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Distribution and diversity of halophilic bacteria in a subsurface salt formation

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Abstract The Waste Isolation Pilot Plant (WIPP) is a salt mine constructed 650 meters below the ground surface by the United States Department of Energy. The facility will be used for permanent disposal of transuranic wastes. This underground repository has been constructed in the geologically stable Permian age Salado salt formation. Of the wastes to be placed into the facility, 85% will be biodegradable cellulose. A 3-year survey of the bacterial populations existing within the facility was conducted. Bacterial populations were found to be heterogeneously distributed throughout the mine. Populations in some mine areas reached as high as 1.0×10^4 colony-forming units per gram of NaCl. The heterogeneous distribution of bacteria within the mine did not follow any recognizable pattern related to either age of the workings or to human activity. A biochemical comparison between ten known species of halophilic bacteria, and strains isolated from both the mine and nearby surface hypersaline lakes, showed the presence of extreme halophiles with wide biochemical diversity, some of which could prove to represent previously undescribed groups. The halophilic bacteria isolated from the mine were found to degrade cellulose and a wide variety of other carbon compounds. When exposed to two types of common laboratory paper, the cellulose-degrading halophiles attached to the substrate within 30 minutes of inoculation. Cultures enriched directly from a brine seep in the mine easily destroyed both papers and produced detectable amounts of oxalacetic and pyruvic acids. The combination of heterogeneity in the distribution of organisms, the presence of a physiologically diverse community, and the relatively slow metabolism of cellulose may explain several long-standing debates about the existence of microorganisms in ancient underground salt formations.

Key words Salt mine · Halophilic bacteria · Halobacterium · Cellulose degradation

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Introduction

During the 20th century there have been at least 106 different reports of the isolation of bacteria from underground geologic formations of various ages (Kennedy et al. 1994). Recent isolations included aerobic and anaerobic bacteria from subsurface sediments (Van Beelen and Fleuren-Kemila 1989; Bone and Balkwill 1988; Balkwill and Ghiorse 1985; Ghiorse and Balkwill 1985a, b; Balkwill 1993), and from several shallow aquifers (Wilson et al. 1983; Beloin et al. 1988). In addition, there have been a variety of reports describing the isolation of non-halophilic, halophilic, and halotolerant bacteria from subsurface salt formations and underground brines (Namyslowski 1913; Dombrowski 1961, 1963; Tasch 1963; Reiser and Tasch 1964; Norton et al. 1993; Denner et al. 1994; Huval and Vreeland 1991).

Despite the high quality of work done in these numerous studies, the data presented have continued to be debated. This controversy has largely arisen from two competing ideas. First is the fact that additional samples taken from the same environments by other scientists have been found to be sterile (W.D. Grant, W.H. Ghiorse, and D. Balkwill, personal communications). All of the experiments conducted to date have involved samples arising either from a single visit to a mine or a remote drilling operation. Under

some conditions, the researchers have had to use samples as large as a kilogram in order to obtain consistent results (W.D. Grant, personal communication).

The second part of the controversy involves the organisms themselves. Whenever samples are taken from a new or unique environment, there is a natural, and logical, expectation of difference. Yet, many of the studies that have reported isolations, have, in fact, described organisms that are similar to bacteria that are relatively easy to find in present day surface environments. This brings up the understandable debate about contamination of the samples. The fact that samples are not easily obtained may result in a relatively limited number of individual bacteria for further study. This has been especially true of halophilic bacteria. In some cases researchers have been forced to conduct their studies on relatively few isolates (Huval and Vreeland 1991) or have reported on only a single species (Denner et al. 1994). Studies in nonsaline environments have generally worked with arger numbers of strains (Balkwill 1993; Jimenez 1990). To date, only one study concentrating on halophilic bacteria found over 50 individual strains (Norton et al. 1993).

The Waste Isolation Pilot Plant (WIPP) is an underground repository built by the United States Department of Energy approximately 650 m below ground surface. The repository is designed for the safe, permanent, underground disposal of defense-related radioactive wastes. It is constructed in a geologically stable, bedded salt formation near Carlsbad, New Mexico. This salt bed, called the Salado Formation, was deposited during the Permian period, approximately 250 million years before the present. The WIPP site, within the Salado formation, possesses a significant number of unadulterated, primary salt crystals (Roedder 1984; Stein 1985a,b), unaltered horizontal salt strata, and a minimal amount of flowing external brine (Roedder 1984; Stein 1985a). These factors indicate that the site has experienced little geologic disturbance since its original deposition.

The wastes to be placed in the WIPP consist of a heterogeneous mixture of celluloses, rubbers, plastics, metals, glasses, and process sludge (Caldwell et al. 1988). Brush (1990) has estimated that celluloses, particularly various types of papers, will comprise a large fraction of the waste materials to be placed in the repository. Ultimately, the WIPP underground repository will contain approximately 4.35×10^6 kg of celluloses (Brush 1990).

The present study was conducted during a 3-year analysis of the distribution of bacteria within the United States Waste Isolation Pilot Plant. Samples were obtained from several different mine regions, and some regions were sampled on more than one occasion. The study involved isolations from both brine and solid salt crystals. This report includes comparisons of nearly 150 isolates, showing that the mine community is composed of a diverse group of organisms. It should be emphasized that the study being reported here was not conducted to show that the isolated bacteria had been trapped within the crystals and are therefore 250 million years old. That work is currently being conducted and will be reported in the future. The goals of this project were to study the distribution of halophilic microorganisms over a large area of a single mine and to study the overall biochemical diversity of any detected population.

