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EXPERIMENTAL ARTICLES

Intracellular Accumulation of the Monomeric Precursors of Polyphosphates and Polyhydroxyalkanoates in *Acinetobacter calcoaceticus* and *Escherichia coli* Cells

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Abstract—The formation of polyhydroxyalkanoates granules in anaerobically grown *Escherichia coli* M-17 cells was found to be preceded by the intracellular accumulation of carbonic acids (predominantly, acetic acid), amounting to 9% of the cytosol. The intracellular concentration of acidic metabolites increased after the lyophilization of the bacterial biomass and decreased after its long-term storage (3.5–13.5 years). The decrease in the concentration of acidic metabolites is likely due to the dehydration of dimeric carbonic acids in the viscoelastic cytosol of resting bacterial cells. The hydrophobic obligately aerobic cells of *Acinetobacter calcoaceticus* IEGM 549 are able to utilize a wide range of growth substrates (from acetate and citrate to hydrophobic hydrocarbons), which is considerably wider than the range of the growth substrates of *E. coli* (predominantly, carbohydrates). The minimal essential and optimal concentrations of orthophosphates in the growth medium of *A. calcoaceticus* were found to be tens of times lower than in the case of *E. coli*. The intracellular content of orthophosphates in *A. calcoaceticus* cells reached 35–77% of the total phosphorus content (P_{total}), providing for the intense synthesis of polyphosphates. The P_{total} of the *A. calcoaceticus* cells grown in media with different proportions between the concentrations of acetate and phosphorus varied from 0.7 to 3.3%, averaging 2%. This value of P_{total} is about two times higher than that observed for fermenting *E. coli* cells. Lowering the cultivation temperature of *A. calcoaceticus* from 37–32 to 4°C augmented the accumulation of orthophosphates in the cytoplasm, presumably owing to a decreased requirement of growth processes for orthophosphate. In this case, if the concentration of phosphates in the cultivation medium was low, they were completely depleted.

Key words: intracellular substances, orthophosphates, polyphosphates, acetic acid, polyhydroxyalkanoates, *Acinetobacter calcoaceticus*, *Escherichia coli*.

The phenomenon of the intracellular accumulation of reserve substances, both mineral (such as polyphosphates, polyPs) and organic (such as polyhydroxyalkanoates, PHAs) is widely spread among prokaryotic and eukaryotic microorganisms [1–3]. The knowledge of the metabolism of such microorganisms may contribute to the elaboration of advanced waste treatment technologies and the development of the ecologically important industry of biodegradable plastics, elastomers, and polyesters [4–6].

It is believed that intracellular PHAs are synthesized in response to oxygen deficiency and are utilized aerobically as a source of carbon and energy [3, 6]. The PHA content of cells may reach 40–80% of the dry biomass. The biosynthesis of PHAs is governed by the carbon source used and the deficiency of nitrogen and sulfur in the cultivation medium [7, 8]. The biosynthesis of polyPs also depends on the composition of the growth medium and the physicochemical conditions of cultivation. For instance, the addition of orthophosphates or short-chain polyPs to a *Propionibacterium shermanii* culture accelerated the synthesis of intracellular polyPs by approximately ten times. In bacterial cells grown on glucose, polyPs are involved in the phosphorylation of this substrate, so that such cells contain two orders of magnitude lower amounts of polyPs than cells grown on lactate [9].

In the *Escherichia coli* cells grown on carbohydrate sources in the presence of excess orthophosphates, polyPs are accumulated with the retention of the intracellular concentration of orthophosphates at a relatively constant level [10]. This suggests that either polyPs maintain the constant intracellular level of orthophosphates in *E. coli* cells or the synthesis of polyPs is regulated by extracellular, rather than intracellular, orthophosphates.

Considerable attention is being given to the investigation of the microorganisms that synthesize both polyPs and PHAs [5, 11, 12]. The balance between the processes of the synthesis and utilization of polyPs and

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PHAs in such microorganisms is determined by a combination of internal and external factors and depends on the intensity of metabolic exchange between the cell and the environment. In gram-negative bacteria, including those of the genera *Escherichia* and *Acinetobacter*, this exchange is provided by the labile system of active transport through the cytoplasmic membrane.

