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Halopeptonella vilamensis gen. nov, sp. nov., a halophilic strictly aerobic bacterium of the family *Ectothiorhodospiraceae*

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Abstract A Gram-negative, halophilic, heterotrophic, rod-shaped, non-spore-forming bacterium (SV525^T) was isolated from the sediment of a hypersaline lake located at 4600 m above sea level (Laguna Vilama, Argentina). Strain SV525^T was strictly aerobic and formed pink-to-magenta colonies. Growth occurred at 10–35 °C (optimum 25–30 °C), at pH levels 6.0–8.5 (optimum 7.0) and at NaCl concentrations of 7.5–25 % (w/v) with an optimum at 10–15 % (w/v). The strain required sodium and magnesium but not potassium ions for growth. Grows with tryptone, or Bacto Peptone as sole carbon and energy source and requires yeast extract for growth. It produced catalase and oxidase. The predominant ubiquinone was Q-8 and the major fatty acids comprised $C_{18:1} \omega$ 7c, $C_{16:0}$ and $C_{18:0}$. The DNA G+C content was

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60.4 mol% and its polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and a phosphoglycolipid. Phylogenetic analysis based on 16S rRNA gene indicated that strain SV525^T belongs to the family *Ectothiorhodospiraceae* within the class *Gammaproteobacteria*. On the basis of phylogenetic and phenotypic data, SV525^T represents a novel genus and species, for which the name *Halopeptonella vilamensis* gen. nov., sp. nov. is proposed. The type strain is SV525^T (=DSM 21056^T =JCM 16388^T =NCIMB 14596^T).

Keywords Halophile · *Ectothiorhodospiraceae* · Hypersaline · Aerobic · Halopeptonella · Andean lakes

Introduction

The family Ectothiorhodospiraceae (Imhoff 1984) represents halophilic and alkaliphilic purple sulfur bacteria that form a separate lineage within the Gammaproteobacteria according to 16S rRNA gene sequence analysis. Initially, this family was created to accommodate purple sulfur bacteria that deposits elemental sulfur outside the cell (Imhoff 2006; Tourova et al. 2007). However, other non-phototrophic chemotrophic species belonging to novel genera were included in the family: Acidiferrobacter (Hallberg et al. 2011), Alkalilimnicola (Yakimov et al. 2001), Alkalispirillum (Rijkenberg et al. 2001), Aquisalimonas (Márquez et al. 2007), Arhodomonas (Adkins et al. 1993), Natronocella (Sorokin et al. 2007), Nitrococcus (Watson and Waterbury 1971), Spiribacter (León et al. 2014), Thioalbus (Park et al. 2011), Thioalkalivibrio (Sorokin et al. 2001), Thiogranum (Mori et al. 2015) and Thiohalospira (Sorokin et al. 2008).

During studies on the diversity of halophilic bacteria from Laguna Vilama, a saline lagoon in Jujuy (Argentine), a Gram-negative bacterium, designated strain $SV525^T$ was isolated. The lagoon is located at 4600 m in the northwestern section of the Andean Argentinean Puna desert (22°35′S 66°55′) and is characterized by wide fluctuations in daily temperatures (-20 to 30 °C), variable pH, high levels of UV radiation, low nutrient availability and high heavy metal concentrations, especially arsenic (Dib et al. 2009).

In this article, we describe the characterization of a novel halophilic bacterium belonging to the *Ectothiorho-dospiraceae* family which represents a novel species of a novel genus for which the name *Halopeptonella vilamensis* gen. nov., sp. nov. is proposed.

Methods

Isolation and cultivation conditions and strains

Strain SV525^T was isolated by serial dilutions in 0.9 % NaCl solution of sediment samples, spread-plated on MGM25 agar and incubated aerobically for 10 days at 30 °C. MGM has the following composition (1⁻¹): 5.0 g tryptone (Oxoid), 1.0 g yeast extract (Oxoid) and 600 ml or 833 ml (for MGM18 or MGM25, respectively) of concentrated salt water (SW30) stock solution (Dyall-Smith 2009). The SW30 solution contained (1⁻¹): 240 g NaCl, 30 g MgCl₂·6H₂O, 35 g MgSO₄·7H₂O, 7 g KCl and 5 ml 1 M CaCl₂·2H₂O. For solid medium 15 g of agar was added. The pH was adjusted to 7.5 ± 0.2, and the medium was autoclaved for 15 min at 121 °C. The strain was routinely grown on MGM18 agar at 30 °C for 5–7 days and maintained at 4 °C for short-term preservation.

