APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1992, p. 260–266
0099-2240/92/010260-07$02.00/0

Vol. 58, No. 1

Isolation and Characterization of *Haloanaerobacter chitinovorans* gen. nov., sp. nov., a Halophilic, Anaerobic, Chitinolytic Bacterium from a Solar Saltern

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Two halophilic anaerobic bacteria, one of which had chitinolytic activity, were isolated from a solar saltern in southern California. These organisms were long, gram-negative, motile, flexible rods. The biochemical and physiological characteristics of these bacteria were very similar but were different from the characteristics of other haloanaerobic bacteria. Both grew at salt concentrations ranging from 0.5 to 5 M and at temperatures ranging from 23 to 50°C. They were sensitive to chloramphenicol but resistant to penicillin, carbenicillin, D-cycloserine, streptomycin, and tetracycline. An analysis of DNAs and whole-cell proteins showed that they were closely related taxonomically and distinguishable from other halophilic anaerobic bacteria. They exhibited 92.3 to 100% DNA homology as determined by DNA-DNA hybridization. The guanine-plus-cytosine contents of their DNAs were 34.8 ± 1 mol%. The two isolates, strains W5C8 and W3C1, differed from other halophilic anaerobic bacteria sufficiently to support establishment of a new genus and species, *Haloanaerobacter chitinovorans*. Strain W5C8 exhibited chitinolytic activity and is designated the type strain. Two chitin-induced extracellular proteins with molecular weights of 38 × 10^3 and 40 × 10^3 were detected in strain W5C8.

A broad spectrum of bacteria inhabit hypersaline environments. Among these halophilic organisms, the anaerobic eubacteria have been studied relatively little compared with the aerobic bacteria. Only three anaerobic genera containing the following five species have been described to date: *Haloanaerobium praevalens*, *Halobacteroides halobius*, *Halobacteroides acetoethylicus*, *Sporohalobacter lortetti*, and *Sporohalobacter marismortui* (14, 16, 17, 20, 27). Oren et al. proposed a new family, the *Haloanaerobiaceae*, for these bacteria on the basis of their unique 16S rRNA oligonucleotide sequences (15). All members of this group are moderately halophilic, heterotrophic bacteria. They ferment various carbohydrates as substrates. However, only *Halobacteroides halobius*, *S. lortetii*, and *S. marismortui* ferment starch (14, 16, and 17). *Haloanaerobium praevalens* and *S. marismortui* also use pectin or glycogen for energy sources (16, 27). None degrades other polysaccharides, such as chitin or cellulose.

In hypersaline environments, species diversity decreases as the salinity increases. Thus, the brine shrimp, *Artemia salina*, and the larvae of the brine fly are the only macroscopically visible organisms that are abundant at salinity levels of 10 to 30%, and the sole phytoplankton at salinity levels of more than 20% is *Dunaliella salina* (21). Consequently, *Artemia salina*, an algal grazer, may attain population densities that are great enough for commercial harvesting (10, 21). Brine shrimp and brine flies are responsible for the major deposition of chitin and other organic matter in these hypersaline environments (18). A mixture of anaerobic halophilic bacteria from salt marsh sediments may use chitin as a carbon and hydrogen source for sulfate reduction and methanogenesis (4). Dense populations of brine shrimp that are present in the solar saltern ponds at Chula Vista, Calif. (10), undoubtedly are the main contributors to the chitinous sediment in this system. In this investigation, using chitin as a substrate, we isolated two halophilic, anaerobic bacteria from this multipond solar saltern. These two strains, strains W5C8 and W3C1, are described and characterized below.

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MATERIALS AND METHODS

Strains. *Halobacteroides halobius* ATCC 35273 was obtained from the American Type Culture Collection, Rockville, Md. *Haloanaerobium praevalens* DSM 2228 was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. Strains W5C8 and W3C1 were isolated from the sediments of salterns having levels of salinity of 20 to 30%.

