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REVIEW

Regulatory Aspects of Ectoine Biosynthesis in Halophilic Bacteria

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Abstract—In this review, the current concepts of metabolic and genetic regulation of the synthesis of ectoine and hydroxyectoine, widely distributed osmoprotectors of halophilic bacteria, are analyzed and generalized. Due to their water-retaining properties, these compounds can be used as multifunctional bioprotectants. The relevance of deciphering ectoine biosynthesis regulation at the levels of enzyme activity and gene transcription in halophiles is related to the search and creation of novel, more efficient producers by deliberate genetic-engineering construction. In spite of the conservativeness of the ectoine biosynthesis pathway in relation to genes and enzymes, indicating the horizontal transfer of *ect* genes, different enzyme properties and mechanisms of transcriptional regulation of the ectoine operon were revealed in various halophilic bacteria. The models of transcriptional regulation of the genes of ectoine biosynthesis in *Chromohalobacter salexigens* and *Methylomicrobium alcaliphilum* are discussed.

Key words: halophilic bacteria, ectoine, hydroxyectoine, biosynthesis regulation

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Microorganisms inhabit aqueous environments with different salinities, from freshwater and marine biotopes to hypersaline water bodies with high NaCl concentrations up to saturation. Osmotic stress is one of the main parameters of all ecosystems. Water freely penetrates through the membrane, and nonadapted organisms quickly lose water in the presence of salts. Therefore, to exist, the cells must maintain turgor pressure in the cytoplasm equal to or exceeding the ambient pressure. To maintain osmotic balance, microorganisms use two main strategies [1–7].

In the first strategy, osmotic balance is maintained by selective accumulation of inorganic ions in the cytoplasm (salt-type osmoadaptation). This strategy is used in two phylogenetically distant groups of microorganisms: aerobic extremely halophilic archaea of the family *Halobacteriaceae* and anaerobic halophilic bacteria of the order *Haloanaerobiales*, acetogenic anaerobes (the species of *Halobacteroides, Sporohalobacter*, and *Acetohalobium),* and sulfate reducers (*Desulfovibrio halophilus, Desulfohalobium retbaense*) [1–3]. During the evolution, the enzymes and other biomolecules of extreme halophiles have been modified to function efficiently at high intracellular salt concentrations. Adaptation of the enzymes consists in the changes in their amino acid composition, in particular, in an increased level of acidic and decreased content of hydrophobic amino acids compensated by the polar amino acids (serine and threonine), thus resulting in the appearance of a strong hydrate envelope around the protein [4].

The second type of osmoadaptation characteristic of most of the moderately halophilic and halotolerant microorganisms is associated with accumulation of specific low-molecular-weight organic substances, osmolytes (or compatible solutes), which are well soluble in water and do not carry charges at physiological pH values. In addition to these common properties, the compatible solutes of halophiles have little in common in their chemical structure [5]. In microorganisms with a nonsalt type of osmoadaptation, intracellular macromolecules do not undergo specific modification. Such a type of osmoadaptation presupposes no drastic genetic, enzymatic, or structural changes and, consequently, provides a more flexible way of cell adaptation to osmotic fluctuations. This probably explains the wide occurrence of the mechanism associated with accumulation of organic substances in the microbial world [5].

The spectrum of compatible solutes found in prokaryotes and eukaryotes is rather broad and diverse [6, 7]. Osmoprotectants belong to different classes of organic compounds and are specific for different groups of halophilic and halotolerant microorganisms. However, biosyntheses of glycerol, glycine-betaine, and ectoines are the most energetically efficient processes, while synthesis of sucrose and trehalose is the least advantageous. Many bacteria can accumulate several osmoprotectants simultaneously, and prevalence of a specific osmolyte is mainly determined by the energetic status of a cell and availability of a carbon and nitrogen source.

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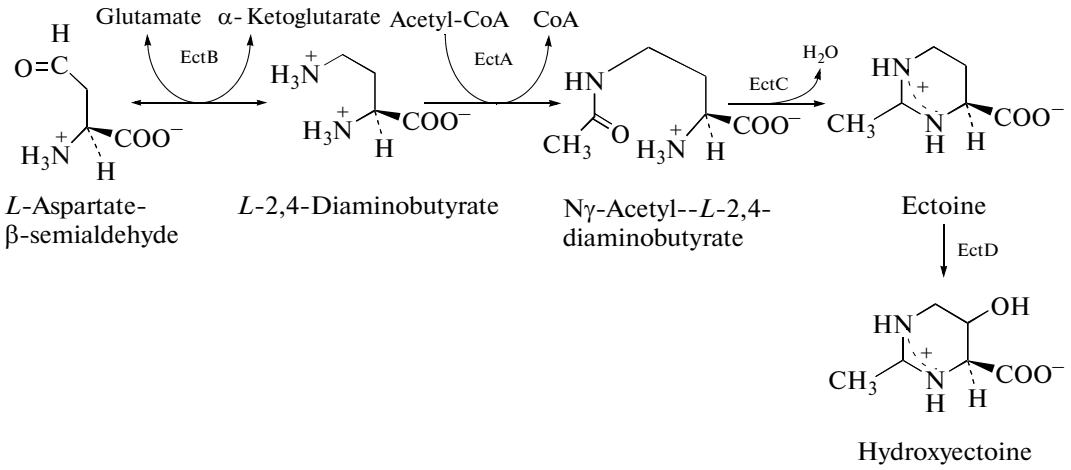

Fig. 1. Ectoine and hydroxyectoine biosynthesis pathways in halotolerant bacteria [5, 13]. EctA, DAB acetyltransferase; EctB, DAB aminotransferase; EctC, ectoine synthase; and EctD, ectoine hydroxylase.

