The biological significance of Halobacteria on nucleation and sodium chloride crystal growth

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ABSTRACT

The participation of halobacteria in halite formation has been previously considered by a number of authors. In fact, the release of bacterial nucleation factors has been suspected but never properly documented. Some studies show that certain chemicals are capable of modifying sodium chloride crystal habit, and this possibility is of the utmost importance for the correct management of salters. For example, from over 500 compounds tested, only 12 have been found able to modify the crystal arrangement of halite. The halobacteria can also get entrapped in fluid inclusions within the crystal and retain viability for several years. Thus, they may play some role in contamination of salt preserved foods. Here we review the subject of halobacteria participation in sodium chloride crystal formation and present some results with regard the influence of cell surface layer (S-layer) components on crystal habit. As appears, the halobacteria may influence crystal growth rate and crystal habit, allowing the optimization of salters management.

1. INTRODUCTION

Cell morphology and cell surface components have been implicated in a wide variety of biological and adhesion phenomena, including mineral and organic crystal formation. Crystallization is used in industry to recover and purify many inorganic and organic materials, but very little work has been done to investigate the feasibility of bulk crystallization for the recovery and purification of proteins and mineral crystals [1]. Crystallization of large molecules, such as proteins, is less known than crystallization of mineral salts, and their mutual effects are poorly understood. For example, it is known that for each protein there appears to be a set of mineral substrates that promote nucleation of protein crystals at lower critical levels of supersaturation than required for spontaneous growth [2,3].

Judged et al. [1] studied the crystallization of ovalbumin in ammonium sulfate and observed that crystal growth could occur without nucleation at a relative supersaturation value of 20. The crystal size distribution was measured and the
crystal growth rate was found to be of second-order depending on ovalbumin supersaturation. There was only a slight effect of the ammonium sulfate concentration at pH above 4.9, while the effect of pH itself allowed a 10th-fold increase in the crystal growth rate constant in the range of 4.6-5.4. Under these conditions, the presence of other protein components did not affect the crystal growth rate of ovalbumin. In general, protein supersaturation can eliminate impurity effects [4] and temperature seems to favor crystallization. As a rule, slow crystallization of proteins is due primarily to impedance of the elementary act of entering the growth site, indicating a decisive role of entropy, not of energy barriers, in the crystallization of biological macromolecules. Pressure may actually inhibit crystal yields since, at equilibrium, two conformations of the protein (in the case of lysozyme) have been postulated: one capable of being incorporated into the nuclei of the crystal, and the other which is not [5]. The addition of polyethylene glycol (PEG) into the crystallization medium favors crystal growth by disordering protein aggregates either, in the medium, or at the surface of the crystal, therefore allowing the formation of much larger crystals [6].

In humans, the organic matrix of renal calculi influence the crystal growth that occurs in such pathological mineral deposits and the use of antibodies against these molecules has allowed to visualize their distribution in a variety of normal and pathological mineralized tissues [7]. In the case of cholesterol, nucleation time referred as the time of the first appearance of cholesterol crystals from isotropic crystal-free bile on light microscopy, is being used to assess the potency of nucleating agents such as immunoglobulins [8]. As demonstrated by this test, biliary IgM (M immunoglobulins), are more potent than biliary IgG (G immunoglobulins) as a potential nucleating agent. A 42 kilo Daltons (KDa) biliary glycoprotein has been also shown to be related with cholesterol crystallization promotion on the pathogenesis of gallstone disease [9]. The protein is an extensively glycosylated (37%) monomer with an isoelectric point, pI, of 4.1, probably due to its sialic content. Enzymatic N-deglycosylation removes the carbohydrate moiety and inactivates the promoting activity. Enzymatic proteolysis results in both a complete structure degradation and functional inactivation.

Biomineralization is the term used to refer to biological metal precipitation, however it should be borne in mind that not necessarily true crystal minerals are formed through this process. Thus, the concept may be misleading. The metal precipitates can exist either intra- or extracellularly, attached or unattached to the cell surface. The adsorption of metals to living or dead cells, which does not involve metabolic energy or transport processess, has been termed “biosorption” [10]. Biosorption has been the metal-microbe interaction most widely studied and covers both the terrestrial and the marine environment. It should be emphasized, however, that biosorption by marine bacteria may have special implications since they occur in a high ionic medium with high concentrations of some metals. Aside of its use for detoxification of heavy metals in aqueous systems [11], very little
information is available about the influence of bacteria in metal crystal formation. One important exception may be the cycling of manganese in the marine environment. In general, metal-precipitating bacteria are abundant in hydrothermal vents and the mechanism of metal precipitation may differ greatly among the different types of microorganisms and metals.

