Genetic and Biochemical Aspects of Ectoine Biosynthesis in Moderately Halophilic and Halotolerant Methylotrophic Bacteria

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INTRODUCTION

The ability of microbial cells to adapt to fluctuations of external osmolarity (osmoadaptation) via activation of specific mechanisms (osmoregulation) is essential for their survival. Halophilic and halotolerant microorganisms have evolved a number of metabolic, enzymatic and regulatory mechanisms that prevent cell dehydration. Osmoadaptation based on accumulation of inorganic ions, mostly K⁺ (salt type of osmoadaptation), is employed in extremely halophilic members of Halobacteriaceae and Halanaerobiales, as well as anaerobic acetogenic species of Halobacteroides, Sporohalobacter, Acetohalobium and sulfate reducers (Desulfovibrio halophilus, Desulfohalobium retbaense) (Zhilina and Zavarzin, 1990; Galinski and Truper, 1994). Structural and metabolic organization of cells of extremely halophilic...
bacteria is well adapted to high intracellular concentrations of ions. Moreover, it often depends on high salinity for optimal operation. For example, malate dehydrogenase of *Halobacterium salinarum* and halophilic archaeon *Haloarcula marismortui* is more acidic and require K⁺ for compact folding and enzyme activity (Marhuenza-Egea and Bonete, 2002; Mevarech et al., 2000). Another osmoadaptation strategy is based on synthesis of small organic molecules (osmolytes). It occurs in a majority of bacteria, archaea, fungi, plants and even animals (Galinski and Truper, 1994; da Costa et al., 1998; Ventosa and Nieto, 1995). Osmolytes (also known as compatible solutes) do not interfere with cellular metabolism. They are represented by compounds of different classes: (i) zwitterions (amino acids and their derivatives including ectoines and betaines), (ii) neutral solutes (sugars and polyols) and (iii) anionic solutes where the negative charge is supplied by a carboxylate, phosphate or sulfate (Galinski, 1995; Roberts, 2004; 2005). Many bacteria, including non-halophilic, accumulate compatible solutes at hyperosmotic conditions either through de novo synthesis or by uptake from surrounding environment.

Ectoine, a cyclic imino acid (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) was originally discovered as a compatible solute in an anoxygenic phototrophs of the *Ectothiorhodospira* group (Galinski et al., 1985). Later, it was found in many aerobic halophilic and halotolerant gram-negative and gram-positive bacteria including *Nocardiopsis*, *Brevibacterium*, *Marinococcus*, *Halomonas*, *Pseudomonas* and *Vibrio* (Ventosa and Nieto, 1995; Galinski, 1995; Kempf and Bremer, 1998; Grant, 2004; Severin et al., 1992). Ectoine and its hydroxylated derivative, hydroxyectoine, are powerful multifunctional bioprotectants that defend against a variety of damaging factors such as heating, freezing, desiccation and UV radiation (Jebar et al., 1992; Buenger and Driller, 2004; Graf et al., 2008). Both compounds are widely used in medicine and cosmetics as brought-range stabilizers. In spite of a rising commercial interest the genetics and biochemistry of ectoine biosynthesis are intensively studied in various bacteria. We refer here to the genetic and enzymatic aspects of the ectoine biosynthesis pathway in aerobic methylotrophic bacteria which utilize single carbon compounds (methane, methanol or methylamines) as the carbon and energy sources. These moderately halophilic and halotolerant methylotrophs have been isolated from soda lakes and (hyper) saline environments. The members of the genera *Methylomicrobium*, *Methylobacter*, *Methylophaga* (Gammaproteobacteria) were shown to synthesize ectoine as an osmoprotectant along with sucrose and glutamate (Khmelenina et al., 1999; Doronina et al., 2003a; 2003b; Kaluzhnaya et al., 2001; 2008). Ectoine was also identified in cells of *Methylarcula* species (Alphaproteobacteria) grown at high salinity (Doronina et al., 2000).

