Diversity of culturable nocardioform actinomycetes from wastewater treatment plants in Spain and their role in the biodegradability of aromatic compounds

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Abstract
Currently, urban and industrial wastewater treatment plants (WWTP) are mainly focused on the reduction of biological oxygen demand and sometimes on the removal of nutrients such as nitrogen and phosphorus. However, there are microorganisms that interfere with the process. In these environments, there is a large diversity of microorganisms that have not been studied in detail and that could provide real and practical solutions to the problems that could be caused by the wastewater treatment process. Among such microorganisms, Gram-positive actinomycete bacteria are of special interest, since they are known for producing secondary metabolites, chemically diverse compounds with a wide range of biological activities and for their capacity to degrade recalcitrant pollutants. Three different media were chosen to isolate actinomycetes from twenty eight wastewater treatment plants (WWTPs) in Spain. A total of 189 activated sludge samples were collected. 126 strains were isolated and identified to belong to one suborder, i.e., Corynebacterineae, and seven genera, i.e., Corynebacterium, Dietzia, Gordonia, Mycobacterium, Rhodococcus, Tsukamurella and Williamsia. Furthermore, 71 strains were capable of biodegrading at least one toxic product, and that 27 of them amplified for catA gene. The results of this study allow to understand the complexity of the foaming forming populations and it shows that WWTPs can be a good source of microorganisms with many potential applications, such as bioremediation or biodegradation of toxic compounds, such as phenol or naphthalene.

Keywords WWTP, actinomycete, activated sludge, mycolata, 16S rRNA gene sequencing, phenol and naphthalene biodegradation

Introduction

The term biodiversity often evokes the image of ecosystems where plant and animal species abound, such as rainforests. However, the organisms that have greater biological and functional biodiversity on our planet are microorganisms (Oren, 2004; Schleifer, 2004; González Pastor, 2010). The fact that there is a wide variety of microorganisms and that many of them have been studied in some detail, opens the possibility to undertake a study for their use in the industry. In this regard, microorganisms belonging to the class Actinobacteria (Gram positive bacteria with a high content of G+C)
generically known as Actinomycetes, are distinguished by their ability to produce significant active compounds (Stackebrandt et al., 1997) and by their various biotechnological applications (Bull et al., 2000).

Activated sludge process is a common aerobic treatment used to reduce the amount of organic matter from wastewater by using microorganisms (Pal et al., 2014), although it poses some problems, being the foaming the most important one. Foaming is a worldwide phenomenon (Wanner et al., 1998; Tandoi et al., 2006) and the greatest problem in solid separation in the activated sludge process (Wang et al., 2015). There is a need to identify the species present in the activated sludge basins to get a better understanding of which are the microorganisms that generate this foam and how to solve it. Foaming is originated in the aeration tanks and it proceeds from the combination of air bubbles, (bio)surfactants and a high presence of hydrophobic filamentous and possibly non-filamentous bacteria (Frigon et al., 2006; Petrovski et al., 2011). Activated sludge foams are characterised by the formation of thick, stable brown-coloured scums which float on the surface of aeration basins and secondary clarifiers in activated sludge sewage treatment plants (Sodell and Seviour, 1990), varying in thickness from a few centimeters to over a meter (de los Reyes, 2010). Filamentous foams cause a variety of operational problems in activated sludge plants, such as the reduction of effluent quality and the loss of biomass (Sodell and Seviour, 1990), and may represent a public health hazard because of the spread of pathogens through the creation of aerosols. Engineering solutions require an understanding of both the taxonomic diversity and ecology of the casual organisms (Goodfellow et al., 1998).

