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Regulation of Acetate Metabolism in a Strain of *Acinetobacter* sp. Growing on Ethanol

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Abstract—Ethanol metabolism in *Acinetobacter* sp. is shown to be limited by the rate of acetate assimilation, a reaction catalyzed by acetyl-CoA synthetase (EC 6.2.1.1). Effects of ions (sodium, potassium, and magnesium), by-products of ethanol and acetaldehyde oxidation (NADH and NADPH), and pantothenic acid on this enzyme are studied (sodium, NADH, and NADPH inhibit acetyl-CoA synthetase; pantothenic acid, potassium, and magnesium act as enzyme activators). Conditions of culturing were developed under which ethanol, acetaldehyde, and acetate in *Acinetobacter* cells were oxidized at the same rates, producing a threefold increase in the activity of acetyl-CoA synthetase in the cell-free extract. The results of studies of acetyl-CoA synthetase regulation in a mutant strain of *Acinetobacter* sp., which is incapable of forming exopolysaccharides, provide a basis for refining the technology of ethapolan production involving the use of C_2 substrates.

Acinetobacter sp. 12S is a strain producing the highly viscous exopolysaccharide (EPS) complex ethapolan when grown in ethanol-containing media [1]. We started to study ethanol metabolism in the mutant strain *Acinetobacter* sp. 1NG, which fails to form EPSs [2], because the cells of the original strain could not be separated from the product and used for enzymological experiments.

Our study demonstrated [2] that oxidation of ethanol to acetaldehyde in *Acinetobacter* sp. 1NG is catalyzed by NAD^+-dependent alcohol dehydrogenase (EC 1.1.1.1). NAD^+ and $NADP^+$ are electron acceptors in the acetaldehyde dehydrogenase reaction. Acetate is involved into the metabolism by acetyl-CoA synthetase (EC 6.2.1.1). The presence of isocitrate lyase (EC 4.1.3.1) indicates that the sequence of anaplerotic reactions replenishing the pool of C_4-dicarboxylic acids in the cells of *Acinetobacter* sp. is the glyoxylate cycle.

In studying specific features of metabolism of C_2 compounds in *Acinetobacter* sp., we focused our attention on the utilization of acetate by the cells. When grown on ethanol, both the original, EPS-producing strain and the mutant require the presence in the medium of a neutral phosphate buffer with sufficient capacity. Culturing of *Acinetobacter* sp. in a buffer-free medium results in its acidification (a decrease in pH to 4.5) due to accumulation of acetate (50–65 mM) [2]. Under these conditions, bacteria ceased to grow and synthesize EPS. Acetate accumulation was also observed when *Acinetobacter* sp. was grown in a buffered Na^+-containing medium in the absence or at low concentrations of pantothenic acid, a precursor of CoA (as mentioned above, acetate in involved into the metabolic pathway by CoA) [2]. It should be noted that *Acinetobacter* sp. exhibits auxotrophy with respect to pantothenic acid [1]. Sodium ions inhibit acetate oxidation and acetyl-CoA synthetase (in intact cells and cell-free extracts, respectively) and limit CoA-dependent metabolism of C_2 compounds [2]. When the bacteria were cultured in a sodium-free medium in the presence of increased concentrations of pantothenic acid (0.0009%), the decrease in pH and acetate accumulation in the culture liquid were not observed [2]. We hypothesized that culturing *Acinetobacter* sp. under such conditions may allow use of buffer-free media; the resulting decrease in the consumption of phosphate salts would reduce the cost of culturing.

In this work, we sought to (1) study the regulation of acetate metabolism in *Acinetobacter* sp. grown in ethanol-containing medium and (2) select conditions of cultivation in the absence of buffer that preclude acetate accumulation in the culture liquid.

MATERIALS AND METHODS

Objects. In this work, we used the mutant strain *Acinetobacter* sp. 1NG, failing to form EPSs [3], and the original, EPS-synthesizing strain *Acinetobacter* sp. 12S, described previously [1].

Cultivation of Acinetobacter spp. Bacteria were grown in liquid mineral medium (A) (having a buffer capacity of 0.05 M) containing (g/l) KH_2PO_4 (6.8), KOH (1.8), KCl (1.4), NH_4NO_3 (0.6), $MgSO_4 \cdot 7H_2O$ (0.4), $CaCl_2 \cdot 2H_2O$ (0.1), and $FeSO_4 \cdot 7H_2O$ (0.001). Medium B had the same components, but some of them (providing buffer capacity) were present at concentrations that were twice as low, compared to medium A (g/l): KH_2PO_4 (3.4), KOH (0.9), KCl (4.4), NH_4NO_3 (0.6), $MgSO_4 \cdot 7H_2O$ (0.4), $CaCl_2 \cdot 2H_2O$ (0.1), and $FeSO_4 \cdot$