The type strain *Spiribacter salinus* LMG 27464^T was obtained from BCCM.

Morphological, physiological, and biochemical characterization

Phenotypic tests were incubated at 30 °C for up to 15 days, unless otherwise stated. Colony morphology, Gram staining, oxidase reaction and catalase activity was performed on 5-day grown cultures on MGM18 agar, according to Menes et al. (2011). Poly- β -hydroxyalkanoate and polyphosphate staining were carried out in cells grown in MGM18, according to Murray et al. (1994). Motility and shape were observed under phase contrast microscopy (Zeiss Axioplan microscope) in cultures at the exponential phase of growth. Production of diffusible or non-diffusible pigments and exopolysaccharide production were observed on MGM18 agar. Growth in anaerobic conditions

was assayed in MGM18 prepared anaerobically in Hungate tubes with oxygen free nitrogen gas phase. To determine the range of substrates used as carbon and energy sources or as carbon, nitrogen and energy sources, we utilized a basal medium, BBSW18 (pH 7.2 \pm 0.2), with the following composition (l^{-1}) : 0.1 g yeast extract (Oxoid), 0.1 g NH₄Cl, 0.08 g KH₂PO₄, 10 ml trace element solution (Menes and Muxí, 2002) and 600 ml SW30 solution (final concentration of total salts 18 %). After sterilization, 1 ml of filter-sterilized vitamin solution was added (Menes and Muxí 2002). Each substrate was added aseptically from a filter-sterilized stock solution to a final concentration of 1 g l^{-1} , except for carbohydrates, which were added to 2 g l^{-1} . The basal medium was prepared without NH₄Cl for amino acid substrates. To study different electron acceptors or fermentation, the basal medium was prepared anaerobically in Hungate tubes as previously explained. Electron acceptors (5 or 10 mM) and substrates (1 g l^{-1}) were added from anaerobic stock solution. Respiration on arsenate, fumarate, nitrate, nitrite was carried out with tryptone (5 g l^{-1}) as substrate or H₂/CO₂ (1 atm in the head space). The utility of BBSW18 as a medium base for SV525^T was checked with tryptone as a positive control substrate in aerobiosis. We also tested other basal media employed for Arhodomonas and Alkalilimnicola species (Adkins et al. 1993; Hoeft et al. 2007; Yakimov et al. 2001) with negative results for SV525^T when tryptone was assayed. Enhancement of pigment production to visible light was performed in MGM18 agar under 2000 lx illumination. Photoheterotrophic growth was checked in anaerobic prepared BBSW18 supplemented with sulfide or thiosulfate (0.2 mM), and pyruvate, lactate or succinate (1 g l^{-1}), and phototrophic growth was checked in the same medium but supplemented with sulfide or thiosulfate (0.2 mM) and NaHCO₃ (0.1 %, w/v). These cultures were incubated under 2000 lx illumination up to 30 days.

Oxidation and fermentation of glucose was determined in OF basal medium (Hugh and Leifson 1953) prepared in SW18 (SW30 diluted 6:10 in water). Urease activity was tested by the procedure recommended by León et al. (2014). API 20 NE kit (bioMérieux) was inoculated according to manufacturer's instructions except that cell suspensions were prepared in SW18 and carbon assimilation tests were prepared using AUX medium supplemented with 0.72 g NaCl, 0.09 g MgCl₂ 6H₂O, 0.10 g MgSO₄ 7H₂O, 0.02 g KCl (total salts 18 % w/v). Carbon source utilization from carbohydrates was tested in API 50CH (bioMérieux) with the cell suspension prepared in BBSW18. The kits were incubated for up to 20 days at 30 °C. Hydrolysis of aesculin, casein, DNA, gelatin, lecithin, Tween 20, Tween 80, starch, tyrosine, and production of phosphatase, pigments form tyrosine and haemolysis was performed in MGM18 agar supplemented with different substrates,

according to Mata et al. (2002). Growth on Trypticase Soy agar (Difco), MacConkey agar (Difco) and cetrimide agar (Difco) was performed in media prepared with SW18.

