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Additional Information

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)

68 generically known as *Actinomycetes*, are distinguished by their ability to produce
69 significant active compounds (Stackebrandt *et al.*, 1997) and by their various
70 biotechnological applications (Bull *et al.*, 2000).
71 Activated sludge process is a common aerobic treatment used to reduce the amount of
72 organic matter from wastewater by using microorganisms (Pal *et al.*, 2014), although it
73 poses some problems, being the foaming the most important one. Foaming is a
74 worldwide phenomenon (Wanner *et al.*, 1998; Tandoi *et al.*, 2006) and the greatest
75 problem in solid separation in the activated sludge process (Wang *et al.*, 2015). There is
76 a need to identify the species present in the activated sludge basins to get a better
77 understanding of which are the microorganisms that generate this foam and how to
78 solve it. Foaming is originated in the aeration tanks and it proceeds from the
79 combination of air bubbles, (bio)surfactants and a high presence of hydrophobic
80 filamentous and possibly non-filamentous bacteria (Frigon *et al.*, 2006; Petrovski *et al.*,
81 2011). Activated sludge foams are characterised by the formation of thick, stable
82 brown-coloured scums which float on the surface of aeration basins and secondary
83 clarifiers in activated sludge sewage treatment plants (Sodell and Seviour, 1990),
84 varying in thickness from a few centimeters to over a meter (de los Reyes, 2010).
85 Filamentous foams cause a variety of operational problems in activated sludge plants,
86 such as the reduction of effluent quality and the loss of biomass (Sodell and Seviour,
87 1990), and may represent a public health hazard because of the spread of pathogens
88 through the creation of aerosols. Engineering solutions require an understanding of both
89 the taxonomic diversity and ecology of the casual organisms (Goodfellow *et al.*, 1998).
90 The occurrence of foam is usually associated with members of the Mycolata, mycolic
91 acid-containing actinomycetes (Sodell and Seviour, 1998; Goodfellow *et al.*, 1996;
92 Seviour *et al.*, 2008) -being *Gordonia amarae* the first filamentous bacterium isolated
93 from foams (Lechevalier and Lechevalier, 1974) and the best-known foam former
94 because of its high frecuency and abundance in foaming wastewater treatment plants
95 (WWTPs) (de los Reyes, 2010)- and *Microthrix parvicella*, a gram-positive
96 nonbranched filamentous bacterium (Sodell and Seviour, 1990; Blackall *et al.*, 1995).
97 Mycolata are members of the order *Actinomycetales* in the phylum *Actinobacteria*
98 (Dworkin *et al.*, 2006). The mycolata isolates in pure culture from foams have been
99 identified as members of the families *Corynebacteriaceae*, *Dietziaceae*,
100 *Mycobacteriaceae* (*Mycobacterium* and *Amycolicicoccus*), *Nocardiaceae* (*Gordonia*,
101 *Millisia*, *Nocardia*, *Rhodococcus*, *Skermania* and *Williamsia*), *Segniliparaceae* and