Metal-microbe interaction can be classified into two classes [12]: a passive process which does not require the direct participation of living organisms and can occur whether the microbes are alive or dead; and an active process, in which some metabolic or enzyme activities are involved. In the first type interaction results as a consequence of the negative charge of microbial cell surfaces and their exopolymers, or through reactions with extracellular complexing agents that can be attached to the cell surface, or released into the medium. This passive sorption of metals is mediated by various functional groups which promote adsorption, ion-exchange, chelation, and/or covalent binding through carboxyl, hydroxyl, sulphydryl, amino, imino, imidazole, sulfate and/or sulfonate groups, present in the cell surface, in the cell wall, or within the cytoplasm, as polysaccharides or glycoproteins. A classical application of this mechanism of sorption is wastewater treatment.

Microorganism that can produce extracellular complexing agents such as "siderophores" do so to trap the metals by a passive mechanism that later are internalized by an active process. The active removal of metals occurs via extracellular precipitation, redox reactions, intracellular accumulation, or volatilization [12]. The biomineralization of gypsum, for example, seems to be a two-step process initiated by the binding of calcium to the cell surface following of the binding of sulfate to the calcium. In photosynthesizing cells, the sulfate is eventually replaced by carbonate to form calcite at the cell surface as the pH increases. Thus, the cells provide essential nucleation sites and the chemical conditions necessary for mineral formation [13].

2. THE PHYSIOLOGICAL ROLE OF CRYSTAL FORMATION

In polar and sub-polar fishes, for example, some glycopeptides protect their body fluids from freezing. Such peptides prevent the growing of ice by a non-colligative process thus inducing the development of unusual and strikingly similar crystal habits suggesting that the peptides show some affinity for similar crystal faces of ice [14,15]. Ideally, the protein has an exact octapeptide repetition and is assumed to have an helical conformation to control crystal formation [3]. Certain bacteria promote the formation of ice in super-cooled water by means of ice nucleators; the opposite effect, inhibition of ice formation, is common for a group of glycoproteins found in different fish and insect species. These substances termed anti-freeze glycoproteins promote the supercooling of water with no appreciable effect on the equilibrium freezing point, or melting temperature, by binding to a growing ice crystal and slowing crystal growth [16]. In a similar fashion, glycosaminoglycans (GAGs) and some sulphated polysaccharides are
involved in preventing urinary stone formation by inhibiting crystal growth and agglomeration, and possibly also nucleation. They can prevent crystal adherence, correct an abnormal oxalate flux, and avoid renal tubular cell damage [17]. Other synthetic polyanions, including peptide analogs of naturally occurring proteins, inhibit the nucleation and growth of calcium salt crystals [18] under physiological conditions.

3. CRYSTAL PROMOTION

Bacteria interact with metals, not only because they are needed as nutrients, but also as important agents in their geochemical cycling. Such metal cycles are driven by diverse chemical and biological processes and may be biotechnologically important. For example, in mining industry the bacteria may participate in the oxidation and solubilization of metal sulfide ores and to recover valuable metals from low grade ores. Metal precipitation by bacteria refers to the transformation of a soluble metal to an insoluble form. Usually, in the first stage of the process the formation of amorphous, highly hydrated, precipitates are obtained, but with aging they can be transformed into crystals [19].

In the crystallization of biomolecules two critical steps, the nucleation of the initial seed and the enlargement of this seed, determine the quality of the final crystal. However, the degree of supersaturation required to nucleate crystals is often higher than the optimal concentration necessary for enlargement. Thus, even under conditions suitable for crystal growth, kinetic factors may prevent the onset of nucleation and crystal growth. Also, spontaneous nucleation may occur at such frequencies that the resulting microcrystalline precipitates are indistinguishable from their amorphous counterparts. It many situations, it is advisable to decouple crystal growth from nucleation in order to grow large, regular crystals. One must not only control the number of seeds, but also reduce the supersaturation level and, therefore, decrease the incorporation of defects detrimental to crystal quality.

Seeding techniques provide a preformed, regular crystal surface onto which further molecules may be aggregated in an orderly form, generally at a lower degree of supersaturation than is required for nucleation. Such techniques are ideally suited to bypass the nucleation step, and hence accomplish the decoupling between nucleation and crystal growth. Three aspects for seeding should be considered: 1) preequilibration of the solution to be seeded and determination of the proper supersaturation level for seeding; 2) the environment and necessary precaution for seeding; and 3) the streak seeding technique and how it can be used in conjunction with microseeding and macroseeding [20].

