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Halococcus salifodinae sp. nov., an Archaeal Isolate from an Austrian Salt Mine

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A novel extremely halophilic archaeon (archaebacterium) was isolated from rock salt obtained from an Austrian salt mine. The deposition of the salt is thought to have occurred during the Permian period (225 × 10^6 to 280 × 10^6 years ago). This organism grew over a pH range of 6.8 to 9.5. Electron microscopy revealed cocci in tetrads or larger clusters. The partial 16S rRNA sequences, polar lipid composition, and menaquinone content suggested that this organism was related to members of the genus *Halococcus*, while the whole-cell protein patterns, the presence of several unknown lipids, and the presence of pink pigmentation indicated that it was different from previously described coccoid halophiles. We propose that this isolate should be recognized as a new species and should be named *Halococcus salifodinae*. The type strain is Blp (= ATCC 51437 = DSM 8989). A chemotaxonomically similar microorganism was isolated from a British salt mine.

Viable halophilic microorganisms have been isolated recently from various ancient salt deposits in England which originated in the Triassic period (195 × 10^6 to 225 × 10^6 years ago) or the Permian period (225 × 10^6 to 280 × 10^6 years ago) (18). We have also been able to cultivate halophilic bacteria from rock salt which was obtained from the Austrian salt mine near Bad Ischl; this salt is believed to have been deposited during the Permian period (30). A preliminary biochemical description of some of the isolates obtained from the British salt deposits as well as the Austrian salt mines has been published previously (24). In this paper we present a detailed characterization of one of the Austrian halophilic coccoid isolates (strain Blp[T] [T = type strain]) which could grow over a wide pH range, and we propose a new species, *Halococcus salifodinae*.

MATERIALS AND METHODS

Culture conditions and bacterial strains. The methods used to isolate bacteria from dry rock salt have been described previously (24). The pH of the growth medium (M2 medium [27]) was 7.4, unless indicated otherwise. When a pH of >8.5 was desired, the medium of Tindall et al. (26) was used, and the pH was adjusted accordingly. Growth in liquid cultures was monitored spectrophotometrically at 600 nm with a Novaspec II instrument (Pharmacia) or at 660 nm (red filter of a Klett-Summerson colorimeter). Utilization of carbohydrates was tested in minimal medium M2A, which contained 50 mM Tris-HCl, 4 M NaCl, 0.1% yeast extract, 1 mM NH_4Cl, 27 mM KCl, 100 mM $MgCl_2$, 1.4 mM $CaCl_2$, 0.1% trace elements solution SL-6 (12), 1% carbohydrate, and 0.002% phenol red as a pH indicator. Growth on carbon sources was determined by monitoring turbidity and was compared with growth of a culture in minimal medium that contained no added carbohydrates. The range of salt concentrations which permitted growth was determined by spreading 100-μl portions of a growing culture on agar plates containing M2 medium supplemented with final NaCl concentrations of 0, 0.5, 1.0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, and 30%. Cultures of strain Blp[T] on agar plates were incubated for 10 to 20 days.

The following archaeal strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen: *Halobacterium salinarium* DSM 668, *Halococcus morrhuae* DSM 1307[T] and DSM 1309, *Halococcus saccharolyticus* DSM 5350[T], *Natronococcus occultus* DSM 3396[T], *Natronobacterium magadii* DSM 3394[T], *Natronobacterium gregoryi* DSM 3393[T], and *Natronobacterium pharaonis* DSM 3395. The strains used to determine the data in Table 1 and for Fig. 5 were obtained from the National Collection of Industrial and Marine Bacteria, Ltd., Aberdeen, Scotland, or from sources described in reference 13. *Halobacterium saccharovorum* M6 (= ATCC 29252), *Halobacterium halobium* R1, *Haloferax denitrificans* ATCC 35960, and *Haloarcula vallismortis* were obtained from L. I. Hochstein, NASA Ames Research Center; *Haloarcula californiae* ATCC 33799 and *Haloarcula hispanica* were gifts from R. H. Vreeland, West Chester University; and *Haloarcula japonica* TR-1 was a gift from K. Horikoshi, The Riken Institute.