ECTOINE AND HYDROXYECTOINE BIOSYNTHESIS

Ectoine as an osmoactive substance was discovered by E. Galinski in the phototrophic purple bacterium *Ectothiorhodospira halochloris* [8]. According to its chemical structure, ectoine may be classified as a partially hydrated imino acid (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylate). Accumulation of ectoines has been shown for various halophilic and halotolerant gram-positive (G^+) and gram-negative (G^-) eubacteria, including the species of *Nocardiopsis, Brevibacterium, Marinococcus, Halomonas, Pseudomonas, Vibrio*, and methylotrophic bacteria. It is interesting to note that nonhalophilic eubacteria under hyperosmotic conditions accumulate ectoine by transporting it from the medium [9–12].

ENZYMES AND GENES OF THE PATHWAY OF ECTOINE AND HYDROXYECTOINE BIOSYNTHESIS

Ectoine biosynthesis is a branch in the pathway for the synthesis of the aspartate family of amino acids. In this pathway, aspartate-β-semialdehyde (ASA) is converted by DAB aminotransferase into L-2,4-diaminobutyrate (DAB) and DAB is acetylated by DAB acetyltransferase to *N*γ-acetyl-2,4-diaminobutyrate (ADAB). Cyclization of ADAB into ectoine is catalyzed by ectoine synthase [13]. This pathway has been confirmed enzymatically for the phototroph *E. halochloris* [13]; the heterotrophs *Halomonas elongata* [14], *Halobacillus dabanensis* [15] and *Bacillus pasteurii* [16]; the methanotroph *Methylomicrobium alcaliphilum* 20Z [17]; and the marine bacterium *Marinococcus halophilus* [18] (Fig. 1).

In *Brevibacterium linens* [19], *Brevibacterium epidermis* DSM 20659 [20], *Brevibacterium* sp. [21], and *Streptomyces parvulus* [22], the pathway of ectoine biosynthesis begins with glutamate. Along with aspartate and glutamate, asparagine can be used as a precursor, as was demonstrated for *Streptomyces griseus* and *Streptomyces clavuligerus* [12].

The genes of ectoine biosynthesis have been characterized in the G^+ moderately halophilic bacteria *M. halophilus* [18], *B. pasteurii* [16] and *H. dabanensis* [15] and in the G^- bacteria *Chromahalobacter salexigens* (previously *Halomonas elongata* DSM 3043) [23] and *H. elongata* [14]; they are located in a single *ectABC* operon, where the *ectA* gene encodes L-2,4-DAB-acetyltransferase and the *ectB* and *ectC* genes encode L-2,4-DAB-aminotransferase and L-ectoine synthase, respectively. However, in the aerobic halo(alkali)philic methanotroph *Mm. alcaliphilum* 20Z, the genes of ectoine synthesis are organized into a four-gene *ectABC-ask* operon comprising an additional gene of aspartate kinase *(ask)* [17]. Thus, the ectoine biosynthesis pathway is rather conservative with respect to enzymes and arrangement of the *ect* genes. In view of the rather high similarity of nucleotide sequences of the *ectA, ectB*, and *ectC* genes, a hypothesis of high conservativeness of the ectoine biosynthesis pathway and distribution of the *ect* genes among bacteria by means of lateral transfer was suggested [16].

Hydroxyectoine is synthesized by direct hydroxylation of ectoine by ectoine hydroxylase (EctD). The *ectD* gene has been presently identified in *Streptomyces chrysomallus* [24], *C. salexigens* [25], and *Salibacillus salexigens* [26]. Theoretically, an alternative pathway is possible, where *N*γ-acetyl-DAB is converted into hydroxyectoine via 3-hydroxy-*N*γ-acetyl-DAB without the involvement of ectoine as an intermediate [27]. However, this pathway has not been confirmed enzymatically.