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$7H_2O$ (0.001). In medium C, these components were present in still lower concentrations, 3.4 times lower than in medium A (g/l): KH_2PO_4 (2.0), KOH (0.55), KCl (5.6), NH_4NO_3 (0.6), $MgSO_4 \cdot 7H_2O$ (0.4), $CaCl_2 \cdot 2H_2O$ (0.1), and $FeSO_4 \cdot 7H_2O$ (0.001). In one variant of the experiment, the content of $MgSO_4 \cdot 7H_2O$ in media A and B was increased to 1.2 g/l. The concentration of K^+ was in all cases equal to 100 mM; pH ranged from 6.8 to 7.0.

Bacteria were also cultured in a medium containing sodium (K^+/Na^+ medium) [2]. Its composition was similar to that of medium A, except that KOH and KCl were replaced by equimolar amounts of NaOH and NaCl.

All media were supplemented with 0.5% (v/v) yeast autolysate and calcium pantothenate (0.0006–0.0012%). Ethanol, which served as the source of carbon and energy, was present at concentrations equal to 0.5% or 1.0% (v/v). The concentration of potassium acetate, used for the same purpose, was 1.6% (which was equivalent, in carbon content, to 1.0% ethanol, v/v). When the initial concentration of ethanol was equal to 0.5%, an addition equivalent to 0.5% ethanol was made in the middle of the exponential stage of bacterial growth. The lower concentration of calcium pantothenate (0.0006%) corresponded to an initial concentration of ethanol equal to 0.5%; when ethanol was present at 1.0% (or potassium acetate, at 1.6%), the concentration of calcium pantothenate ranged from 0.0009 to 0.0012%. In one variant of the experiments, bacteria were cultured in medium A with 1.0% ethanol until the middle of the exponential stage (for 16–18 h), after which potassium acetate was added (0.05–0.5%).

Acinetobacter sp. was grown in flasks, at 220 rpm and 30°C, for 16–96 h. A one-day culture grown in a 1 : 1 mixture of meat–peptone agar and wort agar was used as inoculum.

The biomass was assessed by the optical density of the cell suspension, with subsequent conversion to dry cell weight (using a calibration curve). The maximum value of the specific rate of bacterial growth was calculated as described in [4].

The concentration of EPS was determined by the weight method [1]. The content of acetate in the culture liquid was determined using acetate kinase [5]. The amount of ethanol in the culture liquid was measured by gas–liquid chromatography on a Cvet-4 chromatograph (Czech Republic) equipped with a flame-ionization detector (column, 2 m; solid carrier, celite-545; stationary liquid phase, 20% polyethylene glycol-400; gas carrier, helium; temperature, 150°C).

Cell-free extracts. Extracts were prepared using the cells of *Acinetobacter* sp. 1NG in the exponential phase of growth (16–20 h of cultivation). The cells were separated by centrifugation at 4000 *g* and 4°C for 15 min, washed twice with 0.05 M Tris–HCl (pH 7.0) or 0.05 M potassium phosphate (K^+–phosphate) buffer (pH 7.4) to remove residual medium, and centrifuged under the same conditions. Washed cells were resuspended in 0.05 M Tris–HCl (pH 7.0) or 0.05 M phosphate buffer (pH 7.4) and disrupted by sonication (22 kHz; three cycles of 30 s each at 4°C) in a UZDN-1 disintegrator (Russia). The homogenate thus obtained was centrifuged at 12000 *g* and 4°C for 30 min; the pellet was discarded and the supernatant used as the cell-free extract.

Measurement of enzyme activity. The activities of alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenases (EC 1.2.1.3 and EC 1.2.1.4) were measured by NAD^+ or $NADP^+$ reduction at 340 nm [6, 7].

The activity of acetyl-CoA synthetase was determined from the formation of acetyl-CoA (using its reaction with hydroxylamine, with the formation of acetylhydroxamate) [8]. The product of acetylhydroxamate reaction with ferric chloride was determined spectrophotometrically at 540 nm. The activity of isocitrate lyase (EC 4.1.3.1) was determined by the rate of formation of phenylhydrazone glyoxylate at 324 nm [9].

To study the effects of cations on the activity of acetyl-CoA synthetase in the cell-free extract of *Acinetobacter* sp. 1NG, bacterial cells were washed and disrupted ultrasonically in 0.05 M Tris–HCl (pH 7.0). Cations (K^+ and Mg^{2+}) were introduced into the reaction mixture from concentrated solutions (25% KCl and 10% $MgCl_2 \cdot 6H_2O$). The concentrations of K^+ and Mg^{2+} were within the respective ranges of 10–100 and 2–10 mM. In study of the effects of structural analogues of acetyl-AMP (NADH, NADPH, AMP, adenine, and adenosine) on the activity of acetyl-CoA synthetase, these compounds were introduced into the reaction mixture at 10-mM concentrations.

