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TITLE: *Hydromonas duriensis* gen. nov., sp. nov., isolated from freshwater, in the Douro river, Portugal

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RUNNING TITLE: *Hydromonas duriensis* gen. nov., sp. nov.
CONTENTS CATEGORY: NEW TAXA: PROTEOBACTERIA
FOOTNOTE: The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the isolate A2P5^T is LM653273.

SUMMARY

An aerobic, Gram-negative rod, designated A2P5^T, was isolated from the Douro river, in Porto, Portugal. Cells were catalase- and oxidase-positive. Growth occurred in the range of 15-30 ºC, pH 6-8 and in presence of 1% (w/v) NaCl. The major respiratory quinone was Q8, the genomic DNA had a G+C content estimated to be 47±1 mol% and phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol were amongst the major polar lipids. On basis of the 16S rRNA gene sequence analysis, strain A2P5^T was observed to be a member of the family *Burkholderiaceae*, but could not be identified as member of any validly named genus. The low levels of 16S rRNA gene sequence similarity to other recognized taxa (< 91%), together with the comparative analysis of phenotypic and chemotaxonomic characteristics supported the proposal of a new genus within the family *Burkholderiaceae*. The name *Hydromonas duriensis* gen. nov., sp. nov. represented by the type strain of the genus and of the species is the strain A2P5^T (= LMG 28428^T = CCUG 66137^T), is proposed.

A bacterial strain, designated A2P5^T, was isolated from freshwater of river Douro in Northern Portugal, during a study on the bacterial diversity of drinking water before and after the treatment (Vaz-Moreira *et al.*, 2011, Vaz-Moreira *et al.*, 2013). Based on the 16S rRNA gene sequence analysis, the isolate was identified as a member of the family *Burkholderiaceae*. The highest 16S rRNA gene sequence similarity values were of approximately 90% with members of the genera *Cupriavidus* and *Ralstonia*. Members of these genera comprise bacteria occurring in both environmental and clinical settings, including opportunistic human and plant pathogens, and potential biodegraders of recalcitrant xenobiotics (Coenye *et al.*, 1999, Chen *et al.*, 2001, Cuadrado *et al.*, 2010). To test the hypothesis that strain A2P5^T represents a new genus, the strain was compared with the type strains of the type species of the closest genera, *Cupriavidus necator* LMG 8453^T and *Ralstonia pickettii* LMG 5942^T.

The strain A2P5^T was isolated from river Douro surface water on the culture medium *Pseudomonas* Isolation Agar, after 72h of incubation at 30ºC. Strain A2P5^T was in the water sample at a density of approximately 100 colony forming units per milliliter and was co-isolated with members of taxa such as *Acinetobacter junii*, *Pseudomonas simiae*, *Aeromonas veronii* or *Chryseobacterium* sp. Strain A2P5^T was purified by subculturing on modified Luria-Bertani agar (mLA: 5 g.L^{-1} tryptone, 2.5 g.L^{-1} yeast extract, 1 g.L^{-1} NaCl and 15 g.L^{-1} agar), on which, after 48-72 h of incubation, formed small, convex and beige-colored colonies. The culture was preserved frozen at -80ºC in nutritive broth with 15% (v/v) glycerol. Phenotypic and chemotaxonomic assays were performed simultaneously for strains A2P5^T and the type strains *Ralstonia pickettii* LMG 5942^T and *Cupriavidus necator* LMG 8453^T. Unless stated otherwise bacteria were cultivated on mLA, and incubated at 30 ºC. Colony and cell morphology, Gram staining,