The properties of DAB acetyltransferases of halophilic bacteria

Characteristics	*M. thalassica* [30]	*M. alcalica* [30]	*M. alcaliphilum* [29]	*H. elongata* [28]
pH optimum	9.0	9.5	9.5	8.2
Temperature optimum, °C	30–35	30–35	20	20
Molecular mass (SDS-PAG electrophoresis)	20 kDa	20 kDa	20 kDa	N.d.
Molecular mass (gel filtration)	40 kDa	40 kDa	40 kDa	45 kDa
K_M (DAB)	0.365 mM	0.375 mM	0.465 mM	N.d.
K_M (acetyl-CoA)	76 μM	30 μM	36.7 μM	N.d.
Inhibitors (1 mM)	Zn^{2+} Cd^{2+} Cu^{2+}	Zn^{2+} Cd^{2+} Cu^{2+}	Zn^{2+} Cd^{2+}	N.d.
Optimal concentration of KCl, M	0	0	0.25 M	N.d.
Optimal concentration of NaCl, M	0	0	0.1–0.2 M	0.4 M
Stability	Stable*	Stable*	Stable*	Unstable

* Enzyme preparations (0.5 mg/ml) are stable in 50 mM *tris*–HCl buffer (pH 8.5) for a month at 4 or –70°C; nd, not determined.

PROPERTIES OF THE ENZYMES OF ECTOINE BIOSYNTHESIS

The enzymes of ectoine biosynthesis have been purified from *H. elongata*, the methanotroph *Mm. alcaliphilum* 20Z, and the methylobacteria *M. alcalica* and *M. thalassica* and partially characterized [28–30].

2,4-DAB aminotransferase from *H. elongata* is a homohexamer (~250 kDa). It is a pyridoxal-phosphate-dependent enzyme requiring the presence of K^+ ions for its activity and stability, which is probably due to the presence of specific binding sites for this ion in the protein. Dependence on potassium has been shown for many enzymes of halophilic archaea and eubacteria [31]. DAB aminotransferase of *H. elongata* is specific to L-glutamate as a donor of amino groups (pH_{opt} 8.6–8.7; K_M is 9.1 mM for L-glutamate and 4.5 mM for D,L-aspartate semialdehyde; pI = 6.2).

At present, the conversion of aspartate semialdehyde into DAB is known in several bacteria not synthesizing ectoine: *Xanthomonas* sp. and *Acinetobacter baumanii* [32, 33]. The DAB aminotransferase from *A. baumanii* participates in the biosynthesis of the cell wall component 1,3-diaminopropane. This enzyme is also specific to aspartate semialdehyde as an acceptor of amino groups and to L-glutamate as a donor of amino groups. Its optimal pH values and K_M values for DAB and 2-oxoglutarate are similar to those of the enzyme from *H. elongata* [32]. However, in contrast to the enzymes from *H. elongata* and *A. baumanii*, the DAB aminotransferase from *Xanthomonas* sp. utilizes L-alanine as a donor of amino groups [33].

DAB acetyltransferase from *H. elongata* was purified 400-fold; however, the enzyme could not be obtained in a homogeneous state because of its instability. The molecular mass of the enzyme determined by gel filtration was about 45 kDa [28]. The enzyme preparation had a specific activity of 50 U/mg of protein but proved to be unstable; therefore, it was possible to determine only the subunit protein composition and pH optimum and the increase in enzyme activity in the presence of 0.4 M NaCl and KCl (table).

Homogeneous and stable preparations of DAB acetyltransferases were obtained for the first time by cloning the *ectA* gene from the methanotroph *Mm. alcaliphilum* 20Z and the methylobacteria *M. alcalica* and M. *thalassica* [29, 30]. The DAB acetyltransferase from *M. thalassica* exhibited the maximum activity at pH 8.5–9.0, which decreased upon pH enhancement or replacement of *tris*–HCl buffer for sodium–carbonate buffer in the reaction mixture. On the contrary, the enzymes from the alkaliphiles *M. alcalica* and *Mm. alcaliphilum* 20Z had pH optima 9.5 and were not inhibited by the sodium–carbonate buffer (table), while EctA from *H. elongata* was most active at pH 8.2 [28]. Consequently, there is a correlation between pH values optimal for the activity of DAB acetyltransferases and ecophysiological traits of methylotrophs (alkaliphilic *Mm. alcaliphilum* 20Z and *M. alcalica* and neutrophilic *H. elongata* and *M. thalassica*). There is an analogous correlation with respect to the effect of carbonate ions: inhibition of the enzyme activity of *M. thalassica*, which was isolated from chloride–sodium sources, and the absence of inhibition of DAB acetyltransferase from methylotrophic bacteria isolated from soda lakes with high carbonate concentrations (*Mm. alcaliphilum* 20Z and *M. alcalica*). The adaptation of halophilic bacteria to the ecophysiological conditions of their habitat probably included modification of the enzymes of ectoine synthesis.

The activity of DAB acetyltransferase from *Mm. alcaliphilum* 20Z was maximal at 0.15 mM NaCl and 0.25 mM KCl, whereas the enzymes from methylobacteria were most active in the absence of these salts (table). The stimulating effects of NaCl or KCl on the activity of DAB acetyltransferases may be indirect evidence of the "halophilic" nature of this enzyme of the ectoine biosynthesis pathway in *Mm. alcaliphilum* 20Z and *H. elongata*. On the contrary, the inhibitory effect

of the salts on the enzymes of methylobacteria is probably explained by their adaptation to a relatively constant intracellular ionic strength, which is quite effectively maintained in the cells of neutrophilic strains utilizing methanol as an energy source.