The occurrence of foam is usually associated with members of the Mycolata, mycolic acid-containing actinomycetes (Sodell and Seviour, 1998; Goodfellow et al., 1996; Seviour et al., 2008) - being Gordonia amarae the first filamentous bacterium isolated from foams (Lechevalier and Lechevalier, 1974) and the best-known foam former because of its high frequency and abundance in foaming wastewater treatment plants (WWTPs) (de los Reyes, 2010) - and Microthrix parvicella, a gram-positive nonbranched filamentous bacterium (Sodell and Seviour, 1990; Blackall et al., 1995). Mycolata are members of the order Actinomycetales in the phylum Actinobacteria (Dworkin et al., 2006). The mycolata isolates in pure culture from foams have been identified as members of the families Corynebacteriaceae, Dietziaceae, Mycobacteriaceae (Mycobacterium and Amycolicoccus), Nocardiaceae (Gordonia, Millisia, Nocardia, Rhodococcus, Skermania and Williamsia), Segniliparaceae and
Tsukamurellaceae (Stackebrandt et al., 1997; Zhi et al., 2009). Mycolic acid-containing microorganisms have hydrophobic cell surfaces, a property which has been related to the presence of free long-chain mycolic acids and when present in sufficient numbers they render hydrophobic flocs and hence amenable to the attachment of air bubbles. The air bubble-floc aggregates are less dense than water and float to the surface of activated sludge where they accumulate as foam (Goodfellow et al., 1996; Bendinger et al., 1995). Traditionally, filamentous bacteria in activated sludge have been identified by conventional light microscopy using the descriptions given by Eikelboom (2000) or Jenkins et al. (2004). Currently the taxonomy of mycolata has been revolutionised by the application of modern taxonomic methods (Erhart et al., 1997; Blackall et al., 1998; Marsh et al., 1998; Shen et al., 2007). A positive aspect of the microorganisms belonging to the suborder Corynebacterineae is their metabolic versatility and their ability to degrade toxic compounds (Bell et al., 1998; Arenskötter et al., 2004; Larkin et al., 2005), being Rhodococcus and Gordonia the main genera used to eliminate them (Flavio et al., 1999), since they use many metabolic pathways to degrade them, including the catechol cleavage activity (Veselý et al., 2007; Min et al., 2009; Matera et al., 2010). The catA gene encodes catecol 1,2-dioxygenase and it is involved in the first step of the catalysis of the aromatic ring, carrying out the opening of the ring by means of an ortho-cleavage process (Veselý et al., 2007; Shen et al., 2009).

In Spain sludge foaming is also one of the main problems in WWTPs, although its significance in activated-sludge plants is not well understood and a detailed research on the filamentous bacteria present in such foams has not been implemented yet. Therefore, the main aim of the present study is to determine the taxonomic diversity of mycolic acid-containing actinomycetes. The second aim is to find isolates which are capable of degrading oil derivatives such as phenol and naphthalene because these products are the most common products in the industrial and domestic WWTPs studied.

Materials and methods

Collection of foam samples

The sampled WWTPs were located in different provinces (Alicante, Cádiz, Castellón and Valencia) in Spain. Samples were collected from 28 WWTPs, all of which were
suffering at the time from foaming problems caused by the mycolata. The phenol and
naphthalene (detection of the products was performed by the WWTPs staff) were
common products in the biological reactor of the WWTPs located in Cádiz. The
samples were stored at 4°C for no more than 48 h prior to the selective isolation.

Selective isolation

Ten-fold dilutions of foam samples were made and plated in triplicate onto modified
Czapeck medium supplemented with nalidixic acid (Goodfellow et al., 1996), Sauton
medium and GYEA medium. After 7, 14 and 21 days plates were checked for rough,
wrinkled, dry colonies, with or without aerial mycelium. The isolates were subjected to
Gram and acid-fast staining. The organisms that showed a thin, branched filament with
a tendency to fragment into bacillary and coccoid forms were isolated in pure culture on
yeast extract-malt extract agar (ISP medium 2) (Shirling and Gottlieb, 1966) and
incubated for 2-3 weeks at 28°C. Then, the pure cultures were preserved in 20% (w/v)
stere glycerol solution (Wellington and Williams, 1978) at -80°C for long term
maintenance and on glucose-yeast extract agar at 4°C (Gordon and Mihm, 1962).

