Halomonas zincidurans sp. nov., a heavy-metal-tolerant bacterium isolated from the deep-sea environment

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A Gram-stain-negative, aerobic, rod-like, motile by peritrichous flagella and moderately halophilic bacterium, designated strain B6[T], was isolated a deep-sea sediment collected from the South Atlantic Ocean. The isolate grew with 0.5–15 % (w/v) NaCl, at 4–37 °C and pH 5.0–8.5 and showed a high tolerance to zinc, manganese, cobalt and copper ions. The major fatty acids were $C_{16:0}$, $C_{19:0}$ cyclo $\omega 8c$, $C_{12:0}$ 3-OH and $C_{12:0}$. The predominant ubiquinone was Q-9. The genomic DNA G+C content was 61.1 mol%. Phylogenetic analysis based on 16S rRNA gene comparisons indicated that strain B6[T] belonged to the genus *Halomonas*, and the closest relative was *Halomonas xinjiangensis* TRM 0175[T] (96.1 %). Based upon the phenotypic, chemotaxonomic and genetic data, strain B6[T] represents a novel species from the genus *Halomonas*, for which the name *Halomonas zincidurans* sp. nov. is proposed. The type strain is B6[T] (=CGMCC 1.12450[T]=JCM 18472[T]).

The genus *Halomonas* proposed by Vreeland *et al.* (1980) belongs to the family *Halomonadaceae*, which contains nine additional genera: *Aidingimonas*, *Carnimonas*, *Chromohalobacter*, *Cobetia*, *Halotalea*, *Kushneria*, *Modicisalibacter*, *Salinicola* and *Zymobacter*. At the time of writing, *Halomonas* was the largest genus within this family, comprised of 75 species with validly published names (http://www.bacterio.net/index.html.). Most of these were isolated from saline environments, such as salterns (Kim *et al.*, 2007; Jeon *et al.*, 2007; González-Domenech *et al.*, 2008a; Arenas *et al.*, 2009; Qu *et al.*, 2011; Amjres *et al.*, 2011; Wang *et al.*, 2012), saline soils (Wang *et al.*, 2007a, b, 2008b; González-Domenech *et al.*, 2008b, 2009; Li *et al.*, 2008; Llamas *et al.*, 2011; Zhao *et al.*, 2012; Luque *et al.*, 2012; Poli *et al.*, 2013), a saline well (Xu *et al.*, 2007), a salt pool (Romano *et al.*, 2006), saline lakes (Poli *et al.*, 2007; Romano *et al.*, 2007; Wu *et al.*, 2008;

Wang *et al.*, 2008a; Guan *et al.*, 2010; Guzmán *et al.*, 2010; Menes *et al.*, 2011), a fermented seafood (Kim *et al.*, 2010b); marine animals (Chen *et al.*, 2009); or a renal dialysis machine (Kim *et al.*, 2010a). Here, we present a polyphasic study describing a novel strain of a member of the genus *Halomonas* isolated from a sediment sample collected from the deep-sea environment.

The deep-sea sediment samples were collected from the South Atlantic Mid-Ocean Ridge (13.60° S 14.52° W) at a depth of 2950 m by a TV grab bucket operated from the vessel Da Yang Yi Hao. Aboard the ship, an approximately 100 mg sediment subsample was incubated for 1 h in marine broth 2216 medium (MB; BD). The suspension was plated on marine agar 2216 (MA; BD) containing 20 mM Mn^{2+} using the tenfold dilution-plating technique. After 30 days of aerobic incubation at 28 °C, one cream-coloured colony, designed B6[T], was picked. The isolate was maintained as a glycerol suspension (30 %, v/v) at −40 °C until debarkation. Subsequently strain B6[T] was purified by repeated restreaking; purity was confirmed by the uniformity of cell morphology.

The optimal conditions for growth were determined in HM medium with different NaCl concentrations (0, 0.5, 1, 2, 3, 5, 7.5, 10, 15 and 20 %, w/v) (Ventosa *et al.*, 1982).

