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EXPERIMENTAL ARTICLES

Magnesium Orthophosphate, a New Form of Reserve Phosphate in the Halophilic Archaeon *Halobacterium salinarium*

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Abstract—The accumulation and utilization of reserve phosphate in the extremely halophilic archaeon *Halobacterium salinarium* were studied. The growth of *H. salinarium* was found to depend on the initial concentration of inorganic phosphate (P_i) in the culture medium and its content in the inoculum. Growing cells consumed 85–95% of P_i from the medium. Unlike the reserve phosphates of many other microorganisms, which are mainly polyphosphates, the reserve phosphates of *H. salinarium* cells contain no more than 15% polyphosphates, the rest being magnesium orthophosphate. The excessive consumption of P_i from the medium led to a change in cell morphology and caused the death of part of the cell population. The cells that remained viable could grow in a P_i-deficient medium, utilizing about 70% of the reserve magnesium phosphate as the phosphorus source.

Key words: orthophosphate, polyphosphates, magnesium phosphate, morphology, *Halobacterium salinarium.*

Many microorganisms are able to reserve inorganic phosphate (P_i), an important nutrient whose deficiency may arrest microbial growth. Knowledge of the mechanisms of P_i reservation in different microbial taxa may provide insight into the evolutionary aspects of microbial adaptation to unfavorable environmental conditions and contribute to the development of biotechnological methods of remediation of areas polluted by phosphorus compounds. Microorganisms accumulate P_i mainly as polyphosphates [1]. In actinomycetes, *Penicillium chrysogenum* in particular, intracellular P_i occurs either in a free or in a bound state [2, 3]. In the latter case, P_i is bound by Fe^{3+} ions, with the formation of polymeric iron orthophosphate. The accumulation of P_i in cells and the utilization of intracellular P_i allow microorganisms to survive in media with, respectively, an excess and an insufficient content of phosphorus. In evolutionarily different microorganisms inhabiting different econiches, the mechanisms of accumulation of phosphorus compounds may greatly differ.

The aim of this work was to study the reservation of inorganic phosphate in the halophilic archaeon *Halobacterium salinarium,* which is widely used for the investigation of membrane transport [4] and has been found to accumulate polyphosphates [5].

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MATERIALS AND METHODS

Strain and cultivation conditions. The *Halobacterium salinarium* strain ET 1001 was a generous gift from T.S. Kalebina, Moscow State University.

The strain was maintained at 4°C with monthly transfer onto fresh medium containing (g/l) NaCl, 250; KCl, 2; sodium citrate, 3; $MgSO_4$, 20; peptone (Diakon, Russia), 7; K_2HPO_4, 0.4; and agar, 20.

For study of phosphate accumulation, the strain was grown at 37°C on a shaker (200 rpm) in flasks with 200 ml of liquid medium containing the same concentrations of NaCl, KCl, sodium citrate, $MgSO_4$, and peptone as indicated above and different concentrations of P_i: 0.05 mM (K_2HPO_4 not added), 2.3 mM (K_2HPO_4 added in an amount of 0.6 g/l), and 11.5 mM (K_2HPO_4 added in an amount of 3.0 g/l).

Biomass preparation. Cells grown in the liquid cultivation medium were harvested by centrifugation at 5000 *g* for 40 min and washed twice with the cultivation medium containing no P_i or peptone under the same centrifugation conditions. This made it possible to standardize the NaCl and water contents of the wet biomass, which was used in experiments.

Thin sectioning. Cells were fixed in a 1.5% solution of glutaraldehyde in buffer A (0.05 M cacodylate, pH 7.2, with 250 g/l NaCl and 20 g/l $MgSO_4$) at 4°C for 1 h, washed thrice with buffer A, and refixed at 20°C for 3 h in a 1% solution of OsO_4 in buffer A. After dehydration in a series of alcohol solutions of increasing con-

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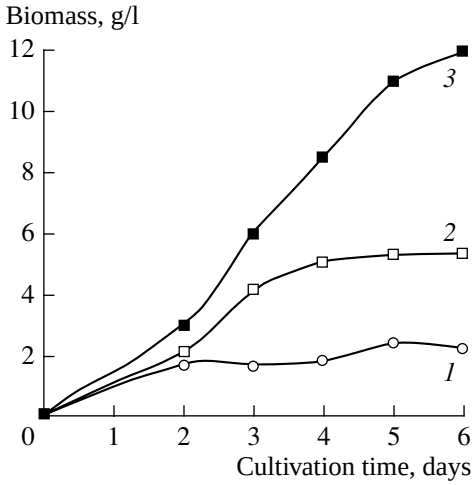

Fig. 1. Growth of *H. salinarium* at different initial concentrations of P_i in the medium: (*1*) 0.05, (*2*) 2.3, and (*3*) 11.5 mM.