cytochrome *c* oxidase and catalase tests were analysed based on the methodologies of Murray *et al.* (1994) and Smibert & Krieg (1994). Cell morphology was observed by transmission electron microscopy, as described before (Vaz-Moreira *et al.*, 2012). Briefly, bacteria were fixed in 2.5% glutaraldehyde and 4% formaldehyde in cacodylate buffer 0.1 M pH 7.2, washed in the same buffer, and post-fixed overnight in 2% OsO4 buffered with cacodylate. After brief washing bacteria were treated with 1% uranyl acetate, dehydrated in increasing concentrations of ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a JEOL 100CXII transmission electron microscope (60 kV). The cells size was determined based on optical microscopy, with the software ImageJ 1.48v. The pH range for growth was examined in modified Luria-Bertani broth (5 $g.L^{-1}$ tryptone, 2.5 $g.L^{-1}$ yeast extract and 1 $g.L^{-1}$ NaCl) containing 12 mM MES (Sigma) to adjust the pH to 5.0, 6.0 and 7.0, 12 mM TAPS (Sigma) to adjust pH to 8.0, and 12 mM CAPS (Sigma) to adjust the pH to 9.0 or 10.0. NaCl tolerance and temperature range for growth were assayed, respectively, in culture medium supplemented with 0.1, 1, 2 and 3% NaCl (w/v) or incubated at 10, 15, 18, 25, 30 and 37 ºC. Biochemical and nutritional tests were performed by using the commercial kits API 20E, API 20NE, API ZYM and API 50CH (bioMérieux SA, France) following the manufacturer's instructions. The API 50CH strips were inoculated with the bioMérieux AUX medium. The growth under anaerobic conditions was tested on mLA incubated in an anaerobic chamber. The ability to reduce nitrate was tested in modified Luria-Bertani broth supplemented with 0.25% agar and 0.1% KNO_3 (w/v), under aerobic and anaerobic conditions.

After 48-72 h of incubation on mLA, strain A2P5^T produced beige-coloured, small and convex colonies. Cells were rods with sizes ranging 0.7-2.2 μm length, with observable electron dense bodies (Fig. 1). In modified Luria-Bertani broth, optimal growth

103 occurred at 30ºC, pH 8 and 0.1% (w/v) NaCl. No growth was observed at 37ºC, in the
104 presence of 3% NaCl or at pH 9.0.
105 Of the 54 carbon sources tested, only D-glucose, D-mannitol and N-Acetyl glucosamine
106 were used as single sources of carbon (Table 1).
107 The nucleotide sequence of the 16S rRNA gene was determined after PCR
108 amplification of total DNA extract using the primers 27F and 1492R as described
109 previously (Ferreira da Silva *et al.*, 2007). The 16S rRNA gene sequence was compared
110 with others available in the EzTaxon database (Kim *et al.*, 2012). Phylogenetic analysis
111 was conducted using the MEGA6 software (Tamura *et al.*, 2013). Sequence similarity
112 was estimated based on the model of Maximum Composite Likelihood and the
113 dendrogram was created with the Neighbor-joining statistical method. The maximum-
114 likelihood method was used to assess tree stability. Non-homologous and ambiguous
115 nucleotide positions were excluded from the calculations and a total of 1248 nt positions
116 were included in the analysis. This analysis showed that the strain A2P5^T belongs to the
117 family *Burkholderiaceae*, with the genera *Cupriavidus* and *Ralstonia* as the closest
118 neighbours, with sequence similarities of 90.3% and 90.6% with the type strains of the
119 type species *Cupriavidus necator* and *Ralstonia picketti*, respectively (Fig. 2). The
120 sequence similarity values with the type species *Polynucleobacter necessarius* subsp.
121 *necessarius* and *Oxalobacter formigenes* were of 87.9% and 88.9%, respectively.
122 Fatty acid methyl esters were extracted and analysed using 48 h cultures on Trypticase
123 Soy Broth Agar, incubated at 28 ºC, as described previously (Vandamme *et al.*, 1992),
124 and separated and identified by using the Sherlock Microbial Identification System
125 (version 3.1; MIDI Inc.). For strain A2P5^T, these analyses were complemented by GC-
126 MS identification of the major fatty acid methyl ester components, using the conditions
127 described previously (Manaia & Moore, 2002), in the equipment Varian 3800 Gas

Chromatograph coupled with a Varian Saturn 2000 ion trap GC-MS workstation software, version 6.9.1.

The polar lipid composition was determined as described previously (Manaia *et al.*, 2004). Determination of the mol% G+C content of the genomic DNA and of the respiratory quinones was performed as described previously (Vaz-Moreira *et al.*, 2007) based on the methods of Mesbah *et al.* (1989) and Tindall (1989), respectively.