Biochemical tests. Catalase activity was determined by placing 1 drop of a 3% (vol/vol) H_2O_2 solution on a lawn of bacteria; the formation of gas bubbles indicated a positive reaction. Oxidase activity was detected by spotting a loopful of a bacterial culture on a paper strip containing *N,N*-dimethyl-1,4-phenylenediammonium chloride and α-naphthol; a blue color revealed the presence of the enzyme. Nitrate reduction was determined as described previously (22). Gelatin liquefaction was assayed in tubes containing M2 broth supplemented with 12% gelatin as described by Tomlinson and Hochstein (27) following incubation at 37°C for 4 weeks.

Analysis of lipids and menaquinones. The preparation of polar lipid extracts and analysis by two-dimensional thin-layer chromatography have been described by Ross et al. (21). Menaquinones were extracted with acetone from lyophilized cells and were purified by thin-layer chromatography (10). Menaquinones were separated on a reverse-phase column

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FIG. 1. High-resolution field emission scanning electron micrograph of Blp^T grown in liquid culture (M2 medium, pH 7.4) to the end of the exponential phase. The accelerating voltage was 3 kV.

(C_{18}) by high-performance liquid chromatography (HPLC) (Waters Instruments), and separation was monitored with a UV detector at 269 nm. A mixture of methanol and 2-chlorobutane (9:1) was used as the eluent; the flow rate was 1 ml/min. Quinones were identified by comparison with the quinones extracted from *Halobacterium saccharovorum* M6.

DNA base composition. Cells were harvested in the early stationary phase of growth, and G+C contents were determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. DNA was isolated by the procedure of Visuvanathan et al. (28) and was analyzed by HPLC. Nonmethylated lambda virus DNA was used for calibration (15).

Sequencing of 16S rRNA and analysis. DNA extraction, amplification of the 16S rRNA gene by the PCR, and sequence analyses were performed as described by McGenity and Grant (13), except that only one of the primers (517 R) was used. A total of 212 bases were sequenced from the 16S rRNA gene of Blp^T and a similar isolate obtained from a British salt mine (strain Br3) (18).

Electron microscopy. Cells were washed three times with a buffer containing 2 mM $MgSO_4$ and 75 mM sodium cacodylate (pH 7.2) and then were fixed with 2% glutaraldehyde in the same buffer for 1 h at room temperature and washed again. The cells were prepared for scanning or transmission electron microscopy as described by Witte et al. (29). Scanning electron microscopy and transmission electron microscopy were performed with a Hitachi model S-4100 field emission scanning electron microscope and a Siemens Elmiscop 101 transmission electron microscope, respectively.

Other methods. Unstained cells were observed with a Leitz Diaplan microscope by using phase-contrast techniques. Gram staining of cells was carried out as described by Dussault (2). Assays to determine antibiotic susceptibility, lysis of halobacterial cells in water, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or isoelectric focusing of whole-cell proteins were performed as described previously (23, 24). In addition to the antibiotic aphidicolin used previously, 40 μg of each of the following antibiotics per disc was used in this study: ampicillin, anisomycin, bacitracin, chloramphenicol, nalidixic acid, novobiocin, streptomycin, and tetracycline. Lysis by bile acids was assayed by monitoring the turbidity of cells suspended in a solution which consisted of 1% Bacto-Peptone (catalog no. 0118-01; Difco) in 20% NaCl, as described by Kamekura et al. (8). In addition, cells suspended in this solution were observed by phase-contrast microscopy for a total of 24 h.

Nucleotide sequence accession numbers. The full 16S rRNA sequence of *Natronococcus occultus* as determined by McGenity and Grant (13) was deposited in the EMBL Data Library under accession number Z28378; partial 16S rRNA

