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ON THE ISOLATION OF HALOPHILIC MICROORGANISMS FROM SALT DEPOSITS OF GREAT GEOLOGICAL AGE

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SUMMARY

From salt sediments of Triassic or Permian age from various locations in the world halophilic microorganisms were isolated. Molecular characteristics of several of the isolates suggested they belong to the archaeobacteria. One group appears to represent novel strains; several properties of one such isolate, strain BIp, are described here.

The existence of viable microorganisms in ancient sediments would have great implications with respect to our notions on evolution, the search for life in extraterrestrial environments and the long-term survival of functional biological structures. Of crucial importance is thus the question if these microorganisms existed in the salt since the time of deposition or invaded at some later date. Some suggestions to address these issues experimentally are discussed.

INTRODUCTION

In all parts of the world salt deposits are found which originate from early periods of the geological history of the Earth. The major such halogenic epochs occurred during the Paleozoic period (Zharkov, 1981). Particularly large sediments were deposited during the Permian and Triassic era, that is, 280 to 195 million of years before present. Microscopic examinations revealed the presence of bacteria in thin sections or dissolved rock salt samples (see Sonnenfeld, 1984 for references). Rather sensational were the claims about thirty years ago that bacteria from Permian or older salt sediments had been brought back to life (Dombrowski, 1963; Reiser and Tasch, 1960). Other workers could not confirm these findings (Bien and Schwartz, 1965). Recently, extremely halophilic bacteria were isolated from an English salt mine, whose deposition appears to have occurred during the Triassic period (Arthurton, 1973). The rock salt samples had been collected right after blasting, and care was taken to exclude any extraneous microbial contamination (Norton, 1988, 1989). Other halophilic bacteria were isolated from a Permian age bedded salt deposit located in New Mexico (R. Vreeland, personal communication). We were able to cultivate halophilic bacteria from rock salt which was obtained from an Austrian salt mine, also of the Permian period. A preliminary characterization of isolates from the English as well as the Austrian salt mines with respect to their antibiotic sensitivity, ATPase enzymes and cellular proteins has been described (Stan-Lotter *et al.*, manuscript submitted for publication). The bacterial isolates fell into two classes; one, which resembled known bacterial strains and one, which did not. Extremely halophilic bacteria belong to the archaeobacteria, a group of microorganisms thought to have diverged early from the main line of prokaryotic evolution (Woese, 1987). A comparison of known archaeobacteria with similar isolates from ancient sediments might provide a time scale for mutational events, since the bacteria, which were included in the salt

sediments, have not evolved for a few hundred million years, in contrast to all other living organisms. The significance of viable organisms from paleozoic times would, besides evolution, extend to other areas of scientific study, for instance, to the search for extraterrestrial life. If it can be proven that bacteria remain viable in a dry state for very long periods, it would be feasible to look for remnants of such life forms in sedimentary formations on other planets, e.g., on Mars.

Here we extend the description of properties of one of the novel isolates, strain BIp, and discuss strategies to determine the age of the microorganisms isolated from rock salt of paleozoic origin.

MATERIAL AND METHODS

Bacteria and Culture Conditions

Samples of rock salt, with varying contents of clay, were obtained from the salt mine in Bad Ischl, Austria. Pieces of about 2 g were dipped in ethanol and flamed. Then they were transferred to 50 ml of sterile complex medium (M2 medium) that was 20% with respect to NaCl and whose pH was 7.4 (Tomlinson and Hochstein, 1976) and incubated at 37 °C with shaking. After about 4 weeks samples of the cultures were streaked on plates containing M2 medium which was solidified by the addition of 2% agar. Colonies appeared after three to four weeks and were purified further by repeated spreading on solid M2 medium. One isolate with pink pigmentation was picked for further characterization and will be referred to as BIp. For some experiments a culture medium with a pH of about 9.5 was used (Tindall *et al.*, 1980). Growth in liquid culture was monitored with a Klett-Summerson colorimeter with a red (No. 66) filter. The following archaebacterial type strains were obtained from the Deutsche Sammlung für Mikroorganismen (DSM): *Halococcus morrhuae* DSM 1307, *Hc. morrhuae* DSM 1309, *Natronococcus occultus*, *Natronobacterium magadii*, *Nb. gregoryi*, *Nb. pharaonis*. *Halobacterium saccharovororum* (ATCC 29252), *Hb. halobium*, *Haloferax denitrificans* and *Hf. vallismortis* were obtained from Dr. L.I.Hochstein, NASA Ames Research Center.

