BACTERIA FROM PALEOZOIC SALT DEPOSITS

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Stimulated by the bacteriological findings in the mineral springs of Bad Nauheim, which carry salts from Permian deposits, I investigated from a bacteriological point of view the Zechstein salts, obtained by means of mining and drilling. Müller and Schwartz (1953), Rippel (1945), and Strong (1956) only achieved the isolation of dead bacteria from Zechstein salts. Reiser and Tasch (1960) reported the living isolation of a diplococcus from Permian salts. We now succeeded in isolating living bacteria. Yet, this achievement seemed rather improbable; for if we had actually extracted living bacteria from Zechstein salts, then we have to assume that we found creatures of the highest individual age ever registered.

The following is a description of the isolating technique we used.

In bacteriological work it is obviously very easy to get unwanted secondary infection. To be sure that this secondary effect would not spoil our results, we used extraordinary precautions. (1) We chose a small research laboratory in which an ultraviolet sterilization lamp was kept burning for four days before the experiment. No one entered this room during these four days. (2) The two researchers entered the laboratory in sterile clothes and sterile rubber gloves after thorough disinfection of their hands and arms. (3) The table and necessary tripods were covered with sterile towels. (4) All necessary instruments, glassware, and apparatus were thoroughly sterilized. (5) The research material, *i.e.*, the piece of salt under consideration, was suspended on thin, sterilized wire from the tripod. (6) This suspended piece of salt was then flamed for one minute with a hot bunsen flame. (7) Immediately afterwards a glass with a culture solution was brought under the piece of salt, so that it was suspended in the solution. (8) The supporting wire was then cut and the glass was closed after sterilizing the rim and the stopper also with the bunsen flame. (9) The cultivation was carried out at a temperature of 40° C. (10) As soon as the culture began to grow, the elaboration to the pure culture proceeded in the usual bacteriological manner.

To working procedure 6, I must add that the necessary time for the surface treatment of the salt with the bunsen flame was ascertained in preliminary experiments. Salt-pieces, which were brought into a fresh suspension of living *Pyocyaneus*—about 80,000 per cm.^{3}—could be sterilized in 45 seconds.

Because salt is a poor heat conductor, the temperature fell rapidly toward the center of the crystal. We heated the surface for 45 seconds. Then 3 cm. from the surface, the temperature rose only by 6.2° C. Thus, we achieved a sterility of the surface and regions close to the surface without producing sterilizing temperatures in deeper layers. Of course, the crystals must be large enough; they must have a diameter of at least 6 cm. Such specimens have a weight of about 250 to 300 gm. A crystal this large saturates about 1 liter of culture solution; a saturated solution is necessary for the cultivation of halophil and halotolerant organisms.

For the duration of this work we set up culture plates on which germs in the air could germinate, which in most cases did not happen. If the germs of the air did germinate, however, they were brought into saline solutions to prove their tolerance to salt. This test always showed an intolerance to salt, so that there was no identity to the bacteria that came from the salt specimens.

In counter-checks we sterilized salt crystals for 4 hours at 200° C., before investigating them bacteriologically in the prementioned manner. These crystals proved to be sterile. We also examined crystals coming up from a depth of more than 4300 m.; in the Mesozoic era these salts lay about 1000 meters deeper than today. At this depth the temperature is at least 160° C., and as expected these salt specimens also showed no sign of life.

Now, how can we find an explanation for the conservation of life over such an extended period of time, that is for over 180 million years? There are two possibilities. First, one is reminded of the method for conserving bacteria that is practiced today, *i.e.*, dehydration at low temperatures. If one extracts almost all the water from the protein of micro-organisms, it is possible to preserve them for years without changing any of their particular characteristics, although there is no metabolic activity whatsoever. We know of certain germs, which lived for more than 30 years, although their metabolism was totally inhibited. Starke and Harrington (1931) consider the vitality of dried bacteria as unlimited. If this is correct, then the hypothesis of finding living organisms in Paleozoic layers could not have received better support, and we would then have found a way of understanding the survival of these organisms over such long periods of time. Second, there is the possibility of reversibly denaturing protein by salification. This method can also be used on higher organisms with good results. For instance, the protein from the eggs of sea urchins can be denaturized in a saturated solution of ammonium sulfate. After months, this process is reversible by simply removing the salts. The eggs retain the ability to be fertilized. Perhaps in our specific case both methods, that of dehydration and that of salification, were in effect.

