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EXPERIMENTAL ARTICLES

Isolation and Phenotypic Characteristics of the *Escherichia coli* Butanol-Tolerant Mutants

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Abstract—Resistance to butanol is a key factor affecting microbial ability to produce economically profitable amounts of butanol. In this study, an *Escherichia coli* strain capable of growth in the presence of 1.5% butanol was isolated. The mutant MG1655 ButR was characterized by increased resistance to ethanol, isopropanol, and bivalent ions but exhibited supersensitivity to osmotic shock. Compared to the wild type strain, the butanol-tolerant mutant was more sensitive to antibiotics inhibiting protein synthesis but was more resistant to membrane-penetrating antibiotics, such as surfactin. Increased content of unsaturated fatty acids was found in the membranes of butanol-tolerant mutants. It was revealed that overexpression of the genes encoding cold-shock proteins decreased butanol tolerance of both mutant and the wild-type strain. It was concluded that butanol tolerance was associated with multiple rearrangements of the cell genetic system, rather than with single mutations.

Keywords: butanol tolerance, phenotypic properties, *Escherichia coli*.

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The steady cost increase of energy carriers together with the limited oil supply dictates great interest in biofuel production from renewable sources, including biomass. Butanol is one of the promising energy compounds. The natural butanol producer *Clostridium acetobutylicum* carries out acetone–butanol fermentation of carbohydrates in two stages: at the first stage, acetate and butyrate are accumulated; at the second stage, glucose utilization continues, and the produced acids are converted into organic solvents, viz., *n*-butanol, acetone, and ethanol [1, 2]. Although this two-stage process provides cells with ATP both during intensive growth and at the phase of preparing for sporulation, it is inconvenient for industrial microbial production of butanol and makes it unprofitable compared to butanol produced from oil stock. Moreover, *C. acetobutylicum* is a strict anaerobe with slow growth and low productivity. The insufficiently studied genetic system and physiology of *Clostridia* makes it difficult to modify the metabolic pathways involved in butanol production [3]. Thereupon, the construction of novel butanol producers from microorganisms for which genetic engineering tools have been elaborated seems to be very promising. It should be emphasized that butanol biosynthesis in microbial cells involves a sequence of completely reversible reactions. This is confirmed by our earlier data on the reversibility of butanol utilization in a genetically modified *E. coli* strain [4]. It has been recently shown that efficient butanol production by recombinant *E. coli* strains requires the construction of irreversible stages of this process, e.g., in the course of butyryl-CoA synthesis [5] or by removing the end product from the cultivation medium. In the latter case, butanol toxicity for the producer is somewhat less important; however, the construction of strains with high resistance to organic solvents remains an urgent task, particularly for *E. coli* strains which are very sensitive to relatively low concentrations of butanol (below 1%) [6].

The studies on toxicity of organic solvents showed that the cell membrane is the most sensitive target and an important element in stress adaptation. There is information on a number of *E. coli* mutants tolerant to various organic solvents (*n*-octanol, *n*-hexane, etc.), and some mechanisms of this resistance have been examined [7]. However, it is difficult to reconstitute the phenotypes of described strains since stress impact provokes a multigenic response [8], which hinders the construction of butanol-tolerant strains. It should be noted that hydrocarbons C_1–C_4 and C_4–C_9 with different hydrophobicity showed different effects on the biological membranes [9]. Short-chain alcohols caused stress by dehydration, whereas long-chain alcohols were embedded into the hydrophobic membrane [10, 11]. Moreover, numerous other important factors influence the viability of microbial cells in the course of biofuel synthesis [12]. For *E. coli*, the etha-

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Table 1. Strains and plasmids of *E. coli* used in the study

Strains and plasmids	Genotype	Source
MG1655	Wild-type strain	VKPM
MG1655 ButR	Butanol-tolerant mutant	This study
pINIII	Amp^R	Provided by K.V. Severinov
pINCspA	pINIII, bearing the gene *cspA*	
pINCspC	pINIII, bearing the gene *cspC*	
pINCspE	pINIII, bearing the gene *cspE*	

nol-caused stress is the best studied [13]; these results were used for the construction of ethanol-producing strains [14]. Recently, isobutanol-resistant mutants of *E. coli* which also exhibited increased *n*-butanol tolerance were obtained and investigated [15]. The studies on identification of the *E. coli* key genes involved in stress response to butanol impact were carried out [12]; however, these results are insufficient to elucidate the adaptation mechanisms emerging in the course of directional evolution of *E. coli* cells via the culture adaptation to increasing concentrations of butanol.