Morphological analysis

Macroscopic -based on the appearance of isolated colonies- and microscopic -to verify
that the strains had the typical nocardioform morphology- observations were made. A
morphological study was performed with reference strains belonging to the suborder
Corynebacterineae, provided by the Colección Española de Cultivos Tipo - CECT and
the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - DSMZ
(Corynebacterium xerosis CECT 4160, Gordonia alkanivorans CECT 7017, Gordonia
amarae CECT 5704, Gordonia paraffinivorans DSMZ 44604, Mycobacterium phlei
CECT 3009, Nocardia asteroides CECT 3051, Nocardia brasiliensis CECT 3052,
Rhodococcus phenolicus DSMZ 44812, Rhodococcus rhodochrous CECT 5749,
Tsukamurela spumae DSMZ 44113 and Williamsia maris DSMZ 45037). All strains
were plated onto ISP medium 2 and incubated for 5-10 days at 28°C. Finally, catalase
test was performed.

Chemotaxonomic analysis
For chemotaxonomic analysis strains were grown on ISP medium 2 for 14 days at 28°C. For the mycolic acids extraction (Hamid et al., 1993), one loopful of fresh biomass was suspended in 1 ml of Tetrabutylammonium hydroxide (TBAH) containing 100 mg of glass beads (<106 μm diameter; Sigma-Aldrich, USA). The suspension was homogenised in a vortex for 30 sec and then placed in a multi-block heater at 100°C for 4 h. After centrifugation (4000 rpm for 5 min), the supernatant was transferred to a new cryotube and 1 ml of dichloromethane and 25 μl of iodomethane were added. After homogenization (40 rpm for 30 min) and centrifugation (2000 rpm for 3 min) the lower layer was transferred to a new microtube, left to dryness in a multi-block heater at 55°C, and 75 μl of petroleum ether added to reconstitute the hydrolysate for the thin-layer chromatography analyses. 10 μl of each sample were employed in the Thin-Layer Chromatography (TLC) aluminium cellulose sheets (Merck, USA) in petroleum ether/acetone (95:5, v/v) until the solvent front was ~1 cm from the top of the plate. Plates were air-dried, sprayed with phosphomolybdic acid (5%, w/v) (Sigma-Aldrich, USA) and heated at 100°C for 5 min for visualization of mycolic acid spots. The extraction of diaminopimelic acid (A2pm) was detected using Staneck and Roberts (1974) procedure. Two loopfuls of fresh biomass were suspended in 500 μl of HCl 6N containing 100 mg of glass beads (<106 μm diameter; Sigma–Aldrich, USA). The suspension was homogenised in a vortex for 5 min at 4000 rpm and then placed in an multi-block heater at 100°C for 4 h. After centrifugation (4000 rpm for 5 min), the supernatant was transferred to a new microtube, left to dryness in a multi-block heater at 100°C, and 500 μl of distilled water added. The suspension was homogenised in a vortex for 30 seconds. After new centrifugation (4000 rpm for 5 min), the supernatant was transferred to a new Eppendorf tube, left to dryness in a multi-block heater at 100°C, and 75 μl of distilled water added to reconstitute the hydrolysate for the thin-layer chromatography analyses. 3 μl of each sample were employed in the TLC on glass cellulose sheets (Merck, USA) in methanol/distilled water/6M HCl/pyridine (32:10,4:1,6:4 v/v) until the solvent front was ~2 cm from the top of the plate. To visualize diaminopimelic acid (DAP) spots, plates were air-dried, sprayed with freshly prepared ninhydrine in acetone solution (0.2%, w/v) and heated at 100°C for 5-10 min. For the whole cell sugar extraction (Hasegawa et al., 1983), one loopful of fresh biomass were suspended in 0,1 ml of HCl 0.25N. The suspension was autoclaved for 15 min at 121°C. 3 μl of each sample were employed in the TLC on cellulose sheets (Merck, USA) in n-butanol/distilled water/pyridine/toluene (10:6:6:1 v/v) until the
The solvent front was ~1 cm from the top of the plate. After that, the TLC on cellulose sheets (Merck, USA) for 2 h. Plates were sprayed with aniline phthalate reagent (Sigma–Aldrich, USA) and heat at 100°C for 4 min.

