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Box-Shaped Halophilic Bacteria

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Three morphologically similar strains of halophilic, box-shaped procaryotes have been isolated from brines collected in the Sinai, Baja California (Mexico), and southern California (United States). Although the isolates in their morphology resemble Walsby's square bacteria, which are a dominant morphological type in the Red Sea and Baja California brines, they are probably not identical to them. The cells show the general characteristics of extreme halophiles and archaebacteria. They contain pigments similar to bacteriorhodopsin which apparently mediate light-driven ion translocation and photophosphorylation.

Walsby (27) observed flat, square bacteria in brine from a coastal sabkha at the western shore of the Gulf of Eilat. The location, geochemistry, and hydrodynamics of the sabkha have been described previously by Gavish (7). The cells had dimensions of 2 to 4 by 2 by 0.25 μm and contained gas vacuoles; they often occurred in the form of sheets comprising 4 to 16 cells or more. Their electron microscopic morphology has been described previously (10, 21, 22), confirming the features seen in a light microscope and revealing a regular cell wall structure similar to that of rod-shaped halobacteria (1, 25). We examined several samples of brine from southern California and Baja California with a light microscope and found numerous morphologically identical square cells in the Baja brines. In addition to rods and coccoid forms, smaller, flat, angular cells without gas vacuoles were also seen in all brine samples. Spectroscopy of cells concentrated from the natural brines showed that in addition to the abundant carotenoids, which caused the reddish color of the brine, a bacteriorhodopsin-like pigment was present. Bacteriorhodopsin is a retinylidene protein so far found only in the cell membranes of extremely halophilic rods, where it forms two-dimensional crystalline patches known as the purple membrane. It enables these cells to use light energy for ATP synthesis and other energy-requiring processes (24).

We have tried to isolate and grow Walsby's square cells in axenic culture. So far, we have obtained cell strains from three brine samples collected at the Red Sea sabkha (Sabkha Gavish; SG), a saltern at Guerrero Negro (GN), Baja California (Mexico), and a saltern at Chula Vista (CV) near San Diego, Calif. The cells are not identical in appearance to Walsby's square cells seen in the natural brines. They are smaller and more variable in appearance and lack the gas vacuoles; however, they show similar flat and angular shapes. We do not believe that they are identical to Walsby's square cells; nevertheless, they are of considerable interest. We describe here the isolation of the strains from SG, GN, and CV and some preliminary observations on their morphology, physiology, and biochemistry. Most of the data pertain to the GN strain; when the limited observations on the other strains differ, this is noted.

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MATERIALS AND METHODS

Enrichment cultures and isolation media. Unless indicated, our standard medium and conditions for growing halobacteria (20) were used throughout this work except that 0.2 g of $CaCl_2$, 3.58 mg of $FeCl_2$, and 0.218 mg of $MnCl_2$ were added per liter of standard medium containing 250 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 3.0 g of sodium citrate, and 10 g of peptone; other modifications used in some of the experiments were as indicated below. Enrichment cultures were obtained by either adding the native brines to complex medium or adding 1.0 to 2.5 g of peptone $liter^{-1}$ to the native brines. These cultures were then incubated in Erlenmeyer flasks on a rotary shaker at 125 rpm and illuminated by a bank of Sylvania cool white fluorescent lamps. The enriched suspension cultures or natural brines were streaked onto agar plates prepared with complex medium or natural brines from which cells had been removed by centrifugation or filtration. The plates were incubated at 37°C in the light, and single colonies were selected according to their cell morphology and transferred to liquid medium in Erlenmeyer flasks. These flasks were incubated under the same conditions as the enrichment cultures. Transfer of cells back to agar plates and reisolation from single colonies were repeated several times.

The growth of the cultures in liquid medium was monitored by measuring the optical density at 750 nm

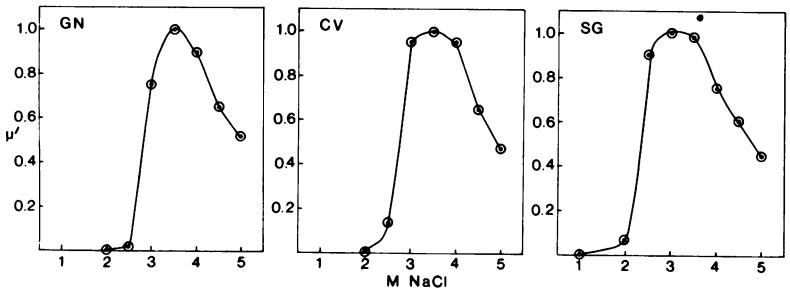

FIG. 1. Growth response to different Na^+ concentrations in complex medium. The growth rates at different salt concentrations are expressed as fractions (μ') of the optimal specific growth rate μ.

in a Beckman DU spectrophotometer equipped with a Gilford power supply. We first determined a standard curve of optical density at 750 nm versus cell number, obtained with Levy or Petroff-Hausser counting chambers, so that the calculated specific growth rate was based on cell counts.

Antibiotics were added to the standard growth medium at concentrations ranging from 1 to 500 μg/ml, and cell numbers were counted after 1, 3, and 5 days of incubation. Cell numbers were expressed as the percentage of cells in control cultures incubated under the same conditions without antibiotics. For penicillin and bacitracin, growth was checked only after 5 days.