Enzyme activities were determined at 28–30°C (the temperature range favoring optimum growth of *Acinetobacter* sp.) and expressed in nmol/min per mg protein. The content of protein in cell-free extracts was calculated by the method of Bredford.

Measurement of substrate oxidation rates in intact cells of Acinetobacter sp. 1NG. Ethanol, acetaldehyde, potassium acetate, and potassium succinate oxidation rates (i.e., respiration rates of intact cells in the presence of the substrates) were determined from the rates of oxygen consumption in the reaction mixtures. Oxygen consumption was measured at 28–30°C using a PPT-1 polarograph (Russia) equipped with a closed electrode. Specific oxygen consumption rates were expressed in nmol O_2 per min per mg cell biomass. The concentration of each substrate was equal to 10 mM.

To decrease the level of endogenous respiration, fasting cells of *Acinetobacter* sp. 1NG were obtained (in 0.05 M K^+–phosphate buffer, pH 7.0). The respiration rates in the presence of the substrates were measured in 0.05 M K^+–phosphate buffer (at pH 5.5 and 7.0).

In studying the effects of cations on the rate of respiration of intact cells of *Acinetobacter* sp. 1NG, 0.05 M Tris–HCl (pH 7.0) was used for washing, fasting, and incubation (at the stage of determining the oxidation rate of the substrates). Cations (K^+ and Mg^{2+})

Table 1. Growth of *Acinetobacter* sp. 1NG in media with varying capacity of phosphate buffer (ethanol, 1.0%; calcium pantothenate, 0.0009%)

Medium	K^+–phosphate buffer, M	Biomass, g/l	pH	Acetate in culture liquid, mM
A	0.050	2.0	7.0	0
B	0.025	1.4	5.9	17.1
C	0.015	1.1	5.5	25.0

were introduced into the incubation mixture as concentrated solutions (25% KCl and 10% $MgCl_2 \cdot 6H_2O$). The concentrations of K^+ and Mg^{2+} were within the respective ranges of 10–100 and 2–10 mM.

RESULTS AND DISCUSSION

When the strain *Acinetobacter* sp. 1NG was grown in medium B or medium C (containing, respectively, 0.015 and 0.025 M K^+–phosphate buffer), acetate accumulation occurred, bringing pH values to 5.9–5.5 (Table 1). Under these conditions, we also noted a decrease in biomass formation. Similar results were obtained with the EPS-producing strain grown in the same media; in this case, the amount of EPSs synthesized was decreased as well. Thus, in spite of the fact that the medium contained no sodium compounds and the concentration of pantothenic acid was increased, decreasing molarity of the buffer was associated with acetate accumulation. Therefore, factors other than those identified previously accounted for the observed decrease in acetate metabolism in the cells of *Acinetobacter* sp. grown in the presence of ethanol.

The study of the oxidation rates of C_2 substrates in intact cells of *Acinetobacter* sp. 1NG grown in K^+/Na^+ medium or medium A (K^+ medium) demonstrated that, following 20 h of fasting, the respiration rate in the presence of ethanol or acetaldehyde was not changed. Under the same conditions, the rate of respiration in the presence of acetate decreased by two and four times, respectively, in the cells grown in medium A and in K^+/Na^+ medium (Table 2). Of note, the ethanol and acetaldehyde oxidation rates were barely, if at all, affected by the composition of the growth medium. Conversely, the acetate oxidation rate was higher in cells grown in medium A (moreover, in this case, the difference in the respiration rates between the cells grown in medium A and K^+/Na^+ became more pronounced after fasting). Our attention was also engaged by the fact that, in fasting cells, the respiration rate in the presence of acetate was 2.5- to 6-fold lower than in the presence of ethanol or acetaldehyde (Table 2).

Analysis of the key enzymes of ethanol metabolism in cell-free extracts of the strain *Acinetobacter* sp. 1NG grown in the presence of 1.0% ethanol and 0.0006% calcium pantothenate demonstrated that the activity of acetyl-CoA synthetase was 3.5- to 5-fold lower as compared to that of alcohol dehydrogenase or acetaldehyde dehydrogenase (Table 3).