In the case of some proteins, crystallization may be affected by the amino-terminal segment that sticks out to interact with a symmetry related molecule through an intermolecular salt-bridge. For example, removal of the residue lysine in position 38 (Lys 38), in the case of an endonuclease from Clostridium thermocellum, or the substitution of its bridge-forming residues by site-directed
mutagenesis, promotes crystal packing arrangements different from the wild type enzyme [21]. Flexible amino- and/or carboxy-terminal extensions, influence crystal nucleation but not crystal growth [21]. Following this idea, an attempt to facilitate crystallization has been the use of engineering cysteins to promote formation of a “back-to-back” dimmer that occurs in different crystal forms of wild-type and mutant lysozymes [22]. The designed double mutant in which the surface residues of asparagine Asn88, and alanine Ala93, were replaced by cysteines, formed dimers in solution and crystallized isomorphously to wild-type but a much faster rate. Overall, the mutant structure remained very similar to wild-type despite the formation of two intermolecular disulfide bridges. The results suggest that the formation of the lysozyme dimer is a critical intermediate in the formation of more than one crystal form and that covalent cross-linking of the intermediate accelerates nucleation and facilitates crystal growth.

Another role seems to be played by polysaccharides in the case of the calcifying algae Pleurochrysis carterae, which produces structures known as coccoliths in homogeneous cell cultures. The polysaccharides PS-1 and PS-2 have been localized in the crystal coats of mature coccoliths, and in electron dense Golgi particles. These polyanions are synthesized in medial Golgi cisternal and co-aggregate with calcium ions into discrete 25 nm particles. The polysaccharides remain with the mineral phase after the coccoliths are extruded from the cells [23].

The effect of many different compounds on calcium stone formation has been evaluated [24]. A few appear to inhibit the nucleation rate, growth and suspension density (crystal mass produced) in proportion to its concentration. On the other hand, glucose, glycerol and certain amino acids which are recognized as osmoregulators, and are produced by halophilic microorganisms, have been also evaluated as halite crystal habit modifiers [25]. In the same way, silica gel as an inert particle, and the ferrocyanide ability to form dendrite crystals of halite, have been already reported [26,27]. Altogether these data point to a dramatic effect of different compounds on mineral and protein crystallization and have induced us to study the influence of halobacteria, and of its components, in the nucleation, shape and growth of NaCl crystals under natural conditions.

4. INFLUENCE OF HALOBACTERIA ON HALITE FORMATION

It seems that since halobacteria have not being connected to any dreadful disease, and because they are easy to grow, albeit very slowly for practical purposes, interest on their biotechnological use is very scant. In fact, their ecological role is still poorly understood [28]. The term halobacteria refers to the halophilic Archaea, not to halotolerant bacteria, and this distinction is of utmost importance. The order Halobacteriales contain six genera and the number of newly found species is increasing. Phylogenetic data indicate that they are among the most modern Archaea, as their strong preference for aerobic life
would suggest. Halobacteria require a minimum of 1.5 M (9%) sodium chloride for growth, and in most cases the optimum lies between 3.5-4.5 M (21-27%) NaCl. Such salt concentration exceeds by far the total saltiness of sea water (which is about 0.6 M or 3.5% of dissolved salts). As a particular feature, halobacteria exhibit active growth and motility in saturated salt solutions, only reduced when entrapped into the salt crystals they bump into. In the absence of salt, all except the coccal forms of halobacteria disrupt promptly and dramatically. In any location where the basic requirement of salt is met, halobacteria will be found. They may become the dominant microflora in what appears to be a classical ecological succession [28].

The halobacteria can be grouped into three major types reflecting their natural source: in the first category are habitats in which the salt mixture derives from evaporated seawater. The commercial salterns consist of shallow evaporating pools containing brines of steadily increasing salinity. They are an ideal ecosystem to observe and study microbial succession. As the brine concentrates under the hot sun and wind, its density and, therefore, thermal storage capacity increase, while its ability to hold dissolved oxygen declines. Certain ions reach saturation earlier than others and precipitate out. For example, calcium and sulfate crystallize out as gypsum. In the less saline stages (<2M salts), halotolerant Prokarya predominate. Between 2-3 M, halobacteria progressively displace Prokarya. At 3 M, halobacteria alone are found as a thriving component of a characteristic hypersaline ecosystem that includes green algae (Dunaliella spp), brine flies, and brine shrimp. Above 3 M, a more subtle succession of progressively more halophilic Archaea appears as the brines evaporate to the saturation point and salt crystal begin to form. On the other hand, underground salt mines reflect their marine origin as evaporitic sediments from past geological era and are composed mainly of sodium chloride and mixed with lesser amounts of potassium, magnesium and calcium salts and layers of fine clay particles. The halobacteria can be recovered from samples of brines that have come to the surface, and also from intact rock salt. Finally, the salt lake habitats are by no means generic. Each is a terminal lake and collects ion-rich runoff from its own drainage area. Chemically, they resemble the composition of surrounding rocks and mineral deposits. Warm, slightly acid to neutral-pH lakes, such as the Great Salt Lake and the Dead Sea, tend to have high sodium and magnesium concentrations. Warm alkaline lakes, also called soda lakes, have high sodium and carbonate concentrations, with very low amounts of magnesium [28].

