Growth Potential of Halophilic Bacteria Isolated from Solar Salt Environments: Carbon Sources and Salt Requirements

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Eighteen strains of extremely halophilic bacteria and three strains of moderately halophilic bacteria were isolated from four different solar salt environments. Growth tests on carbohydrates, low-molecular-weight carboxylic acids, and complex medium demonstrated that the moderate halophiles and strains of the extreme halophiles Haloarcula and Halococcus grew on most of the substrates tested. Among the Halobacterium isolates were several metabolic groups: strains that grew on a broad range of substrates and strains that were essentially confined to either amino acid (peptide) or carbohydrate oxidation. One strain (WS-4) only grew well on pyruvate and acetate. Most strains of extreme halophiles grew by anaerobic fermentation and possibly by nitrate reduction. Tests of growth potential in natural saltern brines demonstrated that none of the halobacteria grew well in brines which harbor the densest populations of these bacteria in solar salterns. All grew best in brines which were unsaturated with NaCl. The high concentrations of Na⁺ and Mg²⁺ found in saltern crystallizer brines limited bacterial growth, but the concentrations of K⁺ found in these brines had little effect. MgSO₄ was relatively more inhibitory to the extreme halophiles than was MgCl₂, but the reverse was true for the moderate halophiles.

Although extremely halophilic bacteria have been intensively studied, few investigations have emphasized their physiology from an ecological point of view. Questions remain concerning natural carbon sources, the relative importance of aerobic and anaerobic processes of energy conversion, and the role of the ionic environment in limiting the distribution and survival of extreme and moderate halophiles in natural brines and salt deposits. Knowledge of the ecology of halophilic bacteria is necessary for predicting microbial activity in the proposed storage of radioactive wastes in buried salts. An understanding of these microorganisms in their natural environments would also provide insight into the genesis and diagenesis of petroleum associated with evaporite deposits and would provide an empirical basis for understanding bacteriostasis by salts.

Hypersaline environments are generally thought to be characterized by low species diversity, probably because eucaryotic organisms are largely or entirely absent. The number and diversity of halophilic bacterial strains and species (i) that exist in nature and (ii) that coexist in local hypersaline environments are unknown. It can be hypothesized that a relatively large diversity of strains, species, or both exists within the bacterial floras, a condition that requires experimental scrutiny rather than the examination of gross morphology used for identifying unicellular and multicellular eucaryotes.

Among the extremely halophilic bacteria, three genera have been described: Halobacterium, Haloarcula (5), and Halococcus (not on the Approved List of Bacterial Names [10]). In the genus Halobacterium, eight or more species have been described. The most intensively studied species, Halobacterium halobium, Halobacterium salinarium, and Halobacterium cutirubrum, are thought to be strains of the same species (23). Because these strains thrive primarily on amino acids, the ability of Halobacterium marismortui (5, 14), Halobacterium volcanii (14), Halobacterium saccharovorum (22), Halobacterium valismortis (6), and Halobacterium sodomense (14) to grow on carbohydrates was originally viewed as highly unusual. Halococcus morrhuae, the only species recognized in this genus, is reported to be unable to thrive on carbohydrates (11). The carbon sources that support the growth of Haloarcula spp. have not been described. A survey of organic substrates metabolized by a representative sample of extreme halophiles isolated on complex medium from different solar salt environments would indicate how versatile these microorganisms are as heterotrophs. Such a survey would indicate whether this classical approach to bacterial taxonomy can be used to define species of halobacteria.

Most extreme halophiles require at least 2.5 M NaCl. In solar salterns, halobacteria color the brines red in NaCl crystallizer ponds with brine densities of ca. 25 to 30° Bé (see Fig. 1) (9). Because of massive NaCl precipitation, the highly concentrated brines between 31 and 32° Bé density have sodium concentrations of less than 2.5 M. Despite the presence of other cations, it can be postulated that the decrease in Na⁺ alone may account for the failure of extreme halophiles to survive in these extremely concentrated brines (the bitterns). However, the effects of the increase in concentrations of the other major ions (Mg²⁺, K⁺, Cl⁻, and SO₄²⁻) should be evaluated.

A survey of moderate halophiles isolated from solar salt ponds showed that at least five genera were represented (24). This study demonstrated that, as a taxonomically diverse group, moderate halophiles have very diverse metabolic requirements and capabilities. They may compete well with halobacteria in some hypersaline environments because they have relatively high growth rates at ambient temperatures (17). Their growth potential in medium that reflects the chemical composition of natural brines should be measured to ascertain the limited success of these microorganisms in the very concentrated brines of solar salterns.

