that CopA was the most abundant protein with a physiological role as an internal membrane ATPase. It was identified in the chromosomes of 70 genera, with few exceptions, such as seven *Xanthomonas* and *Xylella* spp. CopA is an inner membrane pump to extrude copper (Cu⁺) from the cytoplasm to the periplasm and usually works with an external membrane pump that export copper from the periplasm to the extracellular matrix (CusC). Thus, pathogens can control the cell copper levels to avoid damages.

Table 3. Copper homeostasis proteins presence in four plant pathogenic genera (Hernández-Montes *et al.*, 2012). Only proteins with highest abundance within a genus are shown.

| Genera | Proteins for copper homeostasis |
|--------------------|---------------------------------|
| <i>Erwinia</i> | CueO, CopA, CusC |
| <i>Pantoea</i> | YebZ, CueO, CopA, CusC |
| <i>Pseudomonas</i> | CopA, CusC |
| <i>Xanthomonas</i> | CusA, CusC |

Relevance of copA is also supported by Rademacher and Masepohl (2012), who described that upon copper addition all bacteria examined induced ATPase expression, even though different

species utilize structural and functionally different regulators to control ATPase gene transcription. As for the ATPase gene regulation and as a general rule, Gram-negative bacteria activate ATPase gene transcription with increasing copper concentrations, mostly by CueR-like one-component regulator or by CusRS-like two-component systems (Rademacher and Masepohl, 2012). Yamamoto and Ishihama (2005) described that CueR from *E. coli* is the main system regulating copper-response under aerobic conditions. On the other hand, the CusSR system may play an important role in copper tolerance under anaerobic conditions because self-phosphorylation of CusS is activated in that situation.

Other partners playing an important role in copper homeostasis are the multicopper oxidase proteins (MCOs). The most outstanding toxicity of copper is given when Cu^+ is in the cytoplasm, whereas Cu^{++} is less toxic for the cell. In fact, many bacteria synthesize MCOs as additional copper defence determinants. Comparative analysis showed that MCOs can be found in approximately 13% of the bacterial genomes (Ridge *et al.*, 2008). Although the sequence homology among MCOs is low, amino acid alignments show that the overall structures and copper-binding motifs are highly conserved (Reiss *et al.*, 2013). Based on the type of substrates, several types of MCOs can be differentiated (Sakurai and Kataoka, 2007). MCO expression in Gram-negative bacteria is activated by either CueR or CusRS homologues as we mentioned above for copper-ATPase proteins. Although MCOs function occurs in the periplasm, there is no apparent preference for CusRS systems, which sense periplasmic

1.3. Copper friend and foe for bacterial cells

copper concentrations, over CueR sensors, which respond to the cytoplasmic copper status (Rademacher and Masepohl, 2012).

1.3.3. Multicomponent copper efflux systems

Resistance-nodulation-cell division (RND) superfamily efflux systems are responsible for the active transport of toxic compounds from the Gram-negative bacterial cell. Besides copper-ATPases and MCOs, many Gram-negative bacteria synthesize RND-type multicomponent copper efflux systems (Rademacher and Masepohl, 2012). These pumps typically assemble as tripartite complexes, spanning the inner and outer membranes of the cell envelope. These complexes consist of an inner-membrane substrate-binding transporter (or pump), a periplasmic membrane fusion protein (or adaptor), and an outer membrane-anchored channel (Delmar *et al.*, 2013). The assembled tripartite efflux complex is responsible for removing toxic compound out of the cell, and mediating bacterial resistance to these noxious chemicals (Delmar *et al.*, 2013). Taking *E. coli* as a model (Outten *et al.*, 2001; Rensing and Grass, 2003), we know that it harbours the divergently transcribed *cusCFBA* and *cusRS* operons, which encode an RND-type copper efflux system and a copper-responsive two-component system, respectively. Membrane-bound CusS is thought to sense the periplasmic copper status. Upon binding of Cu^+ , CusS is expected to autophosphorylate and donates the phosphoryl group to CusR, which in turn, activates transcription of the *cusCFBA* and *cusRS* operons (Outten *et al.*, 2001; Franke *et al.*, 2003; Rensing & Grass, 2003; Gudipaty *et al.*, 2012). Primarily, these

RND systems excrete copper from the periplasm and, maybe less important for copper export, from the cytoplasm. As it would be expected for bacteria lacking an outer membrane, copper induced RND systems are absent in Gram-positive species (Rademacher and Masepohl, 2012).

1.3.4. Biosynthesis of extracellular polymeric substances (EPS)

A common feature of many bacteria is the biosynthesis of EPS that can offer a protective barrier under environmental stress conditions. These extracellular polymeric substances may protect cells by binding toxic metal ions (Geddie and Suhterland, 1993) and, in fact, bacteria can increase their EPS production in the presence of toxic metals as a defense mechanism (Decho, 1994). Commonly, exopolysaccharides are composed of glucose, fructose, manose, pyruvate and fucose, as well as mannuronic and glucuronic-acid complexes (Brisou, 1995). In addition, the EPS matrix comprises a mixture of heteropolysaccharides, proteins and nucleic acids (Filipe *et al.*, 1999). Exopolysaccharides and other biopolymers exhibit excellent metal-binding properties with varying degrees of specificity and affinity. The binding of cations to bacterial biopolymers generally occurs through electrostatic interaction with negatively charged functional groups such as uronic acids, phosphoryl groups of amino acids, acidic amino acids and phosphate-containing nucleotides (Beech and Sunner, 2004). It has been described that copper increases production of the main exopolysaccharide of *E. amylovora*, amylovoran (Bereswill *et al.*, 1998). Amylovoran and levan contribute to long term survival of

1.3. Copper friend and foe for bacterial cells

this pathogen in a culturable state in presence of copper and can be used as carbon sources under deprivation conditions providing to *E. amylovora* survival advantage (Ordax *et al.*, 2010).

1.3.5. The viable but nonculturable (VBNC) state can be induced by copper

Copper induces a particular physiological state, the VBNC state, in several plant-associated bacteria, such as *Agrobacterium tumefaciens*, *Ralstonia solanacearum*, *E. amylovora*, and *Xanthomonas citri* subsp. *citri* as part of the survival strategies of these microorganisms (Alexander *et al.*, 1999; Grey and Steck, 2001; Ordax *et al.*, 2006; del Campo *et al.*, 2009). Effectiveness of copper to control bacterial plant disease is often measured by the absence of bacterial growth on a solid medium. However, the failure to produce a visible colony may not necessarily mean that the bacterial cell is dead. VBNC state can be induced in bacteria under some stress conditions, losing their culturability on nonselective solid medium but remaining viable and virulent, potentially creating a hidden reservoir of the pathogen. Induction of the VBNC state is a general mechanism to retain viability, at least in a proportion of cells, and it can be considered a long-term dormant-like survival mechanism for non-spore-forming bacteria. Resuscitation of nonculturable cells does not always occur by a simple reversal of the stress that induced the nonculturability, and there appears to be no a universal resuscitation condition (Kell *et al.*, 1998). In the case of *E. amylovora* and *X. citri* subsp. *citri* cells, resuscitation has been reported even after several

months under copper stress (Ordax *et al.*, 2006; Golmohammadi *et al.*, 2013).

1.4. *Erwinia amylovora*, the causal agent

1.4. *Erwinia amylovora*, the causal agent

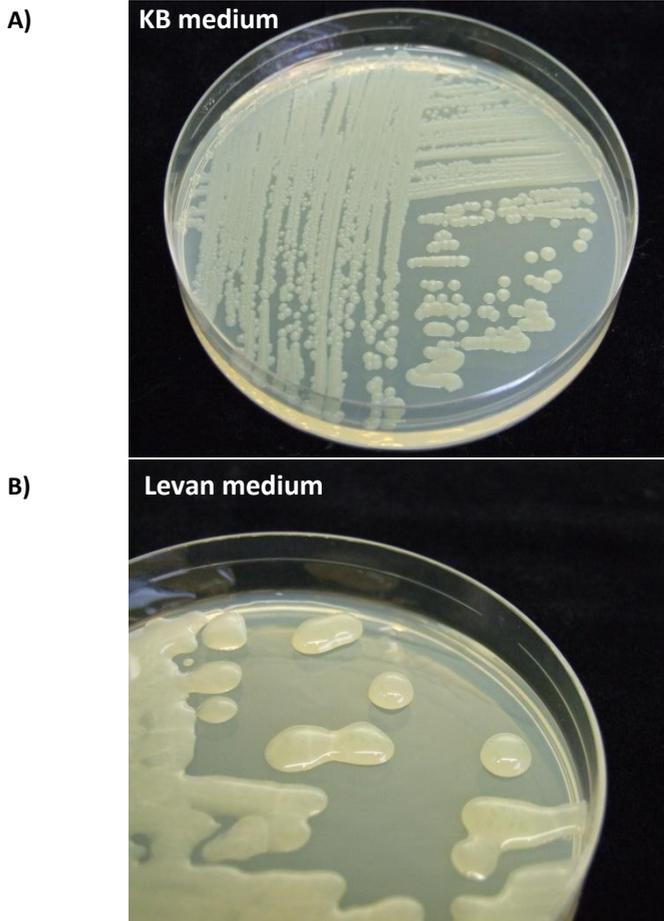
1.4.1. Taxonomy and general features

E. amylovora was the first bacterium for which its pathogenicity to plants was demonstrated, and as a plant pathogenic bacterium was shown to be the causative agent of fire blight (Burrill, 1883). It is a Gram-negative bacterium belonging to the family of *Enterobacteriaceae*, which includes some human and animal pathogens such as *Escherichia coli*, *Yersinia* spp., *Shigella* spp. and *Salmonella* spp. Cells are rod-shaped, have a size of about 0,3µm x 1-3µm, and are motile in culture media by means of two to seven peritrichous flagella per cell (van der Zwet *et al.*, 2012). On the plant surface, the motility of *E. amylovora* is easily expressed (Paulin, 2000), but no motile bacterial cells have been observed in the intercellular spaces of infected plant tissues (Cesbron *et al.*, 2006). *E. amylovora* is a facultative anaerobe and is quoted as being weakly fermentative (Holt *et al.*, 1994). The optimal temperature for its growth is 25-27°C, although it can grow between 3-5°C and up to 37°C (Billing *et al.*, 1961).

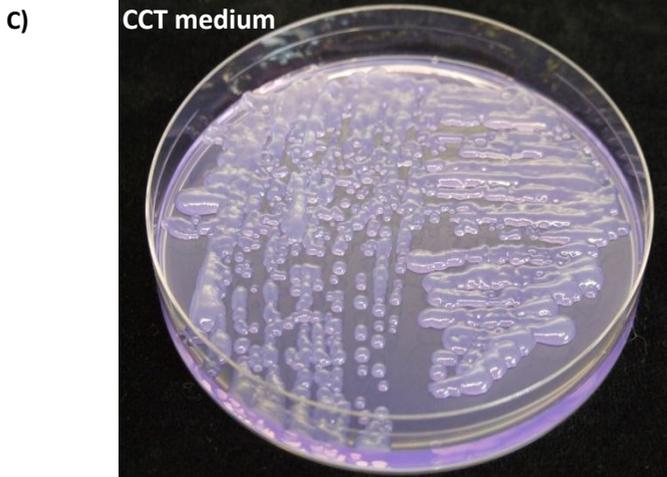
Its appearance on plate depends on the culture medium (Fig. 11). For example in KB medium (EPPO, 2013) *E. amylovora* colonies are visible at 24h and are creamy white, circular and non-fluorescent under UV light color at 366nm after 48h (Fig. 11 A). This allows distinction from fluorescent pseudomonads. Colonies of *E. amylovora* on Levan medium (EPPO, 2013) are apparent at 24h and are whitish,

circular, domed, smooth and mucoid after 48h (Fig. 11 B). *E. amylovora* colonies on CCT medium (EPPO, 2013) are visible at about 48h and are pale violet, circular, highly convex to domed, smooth and mucoid after 72h, showing slower growth than on KB and Levan media (Fig. 11 C). CCT medium inhibits most pseudomonads but not *Pantoea agglomerans* (EPPO, 2013) that is a frequent member of the native microbiota in *E. amylovora* host plants. KB, Levan and CCT are the three media recommended by EPPO to maximum recovery of *E. amylovora* from samples in poor condition.

Figure 11. *Erwinia amylovora* appearance in different solid media. Courtesy of Laboratory of Bacteriology of IVIA, Spain.



1.4. *Erwinia amylovora*, the causal agent



According to phenotypic and genomic studies, the specie *E. amylovora* is very homogeneous at the biochemical and serological levels (Paulin, 2000; Smits *et al.*, 2010), but exhibits differences among strains in several characteristics, including host range, virulence (Norelli *et al.*, 1984; Cabrefiga and Montesinos, 2005; Wang and Zhao, 2009) and presence of different plasmids (Llop *et al.*, 2012).

1.4.2. Virulence factors

Pathogenicity and virulence in *E. amylovora* mainly depend on several factors (van der Zwet *et al.*, 2012): (1) the synthesis of amylovoran and levan, (2) a type III secretion system, (3) the effector protein DspE, (4) the iron-scavenging siderophore desferrioxamine and (5) the presence of plasmids pEA29 and pEI70 (Llop *et al.*, 2011).

Amylovoran is a polymer of a pentasaccharide repeating units that consists mainly of four galactose residues and one glucuronic acid residue (Nimtz *et al.*, 1996). It is produced in the early stationary growth phase and its size varies according to environmental conditions (Bellemann *et al.*, 1994; Geider *et al.*, 1999; Li *et al.*, 2014). Levan is a neutral polyfructan (β -2, 6-D-fructofuranan) synthesized by *E. amylovora* via the secreted enzyme levansucrase from environmental sucrose (van der Zwet *et al.*, 2012). Lack of levan synthesis can result in slow symptom development on shoots of host plants (Bogs and Geider, 2000). *E. amylovora* cells moving through plant vessels result in bacterial aggregate and produce accumulation of EPS, and disruption of water flow (Sjulin and Beer, 1978). The bacterial aggregation causes leakage of the vessels and extrusion of bacteria into the parenchyma, forces bacterial ooze out of the plant surface, and thus causes shoot wilting (Brisset and Paulin, 1991; Vanneste, 1995; Zhao *et al.*, 1996). It has been shown that virulence of *E. amylovora* is dependent on the production of EPS, since strains that do not have the capacity to synthesize EPS are nonpathogenic and are unable to move into plant vessels (van der Zwet *et al.*, 2012).

E. amylovora requires a functional Hrp secretion-translocation pathway, in order to be pathogenic (Klement, 1982; Goodman and Novacky, 1994). The Hrp type III secretion system (TTSS) delivers effector proteins into host plants (He *et al.*, 1994; Kim *et al.*, 1997a, 1997b, 1997c; Bogdanove *et al.*, 1998a, 1998b; Kim and Beer, 2000; Oh *et al.*, 2005). The *hrp/dsp* gene cluster of *E. amylovora* contains

1.4. *Erwinia amylovora*, the causal agent

the *hrp/hrc* and the HEE regions (Oh and Beer, 2005). The *hrp/hrc* region contains 25 genes, including four regulatory genes which control the expression of other genes, and nine *hrc* (hypersensitive response-conserved) genes (Bogdanove *et al.*, 1998b). The HEE region has seven genes including two encoding harpin proteins (*hrpN* and *hrpW*) and two *dsp* genes (Oh and Beer, 2005). *HrpN* is considered to be involved in disease development (Kim *et al.*, 1997a). As for *dsp* genes *DspA/E*, Boureau *et al.* (2006) confirmed that they act as major inducers of cell death during disease and hypersensitive response (HR). *DspA/E*-mediated necrosis may be associated with an alteration of defense responses (Boureau *et al.*, 2006), maybe inhibiting photosynthesis in young leaves.

The production of siderophores provides an efficient strategy to allow pathogens to overcome conditions of iron limitation in host tissues, and also to protect cells against iron toxicity. In iron-limited environments, *E. amylovora* produces trihydroxamate siderophores belonging to the desferrioxamine (DFO) family (Feistner *et al.*, 1993; Kachadourian *et al.*, 1996). Mutants of *E. amylovora* CFBP 1430 lacking a functional high affinity in the iron transport system mediated by DFO are impaired in their ability to initiate fire blight symptoms (Dellagi *et al.*, 1998). Feistner and coworkers (Feistner *et al.*, 1993; Feistner, 1995) identified and characterized the siderophores of *E. amylovora* in which proferrioxamines were found, with desferrioxamines D₂ and E being the major siderophores of all strains studied.

The importance of the role of plasmids pEA29 and pEI70 in the virulence of *E. amylovora* has recently been demonstrated at least for some strains. Llop *et al.* (2011) proved that when pEI70 is introduced into strains with low levels of aggressiveness, the intensity of symptoms increased. On the other hand, after curing this plasmid from strains that showed a standard level of aggressiveness, the intensity of symptoms decreased to levels similar to strains without plasmids or cured of pEA29. Moreover, in this study, 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. In contrast, no significant effect of the introduction of pEA29 was detected on the incidence of infection in strains only harboring pEI70. This plasmid seems to provide some features that compensate for the lack of pEA29 and could explain the standard aggressiveness levels generally observed in the strains harboring it (Llop *et al.*, 2011).

1.5. *Erwinia amylovora* genetics

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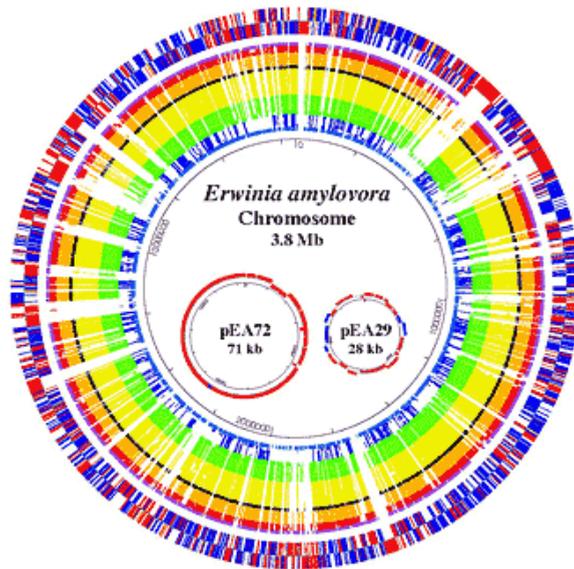
1.5.1. *E. amylovora* genome

The first two published genomes of *E. amylovora* were those of a North American strain isolated from apple, *Ea273* (ATCC 49946) (Sebahia *et al.*, 2010) and the strain CFBP 1430 (Smits *et al.*, 2010), isolated in France in 1972 from *Crataegus*. Afterwards, the draft genome of a *Rubus* strain, ATCC BAA-2158 (Powney *et al.*, 2011) was published. Draft sequences of seven isolates from Maloideae and two additional *Rubus* strains were also generated (Mann *et al.*, 2012), and raw assemblies for eight further Maloideae isolates are available (Smits *et al.*, 2011). The total collection of the genomes of these sequenced strains provides a good overview of the diversity within the species.

The genome of *E. amylovora* ATCC 49946 strain consists of a circular chromosome of 3,805,874 bp and two plasmids, AMYP1 (28,243 bp) and AMYP2 (71,487 bp) (Sebahia *et al.*, 2010) (Fig. 12). Coding regions in the chromosome account for 85.1% of the total sequence, with 3,483 identified coding sequences (CDS) in 2010 (Sebahia *et al.*, 2010). The smaller plasmid, AMYP1, has been reported as pEA29 (McGhee and Jones, 2000), with nearly identical sequences. The larger plasmid, AMYP2, renamed pEA72 for consistency in nomenclature, contains 87 predicted CDSs, with two predicted mobile-element-related CDSs and one pseudogene. Among the CDSs with annotated functions are a cluster of genes (AMYP2_49

to AMYP2_62) that encode a putative type IV fimbrial system (pil genes).

Figure 12. Genome of *Erwinia amylovora* Ea273 and plasmids that harbors. Circular representation. <http://www.plantpath.cornell.edu/labs/beer/Research.html>



The complete genome sequence of *E. amylovora* CFBP 1430 strain consists of the 3,8Mb circular chromosome and the nearly ubiquitous plasmid pEA29. The genome of this strain was assembled against the sequence of strain ATCC 49946 (Smits *et al.*, 2010). Overall, the genome sequences of the European and USA strains were found to be nearly identical (>99.99%), with only a low number of nucleotide differences, reinforcing indications of low diversity within this pathogen. This high genetic-homogeneity suggests that minimal evolution has occurred since *E. amylovora* global dispersal. The *E. amylovora* CFBP 1430 chromosome is 301 bp smaller than that

1.5. *Erwinia amylovora* genetics

of *E. amylovora* ATCC 49946 and this is largely due to intergenomic intergenic transcribed spacer (ITS) variation. This level of conservation between a European and USA strain reflects the relatively recent dispersal of the species and indicates a low rate of significant evolution (Smits *et al.*, 2010).