Materials and methods

Microbiological sampling

The samples used in this project consisted of both crystals and brines flowing into the facility from elsewhere in the formation. Samples were obtained from all mine areas including the major corridors (drifts), waste-holding rooms, and maintenance areas, as well as two brine seeps, one located at the northwestern end of the mine (G-seep) and the other located at the southeastern corner (DHP402A). Crystal samples were taken directly from the mine walls using hammers and screwdrivers that were washed with sterile distilled water before each use. This washing procedure was used since normal aseptic techniques such as sterilization with burning ethanol could not be used in the mine environment because of safety restrictions. Likewise, the use of strong disinfectants was ruled out since these materials could leave residues lowering the perceived numbers of organisms, and many give off fumes that could remain in the mine air for long periods.

During this study, crystal samples were taken only from the surfaces of the mine ribs (walls) that showed no evidence of condensation or brine flow, known as weeping. This type of sampling was acceptable in this case due to the very low humidity (generally $<10\%$) found the New Mexico desert and the fact that the Salado formation has very little brine flow (Stein 1985a). Since the primary goal of the project was to isolate and characterize the halophilic bacterial population present in the mine, no effort was made to surface-sterilize the crystal samples. The samples removed from the mine area were either placed directly into sterilized containers or were added into premeasured 90-ml dilution blanks.

Ten grams of each crystal sample was weighed on a portable balance and added to 90 ml of sterilized 15% (w/v) NaCl brine to form a 1:10 dilution containing 23.5% NaCl. This part of the work was performed in the mine, after which the crystal samples were placed into a dark cooler and allowed to dissolve slowly. The dilutions and solid crystal in sterile bottles were then transported out of the mine and returned to the hotel room where they could be handled under more controlled conditions. This type of field inoculation was used because of the long travel times between the small Carlsbad, NM, airport and Philadelphia, PA. At the hotel, the samples were further diluted and used for a series of plate counts to determine the total numbers of viable halophilic organisms associated with the crystal samples. Brine samples were obtained from G-seep by dipping an open sterile bottle into the seep immediately after removing the cover used to protect G-seep. Samples of

brine from the 15 m deep DHP402A were obtained using a bailer that was permanently installed in the borehole. Five hundred ml of brine was allowed to flow out of the bailer prior to taking the sample.

At the hotel the brine and crystal samples were used directly to produce cellulose-degrading enrichments for additional studies. All brine and crystal samples were diluted, and surface-spread onto two types of agar media (CAS and CG) to determine total viable counts using standard microbiological techniques. The formulation of CAS medium has been described previously (Vreeland et al. 1984; Hesselberg and Vreeland 1995). The CG medium contained $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 20 g; KCl, 4.0 g; K_2HPO_4, 0.5 g; Na citrate, 3.0 g; glucose, 0.5 g; Solka Floc (cellulose), 4.5 g; NaCl, 200 g; Agar, 20 g; deionized water, 1000 ml, pH 7.4 with NaOH. Volumes (500 ml) of the medium were sterilized by autoclaving at 121°C for 20 min. To prevent drying, all plates were incubated in plastic Ziploc bags, containing moistened paper towels, at 35°C until colonies developed. Plates were counted following 21 days' incubation for brine samples and 45 days' incubation for crystal samples.

Culture isolations and phenotypic studies

Single colony isolations were conducted using quadrant streak plates. All colonies were genetically purified by a minimum of two successive single colony isolations. Each colony was transferred to a medium identical to that on which it was initially grown. The purified strains were characterized using 75 phenetic characters for each strain. This characterization used the test series and recommendations of Vreeland (1993). This work compared 200 fresh isolates to 10 known halophilic cultures. A list of previously identified halophiles and their sources is presented in Table 1. In order to handle the surprisingly large number of strains coming from these samples, the testing was performed in 96-well microtiter plates. All media were prepared, sterilized, and aseptically added to the microtiter plates. A single master plate containing 95 individual cultures was prepared and used to inoculate the test plates. Inoculations were performed using a Denley Well Rep II Inoculator (Denley Instruments, Durham NC, USA). In order to ensure adequate inoculation, each test plate was inoculated three times. All medium tests were performed twice. The inoculator tips were sterilized with an alcohol flame after inoculating five test plates. In addition, each test plate contained one uninoculated well to serve as a monitor for contamination. Any plates that showed reactions in the uninoculated well were discarded and the test was repeated. The test results were recorded and analyzed using the NTSYS computer program at the American Type Culture Collection. A phenetic similarity diagram was constructed using the unweighted pair group method with arithmetic averages.

Table 1. Previously described Archaeal halophiles used in this project

Culture	Source
Halorubrum saccharovorum	NCMB 2081
Halococcus morruhae	NCMB 864
Halobacterium salinarium	ATCC 33171
Haloarcula hispanica	Dr. F. Rodriguez-Valera
Haloarcula marismortui	Dr. F. Rodriguez-Valera
Haloarcula japonica	Dr. K. Horikoshi
Haloarcula californiae	Dr. F. Rodriguez-Valera
Haloarcula vallismortis	ATCC 29715
Haloferax gibbonsii	Dr. F. Rodriguez-Valera
Natronobacterium pharaonis	ATCC 35678

Enrichment cultures and attack on cellulose

The enrichment cultures were grown in a medium containing the same salt composition as the CG, supplemented with casamino acids, $0.5\,g \cdot l^{-1}$ as well as $2.5\,g \cdot l^{-1}$ of either laboratory paper towel (Scott Paper, Philadelphia, PA, USA) or Whatman #1 filter paper (Whatman, Clifton, NJ, USA). This medium was designated FP. Enrichment samples with growth were plated onto solid FP medium with filter paper. Plates were screened for positive cellulose utilization by the Congo Red procedure as described by Coughlan and Mayer (1992). The flask with the highest percentage of positive cultures (G-Seep brine) was then diluted with sterile 30% NaCl brine to an optical density of 0.250 at 660 nm. This enrichment sample was used as the inoculum in the following paper degradation experiments.