102 *Tsukamurellaceae* (Stackebrandt *et al.*, 1997; Zhi *et al.*, 2009). Mycolic acid-containing
103 microorganisms have hydrophobic cell surfaces, a property which has been related to
104 the presence of free long-chain mycolic acids and when present in sufficient numbers
105 they render hydrophobic flocs and hence amenable to the attachment of air bubbles. The
106 air bubble-floc aggregates are less dense than water and float to the surface of activated
107 sludge where they accumulate as foam (Goodfellow *et al.*, 1996; Bendinger *et al.*,
108 1995). Traditionally, filamentous bacteria in activated sludge have been identified by
109 conventional light microscopy using the descriptions given by Eikelboom (2000) or
110 Jenkins *et al.* (2004). Currently the taxonomy of mycolata has been revolutionised by
111 the application of modern taxonomic methods (Erhart *et al.*, 1997; Blackall *et al.*, 1998;
112 Marsh *et al.*, 1998; Shen *et al.*, 2007). A positive aspect of the microorganisms
113 belonging to the suborder *Corynebacterineae* is their metabolic versatility and their
114 ability to degrade toxic compounds (Bell *et al.*, 1998; Arenskötter *et al.*, 2004; Larkin *et*
115 *al.*, 2005), being *Rhodococcus* and *Gordonia* the main genera used to eliminate them
116 (Flavio *et al.*, 1999), since they use many metabolic pathways to degrade them,
117 including the catechol cleavage activity (Veselý *et al.*, 2007; Min *et al.*, 2009; Matera *et*
118 *al.*, 2010). The *catA* gene encodes catecol 1,2-dioxygenase and it is involved in the first
119 step of the catalysis of the aromatic ring, carrying out the opening of the ring by means
120 of an ortho-cleavage process (Veselý *et al.,* 2007; Shen *et al.,* 2009).
121 In Spain sludge foaming is also one of the main problems in WWTPs, although its
122 significance in activated-sludge plants is not well understood and a detailed research on
123 the filamentous bacteria present in such foams has not been implemented yet. Therefore,
124 the main aim of the present study is to determine the taxonomic diversity of mycolic
125 acid-containing actinomycetes. The second aim is to find isolates which are capable of
126 degrading oil derivatives such as phenol and naphthalene because these products are the
127 most common products in the industrial and domestic WWTPs studied.
128

129 ## Materials and methods

130

131 ### *Collection of foam samples*

132

133 The sampled WWTPs were located in different provinces (Alicante, Cádiz, Castellón
134 and Valencia) in Spain. Samples were collected from 28 WWTPs, all of which were

135 suffering at the time from foaming problems caused by the mycolata. The phenol and
136 naphthalene (detection of the products was performed by the WWTPs staff) were
137 common products in the biological reactor of the WWTPs located in Cádiz. The
138 samples were stored at 4ºC for no more than 48 h prior to the selective isolation.
139
140 ***Selective isolation***
141
142 Ten-fold dilutions of foam samples were made and plated in triplicate onto modified
143 Czapeck medium supplemented with nalidixic acid (Goodfellow *et al.*, 1996), Sauton
144 medium and GYEA medium. After 7, 14 and 21 days plates were checked for rough,
145 wrinkled, dry colonies, with or without aerial mycelium. The isolates were subjected to
146 Gram and acid-fast staining. The organisms that showed a thin, branched filament with
147 a tendency to fragment into bacillary and coccoid forms were isolated in pure culture on
148 yeast extract-malt extract agar (ISP medium 2) (Shirling and Gottlieb, 1966) and
149 incubated for 2-3 weeks at 28ºC. Then, the pure cultures were preserved in 20% (w/v)
150 sterile glycerol solution (Wellington and Williams, 1978) at -80ºC for long term
151 maintenance and on glucose-yeast extract agar at 4ºC (Gordon and Mihm, 1962).
152
153 ***Morphological analysis***
154
155 Macroscopic -based on the appearance of isolated colonies- and microscopic -to verify
156 that the strains had the typical nocardioform morphology- observations were made. A
157 morphological study was performed with reference strains belonging to the suborder
158 *Corynebacterineae*, provided by the *Colección Española de Cultivos Tipo* - CECT and
159 the *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* - DSMZ
160 (*Corynebacterium xerosis* CECT 4160, *Gordonia alkanivorans* CECT 7017, *Gordonia*
161 *amarae* CECT 5704, *Gordonia paraffinivorans* DSMZ 44604, *Mycobacterium phlei*
162 CECT 3009, *Nocardia asteroides* CECT 3051, *Nocardia brasiliensis* CECT 3052,
163 *Rhodococcus phenolicus* DSMZ 44812, *Rhodococcus rhodochrous* CECT 5749,
164 *Tsukamurela spumae* DSMZ 44113 and *Williamsia maris* DSMZ 45037). All strains
165 were plated onto ISP medium 2 and incubated for 5-10 days at 28ºC. Finally, catalase
166 test was performed.
167 ***Chemotaxonomic analysis***
168