Isolation and culture techniques. The anaerobic techniques of Hungate (9) were used in this study. Culture medium HS-1, which was used for both enrichment cultures and axenic cultures, contained (per liter) 1.0 g of NH_4Cl, 7.0 g of $MgCl_2 \cdot 6H_2O$, 9.6 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $CaCl_2 \cdot 2H_2O$, 3.8 g of KCl, 0.4 g of $K_2HPO_4 \cdot 3H_2O$, 12.0 or 24.0 g of NaCl, 3.0 g of $NaHCO_3$, 1.0 g of Na_2CO_3, 1.0 g of yeast extract, 5.0 g of substrate (glucose, *N*-acetylglucosamine [NAG], chitin, etc.), 0.001 g of resazurin, 0.5 g of L-cysteine, 10 ml of a trace minerals solution (26), and 0.5 g of Na_2S. The pH was adjusted to 7.2 by flushing the medium with CO_2 or N_2. Portions (10%, vol/vol) of pond sediment slurry were inoculated into anaerobic serum bottles containing 50 ml of medium HS-1 supplemented with 0.5% crab chitin (Sigma Chemical Co., St. Louis, Mo.). After 10 to 15 days of incubation at 37°C, the cultures were examined for an increase in turbidity and the disappearance of chitin. Chitin-positive cultures were transferred three times into

fresh chitin-containing medium before axenic cultures were isolated. Roll tubes (9) containing medium HS-1 agar supplemented with NAG were inoculated to isolate chitinolytic strains. Roll tube colonies were first picked and transferred to tubes (18 by 23 mm) containing 5 ml of medium HS-1 supplemented with 0.5% chitin. After 4 to 6 days of incubation, only tubes with dense growth and dissolved chitin were chosen for further isolation attempts. Isolates were routinely maintained in medium HS-1 containing 0.5% NAG or chitin (strain W5C8 only) and kept at 4°C. Otherwise, isolates were stored frozen at −70°C in 50% glycerol and in 50% medium HS-1.

Electron microscopy. Cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and were attached to polylysine-coated glass coverslips by using 0.1% aqueous polylysine. They were rinsed in cacodylate buffer, dehydrated in a 30 to 100% acetone series, and critical point dried in liquid CO_2. Samples were sputter coated with about 10 nM gold and viewed with a Philips model SEM 501 scanning electron microscope. Cells were photographed on Polaroid type 55-P/N film (4 by 5 in. [10.16 by 12.7 cm]) at a magnification of ×5,000.

Physiological and biochemical tests. The effects of temperature, pH, and salt were measured by inoculating each isolate into anaerobic tubes containing 5 ml of medium HS-1 containing the appropriate test concentrations under the appropriate conditions and calculating the growth rates during early exponential growth. Growth rates were determined by measuring the turbidities (optical densities) of cultures at 560 nm. Antibiotic susceptibilities were determined by using the following antibiotics at concentrations of 100 μg/ml: chloramphenicol, carbenicillin, D-cycloserine, penicillin, streptomycin, and tetracycline. Substrate utilization was investigated by using medium HS-1 containing each test compound at a concentration of 5 g/liter as a carbon and energy source and comparing the growth rate with the growth rate on NAG. The criterion for positive substrate utilization was based on achieving a growth rate that was greater than 30% of the maximum growth rate on NAG. Medium HS-1 was modified for the studies in which glucose fermentation and NAG fermentation were investigated by replacing Na_2CO_3 and $NaHCO_3$ with 2 g of $K_2HPO_4 \cdot 3H_2O$ per liter. After 24 h of incubation at 37°C, fermentation products were analyzed as previously described (25). Gram reactions were determined by using smears of liquid cultures that were fixed with acetic acid (6). Catalase and oxidase activities were tested by using the procedure of Holding and Collee (8).

Gas chromatography. CH_4 and CO_2 were analyzed by performing gas chromatography and thermal conductivity detection (1). H_2 was determined by gas chromatography, using a model RGD2 reduction gas detector (3). Acetate and isobutyrate were determined by using gas chromatography and flame ionization detection (1).

G+C contents. To determine guanine-plus-cytosine (G+C) contents, cellular DNAs were extracted and purified by using the method of Liaw and Srinivasan (12). The buoyant density of each purified DNA was measured by ultracentrifugation in a CsCl density gradient (19); the base ratio (in moles percent G+C) was calculated by using the formula of Schildkraut et al. (22).

DNA-DNA hybridization. DNA-DNA hybridization was performed by using the S1 nuclease method and the filter technique (5, 23). ^{3}H-labeled DNA was prepared with [5-^{3}H]dCTP (1 mCi/ml; Du Pont Co., Boston, Mass.) by using a commercial nick translation reagent kit (United States Biochemical Co., Cleveland, Ohio).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by using 12.5% polyacrylamide (11). Samples of whole-cell proteins for electrophoresis were prepared by incubating cells with Laemmli sample buffer (11) in a boiling water bath for 10 min. Extracellular proteins were obtained by concentrating the culture supernatant, using the methanol-chloroform-water mixture of Wessel and Flugge (24). The concentrated protein pellets were dissolved in Laemmli sample buffer for further analysis.