In this investigation, both extremely halophilic and moderately halophilic bacteria were isolated from four different solar salt environments of marine origin. The plating efficiency of native populations was not determined because it is well established that no one medium composition or set of growth conditions can provide the growth requirements of the entire “viable” bacterial flora. Direct microscopic counts (9) may be in error due to the presence of SrSO₄ crystals in suspension with the bacteria (B. Javor, Sixth International Symposium on Salt, in press). The isolates
were screened to establish their physiological diversity and taxonomic relatedness. This communication reports the growth potential of these isolates with different organic substrates and in different salt concentrations of both natural brines and artificial media.

**MATERIALS AND METHODS**

Complex medium (CM), cultivating conditions, and protein measurements have been described previously (10). Technical-grade peptone (Difco Laboratories) was substituted for Inolex peptone. A modification of Sehgel and Gibbons (20) medium, designated SG, was also used. It contained all the salts of CM. The organic source was 7.5 g of Casamino Acids (Difco) plus 5 g of yeast extract (Difco) per liter. Buffered complex medium (CM-B) is described by Tomlinson and Hochstein (21). The buffers, MES [2-(N-morpholino)ethanesulfonic acid], HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), and MOPS [3-(N-morpholino)propanesulfonic acid], provided similar growth rates and yields (unpublished data). In any one experiment, medium with only one of the buffers was used. CM, SG, and CM-B were filtered with GF/C (Whatman) or Whatman no. 1 filters before autoclaving.

Minimal medium (designated MIN) was employed to test growth on individual carbon sources (glycerol, arginine, and acetate). MIN is CM-B without yeast extract and with 200 g of NaCl per liter, 5 mM NH$_4$NO$_3$, and 50 l of Castenholz micronutrients per liter (2). After autoclaving, 1 ml of 0.3 M Na$_2$HPO$_4$ (pH 7.4 to 7.8) was added per liter. One milliliter each of a filter-sterilized B$_2$ solution (0.1 mg/100 ml of distilled water) and a filter-sterilized vitamin mix was added per liter. The vitamin mix solution contained (per 100 ml of distilled water): 1.0 mg of biotin, 10.0 mg of niacin, 5.0 mg of thiamine, 5.0 mg of p-aminobenzoic acid, 2.5 mg of pantothenic acid, 25 mg of pyridoxine hydrochloride, 2.0 mg of folic acid, and 2.5 mg of riboflavin. Organic substrates were added as described for CM-B.

Media prepared from brines from the Exportadora de Sal, S.A., saltern were first clarified by centrifugation. Peptone (Difco technical grade) or peptone and glycerol (1% each) were autoclaved with the brines. The brines are highly buffered naturally. The pH ranged from ca. 7.0 to 7.5.

For comparative screening tests, organic substrates were added aseptically to separately autoclaved CM-B. Organic substrates were added in 1% [wt/vol] final concentrations from 20% sterile stock solutions (except DNA, which was added at a final concentration of 0.5% [wt/vol] from a 10% stock solution). All the carbon sources to be screened were heat sterilized, except the following, which were filter sterilized: d-ribose, α-methyl-d-glucoside, pyruvate, propionate, DNA, and ethanol. Control medium consisted of CM-B to which distilled water was added in a volume equivalent to that of the organic substrates.

For the screening tests, a loopful of culture was inoculated in 3 ml of medium in tubes which were loosely capped. Tubes were agitated at 125 rpm in a New Brunswick incubator at 37°C with a 40-W tungsten bulb and a fluorescent light placed ca. 50 cm away from the tubes. Growth, as shown by optical density at 750 nm (OD$_{750})$, was monitored with a Beckman DU spectrophotometer equipped with a Gilford power supply. Relative specific growth rates for different substrates were determined by comparing the OD$_{750}$ of cells in a set of experiments in which cells were inoculated and harvested at the same times (during logarithmic phase, usually after 2 to 4 days). Comparison with actual growth rates (based on protein determinations of cells harvested at different times in their growth cycle) showed that these two methods gave parallel results (unpublished data).

For fermentation tests, the carbon source (when appropriate) and 2 g of NaHCO$_3$ per liter were added to CM-B or SG immediately after autoclaving. The medium was immediately dispensed aseptically into sterile screwcap tubes, inoculated, and incubated at 37°C. Fermentation was also tested on agar plates with CM-B plus glucose, glycerol, or acetate in a Gas-Pak system (BBL Microbiology Systems) at 37°C.

Nitrate and nitrite reduction were determined in culture tubes with inverted Durham tubes. Medium in which the bacteria demonstrated good aerobic growth was supplemented with 100 mM KNO$_3$ or NaNO$_3$ or 10 mM NaNO$_2$. Medium was used immediately after autoclaving or was steamed just before use. The medium was covered with 1 cm of heavy mineral oil, and the tubes were incubated at 37°C.