**Phenotypic tests**

Micromorphological properties of the isolated strains grown in ISP medium 2 were determined by Gram-staining and acid fast staining reaction. Strains were also examined for a wide range of phenotypic properties using standard procedures (Goodfellow, 1971). Aerial spore-mass colour was determined on the cultures used for morphological examination. Colours were matched to one of the seven colour wheels of Tresner and Backus (Tresner and Backus, 1963) using the methods of Shirling and Gottlieb (1966). Substrate mycelial pigments were observed after 14 days on ISP medium 2. Tolerance to temperature was tested using ISP medium 2. Growth at 28°C and 37°C was controlled after 7 and 14 days, but also after 4 weeks at 10°C. The physiological properties were determined by using tests to determine the hydrolysis of complex substrates (Gordon and Mihm, 1957; Gordon, 1967) as well as tests to determine carbon source utilization according to Yassin et al. (Yassin et al., 1995). The degradation of aesculin (0.1%, w/v) was carried out following the procedures of Williams et al. (1983) and examined after 7, 14 and 21 days; blackening on the test media indicated a positive result. The degradation of tyrosine (0.5%, w/v) was detected into GYEA medium after 7, 14 and 21 days; clearing of the insoluble compound from under and around areas of growth was scored as positive. Urea was performed in test tubes and incubated for 24-48 h at 37°C; when the media turn to red-pink colour indicated a positive result. The ability of the tested strains to use eight carbon compounds (D+lactose, D+maltose, D-arabinose, D+fructose, D-galactose, D-glucose, D-mannitol and meso-inositol) was examined on Stevenson's basal medium (Stevenson, 1967). Carbon sources were added at 1.0% (w/v). Strains were also tested for their ability to use three nitrogen sources at 0.1%, w/v. The Stevenson's basal media was supplemented with either L-alanine, L-histidine or L-proline. Growth of all tests was scored after 7, 14 and 21 days by comparing test plates with both negative and positive controls.

**DNA extraction and PCR amplification of the 16S rRNA gene**
Total genomic DNA from pure culture colonies was extracted using the Gen Elute Bacterial Genomic DNA kit (Sigma-Aldrich, USA) and subjected to PCR amplification with primers 27f and 1492r (Lane, 1991). Reactions were performed in a final volume of 25 µl containing 0.2 mM of each of the four dNTPs (Ecogen, Spain), 0.4 µM of primer 27f and primer 1492r, 1 µl of extracted DNA, 1.5 mM MgCl₂, and 1.25 U BioTaq DNA Polymerase (Ecogen, Spain) with 1× reaction buffer [10× buffer: 160 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween-20]. Amplification was performed in a PTC-100 Peltier Thermal Cycler as follows: after an initial denaturation step (95°C, 5 min), 30 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min), followed by a final extension (72°C, 10 min). PCR products were analyzed by electrophoresis at 90 V for 1 h through 1.2% (w/v) SeaKem LE agarose (FMC Bioproducts, Denmark) in TAE 1X buffer and visualized under UV illumination after staining with etidium bromide. The expected molecular weight of the amplicons was confirmed by comparison to the GeneRuler 100-bp DNA Ladder Plus (MBI, Fermentas, Burlington, Canada). The PCR product was purified with the Gen Elute PCR Clean-up Kit (Sigma-Aldrich, USA) and sequenced with the same sets of primers. The 16S rRNA gene sequences were manually assembled from the combination of separate fragments generated with forward and reverse sequencing primers using the PHYDIT program (Chun, 1995). Isolates were classified to the genus level based on 16S rRNA similarity data. The sequences were presumptively identified using the BLAST (Basic Local Alignment Tool) option of the GenBank web site (http://www.ncbi.nlm.nih.gov/). The almost complete sequences were aligned manually against sequences of references strains of the genus of the suborder Corynebacterineae. Phylogenetic trees were inferred using the neighbour-joining (Saitou and Nei, 1987) tree-making algorithm from the TREECON software suite programs (Van de Peer and de Watcher, 1994) and evolutionary distances matrices were generated for the neighbour-joining method, as described by Jukes and Cantor (Juker and Cantor, 1969). The topologies of the resultant unrooted trees were evaluated in a bootstrap analysis (Felsenstein, 1993) based on 1,000 resamplings of the neighbour-joining dataset using the PHYLIP package (Phylogenetic Inference Package), version 3.5c.

