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EXPERIMENTAL ARTICLES

Peculiarities of Ethanol Metabolism in an *Acinetobacter* sp. Mutant Strain Defective in Exopolysaccharide Synthesis

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Abstract—Activities of the key enzymes of ethanol metabolism were assayed in ethanol-grown cells of an *Acinetobacter* sp. mutant strain unable to synthesize exopolysaccharides (EPS). The original EPS-producing strain could not be used for enzyme analysis because its cells could not to be separated from the extremely viscous EPS with a high molecular weight. In *Acinetobacter* sp., ethanol oxidation to acetaldehyde proved to be catalyzed by the NAD^+-dependent alcohol dehydrogenase (EC 1.1.1.1.). Both NAD^+ and $NADP^+$ could be electron accepters in the acetaldehyde dehydrogenase reaction. Acetate is implicated in the *Acinetobacter* sp. metabolism via the reaction catalyzed by acetyl-CoA-synthetase (EC 6.2.1.1.). Isocitrate lyase (EC 4.1.3.1.) activity was also detected, indicating that the glyoxylate cycle is the anaplerotic mechanism that replenishes the pool of C_4-dicarboxylic acids in *Acinetobacter* sp. cells. In ethanol metabolism by *Acinetobacter* sp., the reactions involving acetate are the bottleneck, as evidenced by the inhibitory effect of sodium ions on both acetate oxidation in the intact cells and on acetyl-CoA-synthetase activity in the cell-free extracts, as well as by the limitation of the C_2-metabolism by coenzyme A. The results obtained may be helpful in developing a new biotechnological procedure for obtaining ethanol-derived exopolysaccharide ethapolan.

Key words: ethanol metabolism, enzymatic activity, respiration rate, regulation.

The bacteria *Acinetobacter* sp. produce a complex polysaccharide preparation (EPS) ethapolan [1]. We have previously developed a procedure for obtaining ethanol-derived ethapolan and new approaches to intensification of EPS synthesis and to the control of its composition and physicochemical properties [1, 2]. However, these studies were based on suppositions about tentative metabolic pathways of the EPS synthesis in *Acinetobacter* sp. No enzymological analysis of the ethapolan-producing strain has been conducted because it was impossible to separate the bacterial cells from the extremely viscous EPS with a high molecular weight.

By means of nitrosoguanidine-induced mutagenesis, mutant strains of *Acinetobacter* sp. unable to synthesize exopolysaccharides were obtained [3]. The wild-type and EPS-mutant strains were indistinguishable in the physiological and biochemical traits (requirement for growth factors, pantothenic acid, and yeast autolysate; utilization of ethanol, mono- and disaccharides; resistance to antibiotics; etc.). The analysis of 16S rRNA demonstrated its identity in the wild-type and mutant stains.

In this work, we attempted to elucidate the major pathways of ethanol metabolism by *Acinetobacter* sp. by using the EPS-mutant strains. We aimed at elucidating the bottleneck of ethanol metabolism to work out methods for their elimination, which would allow us to optimize the procedure of obtaining ethanol-derived ethapolan.

MATERIALS AND METHODS

The mutant strain *Acinetobacter* sp. 1NG unable to synthesize EPS [3] and the wild-type EPS-synthesizing strain *Acinetobacter* sp. 12S described previously [1] were used in this study.

Growth of *Acinetobacter* sp. Bacteria were grown in a liquid mineral medium of the following composition (g/l): KH_2PO_4, 6.8; NaOH, 0.9; NaCl, 1.1; NH_4NO_3, 0.6; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.001 (standard medium 1). The medium was also supplemented with 0.5 vol % yeast autolysate and 0.0003 to 0.0009% calcium pantothenate. Ethanol added at a concentration of 1 vol % served as the carbon and energy source. The bacteria were also grown in liquid mineral medium 2 free of sodium compounds (NaOH and NaCl were replaced by equimolar amounts of KOH and KCl). *Acinetobacter* sp. 1NG cells were cultivated in shake flasks (220 rpm) at 30°C and pH 6.8–7.0 for 16–72 h. *Acinetobacter* sp. 12S cells were grown for 96 h. A one-day-old culture grown on a

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mixture of nutrient agar and wort agar (1 : 1) was used as inoculum.

To determine biomass concentrations, the optical density of a cell suspension measured and converted to dry biomass weight using a calibration curve. The amount of EPS was determined by the weighing method as described previously [1]. Acetate concentration in the culture liquid was assayed enzymatically using acetate kinase [4]. Acetaldehyde was detected in the culture liquid from the reaction with sodium nitroprusside and piperidine [5].