**MATERIALS AND METHODS**

**Genetic aspects of ectoine biosynthesis:** The ectoine biosynthesis represents a branch of the metabolic road for aspartate family amino acids synthesis (Fig. 1) and involves three special enzymes: Diaminobutyric Acid (DABA) aminotransferase (EctB) that catalyses amination of aspartate semialdehyde into diaminobutyric acid, DABA acetyltransferase (EctA) acetylylating DABA into Ny-acetyl diaminobutyric acid and ectoine synthase (EctC) that forms ectoine by cycling of Ny-acetyl diaminobutyric acid (Peters et al., 1990). In various halophilic bacteria studied, the genes coding for the enzymes form a cluster ectABC (Canovas et al., 1998; Louis and Galinski, 1997; Goller et al., 1998; Kuhlmann and Bremer, 2002). Some bacteria possess an additional enzyme that converts ectoine to hydroxyectoine (ectoine hydroxylase, encoded by ectD) (Canovas et al., 1999; Prabhu et al., 2004; Bursy et al., 2007). The ect-genes are usually, but not always, organized into more or less compact operon.

Analysis of complete microbial genomes showed that the ect-genes are widespread among *Proteobacteria* and *Actinobacteria* (Fig. 2). The genetic signature of the ectoine pathway, an ectABC operon, was also identified in genome of *Nitrosopumilus maritimus*, a non-thermophilic archaeon isolated from a salt-water aquarium. Rather high homology of the ectoine biosynthetic genes in microorganisms of different taxonomic position and physiology is a reliable indication of the evolutionary conservation of the biochemical pathway (Kuhlmann and Bremer, 2002).

High homology of the ect-genes in microorganisms allowed identification of the genes in some aerobic methylotrophs by using PCR-surveys (Reshetnikov et al., 2006). ectABC or ectABC-ask clusters were identified in six species of genera *Methylomicrobium*, *Methylobacter*, *Methylophaga* and *Methylarcula*. The organization of the ect-genes (three or four gene cluster) correlates with halotolerance of the host strain. Two halotolerant methanotrophs, *Methylomicrobium kenyense* AM01 and *Methylobacter marinus* 7°C possess three gene operon (ectABC), accumulate ectoine up to 70 mg g⁻¹ of Dry Cell Weight (DCW) and are capable of growth at salinity 4-5% NaCl.
In contrast, methanotrophic species *Methylocaldicrobium alcaliphilum* and methanol- and methylamine-utilizers *Methylophaga alcalica*, *Methylophaga thalassica* and *Methylocarcina marina* possess ectABC-ask operon, can grow at two-fold higher salinity (10-12% NaCl) and accumulate >20 mg of ectoine g\(^{-1}\) of DCW. This implies an important role of the ectoine pathway specific aspartokinase in some methylotrophic species. It was demonstrated that in *Mm. alcaliphilum*, genes ectABC and ask are co-transcribed and thus the aspartokinase must be osmotically controlled (Reshetnikov et al., 2006). One could speculate that the occurrence of the aspartokinase isozyme may provide an advantage at high osmolarity as it makes ectoine biosynthesis independent from complex machinery regulating the amino acid biosynthesis. However, in this case a culture must possess an additional enzyme that could fulfill the pool of amino acid for protein synthesis at low salinity. Indeed, a copy of gene coding for aspartokinase with 77% identity of the translated amino acids is present in *Mm. alcaliphilum* as followed from a draft genome sequence analyses (https://www.genoscope.cns.fr/age/mage).

Analyses of the publicly available complete microbial genomes showed that at least 30 bacterial species harbor homologs of the ectABC-ask genes. Bacteria possessing an ask gene immediately downstream of ectABC are exclusively gram-negative. In the majority of the proteobacterial halophiles, a second, additional copy of the aspartokinase gene was identified. Two and three additional ask genes not linked to the ect-gene cluster occur in *Photobacterium profundum* and *Vibrio cholerae*, respectively. Interestingly, no aspartokinase gene outside of the ect-cluster was found in three species of marine bacteria, *Oceanobacter* sp. RED65, *Sphingomonas* sp. SKA58 and *Lentisphaera araneosa*. In these bacteria the sole ectoine-associated Ask should support both cellular processes-osmoresistance and protein biosynthetic needs. Most likely in these marine bacteria the ect-operon and linked ask gene are constitutively expressed. However, this hypothesis remains to be tested by the comprehensive description of the gene expression and the enzyme properties.

