Biochemistry (Moscow), Vol. 70, No. 8, 2005, pp. 878-883. Translated from Biokhimiya, Vol. 70, No. 8, 2005, pp. 1063-1069.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-216, December 5, 2004.

Cloning, Purification, and Characterization of Diaminobutyrate Acetyltransferase from the Halotolerant Methanotroph *Methylomicrobium alcaliphilum* 20Z

A. S. Reshetnikov[1,2], I. I. Mustakhimov[1,2], V. N. Khmelenina[1], and Yu. A. Trotsenko[1]*

[1]*Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia; fax: (7-095) 956-3370; E-mail: trotsenko@ibpm.pushchino.ru*
[2]*Pushchino State University, 142290 Pushchino, Moscow Region, Russia*

Received July 27, 2004
Revision received August 17, 2004

Abstract—L-2,4-Diaminobutyrate (DAB) acetyltransferase (DABAcT) catalyzes one of the key reactions of biosynthesis of the bacterial osmoprotectant ectoine—acetylation of L-2,4-DAB yielding Nγ-acetyl-2,4-DAB. Gene *ectA* encoding DABAcT was cloned from DNA of the halotolerant methanotroph *Methylomicrobium alcaliphilum* 20Z and expressed in *Escherichia coli* with an additional six His residues at the C-terminus. Homogeneous enzyme preparation with specific activity 200 U/mg was obtained by affinity metal-chelating chromatography. DABAcT was found to be a homodimer with molecular mass 40 kD. The enzyme is most active at pH 9.5 and 20°C, and its activity increased threefold in the presence of 0.1-0.2 M NaCl or 0.2 M KCl. The K_m values of recombinant DABAcT measured at the optimal pH and temperature in the presence of 0.2 M KCl were 460 and 36.6 μM for L-2,4-DAB and acetyl-CoA, respectively. The enzyme is specific for L-2,4-DAB and acetyl-CoA and is also active against propionyl-CoA (20%). Zn^{2+} and Cd^{2+} at 1 mM concentration completely inhibit the recombinant enzyme; 10 mM ATP inhibits 26% of the enzyme activity, whereas EDTA, *o*-phenanthroline, ADP, NAD(P), and NAD(P)H do not significantly effect the enzyme activity. The possible participation of DABAcT in regulation of ectoine biosynthesis in *M. alcaliphilum* 20Z is discussed.

Key words: ectoine, osmoprotectants, halotolerant alkaliphilic methanotrophs, *Methylomicrobium alcaliphilum*, L-2,4-diaminobutyrate acetyltransferase

Many halophilic and halotolerant aerobic bacteria under hyperosmotic stress conditions accumulate ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine-carboxylic acid) in their cells [1-3]. Along with osmoprotectant properties, ectoine acts as a biostabilizer of proteins, DNA, RNA, and their complexes and also cells as a whole; for this reason it is now widely used and microbiological methods for its production are being developed. Studies on regulation of ectoine synthesis in bacteria on the physiological, genetic, and biochemical levels are necessary for development of the scientific basis of corresponding technologies. Bacterial biosynthesis of ectoine is a recently found biochemical pathway that is a side branch of synthesis of amino acids belonging to the aspartate family: it is initiated by the transamination reaction of L-aspartyl semialdehyde into L-2,4-DAB with L-glutamic acid as a donor of the amino group and subsequent acetylation to Nγ-acetyl-L-2,4-DAB and its cyclization into ectoine (Fig. 1) [4].

The enzymes catalyzing these reactions—DAB-aminotransferase (EctB), DABAcT (EctA), and ectoine synthase (EctC)—are partially characterized only in the halophile *Halomonas elongata* OUT30018 [5]. DABAcT is the least studied due to its instability.

Recently we evaluated activities of the enzymes of ectoine biosynthesis and decoded the genes encoding them in the halotolerant alkalophilic methanotroph *Methylomicrobium alcaliphilum* 20Z [6, 7]; this allowed using a method of isolation and purification of subsequent enzymes based on cloning. The goal of this study was cloning, isolation, and characterization of recombinant DABAcT from *M. alcaliphilum* 20Z.

