Micrococcus halobius sp. n.

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A moderately halophilic coccus is described which possesses morphological and physiological characteristics most nearly corresponding to those of members of the genus *Micrococcus*. The organism was isolated from unrefined solar salt and is a moderate halophile; no growth occurs in media without added NaCl, but good growth is observed in media containing 1 to 4 M NaCl or KCl. The characteristics of this organism are sufficiently discrete to suggest that it be placed in a new species, for which the name *Micrococcus halobius* is proposed. The type strain is 28-3 (= ATCC 21727).

A moderately halophilic coccus was isolated from unrefined solar salt and was found to produce a considerable amount of halophilic amylase of unique salt response (17, 18). The present paper describes the morphological and physiological properties of this bacterium, which is sufficiently different from previously described cocci to be recognized as a new species.

MATERIALS AND METHODS

Bacterial strain. A moderately halophilic coccus was isolated from unrefined solar salt of unknown origin obtained from Noda, Japan. The organism was given the strain designation 28-3 and was deposited at the American Type Culture Collection, Rockville, Md., as ATCC 21727.

Measurement of growth. The bacterium was grown in the complex medium (SGC) of Sehgal and Gibbons (20) and in nutrient broth, to each of which was added NaCl, KCl, or glucose. Inocula (0.1 ml) from a 2-day-old culture grown in 2 M NaCl SGC medium were added to 500-ml shake flasks containing 80 ml of medium. The flasks were shaken at 30 C on a reciprocal shaker operating at 140 rev/min with a stroke of 7.5 cm. Turbidity was measured in 10-mm cuvettes at 660 nm against an uninoculated blank.

Media and methods for characterization. Most of the methods used to characterize the organism have been described previously (21), except that 1 M NaCl medium was used unless stated otherwise.

Nutrient broth (1 M NaCl) or agar was used for morphological and cultural observations. Acid production was determined by using MOF medium (12) containing 1 M NaCl. Nitrate reduction was determined by using a medium of the following composition: 0.1% KNO₃, 1.0% polypeptone, 2.0% MgSO₄·7H₂O, 0.2% KCl, 0.3% sodium citrate, 5.8% NaCl, and 2.3 mg% FeCl₂, pH 7.0. Media containing 2, 3, or 4 M NaCl were also em-

ployed. The utilization of NH₄H₂PO₄ was tested by using a medium of the following composition: 0.1% NH₄H₂PO₄, 1.0% glucose, 0.2% KCl, 2.0% MgSO₄·7H₂O, 5.8% NaCl, 0.001% bromocresol purple, 2.3 mg% FeCl₂, 0.4 µg of thiamine per ml, 0.001 µg of biotin per ml, and 1.5% agar, pH 7.0. Vitamin requirements were determined by adding the following nine vitamins to yeast extract-free SGC medium (µg/ml): (thiamine, 0.4; riboflavin, 0.2; vitamin B₆, 0.4; calcium pantothenate, 0.4; *p*-amino benzoic acid, 0.2; nicotinic acid, 0.4; biotin, 0.001; vitamin B₁₂, 0.0002; and folic acid, 0.1). The requirement for a vitamin was determined by preparing nine different media, each lacking one of the vitamins, and checking each medium for growth after inoculation and suitable incubation of the medium.

DNA base composition. The bacterium was aerobically cultivated in 2 M NaCl SGC medium at 30 C for 60 hr. The procedure of Marmur (15) was used for the isolation of deoxyribonucleic acid (DNA). Two methods were employed to analyze the base composition of the isolated DNA: (i) determination of the guanine plus cytosine (GC) content from the melting temperature (*T_m*) by using an automatic recording spectrophotometer (Gilford Instrument Laboratories; see reference 16), and (ii) determination of the GC content according to the ratio *E*₂₆₀:*E*₂₈₀ at pH 3 (6).

Amino acid composition of the cell wall. The bacterium was aerobically cultivated in 2 M NaCl-SGC medium at 30 C for 3 days. Isolation of the cell wall fraction and preparation of the hydrolysate of the cell wall were carried out by the method of Yamada and Komagata (23), except that saline solution containing 1 M NaCl and 2% MgSO₄·7H₂O was used throughout the procedure. Amino acids were detected both by paper chromatography and by an amino acid analyzer (Hitachi 034 liquid chromatograph).

Cellular fatty acid composition. The bacterium was aerobically cultivated in 1 M NaCl-SGC medium at 30 C for 3 days. Methods for the extraction, methylation, and identification of fatty acids were substantially the same as those described by Uchida and Mogi (22).

RESULTS

Morphology. Gram-stained smears showed the bacterium to be a gram-positive coccus, 0.8 to 1.5 μm in diameter (Fig. 1). In unstained preparations, the cells occur singly, in pairs, and occasionally in tetrads and small, irregular clumps. The organism is nonmotile, nonencapsulated, and does not possess endospores.

Cultural characteristics. Colonies on agar are unpigmented, smooth, circular, and opaque. Growth on slants is heavy, smooth, and colorless. In broth, the growth is uniform and colorless; the broth is turbid, and no pellicle is produced. Obligately aerobic. Mesophilic with good growth at

20 and 40 C. Optimum pH 7.0. Good growth occurs between pH 6 and 10.