The mixed cellulolytic population from G-Seep brine and a pure culture of *Haloarcula hispanica* grown in CG medium, with cellobiose as the main carbon source, were also used for an attachment study.

Attachment studies

Initial optical densities of *H. hispanica* and the mixed cellulolytic population from G-Seep brine were measured using a Perkin Elmer UV/Vis lambda 4b spectrophotometer set to 660 nm. Three 15-ml aliquots of each culture were transferred to sterile 20-ml test tubes, each of which contained one sheet of Whatman #1 filter paper with a diameter of 12.5 cm. The tubes were placed in an incubator shaker set to 37°C at 180 rpm. All tubes were sampled every 30 min for 3 h. Replicates were averaged to generate a curve showing a change in optical density over time.

Electron microscopy

After 2 years of incubation, samples of paper from the long-term degradation cultures were prepared for scanning electron microscopy (SEM). For comparison purposes, samples of papers exposed to bacterial attack for 14 days and papers that were suspended in sterile salt brine for 2 years were also prepaped for SEM examination. All paper samples were suspended in concentrated salt brine (30% NaCl) and an equal volume of OsO_4 (4%) was added to the solution to give a final salt concentration of 15% with an OsO_4 concentration of 2%. Papers were fixed in osmium for 2 h, then in NaCl-saturated 3% glutaraldehyde solution overnight. The

samples were then dehydrated through an alcohol series starting with 50% ethanol in 15% NaCl. Other ethanol concentrations were 80%, 90%, 95%, and 100% (3X). The NaCl brine was removed from the ethanol after the 80% step. Paper samples were stored overnight in 100% ethanol to ensure complete removal of water. They were then critical-point dried, mounted onto aluminum stubs, and sputter-coated with gold for 1 min at 1 KV. The stubs were viewed in a Joel SEM310 scanning electron microscope at 10 KV. Photomicrographs were taken using Type 52 Polaroid film. This SEM work and the photographs used in this paper were produced at the Agriculture Research Service, Eastern Regional Research Center in Wyndmoor, PA, USA.

Organic acid production and analysis

The breakdown of individual carbohydrates or sugar polymers such as glucose and cellulose often leads to the production of significant amounts of small molecular weight organic acids. Organic acid production by these bacterial populations was determined by allowing the cellulose-degrading bacterial population, enriched from G-seep brine, to attack two common laboratory papers for a period of up to 64 days. This work was carried out using FP medium supplemented with sterilized Whatman # 1 and Scott brand paper towels. The cultures were incubated at 37°C on a rotating shaker (180 rpm).

The following standard high-purity acids were purchased from either Sigma (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA): oxalic, oxalacetic, citric, α-ketoglutaric, pyruvic, malonic, methylmalonic, succinic, lactic, formic, acetic, proprionic, isobutyric, butyric, isovaleric, valeric, isocaproic, and caproic acid.

Each acid was individually chromatographed to determine: (1) retention time and (2) a reasonable concentration for a standard solution. The standard acids were chromatographed using 20 µl of a 10 mM solution of the acid in 0.007 N sulfuric acid. Following chromatography of the individual acids, a mixed acid standard was prepared in HPLC-grade water and chromatographed. Another standard mix was prepared and extracted from pure G-seep brine to check for the efficiency of extraction and to be sure the medium did not significantly alter the chromatographic behavior of the acids. Since the NaCl-saturated brine only reduced the extraction efficiency of 3 of the 10 organic acids by more than 10% and did not affect the chromatographic behavior of any of the acids, the data are not shown in this paper. Interested parties can contact the corresponding author to obtain these data.

All chemicals used for extraction were also chromatographed to check for contaminant peaks. The ether used for extraction produced a single contaminant peak at 32.55 minutes. This peak was ignored during all subsequent work. HPLC-grade acetonitrile, diethyl ether, and sodium hydroxide were purchased from Fisher Scientific. Sulfuric acid and HPLC-grade water were purchased from Baker (Phillipsburg, NJ, USA).

Analytical procedure

One milliliter of culture supernatant was placed into a 15-ml conical, glass, screw-top test tube and the following reagents were added: 0.2 ml 18 N H_2SO_4, 0.6 gm NaCl, 5 ml diethyl ether, and 25 µl acetonitrile. The mixture was blended on a vortex mixer for 1 min then centrifuged at 1000 rpm for 1 min. All but the final 2 mm of the ether phase was transferred to a clean test tube and 0.2 ml of 0.1 N NaOH was added to the new tube. The solution was mixed gently. A small portion of the NaOH layer was removed with a Pasteur pipette and tested on pH paper. If the pH was less than 9, 1 N NaOH was added, drop by drop, until the pH was 9 or greater. The tube was mixed vigorously for 1 min and then centrifuged for 1 min. Following centrifugation, the ether layer was removed and discarded. Acetonitrile (25 µl) was added, and the tube was allowed to stand for 5 min, after which it was mixed again. Following mixing, 20 µl was introduced into the chromatographic system.

Equipment and chromatographic conditions

Organic acids were analyzed on a Perkin Elmer high performance liquid chromatograph system (Perkin Elmer Norwalk, CT, USA). The system contained a Perkin Elmer Bio 410 pump, a Dupont manually operated column compartment (P.N. 851100-901) with a Rheodyne 7150 injector and 20-µl sample loop, a Perkin Elmer LC 235 UV/Vis diode array detector, and a Perkin Elmer LCI-100 laboratory computer integrator. The mobile phase used throughout the experiment was 5% acetonitrile (Fisher-analyzed HPLC grade) in 0.007 N H_2SO_4, (0.33 ml of Baker grade sulfuric acid per liter of HPLC grade water; Baker). The mobile phase was filtered through 0.22-µm filters and sparged with helium before use. The chromatography was conducted using a flow rate of 0.5 ml/min and a column temperature of 35°C. The total analysis run time was 60 min.