Taken together, the data of Tables 1–3 led us to conclude that the reaction catalyzed by acetyl-CoA synthetase is a rate-limiting step in ethanol consumption. It seems that, in cells of *Acinetobacter* 1NG growing in the presence of ethanol, the rate of acetate formation exceeds that of its involvement into subsequent metabolic steps. Excess acetate is released into the culture liquid, causing its pH to go down. When a buffer is present in the medium, pH oscillations caused by acetate accumulation are decreased. It is conceivable that, under such conditions (i.e., at near-neutral pH values), acetate is transported back into the cells and enters the metabolic pathways. In the absence of a buffer, the inflow of acetate into the culture liquid causes more pronounced changes in pH, which gradually decreases to levels at which cellular uptake of acetate is slowed down or becomes impossible. In this case, gradual accumulation of acetate would inhibit the growth of the culture, not necessarily by decreasing the pH. It was reported [10, 11] that suppression of bacterial growth by high concentrations of acetate in the medium involves a variety of mechanisms, including (1) nonspecific inhibition of enzymatic reactions (caused by acidification of the cytosol) and (2) interference with cellular energy metabolism, primarily via an effect on the electrochemical proton gradient, $\Delta\mu_{H^+}$. Because acetate is a permeating weak acid (similar to benzoate

Table 2. Effect of fasting on the respiration rate of intact cells of *Acinetobacter* sp. 1NG grown in various media in the presence of C_2 substrates

Medium	Duration of fasting, h	Respiration rate, nmol O_2/min per mg cells		
		ethanol	acetaldehyde	potassium acetate
K^+, Na^+ medium	1	149.9	150.3	103.5
	20	152.6	153.5	25.2
K^+ medium (medium A)	1	153.4	157.2	129.3
	20	157.8	159.4	63.5

Note: The concentrations of ethanol and calcium pantothenate in the medium amounted to 1.0% and 0.0009%, respectively; 0.05 M K^+ phosphate buffer (pH 7.0) was used for washing, fasting, and incubation of the cells in the course of respiration rate measurements.

Table 3. Effect of conditions of culturing of *Acinetobacter* sp. 1NG on the activity of the key enzymes of ethanol metabolism

Medium	Calcium pantothenate, %	Mg^{2+}, mM	Ethanol, vol %	Activity, nmol/min per mg protein			
				NAD^+-dependent alcohol dehydrogenase	$NAD^+/NADP^+$-dependent aldehyde dehydrogenase	acetyl-CoA synthetase	isocitrate lyase
K^+, Na^+ medium	0.0006	1.6	1.0	365.7	373.2	74.5	50.5
K^+ medium (medium A)	0.0006	1.6	1.0	354.8	367.3	95.0	98.4
	0.0009	1.6	1.0	349.9	356.7	130.9	130.0
	0.0009	5.0	1.0	359.6	349.7	180.9	185.4
	0.0012	1.6	1.0	353.9	368.4	177.9	183.4
	0.0009	1.6	0.5	265.9	277.9	219.8	225.4
	0.0006	1.6	0.5	279.4	285.7	225.3	230.7

Table 4. Effect of exogenous potassium acetate on the growth of *Acinetobacter* sp. 1NG in medium containing 1.0% ethanol and 0.0009% calcium pantothenate

Concentration of potassium acetate in the medium, mM	pH		Biomass, g/l		Content of acetate in culture liquid after 24 h
	prior to addition of acetate	24 h after addition of acetate	prior to addition of acetate	24 h after addition of acetate	
0	5.5	5.7	0.4	0.7	0
	6.75	6.9	0.35	1.35	0
6	5.5	5.55	0.38	0.48	5.85
	6.75	7.1	0.42	1.40	0
12	5.5	5.5	0.4	0.45	11.8
	6.75	7.25	0.39	1.45	0
24	5.5	5.5	0.42	0.45	22.9
	6.75	7.45	0.45	1.48	0
36	5.5	5.5	0.37	0.4	35.2
	6.75	7.7	0.43	1.35	2.3
48	5.5	5.5	0.43	0.45	46.8
	6.75	8.0	0.4	1.4	5.9

Note: The pH of the culture liquid was adjusted to 5.5 by 6% HCl.

and salicylate), it crosses the bacterial membrane in a protonated form and changes ΔpH.

Our experiments demonstrated that exogenous potassium acetate added into the ethanol-containing medium during the stage of exponential growth of *Acinetobacter* sp. 1NG cells was involved into the metabolism of the bacteria at pH 6.7–6.8, whereas no assimilation took place at pH 5.5. This conclusion was based on the observation that, 24 h after the introduction of potassium acetate into the medium, the pH was increased and no acetate could be detected in the culture liquid (Table 4). The study of the respiration rate of intact cells in the presence of C_2 substrates at varying pH demonstrated that acetate, unlike ethanol or acetaldehyde, is almost not oxidized by the cells at pH 5.5.

