Polyphasic Taxonomy of *Nesterenkonia halobia*

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A phenotypic study has been carried out on six moderately halophilic gram-positive nonmotile cocci isolated from ponds of a saltern located in Huelva, Spain. These strains were examined for 150 morphological, physiological, biochemical, and nutritional traits and showed phenotypic characteristics similar to those of *Nesterenkonia halobia* (formerly *Micrococcus halobius*). The guanine-plus-cytosine (G+C) content of their DNA ranged between 70 and 72 mol%, values quite similar to those described for *N. halobia* (71.5 mol%). The 16S rDNA sequence analysis of one representative isolate showed that it is phylogenetically quite close to *N. halobia*, within the high-G+C-content gram-positive branch. DNA-DNA hybridization experiments showed a high degree of homology (72 to 100%) among the six isolates and the type strain *N. halobia* ATCC 21727. All data demonstrate quite clearly that the six isolates are members of the species *N. halobia*. Since this species was described on the basis of a single strain isolated from unrefined solar salt, and its description is not complete (especially in the utilization of different compounds), our study contributes to a better description of the moderate halophile *N. halobia*.

Moderately halophilic bacteria are microorganisms able to grow optimally in media containing 3 to 15% (wt/vol) salts (7). With respect to their taxonomic distribution, this group is very heterogeneous and includes a great variety of species belonging to *Archaea* (13, 21, 35, 36) and *Bacteria* (27). Within the moderately halophilic heterotrophic bacteria, the gram-positive microorganisms have been studied in less detail than the gram-negative group, which is more abundant in hypersaline environments. Until now only six moderately halophilic gram-positive cocci have been recognized as valid species (3, 25, 26, 28, 29). It has been shown that the majority of moderately halophilic gram-positive cocci isolated from several hypersaline habitats are motile, with *Marinococcus halophilus* being the predominant organism (12, 31); nonmotile species are less common (26, 28, 29, 31, 32).

Nesterenkonia halobia is a moderately halophilic gram-positive nonmotile coccus that was isolated from unrefined solar salt of unknown origin obtained from Noda, Japan, and described originally as *Micrococcus halobius* by Onishi and Kamekura (17). This bacterium produces an extracellular amylase which depends on divalent cations and a high concentration of NaCl or KCl for activity and stability (16).

Since its description in 1972 (17), very few studies have been carried out on this species. Monteoliva-Sánchez et al. (15) determined the fatty acid composition of *M. halobius*, showing that the predominant fatty acid of this organism was the branched anteiso-C_{15:0}; elevated amounts of anteiso-C_{17:0} were also present. In 1994, Koch et al. (6) analyzed the 16S ribosomal DNA (rDNA) sequence of *M. halobius* and showed that this species did not group phylogenetically with the type species of the genus *Micrococcus*, *M. luteus*. Lately, Stackebrandt et al. (26) carried out a phylogenetic and chemotaxonomic analysis of the genus *Micrococcus* and confirmed the results previously obtained by Koch et al. (6). They proposed the inclusion of *M. halobius* in the new genus *Nesterenkonia*, as

N. halobia. The description of this species is based on a single strain, which has not been characterized in detail (17, 26).

In the present work we have isolated and characterized in depth six cocci from hypersaline habitats. Their phenotypic features and DNA base composition were very similar to those described for *N. halobia* (26). In addition, DNA-DNA hybridization experiments were carried out to examine the relatedness of the isolates to each other, as well as to *N. halobia* and to other gram-positive moderately halophilic cocci described. Moreover, almost the complete 16S rDNA sequence of strain RH-59 was determined and compared with those of known species available from databases. The data presented clearly indicate that the six strains studied can be assigned to the species *N. halobia*.

