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ORIGINAL PAPER

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Isolation of the fibrocrystalline body, a structure present in haloarchaeal species, from *Halobacterium salinarum*

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Abstract An organized structure, the fibrocrystalline body (FB), has been isolated from the archaeon *Halobacterium salinarum*. The structure is also present in, and can be isolated from, other extreme halophilic archaea. FB is present in the cytoplasm during the exponential growth and early stationary phases. This structure is affected by vincristine, an antitumoral drug, which targets tubulin. The drug causes fragmentation of the FB, changes in the cell shape, and growth inhibition. Taken together, these results point toward an important role in the life of the cell for this highly organized structure.

Key words Fibrocrystalline body · Extreme halophilic Archaea · Structure isolation

Introduction

Fibrocrystalline bodies (FBs) are cytoplasmatic structures first described 30 years ago in ultrathin sections of the halophilic archaeon *Halobacterium salinarum* (previously *H. halobium*) (Cho et al. 1967; Robertson et al. 1982). In 1967, the electron microscopy studies of Cho and coworkers were the first to describe these structures as organelles comprising "a group of somewhat wavy alternating electron-dense and electron light bands" (Cho et al. 1967). Cho et al. also suggested that these structures were associated with but not surrounded by the plasmatic membrane.

Some years later, Robertson and coworkers developed a specific protocol to study *H. salinarum* by electron microscopy (Robertson et al. 1982). They again observed these structures and gave them the current name of "fibrocrystalline body." By means of image diffraction techniques, they concluded that FBs are composed of groups of tubes arranged in a hexagonal disposition. The dimensions of the structures were 0.7–0.9 μm long and 0.1–0.2 μm thick; they were located in the cytoplasm near the plasmatic membrane. The major axes of both the FB and the cell were oriented in a parallel fashion. Robertson et al. also speculated about a possible composition of polymeric proteins similar to the eukaryotic cytoskeleton proteins actin and myosin. No other data or study about these structures has been published since the work by Robertson and coworkers.

We found that this highly organized structure is also present in other haloarchaea species. In addition, we developed a method for the isolation of FBs from *H. salinarum*. This method is a first step that will allow the study of this structure at a deeper level. It has been observed that the FB is affected by the action of vincristine, an antitumoral drug that acts on tubulin microtubules in eukaryotes and which has been shown to affect different Archaea (Sioud et al. 1987). The data presented here point toward the hypothesis that the FB is a widespread structure in the haloarchaea that could play an important role during the cell cycle.

Materials and methods

Archaeal strains and growth conditions

Archaeal strains *Halobacterium salinarum* R1, *Halorubrum saccharovorum* DSM 1137^{T}, *Haloarcula hispanica* ATCC 33960, *Haloferax volcanii* NCMB 2012 (DS2), and *Natronomonas pharaonis* DSM 2160 were grown as described previously (Rodríguez-Valera et al. 1980), except for *N. pharaonis*, which requires media with low amounts of Mg^{2+} (Soliman and Trüper 1982). The saline buffers used were SW25% (3.34 M NaCl, 0.2 M $MgSO_4$, 0.17 M $MgCl_2$, 70 mM KCl, 8.6 mM $CaCl_2$, 6.3 mM NaBr, 2 mM $HNaCO_3$) and SWi (2.9 M KCl, 1.7 M NaCl, 0.1 M $MgSO_4$, 0.1 M Tris, pH 6.85).

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FB isolation and cell fractions

We found that FBs are extremely labile structures and very sensitive to treatment conditions (results to be published elsewhere). Different methods for cell lysis and extraction were tried, such as lysis by lowering the salt concentration, sonication, biliar salts, and detergents. The method shown here is the optimization of the many different methods tried. Cultures of *H. salinarum* were grown in the exponential phase until they reached an optical density of 0.6–0.7 OD_{540}. Cells were recovered by centrifugation (2,500 *g* for 12 min) and washed with saline solution SW25%. Cells were left for 2 weeks in SW25% to allow the envelopes to become more permeable (Robertson et al. 1982; our unpublished results). After that, cells were resuspended in a solution containing equal parts of SW25% and SWi equivalent to a concentration of 5 units of OD_{540} ml^{-1}, then fixed by adding glutaraldehyde to a final concentration of 0.025% and incubated at 4°C for 25 min.