As one could expect, hypersaline habitats are intensely stressful: first, the halobacteria have to withstand the elevated ionic concentration that no other life form seems to tolerate. All halobacteria require sodium ions for structural integrity. Requirements and tolerances for other ions depends on the strain’s original habitat. To deal with the osmotic challenge that salt concentration imposes, the halobacteria accumulate intracellular substances to balance external stress, for example potassium ions up to 5 M. In spite of being shallow habitats affected by wind, the concentration of dissolved oxygen in brine waters
is low, and becomes even more critical as temperature rises. Midday temperatures in salterns can average 45°C rising to more than 60°C occasionally. To survive under such conditions the halobacteria have adopted several strategies: one appears to be the flexibility of their outer layers which allow the cells to take a variety of flattened shapes including squares, rectangles, flattened discs, and triangles. Such morphologies help the bacteria to exchange nutrients and gases more efficiently. In addition, active flagellar motility is common in the majority of fresh halobacteria isolates, suggesting that such cells use positive taxis to reach oxygen. Other halobacteria have gas vesicles which help them to position in the water column [28].

The ability of halobacteria to survive long dry seasons indicate that brine inclusions are perhaps the most critical strategy of the bacteria for survival. It has been proposed that halobacteria may act as nucleation factors for halite and other minerals [28], in a similar fashion as the nucleation protein of *Pseudomonas syringae* in ice. The halobacteria however, lack peptidoglycan and the non-coccolidal genera exhibit a high-molecular weight complex glycoprotein bearing sulfated oligosaccharides. This protein, as a rule, has an excess of acidic amino acids over basic amino acids, and enables the cell to bind and organize large amounts of cation. The cytoplasm membrane is composed mainly of isoprenyl glycerol diether lipids with chain lengths of 20-25 carbons. Both, sulfated and glycosylated lipids are used as taxonomic criterium.

When sodium chloride crystallizes in natural salterns, the microflora normally present in the ponds influence the overall process in different ways. Some living halobacteria may even get entrapped within the fluid inclusions as the crystal develops, and thus affect the physical characteristics of the product. The microorganism might be able to survive under such conditions for extended periods (about 4 years) and this fact imposes some recommendations for the proper handling and application of the contaminated product. The problem becomes evident when the salt is used for preserving fish, meats and hides: it has been shown that red discoloration of food preserved with salt is due to the halobacteria that remain viable in crude solar salt after harvesting [29]. The survival of the entrapped bacteria is determined by storage conditions, and sometimes they may even reproduce [30]. The water content of fresh solar salt is usually in the range between 2-6% (w/w), and in some cases up to 15% (w/w) [31]. The fluid inclusions are readily observed under low power magnification and may be described as negative cubes, or oblongs, with slightly rounded corners. They contain aqueous solutions and, in some cases, small gas bubbles. They range in size from less then 1 μm in the longest dimension to several millimeters. Most however are in the micrometer size range. If the salt is not exposed to extreme heat, pressure, or recrystallization, inclusion fluids may be considered similar in composition to the evaporitic fluids from which the salt crystals originated. The extent of bacterial entrapment in fluid inclusions has been studied using pure cultures of halobacteria added to saturated salt solutions [32].
With regard to the influence of halobacteria in NaCl crystal habit formation, much less information has been collected in spite of the many investigations on materials affecting the halite crystal habit. About 500 different organic and inorganic compounds have been tested to modify the crystal habit of NaCl and only 12 have shown to be effective [33], among these urea, cysteine, creatinine, papain, monosodium glutamate, some cadmium salts, the sodium hexametaphosphate, aluminon, and the chlorides of Zn\(^{2+}\) and Mn\(^{2+}\), cause the formation of octahedra, while ferrocyanides were capable of modifying the habit formation (US Patent 2 642 335 (16 June 1953) and 3 090 756 (21 May, 1963)). A combination of cubes and octahedrons have resulted from the action of NaOH, boric, phosphoric and hydrochloric acids [34]. The addition of mercuric chloride causes combinations of the cube and dodecahedron crystals, and antimony chloride causes a combination of cubic, octahedron and dodecahedron ones. The greatest effect seems to be played by the ferrocyanide salts which in concentration as low as 10 ppm can cause the formation of vicinal faces that prevents salt bridges between contiguous crystals. By controlling the concentration of ferrocyanide it is possible to favor a particular crystal formation which constitute the basis for the anti-caking and low-bulk density sodium chloride patents. The dendrite crystals formed at high concentrations of ferrocyanide salts are in fact cryptocrystalline extensions of cube corners which grow to lengths of 2.5 cm or more. On the other hand, when a combination of sodium hexametaphosphate and a soluble aluminum salt are used, the formation of octahedra resulting from the action of hexametaphosphate is suppressed and cubic crystals with a basket shape are formed. Such structures have been described as aggregates of a geometrical arrangements of tetrakaidecahedra, a combination of 14-faced of cube-octahedron with eight hexagonal faces and six square faces, all with edges with equal length [26].

