**Halobacterium denitrificans** sp. nov., an Extremely Halophilic Denitrifying Bacterium

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**Halobacterium denitrificans** was one of several carbohydrate-utilizing, denitrifying, extremely halophilic bacteria isolated by anaerobic enrichment in the presence of nitrate. Anaerobic growth took place only when nitrate (or nitrite) was present and was accompanied by the production of dinitrogen. In the presence of high concentrations of nitrate (i.e., 0.5%), nitrous oxide and nitrite were also detected. When grown aerobically in a mineral-salts medium containing 0.005% yeast extract, *H. denitrificans* utilized a variety of carbohydrates as sources of carbon and energy. In every case, carbohydrate utilization was accompanied by acid production. A type culture has been deposited with the American Type Culture Collection, Rockville, Md. (ATCC 35960).

At present, the taxonomy of the halobacteria is controversial. This reflects, in part, the recent characterization of new metabolic types (9, 14, 19, 21, 28, 30) and the apparent biochemical monotony of the group (23). At one time five species were recognized. Two were distinguished from the rest on the basis of gas production from nitrate, and one of these, *Halobacterium marismortui*, produced acid from several carbohydrates (5). *H. marismortui* is no longer recognized as a valid species because of the absence of an extant-type culture (16). A numerical taxonomic analysis (4) recommended that only two halobacterial species be recognized, *Halobacterium salinarium* and *Halobacterium cutirubrum*. Neither one produced gas from nitrate or acid from carbohydrates. Of the currently recognized species, only *Halobacterium mediterranei* (20) and *Halobacterium vallismortis* (9) produce a gas when grown in the presence of nitrate. Since some halobacteria acidify media containing carbohydrates (9, 20, 31) and a number of extreme halophiles are capable of fermentative growth (15), the presence of gas in a medium containing nitrate is not sufficient evidence to conclude that nitrogen was produced or that growth took place as a consequence of denitrification.

Recently, we isolated several bacteria, the characteristics of which suggested that they were members of the genus *Halobacterium*. These organisms grew anaerobically, but only when nitrate was present. Such nitrate-dependent anaerobic growth was accompanied by the production of nitrite, nitrous oxide, and dinitrogen. We believe that these organisms are denitrifying, extreme halophiles and that strain S1 is sufficiently distinctive to warrant its characterization and designation as *Halobacterium denitrificans*.

**MATERIALS AND METHODS**

Enrichment cultures, using either mud or brine from salterns located in the southern portion of San Francisco Bay, were grown in 60-ml serum bottles containing a freshly autoclaved medium. The medium (designated YH medium) contained the following additions (in grams per liter): yeast extract, 5.0; Hy-Case SF, 2.0; NaCl, 176.0; MgCl2·6H2O, 20.0; KNO3, 5.0; KCl, 2.0; CaCl2·2H2O, 0.1. The pH was adjusted to 7.4 at room temperature (22°C) with a low-sodium-error electrode. Bottles that were partially filled with freshly autoclaved medium were inoculated with either mud or brine. The bottles were filled with additional freshly autoclaved medium, sealed with serum caps, and incubated in the dark at 37°C. After vigorous gas formation was observed, fractions were transferred to bottles containing freshly autoclaved YH medium, and the bottles were topped off as described above, sealed with serum caps, and incubated in the dark at 37°C until growth and gas production were observed. After three such transfers, material from the liquid cultures was streaked on YH agar plates (YH medium plus 2% Bacto-Agar [Difco Laboratories, Detroit, Mich.]). The plates were incubated in the dark at 37°C in Gas-Pak jars (BBL Microbiology Systems, Cockeysville, Md.). After about 10 days, several isolated colonies were streaked onto YH agar plates and again incubated anaerobically. Finally, representative colonies were transferred to YH agar slants and incubated aerobically at 37°C. Each of these isolates was subsequently tested for nitrate-dependent anaerobic growth, and those which grew anaerobically only when nitrate was present were saved for further study. We subsequently isolated a number of denitrifying, extremely halophilic bacteria from the Guerro Negro salterns in Baja California using the same procedure.

Unless stated otherwise, the extreme halophiles were grown in the dark at 37°C by the procedure described by Hochstein and Tomlinson (12). Two media were used: the HYH medium, which was YH medium buffered at pH 6.7 with 50 mM N-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES) buffer, and a mineral salts-yeast extract medium (MSY), which contained the following (in grams per liter): NaCl, 176.0; MgSO4·7H2O, 20.0; KCl, 2.0; K2HPO4, 0.1; (NH4)2SO4, 1.0; CaCl2·2H2O, 0.1; FeSO4·7H2O, 0.005; 2-(N-morpholino)ethanesulfonic acid (MES) buffer, 19.5; yeast extract, 0.05. The pH was adjusted to 6.7 at room temperature. Carbon sources were sterilized by filtration through a Nalgene filter (pore size, 0.2 μm) and added to autoclaved medium. Growth was measured with a Klett-Summerson colorimeter with a red (no. 66) filter and corrected when necessary, as described previously (24). Nitrite was determined by the method of Showe and DeMoss (26). Carbon dioxide, nitrous oxide, and dinitrogen were analyzed by gas chromatography with a Poropak Q column (11). Ether-linked lipids were detected chromatographically (22) with cells grown aerobically in HYH medium and harvested in the early stationary phase.