In the present work, we describe the method of directional evolution in butanol-containing media, which allowed us to obtain a mutant of strain *E coli* MG1655 capable of growth in the presence of 1.5% butanol and list the phenotypic characteristics of this mutant, such as its resistance to alcohols, osmotic shock, and antibiotics. We also studied lipid composition of the membranes of the butanol-tolerant mutant and investigated the effect of bivalent cations and overexpression of the cold-shock proteins on butanol tolerance of both the mutant and parent strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1.

Media and cultivation conditions. The cultures were grown aerobically at 37 and 30°C in LB medium containing (g/L): peptone, 10; yeast extract, 5; and NaCl, 5. Butanol-tolerant strains were also grown on solid LB medium supplemented with 2% agar. In some experiments, the cultures were grown under microaerobic conditions in hermetically sealed 15-mL tubes with 10 mL of the medium. Ethanol, isopropanol, and butanol were added in concentrations of 7, 5, and 0.25–2.25%, respectively.

Determination of butanol resistance of the stationary-phase *E. coli* cultures. Strains MG1655 and MG1655 ButR were grown in LB medium to the early stationary phase. The cell suspensions were then transferred into hermetically sealed test tubes (microaerobic conditions) with butanol in concentrations of 1, 1.25, 1.5, and 2%. The control tubes contained no butanol. The cultures were incubated overnight on a temperature-controlled shaker at 30°C. The viability of the cultures was determined from the number of colony-forming units (CFU/mL) on petri dishes with LB agar.

Determination of osmotic tolerance. Osmotic tolerance of strains MG1655 and MG1655 ButR was determined by measuring cell density (OD_{600}) of overnight cultures. The overnight cultures of the parent strain and butanol-tolerant mutant were grown at 37°C in 10 mL of LB medium supplemented with NaCl to the final concentration of 0.5, 0.75, or 1 M.

Antibiotic resistance assay. The antibiotic resistance of strains MG1655 and MG1655 ButR was determined by measuring the zones of growth inhibition in semiliquid LB medium containing 2 and 0.8% of agar. Antibiotics were applied to the filter paper disks in the following concentrations (μg): ampicillin, chloramphenicol, rifampicin, and apramycin, 30; neomycin and cycloserine, 50; surfactin, 12.5.

Analysis of fatty acid composition of the whole cells. Samples for analysis were prepared as described earlier [16]. Analysis was performed on an AT-5973B chromatograph–mass spectrometer (Agillent Technologies, United States). A quadrupole mass spectrometer with a mass range from 2 to 550 amu showed resolving power of 0.5 amu throughout the whole operating range; electron ionization at 70 eV; sensitivity corresponded to 0.01 ng of methyl stearate. Chromatography was performed on a fused quartz capillary column (25 m × 0.25 mm) packed with an HP-5-ms phase (0.2 μm) (Hewlett-Packard, United States). The programmed temperature range from 120 to 280°C was scanned at 5°C/min. The temperature of injector and interface was 280°C. The data were processed using the standard bundled software. Peaks of the compounds were identified using the NIST database of mass spectra.

Overexpression of the cold-shock proteins. Strains MG1655 and MG1655 ButR were transformed with plasmids pINCspA, pINCspC, and pINCspE carrying the genes *cspA, cspC*, and *cspE*, respectively, under the control of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible lac promoter. The overnight cul-