The FAMEs profile of strain A2P5^T was characterized by the predominance of fatty acids identified as summed feature 3 ($C_{16:1}$ *w7c* and/or iso-$C_{15:0}$ 2-OH), in which $C_{16:1}$ *w7c* predominated and summed feature 2 ($C_{12:0}$ aldehyde, an unknown fatty acid with equivalent chain length value of 10.928, and $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I), in which predominated $C_{14:0}$ 3-OH (Table 2). The polar lipids pattern was composed of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol one aminophospholipid and three aminolipids (Fig. 3). The respiratory quinone was ubiquinone 8. The DNA G+C content was 47±1 mol%. These chemotaxonomic characteristics do not exclude the affiliation of strain A2P5^T to the family *Burkholderiaceae* (Yabuuchi *et al.*, 2005).

Confirming the 16S rRNA gene sequence comparative analysis, which suggested that strain A2P5^T could represent a new genus, the phenotypic and chemotaxonomic characterization supported its differentiation from the type strains of the type species of related genera. In particular, in contrast with *Cupriavidus necator* LMG 8453^T and *Ralstonia pickettii* LMG 5942^T, strain A2P5^T was able to assimilate D-mannitol and N-acetyl glucosamine, exhibited β-galactosidase and α-glucosidase activity and was unable to utilize citrate, to assimilate D-fructose, potassium gluconate, potassium 2-ketogluconate, trisodium citrate and some organic acids and to reduce nitrite (Table 1).

The polar lipid profile of A2P5^T was distinct of that of strains *Cupriavidus necator*

153 LMG 8453^T and *Ralstonia pickettii* LMG 5942^T, in particular due the presence of three
154 unknown aminolipids and one aminophospholipid (Fig. 3). Also the FAMEs profile
155 allowed the differentiation of strain A2P5^T from the closest neighbours, showing a
156 higher percentage of the fatty acids summed feature 2 ($C_{12:0}$ aldehyde, and $C_{14:0}$ 3-OH
157 and/or iso-$C_{16:1}$ I) and $C_{12:0}$, and a lower percentage of $C_{16:0}$ and $C_{18:1}$ *w7c* (Table 2).
158 These differentiating characteristics and the unique phylogenetic position (Fig. 2),
159 suggest that strain A2P5^T should be most appropriately allocated to a new genus.
160
161 **Description of *Hydromonas* gen. nov.**
162 Hydromonas [Hy.dro.mo.nas Gr. n. *hydor* water; L. fem. n. monas a unit, monad; N.L.
163 fem. n. *Hydromonas*, a unit (rod) from water]
164
165 Cells are non-spore-forming, Gram-negative rods. Catalase- and cytochrome *c* oxidase-
166 positive. Mesophilic. Chemo-organotrophic with aerobic respiratory metabolism. Poor
167 metabolic versatility, although sugars or thereof derivatives can be used as carbon
168 sources. The respiratory quinone is ubiquinone 8 and the DNA G+C content is 47
169 mol%. Major fatty acids are summed feature 3 ($C_{16:1}$ *w7c* and/or iso-$C_{15:0}$ 2-OH) and
170 summed feature 2 ($C_{12:0}$ aldehyde, and $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I), with $C_{16:1}$ *w7c* and
171 $C_{14:0}$ 3-OH predominating, respectively, in each of those categories. The polar lipids
172 comprise phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and
173 minor unidentified amino(phospho)lipids. Phylogenetically, belongs to the family
174 *Burkholderiaceae*. The type species is *Hydromonas duriensis*.
175
176 **Description of *Hydromonas duriensis* sp. nov.**