Preparation of cell-free extracts. After cultivation of *Acinetobacter* sp. 1NG cells in liquid mineral medium, the culture was centrifuged (4000 *g*, 15 min, 4°C), the cell pellet was washed twice with either 0.05 M Tris–HCl buffer (pH 7.0) or 0.05 M K^+-phosphate buffer (pH 7.4, 4000 *g*, 15 min, 4°C). Washed cells were resuspended in either 0.05 M Tris–HCl buffer (pH 7.0) or 0.05 M phosphate buffer (pH 7.4) to be sonicated (22 kHz) three times for 30 s each time on an UZDN-1 device at 4°C. The resulting suspension was centrifuged (12000 *g*, 30 min, 4°C) and the supernatant obtained served as cell-free extract.

The bacterial cells used to obtain cell-free extracts were taken at the mid-exponential growth phase and at the end of the exponential growth phase (16–20 and 40–44 h of growth, respectively), as well as in the stationary phase (68–72 h).

Enzymatic analyses. Alcohol dehydrogenase (EC 1.1.11.1.) and aldehyde dehydrogenase (EC 1.2.1.3 and EC 1.2.1.4) activities were assayed from either NAD^+ or $NADP^+$ reduction measured at 340 nm [6, 7]. Alcohol dehydrogenase (EC 1.1.99.8) activity was assayed from dichlorophenolindophenol reduction in the presence of phenazine methosulfate (PMS) measured at 600 nm [8]. The activities of acylating acetaldehyde dehydrogenase (EC 1.2.1.10) [9] and that of acetyl-CoA-synthetase (EC 6.2.1.1.) [10] were assayed from acetyl-CoA synthesis detected in the reaction of the acetyl-CoA with hydroxylamine, which resulted in the formation of acetyl hydroxamate. Reaction of the latter with ferric chloride yielded a product determined spectrophotometrically at 540 nm. Acetate kinase (EC 2.7.2.1) [11] activity was determined by the formation of acetyl phosphate interacting with hydroxylamine to produce acetyl hydroxamate.

Isocitrate lyase (EC 4.1.3.1) activity was measured spectrophotometrically at 324 nm from the rate of glyoxylate phenylhydrazine formation [12].

Enzyme activities were assayed at 28–30°C, a temperature optimal for the growth of *Acinetobacter* sp., and were expressed in the amounts of the reaction products (nmol/(min mg protein). The protein content in cell-free extracts was determined by Bradford's method [13]. The Michaelis constant (K_m) was determined from the Lineweaver–Burk plot [14].

To assay alcohol- and aldehyde dehydrogenase activities, *Acinetobacter* sp. 1NG cells were grown on standard medium 1. The activities of acetyl-CoA synthetase, acetate kinase, and isocitrate lyase were assayed in cells grown also on medium 2 containing no sodium ions.

Measurement of the rate of substrate oxidation by intact cells of *Acinetobacter* sp. 1NG. The oxidation rate of ethanol, acetaldehyde (AA), acetate, and succinate was determined polarographically from the rate of oxygen consumption in a reaction mixture using a closed electrode and a PPT-1 polarograph at 28–30°C, a temperature optimal for the growth of *Acinetobacter* sp. The specific rate of oxygen consumption was expressed in nmol O_2/(min mg cells). The substrate concentration was 10 mM. The respiration rate was determined in cells of *Acinetobacter* sp. 1NG grown on standard liquid mineral medium 1 (16–20 h, exponential growth phase), which were harvested by centrifugation (4000 *g*, 15 min, 4°C). The cell pellet was washed twice with either 0.05 M Tris–HCl or phosphate buffer (pH 6.8, 4000 *g*, 15 min, 4°C). Washed cells were resuspended in either 0.,05 M Tris–HCl or phosphate buffer (pH 6.8) and incubated on a shaker, 220 rpm, for 1–20 h at 30°C. The incubation in the buffer (cell starvation) was to reduce the level of endogenous cell respiration. Tris–HCl buffer, either K^+,Na^+-phosphate buffer, or K^+-phosphate buffer (0.05 M, pH 6.8) were used for cell washing, cell starvation, and measurement of the rate of substrate oxidation. In one variant of the experiments, cell starvation was conducted in the presence of pantothenic acid (vitamin B_3) added at a concentration of 0.0003% to the phosphate buffer.