Growth at different temperatures (5–45 °C at intervals of about 5 °C) and pH (5.0-10.0 at intervals of 1.0), was tested in MGM18 with agitation. The final pH was adjusted after autoclaving with HCl or NaOH sterile stock concentrated solutions. Growth at different NaCl concentrations (5-25 % NaCl w/v, equivalent to 7.4-27.4 % total salts) was tested in NaCl-free MGM18 with increasing concentrations of NaCl (5, 7.5, 10, 15, 20 and 25 %). Growth was monitored by measuring OD₆₀₀. Slopes of OD₆₀₀ against time were plotted and averaged from triplicate runs at each temperature and salt concentration to determine optimal growth rates for each condition. For pH experiments growth was checked by microscopic observation since cell aggregation prevent OD₆₀₀ measurement. Requirement of sodium, magnesium and potassium ions were assayed in MGM18 prepared with SW30 without NaCl (for sodium requirement assay) or without magnesium salts (for magnesium requirement assay) or without KCl (for potassium requirement assay). Growth in the presence of arsenic was tested in MGM18 supplemented with increasing concentrations of arsenite or arsenate.

Cells grown in MGM18 for 3 days were prepared for microscopic observations as described (Menes and Muxí 2002) and observed with a JEOL JEM-1010 transmission electron microscope. Pigments were extracted with acetone/methanol (7:1, v/v) from exponential growing cells. The absorption spectrum (from 300 to 900 nm) of these cell free extracts was recorded with a Jenway 6705 spectrophotometer.

Chemotaxonomic analysis

Whole-cell fatty acid composition, polar lipids, respiratory quinones (from cells grow on MGM18 agar for 5 days) and genomic DNA G+C content analyses, were carried out by the Identification Service of DSMZ (Braunschweig, Germany).

Phylogenetic analysis

Extraction of genomic DNA was carried out with the QIAamp DNA Mini Kit (Qiagen) and amplification of nearly full-length 16S rRNA gene fragments was performed using primers 27F (5'-AGAGTTTGATC(A/C) TGGCTCAG-3') and 1492R (5'-ACGG(C/T)TACCTT-GTTACGACTT-3') as described previously (Menes and Muxí 2002). The resulting PCR products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced using an Applied Biosystems automatic sequencer ABI 3730XL at Macrogen Corp., Seoul, Korea.

An almost-complete 16S rRNA gene sequence (1437 nt) was obtained and compared to other released sequences in GenBank using the NCBI-BLAST (Altschul et al. 1997) and megaBLAST (Zhang et al. 2000) against the database containing type strains with validly published prokaryotic names. The top 30 sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon-e server (Kim et al. 2012). For phylogenetic analysis sequences were aligned with clustal W1.8 (Thompson et al. 1994). The resulting multiple sequence alignment was corrected manually. Phylogenetic trees were constructed by the neighbour-joining (Saitou and Nei 1987), the maximum-parsimony (Fitch 1971) and the maximum-likelihood (Felsenstein 1981) methods (with Jukes-Cantor distance correction model), with the software package MEGA version 6.0 (Tamura et al. 2013). A total of 1243 positions were used for the phylogenetic analysis. Bootstrap analyses (1000 replicates) were performed in order to assess the robustness of the topology.

Results and discussion

Morphological, physiological, and biochemical characterization

Cells of strain SV525^T were rod-shaped, occurring singly or in pairs, without flagella and showed a typical Gramnegative cell wall structure (Fig. 1).