It should be mentioned that in gram-positive bacteria ectoine is not the sole osmoprotectant and other organic compatible solutes belonging to the glutamate family aminoacids, glutamate, glutamine and/or proline contribute to osmotic balance (Kuhlmann and Bremer, 2002; Lo et al., 2009; Saum and Muller, 2007; 2008a; 2008b).
This may require a complex regulation since the carbon flow must be also directed to biosynthesis of the osmoprotectants not derived from aspartic acid. It may explain the absence of the ask homologues in the ect-gene cluster of gram-positive bacteria. Remarkably, the halophilic Firmicutes typically possess multiple ask homologues not linked to the ect-genes (Lo et al., 2009).

Phylogenetic relationships of the EctB from bacteria of different physiological groups are shown in Fig. 2. The topology of the EctB tree was similar to those of EctA- and EctC-based phylogenetic trees (data not shown). In general, the Ect proteins from bacteria belonging to the Alpha- and Gammaproteobacteria, Firmicutes and Actinobacteria comprise separate
branches on the trees. This may imply an ancient acquisition of the ect-genes from common ancestor and prolonged evolution inside the respective microbial phylum. Interestingly, the Ect proteins of methane- and methanol-utilizing bacteria of the Methylophilaceae and Methylophilaceae genera are combined in the coherent group on the tree being most closely related to other representatives of the Gammaproteobacteria. On the other hand, the Ect proteins from *Mm. kenyense* AMO1 are more identical to those of the methanol-utilizers than other methanotrophs. Since the bacterium carries the ectABC gene cluster, a recent loss of the ask gene may be proposed. Almost identical Ect proteins (94.8, 98.2 and 98.5% for EctA, EctB and EctC) are present in two strains of the *Mm. alcaliphilum*, 20Z and ML1, isolated from the Tuva soda lake (Russia) and Mono Lake (USA), respectively. Hence, geographical distance of the bacterial habitats has not resulted in significant divergence of the ect genes.

Notably, Ect proteins from *Mb. marinus* 7°C, a methanotroph belonging to the Gammaproteobacteria, are only distantly related to ectoine biosynthetic enzymes of other methylophilous (only 37-51% identities to EctABC sequences of *Mm. alcaliphilum* 20Z) and fall with proteins of the marine representatives of Proteobacteria. Ectoine biosynthesis proteins from *Mb. marinus* 7°C are closely related to those from the marine *Maripropus dens ferrooxydans* (Zetaproteobacteria) (55-80% identities). However, in *M. ferrooxydans* the ectoine biosynthetic pathway is encoded by an ectABC-ask operon, while the ect-cluster from strain 7C lacks ask gene. So far, *Mb. marinus* is the only representative of the Methylobacter genus that is salt-resistant and synthesizes ectoine. Other described species of the genus are non-halophilic. It could be proposed that the ect-operon ubiquity in ancient prokaryotic world that was largely marine, followed by loss in lineages that became adapted to a terrestrial environment (Lo et al., 2009). It is also possible that an ancestor, terrestrial species of Methylobacter adapted to marine ecosystem by acquiring ectoine biosynthesis genes from phylogenetically distant species.

The biosynthesis of hydroxyectoine proceeds via direct hydroxylation of ectoine catalyzed by ectoine hydroxylase (EctD) (Bursy et al., 2007; Reuter et al., 2010). In *Mm. alcaliphilum*, an orf with a high homology to ectD (40-42% identity to ectoine hydroxylases from *Streptomyces avermitilis* MA-4680 and *S. chrysomallus*) was identified 360 bp downstream of the ectABC-ask operon (Reshetnikov et al., 2006). No hydroxyectoine accumulation was detected in *Mm. alcaliphilum* cells grown in the presence of 3 or 9% NaCl. However, it does not exclude that the methanotroph can produce the bioprotectant at some environmental perturbations. NR-Database searches revealed that genomes of 67 proteobacteria and one archaeon, *Nitrosopumilus maritimus*, contain homologues of the *ectD* gene (with identities ranging from 41-55% to that of *V. saleigens*) (Reuter et al., 2010). ectD-like gene is either a part of the ect-cluster or it locates separately. Majority of the microbial genomes contain only one copy of the *ectD* gene, with exception to *Rhodococcus opacus* B4, *Marinobacter aquaeolei* VT8 and *C. saleigens*, that possess two types of EctD-proteins (Reuter et al., 2010). In the case of *C. saleigens*, only one of the EctD-like enzymes contributes to the production of 5-hydroxyectoine (Garcia-Estepe et al., 2006) and the ectD gene is essential for thermoprotection of the bacterium.