169 For chemotaxonomic analysis strains were grown on ISP medium 2 for 14 days at 28ºC.
170 For the mycolic acids extraction (Hamid *et al.*, 1993), one loopful of fresh biomass was
171 suspended in 1 ml of Tetrabutylammonium hydroxide (TBAH) containing 100 mg of
172 glass beads (<106 µm diameter; Sigma-Aldrich, USA).The suspension was
173 homogenised in a vortex for 30 sec and then placed in a multi-block heater at 100ºC for
174 4 h. After centrifugation (4000 rpm for 5 min), the supernatant was transferred to a new
175 cryotube and 1 ml of dichloromethane and 25 µl of iodomethane were added. After
176 homogenization (40 rpm for 30 min) and centrifugation (2000 rpm for 3 min) the lower
177 layer was transferred to a new microtube, left to dryness in a multi-block heater at 55ºC,
178 and 75 µl of petroleum ether added to reconstitute the hydrolysate for the thin-layer
179 chromatography analyses.10 µl of each sample were employed in the Thin-Layer
180 Cromatography (TLC) aluminium cellulose sheets (Merck, USA) in petroleum
181 ether/acetone (95:5, v/v) until the solvent front was ~1 cm from the top of the plate.
182 Plates were air-dried, sprayed with phosphomolybdic acid (5%, w/v) (Sigma-Aldrich,
183 USA) and heated at 100ºC for 5 min for visualization of mycolic acid spots. The
184 extraction of diaminopimelic acid (A2pm) was detected using Staneck and Roberts
185 (1974) procedure. Two loopfuls of fresh biomass were suspended in 500 µl of HCl 6N
186 containing 100 mg of glass beads (<106 µm diameter; Sigma–Aldrich, USA). The
187 suspension was homogenised in a vortex for 5 min at 4000 rpm and then placed in an
188 multi-block heater at 100ºC for 4 h. After centrifugation (4000 rpm for 5 min), the
189 supernatant was transferred to a new microtube, left to dryness in a multi-block heater at
190 100ºC, and 500 µl of distilled water added. The suspension was homogenised in a
191 vortex for 30 seconds. After new centrifugation (4000 rpm for 5 min), the supernatant
192 was transferred to a new Eppendorf tube, left to dryness in a multi-block heater at
193 100ºC, and 75 µl of distilled water added to reconstitute the hydrolysate for the thin-
194 layer chromatography analyses. 3 µl of each sample were employed in the TLC on glass
195 cellulose sheets (Merck, USA) in methanol/distilled water/6M HCl/pyridine
196 (32:10,4:1,6:4 v/v) until the solvent front was ~2 cm from the top of the plate. To
197 visualize diaminopimelic acid (DAP) spots, plates were air-dried, sprayed with freshly
198 prepared ninhidrine in acetone solution (0.2%, w/v) and heated at 100ºC for 5-10 min.
199 For the whole cell sugar extraction (Hasegawa *et al.*, 1983), one loopful of fresh
200 biomass were suspended in 0,1 ml of HCl 0.25N. The suspension was autoclaved for 15
201 min at 121ºC. 3 µl of each sample were employed in the TLC on cellulose sheets
202 (Merck, USA) in n-butanol/distilled water/pyridine/toluene (10:6:6:1 v/v) until the

203 solvent front was ~1 cm from the top of the plate. After that, the TLC on cellulose
204 sheets (Merck, USA) for 2 h. Plates were sprayed with aniline phthalate reagent
205 (Sigma–Aldrich, USA) and heat at 100ºC for 4 min.