Sodium glutamate (up to 0.5 M) had no effect on the activity of DAB acetyltransferases from methylobacteria. It would be logical to suggest that chloride ions have an inhibitory effect (along with the inhibition by high ionic strength).

Dependence of the DAB acetyltransferase reaction rates on the concentration of their substrates (DAB or acetyl-CoA) obeys the Michaelis–Menten kinetics (table). Copper cations in the reaction mixture completely inhibited the activity of DAB acetyltransferase from *M. alcalica* (by 98%), while the enzyme activities of *M. thalassica* and *Mm. alcaliphilum* 20Z in the presence of Cu^{2+} decreased by 47 and 30%, respectively. These differences in the effect of copper correlate with the physiological and biochemical characteristics of bacteria. In particular, since Cu^{2+} participates in methane oxidation [34], the growth and activity of methanotrophs strictly depend on the presence of this cation in the medium.

Ectoine synthase of *H. elongata* was purified to a homogenous state in the presence of 1 mM DAB and 2 M NaCl as stabilizers. The enzyme is a homodimer with a subunit molecular mass of 19 kDa. However, its activity was not observed in the buffer of high osmolarity (0.5 M NaCl) used for gel filtration. Hence, it is unclear whether the native ectoine synthase has the molecular mass of 35 kDa and is a homodimer or nonspecific aggregation of the monomers occurred under high salt concentrations. Analysis of the amino acid composition of ectoine synthase revealed enhanced content of L-aspartate and L-glutamine. The enzyme is very specific to *N*γ-acetyl-DAB; the *N*-acetyl group in the α position is probably not involved in cyclization into ectoine. The isoelectric point (pI) of the enzyme is 4.2–4.4. It is supposed that the limiting stage in the sequence of ectoine biosynthesis is DAB synthesis with the involvement of DAB aminotransferase. This suggestion is confirmed by the fact of DAB absence in the cells of *H. elongata* KS3 [28].

The parameters required for the maximal activity of the above enzymes of the ectoine biosynthesis pathway in *H. elongata* are close: pH 8.2–9.0, t = 15–20°C, and 0.4–0.5 M NaCl. Nevertheless, DAB aminotransferase is more active in the presence of 0.01–0.5 M KCl than at the same concentrations of NaCl [28]. Salt concentrations optimal for the activity of these enzymes are lower than could be supposed based on the halotolerance of *H. elongata* DSM2581, probably due to the relatively low intracellular concentration of free ions. For example, the intracellular level of Na^+ in the cells of the halophilic bacteria *Vibrio costicola* and *Brevibacterium* sp. growing in the presence of high NaCl concentration was 0.04–0.2 M [21, 35].

Ectoine hydroxylase from *Salibacillus salexigens has* been recently purified and partially characterized [26]. The enzyme is a monomer (34 kDa) with pH and temperature optima of 7.5 and 32°C, respectively. The activity depends on 2-oxoglutarate and requires the presence of O_2 in the reaction mixture. Since the active center of ectoine hydroxylase includes one Fe(II) molecule, this protein was assigned to the superfamily of non-heme-containing Fe(II)- and 2-oxoglutarate-dependent dioxygenases (EC 1.14.11). The maximal enzyme activity was 13.8 U/mg; the K_M values for ectoine and 2-oxoglutarate were 3.5 ± 0.2 and 5.2 ± 0.2 mM, respectively [26].

Thus, based on the properties of these enzymes, one may suppose that ectoine biosynthesis is regulated at the level of enzyme activity. It seems that there is a functional adaptation of the enzymes of ectoine synthesis to the ecophysiological characteristics of halophilic bacteria. In addition, it has been shown that, in the presence of chloramphenicol, which blocks protein synthesis, the cells of *H. elongata* synthesize ectoine under hyperosmotic shock similarly to the cells with active protein biosynthesis. This demonstrates that the change in osmolarity of the medium enhances the activity of ectoine biosynthesis enzymes at the posttranslational level [36].

TRANSCRIPTIONAL REGULATION OF ECTOINE BIOSYNTHESIS

In spite of intense research into the molecular mechanisms of osmoadaptation of microorganisms, most of studies deal with identification of osmolytes and the genes responsible for their synthesis. Information on the mechanisms of regulation of the biochemical processes related to the maintenance of cell homeostasis under high osmolarity of the medium is quite fragmentary. It is unknown how osmolarity of the medium determines the level of expression of the genes coding for the enzymes of biosynthesis of osmolytes, in particular, ectoine.

At the first stage, the signal is recorded and transduced into the cell by membrane *sensors*, i.e., proteins with conformation and/or oligomerization depending on the osmolarity of the medium [37–40]. The mechanism of signal transduction is probably analogous to the one proposed for eukaryotes, being a sequence of reactions of sensor phosphorylation (under either osmotic shock or changes in the cell turgor pressure) followed by transfer of the phosphoryl group from the sensor to the transcriptional regulatory protein [40].