The genome of all these *E. amylovora* strains is less than 4Mb long, whereas most free-living enterobacteria, including plant pathogens, have genomes of 4.5 Mb to 5.5 Mb. The *E. amylovora* strains have many more predicted pseudogenes than other enterobacteria with similar lifestyles. It has also revealed clear signs of pathoadaptation to the rosaceous plant environment (Sebahia *et al.*, 2010), since genome contains proteins more similar to other plant pathogens than proteins of closely related enterobacteria. Examples of this probable pathoadaptation are: the type III effectors are homologous to those of plant-pathogenic pseudomonads; a sorbitol-metabolizing cluster that may confer a competitive advantage for survival in rosaceous plants; or the remarkable reduced genome size and erosion or loss of genes involved in anaerobic respiration and nitrate assimilation, relative to other plant- and animal-pathogenic members of the *Enterobacteriaceae* (Sebahia *et al.*, 2010).

Although many of the virulence factors found in *E. amylovora* are present in the genomes of other *Erwinia* species such as *E. pyrifoliae* and *E. tasmaniensis*, a comparative analysis demonstrated that many others are specific for *E. amylovora* and, therefore, may

contribute to the broad-spectrum and severe-disease phenotype of this species (Oh and Beer 2005). Currently, the major factors considered critical for pathogenicity or virulence on rosaceous hosts are those related to Hrp T3SS and the amylovoran biosynthesis (Bernhard *et al.* 1996; Oh and Beer 2005; Oh *et al.* 2005; Zhao *et al.*, 2009).

E. amylovora can be divided into two host-specific groups: strains infecting a broad range of hosts within the *Rosaceae* subfamily *Spiraeoideae* (eg., *Malus*, *Pyrus*, *Crataegus*, *Sorbus*) and strains infecting *Rubus* (raspberries and blackberries). Mann *et al.* (2013) performed a comparative genomic analysis of 12 strains representing distinct populations belonging to each host-specific grouping, with the objective of describing the pan-genome of *E. amylovora*. This pan-genome is highly conserved relative to other phytopathogenic bacteria, comprising on average 89% conserved core genes. Analysis of the annotated sequences revealed that 86% of the average *E. amylovora* genome consists of CDS and has an average CDS density of approximately 1 per kb. Comparison of average amino acid identities (AAI) between the strains indicated that the core genome of *E. amylovora* is highly conserved (>99% AAI among all strains). The pan-genome of *E. amylovora* was calculated to contain 5,751 CDS of which 3,414 CDS were considered as core. The chromosomes of *Spiraeoideae*-infecting strains were highly homogeneous, while greater genetic diversity was observed among

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Rubus-infecting strains, the majority of which was attributed to variable genomic islands.

According to Mann *et al.* (2013), in comparison with other studies, the pan-genome of *E. amylovora* has a high percentage of CDS per individual genome classified as core. This highlights the relatively small amount of intra-species genetic diversity observed in *E. amylovora* even with the inclusion of the more genetically diverse *Rubus*-infecting strains. It has been speculated that *E. amylovora* has a relatively low genetic diversity (compared to other plant pathogens like *P. syringae*) because it undergoes limited genetic recombination and it has a high degree of specialization to a narrow ecological niche. In Spiraeoideae-infecting strains, the genome is exposed to limited selection pressure due to pome fruit breeding strategies favoring high-value varieties that often are highly susceptible to fire blight (McManus and Jones, 1995; Smits *et al.*, 2011).

The number of genomes required to estimate the size of a species' pan-genome has been mathematically modeled (Hogg *et al.*, 2007; Tettelin *et al.*, 2008), leading to the concept of "open" and "closed" pan-genomes. In an open pan-genome new genes are added to the gene repertoire of the species with every new strain sequenced (Tettelin *et al.*, 2008). Based on EDGAR analysis (Blom *et al.*, 2009), using two complete genome sequences and ten draft genome sequences of *E. amylovora*, its pan-genome is predicted to be open. Singleton development analysis estimated that 52 novel CDS (including plasmids) or 40 novel CDS (excluding plasmids) would

be added to the pan-genome with each additional genome of *E. amylovora* sequenced (Mann *et al.*, 2013).

1.5.2. Plasmids

One of the most obvious differences among *E. amylovora* strains is the presence of different plasmids (Llop *et al.*, 2012). Nine plasmids, and someone else of minor size, have been reported in *E. amylovora* up to date. The more studied is plasmid pEA29, that was first described by Merckaert *et al.* (1982) and since then the information about it is very abundant. Its size can actually vary from 27.6 to 34.9 kb (Schnabel and Jones, 1998; Kim and Geider, 1999; McGhee and Jones, 2000), due to a variable number of short sequence repeats harboured. The plasmid encodes a thiamine biosynthesis operon and several candidate genes that could affect virulence and survival in plants (McGhee and Jones, 2000). It 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).

The other less studied plasmids are the following, in decreasing order of size. Plasmid pEA72 was described in strain ATCC 49946, and is considered cryptic (Sebahia *et al.*, 2010).

Plasmid pEI70 (Llop *et al.*, 2006) has characteristics that make it unusual: it is conjugative (Llop *et al.*, 2011), is widespread in 11 European countries, and induces faster development of symptoms when introduced in low virulence *E. amylovora* strains (Llop *et al.*,

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2008 and 2011). This plasmid is thought to provide a fitness advantage to the host bacterium, but its specific role has not been determined yet (Llop *et al.* 2011).

A plasmid called pCPP60 (Steinberg *et al.*, 1990) was renamed as pEA72 in strain ATCC 49946 (AMY P2 in Sebahia *et al.*, 2010), but it could be pEI70 in another ones (Llop *et al.*, 2012).

Plasmid pEA68 was recently discovered in *E. amylovora* strain 692 (LMG 28361), isolated in Poland from *Sorbus* (mountain ash) (Ismail *et al.*, 2014). Although the spread of pEA68 is currently limited to Europe, pEA68 together with pEA72 and pEA78 (both found in North America) belong to a new plasmid family that is present in two continents (Ismail *et al.*, 2014).

Plasmid pEL60 (Foster *et al.*, 2004), reported in strains from Lebanon, may be of environmental significance related to UV radiation tolerance.

Plasmid pEA34 was reported from some strains with resistance to streptomycin (Chiou and Jones, 1991, 1993) and seems to be present only in strains from Michigan (USA) and plasmid pEU30 was reported from USA strains (Foster *et al.*, 2004). Genetic content of pEU30 is similar to that of other plasmids inhabiting plant pathogenic bacteria, with no specific genes apparently related to virulence or fitness.

Plasmid pEA8.7 was reported from a survey in California (USA) looking for strains showing streptomycin resistance (Palmer *et al.*, 1997), and it is closely related, if not identical, to a broad host-range plasmid reported in a wide variety of clinical bacteria.

1.5.3. Genetics of host-pathogen interaction

Extensive genetic studies have been performed to identify and characterize the genes involved in the ability of *E. amylovora* to cause fire blight (Oh and Beer, 2005). The main virulence factors have been described previously in section 1.4.2. which are the protein secretion/translocation pathway, called the *hrp* type III secretion system (*hrp*-T3SS), the type 3 effector DspA/E, and the exopolysaccharide amylovoran (*ams*) and levan (*rls*) required for *E. amylovora* pathogenicity (Oh and Beer, 2005).

Related to the *hrp*-T3SS, was reported that a *hrp*-T3SS mutant and an *ams* mutant can complement each other, and co-inoculation of both mutants restored pathogenicity (Zhao *et al.*, 2009). The *hrp*-T3SS mutant was altered in a basic attack mechanism required at the beginning of the infection process, whereas the *ams* mutant was blocked later on, as amylovoran is required for the bacterial progression in planta through biofilm formation (Koczan *et al.*, 2009).

The first type III effectors identified in *E. amylovora* were harpins and DspA/E. Recent data suggest that harpins HrpN and HrpW could be injected inside the plant cell (Bocsanczy *et al.*, 2008; Boureau *et al.*, 2011), and that HrpN localizes in the plant plasma membrane as

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part of the translocon apparatus (Malnoy *et al.*, 2012). DspA/E is central to the disease process, since it is required to block callose deposition (DebRoy *et al.*, 2004) and to repress PR1 expression (Boureau *et al.*, 2006), suggesting that it contributes to disease development inhibiting salicylic acid-dependent innate immunity (Malnoy *et al.*, 2012).

Many studies, including genome sequencing, have reached the conclusion that only five effector genes (*eop1*, *eop3*, *avrRpt2_{Ea}*, *dspA/E*, and *hopC1*) exist in the genome of *E. amylovora*, which are subjected to direct *hrpL* regulation, a master regulator of T3SS (Zhao *et al.*, 2005; Nissinen *et al.*, 2007; Zhao *et al.*, 2006). A fast transcriptional induction of *hrp* expression was observed within 48 hours in flower challenge as monitored by qPCR (Pester *et al.*, 2012); thereafter, expression of *hrpL* and *hrpA* genes, encoding for major players in T3SS, declines, which correlates well with the onset of transcriptional plant response (Pester *et al.*, 2012), indicating that *E. amylovora* outcompetes plant defense in time during natural flower infections (Pester *et al.*, 2012). Interestingly, *hrp* expression varied between single flowers, and this variability may mimic the natural conditions and thus reflect the epidemiological behaviour of *E. amylovora* (Pester *et al.*, 2012).

It has been recently described by Zeng and Sundin (2014) that small RNAs also regulate multiple virulence determinants. According to these authors, there is an RNA chaperone (Hfq) that enhances the stability of small RNAs and facilitate their regulatory function. Small

RNAs are important post-transcriptional regulators in bacteria and in this sense they found that the two of them, ArcZ and RprA, were required for the full virulence of *E. amylovora*.

1.5.4. *E. amylovora* under stress

Bacteria are constantly exposed to environmental stresses, such as nutritional downshifts, variations in pH and osmolarity, DNA damage by reactive oxygen species (ROS), etc. (Landini *et al.*, 2014). Modulation of expression, as regulated by specific as well as global strategies, has a central role in bacterial adaptation. The global regulation of transcription is a general response to the environmental changes dependent on alternate sigma factors, histone-like proteins, and other transcriptional regulators (Hildebrand *et al.*, 2006).

Copper stress is caused because copper ions, although essential for life, are very toxic when allowed to accumulate to levels beyond cellular needs. The fact that copper resistance mechanisms are frequently found among pathogens, and required for virulence, suggests that this is an important aspect of survival in the host (Festa and Thiele, 2012). Pathogenic microorganisms implement tightly controlled copper homeostatic mechanisms to utilize copper yet resist its toxicity (Festa and Thiele, 2012). Organisms avoid free copper ions within the cell by developing copper translocation routes based in precise sequences of specific protein-protein interactions (Djoko *et al.*, 2007; Bagai *et al.*, 2008). In most cases, expression of the genes for the copper resistance factors acting in the periplasm is

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under the control of a two-component regulatory system (Munson *et al.*, 2000; Franke *et al.*, 2001; Yamamoto and Ishihama, 2005; Gudipaty *et al.*, 2012). In enterobacteria, three mechanisms are used to prevent the toxic action of copper ions in the periplasm: oxidation, sequestration and efflux (Nies and Herzberg, 2013).

Regarding the response to copper conditions of *E. amylovora*, in the context of its survival under this stress, and according to other bacterial models, we can hypothesize the involvement of some genes related with a general stress response (*rpoS*) (Battesti, *et al.*, 2011; Landini *et al.*, 2014), an oxidative stress response (*katG*) (Madar *et al.*, 2013) and a specific copper stress response (*dsbC*) (Hiniker *et al.*, 2005):

-***rpoS***: The general stress response is governed by the alternative sigma factor σ^S . The *rpoS* encoding σ^S is associated with RNA polymerase and, through transcription of genes belonging to the *rpoS* regulon, allows the activation of a “general stress response”, which protects bacterial cells from harmful environmental conditions (Landini *et al.*, 2014).

-***katG***: This gene codes a periplasmic catalase which is under σ^S regulon control. This enzyme removes reactive oxygen species that are produced in some stress conditions and that are detrimental for the bacterial survival (Loewen *et al.*, 1985). The VBNC state has been related with the oxidative stress (Bloomfield *et al.*, 1998).

-*dsbC*: Copper, a redox-active metal, catalyzes periplasmic disulfide bond formation under aerobic conditions. The primary role of DsbC may be to rearrange incorrect disulfide bonds that are formed during certain oxidative stress (Hiniker *et al.*, 2005).

E. amylovora faces to copper exhibiting survival strategies like the VBNC state (Ordax *et al.*, 2006, 2008, 2010), but the molecular level of this interaction is still unrevealed. In other enterobacteria, such as *E. coli*, the transcriptional response under copper stress has been reported, using DNA microarray and *in vivo* and *in vitro* transcription assays (Yamamoto and Ishihama, 2005). In this case, microarray analysis indicated that at least 29 genes (28 up-regulated and one down-regulated) were marked and specifically affected (Yamamoto and Ishihama, 2005), suggesting that copper homeostasis in *E. coli* is maintained mainly by controlling the export of excess copper out of the cells. In this sense, *copA* gene, that codes for Cu⁺-translocating P-type ATPase pump, and *cueO* gene, encoding the enzyme that is considered to convert periplasmic Cu⁺ into the less-toxic oxidative form Cu⁺⁺, were more than threefold up-regulated by copper (Yamamoto and Ishihama, 2005). Copper-responsive genes seem to be organized into a hierarchy of the regulation network, with several regulons which sense and respond to different concentrations of external copper (Yamamoto and Ishihama, 2005).

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1.5.5. Transcriptomic approach for gene expression evaluation

Studying which genes are induced or not in *E. amylovora* under copper stress can help us to understand how this pathogen is affected at molecular level by the presence of this toxic metal. With the development of microarray technology, it is possible to examine how active thousands of genes are at any given time.

According to Pallás *et al.* (2008), in order to build a microarray, first it is necessary to immobilize thousands of molecules of DNA above an inert substrat (generally a glass of a microscope slide size), placed in an organized way (hence the term microarray or micromatrix) and with a high density. Each microarray point belongs to one molecule, generally one gene (or an oligonucleotide whose sequence is contained in a gene). These immobilized acid nucleic points will be subjected to hybridization with labelled molecules of acid nucleics from biological samples. If the immobilized molecule of one microarray point, meet complementary labelled molecules, those will be selectively retained, while those unable to hybridize will be removed during the washed process. The label process is made by union of fluorescent molecules. Afterwards, the hybridized slide must be read by a scann that bombs the slide with a determined wavelength laser and captures the emitted light by the fluorescence of each point. The final result is a table that can provide us an estimation of the expression level of each gene in a particular biological sample. The key of the process lies in using at the same time two kinds of probes labelled with two different fluorofores and

coming from two different physiological situations. Since the scanner can read the slides in more than one wavelength, the final result allows us to compare the relative amount of each gene in the two mentioned situations, considering that this amount is variable depending on the situation, what will be reflected in the degree of hybridization with its complementary sequence. Therefore, we will have the response to the question of what genes are differentially expressed in the two compared situations.

The availability of complete genome sequences of *E. amylovora* has enabled genome-level transcriptomic studies utilizing different types of oligonucleotide microarrays. One of the most used includes 3,483 chromosomal sequences from *E. amylovora* ATCC 49946 and 483 sequences from known *E. amylovora* plasmids (McNally *et al.*, 2012). A *hrpL* mutant of *E. amylovora* Ea1189 was used to validate the array and revealed that 19 genes exhibited positive direct or indirect regulation by HrpL and five genes were negatively regulated (McNally *et al.*, 2012). This work also identified novel genes of the HrpL regulon of *E. amylovora*, including EAM_2938, which encodes a putative membrane protein and has a strong virulence phenotype when mutationally interrupted (McNally *et al.*, 2012).

A second study utilized this same microarray to define the RcsB and RcsC regulons of *E. amylovora* during immature pear infection, identifying 648 differentially regulated genes, which include those related to amylovoran biosynthesis, cell wall proteins, and cell membrane proteins (Wang *et al.*, 2012). Of particular interest was

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the identification that the RcsBCD phosphorelay system regulates the expression of EAM_2938, also regulated by HrpL as described above (Wang *et al.*, 2012).

Microarray experiments greatly facilitate studies focused on a deeper, more comprehensive understanding of *E. amylovora* pathogenesis, a broader identification of virulence gene regulatory circuit, and the discovery of new virulence genes (Malnoy *et al.*, 2012). And, in general, not only limited to pathogenesis issue, microarray technology allows to go further in many other aspects of the *E. amylovora* biology.

2

OBJECTIVES

OBJECTIVES

To accomplish the general objective of determining underlying mechanisms of the *E. amylovora* response facing copper stress conditions, we planned the following partial objectives:

1. To evaluate the expression of *rpoS* gene in the survival of *E. amylovora* strain CFBP1430 in the presence of 0.005mM copper sulfate.
2. To analyze the differential gene expression of *E. amylovora* strain Ea1189 after a copper sulfate shock of 0.5mM through a transcriptomic study.
3. To assess the real role of *copA* gene in *E. amylovora* strain Ea1189 survival during copper exposure *in planta* conditions.

3

MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used are listed in Table 4. In the experiments performed at IVIA, that is, survival studies and the specific evaluation of expression of *rpoS* gene, the French reference strain CFBP 1430 was used. The Spanish strain IVIA-1892 was also included as a control for minimal inhibitory concentration (MIC) determination. For the transcriptomic study performed in Michigan State University, the obtention of mutants and their evaluation, the German strain *Ea1189*, which has been deeply studied there by Dr. Sundin team since long time ago, was selected. To assess the role of some genes differentially expressed in this study, mutants of strain *Ea1189* were built with the helper plasmid pKD46.

Besides *E. amylovora*, a bacterial strain of *E. coli* was used for the cloned process. Strain S-17 of *E. coli* and a total of six genes separately cloned into plasmid pBBR1-MCS-1 were employed for complementation assays of the mutants.

In order to identify the importance of selected genes for *E. amylovora* survival after copper sulfate exposure, one mutant of *E. amylovora* strain CFBP 1430 and eight mutants of strain *Ea1189* were challenged to copper sulfate .

E. amylovora strains were routinely cultured at 26-28°C in King's B (KB) (King *et al.*, 1954) or Luria–Bertani (LB) (Bertani, 1951) media, unless otherwise stated. Mutants were cultured in the same media supplemented with 100µg/mL ampicillin, 20µg/mL chloramphenicol

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or 30µg/mL kanamycin. *E. coli* strain S-17 was cultured in LB at 37°C. Copper sulfate used for all the experiments was pentahydrated (Sigma-Aldrich C7631).

Because of the quarantine status of *E. amylovora* in the EU, the experiments of this thesis performed in Spain, were made inside a P2 security lab.

3.2. Minimal inhibitory concentration (MIC) of copper

To know the copper concentration that constitutes the growth threshold for *E. amylovora* cells in our conditions, we performed an assay to determine the MIC of copper in a non bounding copper solid medium. To select the medium to be employed, first the free copper ion bounding ability of casitone yeast extract (CYE) (Zevenhuizen *et al.*, 1979, Al-Daoude *et al.*, 2009, Sholberg *et al.*, 2001), LB and KB media was compared by using Copper Test MQuant™ strips (Merck; Darmstadt, Germany) which provide a semi-quantitative measure of Cu⁺ and Cu⁺⁺ ion concentrations. Then, for MIC assays, drops of 10µl of bacterial suspensions of approximately 1x10⁸ CFU/ml of *E. amylovora* strains Ea1189, CFBP 1430 and IVIA-1892 were spotted on CYE and KB media containing copper sulfate at different concentrations from 0 to 4mM. Plates were incubated at 26°C for 72h.