177 *Hydromonas duriensis* (du.ri.en.sis, L. neut. adj. duriensis, inhabiting the Portuguese
178 Douro region).
179
180 Colonies are beige-coloured, small (~1 mm diameter), convex and slightly dry on
181 modified Luria-Bertani agar (mLA) after 48-72 h of incubation. Cells are non-spore
182 forming rods (ranging from 0.7-2.2 μm in length and 0.4±0.1 μm wide), with poor
183 growth under anaerobic conditions in the presence of nitrate, Gram-negative, catalase-
184 and oxidase-positive. Good growth occurs on mLA, at 15-30ºC, pH 6-8 and in the
185 presence of up to 1% (w/v) NaCl [optima at about 30ºC, pH 8 and 0.1% (w/v) NaCl].
186 Reduces nitrate to nitrite, but does not reduce nitrite to nitrogen. Simmons citrate is not
187 utilized. Aesculin is not hydrolysed. H_2S, indole and acetoin are not produced. Glucose
188 is not fermented, and none of the API 20E carbon sources leads to acid production
189 under aerobic conditions. Produces β-galactosidase, tryptophan deaminase, alkaline and
190 acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-
191 BI-phosphohydrolase and α-glucosidase, but not the other enzymes present in the API
192 ZYM, 20E or 20NE. Presents a weakly positive reaction for valine arylamidase activity.
193 Assimilates D-glucose, D-mannitol, and N-Acetyl glucosamine, but not the other carbon
194 sources available in the API 50CH or API 20NE systems. The respiratory quinone,
195 major polar lipids and predominant fatty acids are those listed in the genus description.
196 The type strain, A2P5^T (= LMG 28428^T = CCUG not available yet) was isolated from
197 freshwater in river Douro. The DNA G+C content of the type strain is 47±1 mol%.
198
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Table 1. Differentiating characteristics between strain A2P5^T and type strains of the type species of the closest genera: *Ralstonia pickettii* LMG 5942^T and *Cupriavidus necator* LMG 8453^T.

Data are from the present study except where indicated otherwise. ^{w}, weakly positive.

Characteristic	A2P5^T	*Ralstonia pickettii* LMG 5942^T	*Cupriavidus necator* LMG 8453^T
Denitrification (N_2)	-	+	+
Citrate utilization	-	+	+
Assimilation of:			
L-Arabinose	-	+	-
D-Fructose	-	+	+
D-Fucose	-	+^{w}	-
D-Galactose	-	+	-
D-Glucose	+	+	-
D-Xylose	-	+	-
Glycerol	-	+	-
D-Mannitol	+	-	-
N-Acetyl glucosamine	+	-	-
Potassium gluconate	-	+	+
Potassium 2-ketogluconate	-	+	+
Trisodium citrate	-	+	+
Adipic acid	-	+^{w}	+^{w}
Capric acid	-	+	+
Malic acid	-	+	+
Phenylacetic acid	-	+^{w}	+
Enzymes:			
α-Glucosidase	+	-	-
β-Galactosidase	+	-	-
Tryptophane deaminase	+	+	-
Lipase (C14)	-	+	-
Polar lipids	PE, PG, DPG, 3 minor AL and 1 APL	PE, PG, DPG, and 1 minor APL	PE, PG, DPG
DNA G+C content (mol%)	47±1	64 (NMF)*	57±1 (Tm)†
Source of isolation of the type strain	River water	Human clinical specimen	Soil

PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; AL, unidentified aminolipid; and APL, unidentified aminophospholipid.

NMF (determined with the nitrocellulose membrane filter technique); Tm (determined by the thermal melting point)

* (Sahin *et al.*, 2000); †(Makkar & Casida, 1987)

Table 2. Fatty acid composition of strain A2P5^T and members of the closest genera: *Ralstonia pickettii* LMG 5942^T and *Cupriavidus necator* LMG 8453^T.

Those fatty acids for which the amount for all taxa was < 1% are not included. In bold are indicated the fatty acids with amounts >10 %. Therefore, the percentages may not add up to 100%. tr, trace amount (< 1%); ND, not detected.

Fatty acid	ECLs	A2P5^T	*Ralstonia pickettii* LMG 5942^T	*Cupriavidus necator* LMG 8453^T
$C_{10:0}$	10.00	2.4	ND	ND
iso-$C_{11:0}$	10.61	5.2	ND	ND
$C_{12:0}$	12.00	7.1	tr	ND
iso-$C_{13:0}$	12.61	1.2	ND	ND
$C_{14:0}$	14.00	4.1	5.2	2.1
iso-$C_{15:0}$	14.62	1.1	tr	ND
$C_{14:0}$ 2-OH	15.20	ND	1.1	5.1
$C_{16:0}$	16.00	9.0	**27.1**	**20.0**
iso-$C_{15:0}$ 3-OH	16.13	2.9	ND	ND
$C_{17:0}$ cyclo	16.89	ND	2.6	5.1
$C_{16:1}$ 2-OH	17.05	ND	4.0	2.8
$C_{16:0}$ 2-OH	17.23	1.6	tr	1.3
$C_{18:1}$ *w7c*	17.83	7.1	**12.8**	**16.1**
$C_{18:1}$ 2-OH	19.09	ND	3.9	1.7
SF2*	10.92	2.1	0.5	1.9
	15.49	**22.4**	8.7	**15.7**
SF3†	15.82	**32.7**	**31.7**	**28.2**