Results

Distribution of bacteria in the underground formation

Over the course of this study, samples were obtained from nearly all of the long-mined-out corridors (known as drifts) within the underground facility. The data, in Table 2, demonstrate the typical distribution pattern of the bacterial population within the underground mine workings. Probably the most frequent result was finding salt crystals with fewer than 10 bacteria per g of salt. These samples have been presented as "0" in the Table. One problem encountered during this study was an inability to continuously sample a wide variety of sites. This was caused primarily by ongoing mine maintenance, safety concerns, and in the case of the brine samples, a lack of in-flowing brines. Consequently, only three sites within the mine (including both brine sites) were sampled two or more times.

Table 2. Distribution of halophilic bacteria in the Waste Isolation Pilot Plant mine

Site of origin	CAS total	CAS red	CG total	CG red
G-Seep 1	10000	9100	4000	4000
G-Seep 2	7700	3200	ND	ND
DHP402A 1	4900	4900	61000	4000
DHP402A 2	140	30	ND	ND
E0N338.5	6300	5700	16100	3800
E0N61.5	6900	3000	14000	7900
E0C&SH	0	0	0	0
Room 3	225	200	0	0
Room 5	780	600	0	0
Room 7	0	0	0	0
WHS	425	0	1900	1500

The data from G-Seep and DHP402A are expressed as colony-forming units per ml of brine. All other values are expressed in terms of colony-forming units per g of salt.
ND, not done; CAS, CG, media; "Total" total number of viable colony forming units; "Red" number of colony forming units producing RED pigments.

The two brine-containing bore holes that were sampled (G-Seep and DHP402A) were located at opposite ends of the facility and were supplied from very different sources within the Salado formation (L. Brush, personal communication). Both brines produced viable colonies on the two occasions on which they were available for sampling. The number of viable bacteria within these brines was around 4000 colony forming units per ml (CFU/ml). On one occasion DHP402A-1 brine contained 6.1×10^4 CFU/ml. Most of the cultured bacteria produced white colonies resembling halotolerant eubacteria such as the various *Halomonas* species. One G-Seep sample contained 1.0×10^4 CFU/ml which grew primarily on the CAS medium.

Numerous crystal samples taken from various areas of the mine showed that the viable bacterial population was heterogeneously distributed. This heterogeneity did not correlate with the age of the mine region or the amount of human activity. The oldest drift, East 0 (Table 2), contained at least two areas with active bacterial populations. The bacterial populations at the East 0 North 1100 (E0N1100, Table 2), and East 0 North 200 (E0N200, Table 2) sites were remarkably similar. The populations growing on CAS contained 6300–6900 CFU/g salt. Red extreme halophiles made up 90% of the CAS population at E0N1100 but only 40% of the CAS population at E0N200. In contrast, only 25% of the CG population at E0N1100 produced red colonies (Table 2). The salts from E0N200 contained more total and red colonies on CG medium than did the E0N1100 salts. It should be noted that the oldest, and one of the busiest, mine regions, the construction and salt-handling (E0C&SH, Table 2) shaft, proved to have apparently sterile crystals. This was not the only busy region of this drift sampled, however, since the E0N200 site was immediately across from the underground conference and lunchroom.

Within the underground waste handling and containment areas the bacterial population continued to demonstrate a heterogeneous distribution. The largest population detected (1900 CFU/g salt) was found near the bottom of the waste-handling shaft (WHS, Table 2). Within the waste rooms the bacterial populations were very low. The apparent zero sample taken in room 7 was especially noteworthy, since this sample was obtained from the southernmost wall of the facility at a point that was less than 3 m from the DHP402A bore hole. This zero sample was also obtained on the same trip as the DHP402A-1 sample that yielded 6.1×10^4 CFU/ml.

The WIPP facility is aerated by a single large air intake shaft and an exhaust shaft. This system pumps nearly 11328 m^3 of air per min through the mine. Consequently, one possible source for the viable bacterial population present in the facility would be air flow. The viable bacterial counts were probably not due to air circulation, as shown by tests using Petrie plates exposed to the flowing air. The plates were exposed in two ways: several were carried open (medium facing forward) while driving at 8 kph for 360 m along the west 30 mine drift, which represents the mine area with the least amount of air movement. A second set of plates were exposed for several min at the base of the air exhaust shaft which represents the mine region with the highest air flow. No colonies were detected on either set of air-exposed plates.

Phenotypic comparisons of isolates

During the course of this study, over 200 strains were isolated from the WIPP and from a group of hypersaline lakes near the mine. Of these isolates, 121 were red, extremely halophilic organisms. The phenogram (Fig. 1) presents the results of a numerical taxonomic comparison of the isolated, extremely halophilic strains plus some of the known strains. During the course of this work, representatives of 12 known extremely halophilic species were found (Fig. 1). In collapsing the figure for reproduction, five known groups that were composed of a small number of strains (one or two isolates plus a known strain) have been omitted. In addition, several new halophilic genera – *Natrialba* (Kamekura and Dyall-Smith 1995), *Halobaculum* (Oren et al. 1995), and *Halorubrum* (McGenity and Grant 1995) – were described during the analytical phase of this work. Consequently, no representatives of these genera were included in the analysis.