Gram staining, catalase, and oxidase tests have been described previously (7). Arginine dehydrolase, ornithine decarboxylase, and lysine decarboxylase were determined by anaerobic growth in Moller broth base (Difco) plus the salts and trace elements of CM. Amino acids were added to a 1% [wt/vol] final concentration. The tubes were covered with 1 cm of mineral oil.

The methods for ionic analyses have been described (Javor, in press). Lipid analyses were performed by the methods of Ross et al. (19) with the exception that thin-layer chromatography plates were developed in hexane-diethyl ether-acetic acid (74:25:1) and visualized by charting after spraying with 3% cupric acetate in 8% phosphoric acid. Spectral analyses of cell lysates in distilled water were done with a Beckman MVI Acta spectrophotometer. Bacteriorhodopsin was determined by the method of Javor et al. (10).

All reagents were of analytical grade. DNA (degraded free acid type IV herring sperm), penicillin G, and bacitracin were obtained from Sigma Chemical Co., and polymyxin B was purchased from Pfizer Inc.

Halobacterium halobium R1, Halobacterium cutirubrum NRC 34001, Halobacterium salinarium no. 10, and Halobacterium salinitrificans were generous gifts of W. Stoeckenius (University of California, San Francisco). B. Volcani (Scirpps Institute of Oceanography) provided Halobacterium marismortui and Halobacterium volcanii (this strain was pale, not red [12]). L. Hochstein (National Aeronautics and Space Administration) provided Halobacterium saccharovorum.

**RESULTS**

Brine samples were collected from the Exportadora de Sal, S.A., saltern in Guerrero Negro, Baja California Sur, Mexico (designated GN); from the muds of natural salt flats adjacent to this salina (designated GNM); from the La Salina slough in Baja California (designated LS); and from the Western Salt Co. saltern in San Diego Bay, Chula Vista, Calif. (designated WS) (see reference 9 and Sixth International Symposium on Salt, in press, for descriptions of these environments). Because no one medium or culture condition is known to support the growth of all halophilic bacteria, the general-purpose, peptone-based medium was employed for both enrichments and direct plate streaking. Both fast- and slow-growing colonies were selected for eventual study. Strains which subsequently demonstrated poor growth on CM were maintained on SG or CM-B with an appropriate carbon source. All other strains were maintained on CM.

Table 1 summarizes some of the characteristics of the 21 isolates. There are nine strains of extremely halophilic rods (Halobacterium spp.); six strains of extremely halophilic box-shaped bacteria (Haloarcula spp.; strains GN-8 and
GN-9 are questionable; three strains of extremely halophilic, irregular cocci (presumably *Halococcus* spp. or *Halobacterium volcanii*); and three strains of moderate halophiles (possibly *Vibrio costicola*).

All the strains were gram negative, catalase positive, and oxidase positive. Colonies were circular, convex, entire, and smooth. All strains produced opaque colonies except GN-1, GN-3, GN-4, GNM-2, GNM-3, WS-2, and WS-3, which produced translucent colonies. All the strains except GN-9, WS-4, and LS-2 were positive for arginine dihydrolase, ornithine decarboxylase, and lysine decarboxylase (determined after 11 days of anaerobic growth). All strains grew anaerobically on nitrate, and all but *Halocarcula* spp. grew anaerobically on nitrite, although no gas formation was observed. The moderate halophiles grew better anaerobi
cally on nitrate than on nitrate (determined visually). All the extreme halophiles contained bacterioruberin, as demonstrated by the spectra of lysates. The spectra of the lysates of the moderate halophiles had a single peak at 406 nm. Several of the strains produced bacteriorhopdopsin.

The separation of these strains into extreme halophiles (archaebacteria) and moderate halophiles (eubacteria) was determined by lipop analysis. The separation into archaeabacterial and eubacterial strains was confirmed by their growth in the presence of antibiotics: extreme halophiles grew in the presence of 500 U of penicillin G or 300 U of polymyxin B per ml, whereas the moderate halophiles could not. Extreme halophiles could not grow in the presence of 1.4 U of bacitracin per ml, whereas the moderate halophiles did. Strain GN-8 was exceptional in that it could grow in the presence of 1,500 U of polymyxin B per ml (none of the other extreme halophiles could) and in the presence of both 1.4 and 3.4 U of bacitracin per ml.

All the strains were screened for their ability to grow aerobically at the expense of a variety of sugars, sugar alcohols, and low-molecular-weight acids added to CM-B (Table 2). A comparison was made with seven known strains of *Halobacterium*. None of the bacteria could grow on 1% ethanol. Several of the strains were tested for their ability to grow on formate, but none could. Propionate did not support the growth of any of the strains in these screening assays.