* To whom correspondence should be addressed.

0006-2979/05/7008-0878

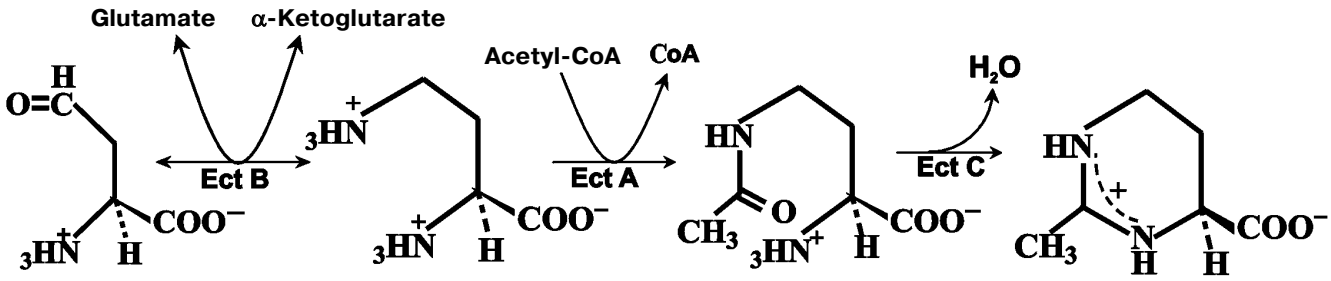

Fig. 1. Pathway of ectoine synthesis in bacteria [2]. EctA, DAB-acetyltransferase; EctB, DAB-aminotransferase; EctC, ectoine synthase.

MATERIALS AND METHODS

Bacteria and culture growth conditions. In this study, we used the moderately halophilic methanotroph *M. alcaliphilum* 20Z [8]. Culture was grown in an air–methane atmosphere on mineral medium at the optimal salt concentration (0.5 M NaCl) as described earlier [9]. *Escherichia coli* XL1-Blue and BL21(DE3) cells (Stratagene, USA) were grown on liquid or agarized (1.5%) LB media from Difco (USA) at 37°C.

Evaluation of DABAcT activity. The enzyme activity was measured at 20°C via formation of 5-thio-2-nitrobenzoic acid ($\varepsilon_{412} = 13.6\ mM^{-1}\cdot cm^{-1}$) resulting from interaction between 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and sulfhydryl groups of CoASH released in the course of the reaction [10]. The reaction medium (1 ml) contained 50 mM Tris-HCl, pH 9.0, 0.1 mM DTNB, 1 mM acetyl-CoA, 0.2 M KCl, and 10 mM DAB. The amount of DABAcT catalyzing formation of 1 μmol of CoASH per min was taken as the activity unit (U). Optical measurements were performed using a Shimadzu UV-160 spectrophotometer (Japan).

Preparation of *E. coli* superproducer of EctA protein. Genomic DNA were extracted from cells and purified as described earlier [11]. Gene *ectA* was amplified by polymerase chain reaction (PCR) from *M. alcaliphilum* 20Z DNA using a Hybaid thermocycler (England). We used direct (5′-TTATCT*CATATG*TTGCCTGATAAAA-3′) and reverse (5′-CCT*CTCGAG*AGCCTGAACCGCTTTGAA-3′) primers containing the recognition sites for *Nde*I and *Xho*I restrictases, respectively. These primers were produced based on the nucleotide sequence of the *ectA* gene determined by us earlier and registered as AY607846 in GenBank.

PCR was performed in 30 μl of reaction mixture containing 68 mM Tris-HCl, pH 8.8, 17 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA from Fermentas (Lithuania), 0.2 mM each dNTP, 10 ng of DNA template, 1 μM corresponding primer, and 2 U BioTag DNA polymerase from Dialat Ltd. (Russia). The following temperature–time profile was used: 94°C for 2 min, 37°C for 30 sec, and 72°C for 1 min; the following 30 cycles: 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min. The PCR products were analyzed by electrophoresis in 1% agarose gel. The DNA fragments were isolated from agarose and purified using a DNA Extraction kit from Fermentas according to the procedure recommended by the producer.