206

207 ***Phenotypic tests***

208

209 Micromorphological properties of the isolated strains grown in ISP medium 2 were
210 determined by Gram-staining and acid fast staining reaction. Strains were also examined
211 for a wide range of phenotypic properties using standard procedures (Goodfellow,
212 1971). Aerial spore-mass colour was determined on the cultures used for morphological
213 examination. Colours were matched to one of the seven colour wheels of Tresner and
214 Backus (Tresner and Backus, 1963) using the methods of Shirling and Gottlieb (1966).
215 Substrate mycelial pigments were observed after 14 days on ISP medium 2. Tolerance
216 to temperature was tested using ISP medium 2. Growth at 28ºC and 37ºC was controlled
217 after 7 and 14 days, but also after 4 weeks at 10ºC. The physiological properties were
218 determined by using tests to determine the hydrolysis of complex substrates (Gordon
219 and Mihm, 1957; Gordon, 1967) as well as tests to determine carbon source utilization
220 according to Yassin *et al.* (Yassin *et al.*, 1995). The degradation of aesculin (0.1%, w/v)
221 was carried out following the procedures of Williams *et al.* (1983) and examined after 7,
222 14 and 21 days; blackening on the test media indicated a positive result. The
223 degradation of tyrosine (0.5%, w/v) was detected into GYEA medium after 7, 14 and 21
224 days; clearing of the insoluble compound from under and around areas of growth was
225 scored as positive. Urea was performed in test tubes and incubated for 24-48 h at 37ºC;
226 when the media turn to red-pink colour indicated a positive result. The ability of the
227 tested strains to use eight carbon compounds (D+lactose, D+maltose, D-arabinose,
228 D+fructose, D-galactose, D-glucose, D-mannitol and meso-inositol) was examined on
229 Stevenson's basal medium (Stevenson, 1967). Carbon sources were added at 1.0%
230 (w/v). Strains were also tested for their ability to use three nitrogen sources at 0.1%,
231 w/v. The Stevenson’s basal media was supplemented with either L-alanine, L-histidine
232 or L-proline. Growth of all tests was scored after 7, 14 and 21 days by comparing test
233 plates with both negative and positive controls.

234

235 ***DNA extraction and PCR amplification of the 16S rRNA gene***

236

237 Total genomic DNA from pure culture colonies was extracted using the Gen Elute
238 Bacterial Genomic DNA kit (Sigma-Aldrich, USA) and subjected to PCR amplification
239 with primers 27f and 1492r (Lane, 1991). Reactions were performed in a final volume
240 of 25 μl containing 0.2 mM of each of the four dNTPs (Ecogen, Spain), 0.4 μM of
241 primer 27f and primer 1492r, 1 μl of extracted DNA, 1.5 mM $MgCl_2$, and 1.25 U
242 Bio*Taq* DNA Polymerase (Ecogen, Spain) with 1× reaction buffer [10× buffer:
243 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25ºC), 0.1% Tween-20].
244 Amplification was performed in a PTC-100 Peltier Thermal Cycler as follows: after an
245 initial denaturation step (95ºC, 5 min), 30 cycles of denaturation (95ºC, 1 min),
246 annealing (55ºC, 1 min), and extension (72ºC, 1 min), followed by a final extension
247 (72ºC, 10 min). PCR products were analyzed by electrophoresis at 90 V for 1 h through
248 1.2% (w/v) SeaKem LE agarose (FMC Bioproducts, Denmark) in TAE 1X buffer and
249 visualized under UV illumination after staining with etidium bromide. The expected
250 molecular weight of the amplicons was confirmed by comparison to the GeneRuler 100-
251 bp DNA Ladder Plus (MBI, Fermentas, Burlington, Canada). The PCR product was
252 purified with the Gen Elute PCR Clean-up Kit (Sigma-Aldrich, USA) and sequenced
253 with the same sets of primers. The 16S rRNA gene sequences were manually assembled
254 from the combination of separate fragments generated with forward and reverse
255 sequencing primers using the PHYDIT program (Chun, 1995). Isolates were classified
256 to the genus level based on 16S rRNA similarity data. The sequences were
257 presumptively identified using the BLAST (Basic Local Alignment Tool) option of the
258 GenBank web site (http://www.ncbi.nlm.nih.gov/). The almost complete sequences
259 were aligned manually against sequences of references strains of the genus of the
260 suborder *Corynebacterineae*. Phylogenetic trees were inferred using the neighbour-
261 joining (Saitou and Nei, 1987) tree-making algorithm from the TREECON software
262 suite programs (Van de Peer and de Watcher, 1994) and evolutionary distances matrices
263 were generated for the neighbour-joining method, as described by Jukes and Cantor
264 (Juker and Cantor, 1969). The topologies of the resultant unrooted trees were evaluated
265 in a bootstrap analysis (Felsenstein, 1993) based on 1.000 resamplings of the neighbour-
266 joining dataset using the PHYLIP package (Phylogenetic Inference Package), version
267 3.5c.
268 ***Biodegradation tests***
269