It should be mentioned that an alternative pathway for transformation of Nγ-acetyl DABA into hydroxyectoine via 3-hydroxy-Nγ-acetyl DABA without ectoine formation step was proposed but this was not enzymatically proved (Canovas et al., 1999).

**RESULTS AND DIACUSSION**

**Some properties of the ectoine biosynthetic enzymes:** In the conditions of hyperosmotic stress (from 0.5-1 M NaCl), *H. elongata* cells with blocked by chloramphenicol protein biosynthesis, accumulated ectoine at the same level as the cells with active protein synthesis. Thus, the regulation of ectoine synthesis can proceed at the enzyme level (Kraegeloh and Kunte, 2002). Three ectoine biosynthesis specific enzymes have been purified from *H. elongata* DSM2581 (Ono et al., 1999). The enzymes are characterized by similar parameters for the maximal activity (pH 8.2-9.0, t = 15-20°C and 0.4-0.5 M NaCl). Since the optimal salt concentrations for the enzymes were lower than those in the medium supporting maximal growth rate of the bacteria, relatively low intracellular concentrations of ions should be maintained (Kraegeloh and Kunte, 2002). Indeed, Na⁺ content of 0.04-0.2 M was found in cells of *Vibrio costicola* u *Brevibacterium* sp. growing at high NaCl concentrations (Gilboa et al., 1991; Nagata et al., 1995).

**DABA aminotransferase (EctB, EC 2.6.1.76):** DABA aminotransferase from *H. elongata* is a homohexameric (~250 kDa) pyridoxal phosphate-dependent enzyme that requires K⁺ for activity and stability. The enzyme is more active in the presence of 0.01-0.5 M KCl, than in the presence of NaCl (Ono et al., 1999). K⁺ requirement is inherent to many enzymes from extremely halophilic
eubacteria and archae with the salt-type osmoadaptation (Marhuenda-Egea and Bonete, 2002; Mevarech et al., 2000; Toney et al., 1995). DABA aminotransferase is specific to L-glutamate, as an amino donor (K_m 9.1 mM) and to D, L-aspartyl semialdehyde (K_m 4.5 mM). The reaction catalyzed by DABA aminotransferase was predicted to be a limiting step of the ectoine biosynthesis pathway thus explaining the absence of DABA in cells of H. elongata KS3 (Ono et al., 1999).

Two putative DABA aminotransferase genes, one in ectABC-ask operon and another in a cluster of genes presumably involved in ectoine degradation, were found in the genome of M. alcaliphilum.

Interestingly, DABA aminotransferase was detected in several bacteria that do not synthesize ectoine. In Acinetobacter baumannii, DABA aminotransferase is involved in biosynthesis of diamonopropane, a component of cell wall peptidoglycan (Ikai and Yamamoto, 1997). In Paenibacillus polymyxa the enzyme is a part of the polymyxin, a peptide antibiotic, formation (Ono et al., 1999). In A. baumannii, it is specific to L-glutamate, whereas in Xanthomonas species to L-alanine as the amino donor (Rao et al., 1969).

**DABA acetyltransferase (EctA, EC 2.3.1.178):**

DABA acetyltransferase from H. elongata was purified (≈400 fold) and was only partially characterized due to low stability of the enzyme (Ono et al., 1999). Histagged DABA acetyltransferases were purified from Mm. alcaliphilum 20Z, M. alcalica and M. thalassica (Reshetnikov et al., 2005; Mustakhimov et al., 2008). The enzyme from these methylotrophs existed as a homodimer with the subunit molecular mass of ~20 kDa and had no requirement for divalent ions.

