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1 ***Pseudonocardia hispaniensis*** **sp. nov., a novel actinomycete isolated from industrial wastewater**
2 **activated sludge**
3
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15
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17
18
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21
22
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26
27

28 **Abstract**

29

30 A novel actinomycete, designated PA3[T], was isolated from an oil refinery wastewater treatment plant,
31 located in Palos de la Frontera, Huelva, Spain, and characterized taxonomically by using a polyphasic
32 approach. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate formed a
33 distinct subclade in the *Pseudonocardia* tree together with *Pseudonocardia asaccharolytica* DSM
34 44247[T]. The chemotaxonomic properties of the isolate, for example, the presence of MK-8 (H_4) as the
35 predominant menaquinone and iso-$C_{16:0}$ as the major fatty acid are consistent with its classification in the
36 genus *Pseudonocardia*. DNA:DNA pairing experiments between the isolate and the type strain of *P.*
37 *asaccharolytica* DSM 44247[T] showed that they belonged to separate genomic species. The two strains
38 were readily distinguished using a combination of phenotypic properties. Consequently, it is proposed
39 that isolate PA3[T] represents a novel species for which the name *Pseudonocardia hispaniensis* sp. nov. is
40 proposed. The type strain is PA3[T] (= CCM 8391[T] = CECT 8030[T]).

41

42 **Introduction**

43

44 The genus *Pseudonocardia* was proposed by Henssen (1957) for mycolateless nocardioform
45 actinomycetes which contained *meso*-diaminopimelic acid and arabino-galactan polymers (wall
46 chemotype IV after Lechevalier and Lechevalier 1970), menaquinones with eight isoprene units as the
47 predominant isoprenologue and a high DNA G+C composition. The description of the genus has been
48 emended repeatedly as new species have been described which show variations in chemotaxonomic and
49 morphological properties (Warwick et al. 1994; Huang et al. 2002; Park et al. 2008). Members of the
50 genus form substrate and aerial mycelia, spore chains by acropetal budding or fragmentation, contain
51 complex mixtures of iso- and anteiso-fatty acids, tetrahydrogenated menaquinones with eight isoprene
52 units (MK_8 [H_4]) as the predominant isoprenologue, iso-branched hexadecanoic acid as the major fatty
53 acid, either phosphatidylethanolamine or phosphatidylcholine as diagnostic polar lipids (polar lipid
54 patterns 2 and 3 *sensu* Lechevalier et al. 1981) and constitute a distinct, albeit heterogeneous, clade in the
55 16S rRNA *Pseudonocardiaceae* gene tree (Huang and Goodfellow 2012). The genus can also be
56 distinguished from other genera classified in this family using a combination of chemotaxonomic and
57 morphological features (Labeda et al. 2011).

58

59 The genus currently encompasses 40 recognised species

60 (http://www.bacterio.cict.fr/p/pseudonocardia.html), most of which have been described in the last five

61 years based upon studies of single strains. Such studies have provided useful information on the

62 evolutionary radiation and distribution of the taxon in natural habitats but have given little insight into the

63 biological properties of pseudonocardia. Single strains representing new species (including several names

64 not validated to date) have been isolated from contaminated industrial sludge (Mahendra and Alvarez-

65 Cohen 2005; Kämpfer et al. 2006), coastal sediment (Liu et al. 2006), indoor environment (Schäfer et al.

66 2009), plant litter (Sakiyama et al. 2010), soil (Park et al. 2008; Qin et al. 2008; Li et al. 2010; Ara et al.

67 2011) and from surface sterilized roots and stems of higher plants (Chen et al. 2009; Duangmal et al.

68 2009; Kaewkla and Franco 2010, 2011; Qin et al. 2010, 2011; Zhao et al. 2011a,b,c).

69

70 There is a need to discover the taxonomic diversity and functions of filamentous actinomycetes in

71 wastewater treatment plants in order to improve operational procedures (Nam et al. 2003; Seviour et al.

72 2008). In the present study a *Pseudonocardia*-like strain was isolated as part of a survey of actinobacterial

73 diversity in activated sludge plants in the south of Spain. A polyphasic taxonomic study of the isolate

74 showed that it represented a new species of the genus *Pseudonocardia* for which the name

75 *Pseudonocardia hispaniensis* sp. nov. is proposed.