Such a mechanism of regulation is typical of the Kdp K^+ transport system of *E. coli* [37]. This system consists of membrane ATPase (*kdp*FABC operon), sensor kinase KdpD, and transcriptional activator protein KdpE. Under osmotic shock, decreased intracellular pressure causes autophosphorylation of KdpD kinase followed by transfer of the phosphate group to aspartate of the regulatory protein KdpE. The phos-

phorylated regulator KdpE activates expression of the *kdp*FABC operon [38, 41].

One more example of the regulation of expression of osmoregulated genes by the mechanism of phosphorylation/dephosphorylation of a transcriptional regulatory protein is the regulation of the OmpC/OmpF transport system [37]. Porins OmpC and OmpF form membrane channels for passive diffusion of a wide range of low-molecular compounds. The quantity of porins is controlled at a level of expression of the *ompC* and *ompF* genes by a two-component EnvZ/OmpR regulatory system consisting of the membrane-bound sensor kinase EnvZ and the cytoplasmic regulatory protein OmpR. Under hyperosmotic conditions, the expression of *ompC* was shown to increase, whereas the expression of *ompF* decreased. Under hypoosmotic conditions, on the contrary, the expression of *ompC* decreased but that of *ompF* increased [42].

The sensor kinase EnvZ phosphorylates or dephosphorylates the regulatory protein OmpR, depending on the osmotic conditions of the medium. The level of OmpR phosphorylation is critical for the interaction with the regulatory regions of the *ompC* and *ompF* genes. In low-osmolarity medium, the level of phosphorylated OmpR decreases and, as a result, the *ompF* gene expression increases. In high-osmolarity medium, the level of the phosphorylated regulatory protein OmpR increases, activating transcription of the *ompC* gene and suppressing expression of the *ompF* gene. In the absence of phosphorylated protein OmpR, the *ompC* and *ompF* genes are not transcribed [43].

At the same time, it should be noted that variations in osmolarity of the medium, cell turgor, membrane fatty acid composition, protein hydration envelope, intracellular concentration of dissolved substances, and activity of extra- and intracellular water are interdependent; therefore, the cell must "control" several signals simultaneously [40].

At present, information on the mechanisms of transcriptional regulation of ectoine biosynthesis genes is rather fragmentary. For the moderately halophilic G^- bacteria *Chromohalobacter salexigens* [44] and *Mm. alcaliphilum* 20Z [17] and G^+ bacteria *Bacillus pasteurii* [6], *M. halophilus* [45], *H. halophilus* [46], and *S. salexigens* [26], it has been shown that the *ect* genes are transcribed as a polycistronic mRNA and the level of their transcription is controlled by osmotic conditions.

In *C. salexigens*, the *ectABC* genes are transcribed from two promoter regions. The first promoter region is located upstream of the *ectA* gene and supposedly consists of four promoters (*PectA1–4*). Two of them (*PectA1* and *PectA2*) show homology with the σ_{70}-dependent promoter of *E. coli*; at the same time, the –10 sequence of *PectA2* is identical to the consensus one (TATAAT). Another promoter (*PectA3*) shows similarity with the σ_S-dependent promoter of *E. coli*. The second promoter region is located at a distance of 25 bp from the starting codon of the *ectB* gene and consists of a single promoter *PectB* homologous to the "heat shock"-dependent sigma factor, $\sigma]_{32}$. It has been shown that the *PectA* and *PectB* promoter regions are osmoregulated and expression from the promoter *PectB* intensifies when the cultivation temperature increases [44].

The measurement of activity of the reporter gene (*lacZ*) under the control of the *PectA* promoter region in different growth phases of *C. salexigens* has shown that *PectA* behaves as a typical σ_S-dependent promoter exhibiting maximum activity during the stationary phase of growth. Moreover, expression of the *lacZ* gene from the promoter region *PectA* was much lower in the *E. coli* JMH0039 mutant defective in the *rpoS* gene encoding the σ_S transcription factor. Together with the low constitutive transcription from the *PectA* and *PectB* promoters under low medium osmolarity, the above observation suggests the involvement of other transcription factors in expression of the *ectABC* operon from the *PectA1* and *PectA2* promoters [44].

On the other hand, weak transcription from the promoter regions *PectA* and *PectB* at low salinity of the medium is probably due to partial constitutive expression from these promoters [47].

The addition of osmoprotectants (ectoine, choline, or glycine-betaine) to the growth medium reduced the basal level of transcription of *PectA-lacZ* and *PectB-lacZ* [44] but increased the growth rate of *C. salexigens* in the whole investigated range of NaCl concentrations [23].

A vegetative σ_A-dependent promoter (equivalent to the σ_{70}-dependent promoter of G^- bacteria) characteristic of *Bacillus subtilis* was found during analysis of the nucleotide sequence upstream the *ectABC* operon in *B. pasteurii* and *S. salexigens* [16, 26].

