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## ***Citreicella manganoxidans* sp. nov., a novel manganese oxidizing bacterium isolated from a shallow water hydrothermal vent in Espalamaca (Azores)**

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### **Abstract**

A Gram-stain negative, non-motile, non-spore forming, aerobic and rod or narrow lemon-shaped bacterial strain, VSW210<sup>T</sup>, was isolated from surface seawater in a shallow water hydrothermal vent region in Espalamaca (Azores). Strain VSW210<sup>T</sup> was found to grow optimally at 30 °C, at pH 7 and in the presence of 2–6 % (w/v) NaCl. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences revealed that strain VSW210<sup>T</sup> clusters with the type strain *Citreicella marina* CK-I3-6<sup>T</sup> (sequence similarity value of 99.6 %), but DNA-DNA hybridization showed DNA-DNA relatedness between the strain VSW210<sup>T</sup> and *C. marina* CK-I3-6<sup>T</sup> to be 55.8 ± 3.2 %. The DNA G+C content of strain VSW210<sup>T</sup> was determined to be 67.4 mol%. The cellular fatty acid profiles of strain VSW210<sup>T</sup> was found to contain C<sub>18:1</sub> ω7c (80.1 %) and C<sub>16:0</sub> (9.2 %). The major polar lipids in strain VSW210<sup>T</sup> were identified as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unidentified phospholipid. Strain VSW210<sup>T</sup> was found to be able to oxidize soluble Mn(II) to insoluble MnO<sub>2</sub>, which was confirmed with LBB staining. Differential phenotypic properties and genetic uniqueness revealed that this strain VSW210<sup>T</sup> is distinguishable from other species of the genus *Citreicella*. On the basis of the data presented, strain VSW210<sup>T</sup> is considered to represent a novel species of the genus *Citreicella*, for which the name *Citreicella manganoxidans* sp. nov. is proposed. The type strain is VSW210<sup>T</sup> (= KCTC 32497<sup>T</sup> = MCC 2286<sup>T</sup>).

**Keywords:** *Citreicella manganoxidans* sp. nov · Polyphasic taxonomy · Seawater · Hydrothermal vent · Azores

## Introduction

The genus *Citricella*, a member of the class *Alphaproteobacteria*, was proposed by Sorokin et al. (2005). At the time of writing, the genus *Citricella* includes only three validly named species, *Citricella thiooxidans* (Sorokin et al. 2005), *Citricella aestuarii* (Park et al. 2011) and *Citricella marina* (Lai et al. 2011). During our investigation of culturable bacterial diversity from a shallow water hydrothermal vent in Espalamaca (Azores, Portugal), several manganese resistant bacteria were isolated (Rajasabapathy et al. 2014a). In this study, we characterised one of these isolates, designated strain VSW210<sup>T</sup>, which was isolated from surface seawater. The aim of the present investigation was to determine the precise taxonomic position of strain VSW210<sup>T</sup> using a polyphasic approach.

## Materials and Methods

### Strain isolation and culture conditions

Surface seawater was collected from a shallow hydrothermal vent in the Espalamaca region (38°33'N; 28°39'W), Azores, Portugal through an Indo-Portugal bilateral program during September 2010. For isolation of heterotrophic manganese oxidizing bacteria, serial dilutions of the samples were spread plated on diluted seawater nutrient agar (dSWNA: peptone 1.25 g L<sup>-1</sup>, beef extract 0.75 g L<sup>-1</sup>, 1.8 % agar prepared in 50 % seawater) amended with 1 mM MnCl<sub>2</sub>. The plates were incubated for up to 3 days at 30 ± 2 °C and morphologically different colonies were isolated. Growth of strain VSW210<sup>T</sup> on marine agar 2216 (MA: M384, Himedia) was confirmed and the strain maintained at 4 °C. Strain VSW210<sup>T</sup> was also stored at -80 °C in dSWNA with 15 % glycerol. The reference strain *C. marina* CK-I3-6<sup>T</sup> was purchased from LMG culture collection (LMG 25230) studied in parallel with strain VSW210<sup>T</sup>.

### 16S rRNA gene sequencing and phylogenetic analyses

Genomic DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions and the 16S rRNA gene was PCR amplified with eubacterial primers 27F/1492R (Lane 1991). The PCR amplified 16S rRNA gene was purified with a PCR cleanup kit (Sigma) and sequenced on an 3130xl Genetic Analyzer (Applied Biosystems). The nearly full length 16S rRNA gene sequence (1373 nt) was subjected to a BLASTn search at the National Center for Biotechnology Information (NCBI) to determine an approximate phylogenetic affiliation and at EzTaxon-e (Kim et al. 2012) to identify similarity values between strain VSW210<sup>T</sup> and closely related taxa. Sequences were aligned using Clustal W (Thompson et al. 1994) and phylogenetic trees were constructed using neighbour-joining (Saitou and Nei 1987), maximum-parsimony (Fitch 1971)

and maximum-likelihood (Felsenstein 1981) methods with MEGA version 5.2 (Tamura et al. 2011). The topology of the phylogenetic trees was estimated by bootstrap analysis with 1,000 replications (Felsenstein 1985).