Strain SV525^T did not utilize any of the substrates employing BBSW18 medium, aerobically, except tryptone and Bacto Peptone in aerobic conditions. No fermentation of D-fructose, D-galactose, glycerol, D-glucose lactose, pyruvate, and succinate was observed. No utilization of any substrates of API 20 NE and API 50 CH detected. Strain SV525^T grows in the presence of up to 2 mM of arsenite and 20 mM of arsenate. No enhancement of pigment production or production of different pigments was observed when strain SV525^T grows under visible light. Photolithoautotrophic and photoheterotrophic growth could not be demonstrated in strain SV525^T. The absorption spectrum of acetone/methanol extracts of cells did not show indications that bacteriochlorophyll or carotenoids were present. Other data are included in the species description.

Chemotaxonomic analysis

The major cellular fatty acids (94.4 %) were: $C_{18:1} \omega 7c$ (66.9 %), $C_{16:0}$ (8.6 %), $C_{18:0}$ (5.1 %), $C_{20:1} \omega 7c$ (3.7 %), $C_{16:1} \omega 7c/15$ iso 2OH (2.9 %), 3-OH $C_{12:0}$ (2.6 %), $C_{19:0}$ cyclo $\omega 8c$ (2.5 %), $C_{12:0}$ (2.1 %), and an unknown fatty acid of ECL 11.799. Traces of $C_{10:0}$, $C_{10:0}$ 3-OH, $C_{18:0}$ iso,

Fig. 1 Transmission electron micrographs of cells of strain $SV525^{T}$. **a** Ultrathin section (*bar* 0.1 µm). **b** Negatively stained preparation (*bar* 0.5 µm)

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences of strain SV525^T and related species using the neighbor-joining method. Bootstrap values above 50 % are shown at the branch points (1000 resamplings). Escherichia coli was used as the outgroup. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximumlikelihood and maximum parsimony algorithms. Bar 0.02 substitutions per nucleotide position



 $C_{18:1} \omega 9c$, and unknown/ $C_{19:1} \omega 6c$ were also detected (each <1 %). The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphoglycolipid and an unidentified lipid (Fig. S3). The predominant quinone was Q-8 (100 %) and traces of Q9 were also detected, and the DNA G+C content was 60.4 mol%.

0.02

Phylogenetic analysis

Phylogenetic analysis based on the nearly complete 16S rRNA gene sequences (Fig. 2; supplementary material) clearly placed strain SV525^T within the family *Ecto*-*thiorhodospiraceae*. The application of different treeing methods and changes of outgroups produced nearly identical tree topologies. The strain SV525^T had a 16S rRNA gene similarity of 95.6 % with *Spiribacter salinus*, 95.3 % with *Arhodomonas recens* and 95.1 % with *Arhodomonas*

aquaeolei but lower sequence similarities (<95 %) to all other species of the family *Ectothiorhodospiraceae* with validly published name. On the basis of sequence divergence, it was obvious that the novel isolate could not be assigned to any of the described genera in this cluster.

- Escherichia coli ATCC 11775^T (X80725)

Thioalkalivibrio jannaschii ALM2^T (AF329083)

The strain SV325^T differed from *Spiribacter salinus* (León et al. 2014) in cell size and morphology, optimum growth temperature, no growth on pyruvate and glycerol as sole carbon and energy source, no urease production, major fatty acids (presence of $C_{18:0}$ and absence of 3-OH $C_{10:0}$) and a different polar lipids profile (absence of phosphoaminoglycolipid) and absence of polyalkanoate inclusion bodies. It also differed from the validly described species of *Arhodomonas: Arhodomonas recens* (Saralov et al. 2012), and *Arhodomonas aquaeolei* the type species of the genus (Adkins et al. 1993) in various features: colony pigmentation, optimum temperature for growth, motility, reduction of nitrate, autotrophic growth on hydrogen, growth

on certain substrates as acetate, Casaminoacids, glycerol, L-glutamate, propionate and pyruvate, and the major fatty acids. Moreover, it has a much lower DNA G+C content than this species (Table 1).

On the basis of this polyphasic taxonomic study we propose that strain SV525^T represents a new species of a novel genus for which we propose the name *Halopeptonella vilamensis* gen. nov., sp. nov.