However, after 3 weeks on MIN plus 1% propionate, *Halobacterium marismortui* demonstrated growth. In all cases, growth on sugars resulted in the slight acidification of the medium (several 10ths of a pH unit), and growth on organic acids resulted in a slight alkalinization.

Several trends were noted among the rods. Strains with a very limited ability to attack carbohydrates included GN-2, GNM-2, GNM-3, and WS-2. These strains resemble the *Halobacterium halobium-Halobacterium cutirubrum-Halobacterium salinarium* group. They were also among the few strains that synthesized bacteriorhopdopsin. However, the GNM strains were less limited in their ability to grow at the expense of low-molecular-weight acids. GNM-2 was the only isolate that grew on 0.5% DNA. Because it could not grow on ribose, these results suggest it grew at the expense of purines, pyrimidines, or both. Strain WS-4, which could not grow on sugars or sugar alcohols, grew best in the presence of pyruvate or acetate. It also grew on MIN supplemented with 1% acetate. It could barely thrive on peptone (CM) or yeast extract plus Casamino Acids (SG). A more complete taxonomic description of this strain is in preparation.

The other rods demonstrated the ability to metabolize some or all of the carbohydrates and many of the low-molecular-weight acids listed in Table 2. There is not enough consistency between the characteristics of any of these isolates (GN-3, GN-5, GN-6, and WS-6) and those of any of the other known strains of *Halobacterium* in the table to make this type of screening of taxonomic value. Strain WS-6 is unusual because it usually grew in clumps, even when it was well agitated. In the clumps, the cells tended to be almost coccoid and encased in multicellular sheathed packets (this made all screening assays difficult). WS-6 is also unusual in that it was the only rod that may have grown at the expense of glycolate.

Among the box-shaped bacteria, strains GN-1, GN-7, GNM-1, and WS-1 thrived at the expense of nearly all the sugars, sugar alcohols, and low-molecular-weight acids listed in Table 2. WS-1 differed from the other strains in its ability to attack lactate and its inability to metabolize α-methyl-d-glucoside (an analog of glucose used in phosphate transport system studies). GN-8 was characterized by rela-
tively low yields and extremely poor growth on CM and SG. GN-9 was characterized by very slow growth rates (up to 1-month for colonies to appear on plates) and low yields. SG produced better growth than did CM. Because strain GN-9 grew so poorly under all the conditions tried, some of the other growth tests were not done with it.

Three strains of extremely halophilic cocci (LS-1, LS-2, and LS-3) were isolated from the La Salina environment. These organisms tended to be irregular in shape, often like flakes. No coccus was ever detected in enrichments or from direct streaking of brines from the other hypersaline environments. No rods or box-shaped bacteria were seen in the microscopic examination of the La Salina brines, on plates from direct streaking, or after enrichments of these brines. There is considerable diversity among the three strains. LS-1 grew on nearly all the carbohydrates and low-molecular-weight acids listed in Table 2. It grew well on peptone. LS-2 grew on most of the substrates listed in Table 2. It grew poorly on peptone alone, but well on SG. LS-3 metabolized many of the carbohydrates and most of the low-molecular-weight acids, but it grew poorly on both CM and SG. LS-3 was unique among the cocci in its ability to metabolize glycolate. The LS isolates showed little lysis in distilled water.

With two exceptions, the three strains of moderate halophiles (GN-4, WS-3, and WS-5) had similar characteristics. Of the substrates listed in Table 2, they could only grow on glucose, sucrose, glycerol, pyruvate, acetate, lactate (except WS-3), and peptone. GN-4 is the only moderately halophilic isolate that grew in minimal medium with 1% glycerol. The moderate halophiles all grew in CM-B with 1% choline, whereas none of the extreme halophiles could. These are probably strains of *V. costicola* (24).

The results of one set of experiments to demonstrate fermentative growth of extreme halophiles under anaerobic conditions are presented in Table 3. Many strains grew fermentatively on CM, carbohydrates, pyruvate, or combinations of these. Most strains that had somewhat better growth on glycerol than on glucose under aerobic conditions had somewhat better growth on glycerol than on glycerol under anaerobic conditions. The *Halobacterium halobium*-like strains (GN-2, GNM-2, GNM-3, and WS-2) grew well anaerobically on SG. Strain WS-4, which grew well aerobically on acetate and pyruvate, failed to show significant anaerobic growth on these substrates. In another experiment, strains GN-3, LS-1, LS-3, and *Halobacterium volcanii* demonstrated significant anaerobic growth on acetate. Other strains that grew on acetate aerobically (GN-1 and GN-6) did not grow on it anaerobically. Fermentative growth in the light was similar to that in the dark. Yields after 6 days of growth were much lower than typical yields during aerobic growth after 2 to 4 days at the same temperature (see the
 ordinates of Fig. 3–6 for comparison). When the experiment was repeated with 12 days of incubation, cell densities were approximately twice those achieved after 6 days. The results of the experiments to show fermentative growth on these substrates under anaerobic conditions in liquid medium were confirmed by growth tests on solid medium in a Gas-Pak system. In addition to these tests, most strains grew anaerobically on arginine, ornithine, and lysine in Møller broth.