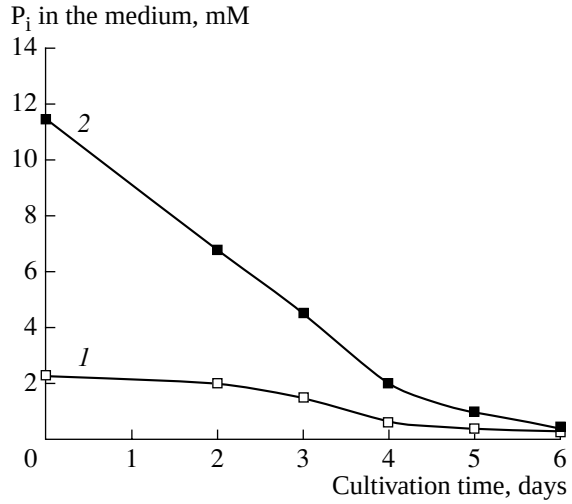

Fig. 2. Dynamics of P_i in the medium during the growth of *H. salinarium* at different initial concentrations of P_i: (*1*) 2.3 and (*2*) 11.5 mM.

centration, the preparation was embedded in Epon 812 epoxy resin. Thin sections cut on an ultratome were mounted on grids, contrasted in a 3% solution of uranyl acetate in 70% ethanol for 30 min and then with lead citrate by the Reynolds method [6], and examined under a JEM-100B electron microscope (JEOL, Japan) operated at 80 kV.

Analytical methods. Polyphosphates were extracted and labile phosphorus and orthophosphate were determined as described elsewhere [7].

High- and low-molecular-weight polyphosphates were precipitated with $Ba(NO_3)_2$ at acidic and alkaline pH, respectively [8]. A solution was rid of nucleotides by their sorption on Norit A activated charcoal [1, 9], the pH of the solution was adjusted to 4.5 by adding 1 N NaOH, and the solution was mixed with a saturated $Ba(NO_3)_2$ solution in cold. The insoluble Ba salts of high-molecular-weight polyphosphates were precipitated by centrifugation. The pH of the supernatant was adjusted to 8.2 with 1 N NaOH, and the supernatant was again mixed with the saturated $Ba(NO_3)_2$ solution in cold. The precipitate formed under such conditions contained low-molecular-weight polyphosphates and barium orthophosphate.

Polyphosphatase activity was determined from the rate of P_i formation in 1 ml of the reaction mixture incubated for 10–40 min [10].

The protein concentration was determined by the method of Lowry *et al.* [11], using bovine serum albumin as the standard.

Magnesium phosphate was isolated by its precipitation from cell lysate by centrifugation at 5000 *g* for 10 min. Cell lysis was induced by placing cells in distilled water [7].

RESULTS AND DISCUSSION

Consumption and accumulation of phosphate by *H. salinarium* cells. The biomass level and the time during which *H. salinarium* shows active growth depended on the initial concentration of P_i in the medium (Fig. 1). Most of the P_i taken up from the medium was accumulated in *H. salinarium* cells. After 6 days of growth in the medium with initial P_i concentrations of 2.3 and 11.5 mM P_i, cells accumulated, respectively, 86.7 and 95.6% of the P_i initially present in the medium (Fig. 2). By comparison, *Saccharomyces cerevisiae* cells consumed no more than 5% of P_i upon an initial concentration in the medium equal to 9 mM [12]. However, the recombinant cells of *Escherichia coli* with an increased capability for P_i consumption accumulated P_i in an amount comprising 16% of the dry weight of cells, about 65% of intracellular P_i being accumulated in the form of polyphosphates [13].