Some differences in the enzyme properties that correlated with eco-physiologies of these bacteria were described. Thus, the DABA acetyltransferase from the neutrophilic H. elongata and M. thalassica was more active at lower pH (pH 8.2 or pH 9.0), than that from alkaliphilic species Mm. alcaliphilum and M. alcalica (pH optima ≥9.5). Unlike enzymes from M. alcalica and Mm. alcaliphilum, DABA acetyltransferase from the marine bacterium M. thalassica was considerably inhibited by carbonates (Mustakhimov et al., 2008). Bearing in mind that growth of the former alkaliphilic methylotrophs is stimulated by carbonates, this feature of the enzyme corresponds to in situ surroundings of the strains. Interestingly, 1 mM Cu^{2+} completely inhibited activity of the DABA acetyltransferase from M. alcalica and 47% that of M. thalassica. In contrast, no inhibitory effect of Cu^{2+} was found for the enzyme from methanotroph Mm. alcaliphilum and this also correlates with an important role of copper in methane oxidation in the culture, since Cu is an essential component of the particulate methane monoxygenase (Murrell et al., 2000).

Another intriguing feature of DABA acetyltransferases from methylotrophic bacteria was the effect of ionic strength on enzyme activity. Like the enzyme from H. elongata, DABA acetyltransferase from methanotroph Mm. alcaliphilum was activated by 0.2 M NaCl implying the “halophilic nature” of the protein. Contrary, the enzymes from methylotrophic cultures, M. thalassica and M. alcalica were inhibited by salts. We may speculate that methanol is more effective substrate for supporting ion extrusion mechanisms in comparison to methane or glucose. As a result, low concentrations of monovalent inorganic ions could be maintained in cytoplasm of the methanol-utilizing bacteria eliminating need for ectoine biosynthetic enzymes that are adapted to high ionic strength.

**Ectoine synthase (EctC, EC 4.2.1.108):**

The homogenous ectoine synthase from H. elongata was purified in the presence of 1 mM Nγ-acetyl-DABA and 2 M NaCl as stabilizing compounds. The molecular mass of the native enzyme remains unclear due to loss of the enzyme activity after gel-filtration at 0.5 M NaCl. The EctC-protein contains enhanced levels of aspartate and glutamate. The enzyme is specific to Nγ-acetyl-DABA, however the N-acetyl group in α-position could not be involved in the cycling process (Ono et al., 1999). The recombinant ectoine synthase was purified from Mm. alcaliphilum 20Z with activity ~64 U mg⁻¹. The protein is a 35 kDa homodimer.

**Ectoine hydroxylase (EctD, EC 1.14.11):**

Was purified from the moderate halophile Virgibacillus (Salibacillus) salexigens (Bursy et al., 2007) and from the Streptomyces coelicolor (Bursy et al., 2008). EctD from V. salexigens is a monomeric protein of molecular mass 34 kDa being a member of the non-heme iron (II)- and 2-oxoglutarate-dependent dioxygenases. The reaction depends on iron (II), molecular oxygen and 2-oxoglutarate. The similar properties were observed for enzyme from Streptomyces coelicolor with respect to optimal pH (pH 7.5), temperature (32°C), K_m values for ectoine (3.5 and 2.6 mM) and co-substrate 2-oxoglutarate (5.2 and 6.2 mM). Contrary to preferential production of 5-hydroxyectoine by S. coelicolor grown at 39°C (Bursy et al., 2007), the high temperature optimum of the EctD enzyme implies that there is no specific thermoactivated regulation of the hydroxylase.
The crystal structure analysis of the V. salexigens EctD (Reuter et al., 2010) showed that the folding of the protein is similar to the human phytanoyl-CoA 2-hydroxylase (McDonough et al., 2005). The core of the EctD structure consists of a double-stranded β-helix forming the main portion of the active site of the enzyme. The positioning of the iron ligand in the active site of EctD is mediated by an evolutionarily conserved 2-His-1-carboxylate iron-binding motif. The side chains of the three residues forming this iron-binding site protrude into a deep cavity in the EctD structure that also harbors the 2-oxoglutarate binding site. Despite high homology and similar reactions catalyzed by the ectoine hydroxylase and L-proline hydroxylase (Hausinger, 2004), the EctD from S. coelicolor had no additional L-proline hydroxylase activity (Bursy et al., 2007).