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

281

282 ***Gene catA molecular detection***

283

284 Gene *catA* is involved in the first step of the catalysis in the hydrocarbon aromatic ring.
285 The detection of the gene cathecol 1,2-dioxeganese was achieved through the
286 amplification via PCR of mentioned gene. In this case, the primers C120f y C120r
287 (Shen *et al.*, 2009) were used. Reactions were performed in a final volume of 25 µl
288 containing 0.2 mM of each of the four dNTPs (Ecogen, Spain), 0.4 µM of primer C120f
289 and primer C120r, 0.5 to 1 µl extracted DNA, 1.5 mM $MgCl_2$ and 1.25 U Eco*Taq* DNA
290 Polymerase (Ecogen, Spain) with 1x reaction buffer [10x buffer: 160 mM $(NH_4)_2SO_4$,
291 670 mM Tris-HCl, 0.1 % Tween-20]. Amplification was performed in a PTC-100
292 Peltier Thermal Cycler as follows: after an initial denaturation step (95ºC, 5 min), 30
293 cycles of denaturation (95ºC, 30 sec), annealing (66ºC, 45 sec) and extension (72ºC, 1
294 min), followed by a final extension (72ºC, 7 min). PCR products were analyzed by
295 electrophoresis at 90 V for 1 h through 1.2% (w/v) SeaKem LE agarose (FMC
296 Bioproducts, Denmark) in TAE 1X buffer and visualized under UV illumination after
297 staining with etidium bromide. The expected molecular weight of the amplicons was
298 confirmed by comparison to the GeneRuler 100-bp DNA Ladder Plus (MBI Fermentas;
299 Burlington, Canada). The PCR product was purified with the Gen Elute PCR Clean-up
300 kit (Sigma) and sequenced with the same sets of primers. The *catA* gene was
301 presumptively identified using the program BLAST (Basic Local Alignment Tool)
302 option of the GenBank web site (http://www.ncbi.nlm.nih.gov/).

303

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 S_1: 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 F_1 to F_7)***. 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 S_2 to S_8)*** belonged to the species *Corynebacterium freneyi* (1 strain), *Corynebacterium variabilis* (1 strain), *Dietzia maris* (1 strain), *Dietzia cercidiphylli* (1 strain), *Gordonia alkanivorans* (2 strains), *Gordonia amarae* (22 strains), *Gordonia araii* (3 strains), *Gordonia bronchialis* (2 strains), *Gordonia cholesterolivorans* (1 strain), *Gordonia effusa* (1 strain), *Gordonia hydrophobica* (9 strains), *Gordonia*