MATERIALS AND METHODS

Organisms and growth conditions. The six strains studied (RH-53, RH-58, RH-59, RH-61, RH-62 and RH-63) were isolated from ponds of a saltern located in Huelva, in southwestern Spain. The isolation medium and the methodology which we used have been described previously (31). The following reference strains were used for comparative purposes: *N. halobia* ATCC 21727^T, *Halobacillus halophilus* DSM 2266^T, *M. halophilus* CCM 2706^T, *Marinococcus albus* CCM 3517^T, *Salinicoccus roseus* CCM 3516^T, and *Salinicoccus hispanicus* CCM 4148^T.

The strains were cultivated in complex medium containing 8.1% (wt/vol) NaCl, 0.7% (wt/vol) MgCl₂, 0.96% (wt/vol) MgSO₄, 0.036% (wt/vol) CaCl₂, 0.2% (wt/vol) KCl, 0.006% (wt/vol) NaHCO₃, 0.0026% (wt/vol) NaBr, 1.0% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.), 0.5% (wt/vol) Proteose Peptone no. 3 (Difco), and 0.1% (wt/vol) glucose (30). The pH was adjusted to 7.5 with 1 M KOH. When required, the medium was solidified with 2.0% (wt/vol) Bacto Agar (Difco).

Phenotypic characterization. Tests for 55 characteristics, including morphological, cultural, physiological, and biochemical features, were carried out (see Table 1). The methodology used has been described previously (2, 22, 30). Nutritional tests were performed with the Biolog (Hayward, Calif.) automatic identification system. Strains were grown on complex medium at 37°C for 24 h and suspended in a sterile solution of 10% (wt/vol) salts (30). Cell density was adjusted to an *A*₅₉₀ of 0.34 to 0.39 with a Biolog photometer, model 21101. Immediately after the cells were suspended in the salt solution, the suspensions were transferred into sterile multichannel pipette reservoirs and the Biolog GP MicroPlates were inoculated with 125 µl of the cell suspension per well by means of an eight-channel repeating pipette. The inoculated Biolog plates were incubated at 37°C for 24 h and the results were read with a MicroPlate Reader by using MicroLog 3.50 computer software to perform automated reading.

DNA extraction and purification. All strains 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

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TABLE 1. Phenotypic characteristics of the six isolates and *N. halobia* ATCC 21727^T, according to this study

Characteristic	No. of strains positive ^a	Results for <i>N. halobia</i> ATCC 21727 ^T
Oxidase	6	+
Catalase	6	+
Acid production from:		
L-Arabinose	0	—
D-Fructose	4	+
D-Galactose	0	—
Glycerol	0	—
D-Glucose	4	+
Lactose	0	—
Maltose	0	—
D-Mannitol	0	—
Sucrose	0	—
D-Trehalose	0	—
D-Xylose	6	+
Indole test	0	—
Methyl red	0	—
Voges-Proskauer	0	—
Nitrate reduction	0	—
H ₂ S production	0	—
Simmons citrate	5	+
Phenylalanine deaminase	0	—
Arginine dihydrolase	0	—
Lysine decarboxylase	0	—
Ornithine decarboxylase	0	—
Urease	0	—
Hydrolysis of:		
Gelatin	6	+
Casein	6	+
Esculin	6	+
Starch	6	+
Tyrosine	6	+
Tween 80	1	—
Phosphatase	0	—
DNase	6	+
Lecithinase	0	—
Hemolysis	0	—
Utilization of:		
α -Cyclodextrin	1	—
β -Cyclodextrin	1	—
Dextrin	6	—
Glycogen	1	—
Inulin	1	—
Mannan	1	—
Tween 40	1	—
Tween 80	0	—
<i>N</i> -Acetyl-D-glucosamine	0	—
<i>N</i> -Acetyl-D-mannosamine	1	—
Amygdalin	1	—
L-Arabinose	3	+
D-Arabitol	2	—
Arbutin	1	—
Cellobiose	2	—
D-Fructose	5	—
L-Fucose	1	—
D-Galactose	1	—
D-Galacturonic acid	1	—
Gentiobiose	1	—
D-Gluconic acid	1	—
α -D-Glucose	6	+
<i>m</i> -Inositol	1	—
α -D-Lactose	1	—