Cell dimensions were determined with a splitting eye piece (Vickers Instruments, Malden, Mass.). The biochemical
tests used for characterization were carried as described by Smibert and Krieg (27) with HY medium. Method 2 was used for all tests, except for gelatin liquefaction and hydrogen sulfide production, for which method 1 was employed. The antibiotic sensitivity of aerobically growing cells was tested on HYH agar plates with disks containing the following amounts of antibiotics: bacitracin (10 μg), streptomycin (10 μg), novobiocin (30 μg), penicillin G (10 U), polymixin B (50 U). Aphidicolin sensitivity was determined in liquid medium to which the antibiotic, dissolved in dimethyl sulfoxide, was added just before inoculation. Dimethyl sulfoxide, at the concentration added with aphidicolin (0.04% [vol/vol]), had no effect on growth. The effect of chloramphenicol and erythromycin was also determined in liquid medium. These antibiotics were added as methanolic solutions. Methanol did not affect growth at the concentrations employed.

Aphidicolin was obtained from Sigma Chemical Co., St. Louis, Mo. Yeast extract was purchased from Difco Laboratories. Hy-Case SF, an acid-hydrolyzed casein, was obtained from the Humko-Sheffield Chemical Co., Norwich, N.Y. The fatty acid, methyl esters standard was purchased from Applied Science, Los Altos, Calif. 

Halobacterium saccharovorum  

Paracoccus halodenitrificans  

FIG. 1. Thin-layer chromatogram of acid methanolysates from lyophilized cells. Lane 1, Strain S1; lane 2, fatty acid, methyl ester standard; lane 3, E. coli; lane 4, P. halodenitrificans; lane 5, H. saccharovorum.

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halodenitrificans (lane 4). The mobility of the major constituent in the acid methanolysates from strain S1 and Halobacterium saccharovorum (lane 5) was similar. The following diphytanoyl ethers were found in the polar lipid fraction from strain S1: phoshatidyl glycerol, phosphatidyl-glycerophosphate, sulfated diglycosyl glycerol diether, and traces of diglycosyl glycerol diether. The principal quinones were MK-8 (76.9%) and MK-8(H2) (20.6%), which agrees with the observation of Collins et al. (3) that these are also the principal quinones found in other extremely halophilic bacteria. Traces of MK-7 and MK-7(H2) were also present. The major squalene derivative was tetrahydro-squalene; only traces of squalene were detected (B. J. Tindall, personal communication).

At 37°C, strain S1 grew aerobically in HY medium containing from 1.5 to 4.5 M NaCl, which was the highest concentration tested. When cells were placed in media containing less than 1.5 M NaCl, they assumed a spherical shape before they lysed. Lysis was essentially immediate when cells were placed in distilled water. Strain S1 was a relatively rapid-growing extreme halophile. A 3-h generation time was observed in HY medium containing from 2 to 3 M NaCl. At higher salt concentrations, the generation time increased to a maximum of 6 h in the presence of 4.5 M NaCl. A generation time of 2 h was observed when cells were grown at 50°C in HY medium containing 3 M NaCl. Growth was observed from 30°C (13-h generation time) to 55°C (4-h generation time). There was no growth at either 20 or 60°C.

To demonstrate carbohydrate utilization, advantage was taken of the ability of strain S1 to grow in a mineral-salts medium containing 0.005% yeast extract (MYS medium). The unsupplemented MYS medium supported a small amount of growth, which in the presence of suitable carbon sources, such as D-glucose or pyruvate, was considerably enhanced (Fig. 2). D-Fructose, maltose, sucrose, D-galactose, lactate, D-glucosat, glycerol, acetate, L-malate, citrate, succinate, and α-ketoglutarate were also used as the sole carbon and energy sources, whereas D-ribose, D-mannose, and lactose were not (data not shown). When MES buffer was omitted from carbohydrate-containing MSY medium, there was less growth, and in those cases in which the sugars were utilized the final pH was 4.9.

The growth of strain S1 was not affected by penicillin, streptomycin, or polymyxin B. Bacitracin and novobiocin inhibited growth; the zones of inhibition were 13 and 15 mm, respectively. In the case of novobiocin, growth beyond the zone of inhibition was not pigmented, suggesting that this antibiotic might be useful for probing the early steps of carotenoid biosynthesis. Chloramphenicol inhibited growth at concentrations greater than 100 μg/ml, while erythromycin was inhibitory at concentrations greater than 400 μg/ml. Aphidicolin, an inhibitor of eucaryotic DNA replication (29) and the growth of various halobacteria, but not eubacteria (6, 25, 29), inhibited the growth of strain S1 at a concentration of 20 μg/ml.