76

77 **Materials and methods**

78

79 *Organisms, maintenance and cultural conditions*

80

81 Strain PA3^T was isolated from a modified Czapek agar (sucrose, 2%, w/v; yeast extract, 0.2%, w/v,

82 $FeSO_4$, 0.001%; KCl, 0.001%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05% ; $NaNO_3$, 0.2%; agar 1.5 %, w/v;

83 distilled water, 1 litre) plate supplemented with nalidixic acid (20 mg l^{-1}) following inoculation with a

84 sample taken from a wastewater treatment plant in Palos de la Frontera, Huelva, Spain. The isolate, which

85 grew as a small colony covered with white aerial hyphae, was purified on yeast extract-malt extract agar

86 (ISP medium 2; Shirling and Gottlieb 1966). The isolate and the type strain of *Pseudonocardia*

87 *asaccharolytica* (DSM 44247^T) were maintained on ISP 2 agar slants at 4ºC and as suspensions of hyphal

88 fragments and spores in 20% (v/v) glycerol at -80ºC. Biomass for all but one of the chemotaxonomic
89 studies was prepared by growing the isolate and *P. asaccharolytica* DSM 44247^T in shake flasks (at about
90 200 rpm) of GYE broth (glucose, 1%; yeast extract 1%; distilled water, 1 liter) for 14 days at 28°C.
91 Similarly, biomass for the fatty acid analyses was harvested from shake flasks of Tryptic Soy Broth
92 (Difco) (150 rpm) after 5 days at 28°C. Biomass for the chemical and molecular studies was washed in
93 distilled water and freeze dried and kept at -20°C until needed.
94
95 *16S rRNA gene sequencing analyses*
96
97 Genomic DNA was extracted from isolate PA3^T using a commercial DNA extraction kit (GenElute,
98 Sigma) and PCR amplification of the 16S rRNA gene achieved using the universal primers 27f and 1492r
99 (Lane 1991), 616V and 699R for a stretch of around 1000 nt close to the 5' end (Arahal et al. 2008), and
100 primers P609D and P1525R (reverse) for a segment of about 750 nt close to the 3' end (Lucena et al.
101 2010). The resultant almost complete 16S rRNA sequence (1434 nucleotides) was compared with
102 corresponding sequences of the type strains of species classified in the genus *Pseudonocardia* using
103 alignments retrieved from SILVA and LTP latest updates as references (Pruesse et al. 2007; Yarza et al.
104 2010); where necessary, additional sequences were retrieved from the DDBJ/EMBL/GenBank databases.
105 Alignments were corrected manually based on secondary structural information. Sequence similarities
106 were calculated in ARB based on sequences without the use of an evolutionary substitution model.
107 Phylogenetic analyses using several treeing methods (distance matrix, maximum-likelihood and
108 maximum-parsimony) and data subsets were examined using the appropriate ARB tools (Ludwig et al.
109 2004) (figure 1).
110
111 *Chemotaxonomy*
112
113 Isolate PA3^T was examined for chemical markers known to be of value in the classification of genera
114 belonging to the family *Pseudonocardiaceae* (Labeda et al. 2011). Standard chromatographic procedures
115 were used to determine the isomers of diaminopimelic acid (Staneck and Roberts 1974), predominant
116 menaquinones (detected by Dr. Brian Tindall of the Identification Service, DSMZ, Braunschweig.
117 Germany), mycolic acids (Hamid et al. 1993), polar lipids (Minnikin et al. 1974), whole-cell sugars