RESULTS AND DISCUSSION

Ethanol oxidation. Ethanol oxidation by alcohol dehydrogenase to acetaldehyde is the first stage of ethanol metabolism in bacteria. Two types of alcohol dehydrogenase are known: $NAD(P)^+$- and pyrroloquinoline quinone-dependent enzymes [6, 8, 15, 16). In the first case, either NAD^+ or $NADP^+$ act as electron acceptors (or as the enzyme prosthetic group), whereas in the second case, pyrroloquinoline quinone (PQQ does). In the assay of PQQ-dependent enzymatic activity, phenazine methosulfate and 2,6-dichlorophenolindophenol serve as artificial acceptors of electrons, because of which the PQQ-dependent alcohol dehydrogenase is also referred to as PMS-dependent. In methylotrophic bacteria, methanol is oxidized by the PMS-dependent methanol dehydrogenase [8, 15], whereas in *Bacillus* sp. C1, methanol is oxidized by the NAD^+-dependent enzyme [16]. PMS-dependent ethanol dehydrogenase was detected in bacteria of the genus *Pseudomonas* [17]. In four strains of *Acinetobacter calcoaceticus*, ethanol is oxidized by the NAD^+-dependent alcohol dehydrogenase [6]. In the 1990s, a new type of nicotine-protein alcohol dehydrogenase that used *N*,*N*'-dimethyl-4-nitrosoaniline as the acceptor of electrons was revealed in some gram-positive bacteria [18].

The PMS-dependent alcohol dehydrogenase was not detected in the cell-free extract of *Acinetobacter* sp. 1NG, but rather the NAD^+-dependent ethanol dehydrogenase activity whose pH optimum was at pH 9.0 (Fig. 1a). During bacterial growth, the activity of NAD^+-dependent ethanol dehydrogenase decreased by 20 to 30% and was 260–270 nmol/(min mg protein) in the stationary phase (Table 1). Thus, in *Acinetobacter* sp., ethanol is oxidized to AA by NAD^+-dependent alcohol dehydrogenase.

Acetaldehyde oxidation. In the cell-free extract of *Acinetobacter* sp. 1NG, the activity of NAD^+-dependent acetaldehyde dehydrogenase was detected, with an optimum at pH 9.0 (Fig. 1a). The alcohol and acetaldehyde dehydrogenase activities showed different affinity to NAD^+: the Michaelis constants were 1 and 3 mM, respectively (Fig. 2). To determine exactly whether one or two different NAD^+-dependent dehydrogenases catalyze ethanol and acetaldehyde oxidation in *Acinetobacter* sp., the enzyme (or enzymes) should be isolated and their physicochemical properties should be studied.

The activity of NAD^+-dependent acetaldehyde dehydrogenase was two times lower than that of alcohol dehydrogenase (Table 1, Fig. 1). Nevertheless, the respiration rate of intact cells in the presence of acetaldehyde did not differ from that in the presence of ethanol (Table 2), and no acetaldehyde was detected in the culture liquid.

Our further experiments with *Acinetobacter* sp. 1NG showed that, in addition to NAD^+, $NADP^+$ can also be the acceptor of electrons in the reaction catalyzed by acetaldehyde dehydrogenase (Fig. 1). Thus, polarographic analysis of AA oxidation by a cell-free extract of *Acinetobacter* sp. 1NG showed that the rate of substrate oxidation was two times higher in the presence of PMS and $NADP^+$ than in the presence of NAD^+ at all pH values, including the optimum pH 9.0 (Fig. 1b). Spectrophotometric measurements of NAD^+- and $NADP^+$-dependent oxidation rates of acetaldehyde yielded similar results (Fig. 1a). Note that $NADP^+$ could not be the acceptor of electrons in the ethanol-dehydrogenase reaction.

Acylating acetaldehyde dehydrogenase is also known to catalyze AA oxidation. Thus, in *Pseudomonas* sp., this enzyme is involved in the formation of acetyl-CoA from acetaldehyde and coenzyme A [19]. However, in the cell-free extract of *Acinetobacter* sp. 1NG, the activity of acetaldehyde dehydrogenase was extremely low (10–15 nmol/(min per mg of protein)) (Table 1).

Acetate metabolism. The rate of ethanol, acetaldehyde, and acetate oxidation by intact cells of *Acinetobacter* sp. 1NG was studied in our further experiments, the necessity of which stemmed from the following. One of the growth requirements of both the wild-type EPS-producing strain and the EPS^- strain of *Acinetobacter* sp. is the availability of a capacious buffer with neutral pH values. When the bacteria are grown in non-