The table shows the distribution of phosphorus over different fractions obtained from *H. salinarium* cells grown for 5 days in media with different initial P_i concentrations. The amount of labile phosphorus in the acid- and alkali-soluble fractions reached, respectively, 3.3 and 10% of the total phosphorus consumed by cells grown in the media with the initial P_i concentrations equal to 2.3 and 11.5 mM. After 3 days of growth in the medium with the initial P_i concentration equal to 2.3 mM, when the content of labile phosphorus in cells was at a maximum, all labile phosphorus could be precipitated by barium nitrate from both acid-soluble and alkali-soluble fractions. This implies that the labile phosphorus represents polyphosphates. The chromatographic analysis of the precipitates by the method described by Kulaev [1] and Kulaev and Vagabov [8] showed that they contained only high-molecular-weight polyphosphates (data not shown). The greatest

content of phosphorus was found in the insoluble precipitate obtained by the centrifugation of cell lysate in distilled water (see table). The chemical analysis of this precipitate showed that it contained magnesium and phosphate ions in a molar proportion of about 3 : 2, suggesting that the major component of the precipitate was $Mg_3(PO_4)_2$ [7].

The accumulation of magnesium phosphate by growing *H. salinarium* cells. Figure 3 shows the accumulation of magnesium phosphate by *H. salinarium* cells grown in the media with initial P_i concentrations of 2.3 and 11.5 mM. It can be seen that the formation of magnesium phosphate occurred throughout the cultivation period and that the amount of magnesium phosphate formed depended on the initial concentration of P_i in the medium.

The pH of the media with the initial concentrations of P_i equal to 2.3 and 11.5 mM did not exceed 6.2 and 6.8, respectively, throughout the cultivation period. Therefore, the formation of the magnesium phosphate precipitate cannot be explained by alkalinization of the medium.

To study the relationship between cell metabolism and the accumulation of magnesium phosphate, we investigated the effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a protonophore which eliminates the electrochemical gradient of H^+ across membranes, on the growth of *H. salinarium* cells, the consumption of P_i from the medium, and the accumulation of magnesium phosphate in the cells (Figs. 4a–4c). In this experiment, *H. salinarium* was grown in the medium with the initial concentration of P_i equal to 2.3 mM. After 1, 2, 3, and 4 days of growth, the medium was supplemented with FCCP to a final concentration of 0.005 mM, which efficiently inhibited culture growth (Fig. 4b). The addition of FCCP in the indicated concentration arrested the growth of *H. salinarium* (Fig. 4a) and stopped the accumulation of magnesium phosphate in cells at the level attained at the moment of FCCP addition (Fig. 4c).

These results suggest that the transport of P_i and Mg^{2+} into cells is energy-dependent, as is the case in many other bacteria [14, 15].

The utilization of reserve magnesium phosphate during the growth of *H. salinarium* in the P_i-deficient medium. *H. salinarium* cells grown for 5 days in the medium with the initial concentration of P_i equal to 11.5 mM contained 2.5 times more magnesium phosphate than when they were grown at 2.3 mM P_i (Fig. 3). When these differently grown cells were inoculated into a P_i-deficient medium containing 0.05 M P_i, the former cells yielded about 2 times more biomass in the stationary growth phase than the latter cells (Fig. 5). During the growth in the P_i-deficient medium of *H. salinarium* cells pregrown at 11.5 mM P_i, the content of magnesium phosphate in the cells gradually decreased (Fig. 6). These experiments clearly showed that *H. salinarium* cells accumulate magnesium phosphate during their growth in the P_i-rich medium and utilize this reserve substance as a source of phosphorus during their growth in the P_i-deficient medium.

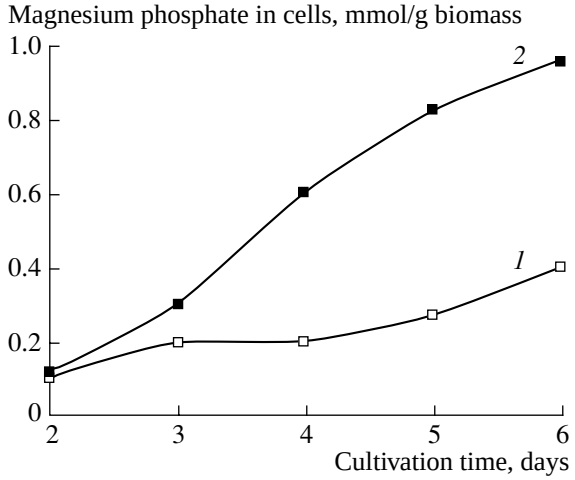

Fig. 3. Dynamics of magnesium phosphate in *H. salinarium* cells grown at different initial concentrations of P_i: (*1*) 2.3 and (*2*) 11.5 mM.