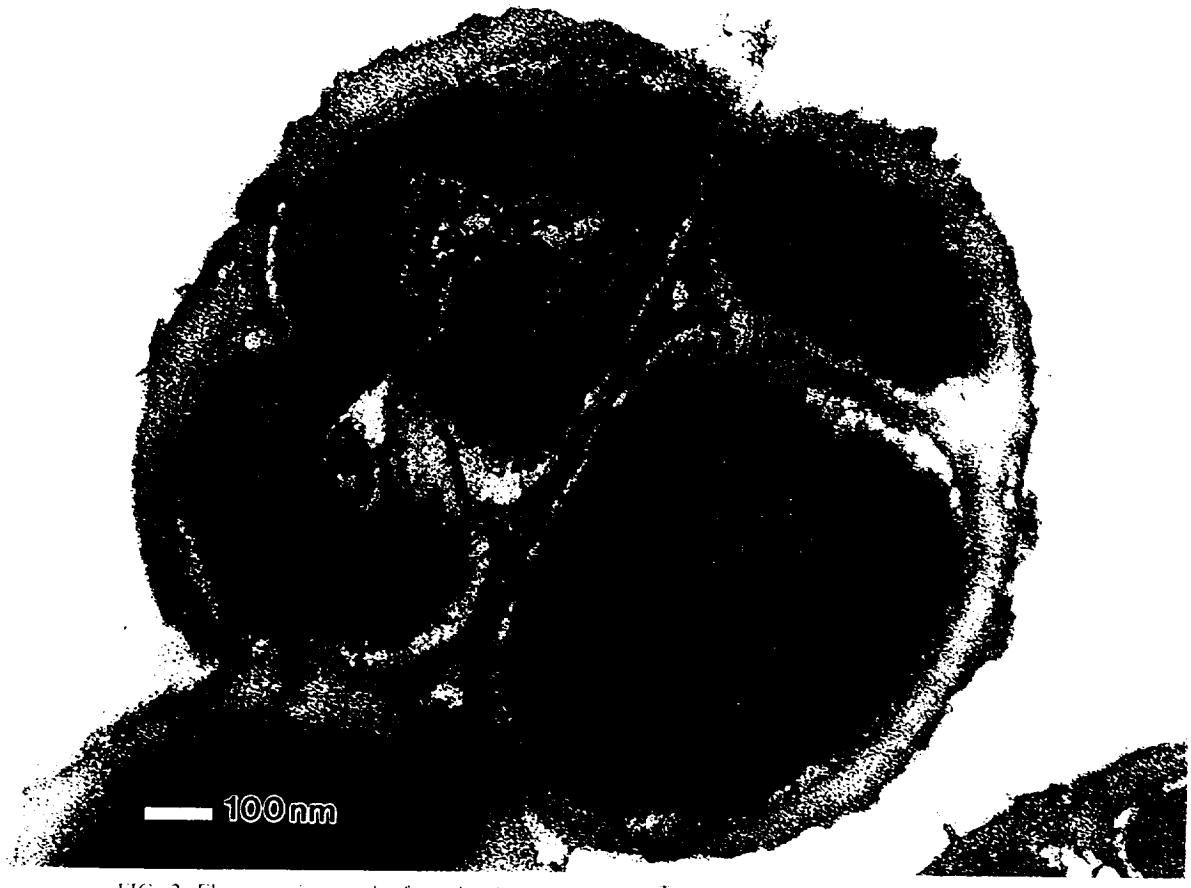

FIG. 2. Electron micrograph of an ultrathin section of Blp[T] grown as described in the legend to Fig. 1

sequences of strains Br3 and Blp[T] (this study) were deposited under accession numbers Z28379 and Z28387, respectively.

RESULTS AND DISCUSSION

Cell and colony morphology. Isolate Blp[T] cells were cocci about 0.8 to 1.2 μm in diameter (Fig. 1 and 2). The cells were uniformly gram negative, whether they were from 3-, 5-, or 14-day-old cultures. Cells grown in liquid medium were nonmotile and occurred in tetrads, in sarcina-like packets or (in older cultures) in large clusters (Fig. 1). Thin sections revealed a thick cell envelope (Fig. 2) with an irregular outer layer. Some of the outermost material appeared to be detachable (Fig. 1) and may have represented an extracellular slime which held the cells together. Colonies on complex medium at pH 7.4 were circular with undulate margins and about 1 to 2 mm in diameter after 10 days of incubation at 37°C and had pink pigmentation; older colonies were brownish. Colonies on medium at pH 9.5 were nonpigmented.