Guerrero Negro appears to be the largest solar saltern of the world and is located on the west coast at the middle of the Baja California Peninsula, in México. Its products, resulting from the evaporitic action of solar and wind energy make a business worth several hundreds of millions of dollars every year and are exported worldwide. Thus, it is just reasonable to become interested in studying the influence of halobacteria in halite production. Our aim was not only supported by a scientific curiosity, but also in view of increasing competition of other countries in the international salt market. Another goal was to consider the possibility of increase production capabilities by taking advantage of the role of halobacteria in halite formation. To determine if halobacteria indeed influence the production of halite, an experiment was designed to study the effect of an endemic isolate (Haloarcula SP8807) and of its Slayer on halite crystal habit, and crystal growth rate.
5. MATERIALS AND METHODS

5.1. Bacteria strains and growth conditions
The bacteria strains tested in this study were Halobacterium halobium NRC 817, Haloarcula vallismortis ATCC 29252, Haloferax mediterranei ATCC 33500, Haloarcula SP8807, Vibrio parahaemolyticus MMF6, Planococcus spp. M6P2, and Azospirillum brasilense Cd DSM 1843. The halobacteria Haloarcula SP8807 was isolated from a commercial saltern in Pichilingue, La Paz, B.C.S., México, while the eubacteria V. parahaemolyticicus, and Planococcus spp. were obtained from an hypersaline ecosystem at Guerrero Negro, B.C.S., México. All the other halobacteria, as well as A. brasilense, which is an organism not found in hypersaline environments, were acquired from an international collection.

The halophilic bacteria were grown in HEC medium formulated as follows in 25% seawater (g/l): NaCl, 195; MgCl₂·H₂O, 16.25; MgSO₄·7H₂O, 25; CaCl₂·2H₂O, 0.6; KCl, 5; NaHCO₃, 0.2; NaBr, 0.6; NH₄Cl, 2.5; FeCl₃·6H₂O, 0.0062; KH₂PO₄, 0.62. The carbon and nitrogen source came from yeast extract (5 g/l) and casein hydrolysate (1.0 g/l) [35]. Incubation was done at 38°C under stirring at 150 rpm. A. brasilense on the other hand, was cultivated in Nutrient Broth (Merck, Germany), and incubated at 30°C under agitation as above.

5.2. Determination of the number and size of halite cubic crystals with scanning electron microscopy
One ml of bacteria culture at the exponential-phase was centrifuged for 5 min., 16,000 x g at 20°C. The pellet was washed three times with sterile 30% NaCl solution, and resuspended in 1 ml of the same solution. From this suspension, 5 μl drops were placed on a gold-coated glass coverslip as described by [25]. The drops were incubated at 24°C without disturbance. After 10, 20, and 30 min., excess of brine was eliminated with a filter paper. The crystals were air-dried and gold-coated at 20 mA during 8 min. The blank consisted of 5 μl drops of sterile 30% NaCl solution treated under the same conditions. The crystals were viewed using a Philips 515 scanning electron microscope at an accelerating voltage of 25 kV.

5.3. Evaluation of effect of strains, s-layer and chemical materials on the crystal form of NaCl
One ml of bacteria culture at the exponential-phase was centrifuged for 5 min., 16,000 x g at 20°C. The pellet was washed three times with sterile 30% NaCl solution, and resuspended in 1.0 ml of the same solution. From this suspension, twenty drops of 5 μl each were placed on a clean microscope slide and kept in a chamber for 24 h at 35°C, and 40% relative humidity. The slides were observed with a phase-contrast microscope (Nikon Labophot, Japan) at low magnifications of 2.5X, 10X and 20X.

The following samples were mixed with sterile 30% NaCl solution: S-layer from Haloarcula SP8807 (20 mg/ml); potassium ferrocyanide, glucose, glycerol, casein hydrolysate, and silica gel (63-200 mm) from Sigma Chemicals Co. St.
Louis, Mo, and amino acids from Merck, Germany (8003-8004), at concentrations between 200 to 2000 mg/ml. Twenty 5-μl drops of each mixture were placed on clean microscope slides, dried, and observed as described above.

5.4. Preparation of s-layer and spheroplast of Haloarcula SP8807

The S-layer was isolated as follows: one ml of bacteria culture in its exponential phase was centrifuged for 5 min., 16,000 x g at 20°C. The pellet was resuspended in 0.1 M MES [2(N-morpholino)ethane sulfonic acid] buffer, pH 7.0, plus 0.5 M sucrose, 0.25 M NaCl, and 0.01 M MgCl₂ [36]. After 30 min., the cells were converted into spherical bodies by a decrease in external sodium chloride and magnesium salt concentration. Under these conditions, the S-layer can be removed and dissociated from the cell surface [37,38].