Continued

TABLE 1—Continued

Characteristic	No. of strains positive ^a	Results for <i>N. halobia</i> ATCC 21727 ^T
Lactulose	1	—
Maltose	3	—
Maltotriose	1	—
D-Mannitol	6	—
D-Mannose	4	—
D-Melezitose	1	—
D-Melibiose	1	—
α -Methyl-D-galactoside	1	—
β -Methyl-D-galactoside	1	—
3-Methyl-glucose	0	—
α -Methyl-D-glucoside	1	—
β -Methyl-D-glucoside	1	—
α -Methyl-D-mannoside	0	—
Palatinose	2	—
D-Psicose	4	—
D-Raffinose	1	—
L-Rhamnose	1	—
D-Ribose	2	—
Salicin	3	—
Sedoheptulosan	1	—
D-Sorbitol	5	—
Stachyose	1	—
Sucrose	1	—
D-Tagatose	1	+
D-Trehalose	2	—
Turanose	1	—
Xylitol	1	—
D-Xylose	5	—
Acetic acid	4	+
α -Hydroxybutyric acid	1	—
β -Hydroxybutyric acid	1	—
γ -Hydroxybutyric acid	1	—
<i>p</i> -Hydroxyphenyl acetic acid	0	+
α -Ketoglutaric acid	2	—
α -Ketovaleric acid	0	—
Lactamide	0	—
D-Lactic acid methyl ester	0	—
L-Lactic acid	1	—
D-Malic acid	1	—
L-Malic acid	1	—
Methylpyruvate	0	—
Monomethyl succinate	1	—
Propionic acid	2	—
Pyruvic acid	2	—
Succinamic acid	1	—
Succinic acid	1	—
<i>N</i> -Acetyl-L-glutamic acid	1	—
Alaninamide	0	—
D-Alanine	1	—
L-Alanine	1	—
L-Alanyl-glycine	1	—
L-Asparagine	1	—
Glutamic acid	1	—
Glycyl-L-glutamic acid	1	—
L-Pyroglutamic acid	1	—
L-Serine	1	—
Putrescine	1	—
2,3-Butanediol	0	—
Glycerol	1	—
Adenosine	2	—
2'-Desoxyadenosine	1	—
Inosine	1	—
Thymidine	5	—
Uridine	1	—
AMP	1	—
TMP	1	—
UMP	1	—

Continued on following page

TABLE 1—Continued

Characteristic	No. of strains positive ^a	Results for <i>N. halobia</i> ATCC 21727 ^T
Fructose-6-phosphate	1	—
Glucose-1-phosphate	0	—
Glucose-6-phosphate	1	—
DL- α -Glycerol phosphate	1	—
Antibiotic susceptibility		
Ampicillin (10 μ g)	6	+
Bacitracin (10 U)	6	+
Cephalothin (30 μ g)	6	+
Chloramphenicol (30 μ g)	6	+
Erythromycin (15 μ g)	6	+
Gentamicin (10 μ g)	0	—
Nalidixic acid (30 μ g)	0	—
Neomycin (30 μ g)	0	—
Novobiocin (30 μ g)	6	+
Polymyxin B (300 U)	0	—
Rifampin (5 μ g)	6	+
Streptomycin (10 μ g)	0	—

^a All strains were gram-positive nonmotile cocci, yellow pigmented, non-spore forming, and strict aerobes. They grew in media containing 3 to 25% (wt/vol) salts, with the optimum at 10% (wt/vol) salts; none grew without salts. Optimum temperature for growth was 37°C, and the strains grew at 20 to 40°C. The pH range for growth was 5.0 to 10.0, with an optimum at pH 7.0 to 7.5.

was extracted and purified by the method of Marmur (9). The purity was assessed from the A_{260}/A_{280} and A_{260}/A_{230} extinction ratios (4).