337 *malaquae* (12 strains), *Gordonia paraffinivorans* (6 strains), *Gordonia*
338 *polyisoprenivorans* (6 strains), *Gordonia rhizosfera* (1 strain), *Gordonia sputi* (11
339 strains), *Gordonia terrae* (3 strains), *Mycobacterium austroafricanum* (3 strains),
340 *Mycobacterium fallax* (1 strain), *Mycobacterium mageritense* (1 strain), *Mycobacterium*
341 *smegmatis* (8 strains), *Mycobacterium vaccae* (2 strains), *Mycobacterium vanbaalenii* (2
342 strains), *Rhodococcus ruber* (3 strains), *Rhodococcus rhodochrous* (1 strain),
343 *Rhodococcus zopfii* (5 strains), *Tsukamurella pseudospumae* (10 strains), *Tsukamurella*
344 *pulmonis* (1 strain), *Tsukamurella spumae* (3 strains), *Tsukamurella tyrosinosolvens* (2
345 strains) and *Williamsia muralis* (1 strain). In all cases, the nucleotide sequences
346 similarity values among the isolated strains and type strains were between 97.47% and
347 100% (Table 1). From the beginning of the 90s, the limit for considering two species as
348 identical was 97% or more. However, a study carried out by Stackebrandt and Ebers
349 (2006) showed that this value was not ideal and it was increased to percentages between
350 98.7% and 99% because rates lower than 98.5% of nucleotide similarity corresponded
351 to rates lower than 70 % in DNA:DNA hybridization. This is the case of D1.2
352 (*Rhodococcus rhodochrous*), CS10.1 (*Gordonia hydrophobica*) and QB19.1 (*Gordonia*
353 *araii*) isolates, with nucleotide similarity percentages of 97.78%, 97.47% and 97.93%,
354 respectively. Therefore different phenotypic tests should be performed.
355 This study shows that not only typical genera and species of activated sludge, such as
356 *Gordonia amarae* (Goodfellow *et al.*, 1994; Klatte *et al.*, 1994) or *Tsukamurella*
357 *spumae* (Nam *et al.*, 2003) were identified, but also others that had not been found to
358 date in these environments such as *Williamsia muralis* (Kampfer *et al.*, 1999) or human
359 pathogens such as *Dietzia maris* (Pidoux *et al.*, 2001), *Gordonia bronchialis*
360 (Tsukamura, 1971), *Gordonia terrae* (Pham *et al.*, 2003) *Tsukamurella pulmonis* or the
361 six environmental *Mycobacterium* species. This indicates that there is still much to
362 study in the microbiological process of wastewater treatment since, traditionally,
363 microorganisms that interfere in the process, especially biological foams producers,
364 have been limited to poorly defined groups. This is the case of morphologies observed
365 in plants with foam problems referred to as *Gordonia amarae* like Organism -GALO-
366 (formerly *Nocardia amarae* like Organism - NALO) without specifying which
367 particular species were responsible. It should be noted that the foam production ability
368 of a microorganism, in our case the mycolata, depends on the abundance and the
369 hydrophobicity of the microorganism. This hydrophobicity is determined by mycolic
370 acids present in all species within the group of mycolata. These compounds vary in

371 length and complexity presenting different patterns of branching, since the longer the
372 carbon chains, the higher hydrophobicity to the microorganism. In this sense, species
373 containing shorter mycolic acids chains belong to the genus *Corynebacterium* (22C -
374 36C) and *Dietzia* (34C - 38C) while species with longer and more complex mycolic
375 acids chains belong to the genus *Mycobacterium* (60C - 90C) and *Tsukamurella* (62C -
376 78C). This indicates that we are overlooking two mycolata genera with high
377 hydrophobicity (*Mycobacterium* and *Tsukamurella*) and therefore with high ability to
378 produce foams. In the present study it has been demonstrated that both genera represent
379 approximately the 26% of the total found genera, so it is relevant to emphasize that
380 *Tsukamurella* and *Mycobacterium* genera have a great importance in Spanish WWTPs.
381
382 ***Phenotypic tests***
383
384 A total of 17 phenotypic tests were performed, as described above. This supported the
385 earlier results obtained in genotypic and chemotaxonomic characterization. Due to the
386 high volume of isolates, we decided to make a representative selection that included all
387 genera and species that were genotypic and chemotaxonomically characterized. These
388 phenotypic tests help us to make a difference between species that are very close or that
389 could be considered as new species, because the percentage of similarity obtained
390 creates some doubts, as in the case discussed in the previous section for the strains D1.2
391 CS10.1 and QB19.1. Generally these tests do not differ greatly between species of the
392 same genus, however, there are always certain tests that provide differentiating elements
393 that can help to improve the characterization of the isolate compared to the
394 phylogenetically closer reference species. However, these are supporting data (not
395 definitive), since the DNA:DNA hybridization and other confirmatory tests such as
396 menaquinones, fatty acids, API-ZYM strip or other tests should be performed in order
397 to differentiate much more clearly our isolates from the species in question. Once the
398 different phenotypic tests were performed, it was checked that all isolates (except for
399 isolates D1.2, CS10.1 and QB19.1) belong to the species obtained in the genotype
400 characterization, since no significant differences in their results were found. For the 3
401 isolates discussed above, differences can be seen in relation to the closest reference
402 species, which together with the low percentage of nucleotide similarity suggests that
403 possible new species could be considered, in the absence of conducting confirmatory