The new cell suspension was centrifuged for 5 min., 16,000 x g at 20°C, yielding a pellet of spheroplasts and a supernatant containing the dissociated S-layer subunits. The S-layer subunits were recovered and dialyzed according to [39]. After dialysis, the S-layer subunits were reassembled in a salt solution composed of 4 M NaCl, 25 mM KCl, and 80 mM MgSO₄, pH 3.2 [37]. The self-assembled S-layer was examined by SDS-PAGE, transmission electron microscopy, and tested in crystal formation studies. For negative staining preparations we used a solution of CaCl₂ 10 mM pH 6 to promote the reassembly of the S-layer subunits [40,41].

5.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was done using gradient PhastGel 4-15% with sodium dodecyl sulfate (SDS) buffered strips (Pharmacia, Uppsala, Sweden). The molecular weight markers were: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa) from bovine milk (Sigma, SDS-7 Dalton Mark VII-L, USA). The gels were stained for protein by the silver impregnation method with PhastGel electrophoresis media (Pharmacia, Sweden), and periodic acid-Schiff (PAS) staining procedure for the detection of glycoproteins using the PhastSystem [38].

5.6. Negative staining for electron microscopy

Negative staining preparations for electron microscopy, EM, were done with fixed and unfixed self-assembled S-layer. Reassembled S-layer was prefixed in 3% (v/v) glutaraldehyde in buffer solution of 4 M NaCl, 25 mM KCl, 80 mM MgSO₄, pH 3.2, during 1 h at 4°C in the dark. After this time the sample was centrifuged for 5 min., 10,000 x g, at 20°C, and the supernatant replaced with clean fixing solution under the same conditions. The fixed S-layer was washed and preserved at 4°C with the same buffer solution.

A few drops (10 μl) of fixed samples containing S-layer fragments were placed on a small sheet of Parafilm and diluted 1:40 with a buffer solution of 2 M NaCl,
12.5 mM KCl, 40 mM MgSO₄, pH 3.2. Immediately 10 μl of sample were transferred to a copper grid previously exposed to glow discharge. After 3 min., each grid with sample was stained with 2-3 drops of 2% of aqueous uranyl acetate for 1 min. Excess stain was removed by blotting with filter paper [39].

10 μl of unfixed samples containing reassembled S-layer in solution of CaCl₂ 10 mM, pH 6, were placed on copper grids previously exposed to glow discharge. After 3 min., each grid with sample was stained with 2-3 drops of 2% of aqueous uranyl acetate for 1 min. Excess of stain was removed by blotting with filter paper [39]. Some samples were micrographed without staining to avoid the dilution of microcrystals. The samples were viewed in a Philips 410 transmission electron microscope at an accelerating voltage of 80 kV.

6. RESULTS AND DISCUSSION

6.1. Crystallization rates

Following the methodology described above, we found no significant differences in the weight of sodium chloride crystals harvested with, or without, halobacteria. That is, in either case the amount of salt crystals produced was about the same so we may conclude that the halobacteria does not improve productivity of salterns. Interestingly, the halobacteria did affect the number and size of the sodium chloride crystals (Fig. 1; for convenience, crystals smaller than 1 mm were disregarded).

![Graph](image)

Figure 1. Influence of halobacteria on the number and size of halite crystals. For convenience, only crystals larger than 1 mm were considered. The data were used to perform a one-way analysis of variance (ANOVA) test with a confidence of p ≤ 0.05. 1 – NaCl (1-8 mm), 2 – Halobacterium halobium NRC 817 (1-25 mm), 3 – Haloarcula vallismortis ATCC 29252 (1-15 mm), 4 – Haloarcula SP 8807 (1-15 mm).
When our data were statistically analyzed by a one-way analysis of variance (ANOVA) to evaluate the effect of halobacteria on crystallization rates, we found that the number of crystals formed in the presence of the bacteria tested, differed significantly from the control (Fig. 1). The blank produced few crystals of similar size, while in the presence of halobacteria many cubic crystals of different size were generated (Fig. 1). The analysis also indicated that the shape, and/or morphology, of the halobacteria provoked different effects on crystal habit [43]. In particular, bacteria with triangular, or square-shape (e.g. _Haloarcula_ SP8807) seem to provide a template and serve as a means of mechanical nucleation to promote crystal formation in saturated solutions [32].

### 6.2. Crystal formation of NaCl

Halite has a crystalline form based on a cubic symmetry [26,27]. The influence of _Haloarcula_ strain SP8807 on NaCl crystal habit growth was observed by scanning electron microscopy (Figure 2). The micrograph illustrates the close association that occurs between the bacteria and halite crystals. In the caption the crystal is shown as if emerging from the cell. This caption is a good physical evidence on the role of halobacteria on crystal growth. By transmission electron microscopy, on the other hand, and in the presence of unfixed and unstained samples of S-layer, symmetric particles of 50 nm of diameter, which could be microcrystals of sodium chloride in their early stage of mineralization, were observed (Fig. 3). These two approaches suggest that either whole cells, and/or their S-layer, can be involved in the nucleation and crystal formation of NaCl.