#### Morphological, physiological and biochemical characterisation

Gram staining (K001, Himedia) and motility (Collee et al. 2008) of strain VSW210<sup>T</sup> was examined using light microscopy (Olympus BX-60) with 1000× magnification. The cell morphology was examined using a scanning electron microscope (JEOL JSM-5800LV). Growth under anaerobic conditions was determined on MA after incubation for 10 days in an anaerobic system with an AnaeroPack (Himedia, Mumbai). The pH range for growth was determined by adjusting the pH of the marine broth 2216 (MB: M385, Himedia) using various buffers to pH 4–5 (citrate buffer), pH 6–8 (phosphate buffer) and pH 9–10 (carbonate buffer) and cell densities for growth were measured at 600 nm using a spectrophotometer (Cary 300) after 3 days of incubation. Temperature dependent growth was tested on MA at 4, 10, 20, 25, 30, 37, 40, 45 and 50 °C. Salinity tolerance of strain VSW210<sup>T</sup> was investigated in nutrient broth (NB: M088, Himedia) prepared in distilled water with NaCl concentrations of 0 – 15 % (w/v; 1 % increments) with and without adding Mg<sup>2+</sup> and Ca<sup>2+</sup> (MgCl<sub>2</sub> 4.53 g L<sup>-1</sup>; MgSO<sub>4</sub> 5.94 g L<sup>-1</sup>; CaCl<sub>2</sub> 1.3 g L<sup>-1</sup>).

Catalase activity was tested using the 3 % (v/v) H<sub>2</sub>O<sub>2</sub> drop method (Smibert and Krieg 1994) and oxidase activity was determined using oxidase discs (DD018, Himedia). Hydrolysis of DNA, urea, starch, xylan, CMC, casein, gelatin, agar, Tweens 20 and 80, tributyrin, xanthine and alginate were determined as described previously (Rajasabapathy et al. 2014b) on MA. Other enzyme activities were tested using API ZYM kits (bioMérieux) according to the manufacturer's protocol. Acid production from carbohydrates was determined using the API 50CH kit (bioMérieux) following the manufacturer's instructions using inoculation medium API CHB/E amended with marine cations supplement (MCS, Farmer and Hickman-Brenner 2006). Antibiotic susceptibility tests were performed on seawater nutrient agar (SWNA: peptone 5.0 g L<sup>-1</sup>, beef extract 1.5 g L<sup>-1</sup>, yeast extract 1.5 g L<sup>-1</sup>, 1.5 % agar prepared in 50 % seawater) using antibiotic discs (Himedia) containing the following concentrations (µg/disc): amikacin (30), amoxicillin (30), ampicillin (25), bacitracin (8 IU), cefadroxyil (30), ceftazidime (30), chloramphenicol (25), chlortetracycline (30), ciprofloxacin (30), kanamycin (30), lincomycin (10), neomycin (30), norfloxacin (10), novobiocin (30), penicillin G (2 IU), polymyxin B (50 IU), rifampicin (15), streptomycin (300), tetracycline (10), tobramycin (10) and vancomycin (5). The results were observed after 3 days incubation at 30 °C.

### Chemotaxonomic analysis

Strain VSW210<sup>T</sup> was cultivated on MA at 30 °C for 2 days and harvested for fatty acid methyl ester (FAME) derivatisation. Fatty acids were saponified, methylated and extracted using the standard protocol of Microbial Identification System TSBA 6.0 (Sasser 1990). Cell biomass of strain VSW210<sup>T</sup> for the analysis polar lipids was obtained from cultures grown for 2 days at 30 °C in MB. Polar lipids were extracted from freeze-dried cells and separated by two dimensional silica gel thin layer chromatography (Merck) as described previously (Collins et al. 1980; Rajasabapathy et al. 2014b). DNA G+C content was determined by reverse-phase HPLC of nucleosides following the protocol described by Mesbah et al. (1989).

### DNA-DNA hybridization

For DNA-DNA hybridization experiments, genomic DNA was extracted and purified according to the method of Marmur (1961). The analysis was done by fluorimetry using a Step One Plus Real-Time PCR system (Applied Biosystems) fitted with 96 well thermal cycling block in 96 well plate (Loveland-Curtze et al. 2011). The reassociation of DNA was carried out at optimum reassociation temperature 78.0 °C [ $T_{or} = 0.51 \times (\% \text{ G+C}) + 47.0$ ] according to De Ley et al. (1970) and Gillis et al. (1970). Resulted DNA-DNA relatedness values are the average of triplicates.

### Manganese oxidation assay

Strain VSW210<sup>T</sup> was inoculated onto dSWNA and dSWNB (media composition the same as dSWNA excluding agar) amended with 5 mM MnCl<sub>2</sub> and incubated in the dark for up to 20 days. Oxidation of soluble Mn(II) to insoluble Mn(III/IV) oxides were observed as brown or black precipitates. Every 5 days, Mn oxidation rates were photometrically measured at A<sub>620</sub> using the leucoberbelin blue (LBB) assay (Krumbein and Altman 1973). A KMnO<sub>4</sub> standard curve was generated to quantify the levels of Mn oxidization in the broth cultures. In the LBB assay, 40 μM KMnO<sub>4</sub> is equivalent to 100 μM MnO<sub>2</sub> (Brouwers et al. 2000).