Strain S1 grew anaerobically in HYH medium in the presence of nitrate or nitrite; no growth was observed when either was omitted or when they were replaced with nitrous oxide. The generation time of cells grown anaerobically at 37°C in the presence of 3 M NaCl was 7.5 h in the presence of nitrate and 10 h with nitrite. Anaerobic growth was accompanied by vigorous gassing in the presence of nitrate. When the nitrate concentration was 0.1%, dinitrogen was
produced concomitantly with exponential growth and was the only product of nitrate dissimilation detected (Fig. 3a). This contrasts with an earlier observation (13) that anaerobic growth in the presence of 0.5% nitrate resulted in the production of dinitrogen, nitrite, and nitrous oxide. Acetylene, which inhibits nitrous oxide reduction by denitrifying bacteria at concentrations ranging from 0.01 to 0.1 atm (1, 33), inhibited the reduction of nitrous oxide by strain S1. When strain S1 was grown anaerobically in the presence of 0.1% nitrate and 0.04 atm of acetylene, the reduction of nitrous oxide was completely inhibited, small quantities of nitrite were also detected (data not shown), the generation time increased from 7 to 23 h, and the growth yield decreased by about 30% (Fig. 3b). The last two observations were unexpected since nitrous oxide did not support the growth of strain S1 and suggest that acetylene acts at a site(s) other than that (those) for nitrous oxide reduction.

**DISCUSSION**

The relatively high NaCl concentration required for growth, lysis at NaCl concentrations less than 1.5 M, the presence of glycerol diethers, and the sensitivity to aphidicolin indicated that strain S1 was a member of the genus *Halobacterium*. The absolute dependence of anaerobic growth on the presence of nitrate (or nitrite) and the concomitant production of dinitrogen with growth confirmed that strain S1 grew by coupling growth to denitrification.

The ability to grow anaerobically is not a singular characteristic among the halobacteria. Certain strains (designated *Halobacterium halobium*, *H. cutirubrum*, and *H. salinarium*) grow anaerobically by fermenting arginine to ornithine, and *H. halobium* can also grow anaerobically to a limited extent by a light-dependent process with preformed bacteriorhodopsin (10). Results of recent studies suggest that the ability of halobacteria to grow in the absence of added nitrate (i.e., fermentatively) may be a widely distributed property (14).

Two closely related extreme halophiles (23) bear at least a superficial resemblance to strain S1. All produce acid from carbohydrates and gas from nitrate. One of these, the putative *H. marismortui*, differs from strain S1 in that *H. marismortui* produces acid from mannose and presumably grows best at 30°C (5). The extreme halophile isolated by Ginzburg et al. (8), and said to correspond to *H. marismortui* (7), differs from strain S1 in that nitrous oxide is the major product of denitrification, with only traces of dinitrogen and nitric oxide being detected (32). The other extreme halophile *H. vallismortis* produces gas when nitrate is present (9). Even though not all strains of *H. vallismortis* will grow anaerobically in the absence of nitrate (23), the likelihood that strain S1 and *H. vallismortis* are identical appears to be slight. They differ in their temperature optima for growth, the minimum NaCl concentration at which growth takes place, and several biochemical properties (Table 1).

The presence of tetrahydrosqualene as the principal squalene derivative in strain S1 suggests that it may belong to a branch of the halobacteria that also includes *H. mediterranei* and *Halobacterium volcanii* (B. J. Tindall, personal
TABLE 1. Distinguishing Characteristics of some gasogenic, acid-producing, extremely halophilic bacteria

<table>
<thead>
<tr>
<th>Property</th>
<th>Strain S1</th>
<th>H. val-</th>
<th>H. medi-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td>lissomor-</td>
<td>terraner-</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>TWEEN 40</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>TWEEN 80</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H_2S from S_2O_3^-</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Indole production</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Maximum growth rate^c</td>
<td>15</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>NaCl (5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>50</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>Temp growth range (°C)</td>
<td>30–55</td>
<td>20–45</td>
<td>25–45</td>
</tr>
</tbody>
</table>

^a Gonzalez et al. (9).
^b Rodriguez-Valera et al. (20).
^c Shortest generation time.

Oxidase and catalase positive. Gelatin liquefied, hydrogen sulfide produced from thiosulfate, and Tween 40 (palmitic acid ester) hydrolyzed. Does not hydrolyze starch, urea, or Tween 80 (oleic acid ester). Indole negative.

Habitat. Solar evaporation ponds (salterns) in the San Francisco Bay area of California and probably in other salterns.

Type strain. Strain S1 has been deposited as Halobacterium denitrificans in American Type Culture Collection, Rockville, Md. (ATCC 35960).

ACKNOWLEDGMENTS

We are indebted to B. J. Tindall, Institut für Mikrobiologie, Rheinische Fredrich-Wilhelms Universität, Bonn, Federal Republic of Germany, for the lipid analysis and helpful comments.

These studies were carried out with the technical assistance of Steven Andersen and Mary Broderick.

LITERATURE CITED