The aim of the present work was to comparatively study the processes of accumulation and utilization of intracellular mineral and organic substances in two bacteria, *Acinetobacter calcoaceticus* and *E. coli.*

MATERIALS AND METHODS

Experiments were carried out with the *Acinetobacter calcoaceticus* strain IEGM 549 isolated from active sludge of a waste treatment plant in Perm and the *Escherichia coli* strain M-17. The latter strain has been used for more than 25 years for the production of colibacterin at the Institute of Vaccines and Antisera, NPO Biomed, Perm. The strains were maintained on nutrient agar at 4°C. *A. calcoaceticus* 549 was cultivated in a basal medium containing (g/l) sodium acetate, 10.0; NaCl, 1.0; Na_2CO_3, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0; Na_2HPO_4, 1.6; KH_2PO_4, 0.2; and 1 ml of Hoagland's trace element solution. *E. coli* M-17 was grown in a basal M9 medium containing (g/l) glucose (or sucrose or other carbon and energy sources), 4.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.25; NH_4Cl, 1.0; Na_2HPO_4, 6.0; and KH_2PO_4, 1.5 [13]. When necessary, the composition of the basal media could be changed. Cultivations were performed aerobically in 250-ml Erlenmeyer flasks on a UMBT shaker at different temperatures. Bacterial growth was monitored by measuring the optical culture density at 670 nm (*E. coli* M-17) or 540 nm (*A. calcoaceticus* 549) and converting the optical data to the dry biomass using calibration curves. The total number of bacterial cells and those containing volutin granules were determined by their direct count on 0.23-μm-pore-size Synpor filters. Cells were stained with Toluidine Blue for 36 h. The refractile intracellular lipoid granules were stained with Sudan Black B or Nile Red [14, 15].

Orthophosphates were analyzed with ammonium molybdate, potassium antimonate–tartrate, and ascorbic acid, by estimating the absorbance of the resultant blue product at 590 nm in a KFK-2 photocolorimeter. Polyphosphates were assayed by measuring the phosphates that were formed during the hydrolysis of polyphosphates in 2% H_2SO_4 at 100°C for 30 min. The total phosphorus was evaluated after the hydrolysis of bacterial biomass at H_2SO_4 for 1 h in a solution containing 2% H_2SO_4 and 1% potassium persulfate [16].

The properties and the accumulation dynamics of intracellular substances were studied using a physicochemical method that employs the phenomenon of decreased freezing, solidification, and melting temperatures of liquid mixtures as compared with the respective temperatures for pure solvents (this is one of the consequences of Raoult's law [17, 18]). Earlier, this method was successfully used to study the yeast *Saccharomyces cerevisiae*, colibacterin (the lyophilized *E. coli* biomass), lactobacterin (the lyophilized *Lactobacillus fermentum* biomass), and bifidumbacterin (the lyophilized *Bifidobacterium siccum* and *B. bifidum* biomass).

The decrease in the freezing temperature (ΔT_{fr}, °C) of a solution is proportional to the molal concentration of the dissolved substance (m): $\Delta T = K_{fr}m$, where K_{fr} is the molal constant of the freezing temperature decrease, or cryoscopic solvent constant, equal to 1.86 for water and 3.90 for acetic acid [18]. The concentration of carbonic acids in the cytosol was calculated using the constant $K_{fr} = 2.5$, which was found from the experimental dependence of T_{fr} on the concentration of acetic acid in aqueous solutions. T_{fr} was measured using a home-made device, consisting of two glass tubes of different diameters inserted one inside the other and placed in a glass with one of the following chilling media: saturated NaCl solution, solid carbon dioxide, and solid carbon dioxide in acetone. A sample (a bacterial suspension or lyophilized biomass with solvent additions) was placed in the smaller tube. The larger tube was necessary to minimize the overcool temperature and to avoid further rapid cooling of the frozen sample.