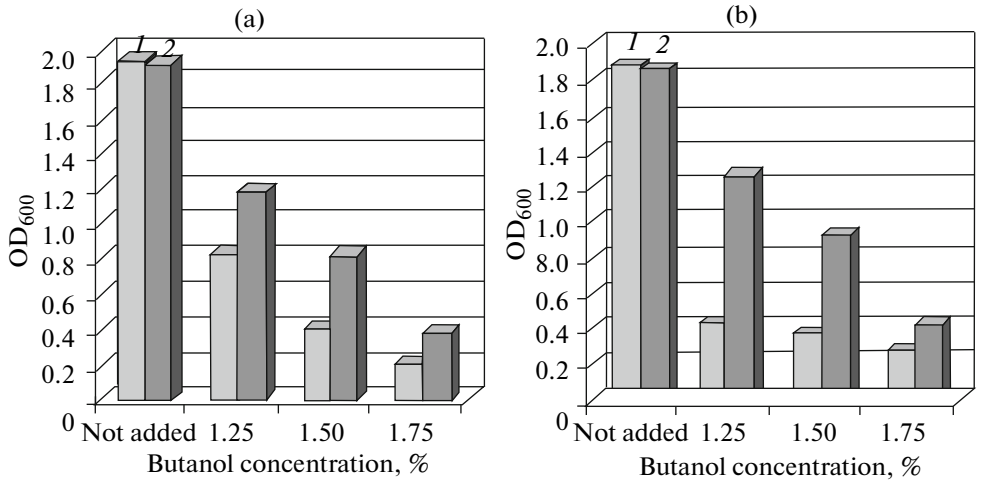

Fig. 1. Optical density of strains MG1655 (*1*) and MG1655 ButR (*2*) grown in LB medium with butanol for 24 h at 30°C (a) and 37°C (b).

tures of cells carrying plasmids pINCspA, pINCspC, and pINCspE were diluted 100-fold and grown at 30°C to OD_{600} of 0.3–0.4. The cell suspension was then plated on petri dishes with LB agar supplemented with ampicillin (50 μg/mL), butanol (0.75, 1, 1.5, and 2%), and IPTG (1 mM).

RESULTS AND DISCUSSION

Directional Microevolution of Strains in Butanol-Containing Media

The study was carried out with the strain *E. coli* MG1655, which was successively transferred into butanol-containing LB media at 37°C. In such a way, a mutant of strain MG1655 capable of growth in the medium containing 0.75% of butanol was obtained. Further selection of butanol-tolerant mutants was performed by a combination of liquid and solid media; after 5–6 successive transfers of the culture in liquid media, the cell suspension was plated onto butanol-containing solid medium and incubated overnight at 37°C. The most rapidly growing (larger) colonies were used for further selection.

The selection of the cultures tolerant to 1% butanol and higher was carried out at 30°C, since at these concentrations elevated temperature increased the toxicity of butanol. Finally, a mutant of strain MG1655 capable of growth in liquid medium with 1.25% butanol and on solid medium with 1.5% butanol was isolated. The ability of this mutant to grow in rich media with different butanol concentrations (1.25, 1.5, and 1.75%) at different temperatures (30 and 37°C) was determined (Fig. 1).

As can be seen from Fig. 1, the selected mutant MG1655 ButR exhibited higher tolerance to butanol than the parent strain, especially at 37°C.

Butanol Resistance of the Stationary-Phase E. coli Cultures

Bacterial cultures in the stationary phase are known to be more resistant to environmental impact [17]. The effect of butanol concentrations on the viability of the stationary-phase cultures of the mutant MG1655 ButR and the wild-type strain MG1655 was therefore studied. Both strains were grown in 100 mL of LB medium on a temperature-controlled shaker at 37°C to the early stationary phase. No difference in the growth rates of strains MG1655 and MG1655 ButR in rich medium was observed. Then the cell suspensions were transferred into hermetically sealed tubes containing 10 mL of the medium supplemented with butanol (1, 1.25, 1.5, and 2%) and incubated overnight under microaerobic conditions. The culture viability was determined by plating the cell suspension on agar medium and enumerating the grown colonies. The results are shown in Fig. 2.

In the stationary growth phase, the most stable membrane lipids (e.g., cardiolipin) are synthesized [17]; therefore, difference in cell viability between the wild-type strain and the butanol-tolerant mutant was low, albeit existent, probably resulting from some changes in the composition of the lipid and protein components of the membranes (Fig. 2). This observation is confirmed by the results shown in Fig. 3.

Mutant MG1655 ButR was distinguished by low cell aggregation. In the course of incubation in test tubes, the cells of the parent strain MG1655 aggregated and formed a precipitate, whereas the cells of the mutant MG1655 ButR remained in planktonic state. The loss of cell ability to aggregate observed in the mutant may be due to a change in the protein and lipid composition of the membranes and, possibly, is associated with increased cell tolerance to butanol.