118 (Hasegawa et al. 1983) and DNA base composition (Mesbah et al. 1984), using appropriate controls.
119 Fatty acid methyl esters were extracted and prepared according to standard protocols as described for the
120 MIDI Microbial Identification System (Sasser, 1990) at the Colección Española de Cultivos Tipo, CECT .
121 Cellular fatty acids were analyzed by GC with an Agilent 6850 chromatographic unit, with the MIDI
122 Microbial Identification System using the TSBA6 method (MIDI, 2008) and identified using the
123 Microbial Identification Sherlock software package. Polar lipids were extracted, examined by two-
124 dimensional TLC and identified using the procedures described by Minnikin et al. (1984). The G+C
125 content of the genomic DNA was determined by HPLC as described by Mesbah et al. (1989).
126
127 *DNA:DNA relatedness studies*
128
129 DNA samples extracted from isolate PA3^T and *P. asaccharolytica* DSM 44247^T using a French pressure
130 cell (Thermo Spectronic) were purified by chromatography on hydroxyapatite (Cashion et al. 1977).
131 DNA:DNA hybridization was carried out, in duplicate, after De Ley et al. (1970), with modifications by
132 Huss et al. (1993), using a model Cary 100 Bio UV/Vis spectrophotometer fitted with a Peltier-
133 thermostated 6 x 6 multicell charger and a temperature controller with an *in situ* temperature probe
134 (Varian).
135
136 *Cultural and morphological properties*
137
138 Cultural characteristics of strain PA3^T and *P. asaccharolytica* DSM 44247^T were determined on modified
139 Czapek's medium, potato dextrose agar (Difco) and standard International *Streptomyces* Project (ISP)
140 media 2-7 (Table 1; Shirling and Gottlieb 1966). Spore chain arrangement and spore surface
141 ornamentation were observed using growth taken from a yeast-extract – malt extract agar (ISP medium 2)
142 plate after 14 days at 28ºC and examined using a JEOL JSM-5410 (JEOL Ltd., Tokyo, Japan) scanning
143 electron microscope operating at 20kv (Alonso et al. 2009). To this end, a loopful of culture was washed
144 in 0.1M sodium phosphate buffer (PBS; pH 7.2) in a 1.5 ml Eppendorf tube, the pellet fixed in additional
145 PBS buffer containing 2.5% (v/v) glutaraldehyde for 3 hours at 4°C and post-fixed with 2% (v/v) osmium
146 tetroxide in 0.1M PBS for an hour at 4°C. The fixed cells were washed in 0.1M PBS, transferred to the

147 surface of a 25 mm Poretics polycarbonate membrane (pore size 0.1 μm; Sigma), immersed in liquid
148 nitrogen, and then coated with gold.
149
150
151 *Phenotypic tests*
152
153 The isolate PA3^T and the *P. asaccharolytica* type strain were examined for a range of phenotypic
154 properties. Their ability to grow at 10, 28 37 and 45°C was determined after 14 days using ISP 2 as the
155 basal medium. Similarly, growth from pH 4-10 (at pH unit intervals adjusted with HCl or NaOH) and in
156 the presence of NaCl (3.5 and 7%, w/v) were examined after 14 days at 28°C. Enzyme activity was
157 established using API ZYM kits (bioMerieux) following the manufacturer's instructions. Growth under
158 autotrophic conditions was tested using mineral media recommended by earlier workers (Okoh et al.
159 2001; Renfuss and Urban 2005; Auffret et al. 2009). Additional biochemical and physiological properties
160 were recorded using previously described procedures (Gordon et al. 1974; Reichert et al. 1998).
161
162 **Results and discussion**
163
164 Isolate PA3^T formed a distinct subclade in the 16S rRNA *Pseudonocardia* tree together with *P.*
165 *asaccharolytica* DSM 44247^T, an association supported by all of the tree-making algorithms and by a
166 98% bootstrap value (Fig. 1). However, the two organisms shared a low 16S rRNA similarity of 97.3%, a
167 value which corresponded to 40 nucleotide differences. The corresponding 16S rRNA similarity values
168 with the remaining pseudonocardial type strains ranged from 94.6 to 96.7%. The DNA:DNA relatedness
169 values for the duplicated assays between strains PA3^T and *P. asaccharolytica* DSM 44247^T were 36.2 and
170 38.0%, well below the 70% cut-off point recommended for the delineation of bacterial species (Wayne et
171 al. 1987).
172
173 Strain PA3^T had chemotaxonomic and morphological properties consistent with its classification in the
174 genus *Pseudonocardia* (Zhao et al. 2011a,b,c; Huang and Goodfellow 2012). The organism formed an
175 extensively branched substrate and aerial mycelia which underwent fragmentation into smooth surfaced
176 coccoid or rod-shaped spores (Figure 2). There is not evidence of acropetal budding, zig-zag morphology