Cultural and biochemical characteristics. Strain Blp[T] grew aerobically with doubling times of 24 to 28 h at 37°C in liquid M2 medium (pH 7.4) with shaking. Catalase, nitrate reductase, and oxidase activities were detected. Gelatin was liquefied. Growth in test tubes containing broth occurred as sediment. Similar growth was observed in media at pH 6.8, 8.5, or 9.5, with no apparent pH optimum in this range. No growth occurred at pH 10.5 or below pH 6.0; growing cells which were suspended in media at those pH values appeared to lyse slowly. Optimal growth occurred at NaCl concentrations of 20 to 25%; no growth was observed at NaCl concentrations below 15%. Like the cells of other halococci, the cells did not lyse within 1 to 2 h when they were suspended in distilled water (5). However, after incubation for 24 h, a portion of the cells had lysed, releasing proteins whose compositions were similar to the compositions shown in Fig. 3, lane 1. No lysis occurred when cells were suspended in a 20% NaCl solution containing bile acids, as was observed with other halococci (8). The temperature range for growth was between 28 and 50°C, and the optimum temperature was 40°C. A minimal medium containing 0.1% yeast extract supported growth when carbohydrates were present. D-Glucose, D-fructose, D-trehalose, D-raffinose, and glycerol were utilized with slight acidification of the medium, whereas utilization of D-galactose, L-rhamnose, D-xylose, and D-arabinose produced more intense acidification. Erythritol was not utilized.

Growth of Blp[T] was strongly inhibited by the antibiotics anisomycin, aphidicolin, bacitracin, and novobiocin. Moderate susceptibility to chloramphenicol and tetracycline was observed. No inhibition of growth occurred with ampicillin, nalidixic acid, or streptomycin.

Gel electrophoresis of whole-cell proteins. SDS-PAGE of whole-cell protein is a rapid method for distinguishing bacterial species, and the level of discrimination of this method is similar to that of DNA-DNA hybridization (6). Strain Blp[T] had a unique protein profile following SDS-PAGE which did not

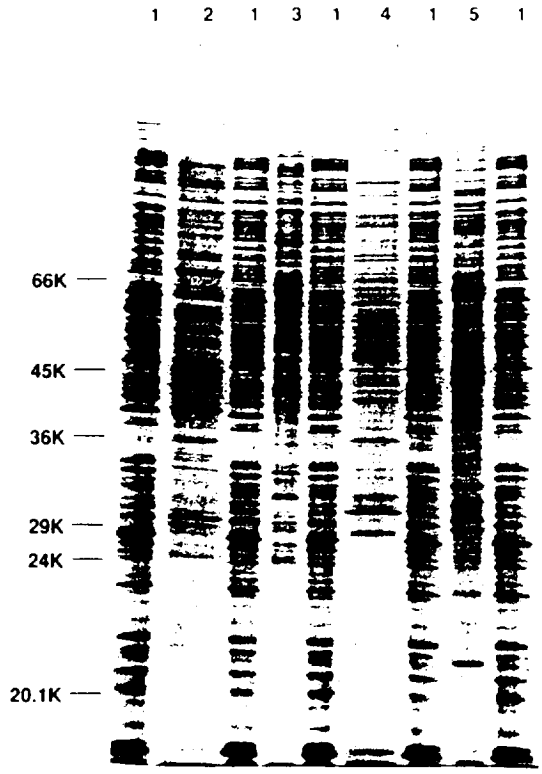

FIG. 3. Whole-cell proteins from various halophilic archaeal strains and Blp^T following separation by SDS-PAGE. Approximately 20 μg of protein was applied per lane following lysis of cells. Proteins were stained with Coomassie blue. Lanes 1, Blp^T; lane 2, *Halococcus saccharolyticus* DSM 5350^T; lane 3, *Natronococcus occultus* DSM 3396^T; lane 4, *Halococcus morrhuae* DSM 1309; lane 5, *Haloarcula californiae*. The positions of molecular mass markers are indicated on the left.