After that, cells were washed twice by centrifugation using SWi buffer. Cellular lysis was carried out by adding sodium taurodeoxicholate (Sigma, St. Louis, MO, USA) to a final concentration of 65 μg ml^{-1} (Kamekura et al. 1988; Kupper et al. 1994). To reduce viscosity, 200 μg ml^{-1} DNase I (Roche, Barcelona, Spain) was added. After gently mixing, cells were allowed to continue incubating at 37°C for 10 min without shaking. Finally, FBs together with envelope fragments were recovered in the pellet after centrifugation at 18,000 *g* for 15 min. The pellet was resuspended in half the volume of lysis in SWi buffer (fraction I). An additional treatment with 0.1% Triton X-100 (Sigma) for 30 min at 37°C and a wash with SWi buffer reduced, but did not eliminate, the amount of cell envelopes in the sample, although it seemed to affect the number and integrity of the FBs.

Electron microscopy methods

The observation of FBs inside the cells by electron microscopy was carried out following the protocol described by Robertson and coworkers (Robertson et al. 1982). The isolated FBs were prepared for electron microscopy by negative staining. Briefly, a 10-μl aliquot of isolated FBs was placed in a copper grid covered with collodion and carbon. After 5 min, the excess of water was eliminated by absorption and the sample was fixed for 2 min in a 2% glutaraldehyde in SWi solution. Samples were washed in distilled water and then stained with 4% uranyl acetate solution for 30 s, and then observed in a transmission electron microscope (model EM-906; Zeiss, Heidelberg, Germany).

Drug treatment

Cytochalasin B and vincristine (Sigma) were dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). Drugs were added to the cultures in the exponential phase. The final concentration of DMSO was 0.1% in all the cultures, independent of drug concentration. Samples were collected at indicated times, and OD_{540} and viable count measures were performed.

Results

The FB is present in exponential-phase cultures but not in stationary-phase cultures of *H. salinarum*

Samples from cultures of *H. salinarum* were taken and processed for observation using electron microscopy (Robertson et al. 1982). As has been described previously by Robertson and coworkers, the transverse section of a FB is a tubular structure ordered in a hexagonal fashion (Fig. 1). We found that FBs are formed by 75 to 90 microtubules (Fig. 1D) and seem to occupy 10% of the cell volume.

To determine whether the FB is a structure that is always present inside the cell or if its presence depends on the growth phase, samples from different times during the growth of *H. salinarum* were taken and processed. Random pictures from ultrathin sections were taken. For each sample, 1,000 sections of cells, both with and without FBs, were counted and the percentage of cells containing FBs was calculated. This percentage is about 30% during the exponential and early stationary phases (not shown). If we consider that the FB occupied only 10% of the cell volume, it seems that this structure is inside all the cells, although we cannot rule out the possibility that this structure has a cycle of polymerization–depolymerization during the life of the cell.

It is important to note that cells containing two or more independent FBs were never found except in the case of cells in the division process. We also found that no FBs were seen inside the sections during the late stationary phase, although we cannot discriminate whether this is caused by a depolymerization of the FB or is an effect of the degradation of its components.

Isolation and characterization of FBs

A panoply of methods designed for isolation of similar structures in other organisms was tried, unsuccessfully in the majority of cases (see Materials and methods). Finally, the protocol described in the Materials and methods section was developed. From the results described in the previous section, cells were taken at 0.7 OD_{540} to allow a maximum of FB content per cell. Cells were then fixed with 0.025% glutaraldehyde to avoid destruction of the FBs during cell lysis. Although it is not essential, based on the results from Robertson and coworkers (Robertson et al. 1982) we found that the starvation of the cells by leaving them in SW25% for 2 weeks resulted in a better fixation of the FB by glutaraldehyde and a greater yield on isolation.