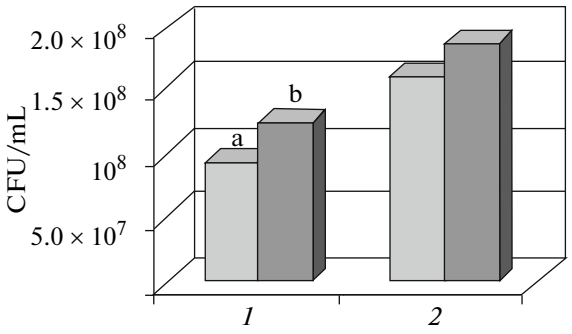

Fig. 2. Viability of the stationary-phase cultures MG1655 (*1*) and MG1655 ButR (*2*) incubated under microaerobic conditions in media containing 1.5% (a) and 1.25% (b) butanol.

Phenotypic Characteristics of the Mutant MG1655 ButR

Alcohol resistance. At the first stage of investigation, the resistance of the mutant MG1655 ButR to more hydrophilic alcohols (ethanol and isopropanol) was tested to evaluate the specificity of the mechanisms of tolerance acquired by the mutant in the course of selection. The mutant and parent strains were grown overnight in LB media containing different concentrations of ethanol or isopropanol. The resistance of strains to alcohols was assayed by measuring the OD_{600} values of overnight cultures. It was revealed that the mutant was more resistant to these alcohols than the wild-type strain (Table 2). Thus, the butanol-tolerant mutant was more resistant than the parent strain not only to butanol, but also to more hydrophilic alcohols.

Sensitivity to antibiotics. Assuming that the mechanism of alcohol resistance in the mutant MG1655 ButR was universal and probably associated with multiple drug resistance [7, 18, 19], we tested the sensitivity of the mutant to various groups of antibiotics, which were different both in the way of penetration into the cell and in the mechanisms of antibacterial action. Strains MG1655 and MG1655 ButR were grown on semisolid agar with antibiotics, and diameters of the growth inhibition zones were measured. The results are summarized in Table 2.

It was revealed that the butanol-tolerant mutant exhibited enhanced sensitivity to antibiotics of the β-lactam series and those inhibiting protein synthesis (chloramphenicol, rifampicin, and aminoglycosides) (Table 2). According to the literature data, increased cell sensitivity to antibiotics may be associated with different types of modifications in the structure of lipopolysaccharides (LPS); these modifications also impaired the association of porins (nonspecific carriers) and affected the functions of other membrane proteins, e.g., the system To1QR–To1B and PAL