**Biodegradation tests**
The pollutants chosen to conduct the biodegradation tests were phenol and naphthalene. This choice was made mainly as they were the toxic compounds with the most impact on the industrial WWTPs studied. Three culture mediums were used. These were the M9 medium (Okoh et al., 2001), the BHM medium (Rhefuss and Urban, 2005) and the MSM medium (Aufrett et al., 2009). Firstly a prior acclimatization of the microorganisms was conducted. For this, the strains were grown in the three culture mediums supplemented with 0.1% (w/v) glucose as the only carbon source and incubated for 14 days at 28°C. Later on, these strains were grown in the same culture mediums but this time supplemented with phenol and naphthalene at 0.1% (w/v). They were incubated at 28°C for 14 and 21 days. The strains grown in the three culture mediums supplemented with glucose were positive controls.

**Gene catA molecular detection**

Gene catA is involved in the first step of the catalysis in the hydrocarbon aromatic ring. The detection of the gene cathecol 1,2-dioxeganese was achieved through the amplification via PCR of mentioned gene. In this case, the primers C120f and C120r (Shen et al., 2009) were used. Reactions were performed in a final volume of 25 μl containing 0.2 mM of each of the four dNTPs (Ecogen, Spain), 0.4 μM of primer C120f and primer C120r, 0.5 to 1 μl extracted DNA, 1.5 mM MgCl₂ and 1.25 U EcoTaq DNA Polymerase (Ecogen, Spain) with 1x reaction buffer [10x buffer: 160 mM (NH₄)₂SO₄, 670 mM Tris-HCl, 0.1 % Tween-20]. Amplification was performed in a PTC-100 Peltier Thermal Cycler as follows: after an initial denaturation step (95°C, 5 min), 30 cycles of denaturation (95°C, 30 sec), annealing (66°C, 45 sec) and extension (72°C, 1 min), followed by a final extension (72°C, 7 min). PCR products were analyzed by electrophoresis at 90 V for 1 h through 1.2% (w/v) SeaKem LE agarose (FMC Bioproducts, Denmark) in TAE 1X buffer and visualized under UV illumination after staining with etidium bromide. The expected molecular weight of the amplicons was confirmed by comparison to the GeneRuler 100-bp DNA Ladder Plus (MBI Fermentas; Burlington, Canada). The PCR product was purified with the Gen Elute PCR Clean-up kit (Sigma) and sequenced with the same sets of primers. The catA gene was presumptively identified using the program BLAST (Basic Local Alignment Tool) option of the GenBank web site (http://www.ncbi.nlm.nih.gov/).
Results and discussion

Isolation of nocardioform actinomycetes from foam samples. Morphological and chemotaxonomic analysis

A total of 126 actinomycete strains were isolated and purified from the isolation plates. They all were filamentous, cocci or irregular rods, Gram positive and catalase positive bacteria. Mucosal colonies had usually cocci morphologies and rough or dull colonies had usually rod morphologies, occasionally with well developed branches in right angle. All the strains contained mycolic acid, meso-diaminopimelic acid as cell wall diaminoacid and arabinose and galactose as whole-cell organism hydrolysates (wall chemotype IV sensu Lechevalier and Lechevalier, 1970) (Fig. 1, Fig. 2 and Fig. 3), therefore all the isolates were well-defined in the suborder Corynebacterineae.