After treatment with *Nde*I and *Xho*I, the DNA fragment was ligated into a pET22b+ vector from Novagen (USA) with the same restriction sites opened under the T7 phage promotor. The recombinant pET*ectA* plasmid thus obtained was transformed to *E. coli* XL1-Blue strain for plasmid production and checking of the insertion structure by sequencing. Then pET*ectA* plasmid was transformed to *E. coli* BL21(DE3) strain expressing RNA polymerase of T7 phage under the control of the *lacUV5* promotor [12]. Colonies were screened for EctA superexpression by SDS-PAGE according to Laemmli [13]. Restriction, ligation, and isolation of plasmids were performed according to the standard procedures [14] using enzyme sets from Fermentas.

Isolation and purification of the recombinant EctA protein. The cells of recombinant *E. coli* BL21(DE3)/pEctA were grown in 0.5 liter of LB medium with ampicillin (50 μg/ml) at 37°C to the optical density $A_{600} = 0.6$-0.7. Protein synthesis was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG); the cells were incubated in a shaker for 3 h at 37°C.

EctA-His_6-tag was isolated from *E. coli* superproducer as described in the Quiagen (Germany) protocols but with minor changes [15]. Biomass was precipitated by centrifugation at 7000*g* for 15 min at 4°C, resuspended in 5 ml of 20 mM Tris-HCl, pH 8.0, containing 150 mM KCl, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and lysozyme (10 mg/ml) and disintegrated using a MSE ultrasonic disintegrator (England) (150 W, 10 Hz, 3 times for 0.5 min with 1-min intervals). Cell lysate was centrifuged at 10,000*g* for 20 min at 4°C, and the supernatant was tested for DABAcT activity and then applied onto a column with 5 ml of Ni^{2+}-nitroacetate (NTA) agarose from Quiagen. After intensive washing with 20 mM Tris-HCl, pH 8.0, containing 150 mM KCl and 70 mM imidazole, the bound EctA-His_6-tag was

eluted with a buffer containing 150 mM KCl and 150 mM imidazole. The protein spectrum of the collected fractions 0.5 ml in volume was analyzed by SDS-PAGE according to Laemmli [13]. The fractions containing EctA-His_6-tag were combined. The buffer was changed for 50 mM Tris-HCl, pH 8.0, on a Sephadex G-25 column. The enzyme solution was applied onto a 1.5 × 7.5 cm column with Blue Sepharose 6FF from Pharmacia (Sweden) equilibrated with 50 mM Tris-HCl, pH 8.0. DABAcT was eluted with a linear gradient of 0-0.5 M NaCl in 50 mM Tris-HCl, pH 8.0; the flow rate was 30 ml/h. The fractions exhibiting DABAcT activity were pooled and concentrated using Microcon YM-10 from Millipore (USA).

Determination of molecular mass. The molecular mass of the native enzyme was determined by gel filtration on a calibrated 1.5 × 100 cm column with Ultragel AcA54 from LKB (Sweden) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.1 M KCl, using an HPLC system from LKB; flow rate 15 ml/h. BSA (66 kD), ovalbumin (45 kD), DNase (31 kD), and cytochrome *c* (12.4 kD) from Pharmacia and Sigma (USA) were used as protein markers. Protein yield was determined via optical absorption at 280 nm using a LINEAR UVIS 200 UV detector (USA). Molecular mass of subunits was determined by electrophoresis in 12% polyacrylamide gel in the presence of SDS according to Laemmli [13] using a kit of protein markers from Fermentas including β-galactosidase (116 kD), BSA (66.2 kD), ovalbumin (45 kD), lactate dehydrogenase (35 kD), endonuclease *Bsp*98I (25 kD), β-lactoglobulin (18.4 kD), and lysozyme (14.4 kD).