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Table 4. Bacterial strains and plasmids used in this thesis.

| Strains and plasmids | Relevant characteristics* | Source or reference |
|--------------------------|--|---|
| Strains | | |
| <i>Erwinia amylovora</i> | | |
| Wild type | | |
| IVIA-1892 | Wild type strain isolated from <i>Pyrus communis</i> | Donat <i>et al.</i> , 2007 |
| CFBP 1430 | Wild type strain isolated from <i>Crataegus sp.</i> | Collection Française de Bactéries Phytopathogènes |
| Ea1189 | Wild type strain isolated from apple | Burse <i>et al.</i> , 2004 |
| Mutants | | |
| CFBP 1430 Δ rpoS | rpoS deletion mutant, Km ^R | Santander <i>et al.</i> , 2014 |
| Ea1189 Δ arcB | arcB deletion mutant, Amp ^R , Cm ^R | This thesis |
| Ea1189 Δ copA | copA deletion mutant, Amp ^R , Cm ^R | This thesis |
| Ea1189 Δ galF | galF deletion mutant, Amp ^R , Cm ^R | This thesis |
| Ea1189 Δ EAM_3469 | EAM_3469 deletion mutant, Amp ^R , Cm ^R | This thesis |
| Ea1189 Δ soxS | soxS deletion mutant, Amp ^R , Cm ^R | This thesis |
| Ea1189 Δ ygcF | ygcF deletion mutant, Amp ^R , Cm ^R | This thesis |
| Ea1189 Δ yhhQ | yhhQ deletion mutant, Amp ^R , Cm ^R | This thesis |
| Ea1189 Δ yjcE | yjcE deletion mutant, Amp ^R , Cm ^R | This thesis |
| <i>Escherichia coli</i> | | |
| S17-1 | Pro ⁻ Res ⁻ Mod ⁺ recA; integrated copy of RP4-Tet::Mu-Kan::Tn7; Mob ⁺ Tp ^R | Simon <i>et al.</i> , 1983 |
| Plasmids | | |
| pKD46 | λ red recombinase helper plasmid; Amp ^R | Datsenko and Wanner, 2000 |
| pBBR1-MCS-2 | RK2-based broad-host-range cloning vector; Km ^R | Kovach <i>et al.</i> , 1995 |
| pArcB | 2,829-pb PCR fragment | This thesis |

MATERIALS AND METHODS

| | | |
|-------|---|-------------|
| | containing the <i>arcB</i> ORF (2,340pb) cloned into pBBR1-MCS-2 that complements the corresponding <i>arcB</i> mutation; Km ^R | |
| pCopA | 2,703pb PCR fragment containing the <i>copA</i> ORF (2,508pb) cloned into pBBR1-MCS-2 that complements the corresponding <i>copA</i> mutation; Km ^R | This thesis |
| pSoxS | 682-pb PCR fragment containing the <i>soxS</i> ORF (333pb) cloned into pBBR1-MCS-2 that complements the corresponding <i>soxS</i> mutation; Km ^R | This thesis |
| pYgcF | 813-pb PCR fragment containing the <i>ygcF</i> ORF (672pb) cloned into pBBR1-MCS-2 that complements the corresponding <i>ygcF</i> mutation; Km ^R | This thesis |
| pYjcE | 2,271-pb PCR fragment containing the <i>yjcE</i> ORF (1,650pb) cloned into pBBR1-MCS-2 that complements the corresponding <i>yjcE</i> mutation; Km ^R | This thesis |
| pYhhQ | 1,009-pb PCR fragment containing the <i>yhhQ</i> ORF (666pb) cloned into pBBR1-MCS-2 that complements the corresponding <i>yhhQ</i> mutation; Km ^R | This thesis |

*Mob⁺ mobilizes transfer of other plasmids; Tp^R, Amp^R, Cm^R and Km^R indicate resistance to trimethoprim, ampicillin, chloramphenicol and kanamidine, respectively.

3.3. Short-term assay for *E. amylovora* survival in AB medium supplemented with different concentrations of copper

Containers with 250mL of AB medium (Alexander *et al.*, 1999) were supplemented or not with copper sulfate at four concentrations chosen below the MIC: 0.005, 0.01, 0.05 and 0.5mM and then inoculated with a suspension of CFBP 1430 *E. amylovora* strain at a final concentration of 10^7 CFU/mL. Containers were incubated for 7 days at 26°C without shaking. Culturability of *E. amylovora* cells after copper exposure was measured by plating serial ten dilutions on KB at different time points and plates were incubated at 26°C for 72h.

3.4. RNA isolation

For the gene expression assays detailed in 3.5., five different commercial kits to extract total RNA were tested with the purpose of selecting that yielding enough and highest quality RNA: RNeasy Mini Kit (Qiagen), PureLink RNA mini kit (Invitrogen), RiboPure bacteria isolation kit (Life technologies), *mirVana* miRNA isolation kit (Invitrogen) and RealTime ready cell Lysis kit (Roche). Total RNA was extracted with each different kit, from a suspension of approximately 10^9 CFU/mL of strain CFPB 1430 grown in KB medium for 72h and then measured with NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific). Since only the last kit included a final step to remove DNA, for the rest of kits we added this step with the TURBO DNA-free kit (Life Technologies). With this exception, in all cases, kits were used according to manufacturer directions.

3.5. Stress-related selected genes. Primers and probes for relative quantitation of gene expression by real-time RT-PCR

For an overview of stress-related genes potentially playing a role in *E. amylovora* survival under copper conditions, we selected *rpoS*, *katG* and *dsbC* genes for expression evaluation assays in presence of copper. These three genes, previously described in section 1.5.4, are involved in the response to general (*rpoS*), oxidative (*katG*) and copper (*dsbC*) stresses, in other bacterial models (Battesti, *et al.*, 2011; Landini *et al.*, 2014; Madar *et al.*, 2013; Hiniker *et al.*, 2005).

Primers and probes (TaqMan) for target sequences of these genes were designed based on the genome sequence of the European reference *E. amylovora* strain CFBP 1430 (Smits *et al.*, 2010) and using Primer Express® Software v.3.0.1. (Applied Biosystems):

- *rpoS* gene: forward primer, 5'-GTTTTGGCCTGTTAGGCTATGAA-3'; reverse primer, 5'-CTCACGGGTCAAACCAATTTC-3'; probe: FAM-CGCCACGCTGGAAGATGTAGGC-TAM.

- *katG* gene: forward primer, 5'-CCCGCTCAATGTGAATTTCA-3'; reverse primer, 5'-CAGTGATTTGAGGTCGGCTTTA-3'; probe: FAM-ATGAGTTCCGCAAACACTGGATTATCCAG-TAM.

- *dsbC* gene: forward primer, 5'-TATCCGGCAGTCGTTAAGCA-3'; reverse primer, 5'-TGGCGAAGGTTGGATCTCA-3'; probe: FAM-ACTGGGCTTACAACAGA-TAM.

The RNA used for real-time RT-PCR assays had different origins. To test the sensitivity of the amplification protocol, RNA was extracted from a suspension of approximately 10^9 CFU/mL of strain CFBP 1430 grown in KB medium for 72h, in the case of the elaboration of standard curve for real-time RT-PCR (section 3.6.), RNA was obtained from a transcription reaction with MEGAscript T7 transcription kit (Life Technologies) and in the case of section 3.4 and 3.7., RNA was extracted from strain CFBP 1430 cells incubated 7 or 50 days in containers with AB medium and different concentrations of copper.

The real-time PCR assay was performed in a 12 μ L reaction mixture containing 2 μ L RNA, 6 μ L 2X TaqMan AgPath-ID MasterMix (with ROX) (Life Technologies), 0.5 μ L M-MLV reverse transcriptase (Life Technologies), 0.12 μ L each of forward and reverse primers (100 μ M), and 0.3 μ L of probe (6 μ M). Amplification and detection were performed with a LightCycler Real-Time PCR detection system (Roche) under the following conditions: RNA was reverse transcribed at 45 $^{\circ}$ C for 10min, followed by PCR activation at 95 $^{\circ}$ C for 10min and 45 cycles of amplification (95 $^{\circ}$ C for 15sec and 60 $^{\circ}$ C for 45sec). Analysis of assays was conducted with LightCycler 480 software (version 1.5; Roche).

3.6. Real-time RT-PCR standard curve for estimating PCR amplification efficiency of *rpoS* gene

The *rpoS* gene was selected to follow amplification efficiency because of its possible potential role in survival of *E. amylovora* under copper stress. To address the performance of the standard curve for *rpoS* gene we used two strategies.

In a first strategy, based on cloning the *rpoS* gene into a vector, the fragment contained the ORF and the entire promoter region of the gene was amplified using new designed external forward primer (5'-TGAAGTCAACGCCAAGCAGC-3') and reverse external primer (5'-ATTCACGCAGACGACGACG-3') from CFBP 1430 strain using Primer Express® Software v.3.0.1. (Applied Biosystems). The PCR product was cloned into pGEM-T easy vector system (Promega) and then introduced in *E. coli* according to the manufacturer instructions. The transformant colonies of *E. coli* were confirmed by PCR. Cloned pGEM-T was extracted from *E. coli* with Real Miniprep turbo kit (REAL) and digested with Not1. The multiple copies of *rpoS* DNA were transcribed to RNA with MEGAscript T7 transcription kit (Life Technologies). Serial 10-fold dilutions of *rpoS* RNA were prepared and stored at -80°C prior to use in standard curve generation.

In a second strategy, *rpoS* gene was amplified using the external forward primer described above but containing the promoter of T7 RNApol (5'-TAATACGACTCACTATAGGGTGAAGTCAACGCCAAGCAGC-3') and the reverse primer as described before. The PCR product was

cleaned with UltraClean PCR Clean-up kit (MoBio). Then, *rpoS* DNA was transcribed to RNA with MEGAscript T7 transcription kit and purified with MEGAclean transcription Clean-up kit (Life Technologies). Serial 10-fold dilutions of *rpoS* RNA were prepared and stored at -80°C prior to use in standard curve generation.

In all cases, the quantity of RNA transcribed was measured with NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific), Quant-iT RiboGreen RNA assay kit (Life Technologies) and Qubit® Fluorometric Quantitation (Life Technologies) device.

The real-time RT-PCR assay was performed as described before in 3.5.

PCR amplification efficiency is the rate at which a PCR amplicon is generated (ABI Guide, 2004), commonly presented as a percentage value. If a particular PCR amplicon doubles in quantity during the geometric phase of its PCR amplification then the PCR assay has 100% efficiency. The slope of a standard curve is commonly used to estimate the PCR amplification efficiency of a real-time PCR reaction. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of C_T value vs. log of input nucleic acid. A standard curve slope of -3.32 indicates a PCR reaction with 100% efficiency (ABI Guide, 2004). A calculation for estimating the efficiency (E) of a real-time PCR assay according to ABI Guide is:

$$E = (10^{-1/\text{slope}}) \times 100$$

3.7. Long-term assay for *E. amylovora* survival in AB medium supplemented with copper sulfate and expression of *rpoS* gene under these conditions

Containers with 250mL of AB medium were supplemented or not with copper sulfate at 0.005mM and then inoculated with a suspension of CFBP 1430 *E. amylovora* strain at a final concentration of 10^7 CFU/mL. This copper concentration was selected when the results of section 3.3. were obtained. Containers were incubated for 50 days at 26°C without shaking. Culturability of *E. amylovora* strain CFBP 1430 cells after copper exposure was measured by plating serial ten dilutions on KB medium at different time points. Plates were incubated at 26°C for 72h. To determine if nonculturable cells were dead or still viable and have entered in the VBNC state, aliquots from containers were stained with the bacterial viability kit LIVE/DEAD (BacLight, Life Technologies) according to manufacturer instructions, and then observed with a Nikon ECLIPSE E800 epifluorescence microscope.

At each time point, an aliquot from containers was saved with glycerol at -20°C. When appropriate, RNA was extracted from those samples with RNeasy Mini Kit (Qiagen), cleaned with TURBO DNA-free kit (Life Technologies) and analyzed by real-time RT-PCR with primers for *rpoS* gene. Primers, probes and protocol to run these RT-PCRs were described before in section 3.5. This experiment was repeated twice.

Data were statistically analyzed using general linear model technique. It is possible to perform this using regression analysis by dichotomizing all factors. This technique is similar to regression models but the idea behind it is a generalization of analysis of variance models. P-value used for rejecting the null hypothesis was 0.05 as is usual in other studies of the discipline.

3.8. Microarray experiment

As a more powerful approach to the objective of studying the gene expression under copper stress conditions, we selected a transcriptomic tool that could provide a general view of the genes affected when *E. amylovora* faces a copper shock.

3.8.1. *Erwinia amylovora* microarray

The microarray used was previously designed at the James Hutton Institute (JHI; formerly Scottish Crop Research Institute (SCRI)) and synthesized by Agilent Technologies, Inc. (Palo Alto, CA, USA), it contains 3,483 target sequences (annotated genes and pseudogenes) of the main *E. amylovora* ATCC 49946 genome (accession NC_013971; Sebaihia *et al.*, 2010), in a design on a single 8 x 15k format slide (McNally *et al.*, 2012).

Each slide contained eight arrays and each array had nearly 12,000 spots, containing the probes in triplicate. Besides the 3,483 target sequences of the *E. amylovora* ATCC 49946 genome, a further 483 target genes or simple gene predictions from five sequenced

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plasmids were present: plasmids 1 and 2 for the same strain (accessions NC_013972, NC_013973; McGhee and Jones 2000; Sebahia *et al.*, 2010), pEL60 and pEU30 (accessions NC_005246, NC_005247; Foster *et al.*, 2004) and pEI70 (accession NC_018999, from the Spanish strain *E. amylovora* IVIA-1614-2a; Llop *et al.*, 2011).

Up to five sense orientation candidate probes per target were designed with the Agilent eArray webtool, using temperature matching methodology, a preferred probe melting temperature of 80°C, no 3' bias and a target length of 60bp. Any short probes were later extended to 60bp using the Agilent linker. BLASTN (Altschul *et al.*, 1997) and Biopython (Cock *et al.*, 2009) were used to identify potential cross-hybridization in order to rank the candidate probes. The selection of one probe per genome target, and up to five probes per plasmid target, allowed all of the probes to be present in triplicate.

We performed experiments using two microarray slides to evaluate the transcriptomic response of *E. amylovora* strain Ea1189 after a copper shock. Each slide contained the RNA extraction of the strain after being treated or not with 0.5mM copper sulfate. Moreover, RNA came from two different bacteria cultures (biological replicates) submitted to the same experimental conditions. Therefore, three independent experiments were carried out with a total of six replicates (two replicates from slide 1 and four replicates from slide 2). The experimental array design of each single 8 x 15k format slide is detailed in Table 5.

Table 5. Experimental design of the array to evaluate *E. amylovora* gene expression after copper shock. NT means non-treated, T means treated, BR means biological replicate. Time of copper exposure, 0 or 5min.

SLIDE 1

| | | | | | | | |
|------|--|--|--|------|--|--|--|
| BR 1 | | | | BR 1 | | | |
| NT0 | | | | NT5 | | | |
| T0 | | | | T5 | | | |

SLIDE 2

| | | | | | | | |
|------|-----|--|--|------|-----|--|--|
| BR 2 | | | | BR 2 | | | |
| NT0 | NT0 | | | NT5 | NT5 | | |
| T0 | T0 | | | T5 | T5 | | |

3.8.2. Experimental set-up for preparation of samples for microarray hybridization

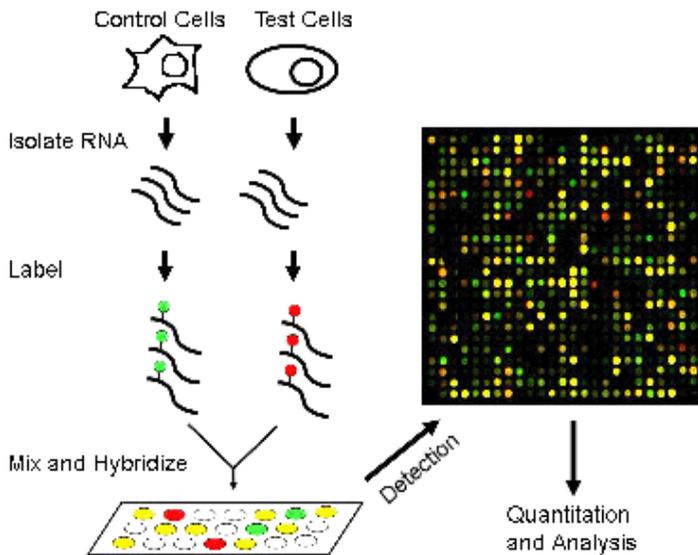
As described, all these molecular assays were run with strain *Ea1189* of *E. amylovora*. A suspension from five colonies coming from a plate of LB medium grown 72h was obtained. Then, a flask with 100mL of LB broth was inoculated with 100µL of the suspension and incubated 16h at 28°C and 200rpm to a density of approximately 10⁹CFU/mL. After that period of time, the culture was split in two fractions of 50mL. One fraction was copper-shock challenged to 0.5mM CuSO₄ for 5 min, whereas the other one was used as control (not copper

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challenged). This copper concentration was selected according to Yamamoto and Ishihama (2005). Cells were then treated with RNAprotect (Qiagen). Total RNA from 1mL culture was extracted by using SV Total RNA Isolation System (Promega) (Fig. 13). It was quantified with NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific) and quality checked using an RNA 6000 Nano Kit on a 2100 Bionalyzer (Agilent Technologies). Fluorescent labelling of total RNA was performed as described by Venkatesh *et al.* (2006) (Fig. 13). The design incorporated a dye-swap and balanced labelling of all samples. Levels and efficiencies of labelling were estimated using the NanoDrop spectrophotometer mentioned before. Microarray hybridization, washing and scanning were performed at the JHI Sequencing and Microarray Facility of Michigan State University (USA) as described previously (Stushnoff *et al.*, 2010) (Fig. 13). They were conducted according to the manufacturer's protocols (Agilent Two-Color Microarray-Based Gene Expression Analysis, version 5.5). Briefly, 20µl labeled samples were added to 5µl 10X blocking agent (Agilent 5188-5242), heat denatured at 98°C for 3 min, and then cooled to room temperature. GE hybridization buffer HI-RPM 2X (25µl) was added and mixed prior to hybridization at 65°C for 17h at 10 rpm. Array slides were dismantled in Wash 1 buffer (Agilent, 5188-5327) and washed in Wash 1 buffer for 1 min, then in Wash 2 buffer (Agilent, 5188-5327) for 1 min, and centrifuged dry. Hybridized slides were scanned using an Agilent G2505B scanner at resolution of 5µm at 532nm (Cy3) and 633nm (Cy5, for two-channel analysis) wavelengths with extended dynamic range (laser settings at 100%

and 10%) (Fig. 13). Microarray images were imported into Agilent Feature Extraction (FE) (v.9.5.3) software and aligned with the appropriate array grid template file (021826_D_F_20081029). Intensity data and quality control (QC) metrics were extracted using the recommended FE protocol (GE2-v5_95_Feb07). Entire FE datasets for each array were loaded into GeneSpring (v.7.3) software for further analysis.

Figure 13. General view of an oligonucleotide microarray performance. http://www.u.arizona.edu/~gwatts/azcc/help_old.html RNA is extracted from control and test cells. Then, the isolated RNA is labeled differently and hybridized with a microarray. The detection of the different fluorescence from different hybridized probes is detected by a scan.



3.8.3. Microarray data analysis

Data were normalized using default settings: for two-channel arrays, data were transformed to account for dye-swaps and data from each array were normalized, according to McNally *et al.* (2012), using the Lowess algorithm to minimize differences in dye incorporation efficiency. Unreliable data flagged as absent in all replicate samples by the FE software were discarded. Significantly changing gene lists were generated from combined replicate datasets for each time point using volcano plot filtering (fold-change ratio >1.5 ; Student's *t*-test with *P* value <0.05). In each independent experiment, technical replicates were analyzed individually. A gene was classified as differentially expressed when it was present at least in one biological replicate of both independent experiments and not in opposite expression in any other replicates. Since fold-change ratios were lower than 1.5, we selected genes with values ≥ 1 for up-regulated genes and values ≤ 1 for down-regulated genes, in a way similar to the study of Mitchell *et al.* (2010). Those genes differentially expressed in both independent experiments were selected for further analysis. Functional classification of the differentially expressed genes was based on the gene ontology using the GPRO v1.1.0 software.

3.9. Validation of differentially expressed genes by quantitative real-time PCR

Twenty-five out of forty-four genes differentially expressed after copper shock were selected for quantitative real-time PCR validation.

These validation assays were performed in collaboration with Estación Experimental del Zaidín (EEZ), Granada, Spain. Those genes were selected attending to its up-regulation and/or product description obtained from data bases, regarding their possible relation with the response of *E. amylovora* to copper stress. The selected genes are described in Table 6.