## Results and Discussion

Cells of strain VSW210<sup>T</sup> were observed to be Gram-stain negative, non-motile, non-spore forming and rod or narrow lemon shaped with tip ends (Suppl. Fig. 1). The colonies of the strain VSW210<sup>T</sup> were observed to be small, non-pigmented, circular, convex and smooth on MA and dSWNA media after 48 h. Strain VSW210<sup>T</sup> did not grow on medium with NaCl as a sole salt and was found to require Mg<sup>2+</sup>. Growth of the strain VSW210<sup>T</sup> was observed at temperatures between 4 and 40 °C with optimum growth at 30 °C and at a pH range of 6.0–9.0 with an optimum pH of 7.0. Differences

between morphological and physiological characteristics of strain VSW210<sup>T</sup> compared with the closely related type strains of the genus *Citricella* are given in Table 1.

Strain VSW210<sup>T</sup> was susceptible to all the antibiotics tested. In assays with API ZYM strips, alkaline phosphatase (weak), esterase (C 4), esterase lipase (C 8), leucine arylamidase, acid phosphatase (weak), naphthol-AS-BI-phosphohydrolase (weak) and  $\alpha$ -glucosidase activities are present, but lipase (C 14), valine arylamidase, cystine arylamidase, trypsin,  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\beta$ -glucosidase, *N*-acetyl-  $\beta$ -glucosaminidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase activities are absent. In the API 50CH test gallery, acids produced from the substrates, glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, methyl- $\beta$ D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, D-sorbitol (weak), esculin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, xylitol, D-fucose, L-fucose and D-arabitol.

Analysis of the 16S rRNA gene sequence of strain VSW210<sup>T</sup> (GenBank/EMBL/DDBJ accession number KC534242) showed that the strain is closely related to *C. marina* CK-I3-6<sup>T</sup> with sequence similarity of 99.6 %. However, DNA-DNA hybridizations between VSW210<sup>T</sup> and *C. marina* CK-I3-6<sup>T</sup> showed a DNA-DNA relatedness of 55.8 $\pm$ 3.2 %, which is lower than 70 %, the recommended threshold value generally considered for prokaryotic species delineation (Stackebrandt and Goebel 1994; Wayne et al. 1987). The 16S rRNA gene sequence similarity values between strain VSW210<sup>T</sup> and other validly named species of the genus *Citricella* in the EzTaxon-e database are 96.9 % (*C. thiooxidans* CHLG 1<sup>T</sup>) and 96.5 % (*C. aestuarii* AD8<sup>T</sup>). Neighbour-joining, maximum-parsimony and maximum-likelihood phylogenetic analysis based on 16S rRNA gene sequences revealed that the strain VSW210<sup>T</sup> formed a separate subclade with *C. marina* CK-I3-6<sup>T</sup> (Fig. 1, Suppl. Fig. 2 and Suppl. Fig. 3).

The polar lipid profile of strain VSW210<sup>T</sup> was found to consist of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unidentified phospholipid (Suppl. Fig. 4). The major fatty acids found in strain VSW210<sup>T</sup> were C<sub>18:1</sub>  $\omega$ 7c (80.1 %) and C<sub>16:0</sub> (9.2 %), a profile which is comparable to those of the type strains *C. marina* CK-I3-6<sup>T</sup> and *C. thiooxidans* CHLG 1<sup>T</sup> (Table 2). However, the fatty acid C<sub>19:0</sub>  $\omega$ 8c cyclo which was found in *C. marina* CK-I3-6<sup>T</sup> (this study) and reported in *C. thiooxidans* CHLG 1<sup>T</sup> and *C. aestuarii* AD8<sup>T</sup> (Lai et al. 2011; Park et al. 2011), was not detected in strain VSW210<sup>T</sup>. In addition, C<sub>10:0</sub> which was observed to be present in strain VSW210<sup>T</sup>, was not observed in the other members of the genus *Citricella*. The genomic DNA G+C content was found to be 67.4 mol% which is very similar to those of other species of the genus *Citricella* (Table 1).

In a plate assay, strain VSW210<sup>T</sup> was found to oxidize Mn(II) within 10 days of incubation, which was confirmed with LBB staining (Suppl. Fig. 5). *C. marina* CK-I3-6<sup>T</sup> was negative for Mn(II) oxidation in this plate assay (data not shown). In liquid medium, up to 10 days, there was no formation of brown colour or of blue colour when treated with LBB. However, the Mn(II) oxidation was started by the 15<sup>th</sup> day, producing 1.1 mM MnO<sub>2</sub> and by 20<sup>th</sup> day 1.7 mM of MnO<sub>2</sub> was observed. Growth of VSW210<sup>T</sup> was also in similar pattern to that of MnO<sub>2</sub> oxidation (Suppl. Fig. 6). Oxidation of Mn<sup>2+</sup> by the strain VSW210<sup>T</sup> may be due to activity of Mn<sup>2+</sup> oxidizing enzymes (Okazaki et al. 1997; Francis et al. 2001) or some unidentified Mn<sup>2+</sup> oxidizing factors. Based on the studies conducted by Adams and Ghiorse (1987) from *Leptothrix*, only part of the Mn(II) oxidizing activity was dependent on Mn<sup>2+</sup> oxidizing protein and part was attributed to as yet unidentified Mn<sup>2+</sup> oxidizing factors.

Based on phylogenetic analysis, phenotypic characteristics, chemotaxonomic data and low level of DNA-DNA relatedness (55.8 ±3.2 %), it can be concluded that strain VSW210<sup>T</sup> represents a novel species of the genus *Citricella*, for which the name *Citricella manganoxidans* sp. nov. is proposed.

