The Role of extreme halophilic Bacteria in Precipitation of Salt

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Natural salt ooids were studied principally through SEM imagery and compared to halite particles obtained in the laboratory by bacterial mediation under controlled conditions. Salt precipitation is induced and oriented by the growth of bacterial colonies. The main bacterial processes involved are the multiplication of cells and the regulation of their internal salt concentration which tends to oversaturate the closely neighbouring environment. Such microbiological processes play a significant role in the quality of marketed solar salt.

1. INTRODUCTION

Occurrences of salt spheroids, dubbed salt ooids or halooids or even halolites, have been discovered in several natural environments. In the continental Dead Sea (Israel), they were considered as resulting from combined crystal growth and rolling processes in salt supersaturated agitated brines [1-2]. Meanwhile, similar halite bodies were discovered in Lake Asal (Republic of Djibouti) and in the solar salt works of Berre (France). They were interpreted as bacterial biocrystalline build-ups [3-4]. Besides, nanoscopic more or less spherical bodies within a bacterial carbonate layer (huntite) were described in Sabkhat el Melah (Tunisia) the interstitial brines of which are of marine origin [5]. These are clusters or colonies of carbonated bacteria in which halite developed as a kind of mineral skeleton adjusted to the size of each colony. Even though the bacterial origin of carbonate ooids is largely admitted, bacterial mediation in salt ooids formation, and more generally in salt precipitation, still remains under question and there was a need to compare such natural sedimentary bodies with salt bacterial products obtained in the laboratory under controlled conditions.

2. CHEMICAL COMPOSITION OF BRINES

Lake Asal is fed with marine originated brines through a recent fault system. Salt halooids form offshore lobate sand bars extending along the edge of the distal salt plain. The solar salt works of Berre are presently fed with brines originated from the dissolution of rock-salt deposits of the Oligocene Manosque basin inside which dissolution cavities were processed for gas and oil stocking. Salt spheroids were occasionally observed in both crystallisors and reservoirs.

As far as chemical compositions are concerned, the terrestrial brine of Berre is not radically different from a marine originated one (Table 1). Meanwhile, since halite spheroids have been observed in salt works and in several natural evaporitic environments with various chemical compositions, the brine chemistry should not be the main factor controlling the formation of halooids.

Table 1
Compositions of Berre and Lake Asal brines and of some brines saturated with NaCl by evaporation at 20°C in the laboratory.

	Berre brine	Lake Asal brine	Brine of marine origin[1]	Solution of pure NaCl
Density	1,210.0	n.d.	1,215.6	1,200.0
H_20 ($g.l^{-1}$)	882.1	n.d.	892.4	882.8
Na^+	123.81	94.0	102.21	124.80
K^+		4.50	4.07	
Ca^{++}	0.76	2.95	0.64	
Mg^{++}	1,87	12.20	12.76	
Cl^-	190.19	185.5	186.33	192.35
SO_4^{--}	10.96	3.10	17.57	
Br^-		n.d.	0.64	

Ionic composition in $g.l^{-1}$. [1] : saturated brine from French Mediterranean solar salt works. n.d. : non determined. Lake Asal brine is below saturation.

3. THE HALITE GRAINS

3.1. Granulometry

The collected halite sands are composed of well rounded grains with very few angular ones. Two or three grains are sometimes grouped in tiny irregular clusters. The round grains are usually spherical or more or less ellipsoidal.

For each sand, the diameters of more than 150 of grossly isodiametric grains were measured by mean of a calliper square. Values (± 0,02 mm) range between 2.00 and 3.80 for Asal halooids (mean diameter: 2.80), and between 1.98 and 4.92, (mean diameter: 3.50) for Berre ones.

Given the good linear correlation between size and weight of particles and assuming that these are strictly spherical, measurements of mean weights gave a mean density of 2.49 for Asal and 1.71 for Berre. Halite density being comprised between 2.1 and 2.6, and even though such a calculation is very approximate, it clearly means that Berre halooids either are composed of pure halite and then are porous (around 20% porosity) or are composed of halite associated with light components (brine of inclusions and/or organic matter) possibly occurring concurrently with some porosity.