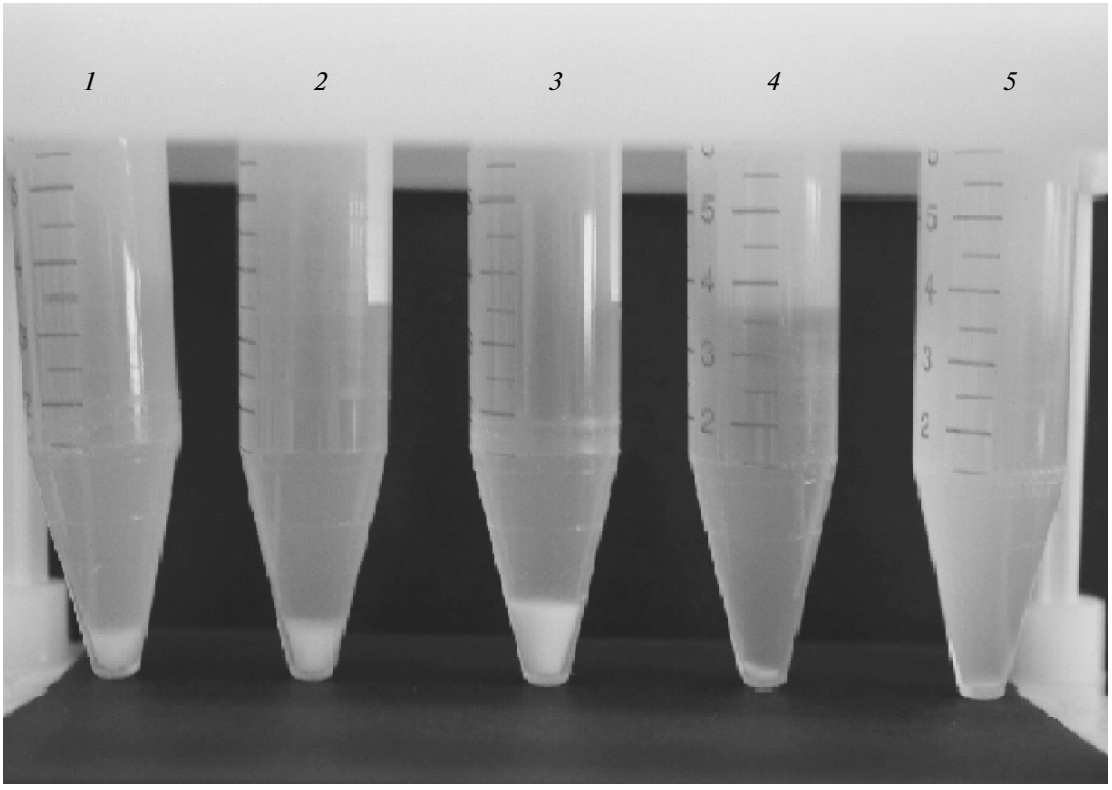

Fig. 3. Images of the stationary-phase cultures grown in LB medium: MG1655, without butanol (*1*); MG1655, with 1% butanol (*2*); MG1655, with 1.5% butanol (*3*); MG1655 ButR, without butanol (*4*); MG1655 ButR, with 1.5% butanol (*5*).

Table 2. Effect of various stress factors on the growth of the butanol-tolerant mutant and the parent strain of *E. coli* on rich media

Strains	MG1655	MG1655 ButR
Antibiotics (per disc)	Diameter of the growth inhibition zone, mm	
Ampicillin, 30 μg	20	25
Chloramphenicol, 30 μg	20	26
Rifampicin, 30 μg	15	17
Apramycin, 30 μg	12	16
Neomycin, 50 μg	8	19
Cycloserine, 50 μg	16	12
Surfactin, 12.5 μg	14	0
Oxidative stress (per disc)	Diameter of the growth inhibition zone, mm	
3% H_2O_2 5 μL	15	15
Paraquat, 50 μg	10	12
Osmotic stress	OD_{600} of overnight cultures	
0.5 M NaCl	1.80	1.54
0.75 M NaCl	1.56	0.475
1 M NaCl	1.496	0.035
Effect of alcohols	OD_{600} of overnight cultures	
7% Ethanol	0.254	1.015
5% Isopropanol	0.305	1.709

which is involved in stabilization of the peptidoglycan–outer membrane complex [20]. However, the mutant MG1655 ButR showed enhanced resistance to antibiotics, which impaired the synthesis of lipids (cycloserine) and disorganized membranes (surfactin) (Table 2).

Oxidative stress. Oxidative stress is one of the factors responsible for the damaging action of organic solvents including butanol. *E. coli* has two major systems regulating cell response to oxidative stress: *oxyR* and *soxRS* regulons. The OxyR protein is a positive transcriptional regulator; its activity can be induced by H_2O_2. The SoxR and SoxS proteins are transcriptional activators of the *soxRS* regulon genes. Gene expression of these proteins is induced by paraquat. According to the literature data, mutations in the genes *soxS* and *soxR* changed the sensitivity to antibiotics (ampicillin, chloramphenicol, tetracycline, etc.). Moreover, the product encoded by the gene *soxR* affected the synthesis of the OmpF membrane protein (nonspecific carrier of small molecules) [21].

Strains MG1655 and MG1655 ButR showed similar sensitivity to the stress caused by H_2O_2. At the same time, the mutant MG1655 ButR was slightly more sensitive to paraquat than the parent strain (Table 2).

Sensitivity to osmotic shock. Osmotic resistance of strain MG1655 and the mutant MG1655 ButR was determined by comparing the OD_{600} values of overnight cultures grown under high osmotic pressure of the media. The cultures were grown at 37°C in LB medium with 0.5–1.0 M NaCl. The viability of the mutant MG1655 ButR decreased sharply under hyperosmotic impact, compared to the parent strain (Table 2). Such enhanced sensitivity may be associated with a change in the amount of nonspecific transmembrane carriers (porins), which in turn may result from considerable rearrangements in the LPS structure of butanol-tolerant mutants, as well as from the changes in the regulation of expression of the membrane protein genes.