Table 6. Selection of *E. amylovora* genes differentially expressed after microarray analysis for validation through quantitative real-time PCR.

| Gene | Product description according to NCBI |
|-------------|---|
| <i>copA</i> | Copper exporting ATPase |
| <i>ygcF</i> | Conserved hypothetical protein |
| <i>yhhQ</i> | Hypothetical protein |
| <i>tatC</i> | Twin-arginine protein translocation system |
| <i>soxS</i> | Transcriptional regulator |
| <i>galF</i> | UTP-glucose-1-phosphate uridylyltransferase |
| <i>apt</i> | Adenine phosphoribosyltransferase |
| <i>ydhC</i> | Inner membrane transport |
| <i>cheA</i> | Chemotaxis protein |
| <i>nodT</i> | RND efflux system, multidrug resistance protein CusA-like |
| EAM_1634 | Hypothetical protein |
| <i>gltI</i> | Glutamate and aspartate ABC transporter |
| <i>rimI</i> | Ribosomal-protein-alanine acetyltransferase |
| <i>arcB</i> | Aerobic respiration sensor-response protein |
| <i>tag3</i> | 3-methyl-adenine DNA glycosylase I |
| EAM_2853 | Putative helicase/relaxase |
| <i>yjcE</i> | Na ⁺ /H ⁺ exchanger |
| <i>uspA</i> | Universal stress protein |
| <i>smdB</i> | Multidrug efflux pump ABC transporter |
| <i>yfcA</i> | Inner membrane protein |
| EAM_3469 | Phage holin |
| <i>tufA</i> | Protein chain elongation factor ET-Tu |
| <i>dfoA</i> | Desferrioxamine siderophore biosynthesis |
| <i>oppA</i> | Oligopeptide ABC |
| <i>ppc</i> | Phosphoenolpyruvate carboxylase |

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Gene expression was measured by quantitative real-time PCR performed in triplicate using new RNA samples taken from cultures of strain *Ea1189* treated with copper as described above for the microarray hybridization assays. RNA was extracted as described in section 3.8.2. and after its quality was confirmed, it was transcribed to cDNA using SuperScript II Reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The optimization of quantitative real-time PCR was set up first using the 16S gene as an internal control at the melting temperature of each primer, using the iQSybrGreen Supermix (BioRad) (Pffaf, 2001). Primers were designed using Primer Express® Software v.3.0.1. (Applied Biosystems) based on the genome sequence of the North American strain isolated from apple, *Ea273* (ATCC 49946) (Sebahia *et al.*, 2010), they were used at 20µM and the sequences are listed in Table 7. All reactions were conducted with a real-time PCR System iQ5 (Bio-Rad) and amplification was carried out with one cycle at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30s and annealing at 63°C for 30s. Nonspecific primer activity was monitored using a dissociation curve and the resulting threshold cycles (C_T) were determined using Bio-Rad iQ5 Software 2.1 (Bio-Rad). The 16S rDNA gene was used as an endogenous control in quantitative real-time PCR to calculate the relative expression. Data of C_T were analyzed quantitatively via the comparative cycle threshold ($\Delta\Delta C_T$) method (Pffaf, 2001) to generate relative fold-change values comparing WT and mutant strains. Each quantitative real-time PCR analysis was performed in triplicate.

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Table 7. Primers used for validation of microarray expressed genes by quantitative real-time PCR.

| Gene | Sequence (5'-3') | |
|-------------|--------------------------------|-------------------------------|
| | Forward | Reverse |
| <i>copA</i> | CCG GCG CAA CAG AGT GA | CAC ATT GTG CAA CGC ATT CC |
| <i>ygcF</i> | CAT TGC ACG GCG GAA AC | CGC ACT AAC GCC TGG TTG A |
| <i>yhhQ</i> | AGC GGC ATG GTG GAT AGC | AAC GGA TCG CTG CTT TGA T |
| <i>tatC</i> | CAC CCC GAT CAA GCT AAC CA | GCG CTC GTG GCG ATA GAG |
| <i>soxS</i> | CGA GCA AAA CGA TTA TGG CTA TC | GAA CAC CCG CTG GAA ACA CT |
| <i>galF</i> | GCT GCA TGA TGA AGC GTT TG | TCG CCG CCA GGT TGT AAC |
| <i>apt</i> | AAC CGG CGG CAC CAT T | TCG CCA CTG AGG TCA AAC AA |
| <i>ydhC</i> | GGG TCT TTC ACC TGC CGA TA | AGC GCA CGA CAG CTA AAG C |
| <i>cheA</i> | GAC GTT GCG CGT CCT GAT A | CAG CAC GCT AAA CCC AAA GG |
| <i>nodT</i> | CGA GCG GGC AAA GAT CAC | CAG ATG CGA TTC GGC AGT AA |
| EAM_1634 | CAG AAA CGC CAA CAC AGG AA | GCC GTC ATG CGT TTG GTT |
| <i>gltI</i> | CGT CCG TCC CCT TCT CCT A | CCT GGA GAT CGG GTT TGT TC |
| <i>rimI</i> | GCG GTA GAT CCG GCA TTT C | CCT CCA GCC ACA GGG TCA T |
| <i>arcB</i> | AGA TGT CGC GGC GTG AA | TCA AGA AAA GAG CGC AGA AAT G |
| <i>tag3</i> | CGG CTC CAC CAC CTG TTA CT | TGT CGG GAT GGC GAA AAC |
| EAM_2853 | TTG CAG GAG GCA CGG TTA C | GCG GAG GAA TAT GGC CTG TT |
| <i>yjcE</i> | GCG GAA CAT ATT GGC GTT TC | CGG AGC ATG GCG AAT AAT G |
| <i>uspA</i> | CGG TGA ATC GCC TGC TAA AA | AAT GCG CAC ATC GAC CAG TA |
| <i>smdB</i> | TGG CAG GTG AGC GAA TTT TC | TCA GGT CGC GAA TGT CGA T |
| <i>yfcA</i> | TGC TGT TTC TCG TGG CGT TA | CAG GGC GGG AAC CGT TA |
| EAM_3469 | TGG CGT TCT GGC TCA ATG T | CCC CGA GAT TTG CCA GAG TA |
| <i>tufA</i> | CCA CGC CGA CTA TGT GAA AA | GGC ATT GGG CCG TCA GT |
| <i>dfoA</i> | AAC GGC GTT TTT CTC GAT CA | GGC GCT TTC CAG CAT CAT |
| <i>oppA</i> | GAA GGC GTA ACG GAA TCG AA | GCC GCC CTC TTT GCT TTC |
| <i>ppc</i> | GGG CCC CGC TGT ATA CCT | GCA CGC CAA AGC ACT TCA C |
| 16S | TCC CTA GCT GGT CTG AGA GGA T | GCT GCC TCC CGT AGG AGT CT |

3.10. Mutant construction and complementation

Standard recombinant DNA techniques were used as described by Sambrook *et al.* (1989). To make specific mutants of *E. amylovora* Ea1189 strain, non-polar chromosomal mutations were generated using the phage λ red recombinase system (pKD46) previously described (Datsenko and Wanner 2000; Zhao *et al.*, 2009; McNally *et al.*, 2012). We decided to make mutants in *copA*, *soxS*, *arcB*, *yjcE*, *ygcF*, *yhhQ*, *galF* and EAM_3469 genes, because their higher expression level as measured by quantitative real-time PCR and/or their interest as potentially related to the response of *E. amylovora* facing copper stress. Single-gene recombinatorial deletions were confirmed by PCR using the primers described in Table 8, based on the genome sequence of the North American strain, Ea273 (ATCC49946) (Sebahia *et al.*, 2010). Afterwards, we selected *copA*, *soxS*, *arcB*, *yjcE*, *ygcF* and *yhhQ* mutants to confirm that their phenotypes were due to the absence of the target gene. Thus, fragments containing *copA*, *soxS*, *arcB*, *yjcE*, *ygcF* or *yhhQ* genes were obtained by PCR amplification of genomic DNA from strain Ea1189 using a specifically designed primer set (see Table 8), and cloned between the XbaI and SacI sites of the broad-host-range vector pBBR1-MCS-2 (Kovach *et al.*, 1995). These fragments contained the ORF and the entire promoter region of each gene (see corresponding plasmids in Table 4). These recombinant plasmids were introduced into the pertinent Ea1189 mutant by bi-parental cross-streak mating using *E.coli* S17-1 harboring the plasmid of

interest as the conjugative donor (Simon *et al.*, 1983; Abarca-Grau *et al.*, 2012). The phenotype of these complemented mutants was checked as described in section 3.11.

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Table 8. Primers used for mutant construction and complementation of genes of *E. amylovora* strain *Ea1189*.

| Name | Sequence (5'-3') | |
|----------------------------|-------------------------------|--------------------------|
| Mutant construction | | |
| arcB_F | AAATATTCGCTCCGATGCAC | |
| arcB_R | GCAGGCGAATGATTCAAAAT | |
| copA_F | TAAAAGCGGATTGCTTTGCT | |
| copA_R | CACCTCTTGCCTTATTTCC | |
| soxS_F | GCC TTTTATCGCGGAGTAGA | |
| soxS_R | TGAAGAGTGCTGTTGCAAGG | |
| ygcF_F | TATCCGTGCAATCTGCCATA | |
| ygcF_R | AACCCGTTCAAGCCCTTTTAT | |
| yhhQ_F | TGAAGGACAGGACTGGGTTT | |
| yhhQ_R | ATC GGT TTT GAT GCT TCA GG | |
| yjcE_F | CGCTTAACGCACTTCCTGTT | |
| yjcE_R | GGGATGGGAGTAGCCATTCT | |
| EAM_3469_F | TGTTCCGTGGAAAGGTAAGG | |
| EAM_3469_R | TTGCTGCCTGTAGTGAATC | |
| galF_F | CGAAGCCCCTGTATTTTT | |
| galF_R | CACGGCTGTGCTCAGATT | |
| Gene cloning | | Product size (pb) |
| copA(XbaI)_F | CCATCTAGAAATGTTTCATGGTTCCCTTG | 2,703 |
| copA(SacI)_R | CCAGAGCTCAATAACCGCAAAGCCATCTG | |
| soxS(XbaI)_F | CCATCTAGACTAAGGCGTAGCTGGCAGAG | 687 |
| soxS(SacI)_R | CCTGAGCTCGCTGTTGCAAGGAGTGCAA | |
| arcB(XbaI)_F | CCATCTAGAAAAAAGGCGACAAGCCATTA | 2,829 |
| arcB(SacI)_R | CCTGAGCTCGGTTACCAATTTCCCTGGTG | |
| yjcE(XbaI)_F | CCATCTAGACCAGTTTGGGGCCGTAATTT | 2,271 |
| yjcE(SacI)_R | CCTTCTAGACTGTTTTCCATGCCAAGCCT | |
| ygcF(XbaI)_F | CCATCTAGAACATCCTGTATGGGGAGACG | 813 |
| ygcF(SacI)_R | CCTGAGCTCATGAGTGGGACTTGACTTG | |
| yhhQ(XbaI)_F | CCATCTAGACGGTAATATGCCGAAAAGT | 1,009 |
| yhhQ(SacI)_R | CCTGAGCTCCTGAAGCCATCGGTTTTGAT | |

3.11. Experiments with mutants of *E. amylovora* to evaluate the role of different genes

3.11.1. The possible role of *rpoS* gene in the survival of strain CFBP 1430

E. amylovora CFBP 1430 Δ *rpoS* mutant was kindly provided by R.D. Santander (Santander *et al.*, 2014). Containers with 4mL of AB medium were supplemented or not with copper sulfate at 0.005, 0.01 and 0.5mM trying to simulate the conditions of section 3.3. and then inoculated with a suspension of mutant strain at a final concentration of 10⁸CFU/mL. Containers were incubated for 64h at 26°C without shaking. Culturability of wild type and mutant strains throughout copper exposure was monitored by plating serial ten dilutions from the containers on KB medium at different time points. Plates were incubated at 26°C for 72h.

3.11.2. Assays to evaluate the possible role of *copA*, *soxS*, *arcB*, *yjcE*, *ygcF*, *yhhQ*, *galF* and EAM_3469 genes of strain Ea1189 after copper shock

3.11.2.1. Copper tolerance *in vitro*. Wild type *E. amylovora* strain Ea1189 was grown 16h in LB broth at 28°C in a shaking incubator to a density of approximately 10⁹CFU/mL and the culture was then split in two fractions as indicated in section 3.8.2. One fraction was copper-shock challenged for 5 minutes to different copper sulfate concentrations ranged from 0.5 to 35mM, to determine the maximal copper concentration that had no impact on its culturability, whereas

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the other fraction was used as control (not copper challenged). Then, an aliquot from each fraction was washed once with PBS and the culturability of wild type was measured by plating serial ten dilutions on LB medium. Plates were incubated at 26°C for 72h.

Then, the concentration of copper sulfate which did not affect the growth of *E. amylovora* Ea1189 (10mM) was selected to test the culturability of selected mutants. Each mutant from a 72h culture was grown 16h in LB broth at 28°C in a shaking incubator to a density of approximately 10⁹CFU/mL and the culture was then split in two fractions, as described before for wild type strain. One fraction was challenged to 10mM copper sulfate for 5 minutes and the other one used as control. An aliquot from each fraction was washed once with PBS and the culturability was measured by plating serial ten dilutions on LB medium supplemented with 100µg/mL ampicillin and 20µg/mL chloramphenicol. Plates were incubated at 26°C for 72h.

3.11.2.2. Effect of introduction of target genes in the mutants. Six out of the eight genes selected to make mutants in *E. amylovora*, were chosen to be introduced in the mutant strains (complemented mutants). We selected those genes because of their product description from data bases and their possible relation with the response of *E. amylovora* to copper stress. After recombinant plasmids containing the ORF and the entire promoter region of each target gene, *copA*, *soxS*, *arcB*, *yjcE*, *ygcF* and *yhhQ* were introduced into each corresponding *E. amylovora* Ea1189 mutant (*Ea1189ΔgeneX*), the phenotypes were tested challenging each

complemented mutant (*Ea1189ΔgeneX*(pGenX)) to 10mM copper sulfate. Thus, *E. amylovora Ea1189* wild type, the six mutants in the target genes, and the new six complemented mutants were grown in separate flasks with LB broth for 16h at 28°C in a shaking incubator. Then, the culture of each strain was split in two fractions. One fraction was copper-shock challenged to 10mM copper sulfate for 5 min, whereas the other one was used as control. An aliquot from each fraction was then washed once with PBS and the culturability was measured by plating serial ten dilutions on LB medium supplemented or not with 100µg/mL ampicillin and 20µg/mL chloramphenicol. Plates were incubated at 26°C for 72h.

3.12. The *copA* case.

As in the quantitative real-time PCR validation assays, *copA* gene exhibited the highest expression of all genes tested, and it is reported that it codes for a copper exporting ATPase, we selected it for further studies.

3.12.1. Expression curve of *copA* in *E. amylovora* after copper-shock induction. Wild type strain of *E. amylovora Ea1189* was grown 16h in LB broth at 28°C with shaking to a density of approximately 10⁹CFU/mL. Then, aliquots of 10 mL were subjected to copper shock with different copper sulfate concentrations ranging from 0.005 to 0.5mM for 5 minutes in a rocking shaker, with one fraction used as untreated control (not copper-challenged). A volume of 0.5 mL of each culture was treated with 1 mL of RNAprotect (Qiagen) and total

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RNA was extracted using the miRNeasy Mini kit (Qiagen) with an on-column RNase-free DNase treatment (Qiagen). cDNA was synthesized from 1 μ g of purified RNA using TaqMan reverse transcription (RT) reagents (Applied Biosystems), according to the manufacturer's protocol. Quantitative real-time PCR was used to determine the relative expression levels of *copA* after induction with the different treatments; 16S rDNA gene was used as an endogenous control. qPCR was performed on a CFX96 Real-Time PCR Detection System (BioRad Laboratories) with the SYBR[®] Green PCR Master Mix (Applied Biosystems). Efficiencies for each primer set were obtained from a standard curve using ten-fold dilution series up to 10⁶ of one cDNA sample of the untreated control at the melting temperature of the *copA* primer. Primers of *copA* and 16S rDNA genes were the same used for validation expression after microarray assay (section 3.9) and the sequences are listed in Table 7. Optimization of primer concentration was carried out by qPCR using combinations of different primer concentrations (50, 300 and 900 nM) and the undiluted cDNA sample of the untreated WT strain. Optimal concentration was selected as the combination with the lowest Ct value. The thermal cycling conditions were one cycle at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30s and annealing at 63°C for 30s. Each biological replicate was run in triplicate. Melting curves were generated to evaluate primer specificity and rule out primer dimer formation. C_T values were analyzed quantitatively via the comparative cycle threshold ($\Delta\Delta C_T$) method (Pffafli, 2001) to generate relative fold-change values

comparing WT and copper-shock induced WT strains. Each quantitative real-time PCR analysis was performed in triplicate.

3.12.2. Copper tolerance *in planta*. Wild type *Ea1189*, mutant *Ea1189ΔcopA* and complemented mutant *Ea1189ΔcopA(pCopA)* were assayed at the same time in this experiment.

Fresh *in planta* pear shoots taken from 8 years old pear trees cv. 'Conference' growing inside a mesh were inoculated in a P2 security lab by immersion in a suspension of approx. 10^9 CFU/mL of each *E. amylovora* strain, potted in closed flask of approx. 13cm diameter and with 1% agar. These flasks were incubated at 25°C in a climate chamber at 8h light-16h darkness for 24h. Then, after 24h inoculation, 4mL of a solution of copper sulfate at 10mM was sprayed on leaves, and the first sampling was made (time 0). Pots were again incubated in the climate chamber and periodically sampled at different time points throughout 29 hours. The procedure of sample processing at each time point was as follows: one leaf was pull out and two square pieces of approximately 1cm^2 were cut with a sterile scalpel. Leaf pieces were crushed in 1mL PBS 0.5X. Culturable cell counts were then determined by plating ten serial dilutions on LB medium and incubating the plates at 26°C. CFU counts were performed after 72h.

Data were statistically analyzed using a generalized mixed model technique (McCulloch and Searle, 2001), where sprout nested in

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experiment was a random effect while the rest of factors were considered fixed. P-value was 0.05.

4

RESULTS

RESULTS

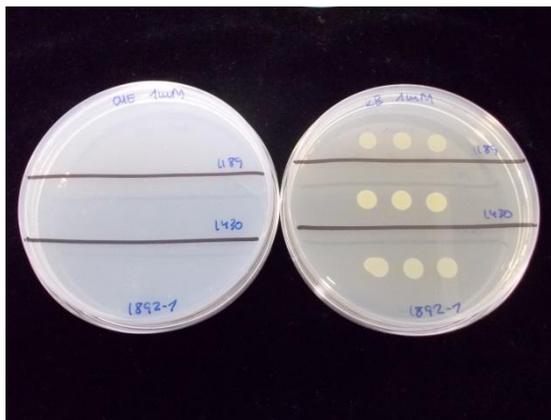
RESULTS

4.1. Minimal inhibitory concentration (MIC) for copper

To evaluate which medium was the most recommendable to perform the MIC assays, we tested three media, CYE, LB and KB for copper bounding ability. Out of the three tested growth media, LB and KB bounded each of them 60% of the copper added, whereas CYE medium did not exhibit ability to bound it, as measured by Copper test Microquant. CYE medium was then selected to assay the MIC for copper sulfate compared to KB medium.

A suspension of each strain of *E. amylovora* (CFBP 1430, Ea1189 and IVIA-1892) was prepared and plotted on CYE and KB media supplemented or not with copper sulfate. All the strains grew at copper sulfate concentrations below 1mM on KB and CYE media, but only the strains plotted on KB medium grew at this concentration (Fig. 14). Therefore, the concentration of 1mM was established as the MIC for copper sulfate in *E. amylovora* under assayed conditions in CYE medium.

Figure 14. MIC for copper in two media. Left side, CYE medium with no growth at 1mM copper sulfate of the strains assayed. Right side, KB medium with growth of all strains at 1mM copper sulfate.



RESULTS

4.2. Short-term assay for *E. amylovora* survival in AB medium supplemented with different copper concentrations

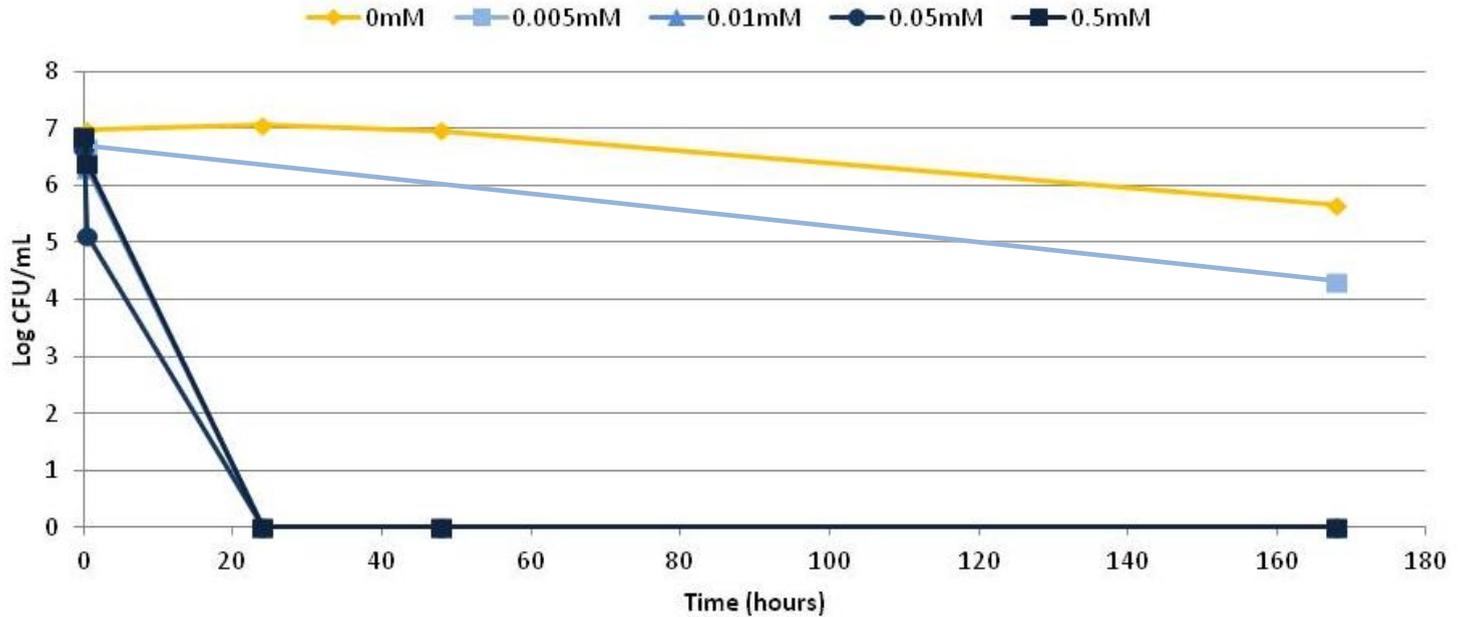
Once determined the MIC for copper sulfate on solid medium, culturability of *E. amylovora* strain CFBP 1430 in liquid AB medium at concentrations lower than 1mM was assayed. As shown in Fig. 15, culturable cells decreased in approximately one and a half logarithmic order through the seven days of the experiment, from 10^7 CFU/mL to 4.5×10^5 CFU/mL, in absence of copper. Treatment with 0.005mM copper sulfate resulted in a loss of culturability of almost three orders of magnitude in the same time period, dropping to 2×10^4 CFU/mL at day seven (Fig. 15). At the other copper sulfate concentrations of 0.01, 0.05 and 0.5mM, culturability of *E. amylovora* CFBP 1430 was under the detection limit just after 24 hours of exposure (Fig. 15).