For a study of the distribution of halophilic bacteria in nature, the isolates were grown in Exportadora de Sal brines supplemented with peptone or glycerol and peptone. The ionic analyses of the brines are presented in Fig. 1. None of the bacteria had optimal growth rates in brines saturated with NaCl (≥25.5° Be). Most of the rods and box-shaped bacteria showed the greatest growth potential in ca. 21 to 22° Be brines (3.0 to 3.5 M Na⁺, 0.42 to 0.45 M Mg²⁺, 4.2 to 4.5 M Cl⁻, 0.16 M SO₄²⁻; Fig. 2a). Strain GN-5 had a wider range of growth potential between ca. 17 and 22° Be (Fig. 2b). Strains GN-6 and GN-8 had a growth potential similar to that of the coccoid strains, with maximum yields in logarithmic phase in ca. 18° Be brines (2.5 M Na⁺, 0.33 M Mg²⁺, 3.3 M Cl⁻, 0.15 M SO₄²⁻; Fig. 2c). Strain LS-1 had a good growth rate in 13° Be brine (1.9 M Na⁺, 0.25 M Mg²⁺, 2.2 M Cl⁻, 0.12 M SO₄²⁻), but strains LS-2 and LS-3 did not. For comparison, several strains of known species of Halobacterium were tested for this medium. Halobacterium marismortui, Halobacterium cutirubrum, and Halobacterium salinarium had a 21 to 22° Be optimum, whereas Halobacterium

### TABLE 3. Growth yields of bacterial isolates by anaerobic fermentation

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>GN-1</th>
<th>GN-2</th>
<th>GN-3</th>
<th>GN-4</th>
<th>GN-5</th>
<th>GN-6</th>
<th>GN-7</th>
<th>GN-8</th>
<th>GN-9</th>
<th>GN-10</th>
<th>WS-1</th>
<th>WS-2</th>
<th>WS-3</th>
<th>LS-1</th>
<th>LS-2</th>
<th>LS-3</th>
<th>Halobacterium halobium</th>
<th>Halobacterium volcanii</th>
<th>Halobacterium marismortui</th>
<th>Halobacterium salinarium</th>
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<tr>
<td>Control</td>
<td>0.068</td>
<td>0.030</td>
<td>0.065</td>
<td>0.040</td>
<td>0.019</td>
<td>0.052</td>
<td>0.009</td>
<td>0.006</td>
<td>0.066</td>
<td>0.057</td>
<td>0.038</td>
<td>0.065</td>
<td>0.030</td>
<td>0.060</td>
<td>0.036</td>
<td>0.061</td>
<td>0.008</td>
<td>0.027</td>
<td>0.096</td>
<td>0.106</td>
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<td>Glucose</td>
<td>0.105</td>
<td>ND</td>
<td>0.120</td>
<td>0.112</td>
<td>ND</td>
<td>0.031</td>
<td>0.067</td>
<td>0.039</td>
<td>ND</td>
<td>0.092</td>
<td>0.051</td>
<td>ND</td>
<td>0.107</td>
<td>ND</td>
<td>0.065</td>
<td>0.093</td>
<td>0.040</td>
<td>0.049</td>
<td>ND</td>
<td>0.143</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.071</td>
<td>ND</td>
<td>0.051</td>
<td>0.067</td>
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<td>0.021</td>
<td>0.021</td>
<td>ND</td>
<td>0.069</td>
<td>ND</td>
<td>0.115</td>
<td>0.022</td>
<td>0.024</td>
<td>0.049</td>
<td>ND</td>
<td>0.146</td>
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<td>Glycerol</td>
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<td>0.042</td>
<td>0.047</td>
<td>0.050</td>
<td>0.006</td>
<td>0.075</td>
<td>0.068</td>
<td>0.024</td>
<td>0.077</td>
<td>0.021</td>
<td>ND</td>
<td>0.126</td>
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<td>0.110</td>
<td>0.157</td>
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<tr>
<td>Pyruvate</td>
<td>0.081</td>
<td>0.042</td>
<td>0.096</td>
<td>0.098</td>
<td>0.046</td>
<td>0.064</td>
<td>0.047</td>
<td>0.031</td>
<td>0.079</td>
<td>0.076</td>
<td>0.046</td>
<td>0.084</td>
<td>0.041</td>
<td>0.071</td>
<td>0.138</td>
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<td>0.061</td>
<td>0.090</td>
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<td>SG</td>
<td>0.068</td>
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<td>0.074</td>
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<td>0.062</td>
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<td>0.040</td>
<td>0.043</td>
<td>0.250</td>
<td>0.073</td>
</tr>
</tbody>
</table>

* a OD₅₆₀ of cells grown for 6 days under conditions described in the text.
* b OD₅₆₀ of cells grown in CM-B plus acetate was 0.054.

