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Pseudonocardia hispaniensis sp. nov., a novel actinomycete isolated from industrial wastewater activated sludge

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Abstract

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

Introduction

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

The genus currently encompasses 40 recognised species (http://www.bacterio.cict.fr/p/pseudonocardia.html), most of which have been described in the last five years based upon studies of single strains. Such studies have provided useful information on the evolutionary radiation and distribution of the taxon in natural habitats but have given little insight into the biological properties of pseudonocardia. Single strains representing new species (including several names not validated to date) have been isolated from contaminated industrial sludge (Mahendra and Alvarez-Cohen 2005; Kämpfer et al. 2006), coastal sediment (Liu et al. 2006), indoor environment (Schäfer et al. 2009), plant litter (Sakiyama et al. 2010), soil (Park et al. 2008; Qin et al. 2008; Li et al. 2010; Ara et al. 2011) and from surface sterilized roots and stems of higher plants (Chen et al. 2009; Duangmal et al. 2009; Kaewkla and Franco 2010, 2011; Qin et al. 2010, 2011; Zhao et al. 2011a,b,c).

There is a need to discover the taxonomic diversity and functions of filamentous actinomycetes in wastewater treatment plants in order to improve operational procedures (Nam et al. 2003; Seviour et al. 2008). In the present study a *Pseudonocardia*-like strain was isolated as part of a survey of actinobacterial diversity in activated sludge plants in the south of Spain. A polyphasic taxonomic study of the isolate showed that it represented a new species of the genus *Pseudonocardia* for which the name *Pseudonocardia hispaniensis* sp. nov. is proposed.

Materials and methods

Organisms, maintenance and cultural conditions

Strain PA3^T was isolated from a modified Czapek agar (sucrose, 2%, w/v; yeast extract, 0.2%, w/v, $FeSO_4$, 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; distilled water, 1 litre) plate supplemented with nalidixic acid (20 mg l^{-1}) following inoculation with a sample taken from a wastewater treatment plant in Palos de la Frontera, Huelva, Spain. The isolate, which grew as a small colony covered with white aerial hyphae, was purified on yeast extract-malt extract agar (ISP medium 2; Shirling and Gottlieb 1966). The isolate and the type strain of *Pseudonocardia asaccharolytica* (DSM 44247^T) were maintained on ISP 2 agar slants at 4ºC and as suspensions of hyphal

fragments and spores in 20% (v/v) glycerol at -80ºC. Biomass for all but one of the chemotaxonomic studies was prepared by growing the isolate and *P. asaccharolytica* DSM 44247^T in shake flasks (at about 200 rpm) of GYE broth (glucose, 1%; yeast extract 1%; distilled water, 1 liter) for 14 days at 28°C. Similarly, biomass for the fatty acid analyses was harvested from shake flasks of Tryptic Soy Broth (Difco) (150 rpm) after 5 days at 28°C. Biomass for the chemical and molecular studies was washed in distilled water and freeze dried and kept at -20°C until needed.

16S rRNA gene sequencing analyses

Genomic DNA was extracted from isolate PA3^T using a commercial DNA extraction kit (GenElute, Sigma) and PCR amplification of the 16S rRNA gene achieved using the universal primers 27f and 1492r (Lane 1991), 616V and 699R for a stretch of around 1000 nt close to the 5' end (Arahal et al. 2008), and primers P609D and P1525R (reverse) for a segment of about 750 nt close to the 3' end (Lucena et al. 2010). The resultant almost complete 16S rRNA sequence (1434 nucleotides) was compared with corresponding sequences of the type strains of species classified in the genus *Pseudonocardia* using alignments retrieved from SILVA and LTP latest updates as references (Pruesse et al. 2007; Yarza et al. 2010); where necessary, additional sequences were retrieved from the DDBJ/EMBL/GenBank databases. Alignments were corrected manually based on secondary structural information. Sequence similarities were calculated in ARB based on sequences without the use of an evolutionary substitution model. Phylogenetic analyses using several treeing methods (distance matrix, maximum-likelihood and maximum-parsimony) and data subsets were examined using the appropriate ARB tools (Ludwig et al. 2004) (figure 1).