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The HM medium contained (l^{-1}): 178.0 g NaCl, 2.0 g KCl, 0.48 g $MgSO_4$ (anhydrous), 0.27 g $CaCl_2$, 0.23 g NaBr, 0.06 g $NaHCO_3$, trace $FeCl_3$, 5.0 g peptone (BD), 10.0 g yeast extract (BD) and 1.0 g glucose (pH 7.5). The pH range for growth was determined by adding MES (pH 4.5–6.0), PIPES (pH 6.5–7.5), Tricine (pH 8.0–8.5) and CAPSO (pH 9.0–10.0) to HM medium at a concentration of 25 mM. The temperature range for growth was determined by incubating at 4, 10, 15, 20, 25, 30, 35, 37, 42, 45 and 50 °C. Unless stated otherwise, the HM medium contained 5 % NaCl (w/v) and pH 7.0. Cell morphology and motility were examined by optical (DM 5000B; Leica), transmission electron (H-600; Hitachi) and scanning electron microscopy (S260; Cambridge).

Phenotypic tests were performed according to the methods of Mata *et al.* (2002) and Arahal *et al.* (2007). Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 solution. Anaerobic growth was carried out with AnaeroPack (Mitsubishi) using nitrate, nitrite or fumarate as potential electron acceptors. Heavy metal tolerance was studied in HM medium supplemented with different concentrations of Mn^{2+} (0, 10, 20, 50, 100, 200 and 800 mM), Co^{2+} (0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5 and 3 mM), Cu^{2+} (0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5 and 3 mM) or Zn^{2+} (0, 0.25, 0.5, 0.75, 1, 1.5, 2, 10, 14, 16, 20 and 30 mM). Exopolysaccharide (EPS) and poly-β-hydroxybutyrate (PHB) production were investigated based on the methods of Poli *et al.* (2007), Stanier *et al.* (1966) and Wang *et al.* (1998). Hydrolysis of aesculin, casein, DNA, gelatin, starch and Tween 20 and 80; nitrate and nitrite reduction; growth on MacConkey and Cetrimide agar medium were tested as described by Mata *et al.* (2002). H_2S production was tested in liquid medium supplemented with 0.5 % (w/v) thiosulfate. Single carbon source assimilation tests were performed by using HM medium without peptone, yeast extract and glucose; and the corresponding filter-sterilized sugars (0.2 %), alcohols (0.2 %), organic acids (0.1 %) or amino acids (0.1 %) was added into liquid medium. Acid production was tested by using modified MOF medium supplemented with 1 % sugars or alcohols (Leifson, 1963; Xu *et al.*, 2008). Susceptibility to antibiotics tests were performed with antibiotic disks on plate at 30 °C and observed 5 days after inoculation. Enzyme activity, acid production and additional phenotypic tests were performed by using API 20 E, 20 NE, 50 CH and ZYM miniaturized systems according to the instructions of the manufacturer (bioMérieux).

Isoprenoid quinones were analysed by reversed-phase HPLC (Komagata & Suzuki, 1987). Fatty acid methyl esters prepared from the lipids extracted from cells grown on HM medium (5 and 10 % NaCl, w/v) at 35 °C were analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID) (Kuykendall *et al.*, 1988). Genomic DNA was obtained using the method described by Marmur (1961). The purified DNA

was hydrolysed with P1 nuclease and the nucleotides dephosphorylated with calf intestine alkaline phosphatase (Mesbah & Whitman, 1989). The G+C content of the resulting deoxyribonucleosides was determined by reversed-phase HPLC and calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) (Mesbah & Whitman, 1989).

The 16S rRNA gene was amplified and analysed as described previously (Xu *et al.*, 2007). The sequence was compared with sequences of closely related reference organisms from the EzTaxon-e service (Kim *et al.*, 2012). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods with the MEGA 5 program package (Tamura *et al.*, 2011). Evolutionary distances were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method. Multilocus sequence analysis was performed according to the methods described by de la Haba *et al.* (2012).