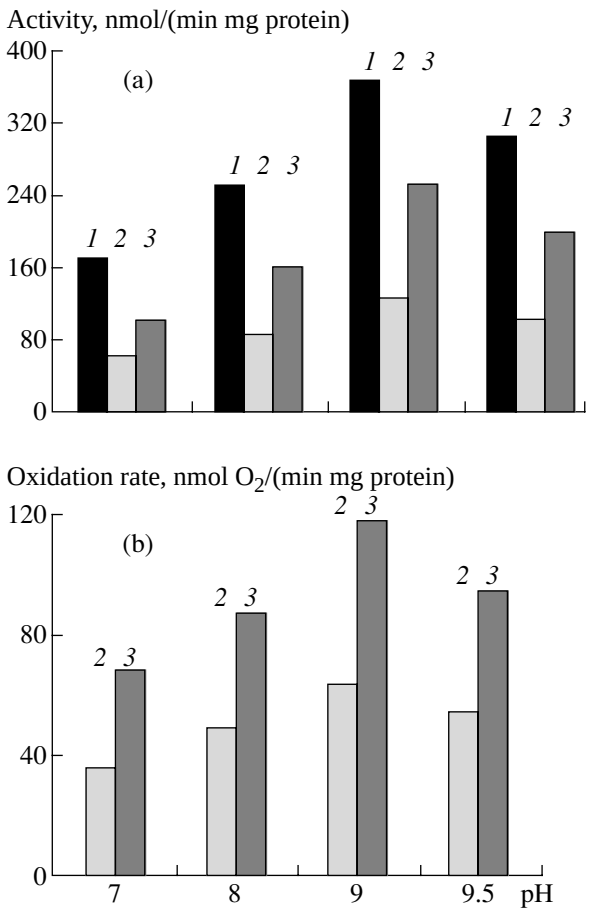

Fig. 1. Effect of pH on the activities of ethanol and acetaldehyde dehydrogenases in cell-free extracts of *Acinetobacter* sp. 1NG: (a) spectrophotometric assay of the oxidation rate of ethanol and acetaldehyde; (b) polarographic assay of the oxidation rate of acetaldehyde in the presence of an intermediate acceptor of electrons, phenazine methoasulfate: *1*, NAD^+-dependent ethanol oxidation; *2*, NAD^+-dependent acetaldehyde oxidation; *3*, $NADP^+$-dependent acetaldehyde oxidation. Cell-free extracts were obtained from bacteria grown to the mid-exponential phase.

buffered medium, a decrease in pH to 4.5 is observed in the culture liquid [1] because of acetate accumulation (50 to 65 mM). Acetate also accumulated in the absence at low concentrations of pantothenic acid (vitamin B_3) in a buffered medium. Vitamin B_3 is a precursor of coenzyme A, which appears to mediate involvement of acetate into metabolism [1, 2]. There was a need, therefore, to elucidate why acetate accumulated in the culture liquid of ethanol-grown *Acinetobacter* sp.

Measurements of the rates of ethanol, AA, and acetate oxidation by intact cells of *Acinetobacter* sp. 1NG showed that the level of cell respiration in the presence of ethanol and AA remained unchanged irrespective of the duration of cell starvation. However, the respiration rate in the presence of acetate decreased significantly with the duration of cell starvation (Table 2). The results obtained also showed that the cells oxidized

Table 1. Changes in the activities of key enzymes of ethanol metabolism during the growth of *Acinetobacter* sp. 1NG

Enzymes	Activity, nmol/(min mg protein) in bacteria grown for (h)		
	24	48	72
NAD^+-dependent alcohol dehydrogenase	365.7	289.5	265.3
NAD^+-dependent acetaldehyde dehydrogenase	119.5	93.7	79.8
$NADP^+$-dependent acetaldehyde dehydrogenase	253.7	197.3	157.1
Acylating acetaldehyde dehydrogenase	14.7	10.9	ND
Acetate kinase	9.8	7.1	ND
Acetyl-CoA synthetase	135.7 (74.5)	134.1	ND
Isocitrate lyase	130.0 (50.5)	144.9 (7.4)	156.3

Notes: (1) Bacteria were grown on standard medium 1 containing 0.0006% B_3.
(2) Activities of acetate kinase, acetyl-CoA synthetase, and isocitrate lyase were assayed in bacteria grown on medium 2 containing 0.0009% B_3. The results obtained with the *Acinetobacter* sp. 1NG grown in standard medium 1 containing 0.0006% B_3 are shown in parentheses.
(3) ND stand for "not determined".
(4) The composition of media 1 and 2 is given in the Materials and Methods section.

potassium acetate at a higher rate than sodium acetate. In these experiments, bacterial cells were incubated in K^+,Na^+-phosphate buffer of the same composition as that of a standard cultivation medium. The respiration rate of intact cells was determined at a potassium or sodium acetate concentration of 10 mM. Thus, an increase of 10 mM in concentration of Na^+ added in the form of acetate led to a 20 to 25% lower respiration rate (after cell starvation for 1–3 h), and this parameter was twice reduced after cell starvation for 20 h (Table 2).