Chemotaxonomy

Isolate PA3^T was examined for chemical markers known to be of value in the classification of genera belonging to the family *Pseudonocardiaceae* (Labeda et al. 2011). Standard chromatographic procedures were used to determine the isomers of diaminopimelic acid (Staneck and Roberts 1974), predominant menaquinones (detected by Dr. Brian Tindall of the Identification Service, DSMZ, Braunschweig. Germany), mycolic acids (Hamid et al. 1993), polar lipids (Minnikin et al. 1974), whole-cell sugars

(Hasegawa et al. 1983) and DNA base composition (Mesbah et al. 1984), using appropriate controls. Fatty acid methyl esters were extracted and prepared according to standard protocols as described for the MIDI Microbial Identification System (Sasser, 1990) at the Colección Española de Cultivos Tipo, CECT . Cellular fatty acids were analyzed by GC with an Agilent 6850 chromatographic unit, with the MIDI Microbial Identification System using the TSBA6 method (MIDI, 2008) and identified using the Microbial Identification Sherlock software package. Polar lipids were extracted, examined by two-dimensional TLC and identified using the procedures described by Minnikin et al. (1984). The G+C content of the genomic DNA was determined by HPLC as described by Mesbah et al. (1989).

DNA:DNA relatedness studies

DNA samples extracted from isolate PA3^T and *P. asaccharolytica* DSM 44247^T using a French pressure cell (Thermo Spectronic) were purified by chromatography on hydroxyapatite (Cashion et al. 1977). DNA:DNA hybridization was carried out, in duplicate, after De Ley et al. (1970), with modifications by Huss et al. (1993), using a model Cary 100 Bio UV/Vis spectrophotometer fitted with a Peltier-thermostated 6 x 6 multicell charger and a temperature controller with an *in situ* temperature probe (Varian).

Cultural and morphological properties

Cultural characteristics of strain PA3^T and *P. asaccharolytica* DSM 44247^T were determined on modified Czapek's medium, potato dextrose agar **(**Difco) and standard International *Streptomyces* Project (ISP) media 2-7 (Table 1; Shirling and Gottlieb 1966). Spore chain arrangement and spore surface ornamentation were observed using growth taken from a yeast-extract – malt extract agar (ISP medium 2) plate after 14 days at 28ºC and examined using a JEOL JSM-5410 (JEOL Ltd., Tokyo, Japan) scanning electron microscope operating at 20kv (Alonso et al. 2009). To this end, a loopful of culture was washed in 0.1M sodium phosphate buffer (PBS; pH 7.2) in a 1.5 ml Eppendorf tube, the pellet fixed in additional PBS buffer containing 2.5% (v/v) glutaraldehyde for 3 hours at 4^{o}C and post-fixed with 2% (v/v) osmium tetroxide in 0.1M PBS for an hour at 4^{o}C. The fixed cells were washed in 0.1M PBS, transferred to the

surface of a 25 mm Poretics polycarbonate membrane (pore size 0.1 μm; Sigma), immersed in liquid nitrogen, and then coated with gold.

Phenotypic tests

The isolate PA3^T and the *P. asaccharolytica* type strain were examined for a range of phenotypic properties. Their ability to grow at 10, 28 37 and 45^{o}C was determined after 14 days using ISP 2 as the basal medium. Similarly, growth from pH 4-10 (at pH unit intervals adjusted with HCl or NaOH) and in the presence of NaCl (3.5 and 7%, w/v) were examined after 14 days at 28°C. Enzyme activity was established using API ZYM kits (bioMerieux) following the manufacturer's instructions. Growth under autotrophic conditions was tested using mineral media recommended by earlier workers (Okoh et al. 2001; Renfuss and Urban 2005; Auffret et al. 2009). Additional biochemical and physiological properties were recorded using previously described procedures (Gordon et al. 1974; Reichert et al. 1998).