The effect of the P_i concentration on the morphology of *H. salinarium* cells. When *H. salinarium* cells were grown to the stationary phase at the initial concentration of P_i equal to 11.5 mM, the cell population was heterogeneous upon centrifugation. Most cells in the upper part of the centrifuge tube were intact (Fig. 7a), although there was an amount of damaged cells. The bottom layer contained mainly magnesium phosphate crystals (Fig. 7b), the number of cells in this layer being small. Thus, the high initial concentration of P_i in the growth medium (11.5 mM) caused the death of a frac-

The effect of the initial concentration of P_i in the medium on its uptake and the content of phosphorus compounds in 5-day-old *H. salinarium* cell biomass

P_i	Initial concentration of P_i in the medium, mM	
	2.3	11.5
P_i, consumed from the medium	100% (0.37 mmol/g biomass)	100% (1 mmol/g biomass)
Polyphosphates (labile phosphorus)	3.3% (0.012 mmol/g biomass)	10% (0.1 mmol/g biomass)
Magnesium phosphate	85% (0.32 mmol/g biomass)	87% (0.87 mmol/g biomass)

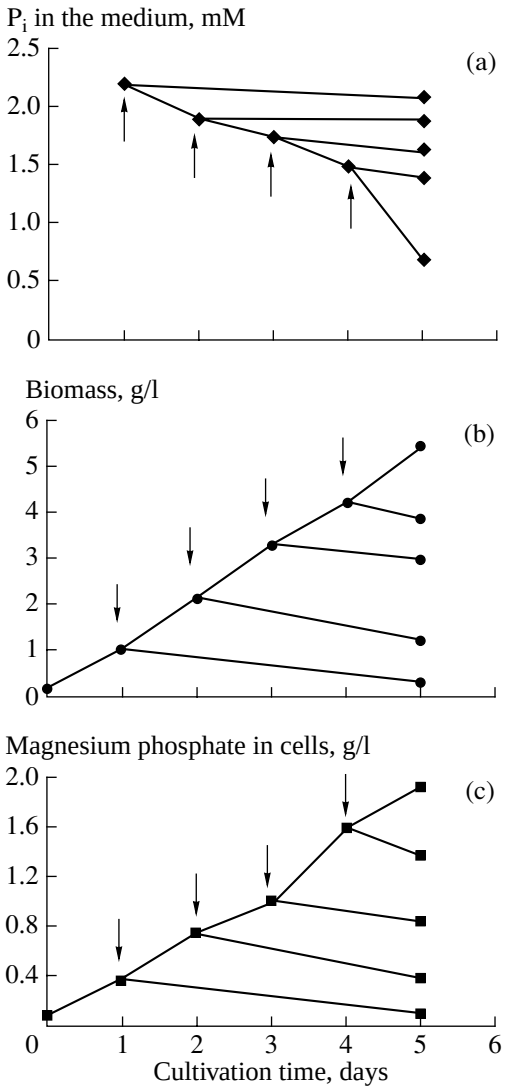

Fig. 4. The effect of FCCP on (a) P_i uptake from the medium, (b) the growth of *H. salinarium*, and (c) the accumulation of magnesium phosphate in cells. The culture was grown at an initial concentration of P_i equal to 2.3 mM. The arrows show the times of FCCP addition.

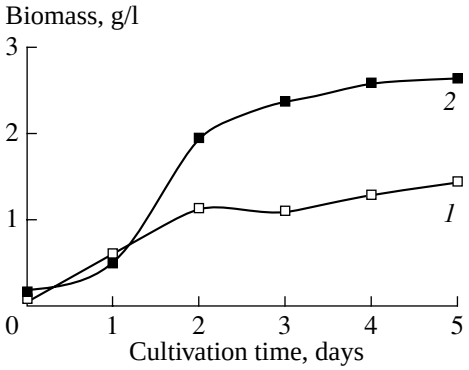

Fig. 5. Growth of *H. salinarium* in the medium deficient in P_i (0.05 mM) from the inoculum grown at (*1*) 2.3 and (*2*) 11.5 mM P_i.