Cells of strain B6[T] stained Gram-negative and were rod-shaped, approximately 0.3–0.5 μm width and 0.7–1.4 μm length after cultivation for 24 h at 30 °C. Cells were motile by means of peritrichous flagella (Fig. S1, available in IJSEM Online). Colonies growing on HM agar at 30 °C were 1–2 mm in diameter, smooth, circular, elevated and cream after 2 days. Cells were oxidase- and catalase-positive. No growth was observed under anaerobic conditions on plates supplemented with nitrate (20 mM), nitrite (10 mM) or fumarate (20 mM). Strain B6[T] was able to grow in liquid containing high concentrations of heavy metals, such as Mn^{2+} (200 mM), Co^{2+} (1.0 mM), Cu^{2+} (2.5 mM) and Zn^{2+} (14 mM). Genomics data indicated that strain B6[T] contained some heavy-metal-resistance genes involved in copper homeostasis and tolerance, cobalt–zinc–cadmium resistance, mercuric reduction and arsenic resistance (data not shown). Zinc is highly toxic to moderate halophiles and its toxicity can be strikingly influenced by the inorganic anionic and cationic components of the medium as well as soluble organic matter (Nieto *et al.*, 1989). The tolerance toward zinc was confirmed on HM, MB and SW-10 plates (Nieto *et al.*, 1989) containing 5 % NaCl (w/v) and different concentrations of Zn^{2+} (0, 1, 10, 15, 20 and 30 mM). *Halomonas xinjiangensis* TRM 0175[T], *Chromohalobacter israelensis* DSM 6768[T] and *Salinicola socius* DSM 19940[T] were used as controls. Strain B6[T] tolerated zinc concentrations up to 20 mM on HM and SW-10 plates and 30 mM on MB plates. None of the reference strains grew on plates containing more than 1 mM Zn^{2+}. The major ubiquinone of strain B6[T] was Q-9 (98.0 %); a small amount of Q-10 (1.2 %) as well as Q-8 (0.8 %) was also detected. Detailed results of morphological and physiological characteristics, nutritional tests, biochemical tests and antibiotic sensitivity tests are given in the species description. Phenotypic characteristics that serve to differentiate strain B6[T] from its closest phylogenetic relative are listed in Table 1 as well as Table S1. The comparison of phenotypic features between

Table 1. Differential characteristics of the novel isolate B6[T] and its closest phylogenetic relative *H. xinjiangensis* as well the type species of the genus, *H. elongata*

Strains: 1. strain B6[T]; 2. *H. xinjiangensis* TRM 0175[T]; 3. *H. elongata* JCM 21044[T]. +, Positive; −, negative; ND, no data available. Data were obtained from this study under identical growth conditions, except where indicated otherwise.

Characteristic	1	2	3
Pigmentation	Cream	Brown–yellow	White
PHB production	−	+	+
NaCl range (%, w/v)	0.5–15	0–20	0–20*
NaCl optimum (%, w/v)	5	10–13	3–8*
pH range	5–8.5	6–9	5–10*
pH optimum	7	7	ND
Temperature range (°C)	4–37	15–50	4–45*
Temperature optimum (°C)	35	37	ND
Hydrolysis of casein	−	+	−
Methyl red test	+	+	−
Utilization of:			
Adonitol	+	−	+
L-Arabinose	+	−	+
Ethanol	+	−	+
D-Ribose	+	−	+
D-Sorbitol	+	−	+
Trehalose	−	+	+
D-Xylose	+	−	+
Acetate	+	−	+
L-Glutamate	+	−	+
Acid production from			
Adonitol	+	−	+
Cellobiose	−	+	+
Ethanol	+	−	−
Maltose	−	+	+
L-Rhamnose	−	−	+
D-Sorbitol	+	−	+
Trehalose	−	+	+
G+C content (mol%)	61.1	60.0†	60.5‡

*Data from Mata *et al.* (2002).
†Data from Guan *et al.* (2010).
‡Data from Vreeland *et al.* (1980); the value was 63.6 mol% from genome sequence.

strain B6[T] and some representative members of the genus *Halomonas* are given in Table S2.

