Microbiology, Vol. 71, No. 2, 2002, pp. 139–142. Translated from Mikrobiologiya, Vol. 71, No. 2, 2002, pp. 166–170.

EXPERIMENTAL ARTICLES

The Role of Low-Molecular-Weight Nitrogen Compounds in the Osmotolerance of *Rhodococcus erythropolis* and *Arthrobacter globiformis*

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Received January 30, 2001; in final form, March 22, 2001

Abstract—Investigations showed that *Rhodococcus erythropolis* E-15 and *Arthrobacter globiformis* 2F cells respond to osmotic shock by increasing the synthesis of free amino acids, primarily glutamic acid (80% of the intracellular free amino acid pool). The osmoprotective role of glutamic acid follows from its beneficial effect on the growth of bacteria in high-salinity media. It was found that the addition of this amino acid to the growth medium at a concentration of 2 mM shortened the lag phase and increased the growth rate and biomass yield of either of the two bacteria. The addition of another osmoprotectant, trehalose, to the high-salinity growth medium of *R. erythropolis* E-15 at the same concentration (2 mM), restored the growth parameters of this bacterium to the control values.

Key words: hydrocarbon-oxidizing bacteria, osmoprotectant, osmotic shock, glutamic acid.

Our investigations of the hydrocarbon-oxidizing bacterium *Rhodococcus erythropolis* showed that it responds to osmotic shock by increasing the synthesis of the disaccharide trehalose [1]. There is evidence that low-molecular-weight nitrogen-containing compounds, amino acids and glycine betaine in particular, may also perform an osmoprotective function in rhodococci [2]. To the best of our knowledge, there have been no attempts to study another hydrocarbon-oxidizing bacterium, *Arthrobacter globiformis*, in this respect.

The aim of the present work was to study the role of low-molecular-weight nitrogen-containing compounds in the osmotolerance of the bacteria *R. erythropolis* E-15 and *A. globiformis* 2F utilizing *n*-alkanes as the sole source of carbon and energy.

MATERIALS AND METHODS

The strains *Rhodococcus erythropolis* E-15 and *Arthrobacter globiformis* 2F used in this work were obtained from the collection of microorganisms at the Department of Hydrobiology, Faculty of Biology, Moscow State University.

The strains were grown at 24°C on a shaker (180 rpm) in 750-ml flasks with 100 ml of either a modified Czapek medium (*A. globiformis* 2F) or Munts medium (*R. erythropolis* E-15) containing 1% liquid paraffin (a mixture of C_{12}–C_{19} *n*-alkanes) as the source of carbon and energy. The modified Czapek medium contained (g/l) KCl, 0.5; $MgSO_4$, 0.5; K_2HPO_4, 1.0; $(NH_4)_2SO_4$, 2.0; $FeSO_4$, 0.01; and $CaCO_3$, 3.0 in tap water. Munts medium contained (g/l) KNO_3, 5.0; $MgSO_4$, 0.5; $MnSO_4$, 0.1; $FeSO_4$, 0.01; KH_2PO_4, 0.6; Na_2HPO_4, 1.15 in tap water. When Munts medium was not supplemented with NaCl, it contained about 0.01 M Na^+ ions. Czapek medium did not contain Na^+ ions at all. The media were inoculated with cells (3 mg by dry weight) washed off from Czapek agar.

Bacterial growth was monitored by determining the dry weight of the biomass. The chalk present in Czapek medium was preliminarily dissolved by acidifying the medium with HCl to pH about 5, and cells were washed with hexane to remove the residual paraffin. Growth curves were constructed from the average results of triplicate measurements.

Free amino acids were extracted from bacterial cells with 70% ethanol at room temperature [3] and analyzed using a Biotronic LC-5001 amino acid analyzer (Germany). The results presented in the paper are the means of duplicate measurements. Glycine betaine was detected in the amino acid extract with Dragendorff reagent [4].

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0026-2617/02/7102-0139$27.00

Table 1. The effect of high salt concentration on the free amino acid pool of *R. erythropolis* E-15 cells grown in Munts medium with liquid paraffin as the carbon and energy source

Amino acid	Standard Munts medium (no NaCl)		2-h incubation in Munts medium with 5% NaCl		Growth in Munts medium with 5% NaCl	
	mg/g cells	% of the total	mg/g cells	% of the total	mg/g cells	% of the total
Glutamic acid	20.19	80.56	26.97	77.56	35.14	75.20
Lysine	1.71	6.82	4.23	12.17	3.09	6.61
Asparagine	1.19	4.75	1.06	3.05	1.90	4.07
Methionine	0.47	1.88	0.32	0.92	0.27	0.58
Threonine	0.44	1.75	0.64	1.84	2.74	5.86
Alanine	0.32	1.28	0.32	0.92	1.04	2.23
Ornithine	0.19	0.77	0.58	1.67	1.16	2.48
Valine	0.11	0.43	0.18	0.52	0.36	0.77
Minor amino acids	0.44	1.76	0.48	1.35	1.03	2.20
Total amino acids	25.06	100	34.77	100	46.73	100

