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Marinococcus hispanicus, a New Species of Moderately Halophilic Gram-Positive Cocci

M. C. MARQUEZ, A. VENTOSA,* AND F. RUIZ-BERRAQUERO

Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Seville, Spain

Five moderately halophilic gram-positive cocci were isolated from soils and ponds of a solar saltern located near Alicante, Spain. These organisms all were nonmotile, nonsporing, reddish orange pigmented, strictly aerobic, and catalase and oxidase positive, had *meso*-diaminopimelic acid in their cell walls, and had DNA base compositions ranging from 45.7 to 49.3 mol%; they constitute a homology group with levels of DNA-DNA homology ranging from 70 to 100%. On the basis of their features, we regard these strains as belonging to a new species of the genus *Marinococcus*, for which we propose the name *Marinococcus hispanicus*. The type strain is strain J-82 (= ATCC 49259 = DSM 5352 = CCM 4148).

Moderately halophilic bacteria are microorganisms that can grow optimally in media containing 3 to 15% (wt/vol) salt (14). This group contains species belonging to the archaebacterial group (*Halomethanococcus doii* [32], *Methanohalophilus zhilinae* [17], and *Methanohalophilus mahii* [23]), as well as many eubacteria. Within the moderately halophilic heterotrophic eubacteria, the gram-negative microorganisms have been studied in more detail (5–9, 11, 13, 19, 20, 24, 25, 30, 33). Until now the gram-positive moderately halophilic bacteria have included only four species, all of which are cocci; these are *Micrococcus halobius* (18), *Sporosarcina halophila* (1), *Marinococcus halophilus*, and *Marinococcus albus* (10).

In this study we isolated five moderately halophilic gram-positive cocci from hypersaline habitats located near Alicante, Spain. These strains were characterized phenotypically, as well as with respect to their DNA base compositions, levels of DNA-DNA homology, cell wall compositions, and quinone systems. On the basis of the results of these studies, the five isolates showed characteristics that were sufficiently different from those of the cocci previously described to justify their inclusion in a new species of the genus *Marinococcus*, for which we propose the name *Marinococcus hispanicus*.

MATERIALS AND METHODS

Bacterial strains. The five strains which we studied were isolated either from hypersaline soils or from the ponds of a solar saltern located near Alicante, Spain. The isolation medium and the methodology which we used have been described previously (29).

The following reference strains were also included in this study: *Micrococcus halobius* ATCC 21727^T (T = type strain), *Marinococcus albus* CCM 3517^T, *Marinococcus halophilus* CCM 2706^T, *Sporosarcina halophila* DSM 2226^T, *Paracoccus halodenitrificans* ATCC 13511^T, *Planococcus citreus* CCM 316^T, *Staphylococcus aureus* ATCC 25923, *Acinetobacter* sp. strain F9-6, *Flavobacterium halmephilum* CCM 2833^T, *Flavobacterium* sp. strain F8-11, *Halomonas elongata* ATCC 33173^T, and *Vibrio costicola* NCMB 701^T.

The strains were maintained on agar slants of 10% (wt/vol) salts complex medium having the following composition: 8.1% (wt/vol) NaCl, 0.7% (wt/vol) $MgCl_2$, 0.96% (wt/vol) $MgSO_4$, 0.036% (wt/vol) $CaCl_2$, 0.2% (wt/vol) KCl, 0.006% (wt/vol) $NaHCO_3$, 0.0026% (wt/vol) NaBr, 1.0% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.), 0.5% (wt/vol) Proteose Peptone no. 3 (Difco), 0.1% (wt/vol) glucose, and 2.0% (wt/vol) Bacto-Agar (Difco). The pH was adjusted to 7.5 with 1 M KOH (28).

Phenotypic characterization. Tests for 150 characteristics, including morphological, cultural, physiological, biochemical, and nutritional tests, were carried out. The methodology which we used has been described previously (8, 24, 28). Unless otherwise indicated, all tests were carried out with 10% (wt/vol) salts (pH 7.5), and incubation was at 37°C in sealed containers.

