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Luís França · Fred A. Rainey · M. Fernanda Nobre
Milton S. da Costa

# *Salinicoccus salsiraiae* sp. nov.: a new moderately halophilic gram-positive bacterium isolated from salted skate

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**Abstract** Two moderately halophilic low G + C Gram-positive bacteria were isolated from a sample of salted skate (Class *Chondrychthyes*, Genus *Raja*). Phylogenetic analysis of the 16S rRNA gene sequence of strains RH1$^T$ and RH4 showed that these organisms represented a novel species of the genus *Salinicoccus*. The new isolates formed pink–red colonies and flocculated in liquid media, with optimum growth in media containing 4% NaCl and pH of about 8.0. These organisms are aerobic but reduce nitrate to nitrite under anaerobic conditions. Acid is produced from several carbohydrates. Oxidase and catalase were detected. Menaquinone 6 was the major respiratory quinone. The major fatty acids of strains RH1$^T$ and RH4 were 15:0 anteiso and 15:0 iso. The G + C contents of DNA were 46.2 and 46.0 mol%, respectively. The peptidoglycan was of A3alpha L-Lys-Gly$_{5–6}$ type. On the basis of the phylogenetic analyses, physiological and biochemical characteristics, we suggest that strain RH1$^T$ (=LMG 22840 = CIP 108576) represents a new species of the genus *Salinicoccus*, for which we propose the name *Salinicoccus salsiraiae*.

**Keywords** *Salinicoccus salsiraiae* sp. nov. · Halophilic · Salted skate

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L. França · M. S. da Costa (✉)
Departamento de Bioquímica,
Centro de Neurociências e Biologia Celular,
Universidade de Coimbra, 3001-401 Coimbra, Portugal
e-mail: milton@ci.uc.pt
Tel.: +351-239-824024
Fax: +351-239-826798

F. A. Rainey
Department of Biological Sciences,
Louisiana State University, Baton Rouge, LA, 70803 USA

M. F. Nobre
Departamento de Zoologia, Universidade de Coimbra,
3004-517 Coimbra, Portugal

## Introduction

Two moderately halophilic species of the genus *Salinicoccus*, named *Salinicoccus roseus* (Ventosa et al. 1990) and *Salinicoccus hispanicus* (Marquez et al. 1990; Ventosa et al. 1992) were isolated from solar salterns in Spain. These strains have optimum growth around neutral pH and grow optimally in the presence of about 8% NaCl. Another organism, named *Salinicoccus alkaliphilus*, represents an additional lineage within the genus *Salinicoccus* was isolated from a soda lake in Inner Mongolia, China (Zhang et al. 2002). This organism is also moderately halophilic but has an optimum pH for growth around 9.5.

We recently isolated two moderately halophilic, aerobic Gram-positive cocci, designated RH1$^T$ and RH4 from salted skate (Class *Chondrychthyes*, Genus *Raja*). Phenotypic and chemotaxonomic characterization and phylogenetic analysis based on 16S rRNA gene sequences showed that these isolates were related to organisms of the genus *Salinicoccus* but represent a new species for which we propose the name *Salinicoccus salsiraiae*.

## Materials and methods

### Isolation and bacterial strains

The novel strains, designated RH1$^T$ (T = type strain) and RH4, were isolated from a sample of salted skate of the genus *Raja*. Salted fish samples (16–18 g) were homogenized in sterile blenders with sterile 5% NaCl solution. One drop of the homogenate was spread on Degryse 162 (Degryse et al. 1978) agar plates containing 5% NaCl. These preparations were incubated at 37°C for up to 5 days. Cultures were purified by sub-culturing and pure cultures were maintained at −80°C in Degryse 162 medium with 5% NaCl and 15% glycerol. The type strains of *S. roseus* (9$^T$ = DSM 5351$^T$) and *S. hispanicus*

(J-82$^T$ = DSM 5352$^T$) were used for comparative purposes.