RESULTS

Enrichment and isolation. Two halophilic, anaerobic strains, designated strains W5C8 and W3C1, were isolated separately from two different ponds of the Western Salt Works solar saltern, which is located in Chula Vista, Calif. Both strains grew on NAG and other carbohydrates. Strain W5C8 also degraded chitin as a carbon and energy source. Strain W3C1 was isolated from a pond having a salinity level of 30%; the pH was 7.0 at the bottom of the pond and 7.1 at the surface. Strain W5C8 was also isolated from a pond having a salinity level of 30%; the pH of this pond was 7.4 at the bottom and 7.7 at the surface. A considerable halophilic flora and fauna, including halobacteria, coccoid cyanobacteria (*Aphanothece halophytica*), brine shrimp, and brine flies, were abundant in these ponds (10). We observed a collection of thick layers consisting mainly of dead brine shrimp in the leeward corners of wind-swept ponds having salinity levels of 20 to 30%. These organisms were deposited in sufficient quantities to elicit a continuous evolution of gas bubbles from the sediment. The gas bubbles and an accompanying odor of volatile fatty acids were indicative of active microbial fermentation by haloanaerobic bacteria; gas evolution and odors were not observed in ponds that did not contain brine shrimp. Samples from the sediments of these solar ponds were inoculated into medium HS-1 containing 24% NaCl and 0.5% crab chitin. After 10 to 15 days at 37°C, dense populations of bacteria grew in these chitin enrichment cultures. The chitin was hydrolyzed completely, and large quantities of H_2 and CO_2 were produced. Inocula (10%) were taken from these enrichment cultures and introduced into fresh medium having the same composition. Morphologically less diverse but faster growing populations of bacteria resulted upon subsequent transfers. Anaerobic bacteria were isolated from these enriched cultures by using the Hungate roll tube technique (9).

Morphology. Isolates W3C1 and W5C8 resembled each other in colony morphology and cell morphology. Young 1-day-old colonies on agar surfaces of roll tube cultures were translucent; older surface colonies were opaque and glossy with entire edges. Colony diameters ranged from 0.5 to 1.0 mm, depending on age. Agar-embedded colonies formed large gas pockets. The cells of both strains were colorless, motile, long, flexible rods. The cells of strain W3C1 ranged from 1.4 to 8.0 by 0.5 μm; strain W5C8 cells ranged from 2.5 to 8.0 by 0.5 μm (Fig. 1a and b). The characteristics of the two isolates were similar to those of *Halobacteroides halobius*, which had long cells in young cultures and short cells which formed blebs and/or spheres (degenerate cells) in older cultures (17). The longer (8.0-μm) motile cells sometimes had a flexible appearance which might have been due to a slight curvature in some rods. In our two isolates,