Other methods. K_m values were calculated according to the Michaelis–Menten equation using the ENZFITTER program. Protein concentrations were determined according to Lowry et al. [16] using BSA as the standard.

RESULTS

Cloning and expression of the *ectA* gene in *E. coli*. To construct the DABAcT superproducer, *ectA* gene was cloned in pET22b+ vector, which determines the synthesis of recombinant protein with additional His_6 residues at the C-terminus. As a result, the pET*ectA* construction was obtained. In *E. coli* BL21 (DE3) cells transformed by pET*ectA* plasmid, after induction by IPTG for 3 h we found by SDS-PAGE a major protein with electrophoretic mobility corresponding to molecular mass ~20 kD in the soluble fraction. Within S.E. limits, this value coincides with the molecular mass calculated from the amino acid sequence of the *ectA* gene (18.9 kD). In control *E. coli* cells grown without inducer the protein band was not detected (data not presented here).

Isolation and purification of recombinant DABAcT. After affinity chromatography of *E. coli* BL21(DE3)/

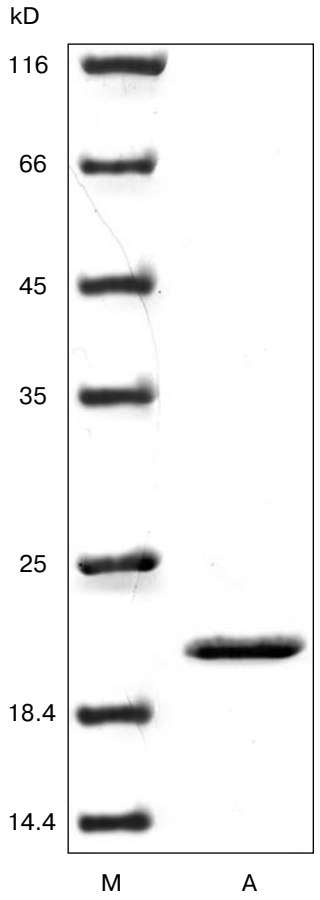

Fig. 2. Electrophoresis of DABAcT in 12.5% polyacrylamide gel in the presence of SDS (A). M, protein markers.

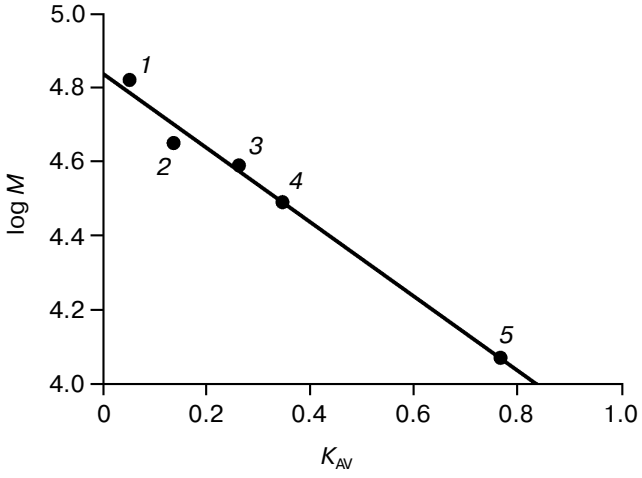

Fig. 3. Determination of molecular mass (*M*) of the native DABAcT on Ultragel AcA54. Proteins: *1*) BSA; *2*) ovalbumin; *3*) DABAcT; *4*) DNase; *5*) cytochrome *c*. K_{AV}, the relative volume of elution.

pEctA lysate on a column with Ni-NTA agarose and elution with imidazole, the protein was additionally purified by dialysis on a column with Sephadex G-25 and chromatography on a column with Blue Sepharose 6FF. Finally, electrophoretically homogeneous EctA-His_6-tag preparation with the specific activity 200 U/mg was obtained (Fig. 2).

Properties of DABAcT. By the gel filtration data, the molecular mass of the native enzyme is 40 kD; this corresponds with dimeric form (2 × 20 kD) of the native enzyme (Fig. 3).