Description of Halopeptonella gen. nov.

Halopeptonella gen. nov. (Ha.lo.pep.to.nel'la. Gr. n. *hals*, halos, salt; N. L. n. *peptonum*, peptone; L. fem. dim. ending -ella; N. L. fem. n. *Halopeptonella*, a halophilic bacterium that grows solely with peptone. Gram-negative, rod-shaped cells occurring singly and in pairs. Older cultures form chains. Cells are non-motile, non-spore-forming, and aerobic. Catalase positive and oxidase positive. The major fatty acids are $C_{18:1} \omega 7c$, $C_{16:0}$, $C_{18:0}$. The DNA G+C content is 60.4 mol% and the polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and a phosphoglycolipid. The type species of the genus is *Halopeptonella vilamensis*.

Description of Halopeptonella vilamensis sp. nov

Halopeptonella vilamensis (vi.lam.en'sis. N. L. fem. adj. vilamensis, pertaining to Laguna Vilama, Jujuy, Argentina).

In addition to properties given in the genus description the species is characterized as follows. Cells are

Table 1	Differential characteristics of strain	SV525 ^T	^r and its closest phylogenetic	relatives
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Characteristic	1	2	3	4		
Cell morphology	Rod	Curved to spiral rod	Rod	Oval rod		
Pigmentation	Pink	Pink	No	No		
Cell size (µm)	$1.0-2.0 \times 0.5-0.8$	$0.8 - 1.8 \times 0.3$	$2.0-2.5 \times 0.8-1.0$	$2.0-2.5 \times 0.8-1.0$		
Polyhydroxyalkanoate inclu- sions	-	+	+	ND		
Temperature for growth (°C)						
Range	10–35	15–40	20–45	20-45		
Optimum	25-30	37	37	37		
NaCl for growth (%, w/v)						
Range	7.5-25.0	10.0-25.0	2.0-25.0	6.0-20.0		
Optimum	10–15	15	10–12	15		
Motility, flagella	_	-	+, 1 polar	+, 1 polar		
Nitrate reduction to nitrite	_	-	+	+		
Autotrophic growth on (with I	NO ₃ ⁻)					
Hydrogen	_	a	+	ND		
Sulfides	_	a	_	ND		
Urease	^a	$+^{a}$	ND	+		
Growth on ^b						
Acetate	_	^a	+	+		
Casaminoacids	_	^a	+	+		
Formate	_	^a	+	-		
L-Glutamate	_	^a	+	+		
Glycerol	_	$+^{a}$	+	+		
Propionate	_	^a	+	+		
Pyruvate	_	$+^{a}$	+	+		
Major fatty acids (>5 %)	$C_{18:1} \omega$ 7c, $C_{16:0}$, $C_{18:0}$	$C_{18:1} \omega_{6c}/C_{18:1} \omega_{7c}, C_{16:0}, 3-OH C_{10:0}, C_{12:0}$	$\begin{array}{c} C_{18:0}, C_{19:0} \text{cyclo}, C_{18:1} \ \omega 7 \text{c}, \\ C_{18:1} \ \omega 9 \text{c}, C_{16:0}, C_{16:1} \ \omega 7 \text{c} \end{array}$	$C_{16:0}, C_{18:1}, C_{19:0}, C_{16:1}, C_{18:0}$		
DNA G+C content (mol%)	60.4	60.0	68.2	67.0		

Data are from this study and León et al. (2014), Saralov et al. (2012), Adkins et al. (1993)

1, SV525^T; 2, Spiribacter salinus LMG 27464^T; 3, Arhodomonas recens IEGM 796^T; 4, Arhodomonas aquaeolei ATCC 49307^T

+, Positive; -, Negative; ND, No data available

^a Obtained in this study

^b Substrates as sole carbon and energy source, or as sole carbon, energy and nitrogen source