If this interpretation was true, then the method should be reproducible in a laboratory experiment. For this experimental reproduction we used *Pseudomonas halocrenaea*, which were isolated from Zechstein salts. This bacterium does not bear spores.

If the nutrient solution in which it started growing is slowly dehydrated, the bacterium will die. This will not happen if one slowly saturates the solution by adding 1 gm. of salt per week. This substratum is now slowly dehydrated, until all salts are completely dry and crystalline. In this dry state it can be kept for long periods of time. When bringing these salts into a fresh nutrient solution again, the original vitality of the bacterium can be re-established.

I would like to point out a further peculiarity: the optimal temperature for many of the germs that we found lies between +45 and +55° C., which is astonishingly high. But, elucidating enough, this temperature corresponds exactly to that temperature which, geologists say, was present when the Zechstein sea was slowly drying up.

I believe that this correspondence of temperatures is certainly not accidental. Because the bacteria were embedded in the crystals, they were assured against

destruction by mechanical pressure. After considering the depth of our findings, we can estimate a maximum of 1400 m. With the normal geothermic gradient, which gives the temperature at a certain level, we get a maximal value of +42° C., which the germs were exposed to during their long latent life. This temperature in no way prevents the preservation of life.

The question of which geological specimen is to be examined is of foremost importance. At first I used all sorts of Zechstein salts, while trying out the bacteriological working procedure. But later, I carefully selected the specimens to be investigated. All specimens, which came from questionable regions, such as near faults or the upper salt level, were discarded. Specimens showing signs of recrystallization were also discarded. We used only pieces which definitely showed signs of being primary Zechstein salts, and of these only those which came from perfectly undisturbed points in the middle of larger successions of rock salt, the layers of which were formed normal-hypidiomorphic to allotriomorphic. Their grain size lies in the order of millimeters. But even with this careful selection of specimens, only about every second culture showed results.

Because it is very probable that the organisms are of primary genesis, we can undertake an estimation of the age of these isolated living bacteria. Because pollen grains were isolated, which served as characteristic fossils, it was relatively easy to establish the age of the bacteria.

We also centered our attention on another aspect of the problem: in undisturbed geological layers the rock salt has practically no pores, if we disregard the lye enclosures. If the salt is taken out from its natural environment, it will not be subject to the pressure of the overlaying strata anymore. It relaxes and thus increases in volume by a few per cent. Due to this loosening, pores begin to form and air can automatically enter the salt. This would make possible the entering of bacterial contamination from the outside. To prove that this was not happening, we prepared petrographic thin sections of the salt. In examining these, we found the bacteria to be embedded in the crystalline structure of the salt and not in the capillary crevices (FIGURE 1).

Contrary to the previously shown Paleozoic microorganisms, this form (FIGURE 2) is a direct decendent of the Paleozoic germ, which was obtained by cultivation, and identified as *Bacillus circulans*. I found this form in three different Zechstein formations. It is a very rare specimen, which has been described only eight times since 1890. A comparison of the Paleozoic and the Recent representatives of this group is of special interest. When the Recent germs are compared from an evolutionary point of view they are neither older nor younger than the Paleozoic ones, but the Recent type has gone through completely different stages of development. They were not preserved in a latent stage of life, but have probably gone through an immensely great number of cell divisions. If it were not for the phenomenon of circular migration, which is peculiar to both the Paleozoic and the Recent type, it would be very difficult to find a relationship between the two.

Comparing them biochemically, we find very distinct differences. Our 3 Paleozoic strains show almost identical biochemical properties. The strain found by Kienholz lost all its saccharolytic characteristics, which its Paleozoic relatives had. The only new characteristic is their ability to liquefy gelatine.