177 or intercalary swellings. In addition, it contains *meso*-A_{2pm}, arabinose and galactose in whole-organism
178 hydrolysates (wall chemotype IV after Lechevalier and Lechevalier 1970), tetrahydrogenated
179 menaquinones with eight isoprene units (MK8 [H_4]) as the predominant menaquinone,
180 diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol,
181 phosphatidylmethylethanolamine and phosphatidylinositol mannosides as major polar lipids
182 (phospholipid type 2 *sensu* Lechevalier et al. 1981) (Supplementary Fig. S1), a high DNA G+C ratio
183 (69.7 mol%), complex mixtures of iso- and anteiso – fatty acids with iso-$C_{16:0}$ as the predominant
184 component , but lacked mycolic acids. The fatty acid profile of *P. assacharolytica* DSM 44247^T was in
185 line with results reported by Reuchert et al. (1998) for four strains assigned to this species.
186
187 Isolate PA3^T and *P. asaccharolytica* DSM 44247^T can be distinguished using a combination of cultural
188 and phenotypic properties (Tables 1, 2 and 3), as exemplified by the ability of the former to produce
189 leucine and valine arylamidases, to grow at 37°C, in the presence of 3%, w/v NaCl, in different pH range
190 and to form distinctive honey coloured, substrate mycelia on glycerol-asparagine and tyrosine agars. In
191 contrast, neither strain produced acid from a broad range of carbohydrates, a result in line with the
192 original description of *P. asaccharolytica* (Reichert et al. 1998). Similarly, the two organisms have
193 qualitatively similar fatty acid profiles though quantitative differences were detected in some components
194 (Table 2). When additional data acquired on isolate PA3^T were compared with corresponding results
195 reported Reichert and his colleagues (1998) it was evident that the two strains can grow autotrophically
196 and have a type 2 polar lipid pattern.
197
198 The chemotaxonomic, morphological and phenotypic data, together with the 16S rRNA sequence and
199 DNA:DNA relatedness findings provide sufficient evidence to support the proposition that isolate PA3^T
200 represents a novel species of the genus *Pseudonocardia* for which the name *Pseudonocardia hispaniensis*
201 sp. nov. is proposed.
202
203 **Description of *Pseudonocardia hispaniensis* sp. nov.**
204
205 *Pseudonocardia hispaniensis* (his.pa.ni’en.sis. L. fem. adj. *hispaniensis*, of or belonging to *Hispania*, the
206 Latin name for Spain, the country where the type strain was isolated).

207 Aerobic, non-motile, Gram-positive, non-acid-alcohol-fast actinomycete which forms extensively
208 branched aerial and substrate mycelia that fragment into smooth surfaced coccoid or rod-like elements.
209 Grows well on ISP media 2-7 forming white aerial mycelia but does not produce diffusible pigments.
210 Grows from pH 5-10 (optimum 7-8) and at 28 and 37ºC, weakly at 45ºC, but not at 10°C. Catalase-
211 positive but oxidase negative. Additional cultural and phenotypic properties are shown in Tables 1 and 2.
212 Aerobic autotrophic growth was observed in mineral medium without a carbon source. The wall diamino
213 acid is *meso*-diaminopimelic acid, the diagnostic sugars arabinose and galactose, and the predominant
214 fatty acid and isoprenoid quinone iso- $C_{16:0}$ and MK8 (H_4), respectively. The polar lipid pattern contained
215 diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol,
216 phosphatidylinositol mannosides and phophatidylmethylethanolamine. The detailed fatty acid profile is
217 given in Table 1. The G+C content of the DNA was 69.7 mol%.
218 The type strain, PA3^{T} (= CCM 8391^{T} = CECT 8030^{T}), was isolated from a sample taken from an oil
219 refinery wastewater treatment plant in Palos de la Frontera, Huelva, Spain. The species description is
220 based on a single strain and hence doubles up as a description of the type species.
221

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224 isolated.
225

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407 **Table 1** Growth and cultural characteristics of strains PA3^T and *P. asaccharolytica* DSM 44247^T after

408 incubation at 28°C for 3 weeks

	Strain PA3^T			*P. asaccharolytica* DSM 44247^T		
Medium	Growth	Substrate mycelium color	Aerial mycelium color	Growth	Substrate mycelium color	Aerial mycelium color
Yeast extract-malt extract agar (ISP medium 2)	+++	Moderate yellow	White	+++	Moderate yellow	White
Oatmeal agar (ISP medium 3)	+++	White	White	++	White	White
Inorganic salts-starch agar (ISP medium 4)	+++	White	White	++	White	White
Glycerol-asparagine agar (ISP medium 5)	++	Honey	None	+	Opaque	None
Peptone-yeast extract iron agar (ISP medium 6)	+++	Yellowish-brown	White	+++	Yellowish-brown	White
Tyrosine agar (ISP medium 7)	++	Honey	None	+	Opaque	None
Modified Czapek's agar	++	White	White	+	White	White
Potato-dextrose agar	++	White	White	+	White	White