Cell lysis was carried out using sodium taurodeoxycholate following a modification of the protocol of Kupper and coworkers for the extraction of flagellar polar heads in

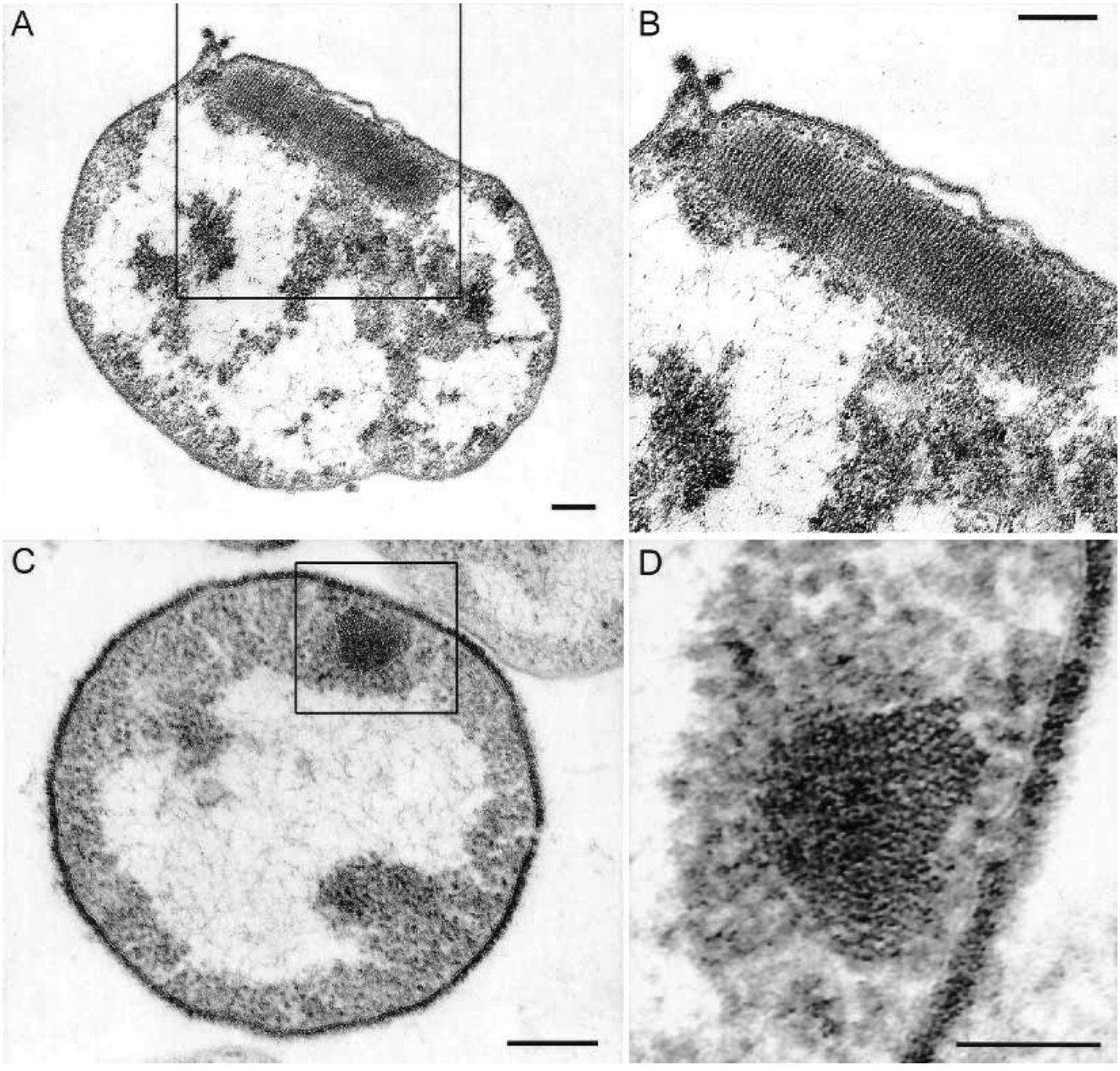

Fig. 1A–D. Sections of *Halobacterium salinarum* cells showing the presence of fibrocrystalline bodies (FBs). **A** Longitudinal section of an FB. **B** Enlargement of the FB. **C** Cross section of an FB. **D** Enlargement of the FB. *Bars* 0.2 μm