#### **Description of *Citricella manganoxidans* sp. nov.**

*Citricella manganoxidans* (man.gan.o'xi.dans. N.L. neut. n. *manganum* manganese; N.L. part. adj. *oxidans* oxidizing; N.L. part. adj. *manganoxidans* manganese-oxidizing).

Cells are aerobic, Gram-stain negative, non-spore forming, non-motile and rods or narrow lemon shaped, with tip ends (0.4–1.1 × 1.9–5.8 μm). Colonies on MA and dSWNA are circular, raised, smooth and non-pigmented after incubation for 48 h at 30 °C. Grows in 0–12 % NaCl (optimum 2–6 %), at pH 6–9 (optimum 7) and at 4–40 °C (optimum 30 °C). Mg<sup>2+</sup> is required for growth. Positive for catalase and oxidase. Anaerobic growth does not occur on MA. Nitrate is not reduced to nitrite. Tween 20, DNA and agar are hydrolysed, but casein, gelatin, Tween-80, xanthine, xylan, CMC and trybutyrin are not. The major fatty acid is C<sub>18:1</sub> ω7c. The major polar lipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unidentified phospholipid. The DNA G+C content of the type strain is 67.4 mol%.

The type strain, VSW210<sup>T</sup> (= KCTC 32497<sup>T</sup> = MCC 2286<sup>T</sup>), was isolated from surface seawater at the shallow hydrothermal vent region in Espalamaca, Azores, Portugal. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain VSW210<sup>T</sup> is KC534242.

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**Table 1.** Differential characteristics between strain VSW210<sup>T</sup> and closely related type strains of species of the genus *Citricella*

Characteristic	1	2	3	4
Cell size (µm)	0.4-1.1×1.9- 5.8	0.9-1×1.4- 1.6 <sup>†</sup>	0.6-0.8×1- 1.5*	1.0-1.4×1.6- 2.2
Motility	-	-	-*	+
NaCl range for growth (% w/v)	0-12	0-12	0.5-16*	1-11
pH range for growth	6-9	6-9	6.5-8.5*	5-9.5
Temperature range for growth (°C)	4-40	4-37	8-35*	15-40
<b>API-CH50</b>				
Glycerol, L-arabinose, D-xylose, D-galactose, methyl-βD-xylopyranoside,	+	+	-	ND
D-lactose, D-melibiose, D-arabitol, L-fucose, L-rhamnose	+	+	-	ND
Erythritol, salicin, D-trehalose, D-lyxose, potassium gluconate, potassium 5-keto gluconate	-	-	+	ND
L-xylose	-	+	+	ND
D-adonitol	-	+	-	ND
L-sorbose	+	-	-	ND
D-sorbitol	w	+	+	ND
Gentibiose	-	w	-	ND
<b>API ZYM</b>				
Alkaline phosphatase	w	+	+	+

Esterase (C4), Esterase lipase (C8)	+	+	w	-
Lipase (C14), Cystine arylamidase, $\beta$ -glucosidase	-	-	w	-
$\alpha$ -chymotrypsin	-	-	w	w
Valine arylamidase	-	-	+	+
$\alpha$ -galactosidase	-	-	-	w
Naphthol-AS-BI-phosphoamidase	w	+	-	+
DNA G+C content (mol%)	67.4	67.5 <sup>†</sup>	67.5-69.2*	67.5

Strains: 1, VSW210<sup>T</sup> (present study); 2, *Citricella marina* CK-I3-6<sup>T</sup> (present study) (<sup>†</sup>Lai et al. 2011); 3, *Citricella thiooxidans* CHLG 1<sup>T</sup> (Lai et al. 2011; \*Sorokin et al. 2005); 4, *Citricella aestuarii* AD8<sup>T</sup> (Park et al. 2011). All strains are positive for catalase and oxidase; leucine arylamidase,  $\alpha$ -glucosidase; acid production from D-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, esculin, D-cellobiose, D-maltose, xylitol and D-fucose. All strains are negative for Gram-staining; enzymes trypsin,  $\beta$ -galactosidase,  $\beta$ -glucuronidase, N-acetyl- $\beta$ -glucosaminidase,  $\alpha$ -mannosidase,  $\alpha$ -fucosidase; acid production from dulcitol, inositol, methyl  $\alpha$ -D-glucopyranoside, methyl  $\alpha$ -D-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, inulin, D-melazitose, D-raffinose, amidon, glycogen, D-turanose, D-tagatose, L-arabitol, and potassium 2-ketogluconate. For column 1 and 2, hydrolysis of Tween 20, DNA and agar are positive and casein, gelatin, Tween-80, xanthine, xylan, CMC and trybutyrin are negative. +, Positive; -, negative; w, weak reaction; ND, not determined