Based on the results obtained, we suggested that sodium ions inhibit acetate oxidation in intact cells of *Acinetobacter* sp. The acetate-activating enzyme (acetyl-CoA synthetase) is known to be inhibited by Na^+ [10]. Since *Acinetobacter* sp. bacteria have a requirement for vitamin B_3, involved in acetate metabolism, this vitamin was assumed to activate acetate oxidation. This suggestion was confirmed in our further experiments. After cell starvation in K^+-phosphate buffer for 3 h, their respiration rate in the presence of both potassium and sodium acetate remained unchanged and was higher than after starvation in K^+,Na^+-phosphate buffer (Table 3). In addition, acetate oxidation by starved cells occurred at a higher rate in the presence of B_3 than in the absence of the vitamin. Cells incubated in either K^+,Na^+-phosphate or K^+-phosphate buffer displayed a higher respiration rate in the presence of potassium than sodium acetate (Table 3). After the addition of 25 to 100 mM of Na^+ to cells incubated in K^+-phosphate buffer, their respiration rate in the presence of acetate decreased 1.3–2-fold (Table 4). The same concentrations of NH_4^+ had no effect on the respiration rate of cells incubated in the presence of acetate. In the presence of ethanol and succinate, both sodium and ammonium ions added at the concentrations studied had no effect on the respiration rate of intact cells of *Acinetobacter* sp. 1NG (Table 4). At the same time, in the presence of ethanol, the respiration rate of cells incubated in K^+-phosphate buffer was somewhat higher than that of cells incubated in K^+,Na^+-phosphate buffer (Tables 2 and 4).

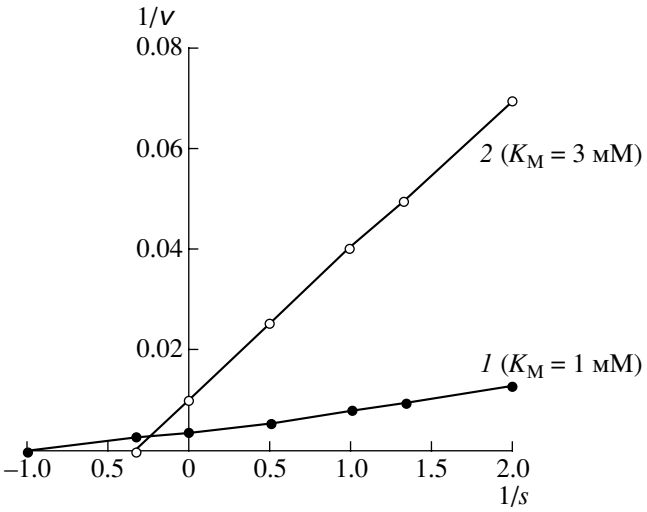

Fig. 2. Rate of (*1*) alcohol and (*2*) acetaldehyde oxidation by dehydrogenases in the cell-free extract of *Acinetobacter* sp. 1NG as dependent on NAD^+ concentration. pH of the reaction mixture was 9.0. The cell-free extracts were obtained from bacteria grown to the mid-exponential phase.

To study the effect of K^+ on the cell respiration rate in the presence of various substrates, we used Tris–HCl buffer (0.05 M, pH 6.8) for cell washing, starvation, and incubation. In the presence of ethanol and succinate, potassium ions at concentrations from 25 to 100 mM had no effect on cell respiration rate, which was 60–70 nmol O_2/(min mg cells). However, in the presence of acetate, the cells incubated in Tris–HCl buffer exhibited an

Table 2. Respiration rates of *Acinetobacter* sp. 1NG cells in the presence of ethanol, acetaldehyde, and acetate, as dependent on the duration of cell starvation

Duration of cell starvation, h	Respiration rate, nmol O_2/(min mg cell)			
	ethanol	acetaldehyde	sodium acetate	potassium acetate
1	120.6	123.3	73.4	88.5
3	132.1	139.6	53.2	65.3
20	129.9	133.5	7.5	18.3

Notes: (1) Bacteria were grown on standard medium 1 containing 0.0006% B_3.
(2) In starvation experiments, cells were incubated in K^+, Na^+-phosphate buffer (0.05 M; pH 6.8).
(3) Respiration rates were determined in K^+, Na^+-phosphate buffer (0.05 M; pH 6.8).
(4) The concentration of ethanol, acetaldehyde, and acetate was 10 mM.

Table 3. Respiration rate of intact cells of *Acinetobacter* sp. 1NG in the presence of acetate as dependent on the presence of Na^+ and pantothenic acid (vitamin B_3)

Phosphate buffer (0.05 M, pH 6.8)	B_3 concentration in the buffer, %	Respiration rate, nmol O_2/(min mg cell)	
		potassium acetate	sodium acetate
KH_2PO_4 + NaOH	0	65.3 (88.5)	53.2 (73.4)
	0.0003	87.8	72.3
KH_2PO_4 + KOH	0	118.2 (119.7)	91.4 (90.3)
	0.0003	134.5	107.2

Notes: (1) Bacteria were grown on standard medium 1 containing 0.0006% B_3.
(2) Cells were starving for 3 h. The results obtained with cells starved for 1 h are shown in parentheses.
(3) Cell starvation and analysis of cell respiration rate were conducted in the same phosphate buffer.

extremely low respiration rate (5–7 nmol O_2/min per mg of cells); therefore, the effect of K^+ on acetate oxidation was difficult to study under these conditions. When the same cells were incubated in K^+-phosphate buffer of equal molarity and pH, their respiration rate in the presence of acetate increased six- to eightfold. In our opinion, the low rate of acetate oxidation by cells incubated in Tris–HCl buffer is a result of impaired transport of this substrate into the cells. Thus, the energy of the proton-motive force generated by ionic gradients on the cell membrane (e.g., by K^+ and H^+ gradients) may account for the active transport of acetate into the *Acinetobacter* sp. cells.