4.3. Efficiency of RNA extraction

In all the experiments, performed with strain CFBP 1430, no differences either in quantity or in quality were found between the five commercial kits used for RNA extraction after repeated trials with each of them and under the same conditions. Then, considering its simplicity and easy to use, we selected RNeasy Mini Kit (Qiagen) for all the RNA extractions, followed by a treatment with TURBO DNA-free kit (Life Technologies) to remove DNA from RNA.

RESULTS

Figure 15. Short-term assay of the *E. amylovora* survival in AB medium supplemented with copper.

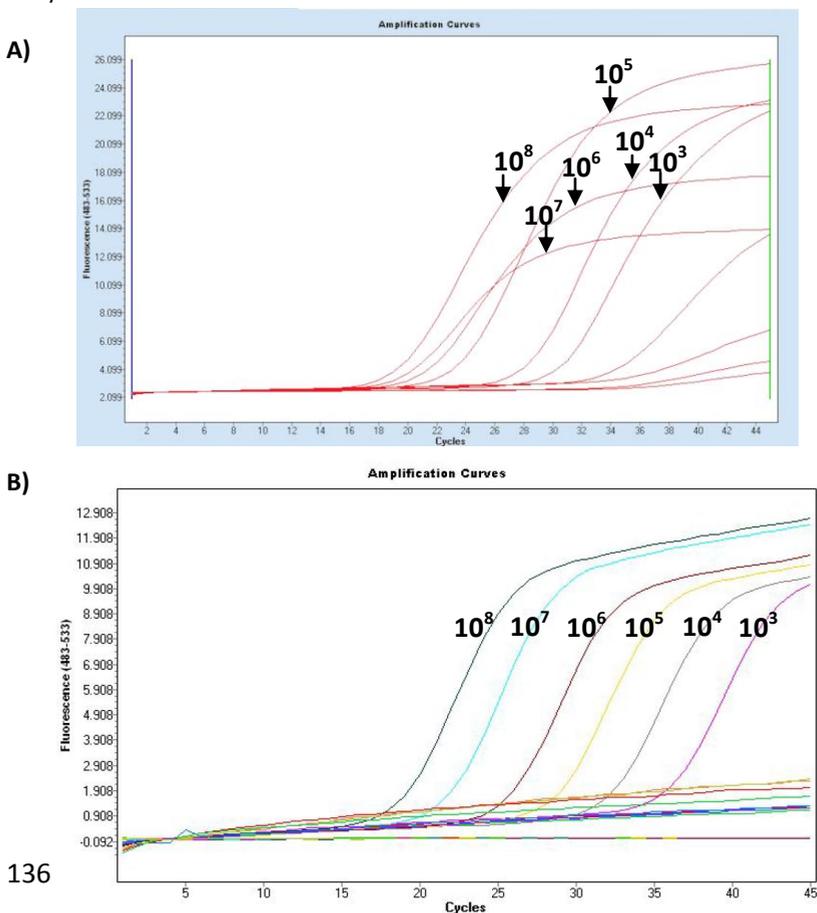


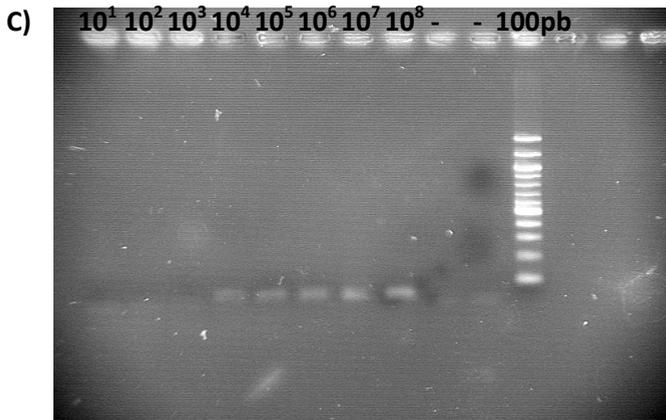
RESULTS

4.4. Sensitivity of *rpoS*, *katG* and *dsbC* primers

The sensitivity of the protocol for detection of strain CFBP 1430 by the primers from *rpoS* and *katG* genes by real-time PCR was 10^3 CFU/mL, as observed in amplification curves of the different cell concentrations (Fig. 16 A and B). The amplification with *katG* primers, however, showed not good reproducibility.

Figure 16. Primers sensitivity of stress-related genes in *E. amylovora* CFBP 1430 strain. A) *rpoS* and B) *katG* by real-time PCR. Fluorescence means wave length emitted by TaqMan probe at each decimal dilution. C) Sensitivity of *dsbC* primers by conventional PCR. In A, B and C, decimal numbers indicate CFU/mL.





For primers of *dsbC* gene, no amplification by real-time PCR was obtained because some problems intrinsic to probe design, but their sensitivity were 10^4 CFU/mL in conventional PCR (Fig. 16 C).

However, due to the problems associated to *katG* and *dsbC* amplification protocols we did not continue to work with them and we focused on the *rpoS* gene, which amplification protocol was running well in sensitivity and reproducibility and besides it is a master regulator of the general stress response.

4.5. Real-time PCR standard curve for *rpoS* gene

The two strategies assayed to generate RNA standards for *rpoS* gene didn't turn out equally efficient. The first strategy, based on cloning *rpoS* PCR product into pGEM-T easy vector system, introducing it into *E. coli*, and then transcribing multiple copies of the target gene, was finally discarded because some problems at the final step of the DNA to RNA transcription that were difficult to solve. Thus, although many

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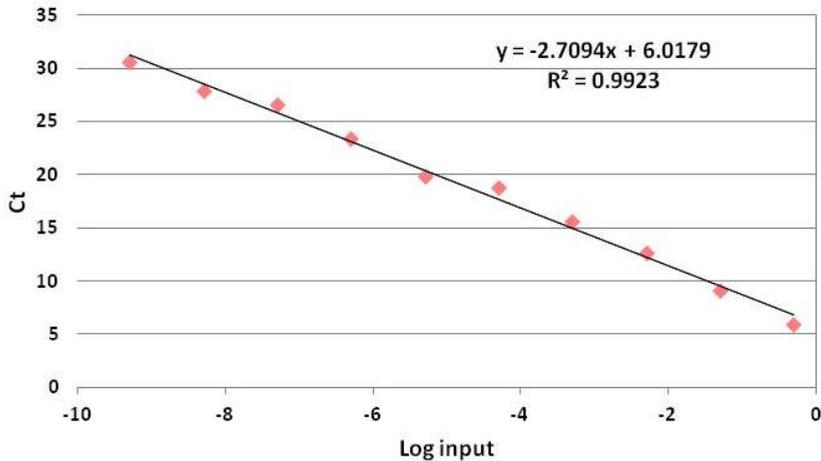
modifications were introduced in the protocol, an insufficient amount of digested plasmid was achieved.

Therefore, we finally chose the second strategy, based on amplification of *rpoS* gene using the external forward primer containing the promoter of T7 RNAPolymerase, which avoids an excessively long clonation process since the amplification product of the gene is directly transcribed to RNA. This strategy worked very well and it did not present any handicap.

The quantity of RNA was comparatively measured with NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific), Quant-iT RiboGreen RNA assay kit (Life Technologies) and Qubit® Fluorometric Quantitation (Life Technologies) device. Finally, the last one demonstrated to have the most reliable measure because of its accuracy based on the detection of RNA-specific fluorescence, and it was used to quantifying RNA in the standard curve for *rpoS* gene.

The semi-log regression line of C_T values versus log of input nucleic acid is represented in Fig. 17. According to this standard curve, the efficiency of PCR amplification for *rpoS* gene was around 82%, since a 100% efficient reaction will yield a 10-fold increase in PCR amplicon every 3.32 cycles during the exponential phase of amplification ($\log_2 10 = 3.3219$), and the slope of the standard curve for *rpoS* gene in our assay was -2.71, more positive than -3.32 characteristic of 100% efficiency.

Figure 17. Calibration curve, using RNA standards for *rpoS* gene, graphically represented as a semi-log regression line plot of Ct (cycle threshold) value vs log of input nucleic acid.

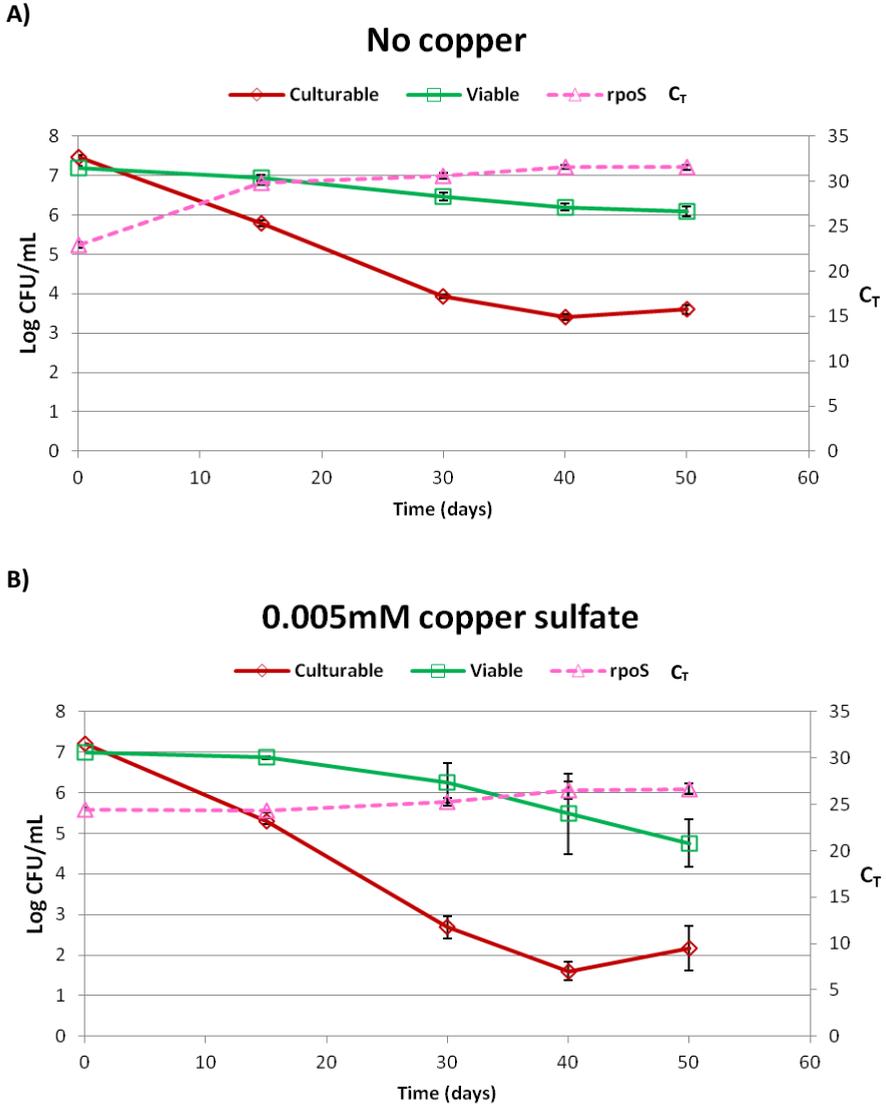


4.6. Long-term assay for *E. amylovora* survival in AB medium supplemented with copper sulfate and expression of *rpoS* gene through time

The lowest copper concentration assayed in short-term survival assays (0.005mM) which allowed the maintaining of culturability in the short-term (Fig. 15), was selected for long-term assays. At long term, culturability of *E. amylovora* CFBP 1430 strain decreased in more than three logarithmic orders after 50 days in AB medium with no copper, whereas viability only went down one log order in the same time period (Fig. 18 A).

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Figure 18. *E. amylovora* strain CFBP 1430 survival in AB medium supplemented with 0.005mM copper sulfate and expression of the *rpoS* gene through time. C_T means cycle threshold.



However, in the presence of copper the reduction in population numbers was more drastic than in absence of copper, both in culturability and viability, since culturable cells decreased in five log orders and viable cells in approximately two from the time 0 (Fig. 18 B). The trend of the respective curves for both culturable ($p=0.0472$) and viable ($p=0.0099$) cells was, in fact, significantly different between copper and no copper treatments.

Throughout this long-term period, expression of *rpoS* gene was evaluated by real-time PCR at each time point. The number of C_T in AB medium with no copper increased the first 15 days from 22.9 to 29.8, and then remained approximately constant until day 50 (Fig. 18 A). In contrast, in the presence of copper the value of C_T , after a slight initial increase, remained approximately constant around 25 (Fig. 18 B). This increase in C_T , regardless the presence of copper, indicated a reduction in *rpoS* expression throughout the time. However, the fact that the value of C_T was lower in presence than in absence of copper all the time assayed ($p<0.0001$) suggests a higher level of expression of the gene under copper stress.

4.7. General characteristics of the *E. amylovora* Ea1189 strain transcriptome in response to a copper shock

After the study of the role of *rpoS* gene in copper survival of *E. amylovora* strain CFBP 1430 and in order to have a more complete picture of the expressed genes by copper stress, a two-color whole genome microarray approach was used to identify differentially

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regulated genes under exposure to copper but in strain *Ea1189*. After microarray hybridization, we decided to analyze data corresponding only to chromosomal genes due to difficulties in finding match sequences for plasmids in databases. The analysis of the results of the first slide revealed 79 induced genes and 46 repressed genes and in the second slide, 85 genes were induced and 120 repressed. The combined analysis of the two experiments as a group (six replicates) identified 44 genes, out of 3,500 chromosomal genes included in the oligonucleotide microarray, that were differentially expressed after a copper shock.

Those 44 differentially expressed genes were classified in six categories according to several features. For this purpose, genes were submitted to databases and analyzed according to their gene ontology with GPRO software. Then, they were classified in transport, stress, metabolism, movement, programmed cell death and catch-all group named as others, as shown in Fig. 19 and Table 9.

Figure 19. Functional categories obtained by *gene ontology* of the 44 differentially expressed genes after a copper shock in *E. amylovora Ea1189* strain.

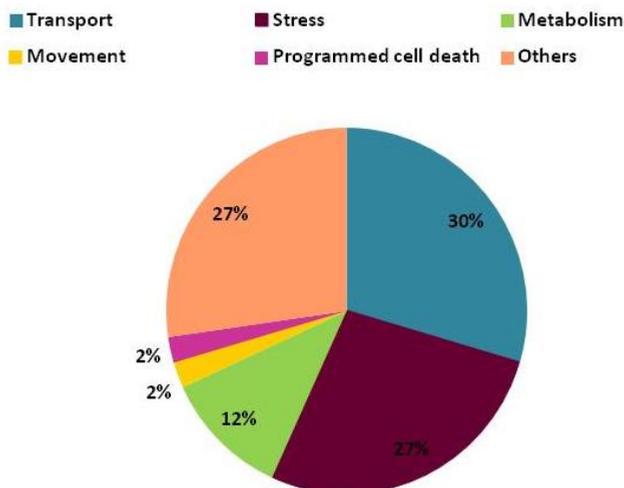


Table 9. Genes differentially expressed in *E. amylovora* Ea1189 in response to a copper shock. Bold words indicate up-regulated genes.

| Functional category ^a | Gene ID ^b | Gene name ^c | Product description | Fold change ^d | |
|----------------------------------|----------------------|------------------------|---|--|------------|
| Transport | EAM_0320 | <i>yjcE</i> | Na ⁺ /H ⁺ exchanger | 1.2 | |
| | EAM_1003 | <i>smdB</i> | Multidrug efflux pump ABC transporter | 1.5 | |
| | EAM_1136 | <i>gltI</i> | Glutamate and aspartate ABC transporter | 1.3 | |
| | EAM_1661 | <i>ydhC</i> | Inner membrane transport protein | 1.6 | |
| | EAM_1897 | <i>oppA</i> | Oligopeptide ABC transporter | 1.3 | |
| | EAM_2340 | <i>yfcA</i> | Inner membrane protein | 1.7 | |
| | EAM_2748 | <i>nodT</i> | RND efflux system, multidrug resistance protein (partial) detox protein CusA-like | 1.3 | |
| | EAM_0222 | <i>secE</i> | Preprotein translocase subunit | -1.3 | |
| | EAM_0954 | <i>proY</i> | Proline-specific permease | -1.2 | |
| | EAM_1044 | <i>copA</i> | Copper exporting ATPase | -1.1 | |
| | EAM_1612 | <i>spy</i> | Periplasmic protein spheroplast protein Y | -1.2 | |
| | EAM_1884 | <i>ompW</i> | Outer membrane protein | -1.2 | |
| | EAM_3433 | <i>tag3</i> | 3-methyl-adenine DNA glycosylase I | -1.3 | |
| | Stress | EAM_0207 | <i>tatC</i> | Twin-arginine protein translocation system | 1.2 |
| | | EAM_0221 | <i>tufA</i> | Protein chain elongation factor EF-Tu | 1.6 |
| EAM_0360 | | <i>dfoA</i> | Desferrioxamine siderophore biosynthesis | 1.1 | |
| EAM_0568 | | <i>rimI</i> | Ribosomal-protein-alanine acetyltransferase | 1.2 | |
| EAM_1023 | | <i>apt</i> | Adenine phosphoribosyltransferase | 1.2 | |
| EAM_2126 | | <i>galF</i> | UTP-glucose-1-phosphate uridylyltransferase | 1.2 | |
| EAM_3348 | | <i>soxS</i> | Transcriptional regulator | 1.2 | |
| EAM_0073 | | <i>rpmB</i> | 50S ribosomal protein L28 | -1.4 | |
| EAM_0127 | | <i>rpmE</i> | 50S ribosomal protein L28 | -1.4 | |
| EAM_1116 | | <i>cspE</i> | Cold shock-like protein | -1.5 | |
| EAM_2370 | | <i>uspA</i> | Universal stress protein | -1.2 | |

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| | | | SERP1273 | |
|------------------------------|----------|--------------------|--|------------|
| | EAM_2853 | | Putative helicase/relaxase | -1.5 |
| Metabolism | EAM_0136 | <i>ppc</i> | Phosphoenolpyruvate carboxylase | 1.2 |
| | EAM_0704 | <i>lpxC</i> | UDP-3-O-acyl N-acetylglucosamine deacetylase | -1.4 |
| | EAM_0861 | | Hypothetical polysaccharide deacetylase | -1.1 |
| | EAM_2964 | | 1-acyl-sn-glycerol-3-phosphate acyltransferase | -1.1 |
| | EAM_3110 | <i>arcB</i> | Aerobic respiration sensor-response protein | -1.5 |
| Movement | EAM_2538 | <i>cheA</i> | Chemotaxis protein | 1.9 |
| Programmed cell death | EAM_3469 | | Phage holin | 1.1 |
| Others | EAM_2701 | <i>ygcF</i> | Conserved hypothetical protein | 1.3 |
| | EAM_0537 | | Hypothetical protein | 1.8 |
| | EAM_2645 | | Hypothetical protein | 1.2 |
| | EAM_2818 | | Hypothetical protein | 1.4 |
| | EAM_3301 | <i>yhhQ</i> | Hypothetical protein | 1.2 |
| | EAM_3421 | | Hypothetical protein | 1.3 |
| | EAM_0684 | | No significant similarities found | -1.7 |
| | EAM_0927 | <i>psiF</i> | Phosphate starvation-inducible protein | -1.3 |
| | EAM_1312 | <i>ybjP</i> | Putative lipoprotein | -1.2 |
| | EAM_1634 | | Hypothetical protein | -1.1 |
| | EAM_2851 | | Hypothetical protein | -1.1 |
| | EAM_3016 | | Hypothetical protein | -1 |

a) Functional category obtained by gene ontology study using GPRO software v1.1.0.

b) Number of the gene according to *E. amylovora* ATCC 49946 sequencing genome.

c) Gene name according to highest similarity query with protein BLAST database. Genes in bold black are those that were up-regulated.

d) Obtained with a *p*-value \leq 0.05.