### Phenotypic characterization

Unless otherwise stated, all morphological examinations, biochemical and tolerance tests were performed as described previously (Santos et al. 1989; Nunes et al. 1992), in 10% MH medium (Ventosa et al. 1982) (http://www.dsmz.de/media/med593.htm) as follows (%, w/v): $MgCl_2$, 0.7; $MgSO_4$, 0.96; $CaCl_2$, 0.036; KCl, 0.2; $NaHCO_3$, 0.006; NaBr, 0.0026; proteose–peptone no. 3, 0.5; yeast extract, 1.0 and glucose, 0.1. The levels of NaCl in the medium varied from 0 to 25%. Routine analysis of strains RH1$^T$ and RH4 was carried out in 10% MH medium containing 4% NaCl.

A sonication bath (Bransonic 12, 50 kHz) was used to disperse clumps of strains RH1$^T$ and RH4 grown in liquid medium. Turbidity was monitored at 610 nm. The NaCl range for growth of the organisms was examined in liquid medium containing 0–26% (w/v) NaCl, in a reciprocal water-bath shaker. The growth temperature range was determined in the same medium between 15 and 50°C. The pH range for growth was determined at 37°C in the same medium with 100 mM MES, HEPES, TAPS and CAPSO.

Enzymatic tests were performed using API ZYM test strips (BioMérieux) as recommended by the manufacturer, but with 4% NaCl (w/v). Results were recorded after 4 h incubation at 37°C. Single carbon source assimilation tests were performed in a basal liquid medium described by Ventosa et al. (1982) to which filter-sterilized yeast extract (0.1 g/l), ammonium chloride (0.5 g/l) and the carbon sources (2.0 g/l) were added. Growth of the strains was examined by measuring the turbidity of cultures incubated at 37°C as described previously (Moreira et al. 2000). Degryse 162 medium basal salts and ABM2 basal medium (Tiago et al. 2004) were also used to assess the assimilation of single carbon sources. API 50 CH test strips (Analytab Products Inc., BioMérieux) were also used to examine the assimilation of carbohydrates and to examine the production of acid using a defined medium (Ventosa et al. 1982) containing the following components per litre: NaCl, 40.0 g (for strains RH1$^T$ and RH4) or 80.0 g (for *S. roseus* and *S. hispanicus*); $MgSO_4 \cdot 7H_2O$, 2.0 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, traces; $KH_2PO_4$, 0.5 g; proteose-peptone no. 3, 1.0 g; yeast extract, 0.1 g; agar, 3.0 g; phenol red, 0.01 g.

### Peptidoglycan, lipoquinone and fatty acid composition

Purified cell wall preparations were obtained and the identification of the peptidoglycan type was performed using previously described methods (Schleifer and Kandler 1972; Schleifer 1985). Lipoquinones were extracted from freeze-dried cells and purified by TLC as described previously (Tindall 1989; Moreira et al. 2000; Freitas et al. 2003). Cultures for fatty acid analysis were grown on plates of modified 10% MH medium containing 8% NaCl in sealed plastic bags submerged in a water-bath at 37°C for 24 h. Fatty acid methyl esters (FAMEs) were obtained from fresh wet biomass; the identification and quantification of the FAMEs and the numerical analysis of the fatty acid profiles were performed by using the standard MIS library Generation Software (Microbial ID Inc., Netwark. Del).

### Determination of G + C content of DNA, 16 rRNA gene sequence and phylogenetic analysis

The DNA for the determination of the G + C content of the DNA was isolated as described by Nielsen et al. (1995). The G + C content of DNA was determined by high-performance liquid chromatography as described by Mesbah et al. (1989). The extraction of genomic DNA for 16S rRNA gene sequence determination, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described previously (Rainey et al. 1996). Purified reactions were electrophoresed using a model 310 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequences were aligned against representative reference sequences of members of the low G + C Gram-positive phylum using the ae2 editor (Maidak et al. 1999). The method of Jukes and Cantor (1969) was used to calculate evolutionary distances. Phylogenetic dendrograms and bootstrap analyses were generated using various algorithms contained in the PHYLIP package (Felsenstein 1993). The 16S rRNA gene sequence of strain RH1$^T$ is available in the GenBank database under the accession number DQ333949.