The computer analyses revealed 8 new phenetic groups that fell within the confines of the family *Halobacteriaceae* (Phenon A, Fig. 1). The computer analysis also separated a second phenon (Phenon B, Fig. 1). This particular group contained a high percentage of isolates from mine samples and was unique in that it contained no known strains. Phenon B was composed of at least 5 phenetically similar bacterial groups. Phenon B also contained several unknown strains originally isolated from other US salt formations by Huval and Vreeland (1991). The largest collection of phenetically similar groups of cultures (unknown groups 11 and 12) contained 9 and 18 strains, respectively. Two-thirds of the strains comprising Group 11 originated directly from the WIPP underground or from another underground formation. The other 3 strains were isolated directly from

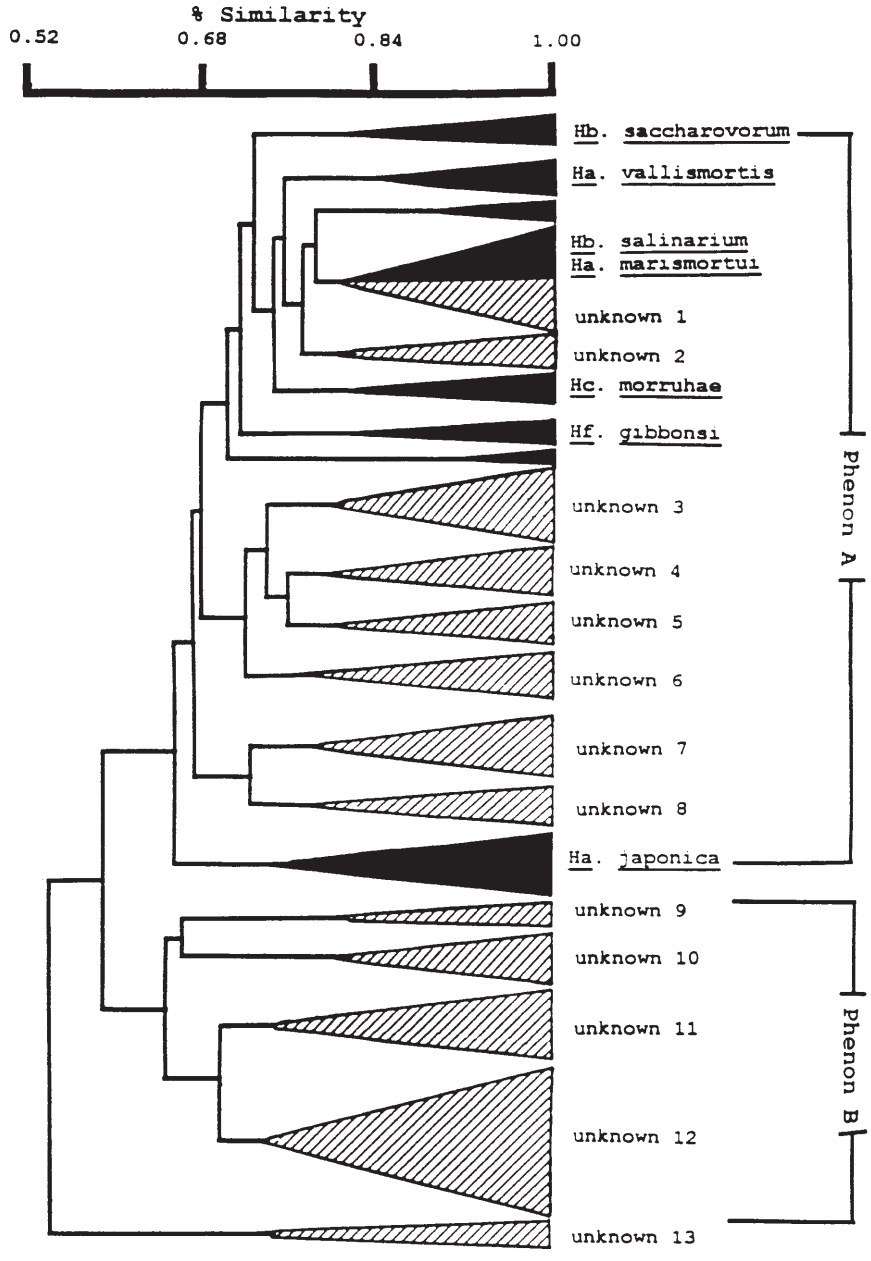

Fig. 1. Phenogram comparing the bacterial cultures isolated from the Waste Isolation Pilot Plant (WIPP) and from nearby hypersaline lakes with various known extreme halophiles. *Ha*, *haloarcula*; *Hb*, *halobacterium*; *Hc*, *halococcus*; *Hf*, *haloferax*

the underground brine outflow of a local potash mine. Complete taxonomic characterization of many of these new bacterial groups is currently being completed. This characterization and formal descriptions of these bacteria will be presented in other publications.

Attachment to cellulose

Figure 2 presents the results of an experiment conducted to determine the rate at which halophilic bacteria growing on an insoluble carbon source, such as cellulose, would attach to the substrate. The optical density of the mixed cellulolytic culture began to decrease within 30 min of exposure to the paper. The optical density of the culture ultimately decreased from 0.93 to 0.29, a change of 0.64 units, after 3 h of incubation (Fig. 2). In contrast, the optical density of the *Ha. hispanica* decreased only 0.16 units during the same period (Fig. 2). The uninoculated, sterile CG medium showed no change in optical density during the experiment. Microscopic examination of individual paper fibers showed a large number of cells associated with the fibers within 30 min of inoculation.