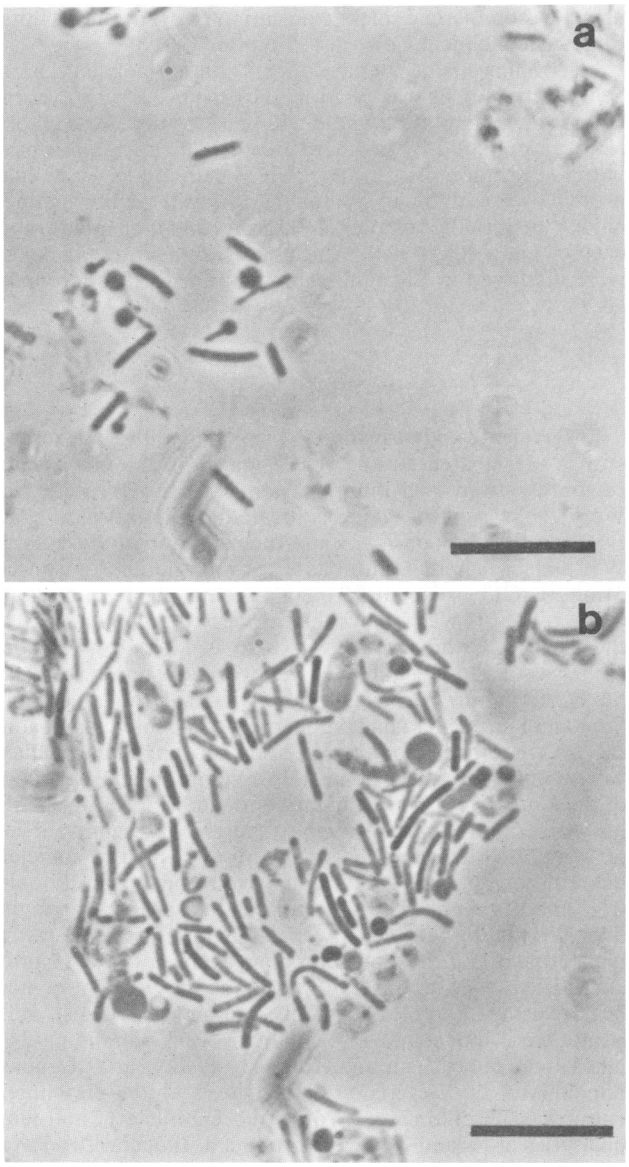

FIG. 1. Phase-contrast micrographs of strain W5C8 (a) and (b) strain W3C1 (b). Long motile cells with a flexible appearance and degenerate cells with blebs (see text) were observed in 24-h cultures grown in medium HS-1. Bar = 10 μm.

spheres emerged from weak spots, usually at the ends of older degenerating cells in hypertonic medium (Fig. 1a and b). Scanning electron photomicrographs showed that the cells were peritrichous (Fig. 2a and b).

Growth and physiology. Both isolates grew in the presence of a broad range of salinity levels. Growth was observed at NaCl concentrations between 0.5 and 5.0 M at 37°C (Fig. 3). Optimal growth was observed at NaCl concentrations between 2 and 3 M. In the presence of 2 M NaCl, strain W3C1 grew optimally at 40 to 50°C, and strain W5C8 grew optimally at 30 to 45°C (Fig. 4). Neither strain grew at temperatures above 60°C. The optimal pH for growth was 7.0 for both isolates. The minimum doubling time for strain W3C1 was 2.9 h in the presence of 2 M NaCl at pH 7.0 and 50°C, whereas the minimum doubling time for strain W5C8 was 2.47 h in the presence of 2 M NaCl at pH 7.0 and 37°C.

Except for the unique ability of strain W5C8 to use chitin as a carbon and energy source, the two strains utilized the same substrates for growth (namely, the carbohydrates D-glucose, D-fructose, D-mannose, D-acetylglucosamine [NAG], D-sucrose, D-maltose, and D-cellobiose). Neither strain utilized acetic acid, glycerol, pyruvate, D-raffinose, cellulose, or pectin. Slight growth was observed on starch and tryptone.

The two strains exhibited the same type of glucose fermentation pattern (Table 1). After 24 h at 37°C, the two strains produced similar quantities of CO_2, H_2, acetic acid, and isobutyric acid from glucose. Strain W5C8 also produced the same types of end products from NAG, but considerably more acetic acid was formed. Both isolates were gram negative. Both were oxidase and catalase negative, and they exhibited the same pattern of antibiotic susceptibility; growth was inhibited by chloramphenicol, but not by carbenicillin, D-cycloserine, penicillin, streptomycin, or tetracycline (each at a concentration of 100 μg/ml). Large spheres like those described in older cultures of *Halobacterioides halobius* by Oren et al. were also observed in our isolates when they were growing on carbenicillin, D-cycloserine, or penicillin. However, the spheres which we observed differed because they were transferable and could grow in the presence of antibiotics. Because these spheres reverted to the normal rod shape after two to three transfers in the presence of carbenicillin, D-cycloserine, or penicillin, they were probably spheroplasts.

Analysis of DNA. An analysis of the DNAs from strains W5C8 and W3C1 indicated that they had the same buoyant density (1.694 ± 0.001 μg/ml), corresponding to a G+C content of 34.8 ± 1 mol%. Further examination by using DNA-DNA hybridization revealed a level of homology of 92.3 to 100% (Table 2). In the same study, the lack of DNA relatedness between our isolates and two representative strains (*Halobacteroides halobius* and *Haloanaerobium praevalens*) was shown by the low levels of homology, which ranged from 0 to 14.8%.