In the experiments that followed, we aimed at identifying factors due to which acetate formation and its subsequent metabolism in the cells of *Acinetobacter* sp. grown in the presence of ethanol occurred at the same rates. We used two criteria for assessing the status of acetate metabolism in the bacteria: (1) the rate of respiration of intact cells in the presence of C_2 substrates (specifically, the rate of acetate oxidation by the cells after long-term fasting) and (2) the activity of alcohol dehydrogenase, aldehyde dehydrogenase, and acetyl-CoA synthetase in cell-free extracts. It should be noted that each criterion has drawbacks of its own. For example, the rate of respiration in the presence of substrates may not be an adequate indication of real metabolic processes occurring in cultured bacteria, because of the effects of substrate transportation into bacterial cells

Table 5. Effect of cations on the activity of acetyl-CoA synthetase in the cell-free extract of *Acinetobacter* sp. 1NG and the rate of respiration of intact cells in the presence of ethanol, acetate, and succinate

Cation	Cation concentration, mM	Respiration rate, nmol O_2/min per mg cells			Activity of acetyl-CoA synthetase, nmol/min per mg protein
		ethanol	potassium acetate	potassium succinate	
Control	0	78.5	40.6	63.5	43.9 (62.3)
K^+	10	76.9	42.4	62.4	90.0
	25	78.3	45.8	64.0	93.2
	50	77.5	52.2	65.3	98.5
	75	79.4	59.7	64.8	103.7
	100	80.5	66.8	65.9	118.9
Mg^{2+}	2	77.9	49.2	60.3	62.3
	5	78.3	56.9	58.7	80.4
	7	79.5	60.2	52.4	87.2
	10	76.4	67.3	39.2	90.5 (130.9)

Note: The bacteria were grown in medium A containing 1.0% ethanol and 0.0009% calcium pantothenate. The respiration rate of the cells and the activity of acetyl-CoA synthetase were measured in 0.05 M Tris–HCl (pH 7.0 and 7.4, respectively). Figures in parentheses designate values measured in 0.05 M K^+–phosphate buffer (pH 7.4). The cells were washed with 0.05 M Tris–HCl (pH 7.0). The duration of fasting was 1 h. In experiments studying the effects of K^+ on the activity of acetyl-CoA synthetase, the concentration of Mg^{2+} in the reaction mixture was equal to 10 mM.

(particularly in cases of active $\Delta\mu_{H^+}$–dependent transport of substrates, which may be influenced by the buffer composition, pH, and other factors). Similarly, the enzymatic activities in cell-free extracts may not necessarily correspond to the rate of the real process in intact cells (which is determined by other factors in addition to the content of enzymes, such as the substrate pool and the regulation of enzymes). Limitations and problems associated with interpretation of data generated in experiments with intact cells and cell-free extracts are dealt with in [12]. The authors note that the results of experiments with intact cells should be compared to the data generated with cell-free extracts and enzymes isolated from them. We assumed that the use of polarographic and enzymatic methods, taken together with data on cell growth, would give us a picture of the processes occurring in the cells that is very close to the real situation. Based on this understanding, we could then develop approaches to regulation of C_2 metabolism in *Acinetobacter* sp.

Evidence available in the literature indicates that acetyl-CoA synthetase is a magnesium-dependent enzyme [13, 14]. Potassium ions serve as activators of the enzyme in many prokaryotic and eukaryotic cells [13–18]. Our experiments demonstrated that K^+ and Mg^{2+} increased both the rate of acetate oxidation in intact cells of *Acinetobacter* sp. 1NG and the activity of acetyl-CoA synthetase in cell-free extracts (Table 5). In the absence of Mg^{2+}, the activity of acetyl-CoA synthetase decreased twofold, but introduction of 100 mM K^+ into a reaction mixture containing 10 mM Mg^+ increased this parameter by 30–35%. The activity of acetyl-CoA synthetase was higher when measured in K^+–phosphate buffer as compared to Tris–HCl. Of note, K^+ had virtually no effect on the rate of respiration of intact bacterial cells in the presence of ethanol or succinate. Magnesium (irrespective of its concentration) had no effect on the rate of respiration in the presence of ethanol; conversely, the rate of respiration in the presence of succinate was decreased at Mg^{2+} concentrations in excess of 5 mM (Table 5).

We reported previously [2] that the activity of acetyl-CoA synthetase decreased 1.3–2 times (as well as the rate of potassium acetate oxidation by intact cells) in the presence of 25–100 mM Na^+. At the same concentrations, NH_4^+ affected neither the respiration rate in the presence of acetate nor the activity of the enzyme in the cell-free extract.