![FIG. 1. Concentrations of major ions in the Exportadora de Sal, S.A., saltern, 13 September 1982. Each curve is defined by the analysis of 26 different brines in the range of 11.1 to 34.5° Be. The curves were extrapolated to normal seawater concentration. (A) Na⁺, Mg²⁺, K⁺, Ca²⁺, Cl⁻, and SO₄²⁻. (B) Sr²⁺.](http://aem.asm.org/)

![FIG. 2. Growth potential of halophilic bacterial isolates in saltern brines plus 1% peptone. Open circles represent repeated experiments.](http://aem.asm.org/)
volcanii had an 18° Bé optimum. The moderate halophiles had optimal growth potentials in 13 to 15° Bé brines (Fig. 2d).

The strains were tested for growth potential with respect to NaCl concentration in SG. Most of the strains showed the greatest growth rate in ≥ 3.5 M NaCl, as shown for strain GN-3 in Fig. 3a. Strains GN-6, GN-8, and WS-4 grew optimally in 3.0 to 3.5 M NaCl (Fig. 3b). Strain LS-1 had a broad tolerance of NaCl (Fig. 3c), although strains LS-2 and LS-3 showed the ≥3.5 M NaCl optimum. The moderate halophiles grew best in SG with 2.5 to 3.5 M NaCl (Fig. 3d).

To determine whether any one cation is particularly detrimental to the survival of the bacteria in highly concentrated natural brines, several strains were grown in peptone-enriched Exportadora de Sal brines to which chloride salts of Na⁺, Mg²⁺, or K⁺ were added in concentrations found in brines up to ca. 28° Bé (the addition of sulfate salts caused a precipitate). A comparison of four strains grown in 16.2° Bé brines (2.2 M Na⁺, 0.29 M Mg²⁺, 2.8 M Cl⁻, 0.14 M SO₄²⁻) is shown in Fig. 4. Strain GN-2 thrived well in high-NaCl brines, but it did rather poorly in Mg²⁺-enriched brines. KCl slightly enhanced growth potential. Strain GNM-3 appeared to require high Mg²⁺ concentrations for greatest growth rates. Strain LS-1 was inhibited nearly equally by the high Na⁺ and Mg²⁺ concentrations found in 28° Bé brines. It was slightly inhibited by the higher KCl concentrations. The moderate halophiles had a response similar to that of LS-1 to increased Na⁺, Mg²⁺, and K⁺ concentrations.

The effects of Mg²⁺ as chloride and sulfate salts were tested on several strains grown in CM with 3.0 M NaCl. Among the extreme halophiles, chloride was better tolerated than sulfate, although the opposite was true for the three moderate halophiles. Among the box-shaped bacteria tested (strains GN-1, GN-7, and GNM-1), the best growth potential was recorded in medium with 0.3 to 0.5 M MgCl₂ or 0.2 to 0.3 M MgSO₄ (Fig. 5a). Strain GN-2, a rod, showed optimal growth potential in medium with ≥0.9 M MgCl₂ (Fig. 5b), whereas strains GNM-2 and GNM-3 thrived best in 0.7 M MgCl₂ (data not shown). There was little change in growth potential in these three strains, as MgSO₄ concentration varied from that of the control medium (81 mM) to nearly 0.9 M. Another rod, strain GN-3, grew optimally in medium with ≥81 mM Mg (Fig. 5c). The LS strains responded in a manner similar to that of GN-3 with respect to MgCl₂ and MgSO₄ concentrations (Fig. 5d). The moderate halophiles also showed maximal growth potential in medium with ≤81 mM Mg (Fig. 5e).

For all the rod- and box-shaped extremely halophilic bacteria tested, an increase in KCl concentration from that of the control medium (27 mM) to 277 mM was accompanied

FIG. 3. Growth potential of halophilic bacterial isolates in SG with different concentrations of NaCl.