Results and discussion

Isolate PA3^T formed a distinct subclade in the 16S rRNA *Pseudonocardia* tree together with *P. asaccharolytica* DSM 44247^T, an association supported by all of the tree-making algorithms and by a 98% bootstrap value (Fig. 1). However, the two organisms shared a low 16S rRNA similarity of 97.3%, a value which corresponded to 40 nucleotide differences. The corresponding 16S rRNA similarity values with the remaining pseudonocardial type strains ranged from 94.6 to 96.7%. The DNA:DNA relatedness values for the duplicated assays between strains PA3^T and *P. asaccharolytica* DSM 44247^T were 36.2 and 38.0%, well below the 70% cut-off point recommended for the delineation of bacterial species (Wayne et al. 1987).

Strain PA3^T had chemotaxonomic and morphological properties consistent with its classification in the genus *Pseudonocardia* (Zhao et al. 2011a,b,c; Huang and Goodfellow 2012). The organism formed an extensively branched substrate and aerial mycelia which underwent fragmentation into smooth surfaced coccoid or rod-shaped spores (Figure 2). There is not evidence of acropetal budding, zig-zag morphology

or intercalary swellings. In addition, it contains *meso*-A_{2pm}, arabinose and galactose in whole-organism hydrolysates (wall chemotype IV after Lechevalier and Lechevalier 1970), tetrahydrogenated menaquinones with eight isoprene units (MK8 [H_4]) as the predominant menaquinone, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylmethylethanolamine and phosphatidylinositol mannosides as major polar lipids (phospholipid type 2 *sensu* Lechevalier et al. 1981) (Supplementary Fig. S1), a high DNA G+C ratio (69.7 mol%), complex mixtures of iso- and anteiso – fatty acids with iso-$C_{16:0}$ as the predominant component , but lacked mycolic acids. The fatty acid profile of *P. assacharolytica* DSM 44247^T was in line with results reported by Reuchert et al. (1998) for four strains assigned to this species.

Isolate PA3^T and *P. asaccharolytica* DSM 44247^T can be distinguished using a combination of cultural and phenotypic properties (Tables 1, 2 and 3), as exemplified by the ability of the former to produce leucine and valine arylamidases, to grow at 37°C, in the presence of 3%, w/v NaCl, in different pH range and to form distinctive honey coloured, substrate mycelia on glycerol-asparagine and tyrosine agars. In contrast, neither strain produced acid from a broad range of carbohydrates, a result in line with the original description of *P. asaccharolytica* (Reichert et al. 1998). Similarly, the two organisms have qualitatively similar fatty acid profiles though quantitative differences were detected in some components (Table 2). When additional data acquired on isolate PA3^T were compared with corresponding results reported Reichert and his colleagues (1998) it was evident that the two strains can grow autotrophically and have a type 2 polar lipid pattern.

The chemotaxonomic, morphological and phenotypic data, together with the 16S rRNA sequence and DNA:DNA relatedness findings provide sufficient evidence to support the proposition that isolate PA3^T represents a novel species of the genus *Pseudonocardia* for which the name *Pseudonocardia hispaniensis* sp. nov. is proposed.

Description of *Pseudonocardia hispaniensis* sp. nov.

Pseudonocardia hispaniensis (his.pa.ni'en.sis. L. fem. adj. *hispaniensis*, of or belonging to *Hispania*, the Latin name for Spain, the country where the type strain was isolated).

Aerobic, non-motile, Gram-positive, non-acid-alcohol-fast actinomycete which forms extensively branched aerial and substrate mycelia that fragment into smooth surfaced coccoid or rod-like elements. Grows well on ISP media 2-7 forming white aerial mycelia but does not produce diffusible pigments. Grows from pH 5-10 (optimum 7-8) and at 28 and 37ºC, weakly at 45ºC, but not at 10ºC. Catalase-positive but oxidase negative. Additional cultural and phenotypic properties are shown in Tables 1 and 2. Aerobic autotrophic growth was observed in mineral medium without a carbon source. The wall diamino acid is *meso*-diaminopimelic acid, the diagnostic sugars arabinose and galactose, and the predominant fatty acid and isoprenoid quinone iso- $C_{16:0}$ and MK8 (H_4), respectively. The polar lipid pattern contained diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and phophatidylmethylethanolamine. The detailed fatty acid profile is given in Table 1. The G+C content of the DNA was 69.7 mol%.