H. salinarum (Kupper et al. 1994). After centrifugation, the pellet (fraction I) was processed for negative staining and observed under electron microscopy. Isolated structures with a striated aspect, similar to FBs found inside the cells, were observed (Fig. 2A). It was not rare to observe these structures associated with envelope remains (Fig. 2B).

To eliminate the possibility that the structures isolated were an artifact of the starvation treatment, cells processed for isolation of FBs were simultaneously processed for electron microscopy ultrathin sections (Robertson et al. 1982). No differences were found in the ultrastructure and the presence of FBs between fresh cells and starved cells. For further characterization of the isolated FBs, aliquots from fraction I were treated with pronase or trypsin. No FBs were observed in the subsequent analysis under the electron microscope, thus indicating their proteinaceous nature. Also, we found that an additional treatment with Triton X-100 eliminates almost all the cellular envelopes (see Fig. 2C). Nevertheless, the electrophoretic analysis of these samples shows that a substantial envelope contamination remains (not shown), and thus further improvement of this isolation protocol is required to achieve molecular analysis of FBs.

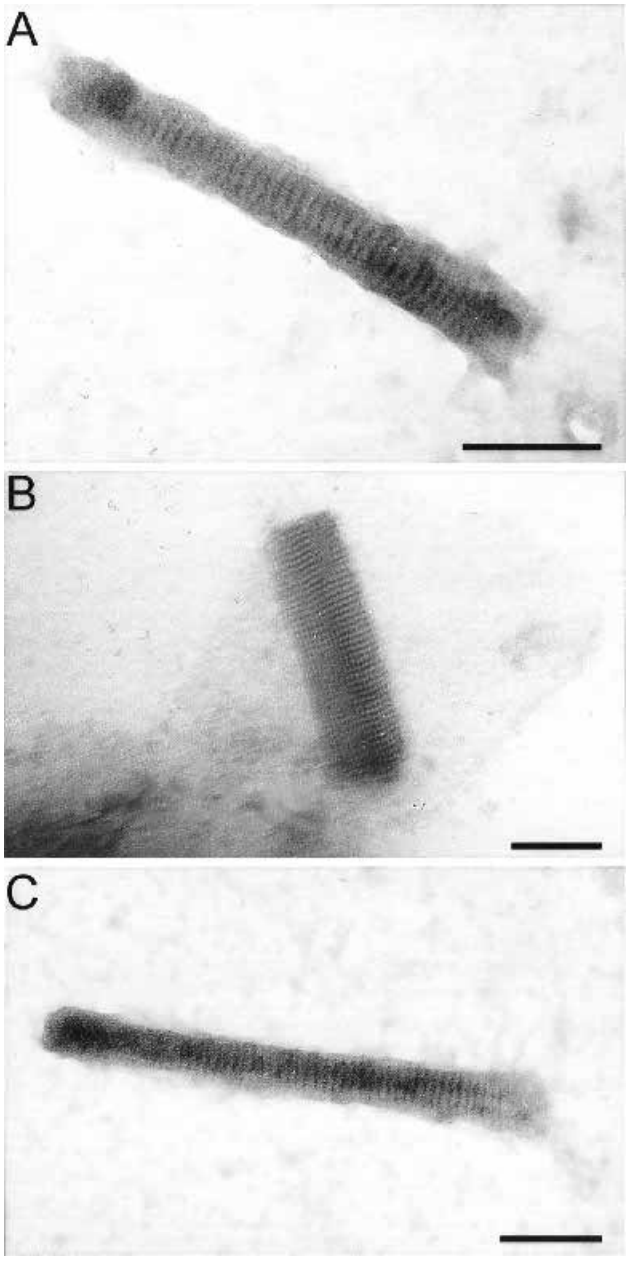

Fig. 2A–C. FBs isolated from *H. salinarum* cultures following the treatment described in Materials and methods. **A** An FB present in fraction I. **B** An FB associated with an envelope present in fraction I. **C** An FB after treatment with 0.1% Triton X-100. *Bars* 0.2 μm