 $1.0-2.0 \times 0.5-0.8 \ \mu m$ and have a strong tendency to clump at 25 % NaCl. Cells are non-motile, non-sporeforming, strictly aerobic and heterotrophic. Do not produce exopolysaccharides, or diffusible pigments, and do not accumulate intracellular polyphosphate granules and poly-β-hydroxyalkanoates. Colonies are pink, circular, smooth, convex, and pinpoint (<0.5 mm in diameter) after 5 days of incubation at 30 °C. Prolonged incubation produced dark pink-to-magenta colonies of irregular margins. Grows at temperatures ranging from 10 to 35 °C (optimum 25-30 °C) and at pH levels 6.0-8.5 (optimum 7.0). The strain is halophilic, capable of growing at NaCl concentrations from 7.5 to 25 % (w/v) with optimum growth at 10-15 % (w/v) in media with 2.4 % (w/v) magnesium and potassium salts. The strain requires sodium and magnesium but not potassium ions for growth and grows in the presence of up to 2 mM of arsenite and 20 mM of arsenate. Grows with tryptone (Oxoid), or Bacto Peptone (Difco) as sole carbon and energy source and requires yeast extract for growth. No oxidation or fermentation of glucose was observed. There is no growth on Tryptic Soy, MacConkey or cetrimide agars supplemented with 18 % (w/v) salts or 15 % NaCl, but grows in Marine agar 2216 (Difco) + 10 % NaCl. No photolithoautotrophic, photoorganoheterotrophic, chemolithoautotrophic or fermentative growth was observed.

Aesculin, casein, DNA, gelatin, lecithin, Tween 20, Tween 80, starch and tyrosine are not hydrolyzed. No pigments are produced from tyrosine. No production of carotenoids pigments. No phosphatase produced. Blood is not haemolysed. Arsenate, fumarate, molybdate, nitrate, nitrite, selenate and tungstate are not reduced with tryptone or hydrogen as electron donor. Arsenite, hydrogen, selenite, sulfide and thiosulfate are not oxidized with nitrate or oxygen as electron acceptors in media supplemented with 10 mM NaHCO₃.

No growth is observed on any of the tested substrates as sole carbon and energy sources: acetate, caproate, citrate, formate, D-fructose, D-galactose, glycerol, D-glucose, DL- β -hydroxybutyrate, lactose, propionate, pyruvate, malate and succinate, D-xylose or as carbon, nitrogen and energy sources: L-arginine, L-alanine, L-aspartate, L-cysteine, L-phenylalanine, L-glutamate, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline L-serine, L-threonine, L-tryptophan and L-valine. No growth is observed with acetate and pyruvate with nitrate and arsenate as electron acceptors.

All tests in API 20 NE are negative: nitrate and nitrite reduction, indol production, glucose fermentation, arginine dihydrolase, urease, aesculin, gelatinase, β -galactosidase, and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl glucosamine, D-maltose, gluconate, capric acid, adipic acid, malic acid, trisodium citrate,

phenylacetic acid. It does not utilize a carbon sources any of the carbohydrates in API 50 CH: *N*-acetyl glucosamine, D-adonitol, aesculin, amygdaline, D-arabinose, L-arabinose, D-arabitol, L-arabitol, arbutine, D-cellobiose, dulcitol, erythritol, D-fructose, D-fucose, L-fucose, D-galactose, gentiobiose, gluconate, D-glucose, glycerol, glycogen, inositol, inuline, 2-ketogluconate, 5-ketogluconate, D-lactose, D-lyxose, D-maltose, α -methyl-D-glucoside, α -methyl-D-mannoside, β -methyl-D-xyloside, D-mannitol, D-mannose, D-melezitose, D-melibiose, D-raffinose, L-rhamnose, D-ribose, salicine, D-sorbitol, L-sorbose, starch, D-sucrose, D-tagatose, D-trehalose, D-turanose, D-xylose, L-xylose and xylitol. The predominant ubiquinone is Q-8.

The type strain, $SV525^{T}$ (=DSM 21056^{T} =JCM 16388^{T} =NCIMB 14596^{T}), was isolated from the sediment of a hypersaline lagoon located at 4600 m above sea level (Laguna Vilama, Argentina).

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