The type strain, PA3^T (= CCM 8391^T = CECT 8030^T), was isolated from a sample taken from an oil refinery wastewater treatment plant in Palos de la Frontera, Huelva, Spain. The species description is based on a single strain and hence doubles up as a description of the type species.

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

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

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.

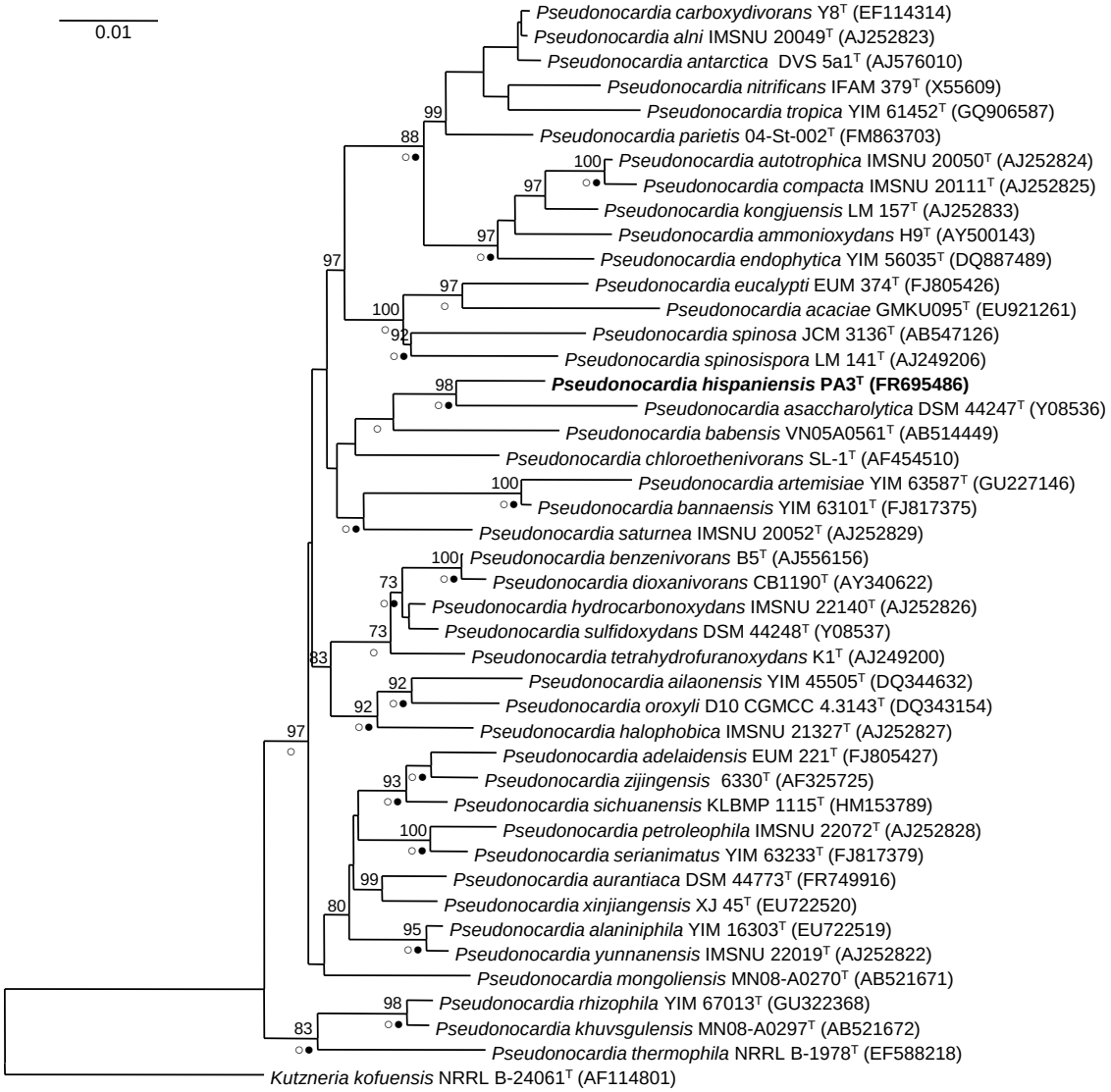
416

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^{o}C	+	-
Growth in presence of 3% w/v NaCl	+	-
pH growth range	5-10	6-9