Biological role of the FB

A feasible hypothesis for the role of FBs is that the existence of a structure so organized and with a relatively large size must have a critical role during the life of the microorganism. Some ultrasections suggest that FBs interact with the chromosome (Fig. 3A). In addition, electron microscopy preparations of cells in the division process during exponential growth show that FBs seem to be duplicated and partitioned between the future daughter cells (Fig. 3B). Preliminary results from our laboratory indicate that FBs interact with DNA in a nonspecific way. FBs were never isolated from cells in the late stationary phase.

Previous work has shown that *H. salinarum* is sensitive to the action of antitumoral drugs that affect the eukaryotic cytoskeleton (Sioud et al. 1987). When we used vincristine, which is a drug that acts on tubulin by preventing its polymerization, growth stopped, and if the dosage was increased, the cells started to die (Fig. 4A,B). Under the electron microscope, the DNA was seen to be dispersed over the cytoplasm; the FBs lost their typical organization and some were fragmented (Fig. 4C,D). Similar studies were done using cytochalasin B; its target is the actin protein, but no effect was observed (results not shown).

The FB can be found in other haloarchaea

To date, FBs have been observed only in *Halobacterium salinarum* (Cho et al. 1967; Robertson et al. 1982). We wondered if this structure is also present in other extreme halophilic Archaea. To address this hypothesis, cells from different haloarchaea species were treated in the same way as *H. salinarum*. The species selected were *Halorubrum saccharovorum*, *Haloarcula hispanica*, *Haloferax volcanii*, and *Natronomonas pharaonis*. Cultures from these species were set up and processed following the protocol of Robertson and coworkers for observation under electron microscopy. Although the protocol was designed specifically for *Halobacterium salinarum*, FBs can be detected in the ultrathin sections of all these extreme halophilic Archaea (Fig. 5A,C,E). Their FBs can be isolated following the protocol described in this work but with a decreased yield. In all cases, the particular striated aspect can be seen (Fig. 5B,D,F). In the case of *Natronomonas pharaonis*, no FB structures were found in ultrathin sections following Robertson's protocol, but FBs were isolated following the isolation protocol (Fig. 5G,H,I).

Discussion

Our results show that an ordered structure, the FB (Robertson et al. 1982), can be observed and isolated from the cytoplasm of *H. salinarum* and other extreme halophilic Archaea. The method described here has been developed for isolation of the FB from *H. salinarum.* Unfortunately, the persistence of contamination from cell envelopes prevents a more exhaustive molecular study at present. During the elaboration of this protocol we found that FBs are very fragile structures. Changes in the concentration of buffers, pH, and temperature affect the structure and organization of the FB during its isolation. We have tried to use both protocols in methanogens and thermophiles but without success. This failure could be explained by the fact that Robertson's protocol and that presented here are developed for extreme halophilic Archaea and their cellular envelopes are different from those of other archaeal groups (Kandler and König 1993). Although we could not identify