## Results

### Morphological and biochemical characteristics

Isolates RH1$^T$ and RH4 formed round pink–red pigmented colonies and coccoid-shaped Gram-positive cells with 1.0–2.5 μm in diameter occurring singly, in pairs, tetrads or clumps and were not motile. Endospores were not formed. Both strains flocculated in the liquid growth media used. The optimum concentration of NaCl for growth was about 4%, growth was observed in medium without additional NaCl and with 22% NaCl (Table 1). The type strains of *S. roseus* and *S. hispanicus* had optimum NaCl concentrations for growth around 8–10% and growth was not observed above 25% NaCl. The growth temperature range of strains RH1$^T$ and RH4 was between 20 and 45°C with an optimum growth temperature of about 37°C. The pH range for growth was between 6.5 and 9.5, with an optimum around 8.0. Strains RH1$^T$ and RH4 were strictly aerobic, but reduced nitrate to nitrite under anaerobic conditions.

**Table 1** Characteristics that distinguish strains RH1[T] and RH4 from the type strains of *Salinicoccus roseus*, *Salinicoccus hispanicus* and *Salinicoccus alkaliphilus*

| Characteristics | RH1[T]/RH4[a] | *S. roseus*[Ta] | *S. hispanicus*[Ta] | *S. alkaliphilus*[Tb] |
|---|---|---|---|---|
| Temperature (°C) for growth | | | | |
| Range | 20–45 | 20–45 | 20–45 | 10–49 |
| Optimum | 37 | 37 | 37 | 32 |
| pH for growth | | | | |
| Range | 6.5–9.5 | 6.5–9.5 | 6.5–9.5 | 6.5–11.5 |
| Optimum | 8.0 | 8.0 | 8.0 | 9.5 |
| NaCl (%, w/v) for growth | | | | |
| Range | 0–22 | 0–25 | 0–25 | 0–25 |
| Optimum | 4 | 8–10 | 8–10 | 10 |
| Degradation of | | | | |
| Esculin | - | - | + | + |
| Gelatin | + | + | + | - |
| Casein | + | + | + | - |
| Presence of | | | | |
| Alkaline phosphatase | + | - | w | nd |
| Esterase lipase | - | w | - | nd |
| Cystine arylamidase | - | - | w | nd |
| *β*-glucosidase | - | - | + | nd |
| Urease | - | - | - | + |
| Acid production from | | | | |
| D-fructose | + | + | + | - |
| D-maltose | + | + | + | - |
| L-arabinose | - | + | - | nd |
| D-ribose | + | - | - | nd |
| D-xylose | - | + | - | nd |
| L-xylose | -/w | - | - | nd |
| D-mannose | - | - | + | nd |
| D-mannitol | - | + | + | nd |
| Methyl-α-D-glucopyranoside | - | - | w | nd |
| Amygdalin | - | - | w | nd |
| Arbutin | - | - | + | nd |
| Esculin | - | - | + | nd |
| Salicin | - | - | + | nd |
| D-melibiose | - | + | - | nd |
| Sucrose | - | + | + | - |
| D-melezitose | - | + | + | nd |
| D-raffinose | - | + | - | nd |
| Starch | - | + (-[c]) | - | nd |
| Xylitol | - | - | w | nd |
| Gentiobiose | - | - | + | nd |
| D-turanose | - | + | + | nd |
| D-tagatose | - | - | + | nd |
| L-fucose | - | - | w | nd |
| D-arabitol | - | - | + | nd |
| Gluconate | - | + | + | nd |
| G + C content (mol%) | 46.2/46.0 | 50.1 (51.2[c]) | 46.4 | 49.6 |