An almost-complete 16S rRNA gene sequence (1477 nt) was determined for strain B6[T]. The 16S rRNA gene sequence comparisons with representative bacteria with validly published names indicated that strain B6[T] was affiliated with the family *Halomonadaceae*. Based on the analysis by the EzTaxon-e service (100 hits), strain B6[T] was most closely related to the genus *Halomonas* (92.9–96.1 % similarity), *Aidingimonas* (93.4 %), *Chromohalobacter* (94.5–96.0 %), *Cobetia* (92.8–93.7 %), *Halotalea* (91.3 %), *Kushneria* (89.6–93.0 %), *Modicisalibacter* (94.4 %) and *Salinicola* (93.4–94.4 %); and showed
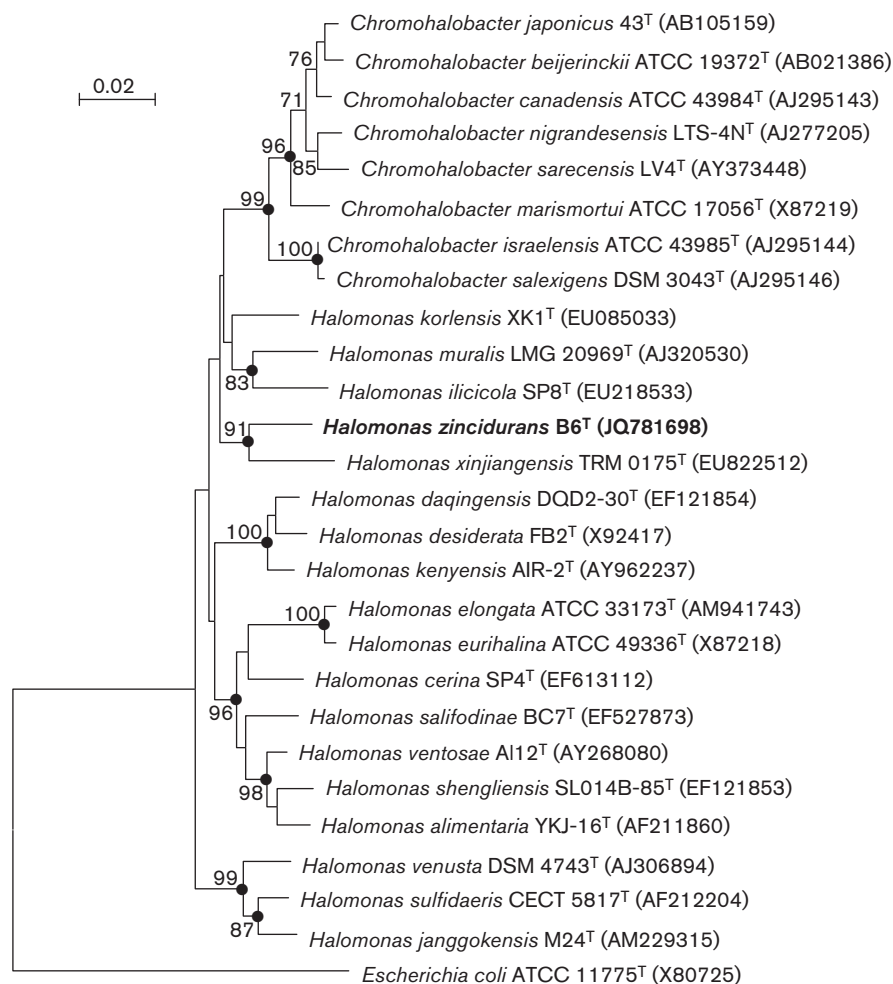
<90 % sequence similarity to other described genera of the family *Halomonadaceae*.