The lyophilized *E. coli* biomass was analyzed using an IFS-66 computer-controlled infrared spectrophotometer (Bruker). Samples were placed between KBr plates. To avoid light scattering, some samples were immersed into mineral oil. When necessary, the lyophilized bacterial biomass was dried at 105°C for 1 h or wetted with water or acetic acid. The absorption bands of the major atomic groups in IR spectra were identified based on the infrared characteristic group frequencies available in the literature [19].

RESULTS

Nonmotile, weakly-charged, hydrophobic, obligately aerobic *A. calcoaceticus* 549 cells are able to utilize a wide range of substrates, from acetate and citrate to hydrophobic hydrocarbons. When its growth is retarded, this species accumulates polyPs, but utilizes them in the phase of logarithmic growth. Under anaerobic conditions, the strain consumes polyPs and does not accumulate PHAs. Like other *Acinetobacter* species [5, 11], *A. calcoaceticus* can accumulate PHAs only under aerobic conditions, for instance, in a weakly alkaline, phosphorus-deficient medium containing 5–10 mg P_i/l and from 1 to 10 g/l sodium acetate. Intracellular PHA granules are soluble in chloroform and stain with lipophilic dyes. When stained with Nile Red, *A. calcoaceticus* colonies fluoresce. Cells of this species actively produce surface-active polysaccharides and interact to form cell aggregates. Conversely, fermenting *E. coli* M-17 cells actively accumulate PHAs but poorly synthesize polyPs.

The biomass of batch *A. calcoaceticus* 549 and *E. coli* M-17 cultures in the stationary growth phase depended on the content of organic matter and mineral sources of nitrogen and phosphorus in the medium (Fig. 1). The maximum yield of the *E. coli* biomass was observed when the medium contained no less than 1700 mg P_i/l. At the same time, the maximum yield of the *A. calcoaceticus* biomass was observed when the concentration of phosphates in the medium was as low as 30–40 mg P_i/l.

The content of P_{total} in the *A. calcoaceticus* cells grown in media with different proportions between the concentrations of acetate and phosphorus varied from 0.7 to 3.3%, averaging 2%. This value of P_{total} is about two times higher than that observed for fermenting *E. coli* cells (Tables 1 and 2). The P_{total} of bacterial cells depended on the incubation temperature. The relative content of orthophosphates in the P_{total} of bacterial cells varied from 35 to 37%. At a cultivation temperature of 4°C, the P_{total} of the bacterial biomass and the polyP/P_{org} ratio were higher than at a normal cultivation temperature (32–37°C). When the concentration of orthophosphates in the growth medium was 4 mg P_i/l, the P_{total} in the dry biomass did not exceed 0.7%. This value, however, reached 2–3% and even more, if the concentration of orthophosphates in the medium was raised to 33 mg P_i/l.

It should be noted that, during the evaluation of P_i in the biomass of *A. calcoaceticus* 549 grown at 4°C, the blue color of the reaction product developed very slowly, presumably due to a high intracellular content of labile pyro- and triphosphate. Together with the phosphates produced from the low-molecular-weight polyP fraction, the content of P_i in the biomass may reach 90% of P_{total}. Lowering the cultivation temperature of *A. calcoaceticus* from 37–32 to 4°C augmented the accumulation of orthophosphates in the cytoplasm, presumably due to a diminished requirement of growth processes for orthophosphate. In this case, if the concentration of phosphates in the cultivation medium was low, they were completely depleted. The subsequent rise of the cultivation temperature to an optimal value of 32°C led to a 1.9-fold increase in the bacterial biomass yield.

The freezing temperature of the wet biomass consisting of the aerobically grown *A. calcoaceticus* 549 and *E. coli* M-17 cells that were precipitated from the culture liquid by centrifugation was 1.2–1.7°C lower than that of the culture supernatant and fresh nutrient medium (Table 3). The freezing temperature of the wet biomass of anaerobically grown *E. coli* M-17 cells was 3–8°C lower than that of the fresh medium. In this case, the freezing temperature of the culture supernatant was 0.3°C lower than that of the fresh medium.