Data of our experiments (Table 5) led us to suggest that changes in the content of K^+ and Mg^{2+} in the medium may be a factor regulating acetate metabolism in the cells of *Acinetobacter* sp. 1NG. In making this assumption, we took into account the fact that the content of these cations in the culture liquid is not necessarily the same as their intracellular concentration. In the medium for *Acinetobacter* sp., K^+ is present at 100 mM (the concentration required for the synthesis of the acylated polysaccharide [19]) and the content of Mg^{2+} is 1.6 mM.

Our experiments (Table 6) demonstrated that, in the presence of potassium acetate, the rate of respiration of intact cells increased with the concentration of Mg^{2+}; i.e., it was higher in the cells from the medium with elevated Mg^{2+} (as compared to those from the standard medium A) and did not decrease after long-term fasting (a twofold decrease was observed under similar condi-

Table 6. Effects of carbon source and Mg^{2+} content in the culture medium on the respiration rate of intact *Acinetobacter* sp. 1NG cells in the presence of potassium acetate

Carbon source	K^+–phosphate buffer, M	Mg^{2+}, mM	Rate of respiration of fasting cells, nmol O_2/min per mg cells	
			1 h	20 h
Ethanol	0.05 (medium A)	1.6	129.3	63.5
		5.0	147.7	149.3
	0.025 (medium B)	1.6	127.9	41.0
		5.0	148.3	150.4
Potassium acetate	0.05 (medium A)	1.6	120.3	122.4

Note: The concentrations of ethanol, potassium acetate, and calcium pantothenate in the medium amounted, respectively, to 1.0, 1.6, and 0.0009%; 0.05 M K^+–phosphate buffer (pH 7.0) was used for washing, fasting, and incubation of the cells in the course of respiration rate measurements.

tions with cells from standard medium A). It is important that the increase in the content of Mg^{2+} in the medium allowed us to decrease by two times the capacity of the buffer (Table 6).

It is worth mentioning that, in *Acinetobacter* sp., acetate is not only an intermediate in ethanol metabolism, but also a growth substrate [1]. As the data of Table 6 demonstrate, fasting did not decrease the respiration rate in the presence of acetate, if the cells of *Acinetobacter* sp. 1NG were grown in acetate-containing medium. It is therefore likely that the decrease in the respiration rate, observed in the presence of acetate in the cells grown in the ethanol-containing medium, was due to inhibitory effects of metabolites of this growth-determining substrate (e.g., NADH) on the activity of acetyl-CoA synthetase.

The mechanism of the reaction catalyzed by acetyl-CoA synthetase has not been studied in sufficient detail. It is believed that the reaction has two stages, with acetyl-AMP (acetyladenylate) being the intermediate [20]:

(1) acetate + ATP $\rightleftharpoons$ acetyl-AMP + PP

(2) acetyl-AMP + CoA $\rightleftharpoons$ acetyl-CoA + AMP

Structural analogues of acetyl-AMP (AMP, NADH, NADPH, etc.) may inhibit the activity of acetyl-CoA synthetase, as shown in a series of prior reports [20–22].

Our experiments demonstrated that, in the presence of NADH and NAHPH (which are formed in the course of NAD^+- and $NADP^+$-dependent oxidation of ethanol and acetaldehyde), the activity of acetyl-CoA synthetase in the cell-free extract of *Acinetobacter* sp. 1NG decreased twofold (Fig. 1). Adenine, adenosine, and AMP inhibited the activity of the enzyme, as well.

In order to eliminate the inhibitory effect of the products of ethanol and acetaldehyde oxidation, we lowered the initial concentration of ethanol in the medium (with subsequent fractional additions made in the course of culturing). A twofold decrease in the concentration of ethanol in the medium increased the rate of cell respiration in the presence of acetate, which took almost the same value as the rates of ethanol or acetaldehyde oxidation (Table 7). Moreover, long-term fasting did not affect this parameter significantly. It is interesting to note that, when the concentration of ethanol in the medium was equal to 0.5%, the content of calcium pantothenate could be reduced from 0.0009 to 0.0006%, without the need for introducing additional Mg^{2+}. Moreover, under these conditions, the bacteria could be culture in medium B, which contains virtually no buffer (Table 7).

