Herminiimonas arsenicoxydans sp. nov., a metalloresistant bacterium

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An arsenite-oxidizing bacterium, designated strain ULPAs1^T, was isolated from industrial sludge heavily contaminated with arsenic. Cells of this isolate were Gram-negative, curved rods, motile by means of a polar flagellum. The strain was positive for oxidase and catalase activities, was able to reduce nitrate to nitrite, used acetate, lactate and peptone as organic carbon sources under aerobic conditions and was able to oxidize arsenite (As[III]) to arsenate (As[V]). 16S rRNA gene sequence analysis and the absence of dodecanoic fatty acids suggested that this strain represents a member of the genus *Herminiimonas* of the family *Oxalobacteraceae*, order *Burkholderiales* in the *Betaproteobacteria*. Genomic DNA–DNA hybridization between strain ULPAs1^T and *Herminiimonas fonticola* S-94^T and between strain ULPAs1^T and *Herminiimonas aquatilis* CCUG 36956^T revealed levels of relatedness of < 10%, well below the recommended 70% species cut-off value. Thus, strain ULPAs1^T (=CCM 7303^T=DSM 17148^T=LMG 22961^T) is the type strain of a novel species of *Herminiimonas*, for which the name *Herminiimonas arsenicoxydans* sp. nov. is proposed.

The arsenic oxanion arsenite (As[III]) can be used by prokaryotes as an electron donor. Cells couple the oxidation of arsenite to the reduction of either oxygen or nitrate and use the energy derived either to fix CO₂ into organic cellular material and achieve growth for chemolithoautotrophic prokaryotes or to help in the resistance to arsenic for heterotrophic organisms (Oremland & Stolz, 2003). The microbiological oxidation of As[III] to arsenate (As[V]) impacts the mobility and the speciation of arsenic in the environment. More than 30 strains representing at least nine genera of the *Bacteria* and *Archaea*, including members of the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deinococcus–Thermus* and *Crenarchaeota*, have been reported to be involved in arsenite oxidation.

Physiologically diverse, these micro-organisms include both heterotrophic arsenite oxidizers and chemolithoautotrophic arsenite oxidizers (Santini *et al.*, 2000; Oremland & Stolz, 2003; Silver & Phung, 2005). Heterotrophic oxidation of As[III] is viewed primarily as a detoxification reaction that converts As[III] encountered on the outer membrane of the cell into the less toxic and less mobile form As[V], perhaps making it less likely to enter the cell (Anderson *et al.*, 1992).

A Gram-negative, aerobic bacterial strain, designated ULPAs1^T, was isolated from an industrial wastewater treatment plant contaminated with arsenic (0.47 mmol kg⁻¹) and other metals (Weeger *et al.*, 1999). This strain was able to tolerate 5 mM As[III] and was able to oxidize it to As[V] either in free suspension (Weeger *et al.*, 1999; Muller *et al.*, 2003) or immobilized in a calcium alginate gel (Simeonova *et al.*, 2005). Moreover, strain ULPAs1^T was able to reduce As[V] to As[III] and to synthesize at least two arsenate reductases (Carapito *et al.*, 2006). Finally, it was also found to be resistant to numerous heavy metals such as Se[IV], Mn[II], Cr[III], Cd[II], Sb[III] and Ni[II] (Muller *et al.*, 2003). Based on partial 16S rRNA gene sequence analysis, strain ULPAs1^T was provisionally identified to be closely related to the species *Duganella zoogloeoides* (former *Zoogloea*

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ULPAs1^T is AY728038.

ramigera IAM 12670) (Weeger *et al.*, 1999) and was tentatively given the name '*Caenibacter arsenoxydans*' (Carapito *et al.*, 2006). The present study completes the phenotypic and genotypic characterization of this strain. Our results show that this bacterium represents a novel species of the recently described genus *Herminiimonas* (Fernandes *et al.*, 2005).

Strain ULPA s_1^T was isolated after aerobic enrichment on a chemically defined medium (CDM) supplemented with 1.33 mM As[III] (Weeger *et al.*, 1999). Briefly, CDM was prepared as follows: 100 ml solution A [81.2 mM MgSO₄·7H₂O (Sigma), 187 mM NH₄Cl (99.8% purity; Merck), 70 mM Na₂SO₄ (99%; Prolabo), 0.574 mM K₂HPO₄ (97%; Prolabo), 4.57 mM CaCl₂·2H₂O (99.5%; Merck), 446 mM sodium lactate (98%; Sigma)], 2.5 ml solution B [4.8 mM Fe₂SO₄·7H₂O (99%; Prolabo)] and 10 ml solution C [950 mM NaHCO₃ (99.5%; Prolabo)] were mixed and made up to 1 litre with water. The final pH of the medium was about 7.2. Cells of strain ULPA s_1^T were Gram-negative, and pale yellow to straw-coloured convex colonies with entire margins were observed when the strain was grown on CDM agar. Transmission electron microscopic observations showed that cells were slightly curved or straight rods with rounded ends, approximately 1–2.5 µm long and 0.5–0.7 µm wide, harbouring a single polar flagellum (Fig. 1). Cell motility was observed on low-concentration agar plates, with a swarming rate of 15–20 mm in 48 h.