Scanning electron photomicrographs of the papers following 2 years of incubation showed extensive degradation of the paper. Figure 3 shows a series of images of representative papers at three stages in the attack of halophiles on this cellulose substrate. The uninoculated control flasks (Fig. 3A,B) show no breakdown or degradation of cellulose due to high salts or long periods of incubation. After 14 days

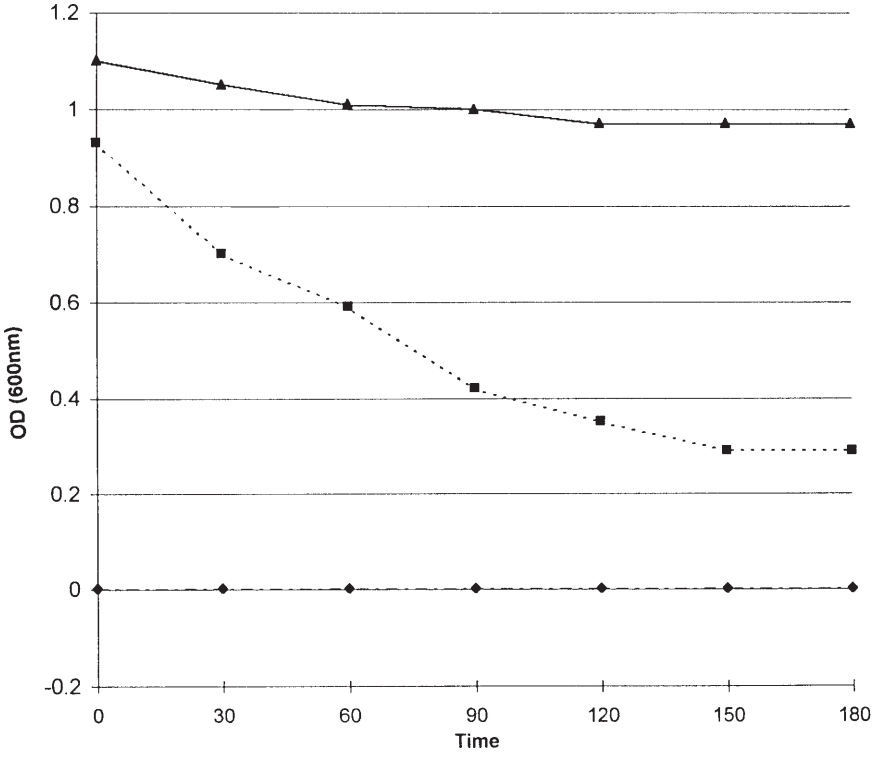

Fig. 2. Attachment of *Haloarcula hispanica* and a mixed culture of WIPP halophiles (originating from G-Seep) to paper substrates. *Diamonds*, control (uninoculated CG medium); *Squares*, mixed culture in CG medium; *triangles*, *Ha. hispanica* in CG medium

of incubation, the paper from the attachment experiment (Fig. 3C,D), shows some initial degradation, especially in amorphic regions (arrow in Fig. 3D) stretching between the fibers. After a 2-year incubation, the cellulose fibers showed evidence of extensive breakdown of the amorphic regions and the fibers (compare Fig. 3E,F with Fig. 3A,B). The individual fibers showed a crust-like appearance (arrow 1, Fig. 3F) and numerous strand breaks (arrow 2, Fig. 3F). The paper section and magnification used in this micrograph have been selected to highlight the alteration of the cellulose fibers. It therefore has relatively few visible cells. Cell masses, but not individual cells, are visible in the lower magnification micrograph shown in Fig. 3C. Some bacterial forms are also visible on the fibers (between arrows 1 and 2 in Fig. 3F). This paper had virtually no remaining structural integrity and easily disintegrated into individual fibers. In contrast, paper stored in sterile concentrated brine maintained its overall structure and strength after two years of suspension (Fig. 3A,B).

Organic acid production during cellulose degradation

Table 3 summarizes the results of the organic acid analyses from the long-term degradation of cellulose by mixed cultures of halophiles originating from G-Seep, growing aerobically. The only organic acids produced were oxalacetic and pyruvic acid. Prior to inoculation, citric acid and isobutyric acid were detected in the medium without cellulose, while formic, acetic, and caproic acids were detected in the media that contained cellulose. After 64 days, these acids were either not detected or were present in decreased concentrations in the flasks that contained cellulose.

Discussion

The information presented in Table 2 illustrates some of the difficulties that would be encountered in assessing the distribution and diversity of bacteria in a subsurface salt formation. These data would help to explain why researchers in some areas need to use large amounts of salt (ca. 1 kg) in attempting their isolations (W.D. Grant, personal communication) while others need only 1–10 g quantities. Basically, the data obtained from the survey of the underground WIPP facility show that the viable and cultivable microbial populations are widely distributed within the salt formation. There are, in fact, more regions with no apparent populations than there are regions containing culturable organisms.

Another reason for a lack of reproducibility may be the amounts of brine present in a given formation. Examination of the data in Table 2 shows this aspect quite clearly. Within the Salado formation, the only samples that could be consistently counted upon to produce viable cultures proved to be the in-flowing brines of G-Seep and DHP402A. This result is identical to that reported by Huval and Vreeland (1991), who isolated several bacteria from brines formed in other regions of the Salado formation and in a different formation entirely.