The enzyme is active in the pH range from 7.5 to 10.5 with the optimum at pH 9.5 (Fig. 4). The enzyme activity increased threefold in the presence of 250 mM KCl or 100-150 mM NaCl (Fig. 5). The optimum reaction temperature is 20°C (Fig. 6).

The enzyme catalyzes condensation of acetyl-CoA with DAB, yielding Nγ-acetyl-2,4-DAB; this was proved by HPLC and ^{1}H-NMR spectroscopy. α-, β-, and γ-aminobutyrate, ornithine, lysine, or aspartate cannot act as the substrates for DABAcT. However, DABAcT exhibited activity with propionyl-CoA (20% activity compared to acetyl-CoA) and negligible activity with acetoacetyl-CoA, malonyl-CoA, and succinyl-CoA (<5%). Activity with methylmalonyl-CoA, hydroxybutyryl-CoA, crotonyl-CoA, and valeryl-CoA was not detected. This indicates specificity of the enzyme for acetyl-CoA and DAB.

The dependence of the reaction rate on DAB and acetyl-CoA concentrations obeys the Michaelis–Menten equation. The calculated K_m values for DAB and acetyl-CoA are 460 and 37 μM, respectively.

Bivalent metal cations at 1 mM concentrations slightly inhibited DABAcT activity, excluding Zn^{2+} and Cd^{2+}, which almost completely inhibited the enzyme activity (Table 1). The inhibitory action of Zn^{2+} was cancelled by 2 mM EDTA.

DABAcT activity was not suppressed by *o*-phenanthroline and EDTA; this indicates the absence of metals in the enzyme molecule. ADP, NAD(P), and NAD(P)H do not significantly effect DABAcT activity. ATP inhibited by 18% the enzyme activity at 1 mM concentration and by 26% the activity at 10 mM concentration. KCl (0.2 M) did not affect the degree of enzyme inhibition by ATP (Table 2).

The purified DABAcT preparation is stable in 50 mM Tris-HCl, pH 8.5, containing 0.2 M KCl at least for one month at 4 and −70°C at protein concentration 0.5 mg/ml.

DISCUSSION

L-2,4-DAB aminotransferase, L-2,4-DABAcT, and L-ectoine synthase, specific enzymes of biosynthesis of the bacterial osmoprotectant ectoine, have been partially purified and characterized only in *Halomonas elongata*

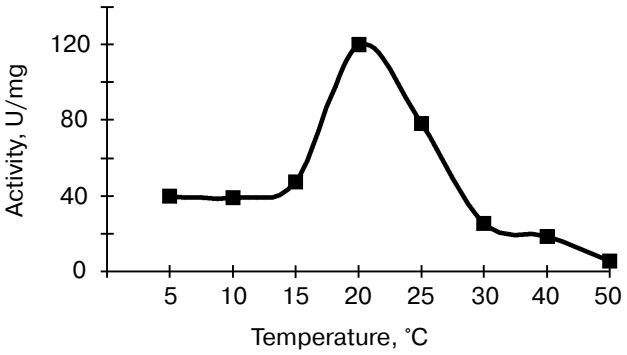

Fig. 4. Temperature dependence of DABAcT activity.

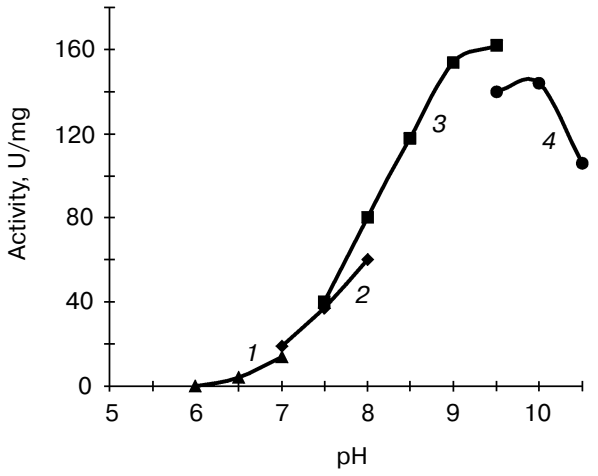

Fig. 5. The pH dependence of DABAcT activity. Buffers: *1*) Na-citrate; *2*) Na-phosphate; *3*) Tris-HCl; *4*) Na-carbonate.