DNA base composition. The guanine-plus-cytosine content of the DNA was determined from the midpoint value of the thermal denaturation profile (10) obtained with a model UV-Vis 551S spectrophotometer (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 (18). The G+C content of reference DNA from *Escherichia coli* NCTC 9001 was taken to be 51 mol% (19).

Preparation of ³H-labeled DNA and DNA-DNA hybridization experiments. DNA was radioactively labeled by the multiprimer system with a commercial kit (RPN 1601Y; Amersham International, Amersham, England) by using [1',2',5-³H]dCTP (Amersham). The average specific activity obtained with this procedure was 8.8×10^6 cpm/ μ g 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 similarity was studied by the competition procedure described by Johnson (4). Competitor DNAs were sonicated (Braun Melsungen, Melsungen, Germany) at 50 W for two 15-s periods. Membrane filters (HAHY; Millipore Corp., Bedford, Mass.) containing reference DNA (ca. 25 μ g cm⁻²) 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 competitor DNA to the concentration of labeled DNA was at least 150:1. The final reaction concentrations were 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 30% formamide, and the final volume was 140 μ l. The hybridization experiments were carried out under optimal conditions, with temperatures ranging between 62.5 and 62.8°C, which is within the limits of validity for the filter method (1). The vials were shaken slightly for 18 h in a water bath (Grant Instruments, Cambridge, England); these procedures were carried out in triplicate. After hybridization the filters were measured with a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.), and the percentage of homology was calculated as described by Johnson (4). At least two independent determinations were carried out for each experiment, and the mean values are reported here.

Amino acid composition of the cell wall. Isolation of the cell wall fractions and preparation of the hydrolysates of the cell walls were carried out according to the method of Schleifer (24). To determine the qualitative amino acid composition of the purified cell walls, the partial acid hydrolysates were spotted onto Schleicher & Schuell 2043b paper in two-dimensional descending paper chromatography. An isopropanol-acetic acid-water (70:10:15, vol/vol) solvent system was applied in the direction of the machine and α -picoline-25% ammonium hydroxide-water (70:2:28, vol/vol) solvent was applied in the other direction. Chromatograms were sprayed with ninhydrin and heated at 100°C for 5 to 10 min.

Isolation of genomic DNA and 16S rDNA sequence analysis. Genomic DNA isolation of strain RH-59, chosen as the representative strain of our isolates, PCR amplification of the 16S rRNA gene, and direct determination of the PCR-amplified 16S rDNA sequences were carried out by methods that have been described previously in detail (14).

For data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and dendrogram constructions for 16S rRNA data, we used a program set written in Microsoft Quick BASIC for use on IBM PC-AT and compatible computers (20). A similarity matrix was constructed from the aligned sequences by using only those positions for which 90% of the strains had data. The similarity matrix was corrected for multiple base changes at single positions by the method of Jukes and Cantor (5). The phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei (23).

Nucleotide sequence accession numbers. The newly determined sequence of the 16S rRNA gene of strain RH-59 has been deposited in the EMBL data library under accession no. Y13857. The accession numbers of the sequences of the reference strains used in the phylogenetic analyses are as follows: *Arthrobacter aureus* DSM 20116, X83405; *Arthrobacter citreus* DSM 20133, X80737; *Arthrobacter crystallopoietes* DSM 20117, X80738; *Arthrobacter globiformis* DSM 20124, X80736; *Arthrobacter ilicis* DSM 20138, X83407; *Arthrobacter nicotianae* DSM 20123, X80739; *Arthrobacter oxydans* DSM 20119, X83408; *Arthrobacter pascens* DSM 20545, X80740; *Arthrobacter protophormiae* DSM 20168, X80745; *Arthrobacter ramosus* DSM 20546, X80742; *Arthrobacter uratoxydans* DSM 20647, X83410; *Arthrobacter ureafaciens* DSM 20126, X80744; *Kocuria kristinae* DSM 20032, X80749; *Kocuria rosea* DSM 20447, X87756; *Kocuria varians* DSM 20033, X87754; *Micrococcus agilis* DSM 20550, X80748; *M. luteus* ATCC 381, M38242; *Micrococcus lylae* DSM 20315, X80750; *N. halobia* DSM 20541, X80747; *Rothia dentocariosa* ATCC 17931, M59055; and *Stomatococcus mucilaginosus* DSM 20746, X87758.