Morphological and biochemical techniques. For light microscopy a Zeiss photomicroscope equipped with phase optics was used. For observation of the cell shape, it is often advantageous to apply a drop of the cell suspensions to a 1- to 2-mm-thick slab of 1 to 2% agar, agarose, or polyacrylamide gel prepared with a salt solution. The cells flatten out on the surface of the gel and are immobilized. The standard electron microscope techniques of using glutaraldehyde and OsO_4 as fixatives and of freezing unfixed cells without cryoprotectant have been described previously (22).

Cells were fractionated after dialysis against 0.1 M NaCl and subsequently against distilled water. The membrane fraction was recovered by differential centrifugation and subfractionated on a sucrose density gradient; details are given in reference 20. For protein determination we used sodium dodecyl sulfate-solubilized samples and the Lowry assay (16), modified to include 1% sodium dodecyl sulfate in solution A. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the cell membrane fractions followed the method of Laemmli (11), but with 16% slab gels 1.5 mm thick. Samples containing 125 μg to 1 mg of protein in 15 to 80 μl of 1% sodium dodecyl sulfate–10% glycerol–0.75% β-mercaptoethanol were applied without prior heating.

For ATP determinations we used the luciferin-luciferase assay as described previously (5). The light-dependent pH changes of cell suspensions were measured in a stainless steel chamber equipped with quartz windows, a thermostat, a combination pH electrode, a temperature probe, and provisions to add drugs to the sample under anaerobic conditions; details are given in reference 2.

Spectroscopy. The spectroscopic techniques are the same as those that have been established in our laboratory for work with the cells and photoactive pigments of *Halobacterium halobium*, using a Cary 14 spectrophotometer with a scattered-transmission accessory or an Aminco DW-2 double-beam spectrophotometer (3). The flash spectrophotometer used to observe time-resolved absorbance changes in the micro- and millisecond range has been described previously (16a, 28a).

Chemicals. All chemicals used, unless otherwise noted, were analytical reagent grade and obtained commercially. Nonactin, rifampin, kanamycin, streptomycin, anisomycin, and bacitracin were purchased from Sigma Chemical Co., penicillin G was purchased from Calbiochem, and carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) was purchased from K & K Laboratories, Inc.

RESULTS

Growth requirements. So far, brines from three locations, SG, GN, and CV, have yielded isolates which somewhat resemble the square bacteria first observed by Walsby, because they are also much smaller in one than in the other two dimensions and are often angular in shape. The three strains were morphologically identical to each other at the light microscope level. The optimal NaCl concentration for all three strains was 3 to 4 M, and the minimum generation time was 4 to 5 h at 37°C. All strains grew also in medium saturated with NaCl (5 M), but only the SG strain still showed some growth in 2 M NaCl medium (Fig. 1). All strains required Mg^{2+}, and the growth response for the GN strain is shown in Fig. 2. The optimal Mg^{2+} concentration was that of the isolation medium, 81 mM, and growth ceased between 1.5 and 2.0 M Mg^{2+}. However, tolerance of high Mg^{2+} concentrations decreased with prolonged culture on 81 mM Mg^{2+} medium. The growth rate of all three isolates did not change when the KCl concentration in the standard medium was increased from 27 to 210 mM. Growth still occurred when KCl was omitted, even upon a second transfer to "KCl-free" medium. It should be noted, however, that the KCl content of the analytical reagent-grade NaCl used is 0.0005%, according to the manufacturer, so that the K^+ content of the KCl-free medium was at least ~ 30 μM.

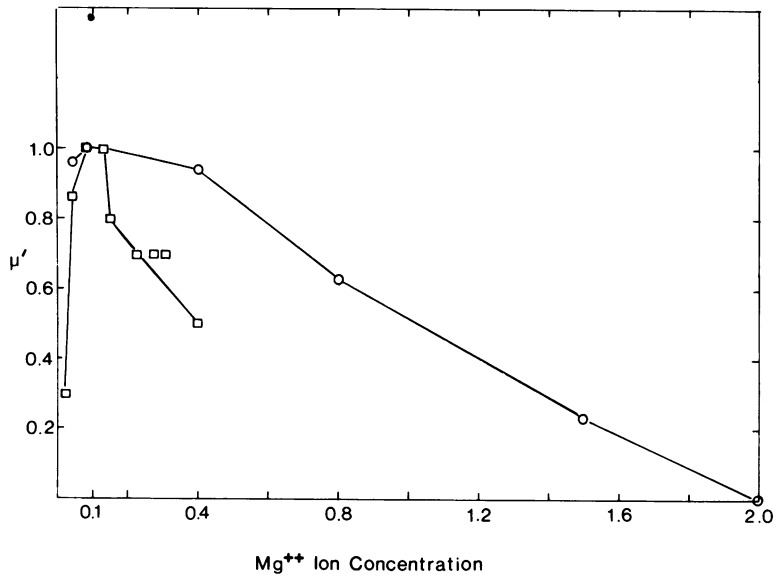

FIG. 2. Growth response of the GN isolate to different Mg^{2+} concentrations. The standard complex medium contains 81 mM Mg^{2+}. For each concentration tested, cells pregrown in either 81 or 800 mM Mg^{2+} were used. Their responses did not differ (○). However, after prolonged growth on standard medium, the Mg^{2+} tolerance had decreased significantly (□). μ', Growth rate expressed as a fraction of the optimal growth rate.