Asal halooids are translucent or even transparent while Berre ones are opaque. This suggests that the Berre ooids had a more rapid growth and thus contain more inclusions and/or impurities than the Asal ones. This also accounts for the greater mean size of Berre ooids as compared to Asal ones since they both form in brines of the same density.

The size distribution diagrams (Figure 1) of grains show that both halooidic sands are composed of several populations the peaks of which are separated by 0.2 to 0.3 mm intervals which could account for a sporadic growth.

3.2. Structure

Under optical microscope and at low SEM magnifications, the fibro-radial organisation of crystallites clearly appears while irregular because the development of the halite elongate crystals (25 to 40 µm large) is awkward due to their cubic system of crystallisation. Some of the grains present an outer optically dark coating 1-1.5 mm thick. At higher magnification, broken parts of crystals and granular laminae appear to be composed of micrometric bodies 1-1.5 µm.

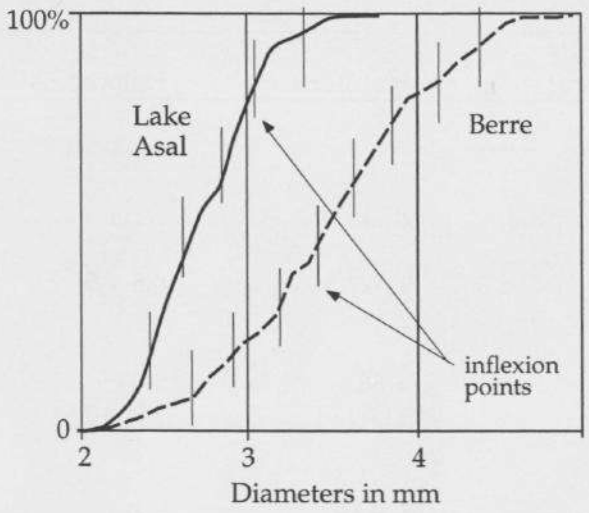

Figure 2. Cumulative size distribution curves of studied halooids.

From their size and shapes, and figures suggesting cell divisions, these tiny particles should be bacterial bodies.

Patches of granular laminae often host young crystals (hoppers) which are nested in their central part. The triangular or rectangular shapes of these new-born (3-5 µm wide) hoppers are closely related to the shapes of the bacterial patches on which they are lying. In some cases, some plain cubic crystals grow up from bacterial mound-like accumulations at the surface of large older crystals. They are composed of tiny micrometric particles which are integrated into the growing crystallite. The shape of mineral material is closely related to the shape of bacterial mounds or colonies.

All ultrastructural observations of halooids strongly suggest that the birth and growth of halite crystals are closely related to the development of bacterial colonies. Such an hypothesis ought to be validated by experiments.

Only a few groups of microbes are able to withstand (or require) high salt concentration. These are filamentous Cyanobacteriaceae and Halobacteriaceae [6]. As far as size, morphology and living conditions are concerned, all observed microbial cells should belong to the order of Halobacteriales i.e. to the group of Extremely Halophilic Archaeobacteria [7].

4. THE HALOBACTERIALES

These bacteria develop only in environments where concentrations in NaCl are usually comprised between 88 and 304 $g.l^{-1}$ NaCl. Some species e.g. Halobacterium salinarium and Halococcus morrhuae, tolerate higher concentrations up to saturation (360 $g.l^{-1}$ NaCl) [8].

All Halobacteriales (Gram -), whatever their genus, are insensitive to those antibiotics that stop the synthesis of peptidoglycan (Penicillin, Chloramphenicol, Streptomycin, Erythromycin and Tetracycline). This is easily understandable when considering that these bacteria do not possess a peptidoglycan layer in their cell wall which is only composed of glycoproteins. The pleiomorphism of most halobacteria is the consequence of this absence. As a matter of fact, peptidoglycan constitutes the rigid "external skeleton" of other bacterial bodies and is thus responsible for their shapes.

It is necessary to point at a remarkable behaviour of halobacteria depending on the concentration of NaCl in environment.