The decrease in ethanol content also intensified the growth of the bacteria: specifically, the duration of the lag was reduced, paralleled by an increase in the rate of substrate consumption and maximum specific growth rate (the time, during which this maximum was

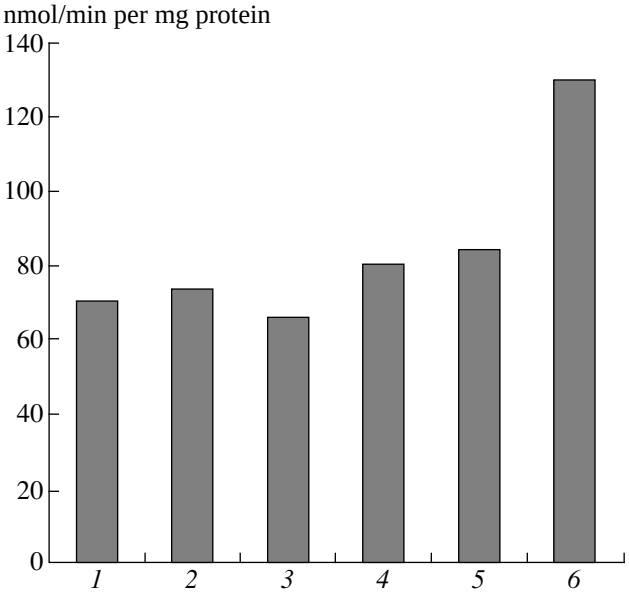

Fig. 1. Effects of structural analogues of acetyl-AMP on the activity of acetyl-CoA synthetase in the cell-free extract of *Acinetobacter* sp. 1NG cultured in medium A containing 1.0% ethanol: (*1*) NADH; (*2*) NADPH; (*3*) AMP; (*4*) adenosine; (*5*) adenine; (*6*) control.

Table 7. Dependence of maximum specific growth rate (μ_{max}) of *Acinetobacter* sp. 1NG culture and respiration rate of intact cells of the bacterium in the presence of C_2 substrates on the initial concentration of ethanol in the medium

Concentration of ethanol, %	K^+–phosphate buffer, M	μ_{max}, h^{-1}	Time of attainment of μ_{max}, h	Respiration rate, nmol O_2/min per mg cells		
				ethanol	acetaldehyde	potassium acetate
0.5	0.05 (medium A)	0.071	42	160.7	176.2	162.1 (140.0)
	0.015 (medium B)	N.D.	N.D.	154.3	158.1	147.9 (125.8)
1.0	0.05 (medium A)	0.045	54	153.4	157.2	129.3 (63.5)
	0.015 (medium B)	N.D.	N.D.	147.4	149.9	63.2 (17.4)

Note: The concentration of Mg^{2+} in the media was equal to 1.6 mM. The duration of cell fasting was 1 h. Figures in parentheses designate values of the cell respiration rate measured in the presence of potassium acetate after 20 h of fasting; 0.05 M K^+–phosphate buffer (pH 7.0) was used for washing, fasting, and incubation of the cells in the course of respiration rate measurements.

attained, and period of culturing, required for the production of maximum amounts of the biomass, decreased correspondingly; Fig. 2, Table 7).

Table 3 lists the values of activity of the key enzymes of ethanol metabolism in *Acinetobacter* sp. 1NG under different conditions of culturing. When the concentration of ethanol in the medium was equal to 1.0% (regardless of other changes in its composition), the activities of alcohol dehydrogenase and acetaldehyde dehydrogenase remained within the range of 340–370 nmol/min per mg protein. If such medium was devoid of sodium, the activity of acetyl-CoA synthetase reached the level of 95 nmol/min per mg protein. An increase in calcium pantothenate and/or Mg^{2+} increased the activity to 130–180 nmol/min per mg protein. When the concentration of ethanol in the medium was decreased to 0.5%, the activity of acetyl-CoA synthetase ranged from 220 to 230 nmol/min per mg protein. In the latter case, the activities of alcohol dehydrogenase and aldehyde dehydrogenase decreased to 260–280 nmol/min per mg protein. Table 3 contains data obtained with cells of *Acinetobacter* sp. 1NG grown in medium A. Culturing in medium B produced similar results.