Beyond this fact, a comparison over such long periods of time gives the following results: (1) The paleozoic strains of the *Bacillus circulans* have quite a lot more biochemical characteristics than those described in the preceding 70

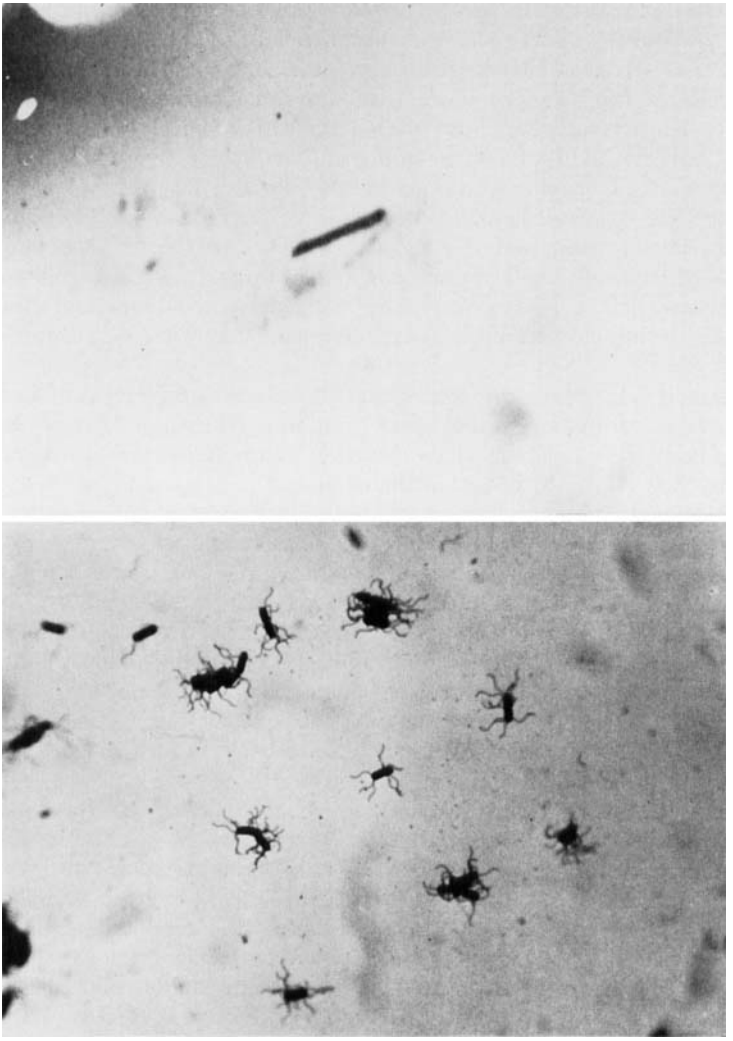

FIGURE 1 (Top). Bacterium in the center of a thin section of a thickness of 15 μ, enlargement 3600:1.
FIGURE 2 (Bottom). *Bacillus circulans* from the Zechstein salt, enlargement 950:1.

years. (2) It seems that the long, latent life of about 180 million years has brought about no loss of characteristics for the Paleozoic species. (3) A loss of characteristics was proved, however, for the Recent representatives of *Bacillus circulans*, which have gone through a vast number of cell divisions. (4) Although the differences in biochemical behavior are very distinct, there is an

absolute accord in the morphological characteristics between the Paleozoic and the Recent representatives of the *Bacillus circulans*. (5) This leads us to believe that the genes responsible for the morphological differentiation are much more stable than those leading to the biochemical characteristics of a species. There is no doubt that this goes for other species as well, but at the moment we are only considering *Bacillus circulans*.

We could not have made these statements, if this species did not have the characteristic of migration. Relying only on the peripherally whipped bacterium and its micromorphology, as with *Bacillus circulans*, any definite determination would have been impossible. Even biochemical investigations and comparisons would lead nowhere, because there are great doubts concerning the question of whether or not characteristics of the Paleozoic germs came to a

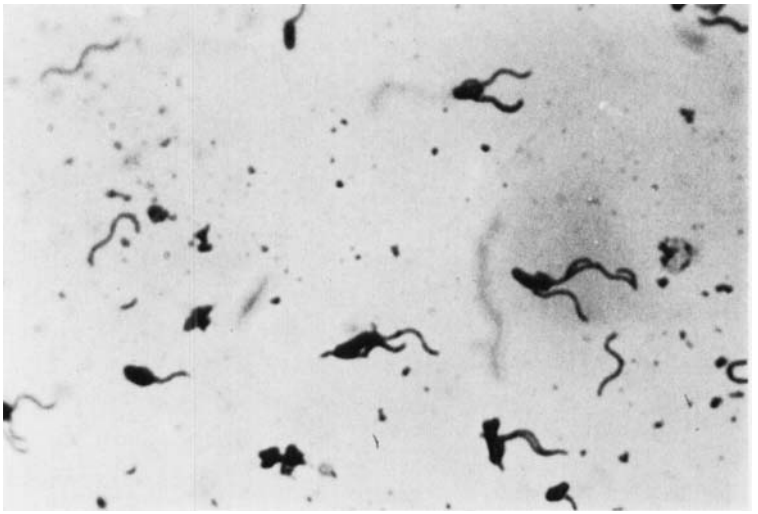

FIGURE 3. Bacterial strain VIII/D from the Middle-Devonian, enlargement 1200:1.

further development in Recent types. Therefore, it should be very difficult to show the identity of other types of bacteria, isolated in mineral salts, with Recent species beyond the probable affinity to a species.