Identification of nocardioform actinomycetes

Comparison of the nearly complete 16S rRNA nucleotide sequences from the isolated strains with corresponding nucleotides sequences of representative’s of the suborder Corynebacterineae (Stackebrandt et al., 1997; Zhi et al., 2009) confirmed that the isolates belong to the following genera (supporting material Table S1: Genbank accession numbers): Corynebacterium - 2 strains, Dietzia - 2 strains, Gordonia - 79 strains, Mycobacterium - 17 strains, Rhodococcus - 9 strains, Tsukamurella - 16 strains and Williamsia - 1 strain (supporting material – Figures F1 to F7). From all the isolates investigated in this work, the most abundant belonged to the genus Gordonia (62.7%), the genus Mycobacterium was the second in abundance (13.49%), followed by Tsukamurella (12.69%) and Rhodococcus (7.14%). Less genera abundant were Corynebacterium (1.59%), Dietzia (1.59%) and Williamsia (0.79%). The isolated strains, based on the results of the nucleotide similarity matrices (supporting material – Tables S2 to S8) belonged to the species Corynebacterium freneyi (1 strain), Corynebacterium variabilis (1 strain), Dietzia maris (1 strain), Dietzia csecretrophyllic (1 strain), Gordonia alkanivorans (2 strains), Gordonia amarae (22 strains), Gordonia aralii (3 strains), Gordonia bronchialis (2 strains), Gordonia choleroliverans (1 strain), Gordonia effusa (1 strain), Gordonia hydrophobica (9 strains), Gordonia
malaquae (12 strains), Gordonia paraffinivorans (6 strains), Gordonia polyisoprenivorans (6 strains), Gordonia rhizosfera (1 strain), Gordonia sputi (11 strains), Gordonia terrae (3 strains), Mycobacterium austroafricanum (3 strains), Mycobacterium fallax (1 strain), Mycobacterium mageritense (1 strain), Mycobacterium smegmatis (8 strains), Mycobacterium vaccae (2 strains), Mycobacterium vanbaalenii (2 strains), Rhodococcus ruber (3 strains), Rhodococcus rhodochrous (1 strain), Rhodococcus zopfii (5 strains), Tsukamurella pseudospumae (10 strains), Tsukamurella pulmonis (1 strain), Tsukamurella spumae (3 strains), Tsukamurella tyrosinosolvens (2 strains) and Williamsia muralis (1 strain). In all cases, the nucleotide sequences similarity values among the isolated strains and type strains were between 97.47% and 100% (Table 1). From the beginning of the 90s, the limit for considering two species as identical was 97% or more. However, a study carried out by Stackebrandt and Ebers (2006) showed that this value was not ideal and it was increased to percentages between 98.7% and 99% because rates lower than 98.5% of nucleotide similarity corresponded to rates lower than 70 % in DNA:DNA hybridization. This is the case of D1.2 (Rhodococcus rhodochrous), CS10.1 (Gordonia hydrophobica) and QB19.1 (Gordonia araii) isolates, with nucleotide similarity percentages of 97.78%, 97.47% and 97.93%, respectively. Therefore different phenotypic tests should be performed.

This study shows that not only typical genera and species of activated sludge, such as Gordonia amarae (Goodfellow et al., 1994; Klatte et al., 1994) or Tsukamurella spumae (Nam et al., 2003) were identified, but also others that had not been found to date in these environments such as Williamsia muralis (Kampfer et al., 1999) or human pathogens such as Dietzia maris (Pidoux et al., 2001), Gordonia bronchialis (Tsukamura, 1971), Gordonia terrae (Pham et al., 2003) Tsukamurella pulmonis or the six environmental Mycobacterium species. This indicates that there is still much to study in the microbiological process of wastewater treatment since, traditionally, microorganisms that interfere in the process, especially biological foams producers, have been limited to poorly defined groups. This is the case of morphologies observed in plants with foam problems referred to as Gordonia amarae like Organism -GALO- (formerly Nocardia amarae like Organism - NALO) without specifying which particular species were responsible. It should be noted that the foam production ability of a microorganism, in our case the mycolata, depends on the abundance and the hydrophobicity of the microorganism. This hydrophobicity is determined by mycolic acids present in all species within the group of mycolata. These compounds vary in
length and complexity presenting different patterns of branching, since the longer the carbon chains, the higher hydrophobicity to the microorganism. In this sense, species containing shorter mycolic acids chains belong to the genus *Corynebacterium* (22C - 36C) and *Dietzia* (34C - 38C) while species with longer and more complex mycolic acids chains belong to the genus *Mycobacterium* (60C - 90C) and *Tsukamurella* (62C - 78C). This indicates that we are overlooking two mycolata genera with high hydrophobicity (*Mycobacterium* and *Tsukamurella*) and therefore with high ability to produce foams. In the present study it has been demonstrated that both genera represent approximately the 26% of the total found genera, so it is relevant to emphasize that *Tsukamurella* and *Mycobacterium* genera have a great importance in Spanish WWTPs.