Protein analysis. Similarities between strain W5C8 and W3C1 were also observed in whole-cell proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5). Almost identical polypeptides were detected in these two isolates, but these polypeptides clearly differed from those of either *Halobacteroides halobius* or *Haloanaerobium praevalens*. In the analysis of extracellular proteins, 38- and 40-kDa polypeptides were found in strain W5C8 grown on chitin (Fig. 6). Higher concentrations of the 40-kDa polypeptide were found in 4-day cultures; however, this polypeptide was not observed (Fig. 6, lane b) in 7-day cultures. On the other hand, the 38-kDa polypeptide was the major extracellular protein present in the 7-day cultures, in which most of the chitin had been hydrolyzed. These two polypeptides were not found in cultures grown on NAG.

DISCUSSION

By using an enrichment technique we isolated a previously undescribed anaerobic, halophilic, chitinolytic bacterium from a sediment sample taken from a hypersaline saltern. Large quantities of chitin were deposited in the sediment by the carcasses of dead brine shrimp and brine flies, which were abundant in this system. Chitinolytic strain W5C8 grew in the presence of a broad range of salinity levels. In the presence of 2 M NaCl, it completely digested 0.5% crab chitin in 7 to 10 days. An analysis of extracellular proteins

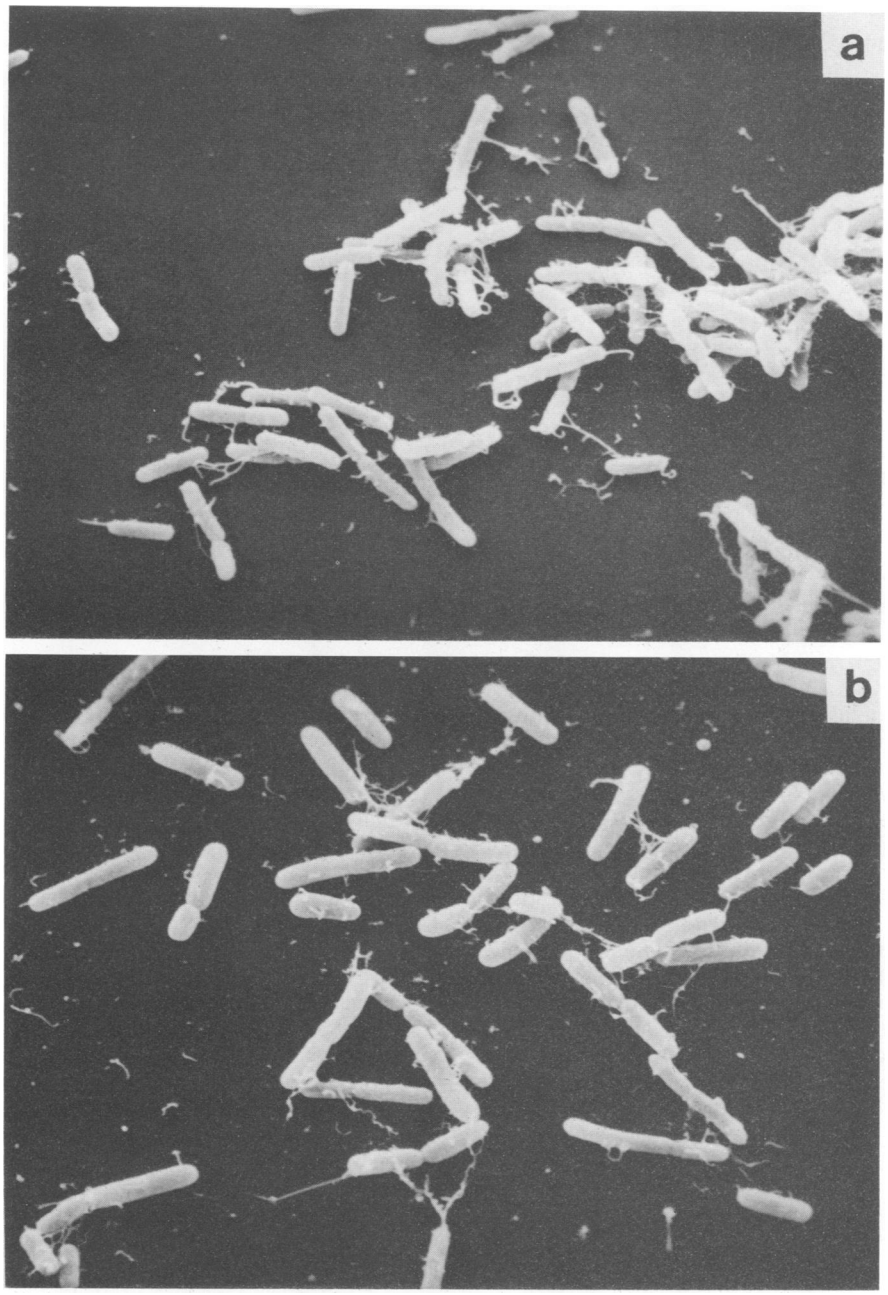

FIG. 2. Scanning electron photomicrographs of gold-coated cells grown in medium HS-1. (a) Strain W5C8. (b) Strain W3C1. Magnification, ×5,000.

from cultures grown on chitin indicated that two polypeptides (38 and 40 kDa) were present; at the end of growth, when all of the visible chitin had been hydrolyzed, only the 38-kDa polypeptide remained. Neither polypeptide was detected when cells were grown in the absence of chitin. Further investigation will be necessary to determine whether either of these two chitin-induced polypeptides exhibit chitinase activity.