Under 88 $g.l^{-1}$ NaCl, most of them die because their wall desegregates into protein monomers and lysis of the cell occurs. For Halobacterium , lysis occurs around 120 $g.l^{-1}$ NaCl. Between 120 and 150 $g.l^{-1}$ NaCl, the cells are not lysed and keep round. This is not the taxonomic form of this genus which is described as a cylindrical rod. This latter form appears at higher concentrations due to a greater rigidity under effect of the concentration of sodium ions inside the proteinaceous wall.

Halobacteriales comprise essentially two groups and six genera. The first group is composed of isolates growing at neutrality or close to neutrality (pH 5-8) with a Mg^{2+} requirement of at least 5 mM (0.122 $g.l^{-1}$). The main characters of genera are given in Table 2.

Table 2.
Main characters of Extremely Halophilic bacteria.

	Halobacterium	Haloarcula	Haloferax	Halococcus
Gram	-	-	-	- (+)
Shape	rods	pleiomorphic	disks	cocci
Size (μm)	0.5-1.2x1-6		1.3x2.3	0.8-1.5
NaCl (g/l)				
requirements	>120	> 88	> 88	> 145
optimum	204.5-263.0	117-175	117-175	204.5-263
Mg^{++} (g/l)				
requirements	> 0.12	> 0.12	> 0.49	> 0.1
optimum	0.12-1.22	0.12-1.22	0.49-1.215	
pH optimum	5.5-8.5	5.5-8.5	5.5-8.5	5.5-8.5
T (°C) optimum	20-40	30-55	35	30-37
Oxygen requirements	aerobes/facultative anaerobes	aerobes/facultative anaerobes	strict aerobes	strict aerobes
Pigments	Bacteriorhodopsin Halorhodopsin	Bacterioruberin Retinian type pigments	Bacterioruberin Retinian type pigments	Carotenoïds Retinian type pigments

Halobacterium. Cells are rods under optimal conditions in young liquid culture. Pleiomorphic and coccoid forms may be present in old liquid culture and in agar-grown cultures. Cells divide by constriction and the time of division is long, between 3 and 6 hours. Cells are motile. Mg^{2+} requirement is moderate. Amino-acids are required for growth. Characteristic sulphated triglycosyl and tetraglycosyl diethers are present. The membrane contains the red pigment bacteriorhodopsin which, when oxygen concentration is low, acts as a proton pump converting light energy to a proton-motive force across the cell membrane which allows survival in anaerobic conditions. Thus, some strains are aerobes-facultative anaerobes. Another pigment, halorhodopsin, acts as a Cl^- pump [9]. The type species is H. salinarium which makes up a significant fraction of the microbial populations of neutral salt lakes and thalassohaline salterns. Proteolytic organisms of this genus are largely responsible for the spoiling of products salted by solar salts.

Haloarcula. Cells are extremely pleiomorphic. Under optimal conditions in liquid media, triangles, rectangles and irregular disks are commonly observed. Cells are motile or not. Mg^{2+} requirement is moderate. Amino-acids are not required for growth. Characteristic triglycosyl diether lipid is present. Bacteria of this genus contain a red pigment, bacterioruberin, and probably other pigments of retinal type in their membrane. Such organisms are found in several thalassohaline and athalassohaline

environments. The type species is H. vallismortis.

Haloferax. Cells are most commonly pleiomorphic rods and flat disks under optimal conditions in liquid media. Cells are motile or not. Mg^{2+} requirement is high. Amino-acids are not required for growth. Characteristic sulphated diglycosyl diether lipid is present. Phosphatidylglycerol sulphate is absent. Bacteria that belong to this genus possess bacterioruberin associated with retinal pigments but may not contain bacteriorhodospin [10]. Cellular division times are long (8 to 10 hours). Such organisms probably make up a significant fraction of the microbial population of the Dead Sea and are found in other Mg-rich environments, notably in salt works thalassohaline salterns. The type species is H. volcanii.