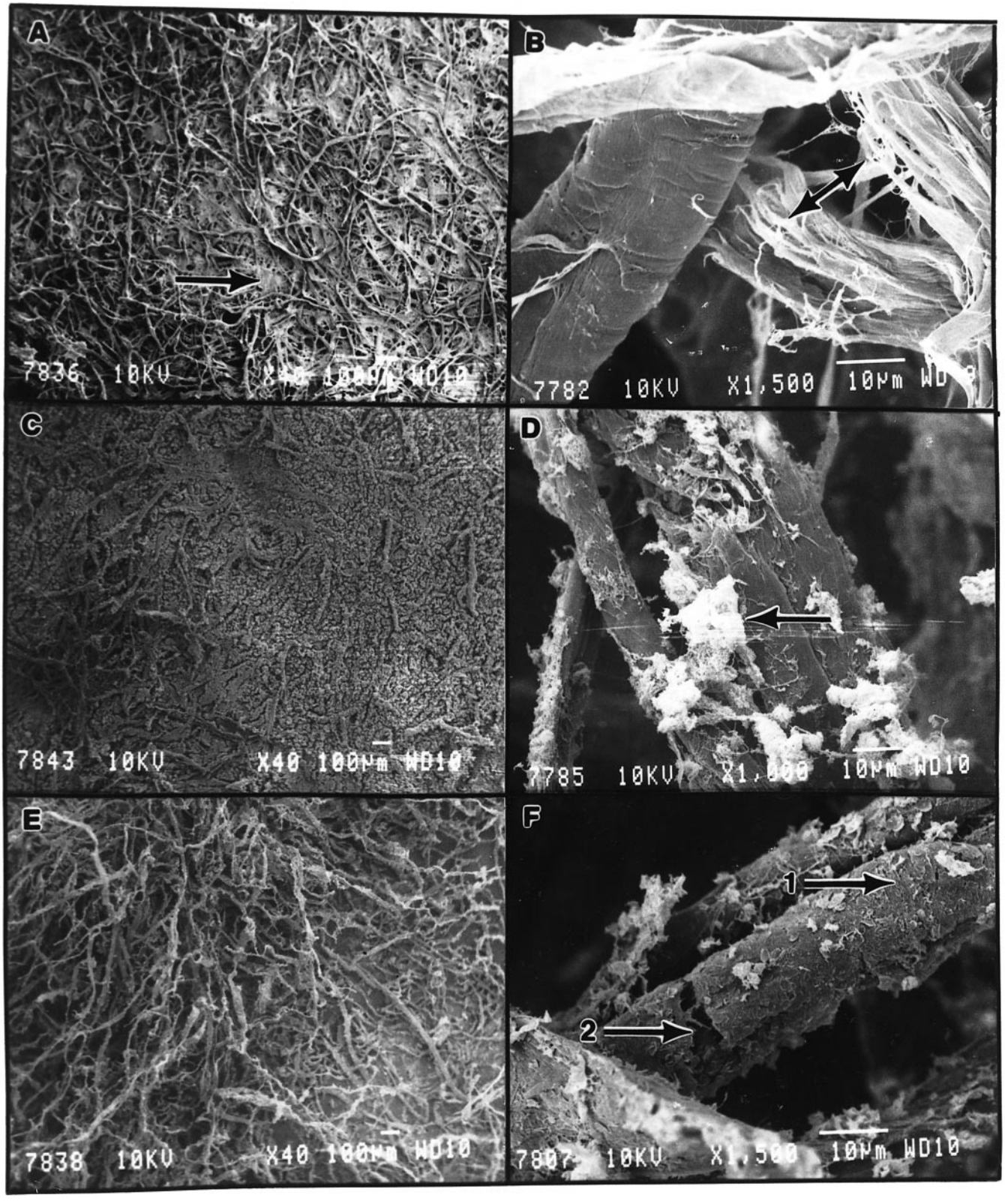

Fig. 3. **A** Scanning electron micrographs of paper after 2 years in sterile brine; **B** higher magnification of paper seen in **A**; **C** paper after 14 days of incubation; with mixed halophile culture originating from G-Seep; **D** higher magnification of paper seen in **C**; (*arrow* points to region of breakdown); **E** paper after two years' incubation with mixed halophile culture; **F** higher magnification of paper seen in **E**. Note crusty appearance (*arrow 1*) and brittleness of the fibers. *Arrow 2* points to a break in the cellulose fiber. *Bar*, 100 µm in **A**, **C**, and **E**. *Bar*, 10 µm in **B**, **D**, and **F**

Table 3. Organic acids produced by mixed cultures of halophiles during aerobic growth on common paper

Organic acid	Day 0			Day 64		
	No paper	Whatman #1	Paper towel	No paper	Whatman #1	Paper towel
Uninoculated media (all values are in millimoles)						
Oxalacetic						
Citric	4.5	4.5	1.4	5.5	5.5	4.9
Pyruvic						
Formic		0.4	0.2		0.3	0.5
Acetic		0.6			0.6	0.9
Isobutyric	2.2	2.2	2.2	2.2		
Caproic		7.5			12.5	2.5
Inoculated media (all values are in millimoles)						
Oxalacetic				13.3	11.1	15.5
Citric	4.2	4.7	4.9	4.1	3.5	3.1
Pyruvic				0.5	0.3	0.5
Formic		0.5	0.5			
Acetic		0.6	0.6			
Isobutyric	2.2	2.2	2.2	2.2	0.2	
Caproic		8.8	7.5			

Treatments were: Control with no paper; Whatman #1 filter paper; and Scott brand 151 paper towel. The experiment was carried out in FP media. The mixed cultures originated from G-Seep brine.

The relationship between underground brines and mine areas is often overlooked during microbial studies involving single-visit samplings. Brine flow is a common characteristic of virtually all underground salt formations (Roedder 1984; O'Neil et al. 1986; Jenyon 1986). This brine may arise from numerous sources including the formation itself, or as a result of the intrusion of rainwater through cracks and fissures in the overlying sediments, or even from accidental events (Gold 1981). Further, the brines move through the formation by a long, complex path, which is impossible to determine. Consequently, while brines would appear to be excellent sources of microbes, most researchers have recognized their limitation regarding determining the exact origin of the bacteria present. However, as brines enter a mine facility, the air flow through the mine causes rapid evaporation resulting in recrystallization of the salts as weeps. Most of these weeps are actually only a few days old. Weep salts have been the source of at least one reported series of isolations (Norton et al. 1993). Further, since brines flow through a formation by a process of dissolution and recrystallization, a formation containing a large amount of flowing brine may contain salt rocks of vastly different ages (Urai et al. 1986; Vreeland and Powers 1998). When these weep salts and recrystallized salts are used as sources of isolations (Norton et al. 1993; Denner et al. 1994) it becomes impossible to determine the exact age of the isolates or even to claim that these cultures arose from the formation itself. This type of problem can only be solved through the careful selection and exclusive use of primary or chevron crystals as described and analyzed by many researchers (Das et al. 1990; Lazar et al. 1988; O'Neil et al. 1986; Roedder 1984; Roedder et al. 1987; Roedder 1990)

One of the most exciting findings in this study was the isolation of a large number of different halophiles (Fig. 1). This was surprising since colonies produced by the individual samples had very low apparent colonial diversity. Finding a very diverse bacterial community in this formation was especially surprising given the age, relative dryness, and overall isolation of the region. The fact that the formation as a whole contained a halophilic population with a high phenotypic diversity is an exciting finding. This type of result might be expected in an environment that has isolated groups of organisms from interaction for eons of time.