Strain W3C1 was isolated from a different pond with a higher salinity level but in the same solar saltern system. Strain W3C1 did not grow on chitin. However, the two strains were similar to each other in most other characteristics. They exhibited similar cell morphologies, optimum salinities and temperatures for growth, substrate ranges, antibiotic susceptibilities, and other biochemical and physiological characteristics. The close relationship between strains W5C8 and W3C1 was also evident from the almost identical polypeptides that resulted from lysis of whole cells. Further study of the DNAs of these strains showed that they belong to the same species (level of DNA homology, 92.3 to 100%) and have G+C contents of 34.8 ± 1 mol%.

Strains W5C8 and W3C1 were morphologically similar to *Halobacteroides halobius*. Their cells were motile, long, flexible rods in young cultures, and short degenerate cells

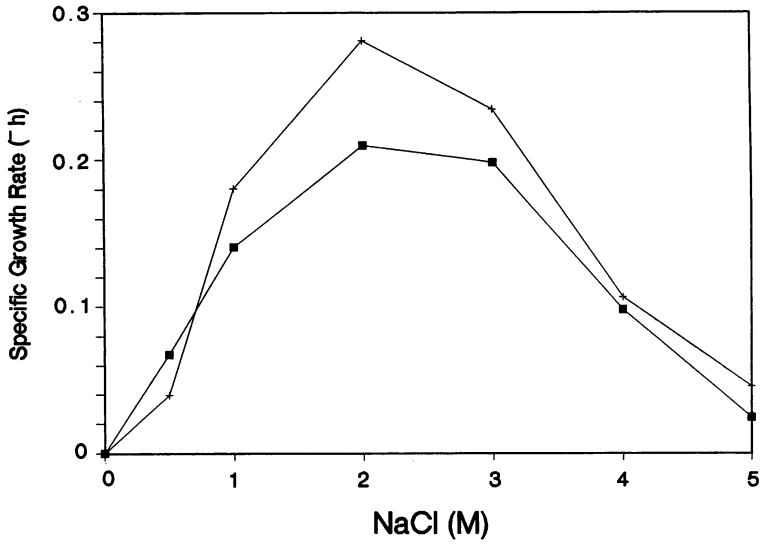

FIG. 3. Growth of strains W5C8 and W3C1 at different NaCl concentrations. The organisms were grown at 37°C in medium HS-1 containing 0.5% glucose and different concentrations of NaCl. The specific growth rates for strain W5C8 (+) and strain W3C1 (■) were determined during early exponential growth.

with extruded spheroplasts were present in old cultures. However, the biochemical and physiological characteristics of these two isolates were considerably different from those of *Halobacteroides halobius* or any other previously described anaerobic halophile. Both strain W5C8 and strain W3C1 grew in the presence of a broader range of salinities (0.5 to 5.0 M NaCl) and at a higher temperature (50°C). Their antibiotic susceptibilities were also different from those of other haloanaerobic isolates. They were resistant to penicillin, D-cycloserine, tetracycline, streptomycin, and carbenicillin. They exhibited little DNA homology or similarity in their whole-cell proteins to two previously described haloanaerobic organisms, *Halobacteroides halobius* and *Haloanaerobium praevalens*. Because of the significant differences between our isolates and other halophilic anaerobes, we propose a new genus and species, *Haloanaerobacter chitinovorans*, for these strains.