Transport category contained 13 genes whose functions were related with exchangers, ABC transporters, membrane proteins and efflux systems. We found several interesting genes as ***copA*** gene, coding for

a copper exporting ATPase; *yjcE* gene, coding for a Na⁺/H⁺ exchanger; two inner membrane proteins **YdhC** and **YfcA** and one outer membrane protein **OmpW**; *smdB* gene, coding for a multidrug efflux pump ABC; *nodT* gene, coding for a RND efflux system; *oppA* gene, coding for a oligopeptide ABC transporter; *gltI* gene, coding for a glutamate and aspartate ABC transporter or *tag3* gene, coding for a 3-methyl-adenine DNA glycosylase I. Fold-changes expression of these genes ranged between -1.1 and 1.7 (Table 9).

Stress category contained 12 genes whose functions were related with translocation systems, elongation factors (EF-Tu), siderophore biosynthesis, transcriptional regulators or stress proteins. We considered the transcriptional regulator *soxS* the most relevant gene of this category. However, we also found other interesting genes such as *dfoA* gene involved in desferrioxamine biosynthesis; *cspE* gene, coding for a cold shock-like protein or *uspA* coding for a universal stress protein; *tatC* gene, coding for a twin-arginine protein translocation system; *tufA* gene, coding for a protein chain elongation factor; *rimI* gene, coding for a ribosomal-protein-alanine acetyltransferase; *apt* gene, coding for a adenine phosphoribosyltransferase; *galF* gene, coding for a UTP-glucose-1-phosphate uridylyltransferase and **EAM_2853** gene, coding for a putative helicase/relaxase. Fold-changes expression range was between -1.5 and 1.6 (Table 9).

Metabolism category was smaller than the previous ones because it only contained 5 genes whose functions were related with Krebs's

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cycle and a respiration sensor-response protein. This last function, coded by *arcB* gene, was apparently the most important founded in this category because it is related to an aerobic-anaerobic switch that could be connected with an answer to redox events produced by copper. Most of the genes in this category were down-regulated with fold-changes ranging from -1.1 to -1.5. Only *ppc* gene, coding for a phosphoenolpyruvate carboxylase that catalyzes the β -carboxylation of phosphoenolpyruvate (PEP), was 1.2fold-change up-regulated. This is a high energetic reaction that supplies dicarboxylic acids to the tricarbolixic acid's cycle (Krebs's cycle) (Table 9).

Only one gene, *cheA*, coding for a chemotaxis protein, was allocated to movement category. And only other gene, **EAM_3469**, was classified in the group of programmed cell death since it codes for a phage holin. Both genes were up-regulated with fold-changes of 1.9 and 1.1, respectively (Table 9).

No function was found for twelve out of the 44 genes differentially expressed after copper shock. We classified them inside a category named "others". Only the sequences for *ygcF*, *yhhQ*, *psiF* and *ybjP* genes were annotated but others like the one for **EAM_1634** gene were not and were classified only as hypothetical proteins. We selected *ygcF* and *yhhQ* for further analysis because they were up-regulated and, in the case of *ygcF*, also due to be conserved. We also selected **EAM_1634** gene because it was down-regulated (Table 9).

4.8. Evaluation of differentially expressed genes under copper conditions by quantitative real-time PCR

To validate gene expression patterns observed in our microarray analysis by a specific and independent technology, the expression of 25 genes, selected among those identified as differentially expressed, was examined by quantitative real-time PCR after optimizing RNAs according to 16S rRNA levels. According to the formula $E=10^{-1/\text{slope}}$, in an ideal situation, the quantity of PCR product should duplicate in each amplification cycle. As it is shown in Table 10, the optimization of each pair of primers for quantitative real-time PCR is around 2, what means they have 100% efficiency. Selection of genes was made according to their probable relation with the function resulted from the annotation with databases and GPRO software, and the putative function in the case of the hypothetical proteins. These genes were: *yjcE*, *smdB*, *gltI*, *ydhC*, *oppA*, *yfcA*, *nodT*, *copA* and *tag3* from transport category; *tatC*, *tufA*, *dfoA*, *rimI*, *apt*, *galF*, *soxS*, *uspA* and **EAM_2853** genes from stress; *ppc* and *arcB* genes from metabolism; *cheA* gene from movement; **EAM_3469** gene from programmed cell death; and *ygcF*, *yhhQ*, and **EAM_1634** genes from “others” category.

Expression of selected target genes by real-time PCR upon exposure to copper was not completely in accordance with the microarray results. The most remarkable divergence was the *copA* gene, which exhibited down-regulation in the microarray analysis but had the highest up-regulation when its expression was measured by

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quantitative real-time PCR, with a value of more than 19-fold increase (Fig. 20). Other genes that showed down-regulation in the microarray but were induced as monitored by PCR were **EAM_1634**, **arcB**, **tag3**, **EAM_2853** and **uspA**, with values of induction of 1.79-, 1.60-, 1.51-, 1.49- and 1.37-fold increase, respectively (Fig. 20).

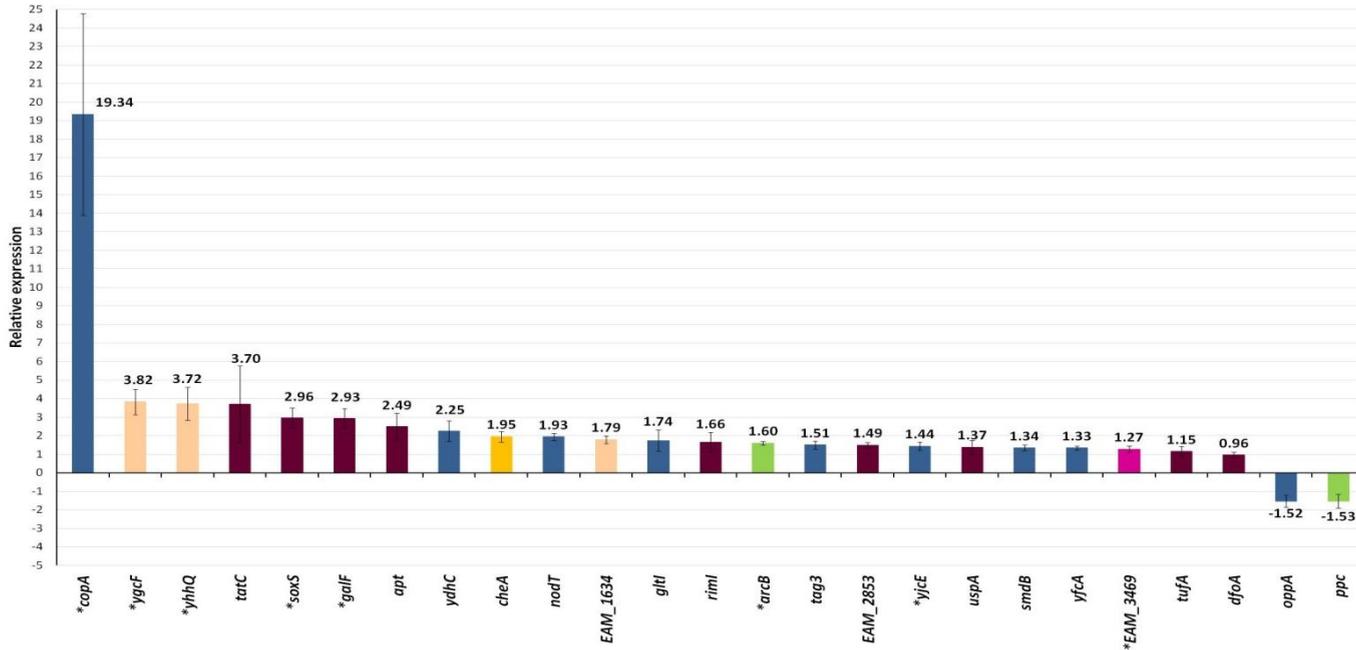
The relative expression of the rest of the analyzed genes ranged from 0.96-fold induction for gene *dfoA* to 3.82-fold induction for gene *ygcF*, and all of them were confirmed as up-regulated (Fig. 20). Finally, only **oppA** and **ppc** genes, both up-regulated in the microarray analysis, were revealed as down-regulated by quantitative real-time PCR in response to copper.

Table 10. Efficiencies of the PCR optimization with the 16S gene as internal control at the melting temperature of each primer.

| Gene name | Target gene efficiency | 16S gene efficiency |
|-------------|------------------------|---------------------|
| <i>copA</i> | 1.984 | 2.029 |
| <i>ygcF</i> | 2.013 | 2.029 |
| <i>yhhQ</i> | 1.989 | 2.040 |
| <i>tatC</i> | 2.022 | 2.029 |
| <i>soxS</i> | 2.029 | 2.040 |
| <i>galF</i> | 1.989 | 2.040 |
| <i>apt</i> | 2.048 | 2.040 |
| <i>ydhC</i> | 2.038 | 2.029 |
| <i>cheA</i> | 1.975 | 2.040 |
| <i>nodT</i> | 2.015 | 2.030 |
| EAM_1634 | 2.044 | 2.040 |
| <i>gltI</i> | 1.979 | 2.040 |
| <i>rimI</i> | 2.006 | 2.029 |
| <i>arcB</i> | 2.036 | 2.040 |
| <i>tag3</i> | 2.040 | 2.040 |
| EAM_2853 | 2.035 | 2.040 |
| <i>yjcE</i> | 2.010 | 2.030 |
| <i>uspA</i> | 2.016 | 2.029 |
| <i>smdB</i> | 2.010 | 2.030 |
| <i>yfcA</i> | 2.011 | 2.029 |
| EAM_3469 | 2.004 | 2.030 |
| <i>tufA</i> | 2.021 | 2.029 |
| <i>dfoA</i> | 2.037 | 2.040 |
| <i>oppA</i> | 2.032 | 2.029 |
| <i>ppc</i> | 2.010 | 2.030 |

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Figure 20. Relative expression of 25 differentially expressed genes in *E. amylovora* Ea1189 after a copper shock. Color indicates the functional category assigned before in section 4.7. For more info also see Fig. 19 and table 8.



The 16S rDNA gene was used as an endogenous control in quantitative real-time PCR to calculate the relative expression. Data of C_T were analyzed quantitatively via the comparative cycle threshold ($\Delta\Delta C_T$) method (Pfaffl, 2001) to generate relative fold-change values comparing WT and mutant strains.

* Inactivated genes for further studies.

4.9. Mutants of selected genes of *E. amylovora*

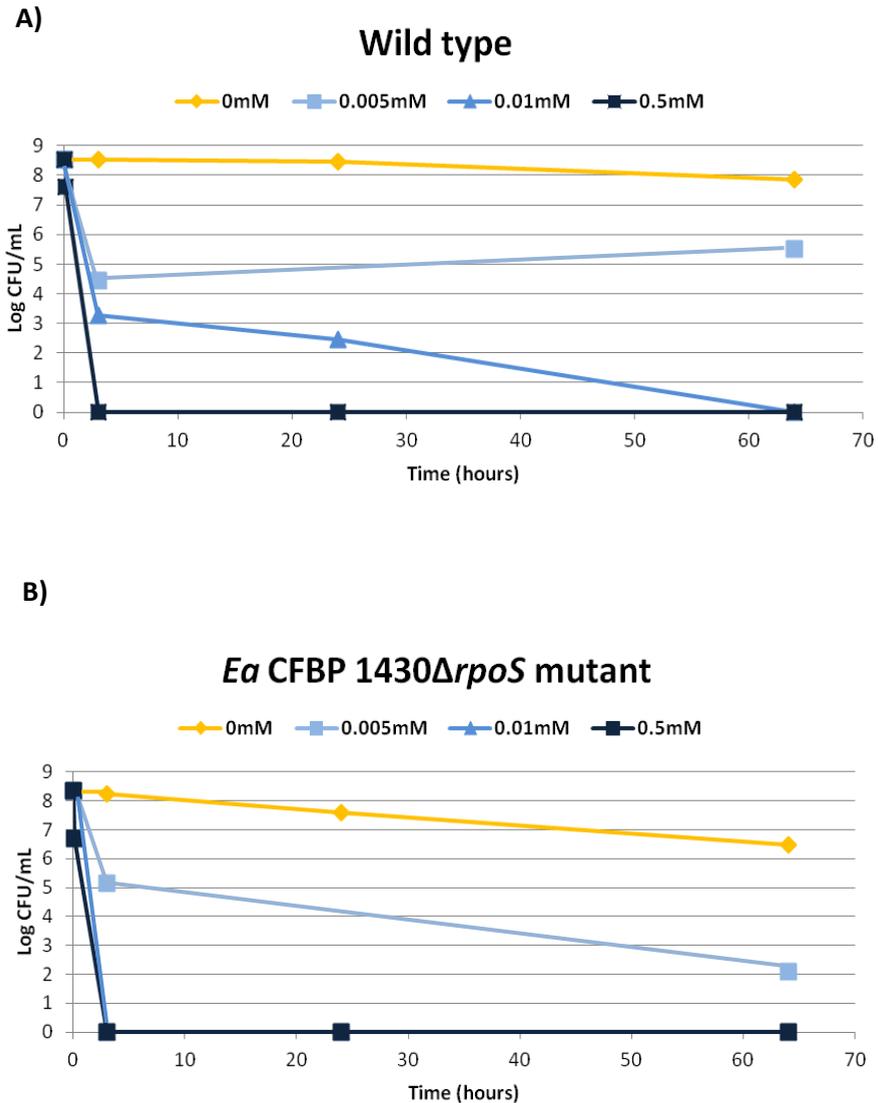
4.9.1. Assays to evaluate the possible role of *rpoS* gene

A mutant of CFBP 1430 strain in *rpoS* gene (Santander *et al.*, 2014), was used to evaluate its role in culturability in the presence of copper. As shown in Fig. 21, mutant culturability was lower than that of wild type, even in AB medium with no copper (Fig. 21). Whereas the wild type strain remained near 10^8 CFU/mL for more than 60 days (Fig. 21 A), the mutant dropped two logarithmic orders in the same period (Fig. 21 B).

The difference between the wild type and the mutant was more pronounced in the presence of copper. At the lowest copper concentration (0.005mM) culturability of the mutant was reduced in more than six log orders, and at 0.01mM it already was under the detection limit at 24h (Fig. 21 B). This was contrasting to the culturability of the wild type, which was decreasing gradually through time (Fig. 21 A). At the highest copper concentration (0.5mM) the culturability of both the wild type and the mutant was under the detection limit already at 24h (Fig. 21).

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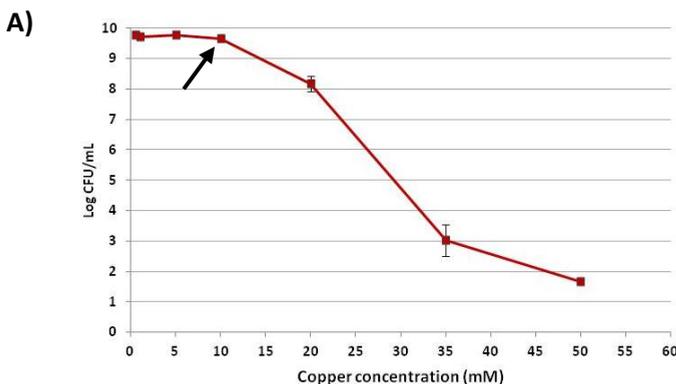
Figure 21. (A) Survival of the *E. amylovora* CFBP 1430 wild type incubated in AB medium supplemented with copper sulfate along 64 hours. (B) Survival of the *E. amylovora* CFBP 1430 *rpoS*⁻ mutant incubated in AB medium supplemented with copper sulfate along 64 hours.



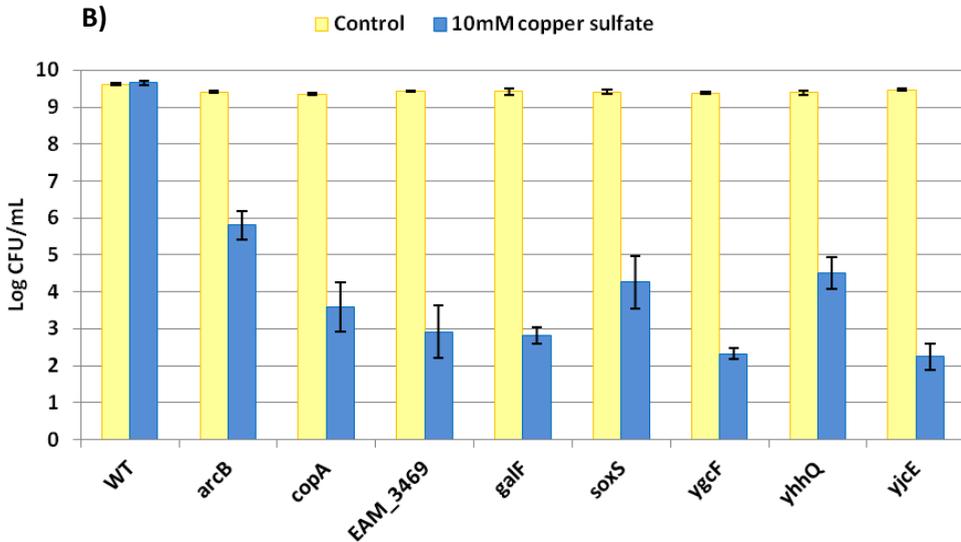
4.9.2. Assays to evaluate the possible role of *copA*, *soxS*, *arcB*, *yjcE*, *ygcF*, *yhhQ*, *galF* and *EAM_3469* genes

4.9.2.1. Copper tolerance *in vitro*. To investigate which genetic systems can operate in *E. amylovora* after a copper shock, *Ea1189* mutants in eight selected up-regulated genes were evaluated for copper tolerance. Selected genes were: *copA*, *soxS*, *arcB*, *yjcE*, *ygcF*, *yhhQ*, *galF* and *EAM_3469*. Culturability was not affected in the wild type strain after treatment with copper sulfate below 20mM, but at 20mM and higher concentrations it was severely reduced (Fig. 22 A). Then, mutants were challenged with the maximal copper concentration with no effect on the wild type strain, that was, 10mM. As shown in Fig. 22 B, the culturability of all the mutants decreased drastically between 4 and 7 orders of magnitude, depending on the inactivated gene, respect to untreated controls.

Figure 22. (A) Culturability of *E. amylovora* strain *Ea1189* growing in liquid media after treatment with copper at different concentrations. Arrow indicates the maximal Cu^{++} concentration with no effect in culturability of the *Ea1189* wild type. (B) Copper sensitivity of selected *E. amylovora* strain *Ea1189* mutants treated with 10mM copper sulfate. Down, the name of the inactivated genes.