Differences in Lipid Composition of the Membranes of Strains MG1655 ButR and MG1655

To determine the possible changes in the membrane structures, the lipid composition of the MG1655 and MG1655 ButR cells was analyzed (Table 3). Fatty acids (FA) of the mutant MG1655 ButR differed from those of the parent strain by the presence of 3-hydroxytridecanoic acid in the former, which probably reflects the changes in the structure of lipid A in the LPS [22–24], as well as by the presence of the other atypical fatty acids including tridecanoic acid and two isomers of pentadecenoic acid (in Table 3, these acids are marked in bold). The mutant was distinguished by increased levels of pentadecanoic, 9-hexadecenoic, heptadecanoic, and 11-octadecenoic acids and decreased amounts of 17- and 19-cyclopropanoic acids (17cyc and 19cyc), as well as of saturated FA with 16 and 18 carbon atoms, palmitic (16:0) and stearic (18:0) acids. Thus, the cells and, consequently, the membranes of the mutant MG1655

Table 3. Fatty acid composition of the cells of strains MG1655 and MG1655 ButR (% of the total FA)

Fatty acid	Symbol	MG1655	MG1655 ButR
Dodecanoic	12:0	1.241	1.329
Tridecanoic	13:0		0.412
Tetradecenoic	14:1	0.188	0.134
Tetradecanoic	14:0	6.212	5.056
7-Pentadecenoic	15:1w8		0.248
9-Pentadecenoic	15:1w6		0.091
Pentadecanoic	15:0	1.966	6.405
3-Hydroxytridecanoic	13:0 3OH		0.224
7-Hexadecenoic	16:1w9	0.165	0.166
9-Hexadecenoic	16:1w7	6.889	12.07
11-Hexadecenoic	16:1w5	0.244	0.345
Hexadecanoic	16:0	37.632	30.167
3-Hydroxytetradecanoic	14:0 3OH	3.249	3.298
Cycloheptadecanoic	17 cyc	24.905	19.259
Heptadecanoic	17:0	1.299	3.672
11-Octadecenoic	18:1w7	6.616	13.356
Octadecanoic	18:0	2.994	0.923
Cyclononadecanoic	19 cyc	6.355	2.843
Total FA		100	99.998

ButR had higher content of unsaturated FA than the wild-type strain MG1655 (26.4 and 14.1%, respectively) (Table 4).

Since there is information in the literature concerning the *E. coli* strains tolerant to organic solvents in which the ratio of unsaturated/saturated fatty acids was changed in favor of the saturated ones [14], the inverse relationship observed in our mutant was surprising. It should be noted that the membranes in which unsaturated fatty acids prevail are more elastic and resistant to mechanical impact, e.g., to stretch, whereas an increase in the amount of saturated fatty acids results in increased membrane rigidity and fragility leading to decreased cell viability.

The Effect of Ca^{2+} Ions on Butanol Resistance

Bivalent cations are known to form bridges between neighboring phospholipids, thus increasing the membrane rigidity [20]. Therefore, the effect of Ca^{2+} concentration on butanol resistance of the mutant and parent strains was studied. The strains were incubated overnight in LB medium with 1.25% butanol supplemented with 2 and 12 mM of $CaCl_2$ (Fig. 4). It was revealed that the addition of Ca^{2+} ions increased cell resistance to butanol, especially in the case of the butanol-tolerant mutant.

The Effect of Overexpression of the Cold-Shock Proteins on Butanol Resistance

It is known that the *E. coli* proteins of the CspA family are RNA chaperons which unwind the hairpin structures at low temperature functioning as transcriptional antiterminators [25]. The overexpression of genes encoding the cold-shock proteins was shown to increase resistance of *E. coli* cells to stressful environmental impact, e.g., to ethanol [20]. To study the effect of overexpression of the proteins belonging to the CspA family on butanol resistance, the cells of strains MG1655 and MG1655 ButR were transformed with the plasmids pINCspA, pINCspC, and pINCspE, in which the genes encoding the cold-shock proteins were under the control of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter [25].