DNA extraction and purification. All of the strains which we studied were harvested, washed, suspended in 0.15 M NaCl–0.1 M EDTA buffer (pH 8.0) (5 g [wet weight] in 50 ml of buffer), and lysed with lysozyme (approximately 10 mg) at 37°C and with sodium dodecyl sulfate at a final concentration of 2% (wt/vol) at 60°C. The DNA was extracted and purified by the method of Marmur (15). The purity was assessed from the A_{260}/A_{280} and A_{230}/A_{260} extinction ratios (12).

DNA base composition. The guanine-plus-cytosine (G+C) content of the DNA was determined from the midpoint value of the thermal denaturation profile (16) obtained with a model UV-Vis 551S spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) at 260 nm; this instrument was programmed for temperature increases of 1.0°C/min. The G+C content was calculated from the thermal denaturation temperature by using the equation of Owen and Hill (21). The G+C content of reference DNA from *Escherichia coli* NCMB 9001 was taken to be 51 mol%.

Preparation of ^{3}H-labeled DNA. DNA was labeled by the multiprime system with a commercial kit (kit RPN 1601Y; Amersham International, Amersham, England), using [1′,2′,5-^{3}H]dCTP (Amersham). The average specific activity obtained with this procedure was 8.8×10^6 cpm/μg of DNA. The labeled DNA was denatured before hybridization by being heated at 100°C for 5 min and then placed on ice.

DNA-DNA hybridization. DNA-DNA homology studies were performed by using the competition procedure of the membrane method described by Johnson (12). Competitor DNAs were sonicated (Braun Melsugen, Melsugen, Federal Republic of Germany) at 50 W for two 15-s time intervals. Membrane filters (type HAHY; Millipore Corp., Bedford, Mass.) containing reference DNA (ca. 25 μg/cm) were placed in 5-ml screw-cap vials which contained the labeled, sheared, denatured DNA and the denatured and sheared competitor DNA. The ratio of the concentration of compet-

* Corresponding author.

itor DNA to the concentration of labeled DNA was at least 150:1. The final reaction concentrations were 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 30% formamide, and the final volume was 140 μl. The hybridization temperature ranged between 49 and 50°C, which is within the limits for the validity of the filter method (4). The vials were shaken slightly for 18 h in a water bath (Grant Instruments, Cambridge, England); these procedures were done in triplicate. After hybridization the filters were washed in 2× SSC at the optimal renaturation temperature. The radioactivity bound to the filters was measured with a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.), and the percentage of homology was calculated by using the method of Johnson (12). At least two independent determinations were carried out for each experiment, and the mean values are reported below. The difference in thermal stability between homologous and heterologous double-stranded DNAs was calculated in some representative strains as described by Johnson (12).

Determination of isomers of diaminopimelic acid. The isomers of diaminopimelic acid in the cell walls of the strains were detected by using the method of Staneck and Roberts (27). Dried cells (approximately 10 mg) were hydrolyzed with 1 ml of 10 N HCl at 100°C for 20 h in a screw-cap tube, and 1 ml of the filtered hydrolysate was chromatographed on cellulose thin-layer plates (E. Merck AG, Darmstadt, Federal Republic of Germany) in parallel with a reference amino acid mixture. For separation, methanol–distilled water–10 N HCl–pyridine (80:26:4:10, vol/vol) was used as the solvent. Then the chromatogram was dried at room temperature, sprayed with a 5% ninhydrin–butanol solution, and heated at 100°C for 3 min.

Quinone system. Quinones were extracted and purified from the cells of the strains by using the method described by Collins (2). The purified menaquinones were examined by reverse-phase partition chromatography, using Merck type HPTLC RP-18F_{254} reverse-phase thin-layer plates (10 by 10 cm) and a polar developing mixture containing acetone and water (99:1, vol/vol). The separated components were detected with 254-nm UV light.

RESULTS

Cell morphology. The cells of the five strains which we studied were gram positive and spherical (diameter, 1.0 to 2.0 μm) and occurred singly, in pairs, in tetrads, and in clumps. These organisms were nonmotile and did not possess endospores.

Cultural characteristics. The colonies of the strains on 10% (wt/vol) salts complex medium were circular, smooth, convex, and reddish orange pigmented. The pigment was nondiffusible, and pigmentation did not vary with different concentrations of salt in the media. Broth cultures were uniformly turbid.