All three strains were resistant to the addition of 500 U of penicillin or streptomycin ml^{-1}, but 20 U of bacitracin or 8 μg of anisomycin ml^{-1} prevented growth. Rifampin showed moderate inhibition at 8 to 30 μg ml^{-1}, and kanamycin showed moderate inhibition at 500 μg ml^{-1}. Higher concentrations of rifampin did not result in stronger inhibition because of its limited solubility in the high-salt medium.

Morphology. None of the colonies on agar plates contained cells with the same light microscopic morphology as Walsby's large, gas-vacuolated cells. However, from plates of the SG, GN, and CV brines, we could select colonies that consisted of cells which had similar flat and angular shapes. The cells retained this morphology when grown in liquid medium. They did not contain gas vacuoles or dark granules visible in the light microscope and did not occur in sheets. They were smaller, more irregular rectangles and squares, with a shorter side of 1.0 μm and a longer one of ~2.0 μm and measuring 0.3 to 0.4 μm in the third dimension. Triangular cells of similar size were often seen, and flat, round, or oval forms were also present. Most of the cells were slightly bent or their edges were slightly curled in, so that the basic angular morphology was obvious only when the cells were adsorbed to the glass or agar surface (Fig. 3, insert). When grown in 5 M NaCl, the cells were larger and more regularly rectangular, whereas in lower concentrations of NaCl, the round and oval shapes became more frequent. When the Mg^{2+} concentration of the medium was high, large, irregular cell aggregates appeared in the suspension cultures.

Scanning electron microscopy confirmed the general shape and dimensions of the cells seen in the light microscope and added little new information (Fig. 3). The sides of the boxlike cells appeared flat, whereas in sectioned material they were convex. We have seen the same difference in appearance of the sides in Walsby's square cells (22). The flat appearance in the scanning electron micrographs of critical point-dried preparations is much less likely to be an artifact than the convex shape seen in sections, and this is the reason the cells were called box shaped. In general, the transmission electron micrographs (Fig. 4) show a less regular morphology; the edges are less sharp and straight, and the regular cell wall structure observed in Walsby's square cells from the natural brine is not obvious. Instead, the cells are bordered by a single dense line ~20 nm thick. Kinks between straight sections or a slightly scalloped contour suggests a rigid, flat wall which has been deformed by embedding and sectioning. Dense cytoplasmic granules seen in Walsby's cells presumably consist of β-hydroxybutyrate and appear as empty vacuoles in the sectioned material. Similar vacuoles were present in the cells of

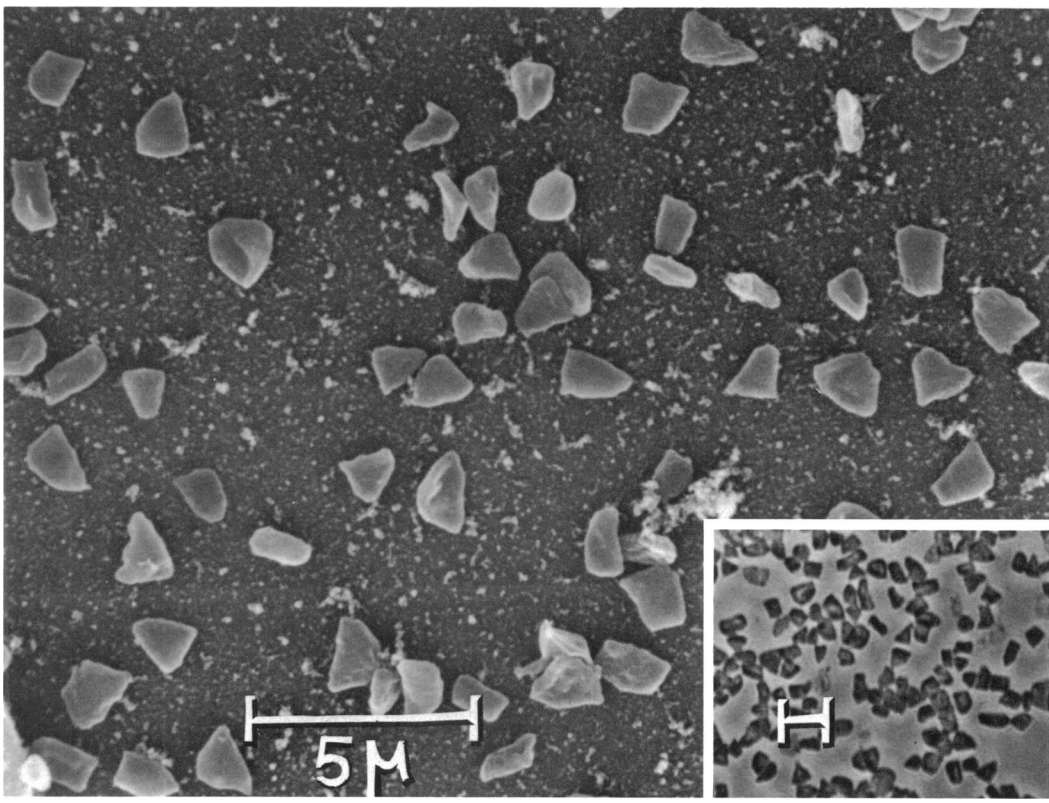

FIG. 3. Scanning electron micrograph of the GN isolate. Cells were washed in 4 M NaCl before fixation with formaldehyde. Magnification, ×6,360. The cells in the light micrograph (insert) are more flattened out through adsorption than are the critical point-dried cells in the electron micrograph. Light micrograph magnification, ×1,290.

the isolated strains but were typically <0.2 μm in diameter and less frequent (Fig. 4).