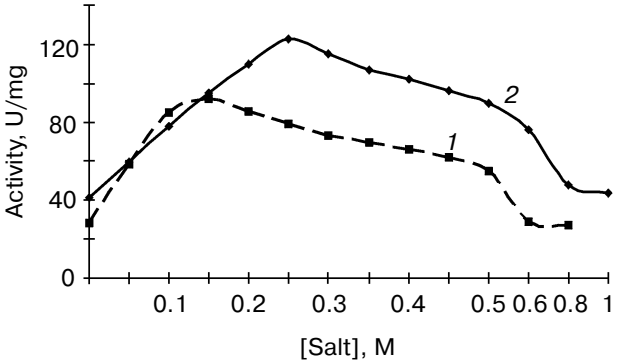

Fig. 6. DABAcT activity versus NaCl (*1*) and KCl (*2*) concentrations.

Table 1. Effect of bivalent metal cations on DABAcT activity in the presence of 0.2 M KCl

Salt, 1 mM	Residual activity, %
Control	100
$MnCl_2$	88
$BaCl_2$	91
$CaCl_2$	89
$NiCl_2$	87
$MgCl_2$	94
$CdCl_2$	0.2
$CoCl_2$	102
$RbCl_2$	88
$CuCl_2$	78
$CuCl_2$ + EDTA (2 mM)	98
$ZnSO_4$	5
$ZnSO_4$ + EDTA (2 mM)	91
$FeSO_4$	84
$FeSO_4$ + EDTA (2 mM)	102

Table 2. Effect of various compounds on DABAcT activity in the presence of 0.2 M KCl

Reagents and measurement conditions	Residual activity, %
Control	100
1 mM EDTA	96
1 mM *o*-phenanthroline	98
1 mM NH_2OH	92
1 mM ATP	80
1 mM ATP*	82
5 mM ATP	78
10 mM ATP	74
10 mM ATP*	74
1 mM ADP	101
1 mM NAD^+	98
1 mM NADH	100
1 mM $NADP^+$	89
1 mM NADPH	95

* Residual activity was measured in the absence of KCl.

OUT 30015 [5]. This is mainly due to difficulties in evaluation of their activity. To measure DABAcT activity in this study, we adapted a well-known procedure applied for citrate synthase detection [10]. In addition, a complete nucleotide sequence of the DABAcT gene in *M. alcaliphilum* 20Z determined by us earlier [8] allowed using methods for protein production based on cloning and expression of the *ectA* gene in *E. coli*; this significantly simplified the procedure for the recombinant enzyme purification by affinity chromatography. As a result, a homogeneous DABAcT preparation with the specific activity 200 U/mg was obtained for the first time. Partially purified enzyme from *H. elongata* had specific activity 50 U/mg protein, but it was unstable and thus was not well studied [5].

Along with common properties, comparison of the DABAcT from two halotolerant strains revealed some distinctions. Their temperature optimum is unusually low, i.e., about 20°C, and they are stimulated by NaCl or KCl (0.1-0.3 M in *M. alcaliphilum* 20Z or 0.4 M NaCl in *H. elongata*). However, DABAcT from the methanotroph has maximal activity at higher pH (pH_{opt} = 9.5) compared with that for the enzyme from *H. elongata* (pH_{opt} = 8.2). These properties of DABAcT correspond well with different dependence of bacterial growth on salt concentration and pH. Thus, *M. alcaliphilum* 20Z grows in 0.05-1.5 M NaCl concentration range (optimal concentration 0.5 M) and pH 9.0, whereas *H. elongata* grows with the maximal rate in neutral medium in the presence of 2 M NaCl.