All strains were catalase and oxidase positive. None of the strains hydrolyzed starch. Strains RH1[T], RH4, *S. roseus*[T] and *S. hispanicus*[T] hydrolyzed arbutin, hippurate and hide powder azure. None of these strains hydrolyzed xylan, elastin and fibrin. Strains RH1[T], RH4, *S. roseus*[T] and *S. hispanicus*[T] were positive to DNAse, esterase (C4), leucine arylamidase, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase and α-glucosidase. Lipase (C14), valine arylamidase, trypsine, acid phosphatase, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase, α-fucosidase were negative. Methyl red, Voges-Proskauer and indol formation tests were negative for all strains. All organisms produced acid from D-glucose. Strains RH1[T], RH4, *S. roseus*[T] and *S. hispanicus*[T] produced acid from glycerol, *N*-acetylglucosamine, D-trehalose, 2-ketogluconate and 5-ketogluconate. None of these strains produced acid from erythritol, D-arabinose, D-adonitol, methyl-*β*-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-α-D-mannopyranoside, D-cellobiose, inulin, glycogen, D-lyxose, D-fucose and L-arabitol- negative, + positive, *w* weakly positive, *nd* not determined

[a]Our results

[b]Results from Zhang et al. (2002)

[c]Adapted from Ventosa et al. (1990)

Catalase, oxidase and DNAse were detected, but amylase and xylanase were not. Arbutine, hippurate, gelatine, casein and hide powder azure were hydrolyzed. These organisms did not hydrolyze elastin, fibrin and esculin. Assimilation of single carbon sources was inconclusive, as we were unable to obtain growth using any of the carbon sources examined on any of the media tested. However, formation of acid on the API 50 CH

test strips showed differences between the new organisms and the type strains of *S. roseus* and *S. hispanicus* (Table 1).

### Chemotaxonomic characteristics

Menaquinone 6 was the major respiratory quinone. The major fatty acids of strains RH1$^T$ and RH4 were 15:0 anteiso (36%) and 15:0 iso (25%). The levels of other fatty acids distinguished the type strains of the species of the genus *Salinicoccus* from each other (Table 2). The major amino acid constituents of the cell wall composition were glycine and lysine, which is in accordance with type A3alpha L-Lys-Gly$_{5-6}$ peptidoglycan described for the genus *Salinicoccus* (Ventosa et al. 1990).

### 16S rRNA gene sequence comparison and guanine plus cytosine (G + C) content of DNA

The G + C contents of the DNA of strains RH1$^T$ and RH4 were 46.2 and 46.0 mol%, respectively. These values were about 4–5 mol% lower than those of the type strain of *S. roseus* (Table 1). The G + C content of the DNA of *S. roseus* was reported as 51.2% (Ventosa et al. 1990); however, our results gave a value of 50.1 (Table 1). Almost complete 16S rRNA gene sequences comprising 1,505 nucleotide positions were determined for strains RH1$^T$ and RH4, which had identical sequences. Pairwise comparison of the 16S rRNA gene sequences showed them to be identical over the 1,505 nucleotide positions determined. Phylogenetic analyses found strains RH1$^T$ and RH4 to group within the radiation of the species of the low G + C Gram-positive genus *Salinicoccus* (Fig. 1). The lineage representing strains RH1$^T$ and RH4 was distinct from those of the previously described species *S. alkaliphilus*, *S. hispanicus* and *S. roseus* and even though the similarity values are as high as 97% the low bootstrap values indicated no specific relationship of the new organisms to any of these three species. Pairwise 16S rRNA gene sequence comparisons showed the sequence of RH1$^T$ to share 95.5, 96.2 and 97.0% similarity to the 16S rRNA gene sequences of *S. alkaliphilus*, *S. hispanicus* and *S. roseus*, respectively.

## Discussion

The phylogenetic analysis based on the 16S rRNA gene sequence of strains RH1$^T$ and RH4 showed them to have identical sequences over the 1,505 nucleotide positions determined and to cluster with the species of the genus *Salinicoccus*. Strains RH1$^T$ and RH4 show 16S rRNA gene sequence similarities in the range 95.5–97.0% to the previously described species of the genus *Salinicoccus* indicating that the new strains constitute a new species of the same genus.