Freeze-fracture electron micrographs show essentially the same fine structure as described for the cells from natural brines (22) with the differences that were expected, i.e., less regular shape, smaller granules, and no gas vacuoles. The membrane fracture faces occasionally show small areas of regular structure, which consist of a few parallel rows of particles (Fig. 5). These are very different in appearance from the crystalline patches of purple membrane seen in *H. halobium* and *Halobacterium cutirubrum* (24).

Pigments. The absorption spectra of the cell suspensions were essentially the same as those of the natural brines (Fig. 1 in reference 22), with three main peaks at 540, 505, and 475 nm and a shoulder at 450 nm, which are characteristic for the main carotenoid of extreme halophiles. A minor peak near 420 nm is probably due to the Soret band of cytochromes. When the cell membrane fraction was isolated, it showed the same spectrum, but less distorted by the scattering artifact. Cells or membrane fractions kept in the dark for several hours showed a small decrease in absorbance near 570 nm which was quickly restored when the cells were illuminated with visible light. This indicates the presence of a light-sensitive pigment similar or identical to bacteriorhodopsin. A difference spectrum of the light effect is shown in Fig. 6 and compared to the difference spectrum for bacteriorhodopsin. The two spectra look almost identical. Assuming an identical pigment, we calculated that one cell would contain ~22,000 bacteriorhodopsin molecules, which is about 2 to 5% of the bacteriorhodopsin content in *H. halobium* R_1 cells grown under the same conditions.

Flash spectroscopy is an even more sensitive indicator of the presence of photoactive pigments and can be used for their characterization. Figure 7 shows the difference spectrum from a cell membrane fraction ~2 ms after a 7-ns flash of 580-nm light. When compared to the corresponding difference spectrum of bacteriorhodopsin, the similarity in the spectra is again