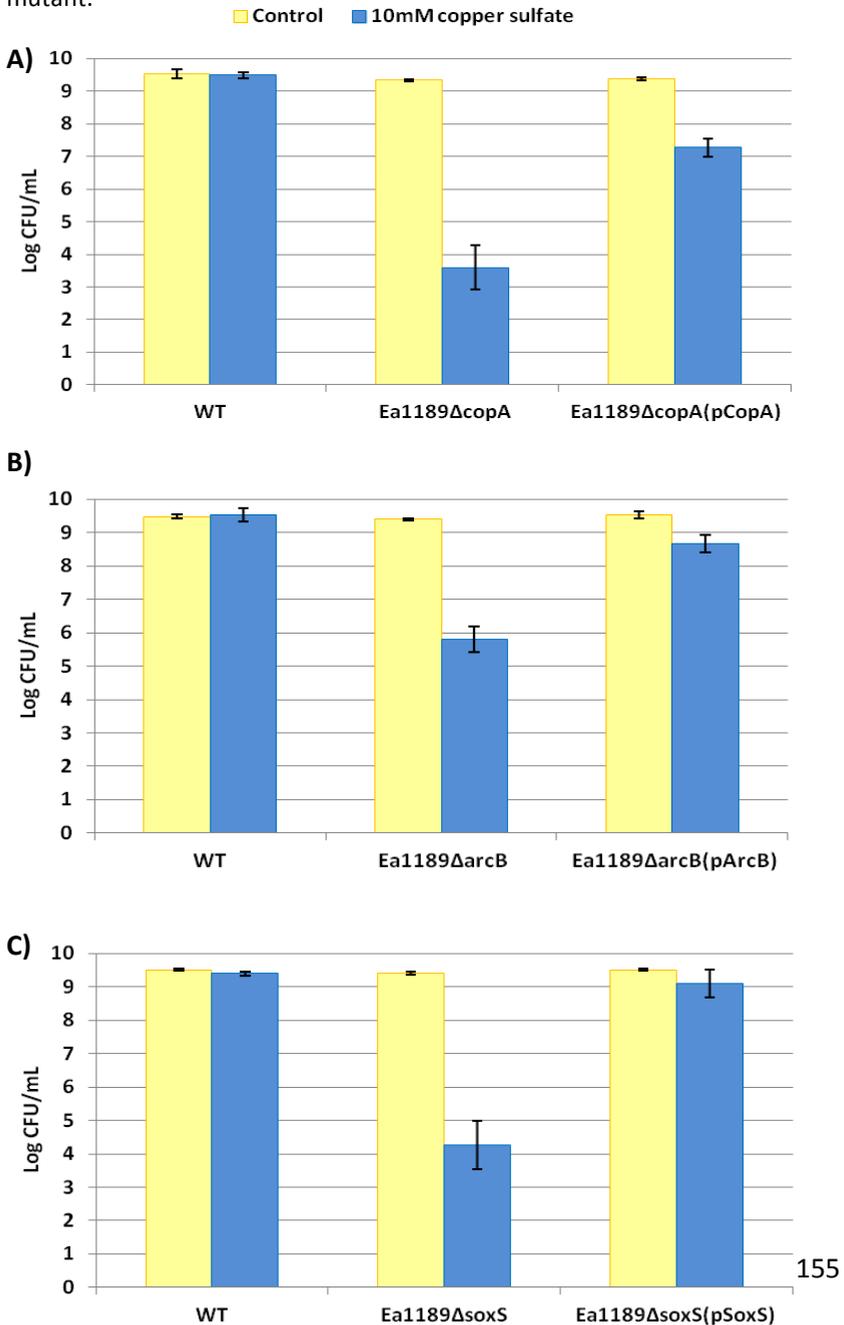


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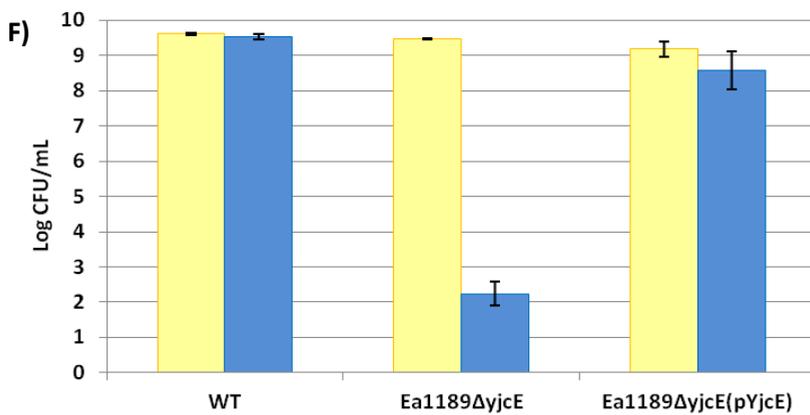
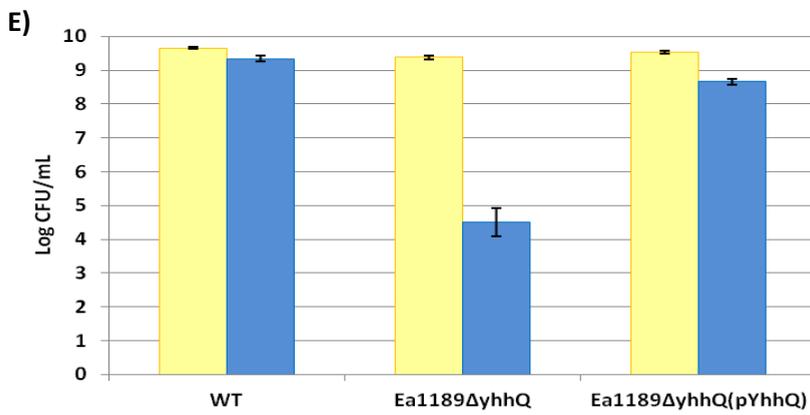
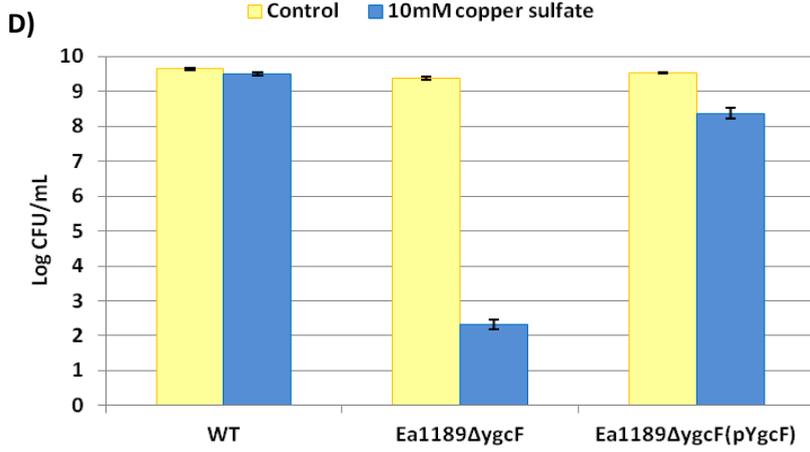


4.9.2.2. Introduction of target genes in the mutants. The ORF of each selected gene as well as the upstream mutagenic region were amplified by PCR, cloned into pBBR1 and introduced into each respective *Ea1189* mutant. The cloned *copA* gene restored almost completely the culturability of the mutant, which dropped more than six orders of magnitude after a copper shock respect to no copper treatment and respect to that of wild type strain under the same copper shock conditions (Fig. 23 A). Culturability of *arcB*, *soxS* and *yhhQ* mutants decreased approximately by two orders of magnitude respect to wild type strain (Fig. 23 B, C, E), whereas that of *ycgF* and *yjcE* mutants was reduced about four orders of magnitude (Fig. 23 D, F). When the respective genes were introduced in each mutant, the wild phenotypes were restored in all cases (Fig. 23), indicating an important role of *copA*, *arcB*, *soxS*, *yhhQ*, *ycgF* and *yjcE* genes for *E. amylovora* survival facing copper shock.

Figure 23. Genes confirmed to have a role in the defense of *E. amylovora* against a copper shock. A) *copA* gene B) *arcB* gene, C) *soxS* gene, D) *ycgF* gene, E) *yhhQ* and F) *ycjE* gene. Down, WT means wild type, *Ea1189ΔgeneX* means mutant strain and *Ea1189ΔgeneX(pGeneX)* means complemented mutant.



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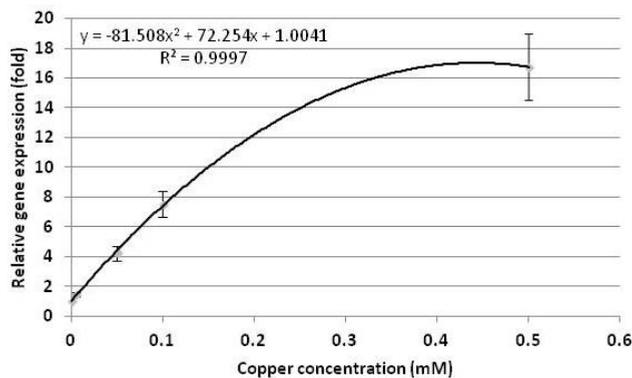
The genes EAM_3469 and galF were not complemented due to lack of time in the period of this thesis but we will keep them in mind for future research.

4.10. The *copA* case

4.10.1. Induced expression of *copA* gene

In order to assess the differential expression of *copA* gene in *E. amylovora* strain Ea1189, a relative quantification by real-time PCR was performed after submitting the strain to a copper-shock induction. As shown in figure 24, *copA* gene was induced in the presence of copper. A very low copper concentration of 0.005mM increased the expression of *copA* 1.5-fold compared with the expression at no copper exposure (1-fold). Interestingly, the relative expression of *copA* gene was observed to raise as soon as the copper concentration was increased, reaching 16.8-fold expression when the strain was challenged with copper sulfate 0.5mM (Fig. 24).

Figure 24. Relative expression curve of *copA* gene in *E. amylovora* after copper-shock induction.



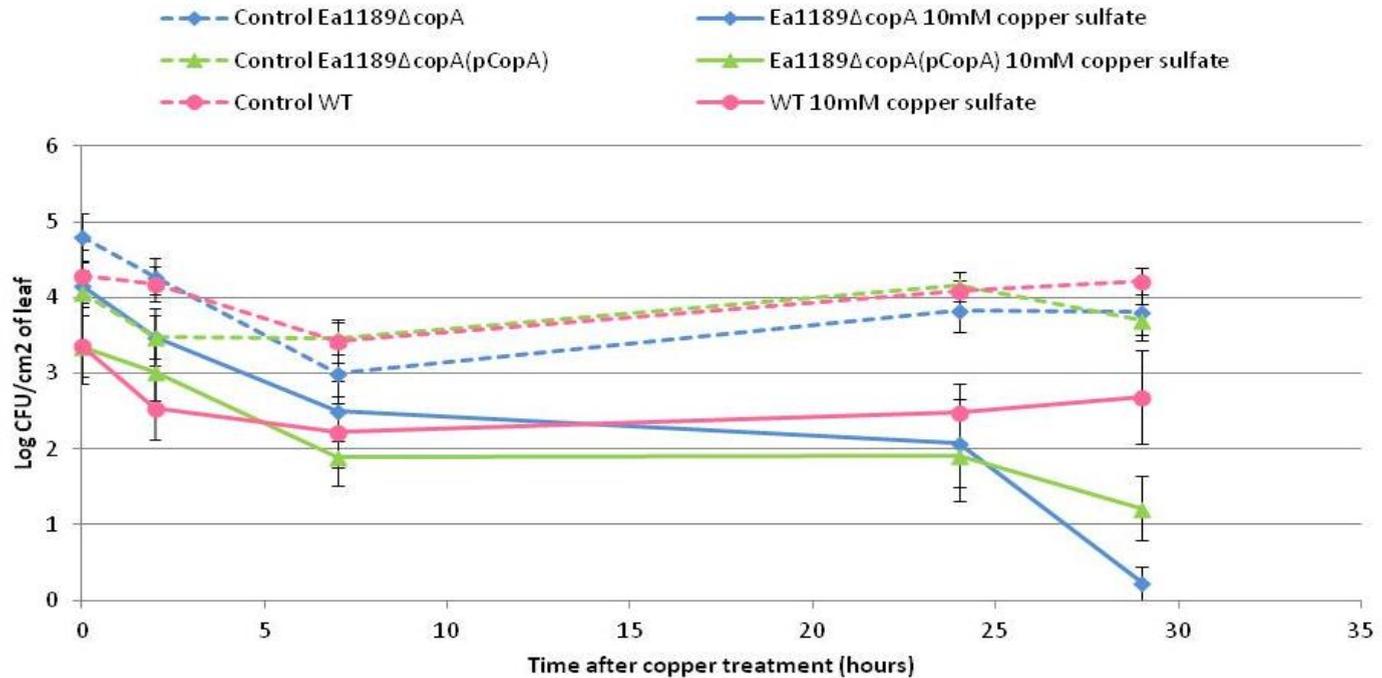
4.10.2. Involvement of the *copA* gene in copper tolerance *in planta*

Due to its high expression level in the presence of copper (Fig. 20), coupled with its demonstrated role in copper tolerance *in vitro* (Fig. 23 A), *copA* gene was selected to further investigate whether it also plays a role in copper tolerance *in vivo* in *E. amylovora* Ea1189. The culturability of *copA* mutant *in planta* was firstly assayed without copper treatment, and it was slightly lower than that of wild type (Fig. 25, blue and pink dashed lines), but the trend of both curves, those of wild type and mutant, through time was not significantly different ($p=0.12$). Interestingly, when the cloned *copA* gene was inserted in the mutant, the numbers of culturable cells recovered those of wild type (Fig. 25, green dashed line). Then, the effect of copper was comparatively evaluated in the three bacterial populations: the wild type, the *copA* mutant and the mutant complemented with *copA* gene. As seen in Fig. 25, copper affected to both mutant (blue continuous line) and wild type (pink continuous line) strains, but this effect was more drastic for the mutant. Thus, culturability of wild type was decreased *in planta* after two hours of copper exposure (Fig. 25, pink continuous versus pink dashed lines), reducing its population almost two orders of magnitude and maintaining at this level throughout the experiment. As for the mutant strain, significant differences in culturability were observed respect to wild type strain at 29h after the copper exposure ($p=0.009$), with a loss of about four orders of magnitude from the inoculation time (Fig. 25, blue continuous line). However at this time,

the culturability of the mutant was restored when the cloned *copA* gene had been introduced (Fig. 25, green continuous line). Although this restoration was only partial, as occurred in *in vitro* assays, the survival of wild type and the complemented mutant was not significantly different ($p=0.25$) through time, confirming the effect of copper in *copA* mutant.

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Figure 25. Role of *copA* gene in *E. amylovora* strain *Ea1189* to 10mM copper sulfate *in planta*. Dashed lines means untreated and continuous lines means after being treated with 10mM of copper sulfate. Wild type strain is represented by a pink circle, mutant *Ea1189ΔcopA* is represented by a blue rhombus and complemented mutant *Ea1189ΔcopA(pCopA)* is represented by a green triangle.



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Copper-based compounds are among the antibacterial agents most widely used in agriculture, and they have been extensively employed for decades to deal with the devastating plant disease fire blight (van der Zwet *et al.*, 2012). But it has also been described that they are not completely efficient to control the disease because, when plants are treated with copper, *E. amylovora* can develop a survival strategy that allows it to persist in time: the viable but nonculturable (VBNC) state (Ordax *et al.*, 2006). In fact, the role of copper as a stress factor for *E. amylovora* was described by the research group in which the present thesis was performed (Ordax, 2008), so the goals of this work were planned as a continuation of the previous findings. Thus, once determined that copper triggers a survival response in *E. amylovora*, we were interested in unraveling the molecular mechanisms involved in this interaction.

First, we addressed this issue by selecting some genes, such as the Sigma factor *rpoS*, that were previously described as important in the bacterial response to several stresses (Battesti *et al.*, 2011; Landini *et al.*, 2014), although not specifically reported in relation to copper. Secondly, we decided to go forward with a transcriptomic study to evaluate the global response of *E. amylovora* to copper, taking advantage of the customized microarray for *E. amylovora* by courtesy of Ian Toth (The James Hutton Institute (JHI), formerly Scottish Crop Research Institute (SCRI)). The experimental design, based on the work of Yamamoto and Ishihama (2005) for another enterobacterium, *E. coli*, consisted on challenging *E. amylovora* to a

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copper sulfate shock and to assess the genes induced under this stressful condition.

All the data obtained from the microarray allowed designing experiments to evaluate the role of some selected genes *in vitro* and lately *in vivo*. We made a selection of genes differentially expressed by copper to validate their expression using quantitative real-time PCR. Then, we constructed *E. amylovora* mutants in those genes exhibiting a higher expression level as measured by quantitative real-time PCR and/or their interest as potentially related to the response of *E. amylovora* facing copper stress. Then, we tested the phenotype of these mutants after challenging them to copper and we complemented the mutants with the introduction of the corresponding gene and verified that the phenotype of the wild type strain was restored. Finally, we selected one of the genes to test its role also *in vivo*. In fact, the validation *in planta* of the role of *copA* gene in the survival of *E. amylovora* to copper exposure is one of the most relevant results of this work. Nevertheless, other remarkable result is the general overview of the genes involved in the survival of *E. amylovora* to copper stress. Up to date, the global transcriptional response of *E. amylovora* against copper had not been elucidated yet. The type of copper stress selected in this study was a rapid shock of 5 min, not only reproducing the conditions of a previous work with *E. coli* (Yamamoto and Ishihama, 2005) but also to avoid the influence of long-term exhaustion of nutrients, the production of toxic metabolites coming from the active metabolism or other factors that could interact with the direct copper effect.

The information provided by this work constitutes a first step in the way of a future research about possible targets for more efficient chemical treatments for fire blight disease. According to the data obtained, it is possible that a treatment against fire blight based on a combination of copper sulfate and an inhibitor of CopA protein would be more effective than copper alone. It is an issue for future research.

After summarizing the global sense and the main achievements of this thesis, the different tasks addressed in the whole work are discussed in detail. As a previous step in the study of the molecular response of *E. amylovora* to copper, the minimal inhibitory concentration (MIC) of this metal was firstly determined to decide the range of copper concentrations to be assayed. On one side, since King's B (KB) and Luria Bertani (LB) media bounded copper at the same extent, we selected KB for comparative purposes because it was used in previous MIC studies with *E. amylovora* (Ordax, 2008). On the other side, we used CYE medium (Zevenhuizen *et al.*, 1979) and our results confirmed those reporting its usefulness for MIC determination since it does not bind any copper (Sholberg *et al.*, 2001; Al-Daoude *et al.*, 2009). In fact, the MIC obtained in CYE, 1mM, was lower than in KB because KB has a greater ability to bind copper, then reducing the amount of free copper that can affect bacterial cells. Ordax (2008) also obtained a MIC higher than 1mM in KB (2.5mM), even in liquid cultures, where all components, including copper, are more accessible to cells, what usually leads to a decrease

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of the MIC, but not in KB due to its copper complexing ability. Therefore, the MIC for the three studied strains was established in CYE at 1mM, pretty similar to that obtained in other studies performed using *E. amylovora* isolates from other geographical origins, such as Syria (Al-Daoude *et al.*, 2009) and British Columbia (Sholberg *et al.*, 2001), which were considered as copper tolerant strains (growth at 1.2mM copper sulfate).

5.1. Survival of *E. amylovora* under several copper conditions and the role of a general stress regulator

The survival of *E. amylovora* in the presence of two copper concentrations below the established MIC (0.005 and 0.5mM) was studied. The lowest copper concentration assayed (0.005mM) was found to cause a significant decrease of two logarithmic orders in CFBP1430 strain population, while the shock with higher copper concentrations (up to 0.5mM) caused a more severe decrease. In long-term survival assays in distilled water without addition of copper, numbers of culturable cells were reduced in approximately three logarithmic orders after 50 days due to the effect of nutrient starvation (Biosca *et al.*, 2006; Ordax *et al.*, 2006). When adding copper (0.005mM), the culturability dropped five log orders in the same time period. In both cases (in absence or presence of copper) a percentage of the population adopted the VBNC state, as previously reported (Biosca *et al.*, 2006; Ordax *et al.*, 2006). This was indicated by the fact that the number of viable cells, according to a culture-independent method (Live & Dead), was not reduced at the same extent as the number of culturable ones. Nevertheless, the fraction of bacterial population in VBNC state was higher in presence of copper due to the joined effect of two stress factors, copper and starvation. The induction of this state by copper has not only been demonstrated in *E. amylovora* (Ordax *et al.*, 2006) but also in other phytopathogenic bacteria (Alexander *et al.*, 1999; Ghezzi and Steck, 1999; del Campo *et al.*, 2009; Um *et al.*, 2013). The present results

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are, therefore, consistent with those previously obtained by Ordax *et al.* (2006). Interestingly, copper at all the assayed concentrations did neither cause cellular lysis nor kill *E. amylovora* cells in other way (Ordax *et al.*, 2006). Bacteria are able to face up to stress through adaptation responses, and many of these responses involve specific pathways that include a given regulator and a set of regulated genes (the regulon) which helps the cell to survive to the stress (Battesti *et al.*, 2011). In consequence, it is very interesting to evaluate the role of these genes to elucidate molecular mechanisms underlying the response of *E. amylovora* to copper stress. As a first approach, we analyzed the expression of the general stress response regulator gene, *rpoS*, because RpoS regulon has been reported as playing a critical role in *E. coli* survival against acid (Small *et al.*, 1994), heat (Hengge-Aronis *et al.*, 1991), oxidative stress (Sammartano *et al.*, 1986), starvation (Lange and Hengge-Aronis, 1991), and near-UV exposure (Sammartano *et al.*, 1986). In *E. amylovora*, an altered expression of this gene under starvation stress has been recently described (Santander *et al.*, 2014), but nothing is known about its relation with copper stress. In other *Enterobacteriaceae* as *E. coli*, the RpoS regulon comprises 10% of its genes (Lacour and Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005; Dong *et al.*, 2008; Dong and Schellhorn, 2009), what gives an idea of its relevance.

We observed that when *E. amylovora* was exposed to copper, the level of *rpoS* expression was significantly higher than in absence of copper. In both cases, with and without copper, there was a

progressively reduction of *rpoS* expression through time, probably because cells needed to save energy since they were maintained in a non chemostat container with mineral medium for a very long period (50 days). However, the reduction in the *rpoS* expression was significantly less pronounced in the presence of copper, indicating that this gene was more active than in absence of the metal. The link between *rpoS* and copper is probably related to oxidative stress provoked by copper excess. In *E. coli*, it has been described that copper overload induces the expression of RpoS, OxyR and SoxRS regulons (Macomber *et al.*, 2007), and in *P. aeruginosa* that there is an increase of a periplasmic protein as a reaction to copper imbalance, leading to oxidative stress (Vijgenboom *et al.*, 1997; Raimunda *et al.*, 2013). Moreover, our results are in accordance with previous works on the role of the RpoS in the defense response of *E. amylovora* (Santander *et al.*, 2014) and also in other phytopathogenic bacteria such as *Xanthomonas campestris* and *Pseudomonas fluorescens* (Rao and Sureshkumar, 2000; Stockwell *et al.*, 2009) in defense against reactive oxygen species (ROS).