Transcriptional regulation of the ectoine biosynthesis genes:

Regulation of the ectoine biosynthesis genes in heterotrophic bacteria: To date, there are only fragmentary data on how an external salinity regulates expression of ectoine biosynthetic genes. In Bacillus pasteurii (Kuhlmann and Bremer, 2002), Marinococcus halophilus (Bestvater and Galinski, 2002), Halobacillus halophilus (Saum and Muller, 2005) and S. salexigens (Bursy et al., 2007), transcription of the ectABC-ask or ectABC genes proceeded as polycistronic mRNAs and subjected to osmotic conditions. The osmoregulated expression of ectABC was also revealed in gram-negative Brevibacterium epidermidis (Orrade et al., 2004), Mm alcaliphilum (Reshetnikov et al., 2006; Mustakhimov et al., 2010) and Chromohalobacter salexigens (Calderon et al., 2004).

In C. salexigens, the ectABC operon is transcribed from several promoter regions. Four putative promoters (PectA1-4) are located upstream of the ectA gene and one internal promoter PectB is upstream of the ectB gene (Calderon et al., 2004). The consensus sequences of the ect promoters closely resembled those of σ70, (PectA1 and PectA2), σ70 (PectA3) and σ32 (PectB) dependent promoters of E. coli. Both PectA and PectB promoter regions were shown to be osmoregulated. Transcription from the PectB was also enhanced upon increasing growth temperature. Expression of the reporter gene lacZ under control of the PectA in recombinant C. salexigens and E. coli cells was considerably increased when the cultures approached stationary growth phase. Moreover, in the mutant E. coli lacking the rpoS gene, expression of the PectA-lacZ fusion was much lower than in the wild type strain. This was consistent with maximal ectoine accumulation in the stationary growth phase (Canovas et al., 1999). It could be reasonable to propose an involvement of the general stress factor σ70, however, no rpoS-like gene has been identified in C. salexigens (Calderon et al., 2004). Participation of different transcriptional sigma factors in expression of the ect-genes was not confirmed by an appropriate genetic study. Supplementation of the growth media with ectoine or glycine betaine decreased the transcription of PectA-lacZ and PectB-lacZ (Calderon et al., 2004). Altogether with constitutive ect-genes transcription from PectA and PectB at low osmolarity, this implies an involvement of other transcriptional factors.

The recent studies have shown a connection between the iron homeostasis and the osmoreponse in C. salexigens (Argandona et al., 2010). An iron homeostasis regulator, Fur, has been described as a potential regulatory link between salinity and iron metabolism. Corresponding six-gene operon cfuABC-fur-hisI-orf6 was identified downstream of the ectABC genes. Fur boxes were found in promoters of the cfuABC-fur-hisI-orf6 and ectABC operons. It was shown that Fur mediates the osmoregulated inhibition by iron of cfuABC-fur-hisI-orf6 expression and functioned as a positive regulator of the ectABC genes under high-salinity conditions.

In gram-positive bacteria, ectoine biosynthesis also seems to be triggered by an osmotic stress. The vegetative σ5-like promoter (equivalent to σ70 like promoter of gram-negative bacteria) was found upstream of the ectABC in Bacillus subtilis, B. pasteurii and S. salexigens (Kuhlmann and Bremer, 2002; Bursy et al., 2007). In Marinococcus halophilus, the transcription of ectABC genes was initiated from three individual σ70/σ5-like-promoters located upstream of each gene (Bestvater and Galinski, 2002). The σ70/σ5-like-promoter sequences were amplified and cloned upstream of gfp in vector pBR322. A linear increase in fluorescence of E. coli cells upon increase of NaCl concentration in the medium was detected, however, the addition of ectoine and betaine decreased the fluorescence signal (Bestvater and Galinski, 2002).

Moderately halophilic Halobacillus halophilus served as a prominent model bacterium to decipher regulatory mechanisms of the osmoresponse. When grown at moderate salinity (1 M NaCl), H. halophilus accumulated glutamate and glutamine as the major compatible solutes whereas ectoine and proline were predominantly produced at very high salinities (Saum and Muller, 2008a; 2008b). Ectoine/proline ratio that was low in cells growing exponentially at different salinities (2 and 3 M NaCl) enhanced more than 1000-fold in the stationary growth phase. The proline
subjected simultaneously to chilling (15°C) and salinity stress (0.7 M NaCl). Transcripts were detected triggered by low growth temperatures (Kuhlmann 2008). The highest level of ectoine and the expression of the respective genes for glutamate dehydrogenase, glutamine synthetase and pyrroline-5-carboxylate reductase. The expression of ecto-genese reached the maximal level when concentration of transcripts for genes for glutamate, glutamine and proline biosynthesis returned almost to the initial levels. The ectABC transcript concentration was highest in the presence of NaNO3. Na-gluconate was as efficient as NaCl while Na-glutamate led only to a minor increase of the transcripts. Hence, transcription of the ecto-genese did not necessarily depend on the presence of chloride anions (Saum and Muller, 2008b).