The delineation of the family *Halomonadaceae* was mainly based on the phylogenetic relationship of 16S rRNA gene sequences (Arahal *et al.*, 2002; Garrity *et al.*, 2005). The phylogenetic trees reconstructed with all three treeing methods indicated that strain B6[T] clustered with *H. xinjiangensis* TRM 0175[T] with high bootstrap resampling values (Felsenstein, 1985) (97 % by the neighbour-joining method, 87 % by the maximum-likelihood method and 90 % by the maximum-parsimony method) (Figs 1, S2 and S3). Therefore, the steady phylogenetic topologies and the low sequence similarities strongly indicated that strain B6[T] is a novel member of the genus *Halomonas*. The maximum-likelihood phylogenetic trees based on the individual gene sequences revealed that strain B6[T] clustered with the members within the genus *Halomonas* (*Halomonas muralis* for 23S rRNA, *rpoD* and *secA* and *Halomonas campaniensis* for *atpA*) (Fig. S2). The maximum-likelihood phylogenetic trees based on the concatenated gene sequences showed that the species of the genus *Chromohalobacter* formed a monophyletic cluster and strain B6[T] was placed outside of the *Chromohalobacter* group (Fig. S3).

Strain B6[T] showed the highest sequence similarities to the type strain of *H. xinjiangensis* (96.1 %) and *Halomonas korlensis* (95.1 %) and less than 95.0 % sequence similarities with other species of the genus *Halomonas* with validly published names. The 16S rRNA gene sequence divergence values between strain B6[T] and the type strains of the species of the genus *Halomonas* exceeded 3 %, a commonly accepted value for the distinction of different genomic species (Stackebrandt & Goebel, 1994). Contrary to a steady monophyletic clade between strain B6[T] and *H. xinjiangensis*, *H. korlensis* clustered with *H. muralis* and *Halomonas ilicicola* and formed an independent clade, which was separate from strain B6[T] in phylogenetic trees and was confirmed by all-species living tree (LTPs_108) (Yarza *et al.*, 2010).

The fatty acids profile of strain B6[T] was different from that of *H. xinjiangensis* TRM 0175[T] (Table S3). The content of $C_{16:0}$ of strain B6[T] (25.3–32.0 %) was higher than that of *H. xinjiangensis* TRM 0175[T] (9.7–9.8 %); whereas the content of $C_{18:1}\omega7c$ (4.6–8.8 %) of the former was less than that of the latter (26.8–35.0 %). Strain B6[T] and *H. xinjiangensis* TRM 0175[T] could be differentiated from each other by different reactions in the tests for utilization of acetate, adonitol, L-arabinose, ethanol, L-glutamate, D-ribose, D-sorbitol, trehalose and D-xylose as sole carbon and energy sources. Further differences included tolerance to heavy metals, production of PHB, formation of acid from sugars and alcohols and the reactions in API miniaturized systems (Table 1 and Table S1).

On the basis of the phylogenetic and phenotypic data presented in this study, strain B6[T] represents a novel species within the genus *Halomonas*, for which the name *Halomonas zincidurans* sp. nov. is proposed.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain B6[T] and related taxa. Bootstrap values are based on 1000 replicates; only bootstrap values above 70 % are shown. Bar, 0.02 substitutions per nucleotide position. Filled circles indicate that nodes recovered with bootstrap values >70 % in maximum-parsimony tree as well as maximum-likelihood tree.

Description of *Halomonas zincidurans* sp. nov.

Halomonas zincidurans (zin.ci.du′rans. N.L. neut. n. *zincum*, zinc; L. part. adj. *durans*, enduring, being insensible; N.L. part. adj. *zincidurans,* zinc tolerating).

Cells stain Gram-negative and are rod-like (0.3–0.5 µm in width and 0.7–1.4 µm in length) and motile by means of peritrichous flagella. Colonies are 1–2 mm in diameter, smooth, circular, elevated and cream-coloured after 2 days. Moderately halophilic. Growth is observed at NaCl concentrations of 0.5–15.0 % (w/v) with optimum growth at 5.0 %. Zinc, cobalt and copper ions are tolerated in millimolar concentrations. The pH and temperature ranges for growth are pH 5.0–8.5 and 4–37 °C (optimum growth at pH 7.0 and 35 °C). Strictly aerobic. Growth is not observed on plates supplemented with nitrate, nitrite or fumarate under anaerobic condition. Oxidase- and catalase-positive. H_2S is produced from thiosulfate. Tween 20