The new species of the genus *Salinicoccus* represented by strains RH1$^T$ and RH4 shares many of the physiological and biochemical characteristics of *S. roseus* and *S. hispanicus*, which clearly indicates that these organisms are closely related to each other and form a homogeneous group of organisms. The type strains of *S. roseus* and *S. hispanicus* were initially reported not to grow in media without NaCl, in contrast to *S. alkaliphilus*, which grew in media without additional NaCl (Zhang et al. 2002). We found that strains RH1$^T$, RH4 as well as the type strains of *S. roseus* and *S. hispanicus* grew in MH 10% medium without additional NaCl; this medium contained only 0.90 mM $Na^+$, however, the growth rate was much lower than in medium containing 4 or 8% NaCl.

Strains RH1$^T$ and RH4 can be distinguished from the type strains of the species previously described of the

**Table 2** Mean fatty acid composition of strains RH1$^T$, RH4 and type strains of *Salinicoccus roseus*, *Salinicoccus hispanicus* and *Salinicoccus alkaliphilus*

| Fatty acid[a] | RH1$^T$/RH4 | *S. roseus*$^T$ | *S. hispanicus*$^T$ | *S. alkaliphilus*$^{Tb}$ |
|---|---|---|---|---|
| 14:0 iso | 1.0 ± 0.1 | 2.1 ± 0.3 | 5.8 ± 1.3 | 4.4 |
| 14:0 | 0.8 ± 0.2 | 1.2 ± 0.3 | 5.3 ± 0.4 | 1.6 |
| 15:0 iso | 26.6 ± 2.2 | 21.4 ± 1.2 | 25.9 ± 3.4 | 22.3 |
| 15:0 anteiso | 35.3 ± 0.7 | 36.0 ± 2.3 | 15.6 ± 3.6 | 27.6 |
| 15:0 | – | 2.1 ± 0.6 | 5.4 ± 0.8 | – |
| 16:1 $\omega$7*c* alcohol | – | 0.8 ± 0.3 | 1.6 ± 0.1 | 5.8 |
| 16:0 iso | 3.5 ± 0.6 | 5.3 ± 0.8 | 5.5 ± 1.0 | 10.1 |
| Unknown[c] | 2.3 ± 0.3 | 2.0 ± 0.4 | 8.2 ± 2.3 | 0.9 |
| 16:1 $\omega$11*c* | – | – | 2.0 ± 0.2 | 2.2 |
| 16:0 | 2.9 ± 0.4 | 3.0 ± 0.7 | 10.3 ± 2.3 | 1.1 |
| 17:1 $\omega$10*c* iso | 3 ± 0.3 | 1.5 ± 0.4 | 0.7 ± 0.1 | 4.0 |
| Sum in feature 5[d] | 0.7 ± 0.1 | 0.5 ± 0.1 | – | – |
| 17:0 iso | 9.5 ± 0.8 | 5.6 ± 0.3 | 4.6 ± 0.9 | 3.3 |
| 17:0 anteiso | 8.5 ± 0.6 | 8.0 ± 1.0 | 1.6 ± 0.7 | 8.9 |
| 17:0 | – | 0.5 ± 0.2 | 0.9 ± 0.3 | – |
| 18:0 | 0.7 ± 0.1 | 1.1 ± 0.2 | 2.4 ± 0.8 | – |
| 19:0 iso | 3.0 ± 0.1 | 4.0 ± 0.3 | 1.1 ± 0.3 | 2.8 |
| 19:0 anteiso | 1.0 ± 0.2 | 2.3 ± 0.5 | – | 1.9 |
| 19:0 | – | 1.0 ± 0.3 | 0.6 ± 0.1 | – |
| 20:0 iso | – | 0.6 ± 0.1 | – | 1.5 |
| 20:0 | 2.1 ± 0.5 | 1.5 ± 0.4 | 2.2 ± 0.2 | 1.3 |