The freezing temperature of the wet biomass of the yeast *C. cerevisiae* containing 44% H_2O was –4.1°C. At the same time, the freezing temperatures of the lyophilized biomasses of *E. coli, L. fermentum, B. bifidum,* and *B. siccum* wetted with small amounts of water or acetic acid were considerably lower than those of pure solvents, i.e, water and acetic acid. For instance, at a moisture content of 26 ± 5% H_2O, the T_{fr} of the biomass was –(20–23)°C (Table 3 and Fig. 2). Raising the moisture content of the biomass to 70 ± 2% led to an increase in its T_{fr} to –(3–8)°C. Further additions of water to the biomass were less effective. It should be noted that after the long-term storage of the lyophilized *E. coli* biomass in sealed ampules, the minimal value of its T_{fr} unexpectedly increased from –22 to –6°C at a biomass moisture content of 37 ± 1%.

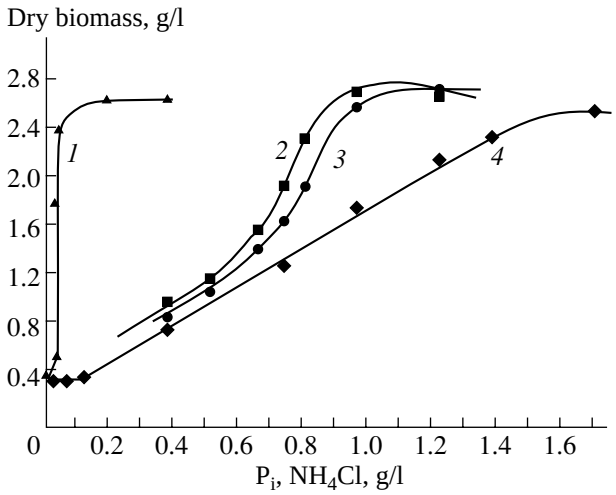

Fig. 1. Dependence of the biomass of the batch cultures of *A. calcoaceticus* IEGM 549 grown in media with 10 g/l sodium acetate and different concentrations of (*1*) phosphorus and (*2*) nitrogen sources and of *E. coli* M-17 grown in media with 4 g/l glucose and different concentrations of (*3*) nitrogen and (*4*) phosphorus sources.

Analysis of the IR spectra of the lyophilized *E. coli* M-17 biomass showed that its long-term storage for 13–16 years resulted in a dehydration of organic matter. This follows from the profound increase in the intensity of absorption bands near 3500 cm^{-1}, which are due to the valence vibrations of the bound OH-groups, and a slight increase in the intensity of characteristic absorption bands within a long-wave region of 1700–600 cm^{-1} (Fig. 3). The hydrogen bonds formed by hydroxyl groups may promote the mutual attraction of various molecules in the viscoelastic cytosol of cells. If so, this must diminish the intensity of the deformational vibration of amide NH-groups (1550 cm^{-1}) and augment the valence vibration of the carbonyl C=O– groups of amides, ketones, and β-ketoesters (1720–1620 cm^{-1}) [19]. Changes in the intensity of the absorption bands at 1740, 1240, and 1120 cm^{-1}, which are due to the interacting C–O– and C–O–C– groups of esters, anhydrides, and acetate, are more difficult to interpret. The IR spectra of the freshly lyophilized samples of colibacterin exhibited a considerable absorption within the spectral region 1100–900 cm^{-1}, which is typical of the deforma-

Table 1. Effect of the cultivation temperature and the orthophosphate concentration in the medium on the phosphorus content of the *A. calcoaceticus* IEGM 549 biomass

Content, mg/l	33 mg P_i/l medium		4 mg P_i/l medium	
	37°C	4°C	37°C	4°C
P_{total} in the culture supernatant	21.45 ± 0.15	14.55 ± 0.15	0.22 ± 0.02	0.11 ± 0.02
Dry biomass	590 ± 10	600 ± 10	590 ± 10	580 ± 10
P_i in the biomass	4.22 ± 0.23	14.57 ± 1.92	1.54 ± 0.15	1.60 ± 0.15
polyP in the biomass	3.76 ± 0.35	2.60 ± 0.12	0.63 ± 0.12	0.78 ± 0.12
P_{org} in the biomass	3.93 ± 0.38	1.67 ± 0.30	1.74 ± 0.26	1.66 ± 0.34
P_{total} in the biomass	11.91 ± 0.37	18.84 ± 1.75	3.91 ± 0.32	4.04 ± 0.39
P_{total}, % of dry biomass	2.02 ± 0.06	3.14 ± 0.29	0.66 ± 0.05	0.70 ± 0.07
P_{org}, % of P_{total}	33.00 ± 2.17	8.86 ± 0.77	44.50 ± 3.03	41.09 ± 4.49
P_i, % of P_{total}	35.43 ± 1.66	77.34 ± 3.03	39.39 ± 0.57	39.60 ± 0.12