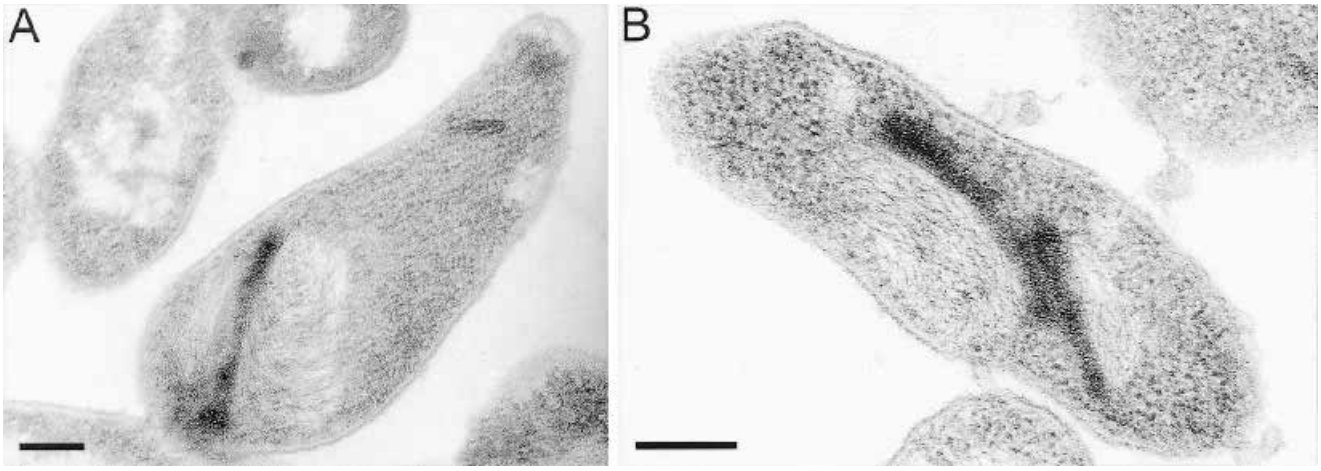

Fig. 3. Sections of *H. salinarum* cells during the division phase show FBs in the intermediate zone between the chromosomes **(B)** and apparently interacting with them **(A)**