Dependence on high ionic strength is typical of most enzymes of halophilic archaebacteria using so-called "salt" type of osmotic adaptation; its essence is accumulation of inorganic ions (mainly K^+ and Cl^-) in the cells [3]. Stimulation of DABAcT activity with NaCl or KCl can indicate that the enzymes of the pathway of ectoine biosynthesis have "halophilic" nature. Although the studied methanotroph accumulates mainly ectoine to equilibrate external osmotic pressure, high K^+ concentrations (0.17-0.36 M) are also detected in the cells; at NaCl concentration in the growth medium from 0.07 to 1 M, this suggests a "mixed" type of osmotic adaptation [6]. The concentration dependence of the KCl stimulating effect of DABAcT activity *in vitro* corresponds well with the intracellular K^+ concentration in this methanotroph.

The inhibitory action of ATP on the activity of DAB acetyltransferase discovered by us accounts for possible regulation of distribution of aspartate flow via two metabolic pathways *in vivo*. ATP is a substrate of aspartokinase catalyzing the first reaction of the pathway of biosynthesis of ectoine and amino acids of the aspartate family; excess ATP level may be a result of deficient aspartate pool in cells. In this case, inhibition of DABAcT diminishes outflow of aspartyl semialdehyde necessary not only for ectoine, but also for lysine, threonine, and methionine biosynthesis.

Thus, it follows from the data that the pathway of biosynthesis of the bioprotectant ectoine providing osmotic adaptation of *M. alcaliphilum* 20Z may be regulated at the level of DABAcT activity.

This work was financially supported by CRDF (grant No. RB1-2509-MO-03), the Russian Foundation for Basic Research (grant No. 01-014-48511), the Russian Academy of Sciences (grant No. 193), and the Ministry of Education of Russia (grant No. E02-60-304). The authors are grateful to A. A. Zimin and A. P. Sokolov for valuable discussion.

REFERENCES

1. Galinski, E. A., Pfeiffer, H. P., and Truper, H. G. (1985) *Eur. J. Biochem*., **149**, 135-139.
2. Galinski, E. A. (1995) *Adv. Microbiol. Physiol*., **37**, 273-328.
3. Sleator, R. D., and Hill, C. (2002) *FEMS Microbiol. Rev*., **26**, 49-71.
4. Peters, P., Galinski, E. A., and Truper, H. G. (1990) *FEMS Microbiol. Lett*., **71**, 157-162.
5. Ono, H., Sawada, K., Khunajakr, N., Tao, T., Yamamoto, M., Hiramoto, M., Shinmio, A., Takano, M., and Murooka, Y. (1999) *J. Bacteriol*., **181**, 91-99.
6. Trotsenko, Y. A., and Khmelenina, V. N. (2002) *Arch. Microbiol*., **177**, 123-131.
7. Reshetnikhov, A. S., Khmelenina, V. N., and Trotsenko, Y. A. (2004) *Dokl. Akad. Nauk SSSR*, **396**, 831-834.
8. Khmelenina, V. N., Kalyuzhnaya, M. G., Sakharovsky, V. G., Trotsenko, Y. A., and Gottschalk, G. (1999) *Arch. Microbiol*., **172**, 321-329.
9. Khmelenina, V. N., Starostina, N. G., Tsvetkova, M. G., Sokolov, A. P., Suzina, N. E., and Trotsenko, Y. A. (1996) *Mikrobiologiya*, **65**, 696-703.
10. Levering, P. R., Binnema, D. J., van Dijken, J. P., and Harder, W. (1981) *FEMS Microbiol. Lett*., **12**, 19-20.
11. Kalyuzhnaya, M. G., Khmelenina, V. N., Kotelnikova, C., Holmquist, L., Pedersen, K., and Trotsenko, Y. A. (1999) *Syst. Appl. Microbiol*., **22**, 565-572.
12. Hochuli, E. (1990) *Genet. Eng*., **12**, 87-98.
13. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
14. Maniatis, T., Frich, E., and Sambrook, J. (1984) *Molecular Cloning* [Russian translation], Mir, Moscow.
15. Henco, K. (1992) *The QIAexpressionist: The High Level Expression and Protein Purification System*, QIAGEN Press, Hamburg.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem*., **193**, 265-275.