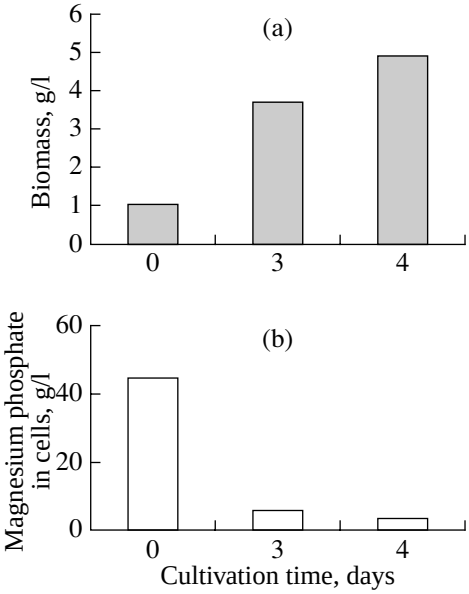

Fig. 6. (a) Growth of *H. salinarium* and (b) the content of magnesium phosphate in cells grown in the medium with 11.5 mM P_i for 5 days and then transferred to the P_i-deficient medium.

tion of cells and induced the formation of magnesium phosphate crystals.

The precipitate obtained by the centrifugation of cell lysate was found to contain 90% magnesium phosphate, 2.82% carbon, 0.49% nitrogen, and an amount of amino acids. It is likely that the adsorbed organic compounds prevent magnesium phosphate from dissolving in the culture liquid after the death of cells. The occurrence of pyrophosphatase activity in the precipitate [5] confirmed the suggestion that the magnesium phosphate crystals resulted from the lysis of a fraction of the population of *H. salinarium* cells.

The stationary-phase *H. salinarium* cells grown at the initial concentration of P_i equal to 2.3 mM remained homogeneous upon centrifugation. Magnesium phosphate crystals were visible neither to the naked eye nor microscopically (data not presented). Precipitate of magnesium phosphate was obtained only from cells whose lysis was induced by placing them in distilled water. It is likely that, at the low initial concentration of P_i in the medium, *H. salinarium* cells with reserve magnesium phosphate remain largely intact.

Electron microscopy showed that *H. salinarium* cells grown to the stationary phase (4 days of growth) at the initial concentration of P_i equal to 0.05 mM were morphologically normal (Fig. 8a and [16]). *H. salinarium* cells grown to the stationary phase (5 days of growth) at the initial concentration of P_i equal to 2.3 mM exhibited changes in their shape, a reduced and com-