**Phenotypic tests**

A total of 17 phenotypic tests were performed, as described above. This supported the earlier results obtained in genotypic and chemotaxonomic characterization. Due to the high volume of isolates, we decided to make a representative selection that included all genera and species that were genotypic and chemotaxonomically characterized. These phenotypic tests help us to make a difference between species that are very close or that could be considered as new species, because the percentage of similarity obtained creates some doubts, as in the case discussed in the previous section for the strains D1.2 CS10.1 and QB19.1. Generally these tests do not differ greatly between species of the same genus, however, there are always certain tests that provide differentiating elements that can help to improve the characterization of the isolate compared to the phylogenetically closer reference species. However, these are supporting data (not definitive), since the DNA:DNA hybridization and other confirmatory tests such as menaquinones, fatty acids, API-ZYM strip or other tests should be performed in order to differentiate much more clearly our isolates from the species in question. Once the different phenotypic tests were performed, it was checked that all isolates (except for isolates D1.2, CS10.1 and QB19.1) belong to the species obtained in the genotype characterization, since no significant differences in their results were found. For the 3 isolates discussed above, differences can be seen in relation to the closest reference species, which together with the low percentage of nucleotide similarity suggests that possible new species could be considered, in the absence of conducting confirmatory
tests such as DNA: DNA hybridization, polar lipids, predominant menaquinones and fatty acids.

**Biodegradation and gene catA molecular detection tests**

Biodegradation tests showed that 71 of the 126 strains were capable of degrading at least one toxic product. The best naphthalene degraders (41 strains) were the *Dietzia*, *Gordonia*, *Mycobacterium*, *Rhodococcus* and *Tsukamurella* strains. For phenol, the best degrading strains (3 strains) were the *Rhodococcus* ones (Fig. 4 and Fig. 5). We have isolated three species, *Gordonia alkanivorans*, *Gordonia paraffinivorans* and *Gordonia polyisoprenivorans* with potential applications in the biodegradation of paraffin, alkanes and polyisoprene. The genus *Gordonia* has attracted much interest due to its ability to degrade xenobiotics and environmental pollutants as well as to transform or synthesize possible useful compounds (Arenskötter et al., 2004). As it can be seen in Table S9, the isolates from *Gordonia alkanivorans* and *Gordonia polyisoprenivorans* are able to degrade naphthalene. The *catA* gene, which encodes catechol 1,2-dioxygenase, were detected in 27 strains, belonging to *Dietzia*, *Gordonia*, *Rhodococcus* and *Williamsia* genera (Table 2). The detection of the amplified fragments was achieved via electrophoresis in the agarose gel at 1,2% (Fig. 6). This result was confirmed using BLAST for these isolates with a percentage of nucleotide similarity between 99% and 100%. It must be highlighted that the number of strains emphasized for gene *catA* does not correspond to the number of strains obtained in the biodegradation trials. This could be due to the isolated strains that proved positive in the trials presenting catabolic routes differing from those of catechol 1,2-dioxygenase, to mutations that had not permitted the correct hybridization of the primers or to horizontal transfers of gene *catA* (Tánsics et al., 2008).

**Conclusions**

To our knowledge, this is the first study about the diversity of mycolata and their biodegradation capacity in WWTPs in Spain. We have isolated 31 different species of mycolata from 28 activated sludge plants, being *Gordonia* genus the most important one regarding the number of identified isolates. However, species belonging to the genera
Rhodococcus, Tsukamurella or Mycobacterium have also been founded, bringing to light that other important genera can also been considered in the formation process of the foaming. Therefore, this result shows a high diversity of mycolata in WWTPs of Spain. The results also show the presence of mycolata species not related with activated sludge process such as Gordonia sputi, Gordonia polyisoprenivorans, Tsukamurella tyrosinosolvens or Williamsia muralis. Furthermore, 71 strains were capable of biodegrading at least one toxic product, and that 27 of them amplified for catA gene. In conclusion, that is why the importance of this work is double; on the one hand, understanding the complexity of the foaming forming populations and, on the other hand, the fact that WWTPs can be a good source of microorganisms with many potential applications, such as bioremediation and biodegradation.

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