It was demonstrated that in C. salexigens and Streptomyces griseus, intracellular levels of ectoine increase in response to high growth temperature (Calderon et al., 2004; Malin and Lapidot, 1996). These findings suggest a role of ectoine as a protectant against the detrimental effects of high temperatures. In contrast, the ectoine biosynthesis in V. pantothenticus was triggered by low growth temperatures (Kuhlmann et al., 2008). The highest level of ectoine and the ectABC transcripts were detected in V. pantothenticus cells subjected simultaneously to chilling (15°C) and salt stress (0.7 M NaCl).

Several additional regulatory elements of hydroxyectoine biosynthesis can be recognized in C. salexigens and S. salexigens. In the latter, mRNA transcribed from the ectD promoter was 900 b.p. being larger than ectD sequence. An orf similar to MarR-family of transcriptional regulators was revealed downstream of the ectD (Bursy et al., 2007). However, the putative regulatory protein is disrupted by two stop codons and no start codon could be found. In C. salexigens, the orf (gene ectR) was found upstream of ectD (Vargas et al., 2008; Garcia-Estepa et al., 2006). Deletion of the ectR gene resulted in decreasing of hydroxyectoine level at high salinity and high temperature, in comparison to the wild type cells. Thus, EctR (presumably belonging to the AraC family of transcriptional regulators) could be an activator of the ectD transcription.

Recent findings in transcriptional regulation of ectoine biosynthesis genes in methylo trophs: In M. alcaliphilum, transcription of the ectABC-ask operon is initiated from two σ70-dependent promoters ectAp1 and ectAp2 (Fig. 3). The predicted -10 (TACTAT) and -35 (TGGACA) regions of ectAp1 showed rather high level of identity with the consensus sequence of the E. coli σ70-recognized promoter (Reshetnikov et al., 2006; Mustakhimov et al., 2010). The putative -10 and -35 sequences of the ectAp2 promoter differ from the respective regions of the E. coli σ70-promoter thus suggesting that expression from ectAp2 may be less effective, than from ectAp1.

Upstream of the ectA gene an orf (gene ectR1) encoding the MarR-like transcriptional regulator with 12-20% identity of translated amino acid sequences was found. Despite low identity, the protein has a structure analogous to the MarR-family regulators: it has the Helix-Turn-Helix (HTH) DNA-binding motif flanking the “wing 1” region (Mustakhimov et al., 2010; Hong et al., 2005; Wilkinson and Grove, 2006). Regulatory function of EctR1 in ectoine biosynthesis was elucidated by characterization of the ectR1 knockout mutant. A promoter-reported system (ectP-gfp fusion) was constructed and introduced into Mm. alcaliphilum wild type and mutant strains. The fluorescence signal in the strain lacking ectR1 was 2-3 folds higher compared to wild type cells. The DABA acetyltransferase activity was also 2-6 times higher in the mutant cells. Thus, EctR1 negatively controlled transcription of the ecto-genese in Mm. alcaliphilum. Expression of the ectABC-ask operon in the ectR1 mutant was still activated by increasing of the medium salinity (from 1-6% NaCl) thus indicating that Mm. alcaliphilum may possess several regulatory systems.
Transcription of the gene ectR1, in turn, was carried out from a single $\sigma^{70}$-like promoter (Fig. 3). The promoter region of ectR1, ectR1p, is located between ectAp1 and ectAp2, suggesting, that its transcription may be controlled by the EctR. The autoregulation was described for some other MarR proteins. The purified EctR1 specifically binds to the promoter region of ectABC-ask operon (Mustakhimov et al., 2010). The EctR1 binding site contains a pseudopalindromic sequence (TATTTAGT-GT-ACTATATA) composed of 8-bp half-sites separated by 2 bp suggesting dimeric association of the EctR1 with the DNA where each protein subunit binds with an inverted repeat (Mustakhimov et al., 2010). Indeed, gel-filtration studies showed that EctR is a dimer both in free solution (m.m. 44-45 kDa) and in DNA binding state (m.m. 50-55 kDa). Hence, it may be proposed that at the low osmolarity, the ectABC-ask genes of *Mm. alcaliphilum* are constitutively transcribed from the weak promoter ectA2p (Fig. 3). EctR1 sterically inhibits the RNA polymerase binding with -10 sequence of the promoter ectA1p and thus represses transcription from the osmoregulating promoter ectA1p. At the elevated external salinity, EctR-DNA complex dissociates making the promoter ectA1p to be accessible for RNA polymerase (Fig. 3).