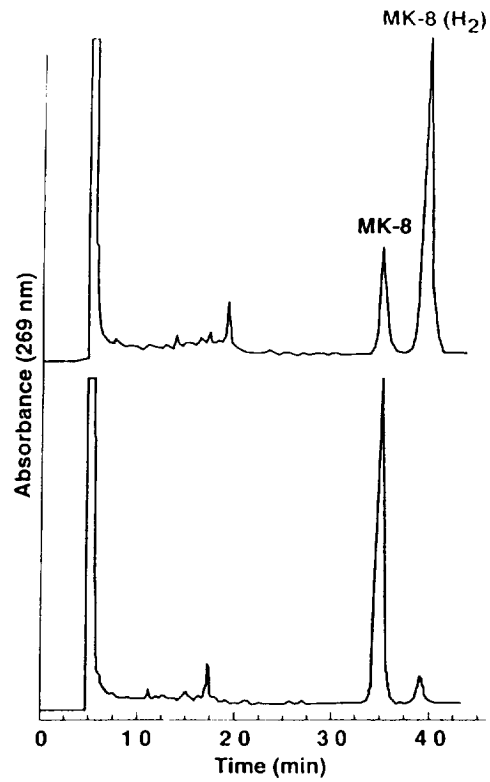

FIG. 4. Separation of menaquinones MK-8 and MK-8(H_2) from Blp^T (upper trace) and *Halobacterium saccharovorum* M6 (lower trace) on a C_{18} reverse-phase column.

resemble the profile of any of the halobacterial type strains which were analyzed. In particular, none of the protein patterns of the halophilic coccoidal archaebacterial strains was similar to the Blp^T pattern (Fig. 3). In addition, the whole-cell protein patterns of *Haloarcula californiae* (Fig. 3), *Haloarcula japonica*, *Haloarcula hispanica*, *Halobacterium saccharovorum*, *Halobacterium halobium* R1, *Halobacterium salinarium*, *Haloferax denitrificans*, *Natronobacterium pharaonis*, *Natronobacterium magadii*, and *Natronobacterium gregoryi* were not similar to the strain Blp^T pattern (data not shown). The protein patterns of Blp^T were identical, whether the strain was grown at pH 7.4 or pH 9.5. A coccoidal halophilic isolate obtained from a British salt mine, Br3 (18), produced a whole-cell protein pattern similar to that of strain Blp^T; there were only minor differences in the relative intensities of some bands (data not shown).

Extremely halophilic bacteria are known to possess acidic bulk proteins (19), whose isoelectric points range between pH 3.6 and 5.0 (23). Isoelectric focusing gels of whole-cell proteins of strain Blp^T revealed almost exclusively acidic proteins with isoelectric points between 3.8 and 4.5. The overall protein pattern observed in isoelectric focusing gels was different from the patterns of all of the halophilic archaeal type strains described previously (data not shown).

Polar lipids and menaquinones. Two-dimensional thin-layer chromatography of lipids revealed $C_{20}C_{20}$ and $C_{20}C_{25}$ archaebacterial core lipids. Phosphatidylglycerol and phosphatidylglycerol phosphate were present, but no phosphatidylglycerol sulfate was detected. Five types of glycolipids were found; one of these was sulfated diglycosyldiphytanylglycerol, and the other four were unknown glycolipids. In the HPLC analysis the quinone extract of Blp^T produced two peaks with the same retention times as peaks detected in an extract of *Halobacterium saccharovorum* (Fig. 4). *Halobacterium saccharovorum* M6 is known to possess two types of menaquinones (25): MK-8, a menaquinone with eight isoprenoid units in the side chain, is the main component, while the dihydromenaquinone MK-8(H_2) has been detected in only minor amounts. Strain Blp^T also possessed both of these menaquinones, although the major quinone (77%) was MK-8(H_2), while MK-8 was present in smaller amounts (23%). The dominance of menaquinone MK-8(H_2) in strain Blp^T indicated that this strain is related to *Halococcus morrhuae*, which is also characterized by the presence of major amounts of MK-8(H_2) and minor amounts of MK-8 (1).

G+C content. The DNA base composition of Blp^T was 62 ± 1 mol% G+C (three determinations). This value is within the range found for *Halococcus morrhuae* (61 to 66 mol%) (11) and similar to the values found for *Halococcus saccharolyticus* (59.5 mol%) (16) and *Natronococcus occultus* (65 mol%) (4).