5.2. Global response against a copper shock

With the aim to dig into the entire system of *E. amylovora* genome-wide response to external copper, we carried out a search for copper-responsive genes by a transcriptomic approach. The experimental design was based on a study performed with copper and the model bacterium *E. coli* (Yamamoto and Ishihama, 2005), another member of *Enterobacteriaceae*, because no previous work with *E. amylovora* on this issue has been reported.

We first carried out a systematic search for copper-responsive genes by using DNA microarray technique. A copper shock markedly activated 23 genes of the pathogen, being ca. 50% of those induced genes classified into the categories of transport (*yjcE*, *smdB*, *gltI*, *ydhC*, *oppA*, *ycfA* and *EAM_2748*) and stress (*tatC*, *tufA*, *dfoA*, *rimL*, *apt*, *galF* and *soxS*), and some also into metabolism (*ppc*), movement (*cheA*), and programmed cell death (*EAM_3469*), remaining 6 as unclassified genes (*EAM_0537*, *EAM_2645*, *ygcF*, *EAM_2818*, *yhhQ* and *EAM_3421*).

In the transcriptional assay previously conducted with *E. coli* (Yamamoto and Ishihama, 2005), 30 copper-shock activated genes were identified and classified into their putative regulons: 5 genes were classified into the CusR regulon, 6 into the CueR regulon, 11 into the CpxR regulon and another 8 genes remained unclassified. That previous study concluded that the copper stimulon is formed by the activated genes under the control of at least four regulons: CueR,

CusR, CpxR and YedW, in a hierarchical regulation network to maintain copper homeostasis. Most of the genes identified in our study differ from those identified in the *E. coli* copper shock microarray study (Yamamoto and Ishihama, 2005). If the conditions of each study are compared, although we isolated mRNA 5 min after the copper shock, as in the *E. coli* study, the growth phase of the challenged cells was different. Transcriptome of *E. coli* was obtained in exponential growth while that of *E. amylovora* was obtained in late exponential growth or beginning of the stationary phase. This difference may have contributed to observed differences in gene expression. However, both our current study with *E. amylovora* and the previous study with *E. coli* included the hitherto identified *copA* gene encoding a Cu⁺-translocating P-type ATPase pump, which exports copper from the cytoplasm to the periplasm and is directly involved in maintaining copper homeostasis in *E. coli* (Rensing and Grass, 2003; Yamamoto and Ishihama, 2005). Besides *copA*, the transcription factor for superoxide response regulon SoxS is copper-activated both in *E. coli* (Yamamoto and Ishihama, 2005; Pletzer *et al.*, 2014) and in *E. amylovora*, as showed in the present work.

In plant pathogenic bacteria, there are no previous studies evaluating transcriptional gene expression after copper stress. The present study raises for the first time the association between copper stress and its effects in the plant pathogenic bacterium *E. amylovora*, copper homeostasis and production of oxidative radicals.

5.2.1. Copper transport genes (copper homeostasis)

As it was described before, copper ion can be a toxic element if it is present over a certain threshold, so metal homeostasis has an essential role in bacterial survival. As a consequence of an aerobic metabolism, copper catalyzes Fenton-like and Haber-Weiss reactions that result in the production of hydroxyl radicals ($\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{HO}^\bullet + \text{HO}^- + \text{Cu}^{++}$; $\text{O}_2^{\bullet-} + \text{Cu}^{++} \rightarrow \text{Cu}^+ + \text{O}_2$) (Halliwell and Gutteridge, 1990, 1984; Rensing and Franke-McDevitt, 2013). The main pool of intracellular copper ions, as reported in *E. coli*, is located in the periplasm (Outten *et al.*, 2001), and the reactive oxygen species (ROS) produced by copper do not reach the cytosolic targets (Macomber *et al.*, 2007; Lemire *et al.*, 2013). However, if the accumulation of ROS in the periplasm is over a certain threshold, a leakage of these toxic species could happen into the cytosol, where they trigger the activation of the ROS defense systems. To avoid these events in cascade, metal efflux and uptake systems are transport mechanisms displayed by bacterial cells to achieve copper homeostasis.

The copper homeostasis protein repertoire described in *E. coli* comprises two systems: one CueR-regulated, which plays a key role in aerobic copper tolerance (Grass and Rensing, 2001; Outten *et al.*, 2001), and other regulated by *cusSR* for anaerobic conditions (Outten *et al.*, 2001). According to Hernández-Montes *et al.* (2012), this dual copper homeostasis protein repertoire is only present in 3% of all organisms tested (among others 268 gamma proteobacteria,

two strains of *E. amylovora* (TAX552 and ATCC 49946), one strain of *E. pyrifoliae* and one strain of *E. tasmaniensis*).

A recent phylogenomic study revealed that the genomes of two *E. amylovora* strains (ATCC 49946 and TAX552) encode at least three periplasmic proteins involved in copper homeostasis: CopA, CueO and an orphan CusC (Hernandez-Montes et al., 2012). While CopA pumps out excess copper from the cytoplasm to the periplasm, CueO oxidizes Cu^+ to Cu^{++} in the periplasm thereby reducing the concentration of the more toxic Cu^+ ion (Grass and Rensising, 2001). In *E. coli*, under anaerobic conditions the two-component regulatory system CusSR activates the transcription of the *cusCBAF* operon that encodes for a complex that pumps Cu^+ from the cytoplasm directly to the extracellular space (Grass and Rensising, 2001). In most bacteria, this complex consists of the inner membrane pump CusA, the periplasmic protein CusB and the outer membrane protein CusC, forming a channel through the periplasm (Hernandez-Montes et al., 2012). In the genome of *E. amylovora*, only an orphan *cusC* gene is present but, surprisingly, it is located in an operon together with two other putative multidrug efflux transporter genes, *emrB* and *emrA*. Thus, it is possible that they may form another complex pump also dedicated to extrude Cu^+ directly from the cytoplasm to the external space, as the *cusCBAF* system does. However, since we did not observe an upregulation of either *emrB* or *emrA*, these multidrug efflux transporter genes may not respond to copper in aerobic metabolism in *E. amylovora*, confirming that described for *E. coli* (Yamamoto and Ishihama, 2005). It is important to note here that, in

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contrast to other enterobacteria, *E. amylovora* is not very efficient in anaerobic metabolism (Sebahia *et al.*, 2010).

In this study, seven genes classified into transport category were over-expressed in the presence of copper. Expression analyses clearly show that *copA* gene is that most remarkably induced by copper. This gene codes for a Cu^+ -translocating P-type ATPase pump that exports copper from the cytoplasm to the periplasm. Although Cu^{++} was added to the medium, it is reasonable to assume that it was reduced to Cu^+ intracellularly, and that Cu^+ was the actual inducer of a response (Rensing *et al.*, 2000). The relevance of *copA* among the mechanisms developed by *E. amylovora* against copper agrees with results observed in previous studies with different gamma proteobacteria: Hernández-Montes *et al.* (2012) found that CopA is the most abundant protein for copper homeostasis in bacteria, with an important physiological role as an internal membrane ATPase. It was identified in the chromosomes of 70 bacterial genera, also the genus *Erwinia* and it was found in all organisms cited before (among others 268 gamma proteobacteria, two strains of *E. amylovora* (TAX552 and ATCC 49946), one strain of *E. pyrifoliae* and one strain of *E. tasmaniensis*) as the core protein for copper homeostasis. It is interesting to note that CopA protein was also found in other genera belonging to *Enterobacteriaceae* family as *Dickeya*, *Pantoea*, *Pectobacterium* or *Pseudomonas*, which contain plant pathogenic bacteria; on the contrary, it is not present in the genome of other phytopathogenic bacteria, such as four strains of *Xylella fastidiosa* and seven out of nine strains of *Xanthomonas* spp. The remarkable

induction of *copA* gene by copper is in agreement with other gene expression studies after copper exposure in non phytopathogenic bacteria, such as *Acidithiobacillus ferrooxidans*, *Streptococcus pneumoniae* or *Listeria monocytogenes* (Salazar *et al.*, 2013; Shafeeq *et al.*, 2011; Corbett *et al.*, 2011). Moreover, recently, Zhao *et al.* (2014) have shown that copper homeostasis also plays an important role in oxidative resistance of *Deinococcus radiodurans* and that this role is played, among others, by *copA*.

We did observe some differences in gene expression between the microarray results and qPCR for a few of the genes examined, most notably *copA*. These differences could be due to the limitations of the microarray technique. The analysis of the global expression of one genome at a specific point in time is the result of all genes from the genetic pool in that particular time. Then, the results of a massive transcriptomic analysis technique should not be taken to do an analysis of the differential expression of each individual gene of the genetic pool, since some discrepancies can rise, as has been described in other bacterial models (Thieme *et al.*, 2008; Karunakaran *et al.*, 2009).

Expression analysis clearly showed that the *copA* gene in *E. amylovora* is not only remarkably induced by copper but also fine-tuned regulated. Three lines of evidence suggest that the expression of *copA* is regulated by copper through the transcriptional regulator CueR. First, sequence analysis outward from *copA* gene revealed that in *E. amylovora* strain Ea1189 both genes are contiguous in the same

locus, and they are transcribed in opposite directions (Figure S1). Second, CueR activates transcription of *copA* in the presence of copper ion in *E. coli* (Outten *et al.*, 2000). And third, the CueR binding site, including the CueR-box sequences, where the Cu⁺-CueR transcriptional factor binds to the *copA* promoter, is known in *E. coli* (Yamamoto and Ishihama, 2005). Sequence analysis revealed that the *copA* promoter of *E. amylovora* strain Ea1189 contains the CueR box consensus sequence, including the inverted repeat with just one mismatch (Figure S2).

Our gene-disruption studies confirmed for the first time a biological role of *copA* gene in *E. amylovora* survival after copper shock because it was partially complemented *in vitro* and *in planta* by addition of the *copA* gene with its native promoter *in trans*. In the *E. amylovora* chromosome, *copA* is located adjacent to *cueR*, the main gene involved in *copA* regulation, and it is possible that the genetic context of *copA* may be important in this case. The 10-100% reduction in survival of the wild type Ea1189 *in planta* upon treatment with copper is a typical result observed following treatment of a copper-sensitive bacterial pathogen on plant surfaces with a copper bactericide (ex. Garrett and Schwartz 1998; Quesada *et al.*, 2010). Further reductions in survival of the $\Delta copA$ mutant could be a reflection of increasing availability of copper on the plant surfaces over time. These results suggest that the *copA* gene plays an important role for survival of *E. amylovora* against copper both *in vitro* and *in planta*.

The present study also showed that other differentially expressed genes classified into the transport family may direct or indirectly be also involved in maintaining copper homeostasis in *E. amylovora*. Concretely, the *ydhC* and *smdB* genes, belonging to drug resistance efflux transporter family, and the transmembrane protein YfcA might act reducing toxicity in the cytoplasm due to ion copper effect. Further gene disruption studies will be required to demonstrate their current role in copper tolerance in *E. amylovora*. This is also the case of *yjcE* gene, coding for a Na⁺/H⁺ exchanger (NHE) with similarity to NHEs in eukaryotes (Verkhovskaya *et al.*, 2001) and having a potential role in eliminating excess acid from cells. Expression analysis and gene disruption studies with *yjcE* show that it should play a role for copper tolerance in *E. amylovora*; this could be accomplished through maintaining pH homeostasis in cells which could reduce the cycling of Cu⁺ and Cu⁺⁺ ions. It has also been demonstrated in human cells (Akram *et al.*, 2006) that an increase in intracellular superoxide anion induces synthesis of an NHE that strongly correlates with the resistance of cells to death.

5.2.2. Oxidative stress (copper toxicity)

The strong relationship between the presence of a stress factor and the production of superoxide radicals has been reported by microarray studies in other bacterial models as *Pyrococcus furiosus*, *Nitrosomonas europaea*, *Herminiimonas arsenicoxydans* or *Bacillus cereus* (Williams *et al.*, 2007; Park and Ely, 2008; Cleiss-Arnold *et al.*, 2010; Mols *et al.*, 2010). In the case of *E. amylovora*, the effect of copper in previous studies on culturability led to conclude that this metal acts as a stress factor also for this pathogen (Ordax *et al.*, 2006). Besides maintaining copper homeostasis, bacteria also have to overcome its toxic effect. As commented before, the main toxicity of copper could derive from the production of reactive oxygen species (ROS), as previously described by Halliwell and Gutteridge (1984) in *E. coli* (see section 1.3.1. of the Introduction), since copper dissociates the oxygen peroxide produced by an aerobic metabolism into hydroxyl radicals. This is genetically supported by the fact that, in the present study, seven differentially-expressed genes were stress-related and they may direct or indirectly be involved in overcoming the toxic effect of this metal in *E. amylovora*.

We identified three ROS related genes that were differentially expressed after a copper shock. A gene-disruption study was performed for the AraC family DNA-binding transcriptional regulator, SoxS, and the Δ soxS mutant was highly affected in copper tolerance, losing more than six orders of magnitude in population numbers. It is well known that copper sulphate is a powerful inducer

of the *soxRS* regulon of *E. coli* through oxidative stress (Greenberg *et al.*, 1990; Kimura and Nishioka, 1997). An intracellular redox signal activates SoxR, a superoxide response sensor protein encoded by *soxR*. It binds to DNA at a specific site activating the expression of *soxS* gene, and SoxS protein is a direct inducer of target genes of bacterial response to oxidative stress (Lushchak, 2011). The *soxS* gene has been already characterized in *E. amylovora* (Pletzer *et al.*, 2014) and its regulon was identified in *E. coli* (Martin and Rosner, 2002). This study shows that copper is one of the signals for activation of *soxRS* system in *E. amylovora* and that some SoxRS-regulated genes of its regulon should play an important role in its copper tolerance.

Another ROS-related gene found in this work was *arcB*. Expression analysis and gene disruption studies with *arcB* show that the two-component regulatory system ArcAB is activated by copper and it is required for copper tolerance in *E. amylovora*. ArcB activates its cognate transcriptional regulator ArcA to repress genes contributing to aerobic metabolism and activates genes necessary for anaerobic metabolism (Loui *et al.*, 2009). Thus, in an anaerobic habitat, bacteria avoid more ROS production and, consequently, oxidative damage. This study shows that copper is one of the signals for activation of ArcAB system and that some ArcAB-regulated genes of its regulon should play an important role in copper tolerance in *E. amylovora*. It seems that, for *E. amylovora*, this strategy to avoid ROS production is not preferred because anaerobic metabolism is not the optimal one for an efficient growth in *E. amylovora*. The fact that this

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gene appears in previous studies related to ROS in the bacterial model *E. coli* (Luchi and Weiner, 1996; Loui *et al.*, 2009) may support the hypothesis that ArcAB system is a metabolic strategy against copper toxicity in *E. amylovora* not described before.

The third ROS-related gene has been described above, *yjcE*. It is also connected with copper homeostasis. Our observation that *E. amylovora* $\Delta yjcE$ mutant was also strongly affected after copper shock supports the hypothesis that pH homeostasis can affect copper sensitivity.

Another interesting gene classified into the stress category is *galF*. The enzyme GalF is involved in the formation of UDP-glucose from α -D-glucose-1P, which is essential for polysaccharide production. Up to date, no direct relation between this gene and copper or others stress factors had been described. However, we observed an increase of almost 3-fold expression in this gene and the reduced culturability of the $\Delta galF$ mutant strain for more than six orders of magnitude after copper exposure, suggesting that this gene is in fact involved, at least indirectly through protection by exopolysaccharide production, in the response of *E. amylovora* against copper stress.

5.3. Response model to copper in *E. amylovora*

The data obtained show that *E. amylovora* responds to copper shock by over-expressing several genes related to two genetic systems that represent two complementary strategies: (i) the control of copper homeostasis through the expression of, at least, the *copA* gene, and (ii) the overcoming of copper toxicity due to oxidative stress through the expression of some ROS-related genes.

All results together allowed us elaborating a putative model of the different genetic mechanisms that probably are involved in the interaction between *E. amylovora* and copper in the assayed conditions (Figure 26). Copper probably crosses the external membrane and enters periplasm via porins, as in *E. coli* (Teitzel et al., 2006) and many others Gram-negative bacteria (de Bruijn, 2016), and then reaches the cytoplasm crossing the inner membrane via certain transport proteins. Once in the cytoplasm, copper generates ROS through Fenton-like reactions. In order to prevent the oxidative damage, *E. amylovora* displays several mechanisms. The most important one seems to be a compendium of actions aimed to maintain copper homeostasis and to face up ROS by the activation of *soxS* (Fig. 26A) and *yjcE* (Fig. 26B) gene expression. The first one activates the expression of other genes related with the elimination of ROS, and the second one contributes to neutralize the acid pH generated by ROS inside the cytoplasm. The activity of these genes is supported by the action of CopA protein (Fig. 26C), which exports copper from inside the cell to the periplasm space. Besides, the

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activation of *arcB* gene (Fig. 26D) allows changing from aerobic to anaerobic metabolism, which would drastically reduce the production of ROS. Finally, the role of the two hypothetical proteins YgcF and YhhQ (Fig. 26E) remains to be elucidated.

The results of this thesis have constituted an approach to the genetic substrate of *E. amylovora* response against copper stress, and they are a starting point to make progress in the knowledge of the complex molecular mechanisms involved in the copper response of the fire blight pathogen.

6

CONCLUSIONS

CONCLUSIONS

This work was designed to improve the knowledge of the molecular mechanisms involved in the response of *E. amylovora* to copper stress, a topic not previously afforded in plant pathogenic bacteria. The new results obtained are summarized in the following conclusions:

1. Copper, even at very low concentrations such as 0.005mM, constitutes a stressful factor for *E. amylovora* and triggers a response, observed at least as a reduced culturability, with a genetic substrate constituted by the coordinated action of several genes belonging to different functional categories.
2. The general stress response regulator gene *rpoS* contributes to the survival of *E. amylovora* under copper exposure, since its expression was consistently higher in presence than in absence of copper when *E. amylovora* was challenged to copper and starvation stressful conditions.
3. A set of at least forty four genes exhibit a differential expression after copper shock. Twenty-five of them belong to functional categories of transport and stress. Genes related to metabolism (five), movement (one) and programmed cell death (one) also show an altered expression. The validation of the differential expression of twenty-five selected genes showed that twenty-three of them increased its expression after copper shock.
4. The genes *arcB*, *copA*, *EAM_3469*, *galF*, *soxS*, *ycgF*, *yhhQ* and *yjcE* are involved in the survival of *E. amylovora* when it is exposed to

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copper sulfate, from concentrations as low as 10mM. The role of six of these genes, *arcB*, *copA*, *soxS*, *ygcF*, *yhhQ* and *yjcE*, was confirmed under *in vitro* conditions, since the complementation of the mutants in those genes restored the wild type phenotype.

5. The differential expression under copper shock of seven genes related to transport suggests its direct or indirect involvement in maintaining copper homeostasis in *E. amylovora*. Genes *ydhC* and *smdB*, that have concern in drug efflux, and YfcA, that is a transmembrane protein, probably act reducing the toxicity of copper present in cytoplasm, whereas *yjcE* gene coding for a Na⁺/H⁺ exchanger probably acts neutralizing the acid pH generated by reactive oxygen species provoked by copper.

6. From the seven transport genes differentially expressed under copper exposure, the *copA* gene, which codes for a Cu⁺-translocating P-type ATPase pump that exports copper from the cytoplasm to the periplasm, is the most remarkably induced by copper and even fine-tune regulated, playing an important role as defense mechanism of *E. amylovora* against copper stress both *in vitro* and *in planta*. The expression of *copA* gene seems to be transcriptionally regulated by copper through the regulator CueR but not through CusSR.

7. The increased expression of *soxS* gene, involved in superoxide resistance, under copper shock confirms its connection with oxidative stress and it also shows, for the first time, that copper can be one of the signals for activation of SoxRS system.

8. The induction of *arcB* gene after activation of ArcAB system triggered by copper suggests another strategy, complementary to the role of *soxS*, to stop reactive oxygen species production through an aerobic/anaerobic switch, since ArcB activates ArcA to repress genes contributing to aerobic metabolism and activates those necessary for anaerobic one.

9. The gene *galF* is also involved in the response of *E. amylovora* against copper probably through an increased production of exopolysaccharide that confers protection to the bacterial cell against copper and other kinds of stress.

10. A putative model of response of *E. amylovora* against copper stress has been elaborated following the experimental evidences from this work. It involves at least two complementary strategies: one aimed to control copper homeostasis, and the other to reduce copper toxicity by counteracting the production of ROS.

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