The EctR1 orthologs were identified in other halophilic bacteria. Our analysis of the DNA fragment containing the ectoine biosynthetic genes in the methanol-utilizing bacterium *M. alcalica* (Mustakhimov et al., 2009) showed the presence of an orf with high homology to the ectR1 gene from *Mm. alcaliphilum* (73% identity of translated amino acids). Moreover, a simple NCBI Blast search revealed several
ectR1-like genes located immediately upstream of the ectoine gene cluster in 17 halophilic bacterial species (Fig. 4). Between them, the orfs of Oceanospirillum sp. (EAR60187), Nitrosococcus oceanis (ABA57535), Saccharophagus degradans (ABD00450), Reinekeea sp. (ZP_01114878) and Oceanobacter sp. (EAT11341) showed the highest identities of translated amino acid sequences with the EctR1 from Mm. alcaliphilum (35.5, 42.2, 45.6, 51.7 and 55.1%, respectively) which are higher than with other MarR-family transcriptional proteins (<20%). We propose that putative EctR1 of higher than with other MarR-family transcriptional proteins (42.2, 45.6, 51.7 and 55.1%, respectively) which are controlling ectoine biosynthesis at the transcriptional level may operate in diverse halophilic and halotolerant bacteria.

So far, it is not clear how changes in external osmolarity may regulate expression of the ectoine biosynthetic genes via the EctR1. It seems logical to propose that DNA binding ability depends on the salinity level may operate in diverse halophilic and halotolerant bacteria.

CONCLUSION

The necessity of unraveling the principles of organization and regulation of the genes and enzymes involved in ectoine biosynthesis by aerobic methylotrophs is conditioned by practical demands for a technology for production of the multifunctional valuable bioprotectants from cheap sources of carbon, such as methane and methanol. Large-scale production of ectoine has been achieved by the “Bitop” and “Merck” (Germany) using heterotrophic bacterium Halomonas elongata. This milking bioprocess is based on the use of the glucose, L-amino acids (glutamate) and highly saline medium (12% NaCl) (Sauer and Galinski, 1998). Aerobic moderately halophilic and halotolerant methylotrophic bacteria when grown at 9% NaCl are capable of accumulation ectoine up to 20% of DCW and therefore, are promising ectoine producers from renewable methane and/or methanol. The differences in ectoine accumulation may be caused by genetically defined regulatory mechanisms in the bacterial producers. Although the enzymology and genetics of the ectoine biosynthesis pathway in methylo trophs are similar to halophilic bacteria, they may have quite different regulatory patterns. For instance, the presence of the osmotically controlled aspartokinase in methylotrophs could make ectoine synthesis rather independent on other amino acids biosynthesis. Such co-ordination of aspartylphosphate synthesis, a common precursor of both ectoine and the amino acids, may provide an advantage during methylotrophic growth at high external osmolarity.

Osmoadaptation of aerobic methylotrophs, besides the osmoprotective compatible solutes biosynthesis (ectoine, glutamate and in some case sucrose), includes other structure-functional mechanisms such as changes in phospholipids fatty acids composition and in bioenergetic machinery. To date, it is not possible to describe whole regulatory cascade from sensing signals and receivers on a cell membrane to the real metabolic and structural rearrangements. Clarifying the nature of initial signals and sensors on a cell membrane and signal transduction to potential transcriptional regulators is a challenging task for future genomic transcriptomic and proteomic studies. The elucidation of cell responses to external perturbations, such as temperature, pH and others will shed more light on still enigmatic mechanisms of cross-adaptation of bacteria to fluctuating environmental conditions.

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