Phylogeny. Table 1 shows a matrix of similarity values for Blp^T and selected archaeal halophilic reference organisms based on a sequence analysis of 212 bases of the 16S rRNA gene. The sequence of isolate Br3 in this region was identical to that of Blp^T. On the basis of the phylogenetic tree constructed from this analysis we concluded that Blp^T represents a distinct lineage within the halophiles and is most similar to

TABLE 1. Matrix showing relationships between species of halobacteria, based on an analysis of 212 bases of the 16S rRNA gene (positions 227 to 438 in the *Halococcus morrhuae* ATCC 17082[T] sequence)

Organism(s)	Avg no. of nucleotide substitutions per site or % similarity[a]							
	Natronococcus occultus NCMB 2192[T]	*Natronobacterium magadii* NCMB 2190[T]	*Halobacterium salinarium* R1	*Haloferax volcanii*	*Halococcus morrhuae* ATCC 17082[T]	*Halococcus morrhuae* NCMB 746	*Halobacterium saccharovorum* NCMB 2081[T]	Blp[T] and Br3
Natronococcus occultus NCMB 2192[T]		94.76	92.38	92.85	91.43	89.95	89.00	89.05
Natronobacterium magadii NCMB 2190[T]	0.0543		89.62	95.75	94.81	93.36	86.19	92.42
Halobacterium salinarium R1	0.0803	0.1117		91.04	88.21	88.15	90.95	89.10
Haloferax volcanii	0.0751	0.0437	0.0954		93.87	92.89	88.10	92.42
Halococcus morrhuae ATCC 17082[T]	0.0910	0.0538	0.1283	0.0640		98.57	87.15	95.26
Halococcus morrhuae NCMB 746	0.1079	0.0695	0.1290	0.0747	0.0144		85.65	93.81
Halobacterium saccharovorum NCMB 2081[T]	0.1190	0.1526	0.0964	0.1296	0.1410	0.1593		88.10
Blp[T] and Br3	0.1184	0.0799	0.1178	0.0799	0.0490	0.0646	0.1296	

[a] The values on the lower left are the average numbers of nucleotide substitutions per site (K_{nuc} values) (7), and the values on the upper right are the percentages of similarity. The EMBL Data Library accession numbers for some of the sequences are given in the text.

Halococcus morrhuae ATCC 17082[T] (Fig. 5). Interestingly, our data indicated that the level of relatedness between Blp[T] and the alkaliphilic coccus *Natronococcus occultus* was lower than the level of relatedness between Blp[T] and the rod-shaped organism *Natronobacterium magadii* or the pleomorphic organism *Haloferax volcanii* (Table 1 and Fig. 5). To date, the following three species of haloneutrophilic cocci and one species of haloalkaliphilic archaeal cocci have been recognized: *Halococcus morrhuae*, *Halococcus saccharolyticus* (16), *Halococcus turkmenicus* (31) and *Natronococcus occultus* (4). Strain Blp[T] could be distinguished from *Halococcus morrhuae* and *Natronococcus occultus* as described above (Fig. 3 and Table 1). Blp[T] differed from *Halococcus saccharolyticus* in pigmentation, the ability to grow at alkaline pH values, utilization of D-raffinose, L-rhamnose, and D-xylose but not erythritol, susceptibility to novobiocin, and whole-cell protein pattern (Fig. 3). A sequence comparison of the same 212 bases of the 16S ribosomal DNA gene indicated that *Halococcus turkmenicus* is phylogenetically distinct (14).