Isolation of strains W5C8 and W3C1 from a solar saltern demonstrated that there are halophilic anaerobic bacteria which differ from those found in the Dead Sea, the Great Salt Lake, and other hypersaline environments (17, 20, 27). The discovery of chitinolytic activity in strain W5C8 adds a potential system for the study of halophilic enzymes other than the halophilic amylases (7, 13). Strain W5C8 also grows under the unusual environmental conditions of anaerobiosis and halophily, which distinguishes it from previously described chitinolytic bacteria. Since strain W3C1, unlike strain W5C8, did not exhibit chitinolytic activity but was similar in all other characteristics, it may serve as an ideal reference organism for future genetic studies.

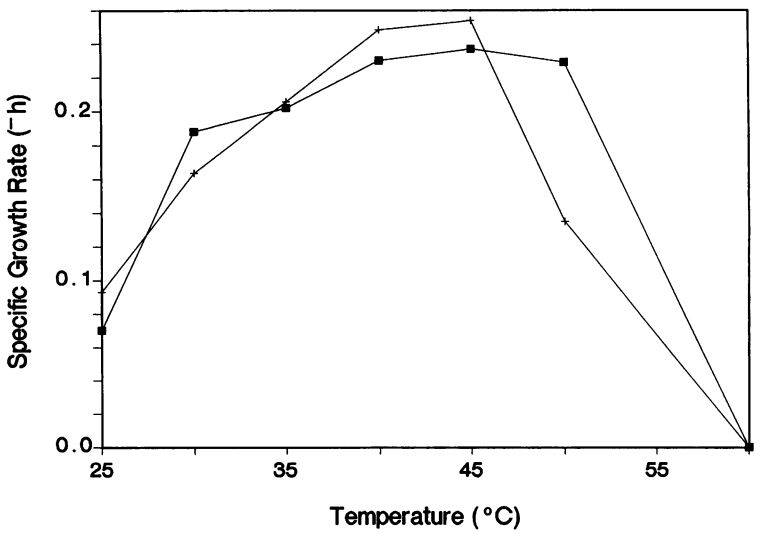

FIG. 4. Growth of strains W5C8 and W3C1 at different temperatures. Cells were grown at different temperatures in medium HS-1 containing 2 M NaCl. The specific growth rates for strain W5C8 (+) and strain W3C1 (■) were determined during early exponential growth.

TABLE 1. Strain WC58 and W3C1 fermentation products from glucose and NAG[a]

Strain	Substrate	Optical density at 560 nm	Final pH	Concn of products (μmol per tube)			
				CO_2	H_2	Acetic acid	Isobutyric acid
W3C1	Glucose	0.37	5.1	75.7	148.1	37.4	0.22
W5C8	Glucose	0.46	5.0	74.3	144.5	44.1	0.20
W5C8	NAG	0.42	5.2	96.9	160.1	129.0	0.22

[a] Cultures grown in modified medium as described in the text were used to inoculate anaerobic culture tubes containing 5 ml of the same fresh medium supplemented with 12% NaCl and 0.5% glucose or 0.5% NAG. Cultures were incubated at 37°C for 24 h before analysis.

Description of *Haloanaerobacter* gen. nov. *Haloanaerobacter* (Ha.lo.an.a.e.ro.bac'ter. Gr.n. *halo*, salt; Gr.pref. *an*, not; Gr.n. *aer*, air; M.L. masc. n. *bacter*, a rod or staff; M.L. fem. n. *Haloanaerobacter*, salt bacterium not living in air) cells are gram-negative, colorless, motile, nonsporulating rods; the cells are long and flexible in young cultures, and short degenerate cells occur in old cultures. Cells are peritrichous.

Surface colonies are opaque and glossy with entire edges. Colony diameters range from 0.5 to 1.0 mm. Agar-embedded colonies form large gas pockets.

Halophilic. Growth occurs at NaCl concentrations of 0.5 to 5 M; optimal growth occurs at an NaCl concentration of 2 to 3 M.

Cultures are susceptible to chloramphenicol.

Obligate anaerobes; oxidase and catalase negative. Strains metabolize only carbohydrates. The major glucose fermentation products are CO_2, H_2, acetic acid, and isobutyric acid.