_Haloarcula_ strain SP8807 also caused the formation of dendrite crystals of halite. The SEM micrograph of Figure 4A shows crystal formations in which dendrite crystals lie outside, and in between, cubic crystals. A high magnification of this caption shows that the dendrite crystals are close to the halobacteria surface, and confirms its ability to modify the crystal habit into irregular shapes (Fig. 4B). The other strains of bacteria tested in our study were also capable of inducing formation of dendrite crystals, except in the examples of _V. parahaemolyticus_ and _Planococcus_ spp. which suggest that not all the microbiological members present in the crystallizer ponds are involved in the modification of crystal habit. This assumption was further supported by the inability of _A. brasilense_, a non-hypersaline eubacteria, to form dendrite crystals.

SDS-PAGE analysis of purified S-layer from _Haloarcula_ SP8807 showed a single protein with a molecular weight of 66 kDa (Fig. 5). Noteworthy, the staining procedure for the detection of glycoproteins failed to reveal any band in the electrophoresis gel. It is well known that the S-layer of most halophytic archaeabacteria appear to be composed of glycoproteins [44], a report on the detailed chemical structure of a glycopeptide of _H. salinarum_ [45], and the primary structure of the cell surface glycoprotein of _H. halobium_ [46], confirmed such observations [47,48]. The cell surface glycoprotein of _H halobium_ has a molecular mass of about 120 kDa (core protein = 87 kDa). Recently, Sumper et al. [49] reported a partial chemical characterization of the S-layer glycoprotein of
*Halofex volcanii* in which the mature polypeptide contains 794 amino acids with a calculated molecular mass of 81 kDa. Although glycosylation is not an obligatory step in S-layer biosynthesis, it represents an important protein-modification reaction which can add a great potential to the diversification of bacterial cell surface properties [50]. In this context, it is interesting to note that upon continuous cultivation under optimal conditions, some bacterial strains loose their ability to glycosylate S-layer proteins [44].

Figure 2. (A) Micrograph of halobacterial cell showing a cubic structure assumed to be a crystal of sodium chloride. The bar represents 1.0 μm. (B) High magnification showing some detail of a sodium chloride crystal on the surface of an halobacterial cell. The bar represents 0.5 μm.
Figure 3. An unstained mount of a mineralized S-layer fragment in which the contrast comes from the sodium chloride crystals. The arrows show symmetric bodies of 50 nm of diameter, probably sodium chloride crystals, on an early stage of formation. The bar represents 0.5 μm.

Negatively stained preparations of S-layer from *Haloarcula* SP8807 strain examined by transmission electron microscopy show structures with a honeycomb appearance of the reassembled S-layer (Figure 6). These are the units suspected to act as templates for crystal nucleation and growth. As in the case of the S-layer protein isolated from *Bacillus coagulans*, which was recrystallized on an air/water interface and on phospholipid films [51], the hydrophobic face of the protein may actually associate with the air/water interface, leaving the negatively charged inner face oriented itself towards the switterionic head groups of the lipids. Hence, individual monocrystalline areas may grow isotropically in all directions, until the front edge of a neighboring crystals is met, forming the honeycomb structure observed in Fig 6. Recently it was proposed that the S-layer may serve also as a template for fine-grained gypsum and calcite formation [52].
Figure 4. Details of dendrite crystals. (A): Dendrite crystals built by flat cubic microcrystals (arrows). Bar represents 5 μm. (B): A high magnification showing the presence of halobacterial cells (arrows) combined with amorphous halite crystals (empty arrow). Bar represents 2 μm.
As shown in Figure 7, the S-layer of *Haloarcula* SP8807 (20 μg/ml) modifies the crystal habit of halite yielding dendrite crystals, as well as cubic crystals (Fig. 7). The dendrite crystals and their branches at micrometer level showed flat cubic microcrystals combined with amorphous crystals and halobacterial cells (Fig. 4). Naked cells, or spheroplasts of *Haloarcula* SP8807, on the other hand, showed to be extremely fragile to influence the crystal production. In fact, partial lysis was observed which translated into many protein bands in SDS-PAGE. A 66 kDa light band from spheroplast samples (Fig. 5) confirmed the observations of Jarrel and Sprott [36] who reported that spherical bodies produced from logarithmic-phase cells contained a cell wall that was thinner than the wall of the original rod shaped halobacterial cells.
Figure 6. Negatively stained (2% uranyl acetate) preparations of S-layer self-assembly products of *Haloarcus* SP8807. (A): Unfixed S-layer reassembled with CaCl₂ 10 mM, pH 6. The arrow shows an area with trace of periodicity in one dimension. The bar represents 100 nm. (B): Self-assembled flat sheet-like S-layer showing a honeycomb appearance of an hexagonal arrangement (circle), stained in presence of 2 M NaCl, 12.5 KCl, 40 mM MgSO₄, pH 3.2. Bar represents 200 nm.