ECLs, Estimated Chain Length

*SF2, summed feature 2 ($C_{12:0}$ aldehyde, an unknown fatty acid with equivalent chain length value of 10.928, and $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I).

†SF3, summed feature 3 ($C_{16:1}$ *w7c* and/or iso-$C_{15:0}$ 2-OH)

For A2P5^T, the SF2 is composed of about two times more $C_{14:0}$ 3-OH than iso-$C_{16:1}$ I, and SF3 includes about four times more $C_{16:1}$ *w7c* than iso-$C_{15:0}$ 2-OH.

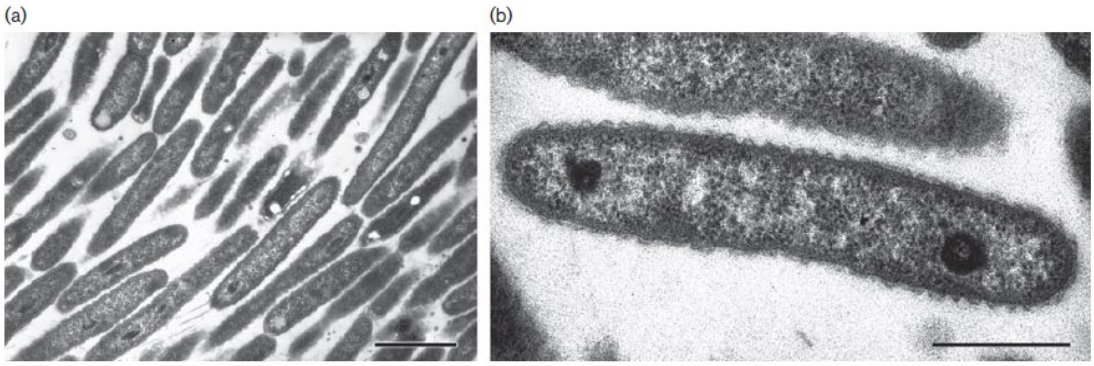

Figure 1. Transmission electron micrographs of cells of strain A2P5^T. (A) Cells after growth for 3 days at 30 ºC on mLA, showing cell morphology. (B) Detail of a cell with two unknown electron dense bodies.