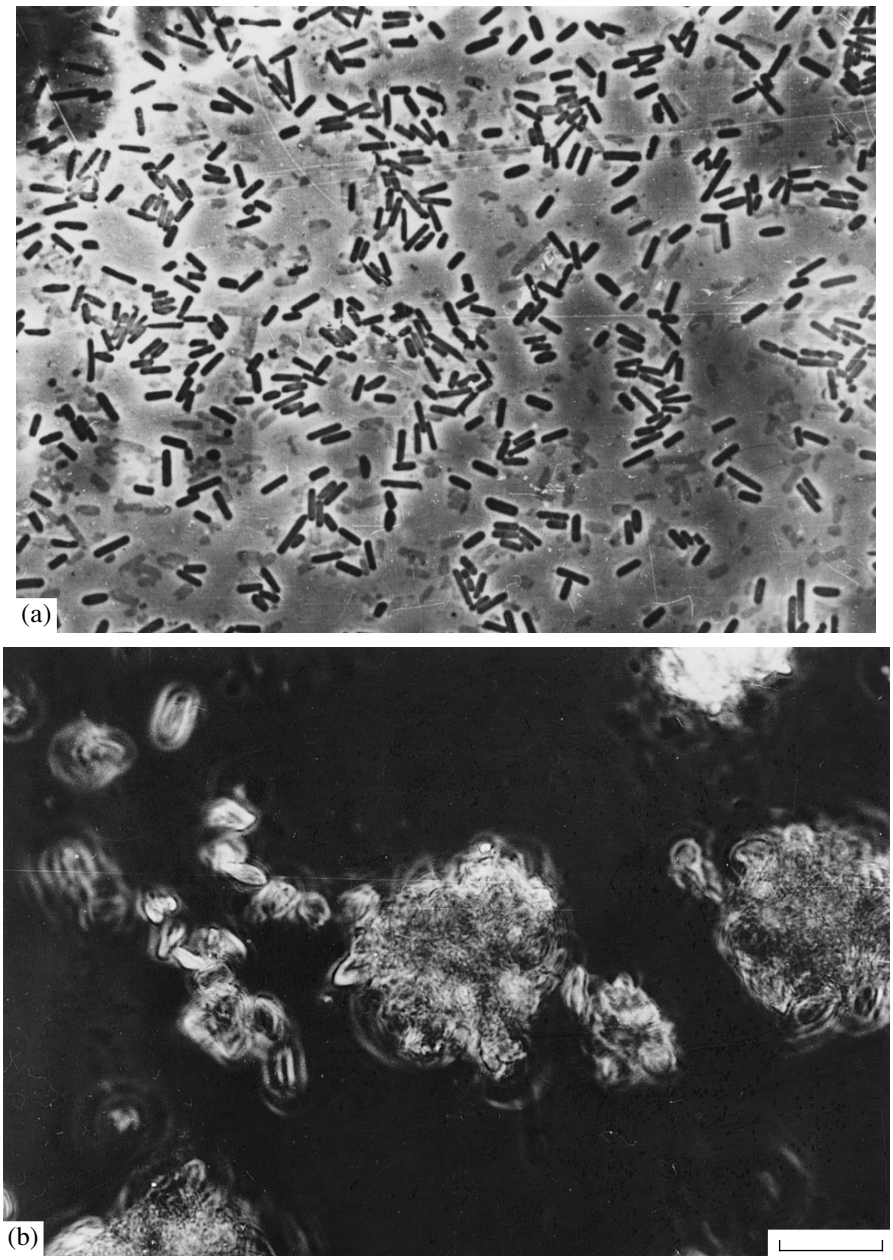

Fig. 7. Phase-contrast microscopy (OPTON ICM 403) of (a) *H. salinarium* cells grown in the medium with 11.5 mM P_i for 5 days and (b) magnesium phosphate crystals formed during culture growth. The scale bar represents 10 μm.

pact cytoplasm zone, an enlarged nucleoid zone, and a damaged cell wall (in some cells) (Fig. 8b). No magnesium phosphate on the cell surface was detected. We believe that these minor changes in cell morphology are due to the accumulation of magnesium phosphate, which is likely bound to cellular proteins and lipids.

Even in the phase of active growth (3 days of growth), *H. salinarium* cells grown at the initial concentration of P_i equal to 11.5 mM had an altered morphology (Fig. 8c), similar to that exhibited by the stationary-phase cells grown at 2.3 mM P_i (altered cell shape, cytoplasm, cell wall, and nucleoid zone). The cytoplasm contained small dissipated polyphosphate granules, which is in agreement with the biochemical data that the concentration of polyphosphates in such cells is at a maximum [7].

After 5 days of growth at 11.5 mM P_i, the morphological changes in *H. salinarium* cells became more significant. The cell wall of many cells was degraded, and the number of lysed cells greatly increased (Fig. 7a). The release of the electron-opaque contents of the cytoplasm into the medium was accompanied by the appearance of a specific regular honeycomb structure in the intracellular space (Fig. 8d), which may represent a