Physiological, biochemical, and nutritional characteristics. The five isolates grew in solid media containing 0.5 to 25% (wt/vol) total salts and grew optimally at a total salts concentration of about 10%. They grew at temperatures ranging from 15 to 37°C; the optimum temperature for growth was 37°C. The pH range for growth was from pH 5.0 to 9.0, with optimum growth at pH 7.5.

The organisms were strictly aerobic and catalase and oxidase positive. Acid was produced from fructose, galactose, D-glucose, glycerol, and mannitol. Nitrate was reduced to nitrite. Gelatin was hydrolyzed; casein and Tween 80 were not hydrolyzed. β-(complete) hemolysis was produced.

TABLE 1. Characteristics which differentiate *Marinococcus hispanicus* strains from one another

Characteristic	No. of strains positive	Reaction of strain J-82^T
Nitrite reduction	3	+
ONPG[a]	1	+
Urease	2	+
Hydrolysis of:		
Esculin	3	+
Starch	1	−
Phosphatase	2	−
DNase	3	+
Lecithinase	2	−
Acid production from:		
Maltose	1	+
Sucrose	2	+
Growth on[b]:		
Arabinose	3	−
D-Gluconolactone	2	−
D-Glucosamine	2	−
Inulin	1	+
D-Melibiose	1	+
Starch	1	+
Glycerol	2	−
D-Mannitol	1	+
m-Inositol	2	−
Citrate	3	+
Fumarate	4	+
DL-Glycerate	4	+
D-Gluconate	2	−
D-Glucuronate	2	−
Glutamate	2	+
Hippurate	2	−
Salicylate	1	−
Growth on[c]:		
L-Alanine	2	−
L-Isoleucine	2	+
L-Proline	1	−
L-Tryptophan	2	+

[a] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.
[b] When supplied as the sole source of carbon and energy.
[c] When supplied as the sole source of carbon, nitrogen, and energy.

Table 1 shows the characteristics which differentiate the five strains which we studied from one another. Other phenotypic characteristics are included below in the species description.

DNA base composition. The G+C contents of the five isolates ranged from 45.6 to 49.3 mol% (Table 2).

DNA-DNA hybridization. The results of the DNA-DNA hybridization studies are shown in Table 2. The levels of relatedness of labeled DNAs from three representative strains to DNAs from the other isolates ranged from 70 to 99%. On the other hand, low levels of relatedness (0 to 38%) were found between the three reference strains and the other strains belonging to related species used for comparison.

Determination of diaminopimelic acid in the cell walls. The cell walls of the five isolates contained *meso*-diaminopimelic acid in the peptidoglycan.

Quinone system. The menaquinones found in the five strains which we studied were MK-7 and MK-8.

DISCUSSION

The five strains studied in this work are very similar in their characteristics, sufficiently similar to warrant placement in the same taxon. These strains are different from the gram-positive moderately halophilic cocci described previously.

TABLE 2. G+C contents of DNAs and levels of DNA-DNA relatedness between *Marinococcus hispanicus* strains and strains of other related species

Unlabeled DNA from:	G+C content (mol%)	% Homology with ^{3}H-labeled DNA from:		
		Strain J-82^T	Strain J-84	Strain J-110
Marinococcus hispanicus strains				
J-82^T	45.7	100 (0)[a]	93	99
J-84	46.8	95 (3.0)	100	95
J-88	45.6	91 (3.0)	94	95
J-109	49.3	70 (3.0)	71	71
J-110	46.7	97 (3.0)	98	100
Marinococcus albus CCM 3517^T	44.9[b]	36	20	28
Marinococcus halophilus CCM 2706^T	46.4[b]	10	13	23
Micrococcus halobius ATCC 21727^T	71.5[b]	0	9	1
Sporosarcina halophila DSM 2226^T	40.8[b]	34	34	28
Paracoccus halodenitrificans ATCC 13511^T	ND[c]	15	0	22
Planococcus citreus CCM 316^T	ND	28	18	23
Staphylococcus aureus ATCC 25923	ND	12	10	19
Acinetobacter sp. strain F9-6	ND	14	1	6
"*Bacillus halophilus*" N23-2	ND	27	5	18
Deleya halophila CCM 3662^T	66.7[b]	30	38	23
Flavobacterium halmephilum CCM 2833^T	62.9[b]	4	21	27
Flavobacterium sp. strain F8-11	ND	1	26	37
Halomonas elongata ATCC 33173^T	60.5[b]	17	38	23
Vibrio costicola NCMB 701^T	49.9[b]	17	32	37

[a] The numbers in parentheses are the differences (in degrees Celsius) between the thermal stabilities of the homologous and heterologous hybrids.
[b] Data from references 1, 7, 8, 10, 18, 24, and 30.
[c] ND, Not determined.