FIG. 4. Growth potential of halophilic bacterial isolates in peptone-enriched saltern brines to which different concentrations of NaCl (●), MgCl₂ (□), or KCl (△) were added.
by a slight increase in potential growth rate (Fig. 5). In the halococcus LS-1 and in all three moderate halophiles, growth potential remained the same for this range of KCl concentrations. Strain LS-2 was inhibited by KCl concentrations greater than 0.1 M (data not shown). Strain LS-3 was not tested.

**DISCUSSION**

**Heterotrophy: general conclusions.** Among the extremely halophilic bacterial isolates, several major groups can be recognized according to the substrates they metabolize: strains that can oxidize a broad range of substrates, and strains that are largely confined to the oxidation of amino acids, carbohydrates, or pyruvate and acetate. Both the Exportadora de Sal and Western Salt solar salt ponds harbor mixed populations of the versatile and “specialist” strains of bacteria. Among the three genera of halobacteria, only members of the genus *Halobacterium* have thus far demonstrated narrow substrate specificity. It remains to be investigated whether there are strains of extreme halophiles that specialize in attacking other classes of organic compounds such as lipids, aromatics, and C1 compounds.

Low-molecular-weight carboxylic acids, particularly the products of glycolysis and the tricarboxylic acid cycle, have generally been excluded in tests of substrate utilization by extremely halophilic bacteria. Pyruvate supported the growth of nearly all the extreme halophiles, and in strain WS-4, it produced the most luxuriant growth of all the substrates tested. The box-shaped bacteria appeared to be the most versatile in metabolizing the four other carboxylic acids listed in Table 2. It is noteworthy that propionate actually inhibited growth of most of the halobacteria, although it has been shown to stimulate CO2 fixation (3, 13).

The inability of some strains to metabolize the substrates tested may be due to an inability to take up the substrate from the medium, or there may be an enzymatic block in the degradative pathway. However, all the enzymes of the tricarboxylic acid cycle have been demonstrated in halobacteria (1). Strains such as GN-8 and LS-2, which grew well on xylene but not at all on ribose, may lack a single isomerase or epimerase.

The taxonomic description of the genus *Halococcus* (11) states that *Halococcus morrhuae* cannot grow on xylose at the expense of carbohydrates. The authors used a medium in which glucose was autoclaved with the salts and yeast extract. Heat sterilization of glucose with the medium makes it unsuitable for bacterial growth (unpublished data). The results with the cocoid strains of this study suggest that they can grow on a wide range of substrates, although strains LS-2 and LS-3 grew poorly on amino acid-based medium (CM). A more rigorous appraisal of strains of *Halococcus morrhuae* would clarify the metabolic diversity found in this species. One or more of the cocoid LS strains might require a new species assignment in this genus.

In the ionic environments in which the moderate halophiles coexist with the extreme halophiles, the two types of microorganisms apparently can compete for many of the same organic substrates. It is noteworthy that the *Vibrio* strains could grow at the expense of choline and none of the halobacteria could. Rafaeli-Eshkol (15) reported that an unidentified moderate halophile oxidized choline to betaine and that choline acted as a protective substance in salt resistance. The ability of moderate halophiles to attack choline may be a useful tool for enrichments of these microorganisms in extremely hypersaline environments.

**Anaerobic growth.** The description of the genera *Halobacterium* and *Halococcus* in Bergey’s Manual of Determinative Bacteriology (5) states that fermentation is never found in organisms of these genera. Tomlinson and Hochstein (21) demonstrated glucose fermentation in *Halobacterium saccharovorum*. Since then, several other species have proved to be capable of carbohydrate fermentation in the presence of air. Additionally, Hartmann et al. (8) showed that *Halobacterium*...
bacterium halobium grew fermentatively at the expense of arginine under anaerobic conditions.

Several general conclusions may be drawn about fermentative growth under anaerobic conditions by the isolates in this investigation: basal medium (CM-B with no added substrates) supported at least some growth in nearly all the isolates; some strains could grow relatively well on one or more of the substrates that supported good growth under aerobic conditions; and all strains had much slower or poorer growth than under aerobic conditions.

Many strains that grew fermentatively on carbohydrates in the presence of air (determined by the acidification of the medium) grew especially well on glucose, totally by substrate-level phosphorylation in the absence of air. Tomlinson and Hochstein (21) demonstrated that O₂ consumption by Halobacterium saccharovorum in the presence of glucose was 18% of the theoretical amount required for its complete oxidation. In the presence of galactose, it was 83%. It is possible that fructose, like galactose, requires more oxygen for its oxidation and therefore was generally a poorer substrate for fermentation. The measurement of O₂ consumption, growth rates, and fermentation products in the presence of glycerol, pyruvate, and acetate by strains that demonstrated relatively good anaerobic growth on these substrates would provide further evidence of the importance of fermentative metabolism in extreme halophiles.