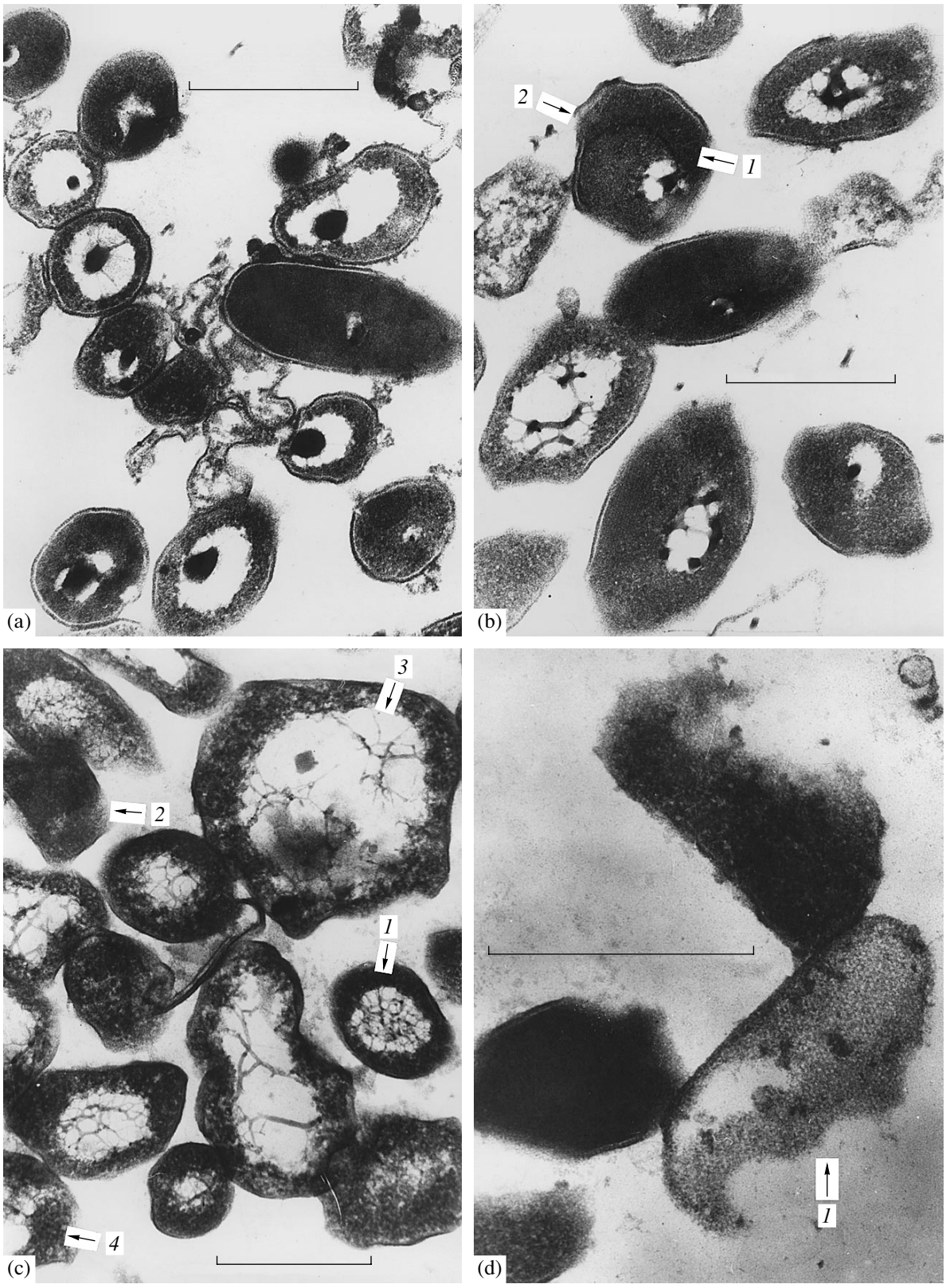

Fig. 8. Electron microscopy of *H. salinarium* cells grown (a) in the medium with 0.05 M P_i for 4 days, (b) in the medium with 2.3 mM P_i for 5 days, (c) in the medium with 11.5 mM P_i for 3 days, and (d) in the medium with 11.5 mM P_i for 5 days. Panel b: *1*, compact cytoplasm; *2*, damaged cell wall. Panel c: *1*, compact cytoplasm; *2*, damaged cell wall; *3*, enlarged nucleoid zone; *4*, polyphosphate granules. Panel d: *1*, a cell with a heavily damaged cell wall and a regular honeycomb structure in the intracellular space. The scale bars represent 0.5 μm.

protein framework formed in the cytoplasm in the process of magnesium phosphate accumulation.

It seems that *H. salinarium* does not possess efficient mechanisms providing for the regulation of P_i uptake and its accumulation in the form of polyphosphates. In spite of the fact that this bacterium has a pool of polyphosphates, its polyphosphatase activity is low and does not depend on the content of P_i in the medium, the amount of polyphosphates, or the culture age [5]. Unlike many other microorganisms, in which polyphosphates serve as reserve substances and play an important role during growth under unfavorable conditions [1, 14, 17, 18], the archaeon *H. salinarium* accumulates magnesium phosphate as the main reserve phosphorus compound. The accumulation of magnesium phosphate is presumably an archaic and primitive form of P_i reservation, which, in general, detrimentally affects particular cells but may promote the survival of a whole archaeon population in a P_i-deficient environment.

ACKNOWLEDGMENT

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