The DNA sequence flanking the *ectABC* genes in *M. halophilus* was characterized as a stress-reactive promoter region bearing the sites homologous to the sequences of σ_{70}- and σ_S-dependent promoters of *E. coli* [45]. Such a sequence was amplified from genomic DNA and cloned upstream of the *gfp* gene into the expression vector pBR322. The measurement of fluorescence of the reporter protein (green fluorescent protein, GFP) in *E. coli* cells grown in the media with different osmolarity has revealed the linear dependence of fluorescence intensity upon NaCl concentration, whereas the addition of ectoine and betaine to the growth medium resulted in a decrease of the fluorescence signal [45].

A similar dependence of expression of the *ectABC* genes on the degree of osmolarity was shown for *Brevibacterium epidermis* [20], *Mm. alcaliphilum* 20Z [17], *Halobacillus halophilus* [46], *C. salexigens* [25], *S. salexigens* [26], and *B. pasteurii* [16]. For example, the method of real-time PCR showed that the quantity of mRNA in the *ectABC* operon of the moderately halo-

philic bacterium *H. halophilus* remained constant when the NaCl concentration in the medium increased up to 1.5 M. On further enhancement of NaCl concentration (up to 3 M), expression of the ectoine biosynthesis genes increased 20-fold. Moreover, expression of the *ect* operon genes reached its maximum 3 h after the hyperosmotic shock, when transcription of the genes of synthesis of glutamate, glutamine, and proline involved in the primary response to hyperosmotic shock decreased to the basal level [46].

Additional regulatory elements involved in the regulation of hydroxyectoine biosynthesis in *S. salexigens* and *C. salexigens* are probably coded by the genes located in close proximity to the *ectD* gene. The Northern blot analysis has shown that the size of mRNA transcribed from the promoter of the *ectD* gene in *S. salexigens* exceeds the gene size by 900 bp. Analysis of the nucleotide sequence has shown that the DNA region downstream the *ectD* gene may contain an open reading frame (ORF), the translated amino acid sequence of which shows similarity with transcriptional regulators of the MarR family [24]. Deletion of the ORF (the *ectR* gene), located upstream the *ectD* gene in the DNA sequence of *C. salexigens*, resulted in diminution of hydroxyectoine synthesis during the growth under high salinity and temperature compared to the wild type strain. Thus, these findings suggest that the protein EctR in *C. salexigens*, which shows similarity with transcriptional regulators of the AraC family, is connected with the promoter region *PectD* and serves as a transcriptional activator of the *ectD* gene [26].

It should be noted that the level of expression of the ectoine biosynthesis genes depends on the nature of the anion, rather than on osmotic pressure as such. The method of real-time PCR showed that the increase in osmolarity of the medium owing to addition of NaCl, $NaNO_3$, or sodium gluconate caused an increase in mRNA quantity of the *ectABC* genes in *H. halophilus*, whereas the addition of sodium glutamate caused an insignificant change in transcription of the genes of ectoine synthesis. After osmotic shock by NaCl, $NaNO_3$, gluconate, glutamate, sucrose, glycine, Na_2SO_4, succinate, or tartrate, the quantity of EctC and ectoine increased in *H. halophilus* cells most significantly on addition of NaCl and $NaNO_3$; if the osmolarity was changed by other salts, the levels of EctC and ectoine increased insignificantly [47].

Until recently, the main working hypothesis was that ectoine biosynthesis is regulated at the transcriptional level due to replacement of sigma factors. The vegetative sigma factor, which supports the low constitutive transcription of the genes of ectoine biosynthesis to maintain the basal level of ectoine, is replaced by the stress sigma factor when the osmolarity of the medium increases. Additional regulatory elements involved in the regulation of hydroxyectoine biosynthesis under temperature stress in *S. salexigens* and *C. salexigens* are probably the products of the ORF located in close proximity to the *ectD* gene (Fig. 2).

TRANSCRIPTIONAL REGULATION OF ECTOINE BIOSYNTHESIS GENES IN AEROBIC METHYLOTROPHIC BACTERIA

The sequencing and analysis of the nucleotide sequence upstream of the ectoine biosynthesis genes in *M. alcalica* (GenBank no. EU315063), *Mm. alcaliphilum* 20Z, and *M. thalassica* revealed the presence of ORF (that were termed as *ectR* genes), the products of which were similar to the known transcriptional regulators of the MarR family. The EctR proteins of methylotrophic bacteria demonstrate low homology (12–20% of identity) with transcriptional regulators of the MarR family. However, in spite of the low homology, EctR of methylotrophic bacteria have a domain structure similar to that of the MarR proteins and contain DNA-binding motives: "HTH" (helix–turn–helix) and "wing" analogous to the respective motives (IPR000835 and IPR011991; InterPro database) of the MarR transcriptional regulators.

Fluorescence measurement of GFP, the gene of which was cloned under control of the *ect* genes promoter, in the mutant strain *Mm. alcaliphilum* 20Z with the *ectR* gene knockout showed the higher activity of the *ectAp* promoter during cell growth in the medium containing 1, 3, and 6% NaCl compared to the wild-type strain. Moreover, the activity of DAB acetyltransferase in the strain *Mm. alcaliphilum* 20Z with a deletion in the *ectR* gene was two to six times higher than in wild-type cells, demonstrating the enhanced level of transcription. Consequently, EctR is a repressor of transcription of the *ect* operon in *Mm. alcaliphilum* 20Z [48].