Thus, the analysis of cation effects on the respiration rate of *Acinetobacter* sp. cells in the presence of various substrates showed that potassium and ammonium cations did not inhibit oxidation of ethanol, acetate, and succinate by intact bacterial cells.

Polarographic analysis allowed us to suggest that sodium ions contained in the standard medium may cause a limitation of C_2-metabolism in ethanol-grown *Acinetobacter* sp. cells, as well as accumulation of acetate in the culture liquid. However, we had to take into account that the concentration of Na^+ in the cells was not necessarily directly correlated with the Na^+ concentration in the medium.

It was demonstrated in this study that in both wild-type and mutant *Acinetobacter* sp. strains grown on medium 2, which contained no sodium compounds, the biomass yield and EPS production (in strain 12S) increased with the increase of the B_3 concentration in the medium. No acetate accumulation or decrease in pH values were observed under these conditions (Table 5). We have previously shown that potassium cations in the incubation medium of the EPS-producing strain had a favorable effect on the physicochemical properties of the polysaccharide synthesized [20].

Note that sodium ions had a lesser adverse effect on the growth of the EPS-synthesizing strain than on the growth of the mutant strain. This phenomenon can be explained by the fact that the univalent cations contained in the medium are involved in structuring of the EPS produced [1, 2]. A portion of sodium ions binds to the EPS molecules to become unavailable for the cells.

Based on this study, the cultivation conditions optimal for the analysis of the enzyme activities involved in acetate metabolism of the EPS^- strain of *Acinetobacter* sp. were worked out.

Acetate is implicated in the bacterial metabolism via two pathways: the pathway involving acetate kinase and phosphotransacetylase [21] and the pathway involving acetyl-CoA synthetase [22, 23]. In many bacterial species, including *Escherichia coli*, both acetate kinase and acetyl-CoA synthetase were detected [23]. Note that in *E. coli* cells, only one of the two pathways of acetate metabolism is operative, depending on the concentration of this substrate in the medium.

In cell-free extracts of *Acinetobacter* sp. 1NG, the acetate kinase activity was not higher than 10 nmol/(min mg protein), and, hence, it was of little consequence for acetate metabolism in these bacteria (Table 1). The activity of acetyl-CoA synthase was found to be at least 10 to 15 times higher than that of acetate kinase (Table 1), and it remained unchanged for 48 h of bacterial growth (till the end of the exponential growth phase). The ammonium ions in the reaction mixture did not affect the activity of acetyl-CoA synthetase, whereas in the presence of sodium ions, this activity was substantially inhibited (Table 4). In the presence of acetate, Na^+ and NH_4^+ had a similar effect on the respiration rate of the intact cells. In the exponential phase, the cells of *Acinetobacter* sp. 1NG grown on a sodium-containing medium displayed an acetyl-CoA synthetase activity as low as 70–75 nmol/(min mg

Table 4. Effect of sodium and ammonium cations on the activity of acetyl-CoA synthetase in the cell-free extract of *Acinetobacter* sp. 1NG and the respiration rate of intact bacterial cells in the presence of ethanol, acetate, or succinate

Cations	Cation concentration, mM	Activity of acetyl-CoA synthetase in the cell-free extract, nmol/(min mg protein)	Respiration rate of intact cells, nmol O_2/(min mg cells)		
			ethanol	potassium acetat	potassium succinate
Without cations	0	135.7	147.8	118.2	125.3
Na^+	25	103.9	145.4	91.8	124.9
	50	87.5	140.9	78.6	125.0
	100	63.1	138.2	59.7	122.1
NH_4^+	25	136.4	148.5	121.9	126.0
	50	133.9	146.4	120.0	128.4
	100	134.8	147.0	118.2	129.3

Notes: (1) The activity of acetyl-CoA synthetase was assayed in bacteria grown to the mid-exponential phase on medium 2 containing 0.0009% B_3.

(2) Respiration rates were determined in bacteria grown on standard medium 1 containing 0.0006% B_3; cells were starved for 3 h in K^+-phosphate buffer (0.05 M, pH 6.8). Respiration rates were determined in K^+-phosphate buffer (0.05 M; pH 6.8).