Antibiotic Sensitivity

Paper disks impregnated with the particular antibiotics were placed on agar plates, which contained 20 ml of solid M2 medium and on which 200 µl of liquid culture had been spread. Zones of inhibition around the disks were recorded after 7 days of incubation at 37 °C. Antibiotics were from Sigma Chemical Company.

Biochemical Tests

Catalase activity was determined by placing a drop of a 3% H₂O₂ solution on a lawn of bacteria. The formation of gas bubbles indicated a positive reaction. Oxidase was detected by spotting a loopful of bacterial culture on a paper strip containing N,N-dimethyl-1,4-phenyldiammoniumchlorid and naphthol. Blue coloration revealed the presence of the enzyme.

Gel Electrophoresis

Sodium dodecyl sulfate (SDS) gel electrophoresis of whole cell proteins was performed as described previously (Stan-Lotter *et al.*, 1989) using the gel system of Laemmli (1970). Isoelectric focussing (IEF) was done in a pH range of 2.5 to 5.0 and 3 to 10, respectively, as described for halobacteria (Stan-Lotter *et al.*, 1989). Ampholytes were from Pharmacia-LKB.

Light Microscopy

Cells were examined with a Leitz Diaplan microscope using phase contrast. Photographs were taken with Ilford film PAN F.

RESULTS

Isolation and Growth Characteristics

Numerous colony types on agar plates were isolated from samples of rock salt, many of them showing pink or red pigmentation. Some of the isolates from an English and an Austrian salt mine, respectively, were similar, but not identical, to classified archaebacteria (Stan-Lotter *et al.*, submitted for publication). Other isolates as represented by strain BIp did not show obvious similarities to known bacteria. These might be novel isolates of potential great interest. The criteria for the characterization of strains included sensitivity towards antibiotics, possession of certain enzymes such as a membrane ATPase and properties of whole cell proteins. In the case of one isolate from the English salt mine, lipid analysis was performed and showed the typical archaebacterial diphanyl-diether (Norton, 1988). More detailed data are, however, necessary to decide upon the novelty of bacterial strains.

BIp grew with a generation time of about 24 h at 37 °C in liquid M2 medium (pH 7.4) with shaking. It reached the stationary growth phase at about 150 Klett units. Similar growth characteristics were observed when BIp was cultivated in medium of pH 9.5. Cells from liquid cultures were coccoid and often growing in tetrads or, particularly in older cultures, in large clusters (see fig. 1). Growth on solid medium was as irregular colonies of pink pigmentation; older colonies turned to a brownish color.

Biochemical Characteristics

Table 1 shows the sensitivity of BIp towards several antibiotics. Growth was strongly inhibited by anisomycin, bacitracin and novobiocin. Moderate sensitivity of BIp was observed against chloramphenicol and tetracyclin. No inhibition of growth occurred with ampicillin, nalidixic acid and streptomycin. These results were generally consistent with previous studies of antibiotic sensitivities of archaebacteria (Hilpert *et al.*, 1981). BIp was catalase positive and oxidase positive.