If all of these considerations were true, then it should be possible to cultivate bacteria from salts of even older origin than those of the Permian age, provided that these salts come from regions where no tectonic movement had occurred since their original formation. These experiments had positive results. In FIGURE 3 are shown bacteria from Middle-Devonian salts from Saskatchewan. All in all we achieved the isolation of six different species from Middle-Devonian salts. We were also fortunate to be able to isolate three different species from Silurian salts, coming from Meyers, New York (FIGURE 4).

Because it was possible to cultivate 2 bacterial species out of Precambrian salt specimens from Irkutsk, we have reached a sort of absolute level of research. It is highly improbable that scientists will find even older individual life than Precambrian, already approximately 650 million years old.

In FIGURE 5 is shown a bacterium from the Precambrian salt after silver

impregnation by the method of Zettnow. Both bacteria found in the Precambrian seem to be closely related to each other.

A list of biochemical data of the isolated germs from paleozoic salts is given in TABLE 1.

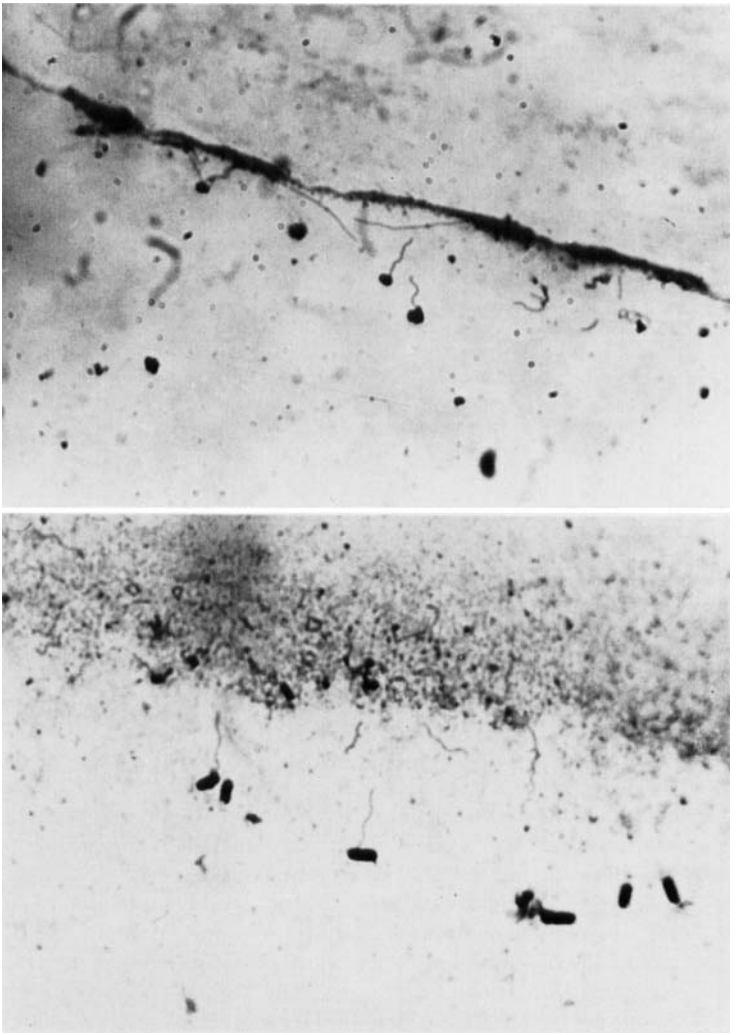

FIGURE 4 (Top). Bacterium from the Silurian, strain XV/1, enlargement 1200:1.
FIGURE 5 (Bottom). Bacterium from the Precambrian salt, strain XXX/1, enlargement 1200:1. (The pictured bacteria are probably the oldest known living organisms with their approximate age of 650 million years.)