(3) Na^+ and NH_4^+ were added to the reaction mixture in the form of chlorides.

Table 5. Accumulation of biomass and exopolysaccharides and changes in pH and in the content of acetate in the culture liquid of *Acinetobacter* sp. 1NG (EPS^-) and 12S (EPS^+) as dependent on the B_3 concentration and on the presence of sodium compounds in the medium

Medium	B_3 concentration, %	Parameters							
		biomass, g/l		exopolysaccharides, g/l		final pH		acetate, mM	
		1NG	12S	1NG	12S	1NG	12S	1NG	12S
Medium 1 (K^+,Na^+-medium)	0.0003	0.6	0.95	0	2.0	4.5	5.5	58.9	35.7
	0.0006	0.9	1.2	0	2.8	5.3	6.0	42.6	15.9
	0.0009	1.1	1.3	0	3.0	5.7	6.2	29.4	8.3
Medium 2 (K^+-medium)	0.0003	1.2	1.3	0	3.2	5.9	6.1	17.1	13.1
	0.0006	1.45	1.4	0	3.85	6.3	6.5	6.4	3.1
	0.0009	1.9	1.45	0	4.3	6.9	6.9	0	0

Note: Bacteria were grown in shake flasks for 72 h (strain 1NG) and 96 h (strain 12S).

protein), whereas in bacteria grown on an Na^+-limited medium, this activity was as high as 130–135 nmol/(min mg protein) (Table 1). Thus, enzymatic analysis confirmed the conclusions from our polarographic studies and growth experiments that sodium ions inhibit acetate metabolism in *Acinetobacter* sp.

In C_2-grown bacteria, the glyoxylate cycle operates as an anaplerotic succession of reactions, which lead to oxalacetate formation [12]. Isocitrate lyase is one of the key enzymes of this cycle. In the cell-free extract of *Acinetobacter* sp. 1NG, isocitrate lyase activity was at the level of 150–160 nmol/(min mg protein) even on the third day of cultivation (stationary growth phase) (Table 1). Note that when *Acinetobacter* sp. 1NG was grown under conditions leading to acetate accumulation in the culture liquid (when sodium compounds are present and the B_3 concentration is low), isocitrate lyase activity decreased to 7–7.5 nmol/(min mg protein) by the end of the exponential growth phase (Table 1).

Thus, our investigation of ethanol metabolism in *Acinetobacter* sp. showed the following: (1) ethanol oxidation to acetaldehyde is catalyzed by alcohol dehydrogenase; (2) the activity of acetaldehyde dehydrogenase can also be detected (both NAD^+ and $NADP^+$ can be the acceptors of electrons; the optimal pH is 9.0); (3) acetate is implicated in the metabolism due to the activity of acetyl-CoA synthetase; (4) in ethanol-grown *Acinetobacter* sp. cells, the glyoxylate cycle operates as an anaplerotic succession of reactions that replenish the

pool of C_4-dicarboxylic acids in the cells; (5) in *Acinetobacter* sp., involvement of acetate into ethanol metabolism is the bottleneck, particularly due to the inhibitation of both acetate oxidation and acetyl-CoA synthetase activity by sodium ions and to the limitation of C_2-metabolism by coenzyme A.

The results obtained will be helpful in developing a new procedure for obtaining ethanol-derived ethapolan.