Although all the isolates grew anaerobically in the presence of nitrate, growth may have occurred by fermentation rather than by nitrate reduction. Assays for nitrate reductase would confirm that the strains can grow by dissimilatory nitrate reduction.

Effects of salts. Although halobacteria are found in the greatest numbers in saltern brines of the density range of ca. 27 to 30° Be (9), they can barely grow in these elevated salinities under culture conditions. These results suggest one or more of the following: (i) the bacteria may be passively concentrated by brine evaporation, (ii) their distribution in lower salinities may be limited by brine shrimp grazing or by competition from other bacteria, or (iii) growth studies under laboratory conditions may not be a good reflection of bacterial activity in nature. This question will be at least partially answered by uptake studies in natural populations.

The Halococcus strains demonstrated salt requirements intermediate between those of Halobacterium and Haloarcula and those of the moderate halophiles. Of the three cocoid strains, LS-1 most closely resembled Halobacterium volcanii in morphology, paucity of carotenoids, substrates metabolized, salt tolerance and optima, and substrates that stimulated and inhibited CO₂ fixation (B. Javor, submitted for publication). Because the epithet bacterium is usually reserved for rods, Halobacterium volcanii might be better classified as Halococcus volcanii. Clarification of the taxonomic status of this species requires further biochemical and metabolic comparisons with known strains of Halococcus.

The abundance of extremely halophilic cocci in the La Salina slough, a body of water whose salinity is that of seawater at times, is explained by the ability of these bacteria to remain viable in dilute solutions (16, 18). Both Halobacterium and Haloarcula lyse in dilute solutions. The reason for the lack of Halococcus in the three other environments sampled is not clear. The inability to synthesize bacteriorhodopsin may be a partial explanation. An ecological study of a solar salt environment where Halococcus competes successfully with Halobacterium and Haloarcula may provide an answer.

The natural brines in which the extreme halophiles demonstrated the greatest growth potential (18 to 22° Be) supported fair growth in the moderate halophiles. Only in the NaCl-saturated brines (≥25.5° Be) did the moderate halophiles fail to demonstrate appreciable growth. Rodriguez-Valera et al. (17) found similar salinity overlaps and limits by plating out saltern bacteria on artificial medium. Thus, although the NaCl-crystallizing ponds of solar salterns are suboptimal environments for halobacteria, they are entirely hostile for the eubacteria.

Studies on the Na⁺, Mg⁺, and K⁺ requirements and sensitivities of the isolates showed that both Na⁺ and Mg⁺ concentrations found in natural brines of up to ca. 28° Be could affect the distribution of individual strains but that K⁺ did not appear to be especially stimulatory or inhibitory. However, it was difficult to separate the effects of Cl⁻ on growth potential. Strains such as GNM-3 demonstrated an extremely high Mg⁺ requirement for optimal growth, a characteristic previously described only in the Dead Sea strain Halobacterium sodomense (14). Most other strains of Halobacterium require 0.1 to 0.5 M Mg, and some are tolerant of higher concentrations (12).

It is noteworthy that chloride salts of magnesium were relatively more inhibitory to the moderate halophiles than were sulfate salts, in contrast to the response of the extreme halophiles. Sulfate has a very low activity coefficient in concentrated brines (4). It is possible that MgSO₄ is less inhibitory than MgCl₂ only because it is relatively less dissociated. Alternatively, the true cell wall of the moderate halophiles may be less permeable to the divalent sulfate ion than to the monovalent chloride ion.

It is difficult to assess the relative importance that each ion plays in preventing halobacteria from thriving in the bitterns brines. Javor (9) suggested that the low water potential of such concentrated brines may be the actual limiting factor. The study of these bacteria with reference to the chemistry of their natural habitats should stimulate future investigations concerning such phenomena as divalent cation transport and regulation and physiological activity at different water potentials.

In an evaluation of the ecological importance of the results of this study, the following conclusions should be considered: (i) in the saltern environments, a fairly wide diversity of species and strains was present (although their respective roles in natural populations were not determined); (ii) all the halobacteria isolated were facultative anaerobes; (iii) classical screening methods (carbon source utilization) can only broadly define species of halobacteria; (iv) halobacteria grew poorly in the concentrated brines which harbored the greatest density of these microorganisms, suggesting that they were passively concentrated by brine evaporation; and (v) Halococcus was only isolated from a curyhaline environment, and conversely, Halobacterium and Haloarcula were only isolated from stenohaline environments. The genus Halococcus needs taxonomic reassessment.

To more fully develop the picture of microbial processes in hypersaline environments, future studies should address the nature of the organic chemistry of natural brines, microbial metabolic and turnover rates in native environments, and the isolation and culture of halophilic bacteria under selective conditions.

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LITERATURE CITED