The type species is *Haloanaerobacter chitinovorans* sp. nov.

Description of *Haloanaerobacter chitinovorans* sp. nov. *Haloanaerobacter chitinovorans* (chi.ti.no.vo'rans. Gr.n. *chiton*, chitin; L. part. adj. *vorans*, devouring, digesting; M.L. part. adj. *chitinovorans*, chitin digesting). Characteristics are the same as those described above for the genus. Peritrichous rods are 1.4 to 8 by 0.5 μm. Growth occurs at temperatures between 23 and 50°C. Optimum growth occurs in the presence of 2 to 3 M NaCl at temperatures between 30 and 45°C. Strains are resistant to penicillin, carbenicillin, D-cycloserine, streptomycin, and tetracycline. Glucose, fructose, mannose, acetylglucosamine, sucrose, maltose, and cellobiose are fermented. The type strain, strain W5C8, uses chitin for growth.

The G+C content of the DNA is 34.8 ± 1 mol%.

Habitat: organic sediment from a solar saltern.

The type strain is strain W5C8 (= OGC 229).

TABLE 2. DNA-DNA relationships of isolates W5C8 and W3C1 and related species

Unlabeled DNA from:	% Homology with ^{3}H-labeled DNA from:			
	Strain W5C8	Strain W3C1	*Halobacteroides halobius*	*Haloanaerobium praevalens*
Strain W5C8	100.0	100.0	3.6	7.8
Strain W3C1	92.3	100.0	1.6	6.4
Halobacteroides halobius	3.6	14.8	100.0	0.0
Haloanaerobium praevalens	0.0	5.2	0.0	100.0

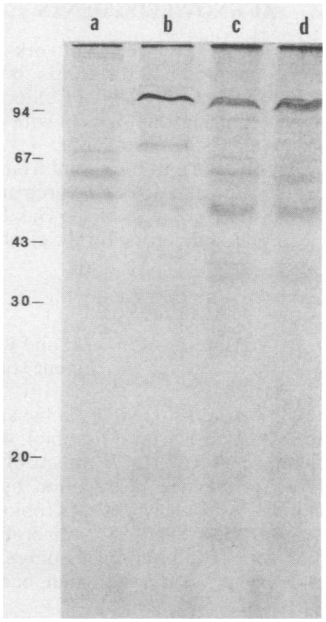

FIG. 5. Whole-cell proteins of strains W5C8 and W3C1, *Halobacteroides halobius* ATCC 35273, and *Haloanaerobium praevalens* DSM 2228. Polypeptides from whole cells were separated on a 12.5% sodium dodecyl sulfate–polyacrylamide gel and stained with Coomassie blue. Lane a, strain ATCC 35273; lane b, strain DSM 2228; lane c, strain W3C1; lane d, strain W5C8. The positions of protein size standards are indicated on the left.

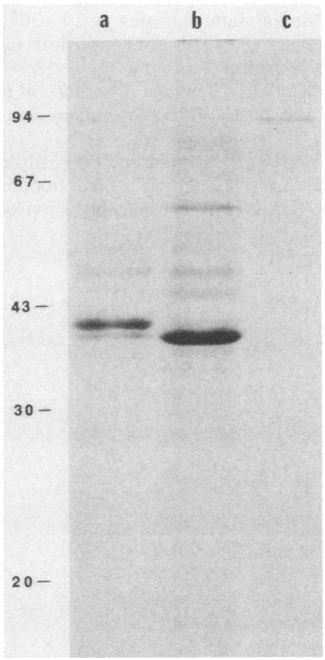

FIG. 6. Extracellular proteins of strain W5C8. Two polypeptides (38 and 40 kDa) were observed in the 4-day (lane a) and 7-day (lane b) chitin cultures, but not in the NAG culture (lane c). The positions of protein size standards are indicated on the left.

ACKNOWLEDGMENTS

We thank Mark McCortney, Western Saltworks Co., Chula Vista, Calif., for his kind cooperation and for access to the solar salterns; J. Pangborn and R. J. Munn, University of California, Davis, for the electron microscopy; Mei Chin Lai for help with culture work; and R. P. Gunsalus for perusing the manuscript.

This study was supported in part by a grant from the University of California Systemwide Biotechnology Research and Education Program and by the IFAS-GRI program of the Gas Research Institute and the Institute of Food and Agricultural Sciences, University of Florida.

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