I have not yet examined salts from the Carboniferous. The bacteria from the Precambrian, Silurian, and some from the Devonian show only few biochemical properties. The "younger" these germs are, the more they are able to perform biochemically, only to lose this ability in later life, as shown in the comparison

TABLE 1

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERS OF PALEOZOIC BACTERIA

Origin (age)	Pre-Cambrian		Silurian			Midde-Devonian						Permian (Zechstein)						
No. of strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Morphology																		
Spore forming	+	+	+	+	+	−	−	+	+	+	+	+	+	−	+	+	+	+
Motile with flagella	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−
Gram	+	+	+	+	+	−	+	±	±	±	−	+	−	−	±	±	±	±
Physiology																		
Starch hydrolysis	+	+	+	+	+	+	−	+	+	+	−	−	+	−	+	+	+	+
Nitrate reduction	(+)	−	−	−	−	−	+	−	+	+	+	+	+	−*	−*	−	−	−
Indol production	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−
Pigment production	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Gelatin liquefaction	+	+	+	+	+	(+)	(+)	−	+	(+)	(+)	−	−	+	−	−	(+)	(+)
H_2S production	+	+	+	+	+	+	+	−	+	+	+	+	+	+	(+)	+	+	+
Salt tolerance	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl red test	−	−	+	+	+	−	−	−	−	−	+	−	−	−	(+)	(+)	+	−
Voges-Proskauer test	+	+	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	−
Hemolysis:	+	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−	−	(+)
	α	α	β	β	β								β	β				β
Acid from:																		
Glucose	±	±	+	+	±	−	−	−	+	−	±	±	+	−	+	+	+	+
Laevulose	−	−	−	(±)	+	−	−	−	(±)	±	±	±	+	−	+	+	+	+
Sucrose	−	−	−	−	−	−	−	−	+	−	+	−	−	−	±	+	+	±
Maltose	−	−	−	−	−	−	−	±	−	+	+	−	−	−	+	−	+	+
Lactose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	+	−	−
Raffinose-hydrate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	±	−	−
Rhamnose-hydrate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
l-arabinose	−	−	−	−	−	(±)	−	−	(±)	(±)	±	−	+	−	+	−	+	+
Salicin	−	−	+	+	+	−	−	−	±	+	±	−	+	−	−	+	+	+
Inulin	−	−	−	−	−	−	−	−	±	±	−	−	−	−	−	−	±	±
Xylose	−	−	−	−	−	(±)	(±)	(±)	(±)	±	(±)	−	−	−	±	±	−	−
Trehalose	−	−	(±)	−	−	−	−	−	±	±	+	−	+	−	−	−	+	±
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mannitol	−	−	−	−	−	−	−	−	+	+	+	−	+	−	−	−	(±)	±

* Gas.

with *Bacillus circulans*. A final proof of my findings is now in preparation. Because it is now possible to find the bacteria in thin sections of the salts, I want to isolate each bacterium individually with a micromanipulator and let it grow in a microculture. During this process it will be kept under constant observation until it shows the germination of spores, or until it starts the first cell division after being dormant for more than 650 million years. I hope soon to be able to show this exciting moment in a motion picture film.

Other institutes are now doing research on the coenzymes and proteins of these Paleozoic bacteria.

Summary

For the first time it became possible to isolate and cultivate bacteria from Permian deposits. The methods of isolation are described in detail and the arguments, which lead to the assumption that the discovered microbes are living representatives of the oldest known individual ages, are summarized. (1) Only such salt deposits were investigated, which showed indications of being of primary genesis. (2) From these salt specimens pollen grains were isolated, which served as characteristic fossils for establishing the age of the deposit. (3) None of the geological prerequisites, such as tectonics, orogenesis, and geothermic gradients, proved to be contrary to the findings. (4) The method of isolation, as well as the precautionary measures and the controlling experiments, are discussed in detail. (5) The results of dehydration at low temperatures and the reversible method of denaturation by salification are pointed out. (6) The embedded bacteria are shown optically in thin sections of the examined salts.

Studies on other salt deposits were made, and living bacteria were isolated from salt deposits from the Middle-Devonian, the Silurian, and the Precambrian. A comparison of the biological characteristics of the Paleozoic germs with Recent bacteria was carried out.

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