Table 2. Effect of the preliminary accumulation of labile phosphates in the inoculum cells incubated at 4°C on the yield of the *A. calcoaceticus* IEGM 549 biomass

Content, mg/l	32°C	4°C
Dry biomass	870 ± 20	880 ± 20
P_{total} (orthophosphates, high-molecular-weight polyPs, organic and condensed phosphorus compounds) in the biomass	15.6 ± 0.4	28.7 ± 1.8
P_{lab} (phosphates and low-molecular-weight polyPs) in the biomass	6.0 ± 0.3	25.9 ± 1.4
P_{total}, % of dry biomass	1.8 ± 0.1	3.3 ± 0.2
P_{lab}, % of P_{total}	38.5 ± 1.9	90.2 ± 4.9
Biomass yield after 30 h of growth	2500 ± 200	4700 ± 300

Table 3. Minimal freezing temperatures of some solutions, media, and bacterial biomasses

Sample	T_{fr}, °C
Physiological saline solution (0.9% NaCl)	−0.5
Fresh sterile cultivation media of *A. calcoaceticus* and *E. coli*	−0.3
Culture supernatant of *A. calcoaceticus*	−0.3
Wet biomass of *A. calcoaceticus*	−1.5
Culture supernatant of aerobically grown *E. coli*	−0.5
Wet biomass of aerobically grown *E. coli*	−2.0
Culture supernatant of anaerobically grown *E. coli*	−0.6
Wet biomass of anaerobically grown *E. coli*	−8.1
Lyophilized *E. coli* biomass (280.6 mg; 3.2% moisture; 5-month storage) wetted with 0.1 ml H_2O	−22.0
Lyophilized *E. coli* biomass (179.0 mg; 3.8% moisture; 13.5-year storage) wetted with 0.1 ml H_2O	−5.8
Lyophilized *S. cerevisiae* biomass (501.0 mg; 8.5% moisture) wetted with 0.4 ml H_2O	−4.1
Lyophilized *L. fermentum* biomass (343.4 mg; 16.5% moisture) wetted with 0.1 ml H_2O	−20.2
Lyophilized *B. bifidum* biomass (354.9 mg; 10.0% moisture) wetted with 0.1 ml H_2O	−22.1
Lyophilized *B. siccum* biomass (398.1 mg; 15.5% moisture) wetted with 0.2 ml H_2O	−23.0