Growth in media of different NaCl concentrations. The best growth was obtained in media containing 1 or 2 M NaCl (Fig. 2); these gave a maximum yield after 2 to 3 days of cultivation. Growth was fairly good in 3 M NaCl. Moderate growth was observed in 4 M NaCl after a lag of 2 to 3 days. However, growth did not occur in media without NaCl even after 7 days. Similar growth patterns were shown in media containing KCl instead of NaCl, but no growth was observed in media containing 1, 2, or 3 M glucose but no added NaCl. Thus, the bacterium is a moderate halophile.

Physiological characteristics. Using the MOF medium of Leifson (12), acid was produced from glucose, galactose (slow), xylose, maltose, sucrose, lactose, starch, raffinose, glycerol, and mannitol aerobically but not anaerobically. In other words, carbohydrate metabolism is oxidative and not fermentative. Only 0.08% total acid (calculated as acetic acid) was produced from glucose by shake culture at 30 C for 7 days. Trehalose and inulin were not attacked. No gas was produced from the sugars tested. Nitrate was not reduced. Gelatin was not liquefied, and the catalase reaction was strongly positive. Coagulation but no acid was produced in milk. Indole and H_2S not produced. Urease production, negative. Acetoin production, positive. Chitin is not decomposed. $\text{NH}_4\text{H}_2\text{PO}_4$ is very weakly utilized. Thiamine is essential and biotin is stimulatory for growth.

FIG. 1. Gram stain of *Micrococcus halobius* 28-3 showing various cell arrangements. $\times 1,070$.

A Age of culture (days)

B Age of culture (days)

FIG. 2. Growth curves of *Micrococcus halobius* 28-3 in media containing various concentrations of NaCl. Symbols: Δ , 0 M NaCl; $\square$, 1 M NaCl; $\blacksquare$, 2 M NaCl; $\circ$, 3 M NaCl; $\bullet$, 4 M NaCl. A, Sehgal and Gibbons medium without added NaCl contains 182 mg of Na^+ per 100 ml originating from vitamin-free Casamino Acids (Difco) and sodium citrate. B, Nutrient broth.

TABLE 1. *Composition of cellular fatty acids of Micrococcus halobius* 28-3 as determined by gas chromatography^a

Fatty acid	Percent composition
iso-C ₁₄	0.2
iso-C ₁₅	2.7
anteiso-C ₁₅	31.7
iso-C ₁₆	16.7
n-C ₁₆	0.4
iso-C ₁₇	3.0
anteiso-C ₁₇	45.3

^a Gas chromatograph, Hitachi K-53; detector, FID; column, 20% diethylene glycol succinate polyester/Chromosorb W DMCS 80 to 100 mesh, 3 mm by 2 m (dual); carrier gas, N₂; flow rate, 30 ml/min; over temperature, 160 C.

DNA base composition. The GC content of the DNA of the bacterium was 71.5 and 70.0 moles percent by the methods of Marmur and Doty (16) and Frédéricq et al. (6), respectively.

Amino acid composition of cell wall. Alanine, glutamic acid, glycine, and lysine were the major amino acid constituents. Diaminopimelic acid could not be detected.

Cellular fatty acid composition. As shown in Table 1, the fatty acid composition of the bacterium was characterized by a high content of anteiso acids (77% of the total fatty acids). The pattern resembles that of *Micrococcus* (7, 8, 13, 14) but differs markedly from that of salt-tolerant *Pediococcus halophilus* (22).

DISCUSSION

On the basis of its general morphology, oxidative metabolism, relatively low acid production, and production of catalase, the bacterium described here belongs to the genus *Micrococcus* Cohn, according to *Bergey's Manual* (3).

Recently the DNA base compositions of micrococci (1), marine and halophilic micrococci (2), gram-positive cocci (9), and extremely halophilic cocci (10) were determined. It was confirmed by Boháček et al. (1) that the GC content of the DNA of organisms in the genus *Micrococcus* is in the range of 65 to 75%. The GC content of the DNA of strain 28-3 falls within this range.

It has also been demonstrated that the major fatty acid components of micrococci were odd-numbered and branched-chain (7, 8, 13, 14). Seventy-seven percent of the total fatty acids of strain 28-3 was anteiso C₁₅ and C₁₇ acid.

Thus the DNA base and fatty acid compositions of strain 28-3 agree with those of members of the genus *Micrococcus*.

Concerning halophilic or halotolerant *Micrococcus* species, only *M. morrhuae* (3), *M. halodenitrificans* (19), and *M. halodurans* (4) have so far been described. *M. halodurans* has a 32.0% GC content in the DNA, similar to that of members of the genus *Staphylococcus* (2), and it was considered by Boháček et al. (2) to be an aberrant strain of *Staphylococcus epidermidis*. *M. halodenitrificans* is gram negative, and its fatty acid composition differs significantly from that of gram-positive micrococci (7). *M. halodenitrificans* has tentatively been placed in the genus *Paracoccus* (5). *M. morrhuae* is a red bacterium which reduces nitrate to nitrite.

Strain 28-3 differs in a number of respects from all previously named species of *Micrococcus*, and it is therefore here regarded as being a member of a new species for which the name *Micrococcus halobius* is proposed (Gr. noun *hals* salt; Gr. noun *bios* life; M.L. adj. *halobius* living on salt). The type strain of *M. halobius* is 28-3 (= ATCC 21727). Because this was the only strain of this new species that was isolated, the description of the type strain is the same as that given above for the species.

It is realized that the description of *M. halobius* will ultimately be modified as additional strains are found, as is the case of all newly named and described species, irrespective of the number of strains on which the original description was based.

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