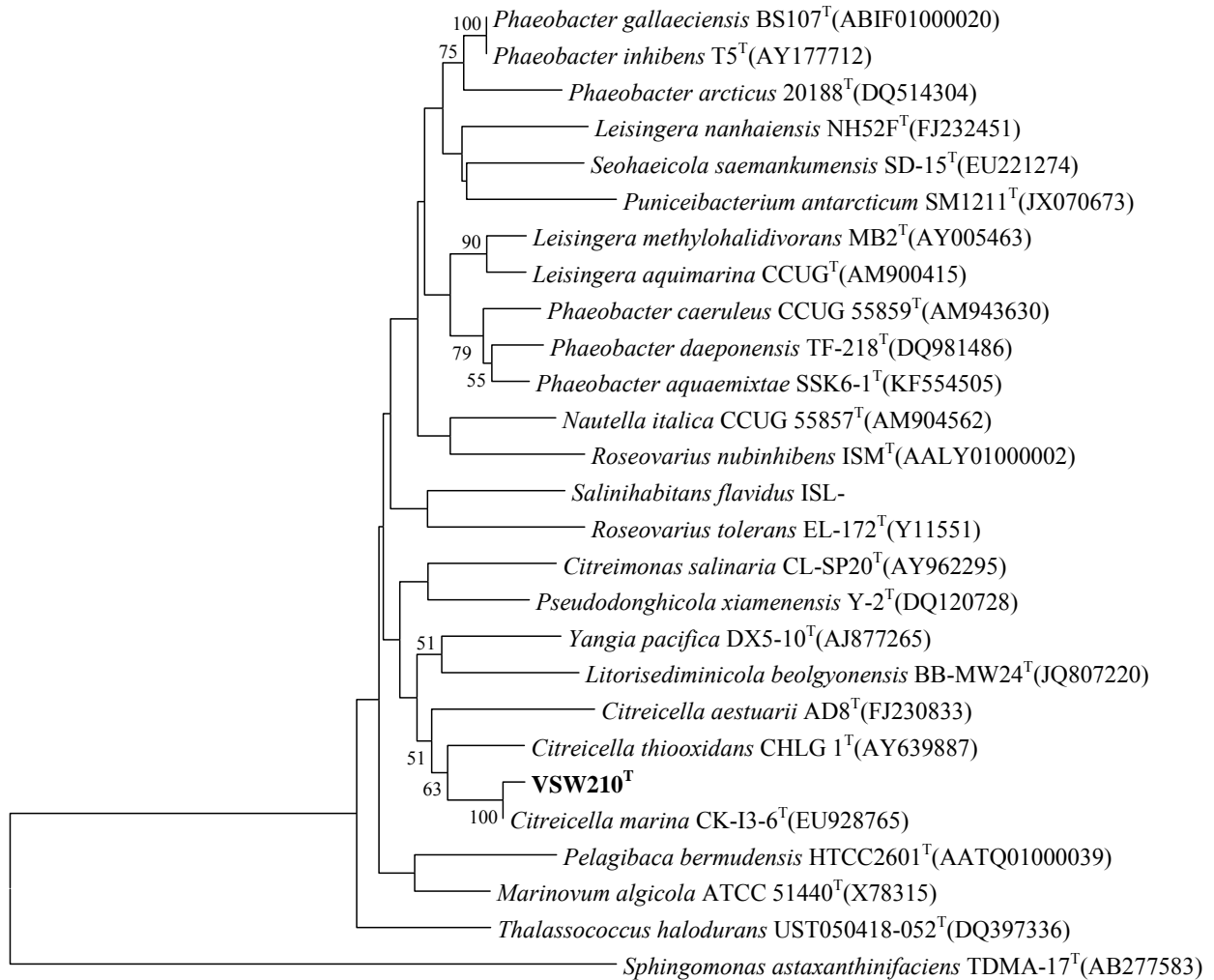
**Table 2.** Fatty acid profile of strain VSW210<sup>T</sup> and closest type strains of species of the genus *Citricella*

Strains: 1, VSW210<sup>T</sup> (present study); 2, *Citricella marina* CK-I3-6<sup>T</sup> (present study); 3, *Citricella thiooxidans* CHLG 1<sup>T</sup> (Lai et al. 2011); 4, *Citricella aestuarii* AD8<sup>T</sup> (Park et al. 2011). Only fatty acids amounting to  $\geq 1$  % of the total are shown. -, not detected; tr, trace amount (< 1 %).

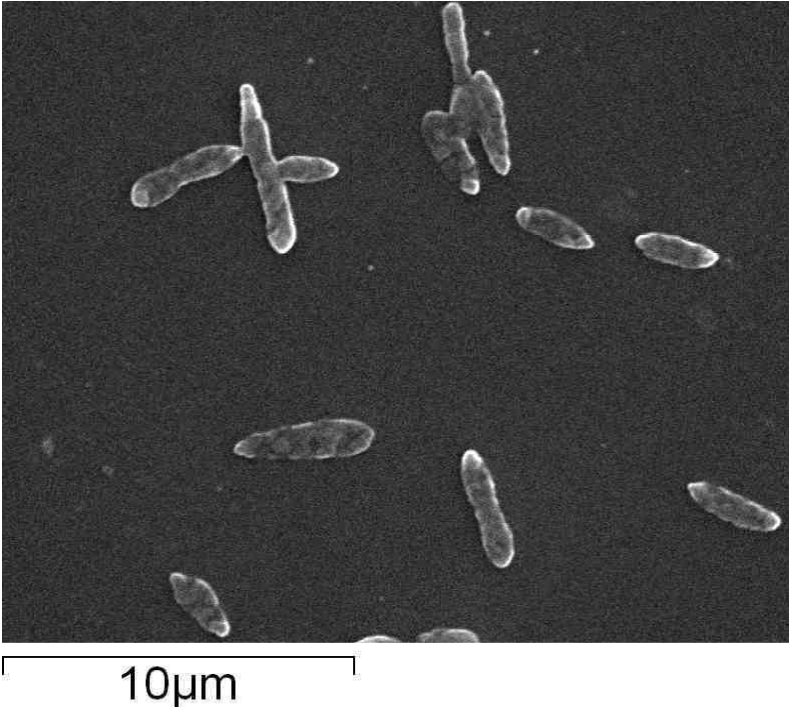
Fatty acids	1	2	3	4
C <sub>12:1</sub> 3-OH	2.8	2.4	tr	-
C <sub>12:0</sub> 3-OH	-	-	1.7	-
C <sub>10:0</sub>	1.7	-	-	-
C <sub>16:0</sub>	9.2	11.7	8.6	10.2
C <sub>17:0</sub>	1.1	-	tr	-
C <sub>18:1</sub> $\omega 7c$	80.1	76.9	74.6	57.0
C <sub>18:0</sub>	1.3	2.9	2.6	-
C <sub>18:1</sub> $\omega 7c$ 11-methyl	-	-	tr	2.3
C <sub>19:0</sub> $\omega 8c$ cyclo	-	2.1	6.6	15.5
Summed feature 3*	3.9	4.1	1.9	11.4