Many of the arguments against the idea of microbes surviving in geologically old formations have revolved around the fact that researchers have generally found more previously identified species than they have found unique strains. Based upon the data obtained from this study (Fig. 1) it would appear that the lack of new strains relates more to the use of relatively few samples than it does to any inherent lack of new phenotypes within the geological arena. This is especially true in relation to the cellulose- and carbohydrate-utilizing aerobic halophiles present in these samples (Table 2).

A cellulose-utilizing, extremely halophilic bacterium was first reported by Bolobova et al. (1992). The organism, named *Hallocella cellulolytica*, is obligately anaerobic and is able to utilize cellulose as a sole carbon source. The Salado salt formation also contained cellulose-utilizing aerobic bacteria. In many areas of the formation, cellulose/carbohydrate-utilizing bacteria outnumbered the non-carbohydrate-utilizing organisms (Table 2). In order to utilize these insoluble materials effectively, the organisms must first attach to the substrate. Apparently, halophiles are able to accomplish this within the first 30 min of exposure to the cellulose (Fig. 2), although the exact mechanism of attachment was not determined. In both the *Halocella cellulolytica* and the Salado cultures, the attachment process appears to be affected by the presence of other carbon

sources in the medium. Separate experiments using different amounts of casamino acids (data not shown) indicated that higher organic contents actually inhibited cellulose breakdown in these cultures.

Finding a relatively large number of cellulolytic cultures within the Salado salt beds may help to explain one aspect of these underground formations. Virtually all hypersaline lakes contain large amounts of dead plant material (R.H. Vreeland, personal observation) Most of this plant matter, which generally consists of small to medium sized branches, is covered with salt crystals. Frequently, these salt-covered branches can be found embedded in the crystal pan at the bottom of the lake area. Despite this, there is literally no sign of fossilized or salt-preserved organic material in the nearly 8 km of mine tunnels excavated in the Salado or in any of the other underground formations that have been sampled by the senior author (RHV). An attractive hypothesis to explain the presence of detectable populations of cellulose-degrading bacteria in a formation that has no visible plant matter would be the presence, early in the life of the formation, of a slowly degradable carbon source such as dead plant matter. This would also be consistent with the heterogeneous distribution of the bacterial population found during this survey.

The pattern by which halophilic bacteria might attack a cellulosic material is perhaps most noticeable in the attachment study (Fig. 2) and the scanning electron microscopy presented in Fig. 3A–F. Based upon these data the halophiles would rapidly attach to the substrate once it had been completely wetted by the brine. In the inoculated cultures, the halophiles attached to the paper had a noticeable effect on the substrate after only 14 days of incubation (Fig. 3D). Most of this early degradation is focused on amorphic regions of the fibers. After longer incubation (2 years in this study), both the amorphic and crystalline cellulose fibers show evidence of breakdown (Fig. 3F). Nevertheless, most of the original paper was still present after 2 years. This sequence of events is consistent with those described by Klyosov (1990) for nonhalophilic cultures. However, the overall process is certainly slower. Because of the relatively plastic nature of underground salt formations (Urai et al. 1986), any cavities left after the degradation of the material would inevitably be filled in, leaving little to no trace of the original substrate. Since the salts would most likely form a complete layer cutting off oxygen from the system, much of this degradation activity would probably be fermentative with the resulting production of various small molecular weight organic acids. If such end-products were available in great abundance within the salt crystals, it might be possible to use published procedures to extract the acids from samples of salt crystals as metabolic markers (Das et al. 1990; Lazar and Holland 1988; Roedder et al. 1987; Roedder 1990).

For instance, fermentative halophilic bacteria produce acetate, H_2, CO_2, ethanol, butyrate, lactate, formate, and propionate when these anaerobes are growing on glucose (Rainey et al. 1995). In contrast, the aerobic carbohydrate-utilizing halophile, *Halorubrum* (*Halobacterium*) *saccharovorum* accumulates acetic and pyruvic acids in its medium during growth on glucose (Hochstein 1988). The mixed aerobic cultures of halophiles isolated from the Salado produced relatively low amounts of oxalacetic and pyruvic acid when grown on cellulose (Table 3). Consequently, while slow microbial growth on cellulose could provide a survival mechanism for bacterial populations trapped in salt, the amounts of detectable by-products may be too low to provide a quick mechanism for detecting salt regions which may contain viable bacteria.

In summary, areas of the Permian age Salado formation contain viable populations of halophilic microorganisms. These populations are, however, scattered heterogeneously throughout the available mine areas. Some regions of the formation continuously yielded no viable bacteria while other regions frequently possessed viable bacterial populations. In some mine regions, the detectable population was composed primarily of red, archaeal-type halophiles while samples from other areas contained a large number of nonpigmented bacteria in addition to the red halophiles. While the total bacterial population does appear to be relatively small, the diversity of bacterial types is rather surprising. A detectable number of cellulose-degrading bacteria were also associated with the formation salts. This was another surprising result, given the apparent lack of plant material trapped within the salt formation.

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