404 tests such as DNA: DNA hybridization, polar lipids, predominant menaquinones and
405 fatty acids.
406
407 ***Biodegradation and gene catA molecular detection tests***
408
409 Biodegradation tests showed that 71 of the 126 strains were capable of degrading at
410 least one toxic product. The best naphthalene degraders (41 strains) were the *Dietzia*,
411 *Gordonia*, *Mycobacterium*, *Rhodococcus* and *Tsukamurella* strains. For phenol, the best
412 degrading strains (3 strains) were the *Rhodococcus* ones (Fig. 4 and Fig. 5). We have
413 isolated three species, *Gordonia alkanivorans*, *Gordonia paraffinivorans* and *Gordonia*
414 *polyisoprenivorans* with potential applications in the biodegradation of paraffin, alkanes
415 and polyisoprene. The genus *Gordonia* has attracted much interest due to its ability to
416 degrade xenobiotics and environmental pollutants as well as to transform or synthesize
417 possible useful compounds (Arenskötter *et al.*, 2004). As it can be seen in **Table S9**, the
418 isolates from *Gordonia alkanivorans* and *Gordonia polyisoprenivorans* are able to
419 degradate naphthalene. The *catA* gene, which encodes catechol 1,2-dioxygenase, were
420 detected in 27 strains, belonging to *Dietzia*, *Gordonia*, *Rhodococcus* and *Williamsia*
421 genera (Table 2). The detection of the amplified fragments was achieved via
422 electrophoresis in the agarose gel at 1,2% (Fig. 6). This result was confirmed using
423 BLAST for these isolates with a percentage of nucleotide similarity between 99% and
424 100%. It must be highlighhted that the number of strains emphasized for gene *catA* does
425 not correspond to the number of strains obtained in the biodegradation trials. This could
426 be due to the isolated strains that proved positive in the trials presenting catabolic routes
427 differing from those of catechol 1,2-dioxygenase, to mutations that had not permitted
428 the correct hybridization of the primers or to horizontal transfers of gene *catA* (Tánsics
429 *et al.*, 2008).
430

431 Conclusions

432
433 To our knowledge, this is the first study about the diversity of mycolata and their
434 biodegradation capacity in WWTPs in Spain. We have isolated 31 different species of
435 mycolata from 28 activated sludge plants, being Gordonia genus the most important one
436 regarding the number of identified isolates. However, species belonging to the genera

437 *Rhodococcus*, *Tsukamurella* or *Mycobacterium* have also been founded, bringing to
438 light that other important genera can also been considered in the formation process of
439 the foaming. Therefore, this result shows a high diversity of mycolata in WWTPs of
440 Spain. The results also show the presence of mycolata species not related with activated
441 sludge process such as *Gordonia sputi*, *Gordonia polyisoprenivorans*, *Tsukamurella*
442 *tyrosinosolvens* or *Williamsia muralis*. Furthermore, 71 strains were capable of
443 biodegrading at least one toxic product, and that 27 of them amplified for *catA* gene. In
444 conclusion, that is why the importance of this work is double; on the one hand,
445 understanding the complexity of the foaming forming populations and, on the other
446 hand , the fact that WWTPs can be a good source of microorganisms with many
447 potential applications, such as bioremediation and biodegradation.
448

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450
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454

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