\*Summed features contain fatty acids that cannot be separated using standard procedures. Summed feature 3 contained C<sub>16:1</sub>  $\omega 7c$ / C<sub>16:1</sub>  $\omega 6c$ .

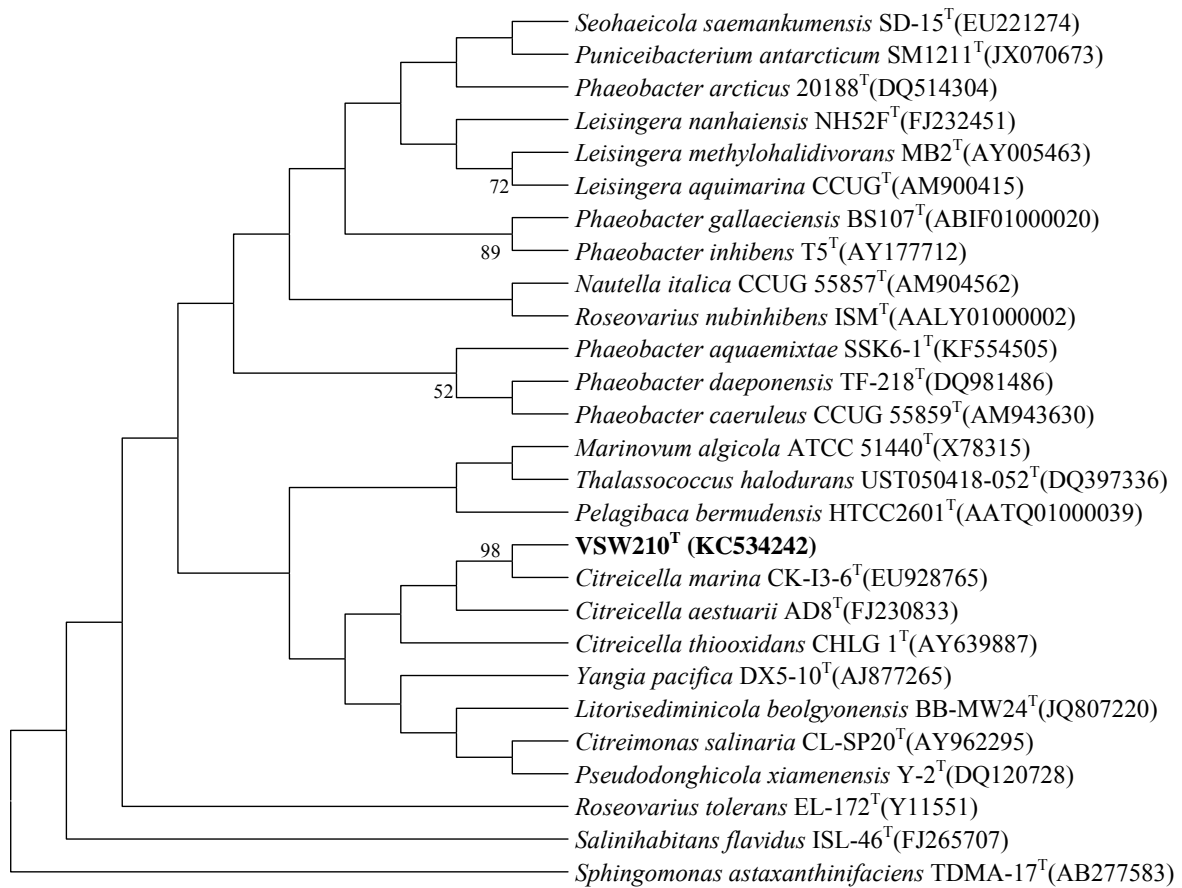
**Fig. 1.** 16S rRNA gene sequence based neighbour-joining tree showing the phylogenetic relationship of strain VSW210<sup>T</sup>. Numbers at the nodes are bootstrap values >50 %. *Sphingomonas astaxanthinifaciens* TDMA-17<sup>T</sup> (AB277583) was used as an out-group. Bar, 0.02 substitutions per nucleotide position.