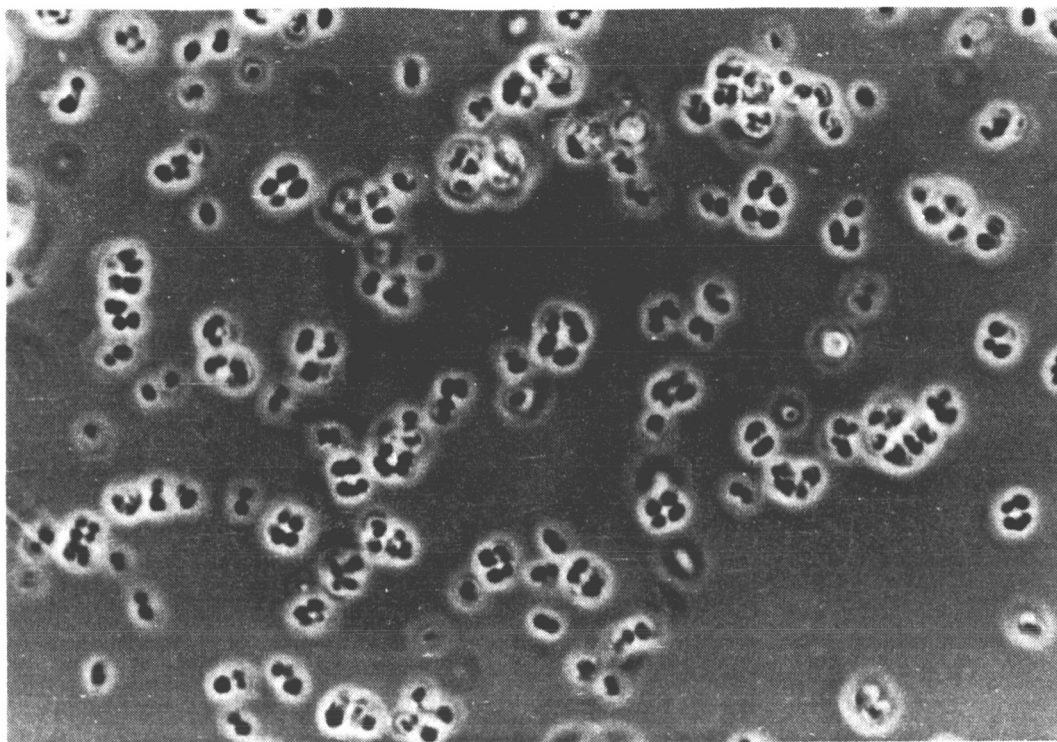


Figure 1. Phase-contrast photomicrograph of strain BIp, x 3550.

Table 1. Effect of antibiotics on growth of strain BIp.

	Concentration (μg per disk)	Sensitivity
Ampicillin	40	- *
Anisomycin	50	+++
Bacitracin	40	+++
Chloramphenicol	40	+
Nalidixic acid	40	-
Novobiocin	40	+++
Streptomycin	40	-
Tetracyclin	40	++

*-, no inhibition; +, ++, +++, zone of inhibition ≤ 1 mm, ≤ 3 mm, > 10 mm, respectively.

Gel Electrophoresis of Whole Cell Proteins

SDS gel electrophoresis of whole cell proteins is a rapid method for distinguishing bacterial species (Jackman, 1985). We have used this method to identify wild type strains and mutants of halobacteria (Stan-Lotter *et al.*, manuscript in preparation). Strain BIp showed a unique protein

pattern following SDS gel electrophoresis which did not resemble that of any of the halobacterial type strains which we tested (data not shown). In particular, none of the known coccoid archaeobacterial strains (*Halococcus*, *Natronococcus*) were similar to BIp. The protein patterns of strain BIp were identical, whether the strain was grown at pH 7.4 or pH 9.5.

Extremely halophilic bacteria are known to possess almost exclusively acidic proteins (Reistad, 1970), with isoelectric points ranging between pH 3.6 and 5.0 (Stan-Lotter *et al.*, 1989). On isoelectric focusing gels, strain BIp showed acidic proteins between pH 3.8 and 4.5. The overall protein pattern on IEF gels was again different from that of any of the archaeobacterial type strains (not shown).