Key: +, positive; -, negative. *Result not congruent with that reported by Reichert et al. (1998). Neither of the strains hydrolyzed urea or produced acid from adonitol, L-arabinose, *meso*-erythritol, fructose, galactose, glucose, lactose, inositol, inulin, maltose, mannitol, mannose, melezitose, rhamnose, saccharose, sorbitol, trehalose or xylose.

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



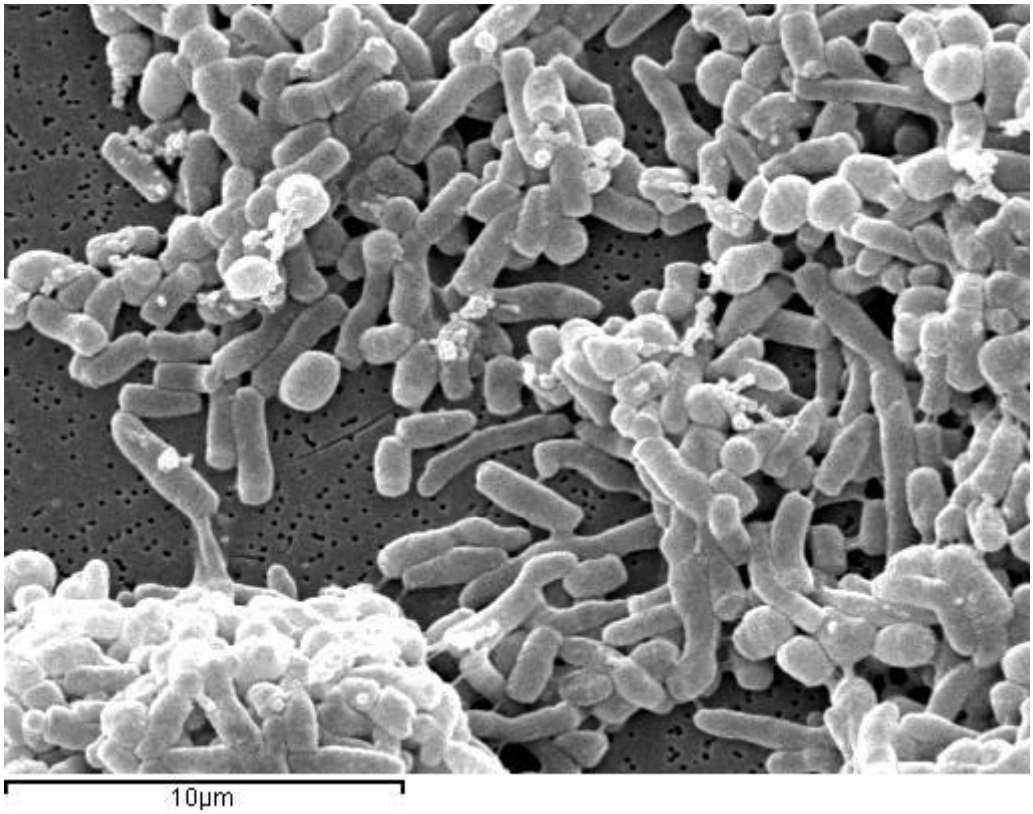


Figure 2: Scanning electron micrograph of a 14-days old culture of strain PA3^{T}.

Supplementary Figure S1. Two-dimensional thin-layer chromatogram of polar lipids of strain PA3^{T}, detected with the spray reagent molybdatophosphoric acid. Abbreviations: PME, phosphatidylmethylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PE, phosphatidylethanolamine. First dimension, left to right; second dimension bottom to top.