Halococcus. Cells are invariably coccoid occurring in pairs, tetrads, sarcinae or irregular clusters. Cells are non motile and are not lysed in distilled water. The wall material contains a complex highly sulphated or sulfonated heteroglycan which is apparently responsible for the rigid structure of the wall and for the resistance against lysis in hypotonic solutions [11,12]. At least some cells are stain Gram-positive. Cells contain several orange or red carotenoids and retinal pigments. The best recorded generation time is 14 hours. They are chemoorganotrophs with a strong demand in amino-acids, purines and pyrimidines and develop well between 30 and 37 °C. The type species is H. morrhuae. Halococcus species are encountered in the same places as other halobacteria, in strongly saline natural lakes and marine salterns, and have been shown to cause spoilage of salted fish. They have also been isolated from sea water [13] and hypersaline soils [14].

The second group is composed of Haloalkaliphilic isolates that grow only at high pH (8.5-11.0) with a very low Mg^{2+} requirement (<1mM or 0.024 $g.l^{-1}$). It comprises two genera Natronobacterium and Natronococcus which are found in continental soda lakes such as Wadi Natrun or Lake Magadi [15]. Both genera should not be involved in presently studied crystallisation.

5. LABORATORY EXPERIMENTS

The halobacterial populations of several samples of cooking salt coming from solar salt works of several countries were first studied. The bacterial populations were seeded into a numeration liquid medium containing 225 $g.l^{-1}$ NaCl that is below halite saturation. Such media are used to carry out halobacteria counting by the Most Probable Number method. After two weeks incubation at 35°C, the presence of halobacteria is depicted by the presence halite grains in the liquid [16]. Halite precipitation does not occur in proof tubes placed under the same incubation conditions. In our experiments small (0.5 mm approximately) rounded grains appeared in seeded tubes. Out of curiosity, some of the grains were gently crushed on a glass plate and examined through optical microscopy. They appeared to be composed of bacteria and halite fibres with a radial structure. Unfortunately, these colonies were not studied further.

More recently a solid medium was used (Table 3) in order to examine more easily the solid particles produced by the halobacterial populations of Guérande (France) cooking salt. Seeded and proof plates were enveloped in folded plastic bags in order to avoid desiccation as far as possible, and placed for two weeks in an incubator at 35°C. The composition of the medium (225 $g.l^{-1}$ NaCl) allows the growth of EHB only. Thus, after purification and Gram staining, the shapes of strains may be observed under optical microscopy allowing generic identification.

After incubation, nothing appeared on proof plates. On seeded plates, bacterial colonies developed and were rapidly covered by salt crusts visible to the naked eye. These crusts developed strictly over colonies and never elsewhere. Hence, salt crystallisation is closely related to bacterial development.

Table. 3
Composition of culture solid medium for bacterial salt precipitation experiments.

Peptone	5 $g.l^{-1}$
Yeast extract	5 $g.l^{-1}$
NaCl	225 $g.l^{-1}$
$MgSO_4$, $7H_2O$	25 $g.l^{-1}$
KH_2PO_4	3.5 $g.l^{-1}$
Trisodiumcitrate	3 $g.l^{-1}$
$(NH_4)_2Fe(SO_4)_2$,$6H_2O$	70 $mg.l^{-1}$
$MnCl_2$, $4H_2O$	0,5 $mg.l^{-1}$
Agar	15 $g.l^{-1}$
Demineralised H_20	1000 ml
pH	7,5 before sterilisation
Incubation temperature	35 °C
Incubation time	15 days

Such a composition fits with the requirements in NaCl and amino-acids of EHB. When such a medium is used, the numeration threshold is 5 EHB per gram of solid halite. It is worth mentioning the necessity to use dilution tubes which already contain more than 125 $g.l^{-1}$ NaCl in order to avoid bacterial cells lysis.

Usual forms were Halococcus and Halobacterium. In most cases, the bacterial strengths were rather high and often reached or overstepped 10^4 living cells per gram of analysed solid salt.

The salt crusts lying over bacterial colonies were sampled and their upper surface was observed and analysed through SEM after gold metallisation as only preparation. The most striking structural features of such crusts are the following.

The flat parts of the surface of colonies are made of bacterial bodies encrusted with halite as revealed by microprobe analyses. They are also covered with tiny hollow hoppers of approximately the same size as those observed in natural ooids. These hoppers, sometimes polycrystalline, are always located on top of tiny bacterial clusters growing up from the surface.