In a previous study, Hao et al. (10) studied taxonomically a group of 14 gram-positive moderately halophilic cocci and, on the basis of the characteristics of these organisms, suggested their inclusion in a new genus, *Marinococcus*, with two species, *Marinococcus albus* and *Marinococcus halophilus*. All of these bacteria had *meso*-diaminopimelic acid in their cell walls, DNA base compositions ranging from 43.9 to 46.6 mol%, and menaquinone systems with MK-7.

Phenotypically, our isolates had some characteristics that were similar to those of the species belonging to the genus *Marinococcus*; however, they were different in many respects from *Marinococcus halophilus* and *Marinococcus albus* as described by Hao et al (10) (Table 3).

The DNA base compositions of our isolates (45.6 to 49.3 mol%) were similar to that of *Marinococcus halophilus* (46.4 mol%) and somewhat higher than the value described for *Marinococcus albus* (44.9 mol%) (10). The cell wall analysis of our strains showed that they contained murein of the *meso*-diaminopimelic acid direct type, as found in *Marinococcus halophilus* and *Marinococcus albus* (10). The menaquinones found in our isolates were MK-7 and MK-8; however, in *Marinococcus halophilus* and *Marinococcus albus* only MK-7 was described as a major menaquinone (10).

Our new isolates are clearly different from *Micrococcus halobius*, the only validly published moderately halophilic gram-positive coccus described as nonmotile, in the following phenotypic and chemotaxonomic characteristics: pigmentation, acid production from different sugars, Voges-Proskauer test, nitrate reduction, hydrolysis of gelatin, DNA base composition, cell wall type, and menaquinone system (3, 18).

Our isolates are also phenotypically and chemotaxonomically different from *Sporosarcina halophila*. The phenotypic differences are in the following characteristics: motility, spore production, pigmentation, acid production from the sugars studied, nitrate reduction, and hydrolysis of casein. Chemotaxonomically, *Sporosarcina halophila* contains murein of the Orn–D-Asp type and MK-7 as the main menaquinone and has a DNA G+C content that is somewhat lower (40.1 to 40.9 mol%) than the values for our isolates (1).

The traits which distinguish the strains which we studied from other moderately halophilic gram-positive cocci described previously are shown in Table 3.

The DNA relatedness experiments showed that our five isolates form a single DNA homology group with homology values equal to or greater than 70%. The majority of authors suggest that among strains of the same species the levels of DNA homology should be equal to or greater than 70% (22, 31). Thus, on the basis of DNA relatedness results, our isolates form a group of organisms that are sufficiently related to constitute a genospecies. On the other hand, low levels of DNA homology were obtained between the three representative strains and all of the moderately halophilic, marine or nonhalophilic bacteria which we studied.

Recently, Fendrich (5) isolated a nonmotile, gram-positive, halophilic coccus from Great Salt Lake, Utah. This coccus differs from our isolates in several phenotypic features, as well as in DNA base composition and cell wall type.

With respect to the gram-positive, nonhalophilic cocci described previously, our isolates are clearly different in their phenotypic and chemotaxonomic characteristics (26).

For these reasons, we propose that our five isolates should be placed in a new species of the genus *Marinococcus*, for which we propose the name *Marinococcus hispanicus*.

Description of *Marinococcus hispanicus* Márquez, Ventosa, and Ruiz-Berraquero sp. nov. *Marinococcus hispanicus* (his.pa'ni.cus. L. adj. *hispanicus*, Spanish). Gram-positive, spherical cells (diameter, 1.0 to 2.0 μm) occur singly, in pairs, in tetrads, or in clumps. Nonmotile and nonsporing. Colonies are round and smooth and form a reddish orange, nondiffusible pigment. Broth cultures are uniformly turbid.

The optimal salt concentration for growth is 10% (wt/vol) at 37°C; the organism grows at salt concentrations between 0.5 and 25% (wt/vol). No growth occurs in the absence of NaCl.