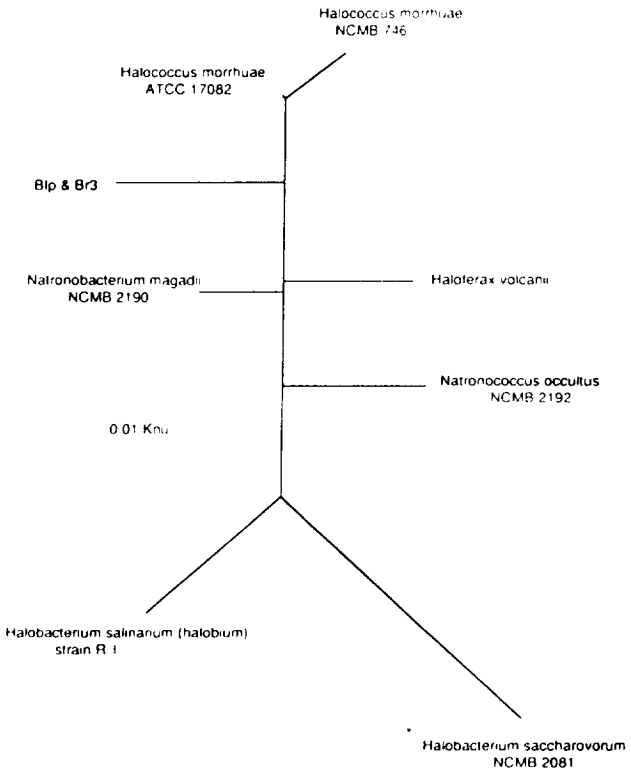

FIG. 5. Unrooted phylogenetic tree of halobacterial species, obtained from the K_{nuc} values shown in Table 1 by using the least-square algorithm of Fitch and Margoliash (3).

Conclusions. On the basis of polar lipid content, antibiotic susceptibility, and acidic bulk proteins, strain Blp[T] was identified as a halophilic archaeon (archaebacterium). The morphology, slow growth, and menaquinone content of this organism were similar to *Halococcus morrhuae* characteristics (11). However, Blp[T] differed from *Halococcus morrhuae* with respect to whole-cell protein pattern, lipid composition, partial 16S ribosomal DNA sequence, pigmentation, and pH range for growth (strain Blp[T] grew at pH 6.8 to 9.5). We believe that these data justify our proposal of a new species, *Halococcus salifodinae*.

Both Blp[T] and a similar organism (Br3) were isolated from salt mines which contain paleozoic deposits (Alpine basin and Zechstein succession, respectively) (30). When grown in a laboratory, these and other halophilic mine isolates (18, 24) were similar in many respects to typical halobacterial inhabitants of evaporitic brines (20). In present-day England and Austria no major saline evaporites are formed, as was the case during the late Permian and early Triassic periods. Taking continental drift into account (30), the two locations were close to the paleoequator during those times, implying that the average temperature was higher and the environment was arid. Disseminative forms such as spores have not been found in any archaebacterium, although halocysts, which might function as resting stages, are produced by some soil-inhabiting species (9). However, several extreme halophiles among the *Archaea* appear to survive embedding in salt crystals (17) and may be

capable of dormancy in this state. If the halophilic bacterial mine isolates are the remnants of populations that originally inhabited the paleozoic brines, they might hold great promise as organisms which could enhance our understanding of evolution and bacterial phylogeny. However, direct determination of the age of the salt-embedded microorganisms is not feasible at the present time because of a lack of suitable dating methods. Further experimental approaches will be necessary to establish if a paleozoic origin of the salt mine bacteria can be considered.

Description of *Halococcus salifodinae* sp. nov. *Halococcus salifodinae* (sa.li.fo.di'nae. L. adj. *salifodinae*, of a salt mine, referring to the source of the isolate). Cocci are 0.8 to 1.2 μm in diameter and occur in tetrads, sarcina-like packets, and irregular clusters. Gram negative; nonmotile; aerobic. Small colonies (1 to 2 mm in diameter after 1 week of incubation at 37°C) on complex medium at a neutral pH value are pink and circular, with undulate margins; colonies are nonpigmented at pH 9.5. The optimum temperature is 40°C; the pH range for growth is 6.8 to 9.5. The optimal NaCl concentration for growth is 20 to 25%; no growth occurs at NaCl concentrations below 15%. Oxidase and catalase positive. Grows in minimal medium containing 0.1% yeast extract and D-galactose, L-rhamnose, D-xylose, D-arabinose, D-glucose, D-fructose, D-trehalose, D-raffinose, or glycerol as carbon sources.

Nitrate is reduced to nitrite. Gelatin is liquefied.

Susceptible to anisomycin, aphidicolin, bacitracin, and novobiocin; slightly susceptible to chloramphenicol and tetracycline; not susceptible to ampicillin, nalidixic acid, and streptomycin.

The G+C content of the DNA is 62 ± 1 mol%.

The main polar lipids are $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate. Menaquinones MK-8(H_2) and MK-8 are present.

Whole-cell proteins are acidic with isoelectric points predominantly between 3.6 and 4.5.

Natural habitat: unknown.

The type strain is Blp, which has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 8989 and in the American Type Culture Collection as ATCC 51437.

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