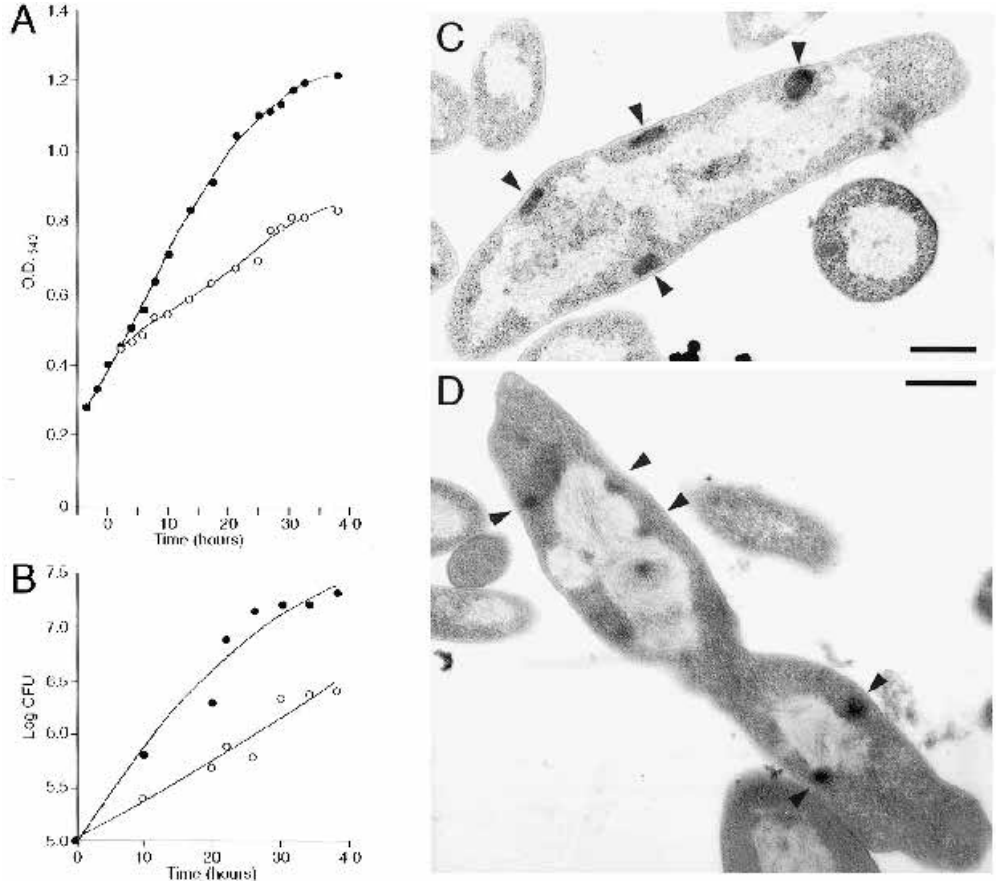

Fig. 4A–D. Effects of vincristine in cultures of *H. salinarum*. A culture of *H. salinarum* was kept growing exponentially. At time 0, the culture was split. One culture was allowed to continue to grow (●), and vincristine (25 μg ml^{-1}) was added to the other (○). Samples for OD_{540} measurements and viable count were withdrawn at the times indicated in each graph and processed as described in Materials and methods. **A** Growth curve. **B** Viable counts. **C** Sections of an individual cell. **D** Section of a cell in the process of division. *Arrowheads* indicate the presence of FBs. *Bars* 0.5 μm

any component of the FB, the sensitivity to pronase indicates that this structure is composed of proteins. Improvement of the isolation protocol to achieve a complete purification of the FB for its molecular characterization is now in progress.

The FB is present in all the cells during the exponential growth phase and in the early stationary phase. We also found that FBs cannot be visualized or isolated during late stationary phase, although we do not know if this results from depolymerization of the structure or from degradation of its components. Experiments designed to discriminate between these options are now being conducted in our laboratory. In addition, FBs seem to be duplicated during the cell division process. Preliminary data from our laboratory suggest that FBs interact nonspecifically with DNA. Although Archaea have been described to have