Table 2. The effect of glutamic acid and trehalose on the growth parameters of *R. erythropolis* E-15 and *A. globiformis* 2F in media with various salinity values

Bacterium	Medium	Lag phase, days	Specific growth rate (μ), h^{-1}	Biomass yield, g dry cells/l
Rhodococcus erythropolis E-15	Munts medium (control)	<0.5	0.020	2.79
	Munts medium with 2 mM glutamic acid	<0.5	0.022	3.49
	Munts medium with 5% NaCl	7	0.015	1.88
	Munts medium with 5% NaCl and 2 mM glutamic acid	4	0.021	3.19
	Munts medium with 5% NaCl and 2 mM trehalose	<0.5	0.017	2.64
Arthrobacter globiformis 2F	Czapek medium	<0.5	0.025	4.87
	Czapek medium with 2 mM glutamic acid	<0.5	0.027	5.65
	Czapek medium with 5% NaCl	5	0.013	2.06
	Czapek medium with 5% NaCl and 2 mM glutamic acid	1	0.023	4.4

RESULTS

According to Kushner [5], both *R. erythropolis* and *A. globiformis* are moderately halotolerant bacteria, so that 7% NaCl present in the medium can completely inhibit their growth. In our experiments, NaCl was used at a concentration of 5%, which still allows the bacteria to grow.

The bacterium *R. erythropolis* E-15 was cultivated under the following growth conditions: (1) cells were grown in standard Munts medium without adding NaCl (control); (2) cells grown in standard Munts medium were transferred to Munts medium containing 5% NaCl and incubated for 2 h (osmotic shock); and (3) cells were from the outset grown in Munts medium supplemented with 5% NaCl (osmotic stress).

Analysis showed that the intracellular free amino acids of the control *R. erythropolis* E-15 cells were dominated by glutamic acid, which amounted to more than 80% of the total free amino acid pool (Table 1). The second amino acid, in order of decreasing intracellular content, was lysine (about 7% of the total). Osmotic shock led to an increase in the content of glutamic acid and lysine by 33 and 148%, respectively. In this case, the fraction of glutamic acid in the free amino acid pool of cells slightly decreased, whereas that of lysine increased almost twofold. Osmotic stress led to an increase in the intracellular content of glutamic acid, lysine, asparagine, and threonine by 74, 80, 60, and 519%, respectively. The total amount of free amino acids in the cells exposed to osmotic shock and osmotic stress increased by 38 and 86%, respectively.

In all three experimental variants (control, osmotic shock, and osmotic stress), the total fraction of the minor amino acids serine, glycine, cysteine, isoleucine, leucine, tyrosine, phenylalanine, histidine, and arginine did not exceed 2% of the free amino acid pool of *R. erythropolis* E-15 cells. Proline and glycine betaine were not revealed.

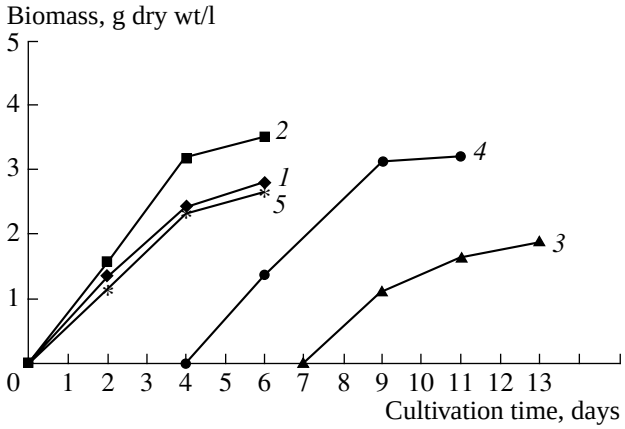

Fig. 1. The protective effect of trehalose and glutamic acid on the growth of *R. erythropolis* E-15 in paraffin-containing Munts medium: (*1*) Munts medium (control); (*2*) Munts medium with 2 mM glutamic acid; (*3*) Munts medium with 5% NaCl; (*4*) Munts medium with 5% NaCl and 2 mM glutamic acid; and (*5*) Munts medium with 5% NaCl and 2 mM trehalose.

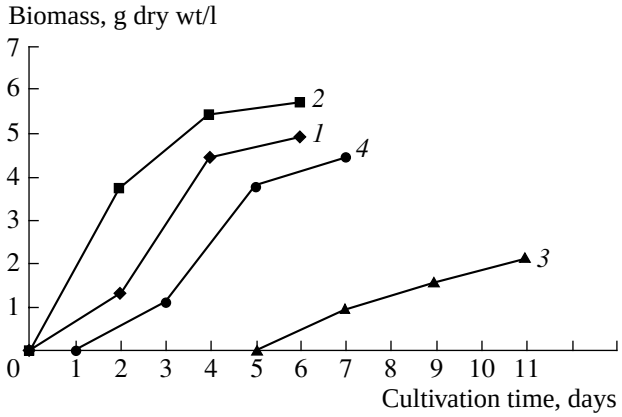

Fig. 2. The protective effect of glutamic acid on the growth of *A. globiformis* 2F in paraffin-containing Czapek medium: (*1*) Czapek medium (control); (*2*) Czapek medium with 2 mM glutamic acid; (*3*) Czapek medium with 5% NaCl; and (*4*) Czapek medium with 5% NaCl and 2 mM glutamic acid.