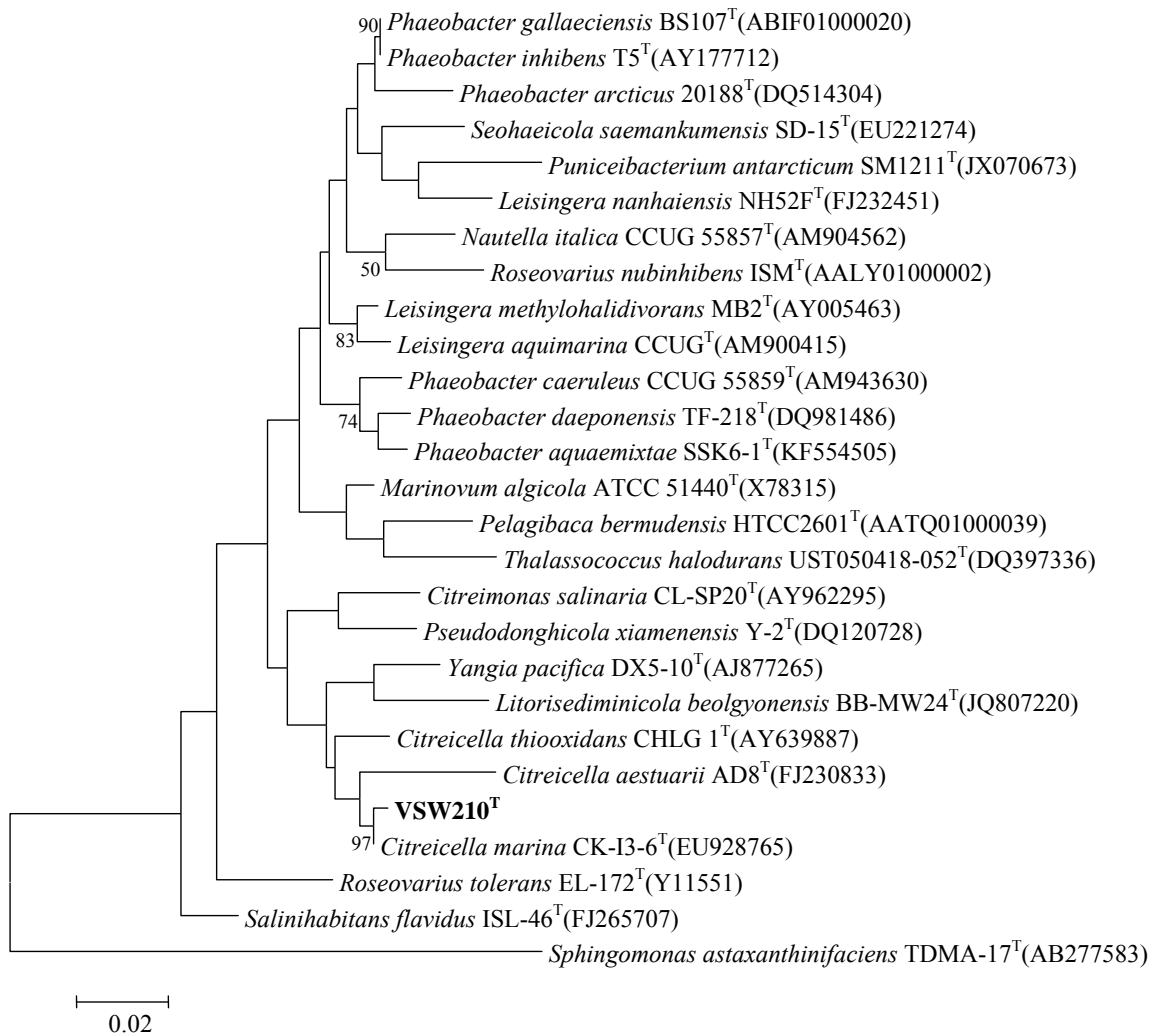
**Supplementary Fig. 1.** Scanning electron microscopic picture of strain VSW210<sup>T</sup> showing rod/narrowed lemon shaped, with tip ends cells



**Supplementary Fig. 2.** Maximum-parsimony tree based on 16S rRNA gene sequence showing the phylogenetic position of the strain VSW210<sup>T</sup> and related members.

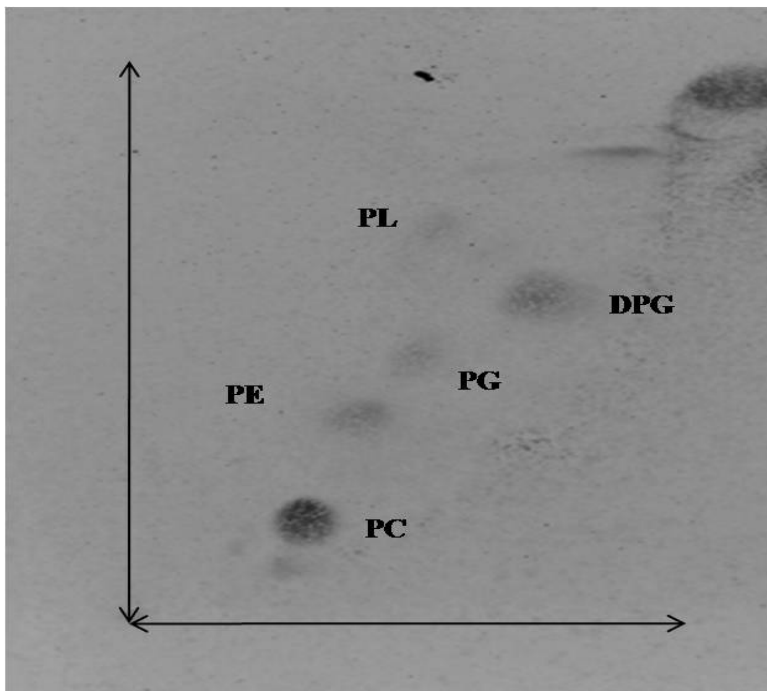


**Supplementary Fig. 3.** Maximum-likelihood tree based on 16S rRNA gene sequence showing the phylogenetic position of the strain VSW210<sup>T</sup> and related members