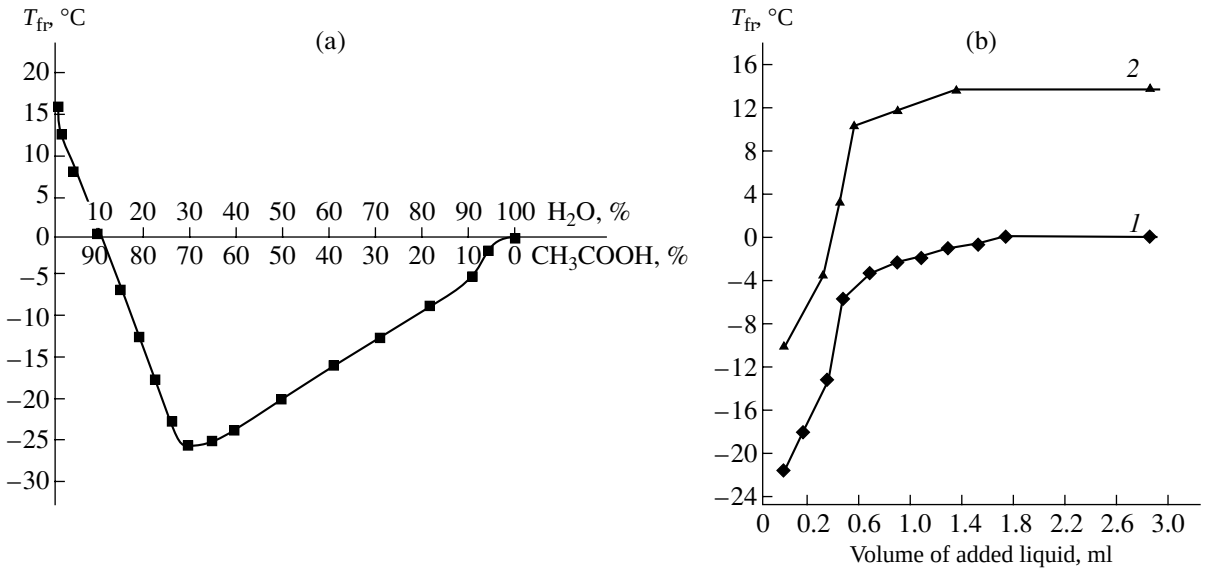

Fig. 2. Freezing temperatures of (a) acetic acid solutions and (b) lyophilized *E. coli* M-17 biomass (288.1 ± 7.5 mg) wetted with (*1*) water and (*2*) acetic acid.

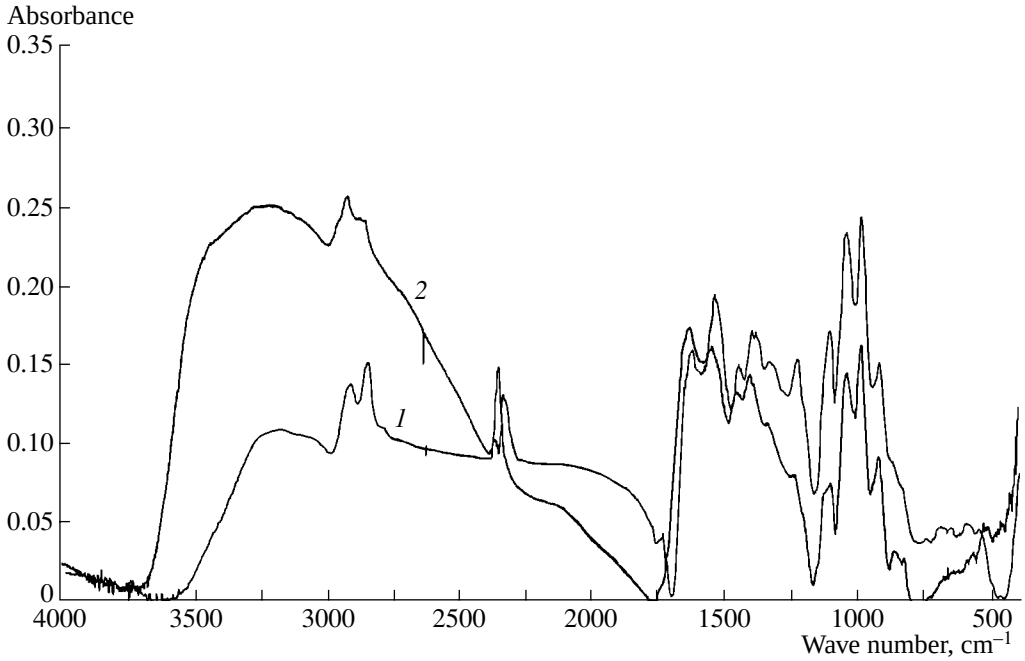

Fig. 3. The IR spectra of the lyophilized *E. coli* M-17 biomass stored for (*1*) 5 months and (*2*) 13.5 years.

tional vibration of unoxidized CH– groups and the valence vibrations of C–C– groups.

Cytochemical analysis showed that 15–20% of cells in fresh colibacterin samples have PHA granules, which are soluble in chloroform, whereas almost 100% of both viable and nonviable cells in the long-stored colibacterin samples have a lipoid substance spread in the cytosol. Up to 99.31% of cells in lyophilized colibacterin samples lost their viability within 3.5 years of storage. In the next 10 years of storage, the percentage of nonviable cells in colibacterin increased to 99.98%.