Different carbon substrates were tested and the results are listed in Table 1. Growth was not affected when the medium was supplemented with As[III] (1.3 mM). Chemolithoautotrophic growth was tested on CDM in the absence of lactate or other organic carbon sources; no growth was observed, indicating that strain ULPA s_1^T possesses a chemoorganotrophic metabolism. Moreover, under anaerobic growth conditions (BBL GasPak pouch; Becton Dickinson) on

CDM containing a carbon source (lactate or acetate) and one metal (2.5 mM selenate, 2.5 mM arsenate), no growth was observed after 2 weeks, indicating that these metals are not used as electron acceptors. Finally, strain ULPA s_1^T showed mesophilic growth between 4 and 30 °C with optimum growth at 25 °C. Above 30 °C, growth was inhibited. Optimum pH for growth was between 7 and 8.5. Antibiotic resistance was tested by the disc diffusion method (Courvalin *et al.*, 1985). Growth of the isolate on CDM agar was not inhibited by tetracycline or ampicillin, but was inhibited by kanamycin, chloramphenicol, streptomycin and trimethoprim-sulfamethoxazole.

16S rRNA gene fragments were amplified by PCR from DNA extracts by using the eubacterial universal primers specific for this gene (P8: 5'-AGAGATTTGATCCTGGC-TCAG-3'; Pc1544: 5'-AAGGAGGTGATCCAGCCGCA-3'). Phylogenetic analyses based on the 16S rRNA gene sequence (1455 nt) further supported the conclusion that the strain belongs to the *Betaproteobacteria* and that its nearest phylogenetic relatives are members of the family *Oxalobacteraceae* in the order *Burkholderiales* (Garriety *et al.*, 2001): *Herbaspirillum huttiense* ATCC 14670 T (96% sequence similarity), *Herbaspirillum rubrisubalbicans* ATCC 19308 T (96%), uncultured *Duganella* clone CTHB-18 (96%), *Paucimonas lemoignei* LMG 2207 T (95%), *Duganella zoogloeoides* IAM 12670 T (94%) and *Telluria mixta* ACM 1762 T (93%).

Strain ULPA s_1^T exhibited 16S rRNA gene sequence similarity of 98.56% to the recently described species *Herminiimonas fonticola* S-94 T (Fernandes *et al.*, 2005), 98% to *Herminiimonas aquatilis* CCUG 44693 T (Kämpfer *et al.*, 2006) and 98.6% to the partially characterized strain ND5, isolated from a soil in Tokyo (Iizuka *et al.*, 1998). A phylogenetic tree based on 16S rRNA gene sequences detailing the relationship between strain ULPA s_1^T and its closest relatives is shown in Fig. 2. In order to determine further the position of strain ULPA s_1^T , DNA–DNA hybridization experiments were performed as described by Riegel *et al.* (1994). Strain ULPA s_1^T showed levels of DNA–DNA hybridization of 3% with *H. fonticola* S-94 T , 5% with *H. aquatilis* CCUG 36956 T and 11% with strain ND5. These values are clearly lower than the recommended 70% cut-off value used to delineate genomic species (Sneath, 1984; Stackebrandt & Goebel, 1994).

Phenotypic data were obtained by classical methods of carbohydrate utilization determined either on nutrient broth or with the API 20NE and API 50CH systems. As with the two recently described *Herminiimonas* species, strain ULPA s_1^T utilized few compounds as unique carbon sources, whereas strain ND5 was able to assimilate a greater variety of carbon sources (Table 1). *H. aquatilis* CCUG 36956 T and strain ND5 have been shown to grow on nutrient-rich media (Iizuka *et al.*, 1998; Kämpfer *et al.*, 2006), but these media did not support the growth of strain ULPA s_1^T . The minimal inhibitory concentration (MIC) of different metals was determined by following the procedure described by Lim & Cooksey (1993). Briefly, bacterial suspensions were

Fig. 1. Electron micrograph of a negatively stained cell of strain ULPA s_1^T , stained as described by Bertin *et al.* (1994). Bar, 500 nm.