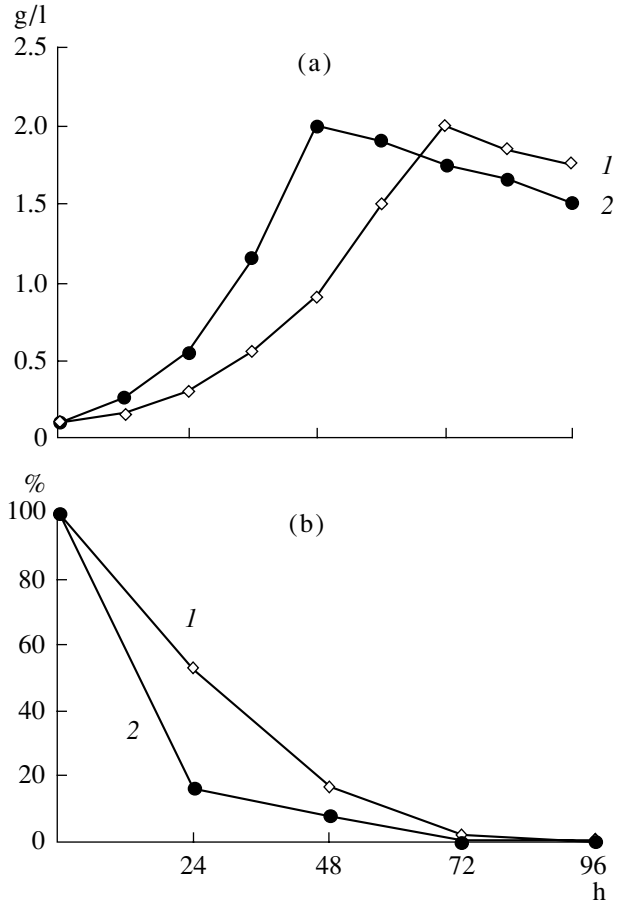

Fig. 2. Effect of ethanol, present in the culture medium (medium A) at an initial concentration of (*1*) 1.0% or (*2*) 0.5%, on (a) the accumulation of *Acinetobacter* sp. 1NG biomass and (b) the consumption of the substrate.

Thus, we succeeded in selecting conditions of *Acinetobacter* sp. 1NG culturing under which the activity of acetyl-CoA synthetase increased three times, maintaining almost equal rates of formation and metabolism of acetate in cells grown in the presence of ethanol. Of interest, changes in the activity of isocitrate lyase, which occurred on modifying the conditions of culturing, were similar to those of acetyl-CoA synthetase. It is conceivable that acetyl-CoA, originating from C_2 compounds, acts as an inducer of isocitrate lyase in the bacterial cells.

Finally, we checked whether the patterns revealed in experiments with the EPS mutant (*Acinetobacter* sp. 1NG) are reproduced in the original, EPS-producing strain cultured under the conditions selected (Table 8). Our experiments demonstrated that, with both strains of *Acinetobacter* sp., the drop in pH and the accumulation of acetate in the culture liquid were prevented by decreasing the concentration of ethanol to 0.5%. This allowed culturing the bacteria in buffer-deficient media. Under the conditions selected, the amount of EPSs increased by 20–25%, which corresponded to a yield increment of 1.5 times (relative to the amount of biomass); moreover, the time of culturing was reduced.

Table 8. Production of exopolysaccharides (EPSs) by *Acinetobacter* sp. 12S under various conditions of culturing

Ethanol, %	Calcium pantothenate, %	Buffer capacity, M	pH_{fin}	EPS, g/l	EPS yield, g per g biomass	Duration, h
0.5	0.0006	0.05	6.95	5.10	3.47	72
	0.0006	0.015	6.85	5.04	3.32	72
1.0	0.0009	0.05	6.90	4.30	2.40	96
	0.0009	0.015	6.80	4.40	2.35	96

Note: When ethanol was present at a concentration of 0.5%, its addition equivalent to 0.5% was made in the middle of the exponential growth phase. The bacteria were cultured in media A (buffer capacity, 0.05 M) or B (buffer capacity, 0.015 M) in the presence of 1.6 mM Mg^{2+}.

Thus, our results demonstrate that acetyl-CoA synthetase catalyzes a rate-limiting reaction in *Acinetobacter* sp. 1NG; the enzyme is inhibited by Na^+ and products of ethanol and acetaldehyde oxidation (NADH and NADPH). Similar inhibitory effects are exerted by adenine, adenosine, and AMP, suggesting that the reaction involves two stages, with the formation of acetyl-AMP as an intermediate. Pantothenic acid, Mg^{2+}, and K^+ activate the enzyme.

Conditions under which the oxidation rates of ethanol, acetaldehyde, and acetate in intact cells were nearly equivalent (and the activity of acetyl-CoA synthetase in the cell-free extract increased threefold) were achieved by (1) decreasing the initial concentration of ethanol from 1.0 to 0.5% (with subsequent introduction of 0.5% ethanol in the middle of the exponential phase of growth), (2) omitting Na^+, and (3) introducing 100 mM K^+. These changes in the composition of the medium allowed us to culture *Acinetobacter* sp. at a 3.4-fold lower buffer capacity than that characteristic of standard media. In order to achieve a similar result at 1.0% ethanol (in the absence of Na^+ and in the presence of 100 mM K^+), the content of pantothenic acid should be increased to 0.0009–0.0012% and that of Mg^{2+} to 5 mM. The study of the regulation of acetyl-CoA activity in the mutant strain *Acinetobacter* sp. 1NG (which fails to form EPSs) provides a basis for refining the technology of ethapolan production involving the use of ethanol-containing media.

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