Experiments showed that the wetting of the long-stored colibacterin samples with a 3% solution of acetic acid in phosphate buffer augmented the survival rate of lyophilized *E. coli* cells to a larger extent than their wetting with physiological saline solution (0.9% NaCl),

which is commonly used for the resuscitation of lyophilized cells. This concentration of acetic acid is about 3.5 times higher than the concentration of acetic acid in the cultivation medium that suppressed the growth of batch *E. coli* cultures [20].

DISCUSSION

The transport systems of phosphate ions in *A. calcoaceticus* IEGM 549 cells are saturated with phosphate concentrations in the medium that are 50 times lower than those observed for *E. coli* M-17 cells (Fig. 1 and Tables 1 and 2). The more efficient phosphate transport system of *A. calcoaceticus* seems to be necessary to provide phosphates for an intense synthesis of high-molecular-weight polyPs in this bacterium. Therefore, first, the accumulation of polyPs in *A. calcoaceticus* cells is most likely regulated not only by the extracellular (as in *E. coli* cells [10]) but also by intracellular pool of orthophosphates. Second, the role of polyPs in the phosphorylation of hexoses and the maintenance of a constant level of intracellular phosphate ions in *A. calcoaceticus* cells is not so essential as in the case of *E. coli* cells.

The freezing temperature T_{fr} of the wet *A. calcoaceticus* biomass is only 1.2°C lower than that of the culture supernatant (Table 3). Assuming that old cells contain 80% water [14, 17] and that P_i comprises about 50% of P_{total}, the contribution of sodium, potassium, and ammonium phosphates to the decrease in the T_{fr} of the cytosol will be 0.6–0.7°C, while the contribution of NH_4Cl and other nitrogen-containing solutes will be 0.5–0.6°C. Therefore, growing *A. calcoaceticus* 549 cells accumulate acetate in amounts that are too small to activate the synthesis of PHAs.

The T_{fr} of the wet biomass of aerobically grown *E. coli* cells is 0.5°C lower than that of aerobically grown *A. calcoaceticus* cells. In the case of the anaerobically grown cells of these two bacteria, the respective decrease in T_{fr} may reach 3.8 ± 1.0°C owing to the intracellular accumulation of acidic metabolites. Therefore, the concentration of acetate in the anaerobically grown cells of these bacteria may be 7.6 ± 2.0 times (in some cases, even 13.2 times) higher than in anaerobically grown cells. Calculations also show that anaerobically grown *E. coli* cells may contain 9.1 ± 2.4% carbonic acids in their cytosol, which is equivalent to 1.5 ± 0.4 M acetic acid. Taking into account these calculations, it is not surprising that the 3% solution of acetic acid in the phosphate buffer turned out to be optimal for the resuscitation of lyophilized bacterial cells.

During the long-term storage of lyophilized bacterial biomass, its freezing temperature considerably increased (Table 3 and Fig. 3), indicating that acidic metabolites in the viscoelastic cytosol of *E. coli* cells were transformed with the liberation of water and formation of a lipoid substance stainable with lipophilic dyes. This substance is most likely acetic anhydride $(CH_3CO)_2O$, which is formed through the dehydration of acetic acid dimers.

Thus, the heterotrophic, gram-negative bacteria *E. coli* and *A. calcoaceticus* differ in their metabolic pathways which implement the synthesis of polyPs and PHAs from endogenous and exogenous precursors. Actively fermenting hydrophilic *E. coli* cells are able to utilize simple carbohydrates (which are not utilizable by *A. calcoaceticus*), produce carbonic acids (predominantly, acetic acid), and accumulate them in the cytoplasm, which is a prerequisite for an intense synthesis of PHAs. At the same time, acetate-oxidizing hydrophobic *A. calcoaceticus* cells growing aerobically in the presence of low concentrations of orthophosphates in the medium efficiently accumulate phosphate ions in the cytoplasm (up to 77% of P_{total}), which is a prerequisite for an intense synthesis of polyPs.

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