RESULTS AND DISCUSSION

The six strains isolated showed morphological, physiological, biochemical, and nutritional characteristics similar to those described for *N. halobia* (17). The description of this species is based on the results obtained for a single strain on which a limited number of phenotypic tests were performed. The phenotypic features of our isolates, as well as those of the type strain *N. halobia* ATCC 21727, obtained in this study are summarized in Table 1. Of the total of 150 tests performed, only 38 characteristics were described by Onishi and Kamekura (17) for *N. halobia*. In 29 tests their results were similar to ours; in the other 9 common tests, however, we found differences between the results reported by Onishi and Kamekura (17) for *N. halobia* ATCC 21727^T and those obtained with our isolates. The results obtained in this study for the type strain of *N. halobia* were similar to those obtained for the majority of the fresh isolates.

With respect to the data reported by Onishi and Kamekura (17) for *N. halobia* ATCC 21727^T and those obtained in this study for this reference strain, differences were observed in acid production from galactose, glycerol, lactose, maltose, mannitol, and sucrose; Voges-Proskauer and Simmons citrate tests; and hydrolysis of gelatin.

TABLE 2. G+C content of DNA and DNA-DNA relatedness among *N. halobia* and related species

Strain	G+C content (mol%) of unlabeled DNA	% Homology of unlabeled DNA with ³ H-labeled DNA from:		
		<i>N. halobia</i> ATCC 21727 ^T	Strain RH-53	Strain RH-59
<i>N. halobia</i> ATCC 21727 ^T	71.5	100	73	72
RH-53	71.1	79	100	95
RH-58	71.8	85	90	98
RH-59	72.0	81	100	100
RH-61	71.3	80	100	100
RH-62	70.4	84	98	92
RH-63	71.9	94	92	83
<i>H. halophilus</i> DSM 2266 ^T	40.8 ^a	32	0	25
<i>M. halophilus</i> CCM 2706 ^T	46.4 ^a	22	17	18
<i>M. albus</i> CCM 3517 ^T	44.9 ^a	0	0	2
<i>S. roseus</i> CCM 3516 ^T	51.2 ^a	36	19	17
<i>S. hispanicus</i> CCM 4148 ^T	45.7 ^a	33	29	4

^a Data from references 3, 25, 28, and 29.

TABLE 3. Characteristics useful in distinguishing *N. halobia* from other moderately halophilic gram-positive cocci^a

Characteristic	<i>N. halobia</i>	<i>H. halophilus</i>	<i>M. halophilus</i>	<i>M. albus</i>	<i>S. roseus</i>	<i>S. hispanicus</i>
Motility	—	+	+	+	—	—
Spore production	—	+	—	—	—	—
Pigmentation	None	Orange	Yellowish orange	Creamy white	Pink red	Reddish orange
Oxidase	+	+	—	+	+	+
Acid production from:						
D-Fructose	—	—	—	—	—	+
D-Galactose	—	—	—	—	—	+
Glycerol	—	—	+	—	—	+
D-Glucose	+	—	+	—	—	+
D-Mannitol	—	—	+	—	—	+
D-Trehalose	—	—	+	—	—	—
D-Xylose	+	—	+	—	—	—
Nitrate reduction	—	—	—	+	+	+
Urease	—	—	—	+	—	+
Hydrolysis:						
Casein	+	+	+	—	+	—
Esculin	+	—	+	—	—	+
Gelatin	+	+	+	—	+	+
Starch	+	+	—	—	+	—
DNase	+	+	—	+	+	+
Hemolysis	+	ND	+	—	—	+
G+C content (mol%)	70.4–72.0	40.1–40.9	46.4	44.9	51.2	45.6–49.3
Menaquinone system	MK-9, MK-8, MK-7	MK-7	MK-7	MK-7	MK-6	MK-6
Cell wall type	L-Lys–Gly–L-Glu	Orn–D-Asp	<i>m</i> -Dpm	<i>m</i> -Dpm	L-Lys–Gly ₅	L-Lys–Gly ₅