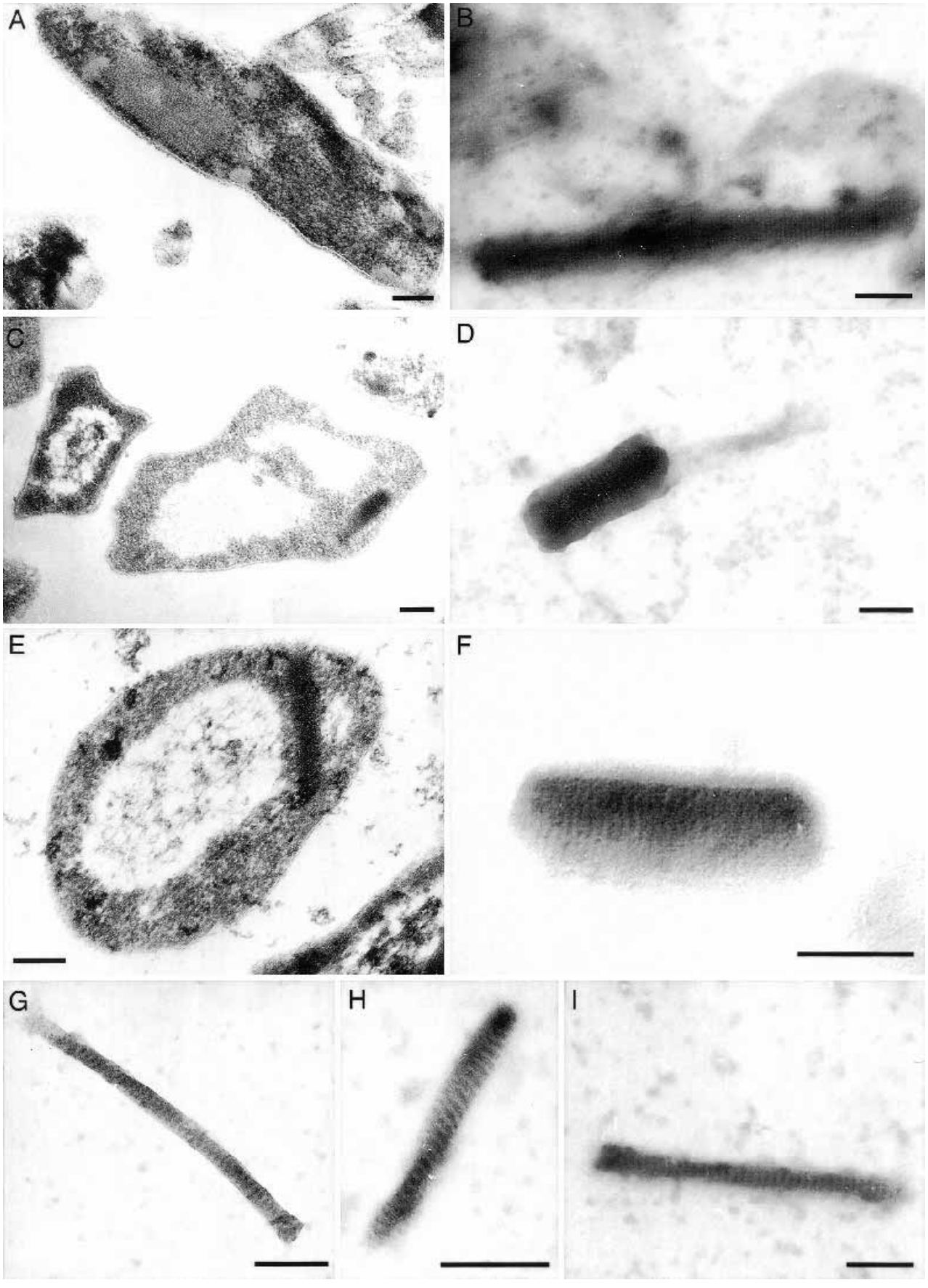

Fig. 5A–I. Presence of FBs in other extreme halophilic Archaea. **A,B** *Halorubrum saccharovorum*: (**A**) section of a cell; (**B**) isolated FB. **C,D** *Haloarcula hispanica*: (**C**) section of a cell; (**D**) isolated FB. **E,F** *Haloferax volcanii*: (**E**) section of a cell; (**F**) isolated FB. **G,H,I** *Natronomonas pharaonis*: isolated FBs. *Bars* 0.2 μm

homologues to bacterial cell division genes (Baumann and Jackson 1996; Margolin et al. 1996), the work of Bernander and Poplawski (Bernander and Poplawski 1997; Bernander 1998; Poplawski and Bernander 1997) shows that the archaeal cell cycle is more similar to that of eukaryotic cells than that of bacteria.

We speculate that the FB can be an analogue of the eukaryal cytoskeleton. The existence of a cytoskeleton in prokaryotes has been discussed (Baumann and Jackson 1996; Bermudes et al. 1994; Erickson 1995; Hixon and Searcy 1993; Trent et al. 1997), and a recent review (Bernander 2000) opens the question of the structure of the partition/mitotic apparatus in Archaea. Nevertheless, the molecules proposed to be involved in that structure vary greatly. In eukaryotes, the cytoskeleton is a network composed of many different proteins that is involved in maintenance of cell shape, intracellular transport, genetic material segregation, cell division, and other essential cellular processes.

The FB is an attractive candidate as a kind of prokaryotic cytoskeleton. It is an ordered structure, and its presence in other species of extreme halophilic Archaea could indicate its existence in other members of the archaeal domain. Nevertheless, *H. salinarum* movement results from presence of a flagellum, so clearly the FB is not involved in cell movement in this case. Also, although the FB structure is very different from a network, we cannot exclude the existence of a reorganization cycle of the FB components during cell life. The use of vincristine, a drug that acts on tubulin, is an inital step in the study of the biological role of the FB. Although we cannot rule out the possibility that the observed effect of vincristine on cell viability is independent of its effect on FB structure, the observation of both effects could indicate that this structure plays an essential role during cell life and that some of its functions can be analogous to those performed by the eukaryal cytoskeleton. Further studies are already being conducted in our laboratory to address this question.

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