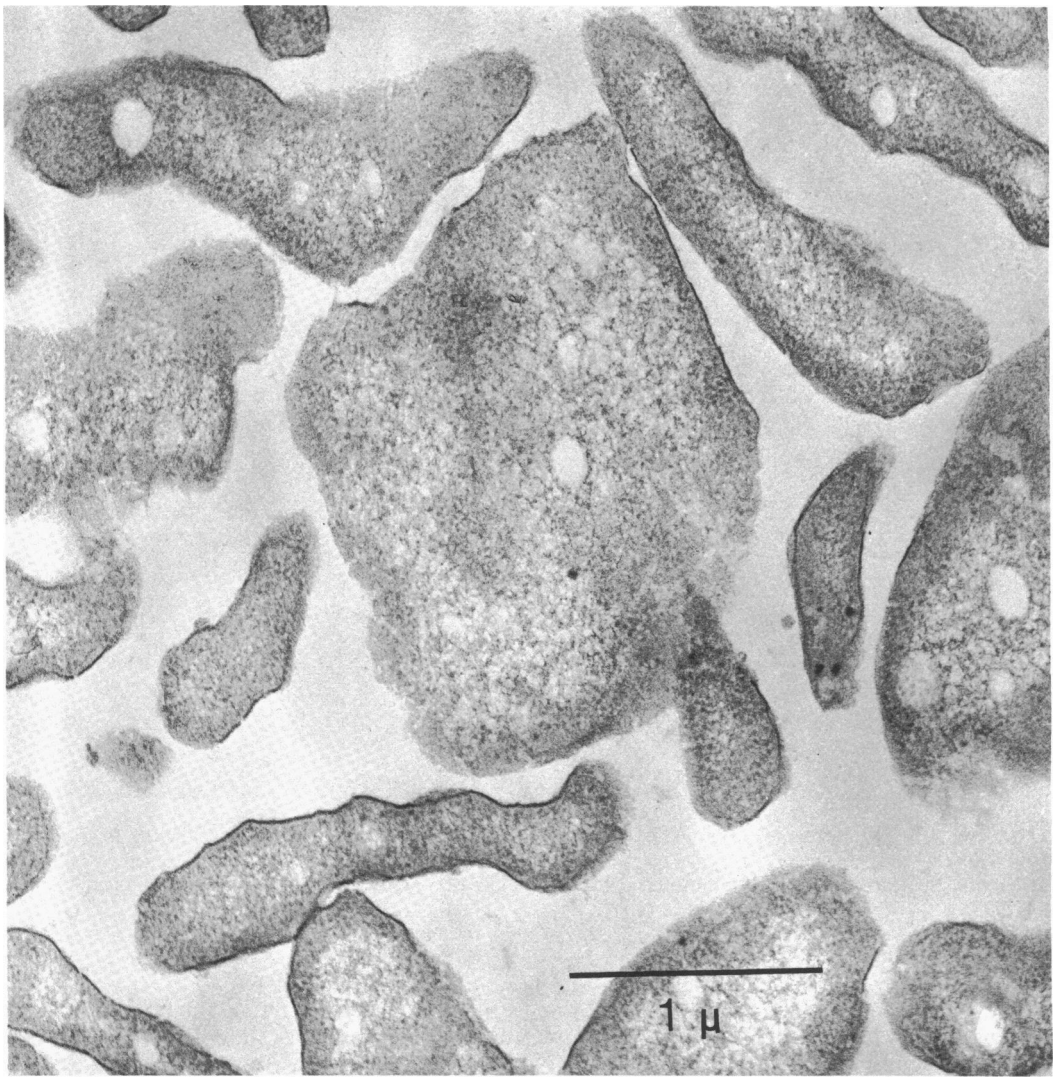

FIG. 4. Transmission electron micrograph of GN cells fixed in glutaraldehyde followed by OsO_4 and stained with lead citrate. Magnification, ×36,000.

striking; only the short-wavelength peak appears to be slightly shifted to a shorter wavelength in the new isolate. The long-wavelength peak appeared ~5 ms after the flash. A similar peak is seen in bacteriorhodopsin, but would be less pronounced under the same conditions. The pigment returned to its original absorption spectrum, i.e., completed its photoreaction cycle, in ~10 ms.

Bacteriorhodopsin usually is found in a specialized region of the cell membrane, the purple membrane, which can be isolated on sucrose gradients as a single dense band after lysis of the membrane in distilled water. We applied the same technique to the GN strain and obtained five bands, all of which contained the bacteriorhodopsin-like pigment by our spectroscopic criteria.

Physiology. The presence of a bacteriorhodopsin-like pigment in these cells suggests that they might be photosynthetic. Cells kept under anaerobic conditions for 30 min maintained a low level of ATP in the cytoplasm. Upon illumination with light of wavelengths > 520 nm, the ATP concentration rapidly tripled, then dropped off sharply when the light was shut off (Fig. 8). Giving the cells access to air had qualitatively and quantitatively the same effect. These results are practically identical to those obtained with *H. halobium* (5) and strongly suggest that the new