There are also some kinds of biomineral mounds or domes (10 to 20 µm wide) standing on circular assemblages of hoppers. On top of some mounds, new hollow hoppers may also appear. Added to experimental conditions, these observations strongly suggest that halite crystals formation is related only to the growing parts of bacterial colonies.

More complex crystalline assemblages were also observed on Halococcus colonies. They form around an initial hopper by the lateral growth of halite elongate crystals more or less perpendicularly to the sides of initial hopper. These halite fibres are sometime curved and give to the whole assemblage a fibro-radial, i.e. an ooidal, structure. Bacterial bodies are often still recognisable between fibres. Such a biocrystalline assemblage keeps a roughly cubic outer shape. The largest observed assemblages form hemispheroids 60 µm in diameter. They are composed of halitised bacterial bodies which are organised and arranged following the cubic frame of halite. Some crystalline edges appear toward the top of the structure. Let us notice that such structures were obtained after only two weeks incubation which partly accounts for the high deposition rate of rocksalt, as largely known from numerous geological evidences [17].

While somewhat disordered due to the particular shape of bacterial cells (rods), similar figures were observed with

Halobacterium, notably hoppers and more or less rounded fibro-radial fabrics growing from a central hopper.

6. THE MECHANISMS OF BACTERIAL SALT PRECIPITATION

The question arises whether the role of bacteria is passive, as nucleation sites, or active and then really linked to biological processes. All observations clearly show that the crystallisation of particles and the structuration of halite assemblages are closely and only related to the growing parts of bacterial colonies and not to bacterial bodies. No crystal appeared on unseeded proof plates after 15 days incubation.

Besides, experimental crystallisation was carried out in a medium (225 $g.l^{-1}$ NaCl) which was initially approximately 100 $g.l^{-1}$ NaCl below saturation. Hence, halite precipitation first occurs because in the closest environment of growing colonies, the medium reaches saturation and even oversaturation. As a matter of fact, one of the properties of Halobacteria is their ability to maintain an intracellular concentration of NaCl lower than the extracellular one. For example, the intracellular/extracellular concentrations ratio is 0.8 for H. morrhuae and 0.3 for H. salinarium when the NaCl extracellular concentration is 236 $g.l^{-1}$ [18]. Such a phenomenon is linked to the bacterial metabolism since it depends upon ionic pumps which are under control of pigments activity. This obviously should induce an increase of salinity in the near vicinity of growing cells and colonies, and local saturation, and even oversaturation, if the medium is already highly concentrated. This explains why the embryonic salt structures (hoppers) always appear on the growing, active parts of bacterial colonies and that the outer shapes of halite assemblages are dependent upon the shapes and volumes of growing colonies. Such a biologically induced phenomenon does not preclude a passive role of bacteria as nucleation sites when the whole brine body has reached saturation.

In brines, the bacterial osmoregulation and the growth of bacterial colonies living on the surface of the halite grains must be responsible for the radial salt accretion around the grains. The *bio-mineral growth* of the grains is thus controlled by the radial expansion of microbial communities in an isotropic environment. Indeed, the medium is approximately chemically isotropic. Furthermore it is even isotropic for energy (light and temperature) because of the high reflectance of the halitic sediments at the bottom.

The size distributions of salt grains suggest that the biomineral growth of ooids is not continuous but occurs sporadically. A simple explanation could be that individual ooids undergo alternating immersion and exondation periods, growth occurring only during immersion. However, this explanation hardly accounts for the size distribution pattern of the population of ooids. The biomineral accretion phases more probably represents the bacterial exponential response to an increase in organic nutrients in the medium. Such a phenomenon has already been demonstrated for carbonate ooids [19]. It most likely occurs following the decay of phytoplanktonic sporadic blooms that could be either local or generalised over the whole basins and related to seasonal variations (especially temperature and water motions). Comparison of aspects and densities of Berre halooids and Asal ones suggests that the former should be rather rapidly formed in generally eutrophic conditions whereas the latter should form more slowly or sporadically in overall oligotrophic conditions.