Table 1. Physiological characteristics that differentiate strain ULPAs1^T and related strains

Strains: 1, strain ULPAs1^T; 2, *H. fonticola* S-94^T (data from Fernandes *et al.*, 2005); 3, *H. aquatilis* CCUG 44693^T (Kämpfer *et al.*, 2006); 4, strain ND5 (data from this study unless indicated). With the exception of microscopic observations and metal resistance tests, all characteristics were tested by using the API 20NE and API 50CH systems for strains ULPAs1^T and ND5. Arsenite oxidase activity was tested by AgNO₃ staining (Lett *et al.*, 2001; Simeonova *et al.*, 2004). All four strains are positive for oxidase activity and assimilation of lactate. Strains ULPAs1^T and ND5 showed negative results for glucose fermentation, aesculin hydrolysis, gelatin hydrolysis, β -galactosidase activity and assimilation of mannose, mannitol, *N*-acetylglucosamine, caprate, citrate, glycerol, erythritol, arabinose, D-ribose, xylose, methyl β -D-xylopyranoside, galactose, fructose, sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, amygdalin, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-trehalose, D-melezitose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, fucose, arabinol, gluconate, 2-ketoglutarate and 5-ketoglutarate. +, Positive; –, negative; ND, no data.

Characteristic	1	2	3	4
Cell morphology	Curved rods	Rods	Rods	Rods*
Cell size (μ m)	1–2 $\times$ 0.5–0.7	1–4 $\times$ 0.7	3–4 $\times$ 1–1.5	0.5 $\times$ 0.1–0.2*
Colony aspect	Opaque yellow to straw coloured	Transparent yellowish	Yellowish	Small, white
Anaerobic growth	–	–	ND	–
Growth temperature (°C)				
Range	4–30	10–35	ND	ND
Optimum	25	30	25	30
Optimum pH for growth	7.2–8.5	8.0	ND	ND
DNA G+C content (mol%)	54.3	52	ND	ND
MIC of metal ions:				
Cd ²⁺	630 μ M	20 μ M	80 μ M	ND
Mn ²⁺	> 10 mM	10 mM	5 mM	ND
Se ²⁺	> 10 mM	1.25 mM	5 mM	ND
As[III]	5 mM	0.63 mM	1.25 mM	2 mM
As[V]	> 10 mM	5 mM	> 10 mM	> 10 mM
Arsenite oxidation	+	–	–	–
Nitrate reduction	+	–	ND	+
Catalase	+	+	ND	+
Indole production from tryptophan	–	–	ND	+
Urease activity	–	–	ND	+
Assimilation of:				
Acetate	+	+	–	+
Citrate	–	+	–	–
Malate	–	+	–	+
Phenylacetate	–	ND	–	+
Adipate	–	ND	ND	+
Gluconate	–	ND	–	+
Glucose	–	–	–	+
Maltose	–	–	–	+
Sucrose	–	–	–	+
Inulin	–	ND	ND	+
Peptone	+	ND	ND	+
Alanine	–	+	–	ND
Raffinose	–	ND	–	+

*Data from Iizuka *et al.* (1998).

transferred in triplicate from microtitre plates to solid CDM plates supplemented with increasing concentrations of metals. The MIC, defined as the metal concentration that inhibited confluent growth on plates after 3 days at 30 °C, was then determined (Table 1). Growth of *H. fonticola*, *H. aquatilis* and strain ND5 was inhibited by 2 mM As[III],

whereas strain ULPAs1^T exhibited growth at up to 5 mM As[III]. Differences in MIC for different metals allowed the differentiation of ULPAs1^T from *H. fonticola* and *H. aquatilis* (Table 1). Taken together, these data demonstrate that, despite a significant level of conservation in their 16S rRNA gene sequences, some significant phenotypic

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences, showing the relationship of strain ULPAs1^T with its closest relatives within the *Oxalobacteraceae*. The sequences obtained were compared with those in the EMBL/GenBank databases, and aligned by using the CLUSTAL method (Higgins & Sharp, 1988) with DNASTar software. The 16S rRNA gene sequence was aligned with reference sequences by using the CLUSTAL W program (Thompson *et al.*, 1994). The tree was constructed from an evolutionary distance matrix calculated by the neighbour-joining method (Saitou & Nei, 1987), with *Cupriavidus metallidurans* CH34^T and *Ralstonia solanacearum* ATCC 11696^T as the outgroup. GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position.

differences allowed the differentiation of strain ULPAs1^T from *H. fonticola*, *H. aquatilis* and the partially characterized strain ND5 (Table 1).