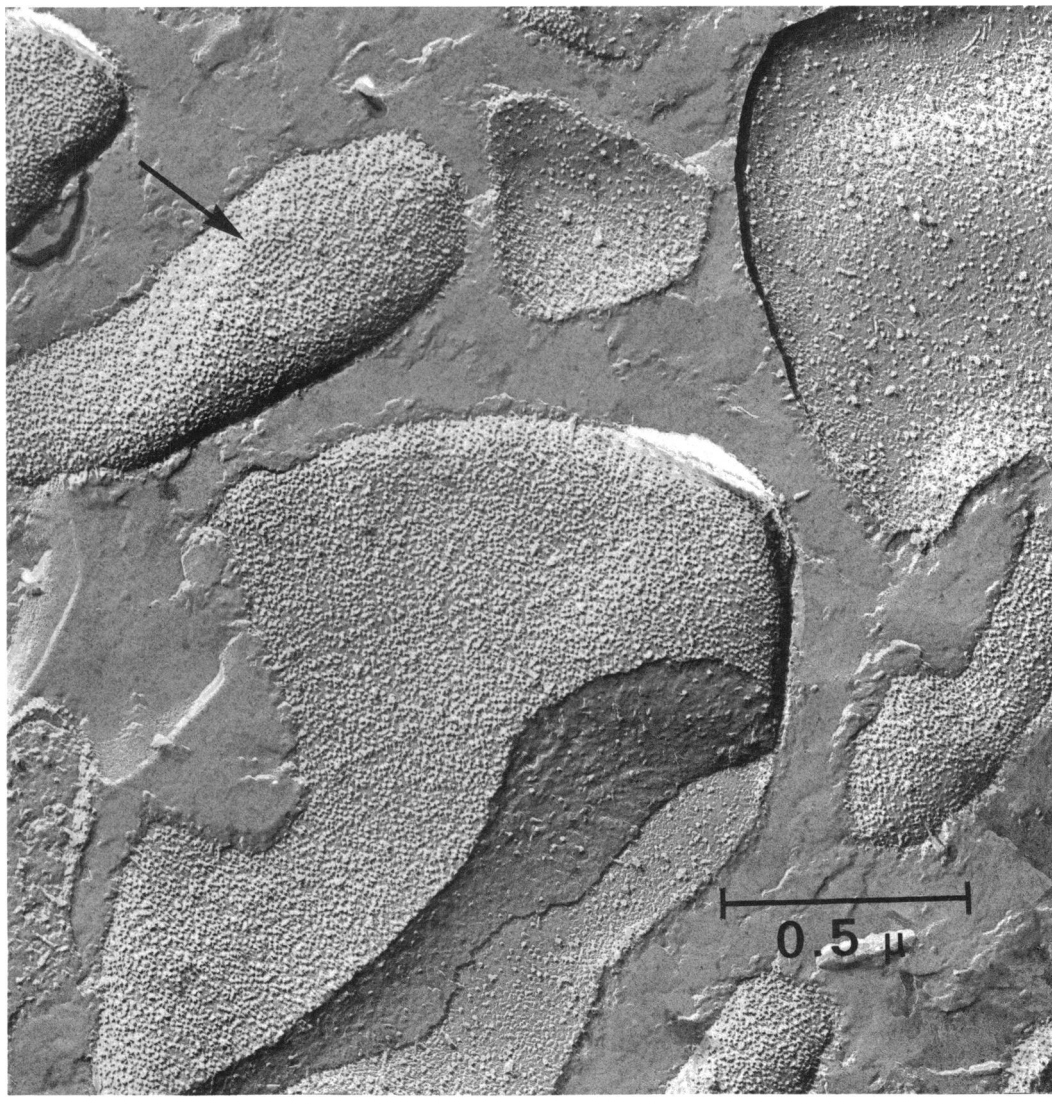

FIG. 5. Freeze-fracture electron micrograph of GN cells. The cytoplasmic membrane fracture face of the cell above and to the left of center shows a small ordered array of membrane particles (arrow). Magnification, ×70,000.

isolate also can use both respiration and light energy for ATP synthesis.

Bacteriorhodopsin converts light energy into an electrochemical gradient by ejecting protons from the cell. Illumination of a cell suspension of the GN isolate in basal salt (the standard growth medium without peptone) resulted in a small transient alkalization of the suspension followed by a larger acidification, which was sustained until the light was turned off; the pH then returned to the original level (Fig. 9A). This is the same response observed in an *H. halobium* cell suspension (2) and is attributable to the light-driven proton ejection by bacteriorhodopsin. The addition of the uncoupler CCCP converted the light-induced acidification into an alkalization (Fig. 9B). *H. halobium* mutants which contain little or no bacteriorhodopsin or cell envelope subfractions poor in bacteriorhodopsin also show light-induced, CCCP-enhanced alkalizations. This effect has been attributed to the presence of a second retinal pigment similar to bacteriorhodopsin but present at a much lower concentration. It has been named halorhodopsin, or P_{588}, and is thought to function as a light-driven pump for an ion other than

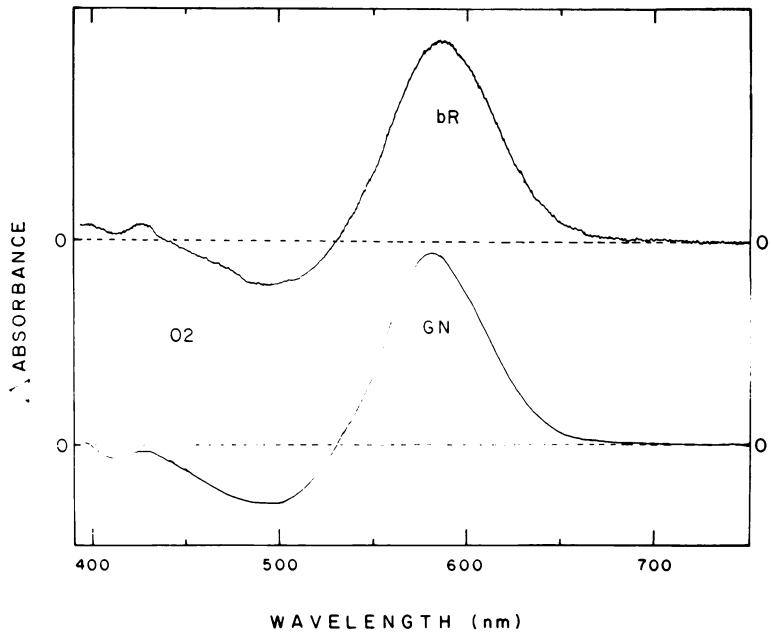

FIG. 6. Light-adapted minus dark-adapted difference spectra for bacteriorhodopsin (bR) and for the GN isolate. We compared the difference spectrum of monomeric bacteriorhodopsin in asolectin vesicles (for details, see reference 4) with that of the GN cell membrane fraction. Both preparations were kept in the dark overnight and then exposed for 3 min to 3×10^3 ergs of white fluorescent light $cm^{-2}\ s^{-1}$.

H^+. The outside-positive membrane potential generated by this pump drives protons into the cell and results in a net alkalization of the medium, if the proton permeability is enhanced by CCCP (15, 17). Consequently, if the increase in membrane potential is inhibited by the addition of nonactin, which increases the K^+ permeability of the cells, the light-induced alkalization in the presence of CCCP should also be inhibited, and this was the effect that we observed (Fig. 9C). We may tentatively conclude that our isolate, in addition to a bacteriorhodopsin-like pigment that functions as a light-driven proton pump, also contains a second light-driven ion pump similar to P_{588} of *H. halobium*.

DISCUSSION

The aim of the work reported here was the isolation and axenic culture of the square bacteria first observed by Walsby. However, the morphologies of the isolated strains, although similar, differ significantly from that of Walsby's square cells in the natural brines. This could, of course, be due to the different growth conditions in the natural brine pools and in the laboratory. However, we have also seen cells of the same shape and size as those of our isolates in the natural brines, but they are less prominent than the square cells described by Walsby (see Fig. 2 in reference 22). We believe that these are the cells that we isolated and that they represent at least a different species. Nevertheless, their unusual shape and photoactive pigment(s) make them interesting in their own right.