In *Mm. alcaliphilum* 20Z and *M. thalassica*, the *ectABC-ask* genes are transcribed from two promoters showing homology with the σ_{70}-dependent promoter of *E. coli*; the –10 and –35 sequences of *ectAp1* promoters in the methanotroph and in the methylobacterium are identical. Since the sequences of the *ectAp1* promoter are the closest to the canonic ones, it is possible that expression of the *ect* operon from this promoter is more efficient than from the *ectAp2* promoter. Transcription from the stronger promoter *ectAp1* is probably regulated by the osmolarity of the medium, while the *ectAp2* promoter carries out weak constitutive transcription of the *ect* genes. At the same time, promoter regions of the *ectABCask* operon in methylotrophic bacteria demonstrated no sequences homologous to the σ_S-dependent promoter of *E. coli*.

The *ectR* gene in *Mm. alcaliphilum* 20Z is transcribed from the only promoter, also showing similarity with the σ_{70}-dependent promoter of *E. coli*. On the contrary, the *ectR* gene in *M. thalassica* is transcribed from three promoters and the –10 and –35 sequences of only one of them are homologous to the canonic

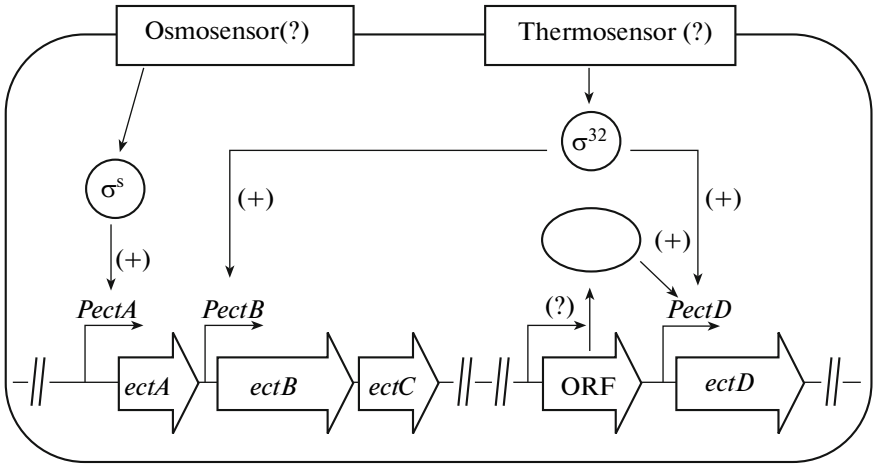

Fig. 2. The modern concept of the regulation of ectoine and hydroxyectoine biosynthesis in *C. salexigens*. Cited from [47].

sequences of the σ_{70}-dependent promoter of *E. coli*, while the respective sequences of other two promoters are degenerate.

Thus, in *Mm. alcaliphilum* 20Z, the promoter region (*ectR1p*) of the *ectR* gene is located between the *ectAp1* and *ectAp2* promoters of the *ect* operon, indicating that transcription from the *ectR1p* promoter may be controlled by its own product, the protein EctR. In the methanotroph, this protein seems to be an autoregulator similar to some representatives of transcriptional regulators of the MarR family.

On the contrary, in *M. thalassica*, the *ectR1p1, ectR1p2*, and *ectR1p3* promoters of the *ectR* gene and the promoter region of *ect* operon do not overlap. The EctR binding site has not been found in promoter regions of the *ectR* gene. It is likely that the *ectR* gene in *M. thalassica* is transcribed constitutively but at a low level.

It has been ascertained that the addition of EctR from *Mm. alcaliphilum* 20Z results in a decrease in electrophoretic mobility of the PCR fragment carrying the promoter region of the *ect* operon, both in the presence and absence of heterologous DNA. Consequently, EctR specifically binds to the promoter region of the *ect* operon. Mapping of the protein recognition site using DNAse I shows that EctR of *Mm. alcaliphilum* 20Z protects the extensive asymmetrical DNA region bearing the –10 sequence of the *ectA1p* promoter of the *ect* operon. Moreover, within the nucleotide sequences protected by EctR, a pseudopalindrome may be distinguished consisting of a degenerate inverted repeat (shoulder) of 8 bp in length. It implies that the protein is associated with DNA as a dimer, and inverted repeats are the binding sites for each subunit. Gel filtration showed that EctR from *Mm. alcaliphilum* 20Z is a dimer both in the free (molecular mass of 44–45 kDa) and DNA-bound (50–55 kDa) states.

Consequently, it may be supposed that the *ect* operon of *Mm. alcaliphilum* 20Z is constitutively transcribed from the weak promoter *ectAp2* at a low ionic strength of the growth medium. Transcription from the strong osmoregulated promoter *ectAp1* is repressed by EctR, which sterically inhibits the binding of RNA polymerase to the –10 sequence of the *ectAp1* promoter. Under increasing osmolarity of the medium, the EctR–DNA complex dissociates, making the *ectAp1* promoter accessible for RNA polymerase (Fig. 3).