DISCUSSION

Several properties of strain BIp which are described here suggested that it is an archaeobacterium. The sensitivity towards antibiotics (table 1) was similar to that of other archaeobacteria and its whole cell proteins were acidic, as is the case for proteins from all halophilic archaeobacteria. Its rather slow growth is similar to that of other halophilic archaeobacteria, particularly *Halococcus* (Staley *et al.*, 1989). However, none of the two types of coccoid aerobic archaeobacterial isolates known to date, *Halococcus* and *Natronococcus*, showed similarities to BIp with respect to cell protein patterns and pigmentation. In addition, the wide growth range of BIp, from pH 7.4 to 9.5, is unlike that of other coccoid archaeobacteria. A clear distinction of archaeobacteria from eubacteria and among archaeobacterial strains can be made by sequencing nucleic acids, e.g. 16 S rRNA (Woese, 1987) and by the analysis of lipids (Ross *et al.*, 1985). These methods are in progress with strain BIp. In summary, BIp probably belongs to the halophilic archaeobacteria and might represent a novel isolate.

From several different locations of salt sediments of great geological age halophilic microorganisms have now been isolated. The question arises if these bacteria were deposited at the time of sedimentation. Alternatively, they may have entered the salt sediments at some later date, or, thirdly, they may represent present-day bacterial contaminants which were introduced during handling of the samples. If the first scenario is correct, these organisms would provide a unique repository of biomolecules, which was not changed by mutational events experienced by all other living organisms. Many evolutionary problems could be addressed by the study of such ancient bacteria. Moreover, the possibility of long term survival of bacteria would have to be taken into consideration when looking for extraterrestrial forms of life. In lunar soil, minerals such as halite (NaCl) and sylvite (KCl) have been detected (Ashikmina *et al.*, 1978); on Mars, surface features were seen which suggested the presence of a liquid, probably water, at some earlier period of its history (Carr, 1987). Thus, the possibility of "halophilic life" in extraterrestrial environments might be realistic and should be worth of further exploration.

Direct determination of the age of microorganisms from rock salt is not easy because of the scarcity of organic material in the samples and the lack of suitable isotope dating methods. An indirect method is the analysis of pollen and spores from extinct plants, which has been performed with Austrian salt sediments (Klaus, 1974) and revealed a Permian origin of the rock salt. However, the possibility that the bacteria in the same sediment entered at a later time by unknown processes cannot be excluded at present. Here, geological investigations would be necessary which could prove

that the salt sediments consist of primary crystals, which have not been extensively altered during later times. The third possibility mentioned above, contamination with present-day halophilic microorganisms, can be excluded with proper isolation techniques, such as flaming the salt samples and/or treatment with bactericidal agents. In addition, halophilic archaebacteria are, in the experience of most laboratories, not likely to occur as air- or dust-borne contaminants, due to their complex nutritional requirements.

A different approach to the problem of long term survival of halophilic microorganisms was taken by Norton and Grant (1988), who showed that cells remained viable after at least six months of storage in fluid inclusions of salt crystals. This type of experiment could be extended to include crystallization and storage of bacteria-containing brines under conditions, e.g., atmospheric composition, temperature etc., which are thought to prevail on Mars.

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BIOGRAPHY

Helga Stan-Lotter holds a M.Sc. degree in Microbiology and a Ph.D. degree in Science, both from the Technical University of Munich, Germany. Postdoctoral studies were on bacterial membrane pore proteins at the University of Calgary, Alberta, Canada. She then obtained a faculty position as research associate at the University of Vancouver, Canada, working on microbial bioenergetics. Under an NRC associateship she joined the laboratory of L. Hochstein at Ames Research Center, where she is now a Principal Investigator with the SETI Institute. Research interests are the evolution of bioenergetics and archaeobacteria from salt sediments. She also holds teaching appointments in Microbiology at the University of Vienna, Austria.