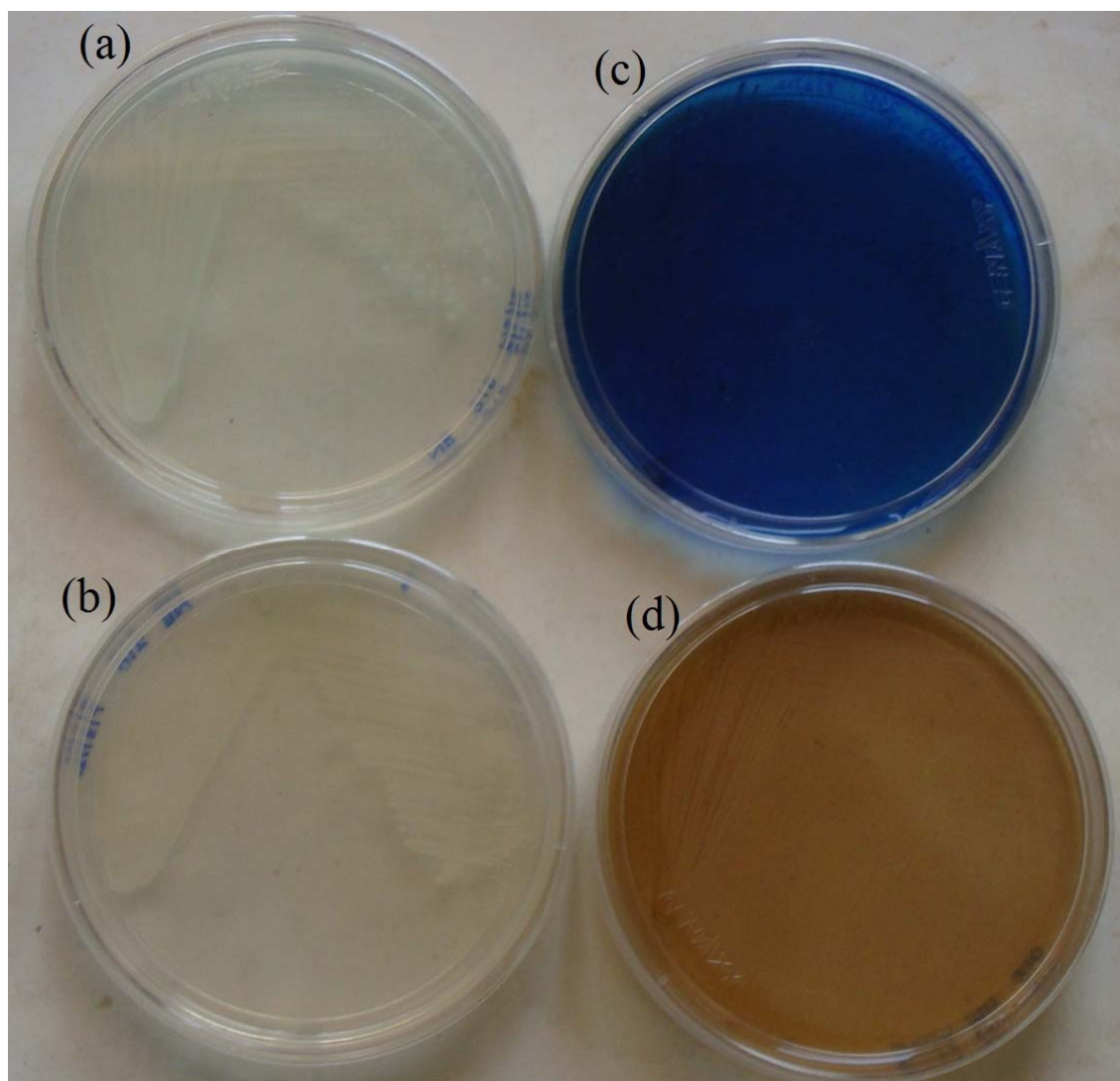




**Supplementary Fig. 4.** Two-dimensional TLC of polar lipids of the strain VSW210<sup>T</sup>. PL, unidentified phospholipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol



**Supplementary Fig. 5.** Strain VSW210<sup>T</sup> oxidized Mn(II) on plates which was detected as brown colour formation and confirmed with LBB staining. Plate (a) and (b) are controls (media plates without MnCl<sub>2</sub>); (c) after addition of LBB stain; (d) Mn(III/IV) oxides as dark brown precipitates before adding LBB



**Supplementary Fig. 6.** Growth and Mn(II) oxidation of potential hydrothermal vent strain VSW210<sup>T</sup>. Data shown are representative of 3 experiments