The bacterium *R. erythropolis* E-15 exposed to high salt concentration exhibited an extended (by 7 days) lag phase and decreased specific growth rate and biomass yield (Fig. 1 and Table 2). The addition of glutamic acid to the growth medium at a concentration of 2 mM restored the specific growth rate and the biomass yield to the control values, leaving, however, the lag phase increased by 4 days. It should be noted that glutamic acid also enhanced the biomass yield of the control *R. erythropolis* E-15 culture. In accordance with the osmoprotective role of trehalose established in our earlier study [1], the addition of this disaccharide to the growth medium of *R. erythropolis* E-15 at a concentration of 2 mM almost completely removed the inhibitory effect of 5% NaCl on the growth of this bacterium.

As in *R. erythropolis* E-15 cells, glutamic acid was found to be the dominant amino acid in *A. globiformis* 2F cells grown in all three experimental variants, comprising more than 80% of the total free amino acid pool (Table 3). Arthrobacter cells contained cysteic acid, which was absent from rhodococci. The minor amino acids serine, glycine, valine, methionine, isoleucine, leucine, histidine, arginine, and ornithine amounted to 3–5% of the total free amino acid pool. The effects of osmotic shock and osmotic stress on the free amino acids of arthrobacters were similar to their effects in the case of rhodococci: the intracellular content of all amino acids, including dominant ones (glutamic acid, asparagine, and threonine) increased in response to high salt concentration. In addition, a considerable increase in the content of lysine was observed.

Table 3. The effect of high salt concentration on the free amino acid pool of *A. globiformis* 2F cells grown in Czapek medium with liquid paraffin as the carbon and energy source

Amino acid	Czapek medium without NaCl		2-h incubation in Czapek medium with 5% NaCl		Growth in Czapek medium with 5% NaCl	
	mg/g cells	% of the total	mg/g cells	% of the total	mg/g cells	% of the total
Glutamic acid	13.80	80.32	16.67	81.40	25.58	80.84
Threonine	0.82	4.77	1.01	4.93	1.89	5.97
Cysteic acid	0.51	2.97	0.27	1.32	0.55	1.74
Asparagine	0.46	2.68	0.64	3.13	1.22	3.86
Alanine	0.25	1.46	0.24	1.17	0.36	1.14
Tyrosine	0.22	1.28	0.13	0.63	0.26	0.82
Lysine	0.18	1.05	0.66	3.22	0.40	1.26
Cysteine	0.12	0.70	0.22	1.07	0.35	1.11
Minor amino acids	0.82	4.77	0.64	3.13	1.03	3.26
Total amino acids	17.18	100	20.48	100	31.64	100

Both osmotic shock and osmotic stress lengthened the lag phase of *A. globiformis* 2F and decreased its specific growth rate and biomass yield by about two times (Fig. 2 and Table 2). The addition of glutamic acid to the growth medium at a concentration of 2 mM restored all growth parameters to the control values.

DISCUSSION

The enhanced biosynthesis of low-molecular-weight nitrogen-containing compounds, amino acids and glycine betaine in particular, is one of the most widespread mechanisms of defense against osmotic stress in prokaryotes [2, 5]. The present investigation showed the functioning of the same osmoprotective mechanism in the bacteria *R. erythropolis* E-15 and *A. globiformis* 2F grown on paraffin. It was found that the free amino acid pool in these bacteria grown at high salinities increased almost twofold. In agreement with the observations that the free amino acids of hydrocarbon-oxidizing mycobacterial cells are dominated by glutamic acid [6, 7], the content of this amino acid in *R. erythropolis* E-15 and *A. globiformis* 2F cells grown on paraffin was found to be high, comprising from 20 to 35 mg/g cells. The fraction of glutamic acid in the intracellular pool of free amino acids in arthrobacters and rhodococci did not change in response to osmotic shock and osmotic stress. We failed to reveal proline and glycine betaine in these bacteria, although some researchers believe that these compounds perform an osmoprotective function in hydrocarbon-oxidizing bacteria [2, 3].

When added exogenously, glutamic acid and trehalose restored the specific growth rate and biomass yield of *R. erythropolis* E-15 exposed to high salt concentration to the control values and shortened the lag phase either completely (trehalose) or to 40% of its duration in the control cells (glutamic acid).

Trehalose is an essential component of the glycolipids and mono- and dimycolates that constitute the cell wall of rhodococci [8]. In response to osmotic stress, *R. erythropolis* cells enhance the synthesis of trehalose and amino acids, glutamic acid in particular, in order to increase intracellular osmotic pressure. Lacking trehalose, *A. globiformis* cells respond to osmotic stress by enhancing the synthesis of only amino acids, including glutamic acid.

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