The structural basis for their angular morphol-

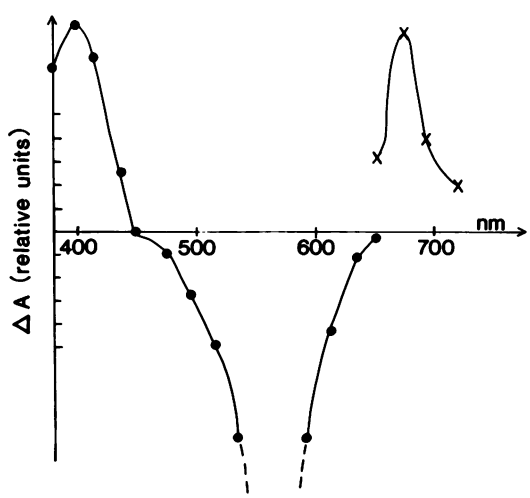

FIG. 7. Difference spectra at room temperature for a cell suspension of the GN isolate after a 7-ns flash of green light. Absorption spectra 2 ms (●) and 6 ms (×) after the flash minus the dark spectrum are shown. A, Absorbance.

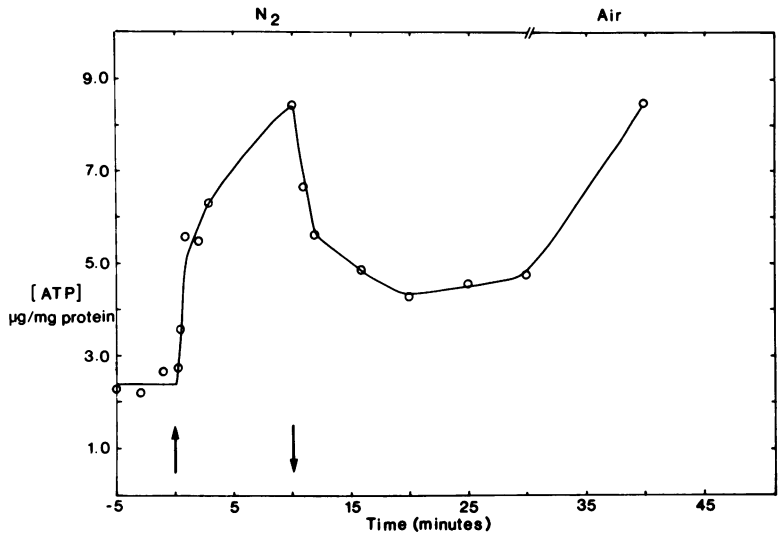

FIG. 8. Light and respiration effects on intracellular ATP content. Cells were kept in the dark under anaerobic conditions for 20 min until the ATP content had dropped to a low and constant level. The illumination period is marked by arrows, and the transition from anaerobic to aerobic conditions is indicated in the upper margin.

ogy is not known. If they are similar to other halobacteria, it should reside in the glycoprotein, which is the only constituent of the wall in the extremely halophilic rods and determines their shape (18, 29). Walsby has pointed out that in halobacteria osmotic forces do not dictate a shape favorable for withstanding internal pressure, i.e., spheres, ovoids, or rounded-off cylinders, because in halobacteria the internal salt concentration equals the outside concentration (27). However, in their natural environment these cells are more likely than most other procaryotes to experience abrupt large increases in internal osmotic pressure when rain or high tides dilute the salt ponds. We would rather argue that their flat shapes and relatively soft cell walls allow a large increase in their internal volume with a relatively small change in their cell envelope shape.

The isolates may tentatively be identified as extreme halophiles because of the high salt concentrations that they require for growth and maintenance of shape and because of their pigmentation. Their simple cell wall structure and lysis at low ionic strength, the high guanine-plus-cytosine content of their DNA (68%), and the presence of large, more adenine-plus-thymine-rich plasmids (F. Pfeifer, personal communication) also fit this assignment. They appear to have an unusually low requirement for potassium; other extreme halophiles require millimolar concentrations. (For general reviews of the properties of extreme halophiles, see references 8 and 12 to 14). The extreme halophiles belong to the new kingdom archaebacteria (6), and this is consistent with the lipid composition and sensitivity to antibiotics of our isolates. All lipids of the GN strain appear to be isoprene derivatives, and the polar lipids bear ether-linked phytanyl chains (M. Kates, personal communication). The sensitivity to bacitracin may appear surprising because bacitracin is mainly known for its effectiveness against gram-positive bacteria, where it is thought to act mainly through inhibition of peptidoglycan synthesis (26). However, Mescher and Strominger (18) demonstrated that it also strongly inhibits halobacteria. It has been shown to prevent glycosylation of one site in the cell wall glycoprotein of *H. halobium* (29).