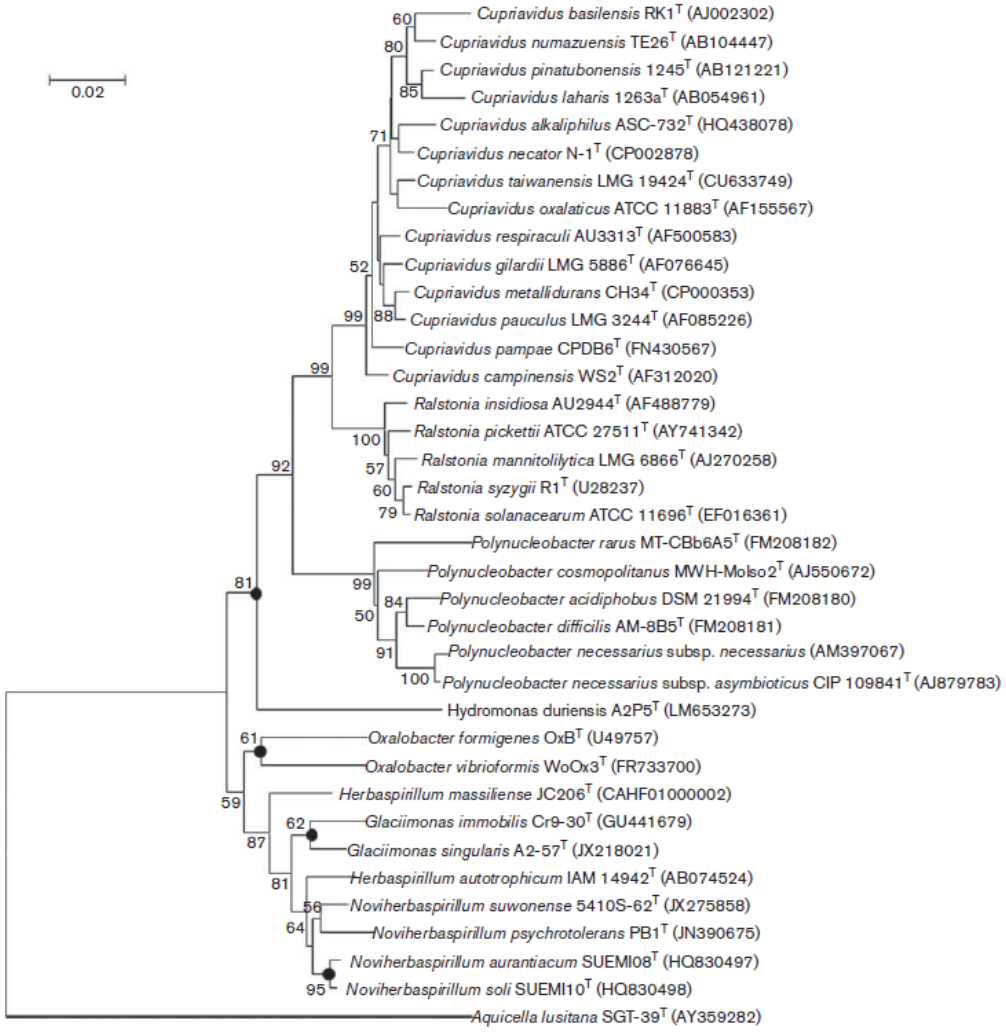

Figure 2. Phylogenetic tree derived from 16S rRNA gene sequence analysis, showing the relationship of strain A2P5^T with members of the family *Burkholderiaceae* and *Oxalobacteraceae*. The species *Aquicella lusitana* was used as outgroup.
The tree was generated by the Neighbor-joining method. Bootstrap values, generated from 1000 re-samplings, at or above 50% are indicated at branch points. Closed circles indicate branches

also recovered by the maximum-likelihood method. Bar, 1 substitution per 50 nucleotide positions.

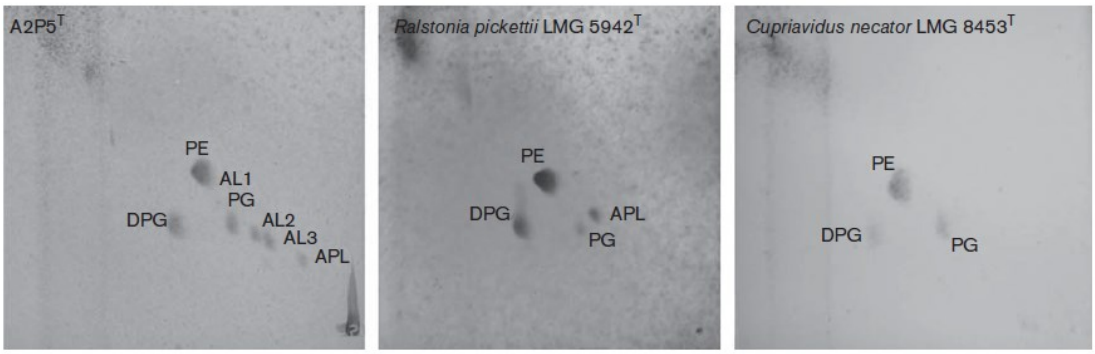

Figure. 3. Polar lipid patterns of strain A2P5^T and the closest neighbours *Ralstonia pickettii* LMG 5942^T and *Cupriavidus necator* LMG 8453^T.
PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; AL, unidentified aminolipid; and APL, unidentified aminophospholipid.