is hydrolysed. Aesculin, agar casein, DNA, gelatin, starch and Tween 80 are not hydrolysed. Nitrate is reduced to nitrite, but nitrite is not reduced. Does not grow on MacConkey agar or Cetrimide agar. Positive for arginine dihydrolase, methyl red test and Voges–Proskauer test, but negative for EPS and PHB production, indole formation, lysine and ornithine decarboxylases, ONPG test, phenylalanine and tryptophan deaminases and urease. The following constitutive enzyme activities are detected in API ZYM tests: acid and alkaline phosphatases, esterase (C4), esterase lipase (C8) and leucine and valine arylamidases; but the species is negative for *N*-acetyl-β-glucosaminidase, α-chymotrypsin, cystine arylamidases, α-fucosidase, α- and β-galactosidases, β-glucuronidase, α- and β-glucosidases, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase and trypsin. The following compounds are used as sole carbon and energy sources: adonitol, L-arabinose, cellobiose, ethanol, D-fructose, D-glucose, glycerol, maltose, mannitol, D-mannose,

D-ribose, D-salicin, D-sorbitol, starch, D-xylose, acetate, citrate, D-gluconate, propionate, pyruvate and succinate. The following compounds are not used as sole carbon and energy sources: aesculin, formate, fumarate, D-galactose, myo-inositol, α-lactose, malate, malonate, melezitose, L-rhamnose, raffinose, sorbose, sucrose and trehalose. The following compounds are used as sole carbon, nitrogen and energy sources: L-alanine, L-arginine, glycine, L-glutamate, L-lysine, L-ornithine and L-serine. Acids are produced from adonitol, L-arabinose, ethanol, D-fructose, D-galactose, D-glucose, glycerol, mannitol, D-mannose, D-ribose, D-sorbitol and D-xylose, but not cellobiose, myo-inositol, α-lactose, maltose, melezitose, raffinose, L-rhamnose, D-salicin, sorbose, sucrose and trehalose. According to the results from the API 50 CH test, acid is produced from: L-arabinose, D-fucose, D-galactose, D-glucose, glycerol, ribose and D-xylose; results are weakly positive for the production of acid from D-adonitol, D-arabinose, D-fructose, D-mannose and D-mannitol; but negative for N-acetylglucosamine, aesculin, amygdalin, D-arabitol, L-arabitol, arbutin, cellobiose, dulcitol, erythritol, L-fucose, β-gentiobiose, glycogen, gluconate, inulin, inositol, 2-ketogluconate, 5-ketogluconate, lactose, maltose, melibiose, melezitose, methyl-α-D-glucoside, methyl-α-D-mannoside, methyl-β-D-xyloside, raffinose, L-rhamnose, D-salicin, L-sorbose, starch, sucrose, D-tagatose, trehalose, turanose, xylitol, D-lyxose and L-xylose. Acid production is negative from D-sorbitol in the API 50 CH tests, but positive when tested according to the method of Leifson (1963). Susceptible to (μg per disc unless otherwise stated) amoxicillin (10), ampicillin (10), carbenicillin (100), cefotaxime (30), cephalothin (30), chloramphenicol (30), erythromycin (15), nalidixic acid (30), nitrofurantoin (30), novobiocin (30), penicillin (10), polymyxin B (300 IU), rifampicin (5), sulfamethoxazole (300) and tetracycline (30), but not susceptible to bacitracin (0.04 IU), kanamycin (30) and tobramycin (10). The predominant ubiquinone is Q-9. Major fatty acids (>10 %) are $C_{16:0}$, $C_{19:0}$cyclo-$\omega 8c$, $C_{12:0}$ 3-OH, $C_{12:0}$.

The type strain is $B6^T$ ($=CGMCC\ 1.12450^T=JCM\ 18472^T$), isolated from a sediment sample collected from the South Atlantic Ocean. The DNA G + C content of the type strain is 61.1 mol% (by HPLC).

Acknowledgements

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