Standard procedures were used to determine the G+C content of the genomic DNA of strain ULPAs1^T. The DNA G+C content determined was 54.3 mol%, similar to the value for *H. fonticola* (52%; Fernandes *et al.*, 2005). Fatty acid analysis was performed by the Belgian Co-ordinated Collections of Microorganisms (BCCMTM/LMG, University of Gent, Belgium) and the results are presented in Table 2. The cellular fatty acid compositions of strain ULPAs1^T, *H. fonticola* and *H. aquatilis* differ notably from those of other members of the '*Oxalobacteraceae*' by the absence of dodecanoic fatty acids. As with *H. fonticola* and *H. aquatilis*, strain ULPAs1^T contained large amounts of C_{16:0} and C_{16:1ω7c} fatty acids. Strain ULPAs1^T differed from *H. aquatilis* by producing C_{17:0} cyclo and C_{14:0}. Differences in cellular fatty acid composition allowing the differentiation of strain ULPAs1^T from *H. fonticola* and *H. aquatilis* are shown in Table 2.

Taken together, our results suggest that strain ULPAs1^T is a member of the *Betaproteobacteria*, order *Burkholderiales*, and is the type strain of a novel species of the genus *Herminiimonas*, for which the name *Herminiimonas arsenicoxydans* sp. nov. is proposed.

Description of *Herminiimonas arsenicoxydans* sp. nov.

Herminiimonas arsenicoxydans (ar.se.nic.ox'y.dans. N.L. n. *arsenicum* arsenic; N.L. v. *oxydare* to oxidize; N.L. part. adj. *arsenicoxydans* arsenic-oxidizing).

Cells are slightly curved or straight rods with rounded ends, approximately 1–2 µm long and 0.5–0.7 µm wide. Cells stain Gram-negative and harbour a single polar flagellum. Cells do not form spores. Colonies on CDM agar are convex with entire margins and are pale yellow to straw coloured.

Table 2. Fatty acid composition of *Herminiimonas* species

Strains: 1, strain ULPAs1^T; 2, *H. fonticola* S-94^T (data from Fernandes *et al.*, 2005); 3, *H. aquatilis* CCUG 44693^T (Kämpfer *et al.*, 2006). Data are percentages of total fatty acids.

Fatty acid	1	2	3
C _{9:0}	—	—	0.2
C _{10:0}	—	0.5	—
C _{10:0} 3-OH	8	6.0	5.2
C _{14:0}	5	4.8	—
C _{15:0}	—	—	2.8
C _{15:1ω6c}	—	—	3.6
C _{16:1ω7c}	52.7	45.6	47.7
C _{16:1ω5c}	—	0.8	1.5
C _{16:0}	20.4	26.1	11.9
C _{17:0} cyclo	3.3	7.9	—
C _{17:1ω6c}	—	—	16.0
C _{18:1ω7c}	6.6	7.3	9.1
C _{18:0}	—	0.3	—
C _{19:0} cyclo ω8c	—	0.4	—
Summed feature 6*	1.5	—	—

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 6 comprises C_{18:2ω6,9c} and/or anteiso-C_{18:0}.

Positive for oxidase and catalase activity. Most alcohols (e.g. ethanol, methanol), sugars (e.g. fructose, glucose) and sugar acids (e.g. gluconate) and most rich media (e.g. gelatin, trypticase soy, MRS and Luria–Bertani broths) do not support growth. Phototrophic or chemolithotrophic growth is not observed. Exhibits aerobic chemo-organotrophic metabolism using oxygen as a terminal electron acceptor. Optimal growth occurs at between pH 7 and 8.5. Growth occurs at 4–30 °C, optimal growth being at approximately 25 °C. Major fatty acids include C_{16:0}, C_{14:0} and cyclo C_{17:0}. The hydroxylated fatty acid C_{10:0} 3-OH is also present but dodecanoic acid is not. Cells are resistant to tetracycline and ampicillin and to heavy metals: As[III] (5 mM), As[V] (>50 mM), Se[IV] (>10 mM) and Mn[II] (>10 mM). Able to oxidize arsenite to arsenate as well to reduce arsenate to arsenite. The DNA G + C content is 54.3 mol%.

The type strain, ULPAs1^T (=CCM 7303^T=DSM 17148^T=LMG 22961^T), was isolated from an enrichment culture inoculated with a liquid sample from an industrial wastewater treatment plant contaminated with arsenic in Germany.

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