– not detected
[a]Values for fatty acids present at levels of less than 0.5% are not shown
[b]Results from Zhang et al. (2002)
[c]Unknown fatty acid with an equivalent chain length 15.670
[d]Group of fatty acids (17:1 iso I and/or 17:1 anteiso B) that could not be separated by this method

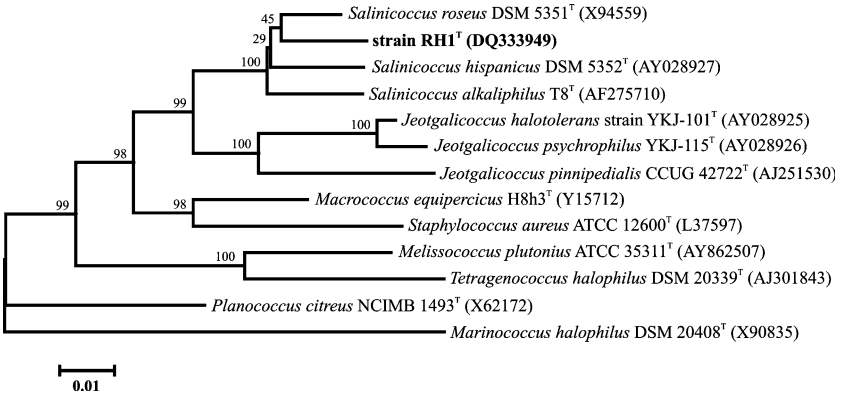


**Fig. 1** 16S rRNA gene sequence based phylogeny showing the relationships of strain RH1$^T$ and related taxa. The dendrogram was constructed from distance matrices using the neighbour-joining method. Numbers at branching points represent bootstrap values from 1,000 replicates. Scale bar, one inferred nucleotide substitution per 100 nucleotides

genus *Salinicoccus* by the lower optimum NaCl concentration for growth, by acid production from different carbohydrates and by differences in fatty acid composition. The higher pH range of *S. alkaliphilus* also serves to distinguish this species from the other species of the genus, which includes the new organisms isolated from salted skate.

On the basis of the results presented in this study, we are of the opinion that strains RH1$^T$ and RH4 represent a new species of the genus *Salinicoccus* for which we propose the name *S. salsiraiae*.

## Description of *Salinicoccus salsiraiae*

*Salinicoccus salsiraiae* (sal.si.ra'i.a.e. L. adj. *salsus*, salted; L. n. *raia*, a ray; N.L. gen. n. *salsiraiae*, of a salted ray). *S. salsiraiae* forms spherical cells, Gram-positive, 1.0–2.5 μm in diameter, occurring singly, in pairs, tetrads or clumps. The cells are not motile and non-spore forming. Strain RH1$^T$ forms pink–red colonies and flocculates in liquid media. The optimal salt concentration for growth is 4% (w/v), growth occurs in medium without additional salt and in medium containing 22% NaCl. Growth occurs at 20 and 45°C; the optimum growth temperature for strain RH1$^T$ is about 37°C. The optimum pH is about 8.0; growth occurs between pH 6.5 and 9.5. The major fatty acids are 15:0 iso and 15:0 anteiso. Menaquinone 6 is the major respiratory quinone. The peptidoglycan is of the A3alpha L-Lys-Gly$_{5-6}$ type. Strain RH1$^T$ is strictly aerobic and reduces nitrate to nitrite. Cytochrome oxidase, catalase and DNAse are produced; arbutin, hippurate, gelatine, casein and hide powder azure are degraded. This organism produces acid from D-ribose, glycerol, D-glucose, D-fructose, *N*-acetylglucosamine, D-maltose, D-trehalose, 2-ketogluconate and 5-ketogluconate.

The DNA of strain RH1$^T$ has a G + C content of 46.2 mol%. This bacterium was isolated from a salted ray (skate). Strain RH1$^T$ has been deposited in the Universiteit Gent, Laboratorium voor Microbiologie, Gent, Belgium as strain LMG 22840 and the Collection de l'Institut Pasteur, Paris, France as strain CIP 108576.

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