The effect of overexpression of the genes encoding the cold-shock proteins on butanol resistance of strains MG1655 and MG1655 ButR is shown on Fig. 5. The activation of expression of the genes

Table 4. Comparative lipid composition of strain MG1655 and its mutant MG1655 ButR (% of the total FA)

Strain	Saturated	Unsaturated
MG1655	51.3	14.1
MG1655 ButR	47.8	26.4

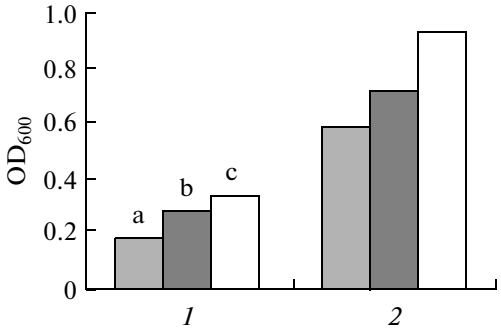

Fig. 4. Effect of Ca^{2+} ions on butanol resistance of the parent strain MG1655 (*1*) and the mutant MG1655 ButR (*2*). The cultures were grown in LB medium containing 1.25% butanol without $CaCl_2$ (a) and with addition of $CaCl_2$: 2 mM (b) or 12 mM (c).

encoding proteins of the CspA family resulted in decreased butanol resistance in both the parent strain and the butanol-tolerant mutant MG1655 ButR that is especially clearly defined in the case of proteins CspE and CspC (Fig. 5b *1*, *1'* and *2*, *2'*, respectively). Thus, the effect of overexpression of the cold-shock genes on bacterial resistance to butanol was diametrically opposite to that observed in the case of ethanol, indicating that considerably different mechanisms are probably involved in bacterial resistance to these alcohols.

The obtained results allow us to conclude that the isolated butanol-tolerant mutant MG1655 ButR exhibits a number of phenotypic characteristics (resistance to ethanol and isopropanol, the absence of cell aggregation in butanol-containing medium, resistance to surfactin, changed fatty acid composition, and hypersensitivity to osmotic shock) which are indicative of the structural rearrangements in the cell membranes including both the protein and the lipid components. According to the literature data, butanol had considerable effect on the growth of *E. coli* strains via different mechanisms, such as an increase in the membrane fluidity, imbalance of electrolytes, a change in fatty acid composition, and modulation of synthetic pathways [5]. The applied selection of *E. coli* strains based on resistance to increasing concentrations of butanol allowed us to conclude that butanol tolerance was associated with multiple rearrangements of the cell genetic system rather than with single mutations. Detailed analysis of the microevolution of protective mechanisms responsible for butanol resistance in *E. coli* cells will make it possible to rationalize the construction of butanol-producing strains on the basis of *E. coli*.

ACKNOWLEDGMENTS

We are grateful to K.V. Severinov for providing the plasmids bearing the cold-shock genes.

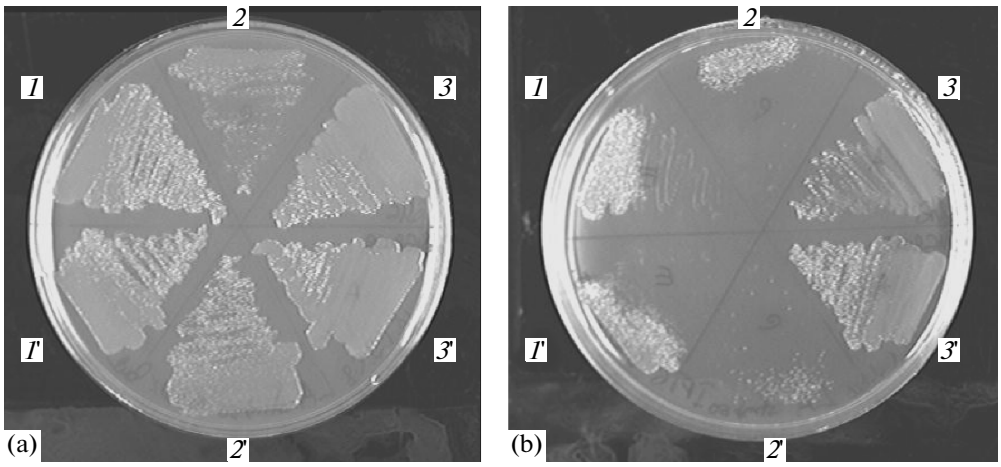

Fig. 5. Effect of overexpression of the proteins CspE, CspC, and CspA without induction (a) or with induction (b) on resistance to butanol (0.75%) in strains MG1655/pIN CspE (*1*), MG1655/pIN CspC (*2*), MG1655/pIN CspA (*3*), MG1655 ButR/pIN CspE (*1'*), MG1655 ButR/pIN CspC (*2'*), and MG1655 ButR/pIN CspA (*3'*).

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