409
410 Key: +++, abundant; ++, moderate; +, poor growth. Diffusible pigments were not formed on any of the
411 media.
412

413 **Table 2** Fatty acid composition (%) of strains PA3^T and *P. asaccharolytica* DSM 44247^T. -, not

414 detectable; tr, trace amount (<1 %).

Fatty acids	Strain PA3^T	*P. asaccharolytica* DSM 44247^T
Hydroxy fatty acid:		
$C_{16:1}$ 2OH	1.4	-
Saturated fatty acids:		
$C_{14:0}$	-	1.0
$C_{17:0}$	-	3.1
$C_{16:0}$	tr	6.3
Unsaturated fatty acids:		
$C_{17:1}$ ω8c	tr	5.8
$C_{17:1}$ ω6c	9.9	5.5
$C_{18:1}$ ω9c	tr	2.1
Branched fatty acids:		
iso-$C_{14:0}$	tr	2.2
iso-$C_{15:0}$	12.3	14.5
iso-$C_{16:0}$	23.5	23.8
iso-$C_{17:0}$	14.7	11.5
iso-$C_{18:0}$	tr	-
iso-$C_{16:1}$ H	12.1	6.2
anteiso-$C_{17:0}$	5.9	4.3
$C_{17:0}$ 10-methyl	3.4	2.7
$C_{18:0}$ 10-methyl	tr	-
Sum In Feature:		
3 ($C_{16:1}$ ω7c/ $C_{16:1}$ ω6c)	2.9	7.3
4 ($C_{17:1}$ iso I/ anteiso B)	tr	-
9 (iso-$C_{17:0}$ ω9c/ $C_{16:0}$ 10-methyl)	10.3	3.6

415 Key: -, not detectable.

416

417 **Table 3** Phenotypic properties which distinguish strain PA3^T from the type strain of *P. asaccharolytica*

Characteristic	Strain PA3^T	*P. asaccharolytica* DSM 44247^T
API enzyme tests:		
Acid phosphatase	-	+
Cysteine arylamidase	+	-
Esterase (C4)	-	+
Leucine arylamidase	+	-
Valine arylamidase	+	-
Degradation of tyrosine	-	+*
Nitrate reduction	-	+
Tolerance tests:		
Growth at 37°C	+	-
Growth in presence of 3% w/v NaCl	+	-
pH growth range	5-10	6-9