REFERENCES

1. Grinberg, T.A., Pirog, T.P., Malashenko, Yu.R., and Pinchuk, G.E., *Mikrobnyi sintez ekzopolisakharidov na C_1–C_2-soedineniyakh* (Microbial Synthesis of Exopolysaccharides on C_1–C_2 Compounds), Kiev: Naukova Dumka, 1992.
2. Pirog, T.P., Fundamentals of the Regulation of the Composition and Physicochemical Properties of Exopolysaccharides Produced by *Acinetobacter* sp., *Doctoral (Biol.) Dissertation*, Kiev, 1999.
3. Pirog, T.P., Stolyar, S.M., and Malashenko, Yu.R., Isolation and Characterization of *Acinetobacter* sp. Mutants Defective in Exopolysaccharide Biosynthesis, *Mikrobiologiya*, 2000, vol. 69, no. 5, pp. 674–680.
4. Krishtab, T.P. and Sokolov, I.G., An Enzymatic Method for Quantitative Determination of Acetic Acid in the Cultural Liquid, *Mikrobiol. Zh.*, 1985, vol. 47, no. 1, pp. 86–88.
5. Faigl', F., *Kapel'nyi analiz organicheskikh veshchestv* (Spot Analysis of Organic Compounds), Moscow: Goskhimizdat, 1962.
6. Beardmore-Gray, M. and Anthony, C., The Absence of Quinoprotein Alcohol Dehydrogenase in *Acinetobacter calcoaceticus*, *J. Gen. Microbiol.*, 1983, vol. 129, no. 10, pp. 2979–2983.
7. Hommel, R., Kurth, J., and Kleber, H.P., $NADP^+$-Dependent Aldehyde Dehydrogenase from *Acinetobacter rancens* CCM 1774: Purification and Properties, *J. Basic Microbiol.*, 1988, vol. 28, no. 1/2, pp. 25–33.
8. Anthony, C. and Zatman, L.J., The Microbial Oxidation of Methanol: Purification and Properties of the Alcohol Dehydrogenase of *Pseudomonas* sp. M27, *Biochem. J.*, 1967, vol. 104, pp. 953–955.
9. Burton, R.H. and Stadtman, E.R., The Oxidation of Acetaldehyde to Acetyl Coenzyme A, *J. Biol. Chem.*, 1953, vol. 202, no. 2, pp. 873–890.
10. Beinert, H., Green, D.E., Hele, P., Hift, H., Von Korff, R.W., and Ramakrishnan, C.V., The Acetate Activating Enzyme System of Heart Muscle, *J. Biol. Chem.*, 1953, vol. 203, no. 1, pp. 35–45.
11. Rose, I.A., Grunberg-Manago, M., Korey, S., and Ochoa, S., Enzymatic Phosphorylation of Acetate, *J. Biol. Chem.*, 1954, vol. 211, no. 2, pp. 737–756.
12. Dixon, G.H. and Kornberg, H.S., Assay Methods for Key Enzymes of the Glyoxylate Cycle, *Biochem. J.*, 1959, vol. 72, no. 1, pp. 195–196.
13. Bradford, M., A Rapid Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein–Dye Binding, *Anal. Biochem.*, 1976, vol. 72, pp. 248–254.
14. Dixon, M. and Webb, E., *Fermenty* (Enzymes), Moscow: Mir, 1980, vol. 1.
15. Anthony, C., *The Biochemistry of Methylotrophs*, New York: Academic, 1982.
16. Arfman, N., de Vries, K.J., Moezelaar, H.R., Attwood, M.M., Robinson, G.K., van Geel, M., and Dijkhuizen, L., Environmental Regulation of Alcohol Metabolism in Thermotolerant Methylotrophic *Bacillus* Strains, *Arch. Microbiol.*, 1992, vol. 157, no. 3, pp. 272–278.
17. Diehl, A., von Wintzingerode, F., and Gorisch, H., Quinoprotein Ethanol Dehydrogenase of *Pseudomonas aeruginosa* Is a Homodimer: Sequence of the Gene and Deduced Structural Properties of the Enzyme, *Eur. J. Biochem.*, 1998, vol. 257, no. 2, pp. 409–419.
18. Nagy, I., Verheijen, S., De Schrijver, A., Van Damme, J., Proost, J., Schoofs, G., Vanderleyden, J., and De Mot, R., Characterization of the *Rhodococcus* sp. N186/21 Gene Encoding Alcohol: *N,N'*-Dimethyl-4-Nitrosoaniline Oxidoreductase Inducible by Atrazine and Thiocarbamate Herbicides, *Arch. Microbiol.*, 1995, vol. 163, no. 6, pp. 439–446.
19. Powlovski, J., Sahlman, L., and Shingler, V., Purification and Properties of the Physically Associated Meta-Cleavage Pathway Enzymes 4-Hydro-2-Ketovalerate Aldolase and Aldehyde Dehydrogenase (Acylating) from *Pseudomonas* sp. Strain CF600, *J. Bacteriol.*, 1993, vol. 175, no. 2, pp. 377–385.
20. Pirog, T.P., Grinberg, T.A., Buklova, V.N., Votselko, S.K., and Malashenko, Yu.R., Production of Exopolysaccharides by *Acinetobacter* sp. in Batch Cultures with Different Potassium Concentrations, *Mikrobiologiya*, 1995, vol. 64, no. 1, pp. 51–54.
21. Gottschalk, G., *Bacterial Metabolism*, New York: Springer-Verlag, 1979. Translated under the title *Metabolism bakterii*, Moscow: Mir, 1982.
22. Ampe, F. and Lindley, N.D., Acetate Utilization Is Inhibited by Benzoate in *Alcaligenes eutrophus:* Evidence for Transcriptional Control of the Expression of *acoE* Coding for Acetyl Coenzyme A Synthetase, *J. Bacteriol.*, 1995, vol. 177, no. 20, pp. 5826–5833.
23. Kumari, S., Tishel, R., Eisenbach, M., and Wolfe, A.J., Cloning, Characterization, and Functional Expression of *acs*, the Gene Which Encodes Acetyl Coenzyme A Synthetase in *Escherichia coli*, *J. Bacteriol.*, 1995, vol. 177, no. 10, pp. 2878–2886.