It is still unclear how osmolarity of the medium regulates the expression of ectoine biosynthesis genes via the regulatory protein EctR. It would be logical to suggest that transcription of the *ect* operon is regulated due to changes in the DNA-binding activity of EctR, which, in turn, depends on external salinity. However, this activity of EctR cannot be directly regulated by salt concentration in the medium, because the *ect* operon is also expressed in *Mm. alcaliphilum* 20Z in a low-salinity medium. It would be reasonable to suppose that transcriptional regulation of ectoine biosynthesis is carried out due to posttranslational modification of EctR, e.g., via phosphorylation/dephosphorylation, resulting in changes in its conformation and, accordingly, in its DNA-binding activity. Similar regulation depending on the osmolarity of the medium has been shown for Kdp and OmpC/OmpF transport systems [37]. However, the strict interrelation between variations in the ambient salinity of, regulation of ectoine synthesis, and total cell response is indicative of a more complex regulatory mechanism not confined to replacement of sigma factors and involvement of the transcriptional repressor EctR.

CONCLUSIONS

The necessity of understanding the principles of organization and regulation of the genes and enzymes

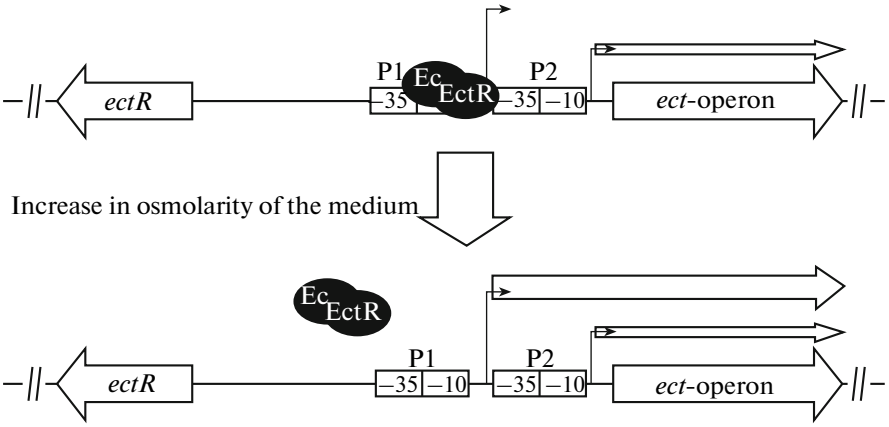

Fig. 3. Transcriptional regulation of the ectoine biosynthesis genes in *Mm. alcaliphilum* 20Z. P1 and P2 correspond to the promoters *ectAp1* and *ectAp2*.

of ectoine biosynthesis in aerobic methylotrophs is dictated by the practical tasks of developing a technology for production of this bioprotecant, which is increasingly used in medicine, cosmetics, and research practice as a stabilizer for biomolecules and whole cells and a water-retaining agent [49]. The method of ectoine production implemented at the Biomol company (Germany) relies on the heterotrophic bacterium *Halomonas elongata* on the medium with glucose, L-amino acids, and 12% NaCl. Moderately halophilic methanotrophs growing on the medium with 6% NaCl can accumulate up to 20% ectoine, i.e., more than heterotrophic producers growing with 12% NaCl [50, 51]. Hence, halophilic methanotrophs can be considered as potential ectoine producers. Different levels of the osmoprotectant may result from genetically determined regulatory mechanisms of ectoine biosynthesis. For example, ectoine is synthesized by halophilic methanotrophs via a biochemical pathway similar to that in heterotrophic halophilic bacteria [17]. *Mm. alcaliphilum* 20Z, *M. alcalica* and *M. thalassica* are characterized by substantially different organization of the ectoine biosynthesis genes, since they form a four-gene *ectABCask* cluster including an additional aspartate kinase gene. This suggests the presence of a specific aspartate kinase isoform, which is probably responsible for the synthesis of ectoine precursors (aspartyl phosphate and aspartyl semialdehyde) relatively independent of the main constructive metabolism.

Osmoadaptation of halophilic bacteria, in addition to the synthesis of osmoprotectants (ectoine, glutamate, and sucrose), includes significant structural and functional changes (in the fatty acid and phospholipid membrane composition) [50]. At present, it seems impossible to describe the whole regulatory cascade, beginning from the perception by the cell of signals of the changes in osmotic conditions and finishing with the structural and functional rearrangements. It is necessary to ascertain the nature of the primary signal and determine the osmosensors and mechanisms of signal transduction from the cell membrane to possible transcriptional regulators. Moreover, it is extremely important to identify other osmoregulatory genes and to reveal the relations between the systems of response to various stress factors, such as temperature, osmolarity, pH, etc., which would allow us to understand and interpret the complex mechanism of cross adaptation of bacteria to the varying environmental conditions.

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