Growth occurs at 15 to 37°C and pH 5 to 9; optimal growth occurs at 37°C and pH 7.5.

Strictly aerobic. Catalase and oxidase are produced. Acid is produced from fructose, galactose, glycerol, D-glucose, and D-mannitol; acid is not produced from arabinose, lactose, D-trehalose, and xylose. Gelatin is hydrolyzed; casein, Tween 80, and tyrosine are not hydrolyzed. The methyl red test is positive. Nitrates are reduced to nitrites.

Voges-Proskauer, indole, Simmons citrate, phenylalanine deaminase, H_2S production, arginine, and lysine and ornithine decarboxylase tests are negative.

The following compounds are utilized as sole carbon and energy sources: D-glucose, D-mannose, L-raffinose, L-rhamnose, ribose, salicin, and sucrose.

The following compounds are not utilized as sole carbon and energy sources: amygdalin, D-cellobiose, D-fructose, D-fucose, D-galactose, D-galactosamine, lactose, maltose, D-trehalose, D-xylose, adonitol, dulcitol, erythritol, ethanol,

TABLE 3. Characteristics useful in distinguishing strains of *Marinococcus hispanicus* from other gram-positive, moderately halophilic cocci

Characteristic	*Micrococcus halobius*[a]	*Sporosarcina halophila*[a]	*Marinococcus halophilus*[a]	*Marinococcus albus*[a]	*Marinococcus hispanicus*
Motility	−[b]	+	+	+	−
Spore production	−	+	−	−	−
Pigmentation	None	Orange	Yellowish orange	Creamy white	Reddish orange
Oxidase	+	+	−	+	+
Acid production from:					
Fructose	ND	−	−	−	+
Galactose	+	−	−	−	+
Glycerol	+	−	+	−	+
D-Glucose	+	−	+	−	+
Maltose	+	−	+	−	D
D-Mannitol	+	−	+	−	+
Sucrose	+	−	+	−	D
D-Trehalose	−	−	+	−	−
Xylose	+	−	+	−	−
Voges-Proskauer test	+	−	−	−	−
Nitrate reduced to nitrite	−	−	−	+	+
Urease	−	−	−	+	D
Hydrolysis of:					
Casein	ND	+	+	−	−
Esculin	ND	ND	+	−	D
Gelatin	−	+	+	−	+
Starch	ND	+	−	−	D
DNase	ND	+	−	+	D
Hemolysis	ND	ND	−	−	+
DNA base composition (mol%)	70–71.5	40.1–40.9	46.4	44.9	45.6–49.3
Menaquinone system	MK-8, MK-7, MK-6	MK-7	MK-7	MK-7	MK-8, MK-7
Cell wall type	Ala, Glu, Gly, Lys	Orn-D-Asp	*m*-Dpm[c]	*m*-Dpm	*m*-Dpm

[a] Data from references 1, 3, 10, and 18.
[b] +, Positive; −, negative; D, differs among strains; ND, not determined.
[c] *m*-Dpm, *meso*-Diaminopimelic acid.

propanol, D-sorbitol, *N*-acetylglucosamine, DL-α-aminobutyrate, δ-aminovalerate, benzoate, butyrate, caprylate, lactate, DL-malate, oxalate, propionate, quinate, D-saccharate, succinate, and D-tartrate.

L-Glutamine is used as a sole source of carbon, nitrogen, and energy.

The following compounds are not utilized as sole carbon, nitrogen, and energy sources: L-allantoin, DL-arginine, L-asparagine, aspartic acid, betaine, creatine, ethionine, phenylalanine, glycine, glutamic acid, L-histidine, L-leucine, DL-lysine, L-ornithine, putrescine, sarcosine, L-serine, L-threonine, and L-valine.

Other phenotypic features of this species are shown in Table 1.

DNA base composition ranges from 45.6 to 49.3 mol%.

The cell wall contains peptidoglycan of the *meso*-diaminopimelic acid type.

The major quinones are MK-8 and MK-7.

The habitat is solar saltern and saline soils.

The type strain is strain J-82 (= ATCC 49259 = DSM 5352 = CCM 4148).

Description of the type strain. The description of the type strain is the same as that of the species. The base composition of its DNA is 45.7 mol% G+C, as determined by the thermal denaturation method. Other characteristics are shown in Table 1.

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