In addition to the above, we studied the effect of different compounds on the induction of dendrite crystals of halite, as shown in Table 1. Again, ferrocyanide was found the most active agent inducing the formation of dendrite crystals and at high concentration (250 µg/ml) caused dendrite growth of sodium chloride crystals entrapped within cubic crystals. Casein hydrolysate (1200 to 2000 µg/ml), and some aminoacids tested, did not induce the production of dendrite crystals (Table 1). Similarly, the absence of dendrite crystal formation in the presence of silica gel demonstrates that this phenomenon is not the result of an inert particle effect.
Figure 7. Dendrite halite crystals caused by S-layer (20 µg/ml) from *Haloarcula* strain SP8807. Bar represents 50 µm.

### Table 1

Effect of dissolved and suspended materials on the formation of halite crystals

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/ml)</th>
<th>Shape</th>
<th>Relative abundance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Frequency (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrocyanide</td>
<td>250</td>
<td>Dendritic&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(+++)</td>
<td>100</td>
</tr>
<tr>
<td>Glucose</td>
<td>250</td>
<td>Cubic</td>
<td>(+++)</td>
<td>100</td>
</tr>
<tr>
<td>Glycerol</td>
<td>250</td>
<td>Cubic</td>
<td>(+++)</td>
<td>100</td>
</tr>
<tr>
<td>Amino acids&lt;sup&gt;e&lt;/sup&gt;</td>
<td>200-2000</td>
<td>Cubic</td>
<td>(+++)</td>
<td>100</td>
</tr>
<tr>
<td>Casaminoacids</td>
<td>200-600</td>
<td>Cubic</td>
<td>(+++)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>Cubic</td>
<td>(+++)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>Dendritic&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(+++)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dendritic</td>
<td>(+)</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cubic</td>
<td>(+)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dendritic&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(+++)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dendritic</td>
<td>(+)</td>
<td>40</td>
</tr>
<tr>
<td></td>
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<td>(+)</td>
<td>40</td>
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<tr>
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<td>Cubic</td>
<td>(+)</td>
<td>10</td>
</tr>
<tr>
<td>Silica gel</td>
<td>100-2000</td>
<td>Cubic</td>
<td>(+++)</td>
<td>100</td>
</tr>
<tr>
<td>Halobacteria cells</td>
<td>200&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Dendritic&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(+++)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cubic</td>
<td>(+)</td>
<td>50</td>
</tr>
<tr>
<td>S-layers</td>
<td>20&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Dendritic&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(+++)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cubic</td>
<td>(+)</td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup> (+++) Abundant; (+) common; (+) present

<sup>b</sup> The frequency of occurrence of the crystals.
b= Frequency found in 60 experiments
c= Inside the cubic crystals (50 μm average width of the branches)
d= Between the cubic crystals (10 μm average width of the branches)
e= D-L alalnine, L(-) proline, glycine, D-L scrin, L cysteine, D-L threonine, L(-) tyrosine, L histidine, L(+)- lysine, and L glutamic acid
f= On basis of dry weigh
g= On basis of Coomassie protein determination.

In conclusion, the observation of cubic structures associated with halobacterial cells and S-layer samples suggests that they may serve as templates in the nucleation and halite formation. The modification of halite crystal habit, resulting in dendrite shape, was attributed to the proteinaceous component of the S-layer of the halophytic archaeabacteria. Monitoring the types and concentration of dissolved organic carbon compounds, and of halobacteria, in natural solar salterns may be an important biotechnological tool in the operation of salterns.

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REFERENCES
14. J.A. Raymond and A.L. DeVries, ACS-Symposium-series-American-Chemical-
Society, USA, 444 (1991) 249.
16. A. Parody-Morreale, K.P. Murphy, E. Di-Cera, R. Fall, A.L. DeVries and
17. E.R. Boeve, L.C. Cao, C.F. Verkoelen, J.C. Romijn, W.C. De-Bruijn and
21. V. Chitarra, H. Souchon, S. Spinelli, M. Juy, P. Beguin and P.M. Alzari,
24. K. Kohri, T. Umekawa, M. Kodama, Y. Katayama, Y. Ishikawa, M. Takada,
25. A. López-Cortés, J.L. Ochoa and R. Vázquez-Duhalt, Geomicrobiol. J.,
35. M.F. Torreblanca, F. Rodríguez-Valera, G. Juez, A. Ventosa, M. Kamekura
40. M. Kessel, I. Wildhaber, S. Cohen and W. Baumeister, EMBO J.,