Most halobacteria are pink or reddish due to the presence of bacteriorhodopsin and other retinal-based pigments as patches in the cell membrane (purple membrane) [20]. These pigments are partly responsible for the usual colour of salt depositing brines in salt works crystallisors. They are only synthesised under low oxygen tension, which is not the case for Berre and Lake Asal brines which stay around

halite saturation, and partly explains the whiteness of ooids.

Halolites appear to be biocristalline build-ups formed during the early steps of halite precipitation i.e. in brines reaching saturation, fairly oxygenated and rather rich in organic matter. In highly concentrated salterns, the mineral crystallisation rules should overcome the biological trends.

7. CONCLUSION

This study confirms that Extremely Halophilic bacterial colonies are active in the formation and growth of halooids through their metabolism which keeps local saturation and their spatial development which guides the growth of bio-mineral assemblages. Bacterial bio-precipitation should be an usual process in geological salt deposits even though it leaves no trace in fossil formations due to diagenetic recristallisations. Of course salt bio-precipitation requires high salinities which are only obtained in evaporitic environments but such a phenomenon should considerably help for salt deposition because saturation of global environment is not fully required. It is even probable that salt production would be considerably lowered, and some year nil, in salt works of Brittany, if halobacteria were absent from brines in which saturation is hardly obtained due to climatic conditions. On another hand, the rapid bio-precipitation of salt should induce the presence of impurities and inclusions which could lower the quality of salt for chemical uses.

REFERENCES

1. Y. Weiler, E. Sass, I. Zak, Sedimentology, 21 (1974) 623-632.
2. P. Sonnenfeld, Brines and evaporites. Academic Press, Inc. Orlando, 1984.
3. S. Castanier, J.-P Perthuisot, J.-M. Rouchy, A.Maurin, O. Guelorget, Geobios, 6 (1992) 811-821.
4. S. Castanier, J.-P. Perthuisot, M. Matrat, J.-Y. Morvan, Sedimentary Geology 125 (1999) 9-21
5. J.-P. Perthuisot, S. Castanier, A. Maurin, Bull. Soc. Géol. Fr., 4 (1990) 657-66.
6. J.T. Staley, M.P. Bryant, N. Pfennig, J.G. Holt, (Eds), Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, Hong Kong, London, Sydney, (1984) 261-267.
7. J.T. Staley, M.P. Bryant, N. Pfennig, J.G. Holt, (Eds), Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, Hong Kong, London, Sydney, (1989) 2216-2233.
8. R.Y. Stanier, J.L. Ingraham, M.L. Wheelis, P.R. Painter, General Microbiology, MacMillan Education, London, 1987.
9. J. Pelmont, Bactéries et environnement. Adaptations physiologiques, Presses Universitaires de Grenoble, 1993.
10. A. Oren, and M. Shilo, Arch. Mikrobiol., 130 (1981) 185-187.
11. R. Reistad, Arch. Mikrobiol., 102 (1975) 71-73.
12. J. Steber, and K.H. Schleifer, Arch. Mikrobiol., 105 (1975) 173-177.
13. F. Rodriguez-Valera, F. Ruiz-Berraquero, A. Ramos-Cormenzana, Appl. Environ. Microbiol., 38 (1979) 164-165.
14. E. Quesada, A. Ventosa, F. Rodriguez-Valera, A. Ramos-Cormenzana, J. Appl. Bacteriol., 53 (1982) 155-161.
15 B.J. Tindall, H.N.M. Ross, W.D. Grant, Syst. Appl. Microbiol., 5 (1984) 41-57.
16. H. Onishi, M.E. McCance, N.E. Gibbons, Can. J. Microbiol., 11 (1965) 365-373.
17. P. Sonnenfeld and J.-P. Perthuisot, Short Course in Geology, 3, 28th Int. Geol. Congress, Amer. Geoph. Union, Washington, 1989.
18. J.H.B. Christian and J.A. Waltho, Biochem. Biophys. Acta, 65 (1962) 506.
19. S. Castanier, A. Maurin, J.-P. Perthuisot, Bull. Soc. Geol. Fr., 3 (1989) 589-595.
20. L.M. Prescott, J.-P. Harley, D.A. Klein,. Microbiology (Second edition). W.C. Brown Communications, Inc. A Time Mirror Cy., 1993.