418 Key: +, positive; -, negative. *Result not congruent with that reported by Reichert et al. (1998). Neither of

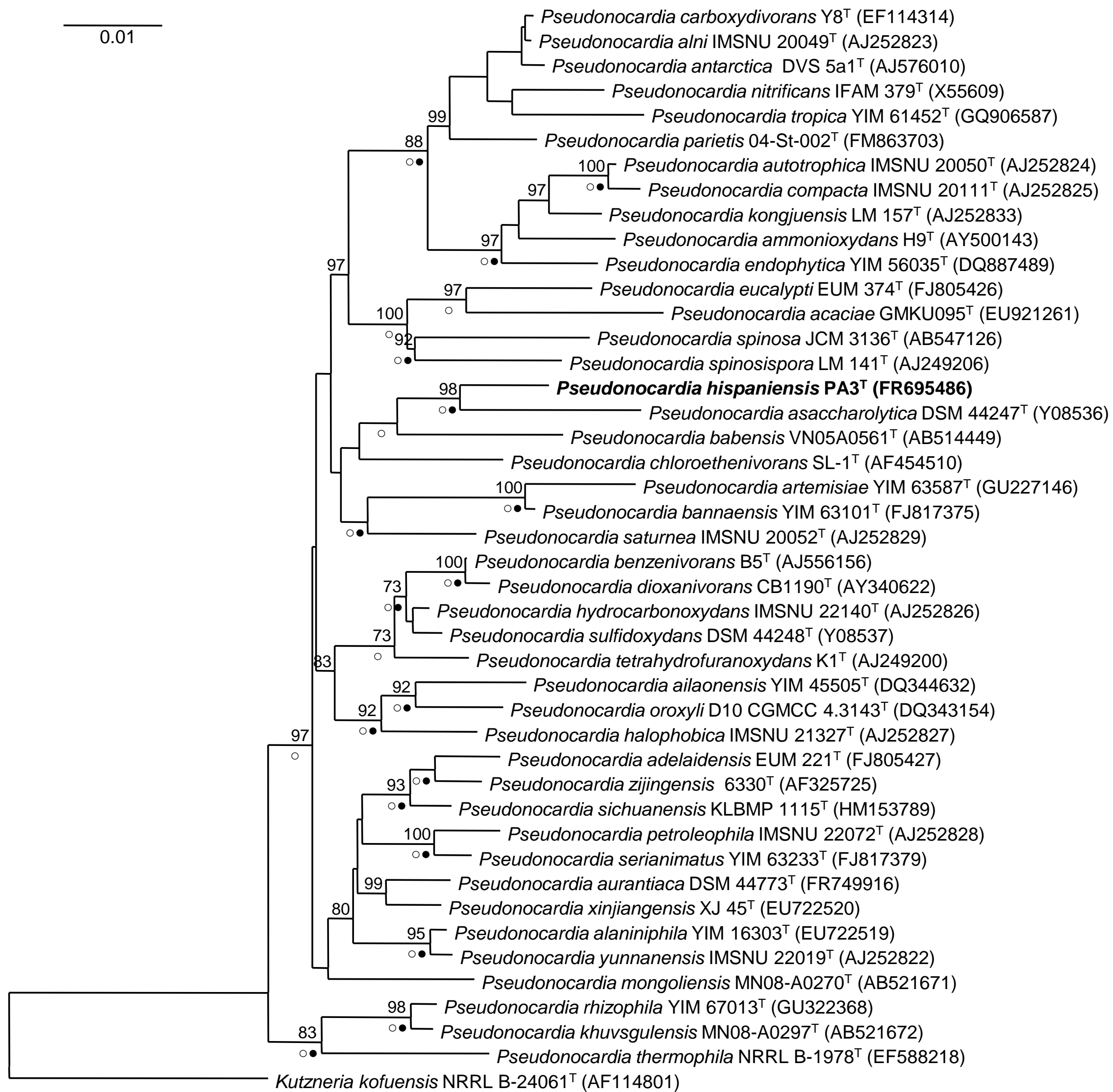
419 the strains hydrolyzed urea or produced acid from adonitol, L-arabinose, *meso*-erythritol, fructose,

420 galactose, glucose, lactose, inositol, inulin, maltose, mannitol, mannose, melezitose, rhamnose,

421 saccharose, sorbitol, trehalose or xylose.

422

423 Figure 1. Neighbor-joining phylogenetic tree based on almost-complete 16S rRNA gene sequences (1422
424 sites used) showing the position of isolate PA3^{T} in the *Pseudonocardia* genus. Bootstrap values (> 70 %)
425 were based on 1000 resamplings. Circles indicate the corresponding nodes recovered in trees generated
426 with the maximum-parsimony (open circles) or the maximum-likelihood (filled circles) methods. Bar, 1
427 substitution per 100 nucleotide positions.