^a Data for species other than *N. halobia* are from references 3, 25, 28, and 29. ND, not determined; MK-9, -8, -7, and -6, menaquinone with 9, 8, 7 and 6 isoprene units, respectively (26); *m*-Dpm, *meso*-diaminopimelic acid.

The DNA base composition of our isolates is shown in Table 2. The range obtained for the six isolates (70.4 to 72.0 mol%) includes the value reported for *N. halobia* (71.5 mol%) (17) and is very different from those described for *H. halophilus* (40 to 40.9 mol%) (25), *M. halophilus* (44.9 to 46.6 mol%), *M. albus* (43 to 44.9 mol%) (3), *S. roseus* (51.2 mol%) (28), and *S. hispanicus* (45.6 to 49.3 mol%) (11). The results of the DNA-DNA hybridization experiments are also presented in Table 2. The DNA relatedness experiments show a high degree of homology between the six isolates and the type strain of *N. halobia*, ranging from 72 to 100%. Since it is generally accepted that among strains of the same species the levels of DNA homology should be equal to or greater than 70% (19, 33), on the basis of DNA relatedness results, our isolates and the type strain *N. halobia* ATCC 21727 constitute a genospecies. On the other hand, low levels of DNA homology (0 to 29%) were obtained between the two ³H-labeled representative strains and all other gram-positive moderately halophilic cocci studied.

The cell wall analysis of the six isolates showed that they contained murein of L-Lys–Gly–L-Glu type, variation A4α. This murein type is the same that has been found in *N. halobia* (26). These results were confirmed by Weiss (34). Table 3 shows the phenotypic characteristics useful for the differentiation of *N. halobia* from other gram-positive moderately halophilic cocci previously described.

We determined almost the complete 16S rRNA gene sequence of isolate RH-59 (positions 16 to 321 and 362 to 1362; *E. coli* 16S rRNA gene sequence numbering). That sequence was compared with more than 4,000 16S rRNA/rDNA sequences deposited in the databases (8). The sequence of strain RH-59 presents the highest level of similarity with the 16S rRNA sequence of *N. halobia* (99.1%). However, 16S rRNA/rDNA sequence similarities equal to or lower than 95.6% were obtained between strain RH-59 and the other species included

FIG. 1. Unrooted phylogenetic tree derived from analysis of the 16S rRNA/rDNA sequences of strain RH-59 and related species belonging to the branch of gram-positive bacteria with a high G+C content.

in the gram-positive phylum. The 16S rRNA/rDNA sequence similarity between strain RH-59 and other previously described moderately halophilic gram-positive cocci was also very low: 81.9% for *S. roseus*, 81.2% for *M. albus*, and 81.1% for *M. halophilus* and *H. halophilus*.

Figure 1 shows the general structure of a phylogenetic tree obtained from the similarity matrix. Strain RH-59 and *N. halobia* constituted a cluster clearly separated from other species of the genera *Micrococcus*, *Arthrobacter*, and *Kocuria*, in agreement with the study of Stackebrandt et al. (26). Our phylogenetic analysis agrees with the DNA homology study and, overall, our results clearly show that our isolates are phylogenetically and genotypically closely related to the species *N. halobia*.

Taken together, the data present clear evidence that the six strains isolated from hypersaline habitats should be classified within the species *N. halobia*. Thus, this work contributes to a more complete description of this species, especially with respect to their nutritional capabilities. Besides, since its original description was based on a single strain isolated from a salt sample, and thus its natural habitat is unknown, our study shows that although this species does not constitute a high proportion of the microbial population of salterns, it is found in this hypersaline environment.

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