The morphologies of our isolates resemble those of *Halobacterium volcanii*, but our isolates are apparently less pleomorphic. *H. volcanii* under optimal growth conditions has been described as disk shaped but with variations in individual cells ranging from round to oval, square, rectangular, triangular, and often bent into cup shape. Although most of the properties of *H. volcanii*, e.g., amino acid composition, cell wall structure, and lipids, are similar to those of the most studied extremely halophilic rods, its NaCl requirement is lower and its $MgCl_2$ tolerance higher, which is not surprising since it has been isolated from the Dead Sea (19). Cup- and disk-shaped cells have also been described as the dominant morphological species in the surface water of the Dead Sea (9). Other strains

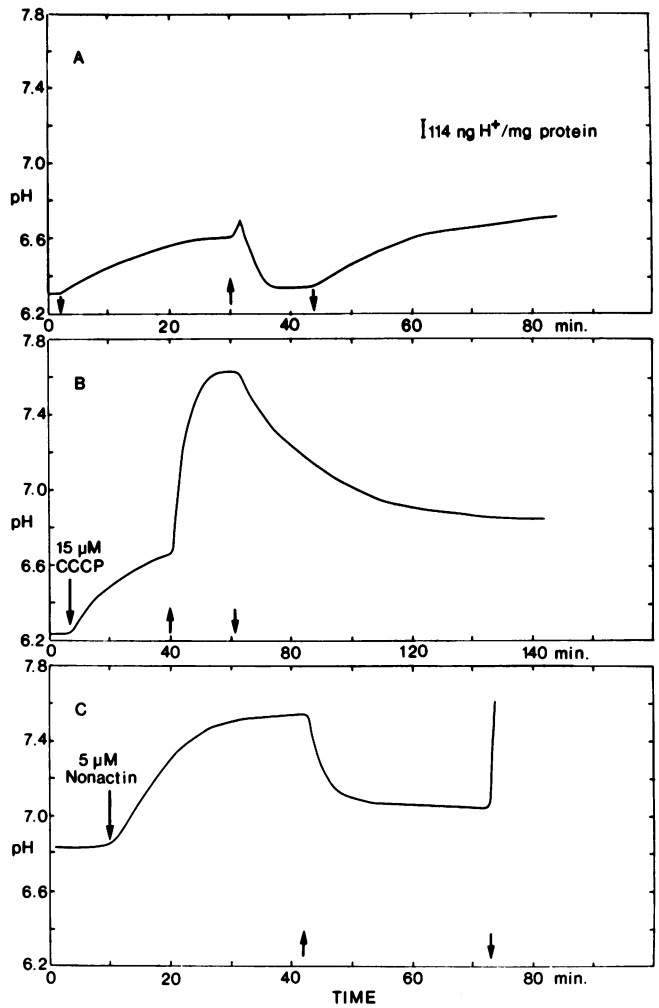

FIG. 9. Light-dependent pH changes in an anaerobic suspension of GN cells in basal salt. Light on (↑) and light off (↓) are marked. In (B), the proton ionophore CCCP was added to increase the permeability to protons; in (C), nonactin was added to abolish the membrane potential.

with the same or a very similar morphology have recently been isolated from the Dead Sea (A. Oren and M. Shilo, personal communication) and from the SG (Y. Cohen, personal communication).

The new isolates apparently are capable of photophosphorylation and can use light energy to generate an electrochemical proton gradient. Spectroscopically, we have not found any chlorophyll but rather a bacteriorhodopsin-like pigment, which appears to be the light energy converter. These tentative conclusions certainly need further experimental corroboration; however, the observations are so strikingly similar to the results obtained with *H. halobium* that we may assume the existence of a similar photosynthetic system. This makes the small differences observed most interesting. We have not found any purple membrane in the new isolates; the bacteriorhodopsin-like pigment is apparently present in relatively low concentrations and dispersed as monomers or small aggregates over the cell membrane. There are indications that in *H. halobium*, in addition to bacteriorhodopsin in purple membrane, a similarly dispersed component may also be present. However, it is not readily observed there because the much higher concentration of bacteriorhodopsin in the purple membrane patches masks its presence (23). Our isolates may help to determine the physiological role of the dispersed pigment.

We have detected small differences in flash spectra between bacteriorhodopsin and the new pigment(s). This may indicate that we are deal-

ing with slightly different pigments. However, the reversal of the light-induced pH response when an uncoupler is added and the inhibition of that response by nonactin indicate the presence of P_{588} (halorhodopsin) or a similar pigment. P_{588} also undergoes a photoreaction cycle (28), and if both pigments are present in comparable concentrations, the transient absorbance changes would show a mixture of both. The new isolates, therefore, may contain the same two pigments, but with less of the bacteriorhodopsin component than does *H. halobium*.

The distinct flat and angular shape of the new isolates distinguishes them from other procaryotes. We have proposed in our earlier paper (22) that such box-shaped procaryotes should be named *arcula* (Latin for a small box). In the meantime, Walsby (21) has proposed *quadra* for the large square cells that he first observed and that we believe are different from the isolates described here. We therefore withdraw our earlier suggestion. However, should further characterization of our isolates show that a new genus is required, we now propose to name the strain isolated from SG *Haloarcula sinaiiensis* and the strain from GN *Haloarcula californiae*. Preliminary results from restriction enzyme cleavage patterns of the DNA indicate that the GN and CV strains are closely related or identical, whereas the Red Sea strain, SG, is distinctly different (F. Pfeifer, personal communication). The 5*s* RNA cleavage pattern from the GN isolate is nearly identical to those of *Halobacterium vallismortis* (ATCC 29715) and *Halobacterium marismortui* (the Ginzburg strain) but distinctly different from those of *H. cutirubrum* and *Halobacterium morrhuae*, consistent with the placement in a separate genus (D. E. Nicholson and G. E. Fox, personal communication).

ACKNOWLEDGMENTS

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