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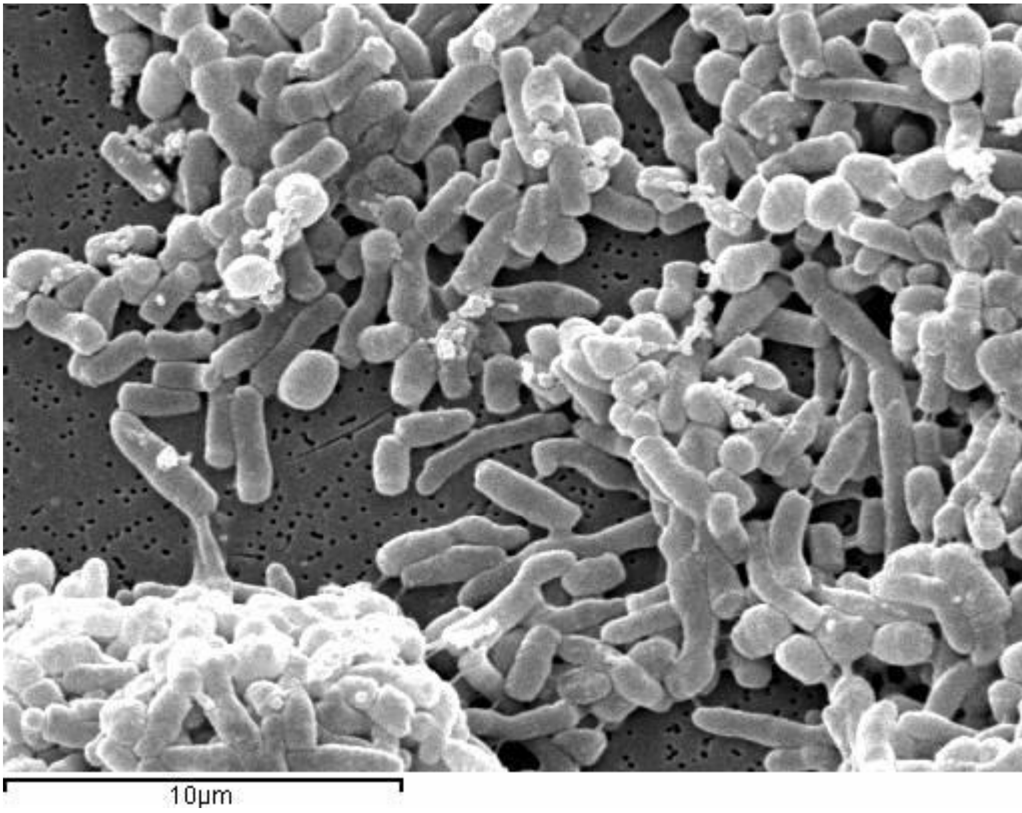

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436 Figure 2: Scanning electron micrograph of a 14-days old culture of strain $PA3^T$.

437

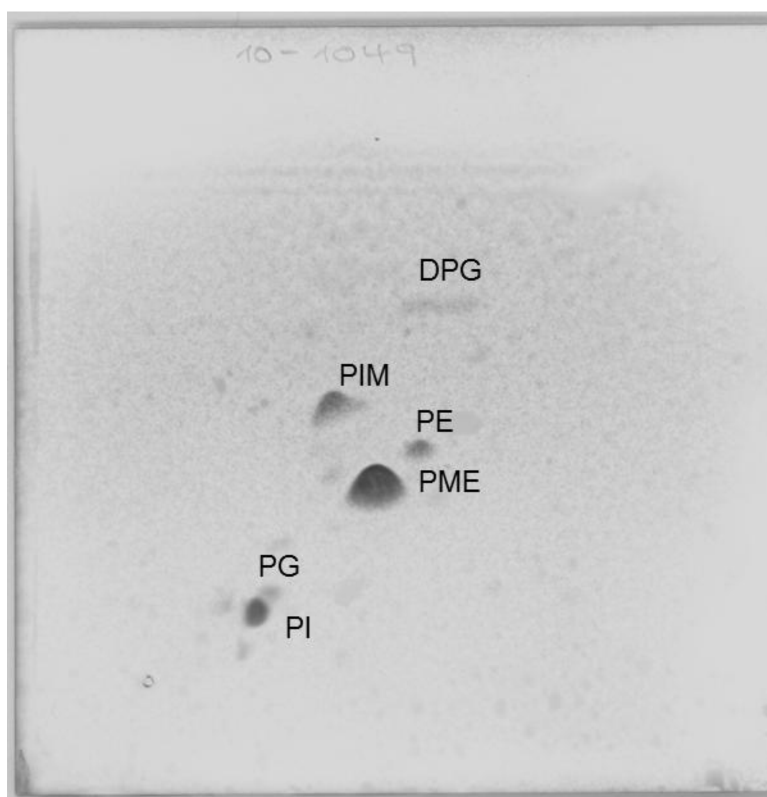
438 Supplementary Figure S1. Two-dimensional thin-layer chromatogram of polar lipids of strain PA3^T,

439 detected with the spray reagent molybdatophosphoric acid. Abbreviations: PME,

440 phosphatidylmethylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PI,

441 phosphatidylinositol; PIM, phosphatidylinositol mannoside; PE, phosphatidylethanolamine. First

442 dimension, left to right; second dimension bottom to top.



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