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SHORT COMMUNICATIONS

Identification of Ectoine Synthesis Genes in a Moderate Halophilic Alphaproteobacterium *Methylarcula marina*

A. S. Reshetnikov, V. N. Khmelenina, and Yu. A. Trotsenko[1]

Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia

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Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid), an osmolyte widespread among halophilic and halotolerant prokaryotes, is also a polyfunctional bioprotectant. Ectoine biosynthesis from aspartate requires a number of specific enzymes including L-2,4-diaminobutyrate (DAB) aminotransferase (EctB), DAB acetyltransferase (EctA), and ectoine synthase (EctC). The genes coding for the enzymes in most of the studied ectoine producers are organized into the clusters *ectABC* or *ectABC-ask*, the latter encoding also a specific isoform of aspartate kinase (Ask) [1, 2].

Earlier, ectoine and its biosynthesis genes were revealed in methane- or methanol-consuming halophilic methylotrophic gammaproteobacteria of the genera *Methylomicrobium* and *Methylophaga* [2, 3]. Apart from ectoine, these methylotrophs accumulate glutamate and sucrose as additional osmolytes. Search and study of new methylotrophic producers of ectoine is in demand due to the practical needs to produce this bioprotectant from single-carbon substrates (methane, methanol, etc).

The aerobic methylobacterium *Methylarcula marina* hal1 grows on methylamine at salinity range of 1–10% NaCl and synthesizes ectoine as an osmoprotectant (up to 20% to dry cell weight) but does not accumulate sucrose [4]. Inability of *M. marina* to synthesize sucrose provides several advantages since it implies a more efficient conversion of a single-carbon substrate into the end product and excludes a number of additional ectoine purification steps. In contrast to the previously studied methylotrophs, *M. marina* belongs to the *Alphaproteobacteria*. Although the genes and enzymes responsible for ectoine biosynthesis have not been investigated in representatives of the *Alphaproteobacteria*, their characterization is important for better understanding of osmoadaptation mechanisms and efficient use of the ectoine producers.

[1] Corresponding author; e-mail: trotsenko@ibpm.pushchino.ru

The aim of the present work was to identify the ectoine biosynthesis genes in *M. marina*.

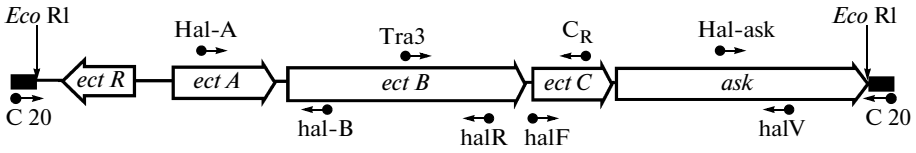
M. marina was cultured at 29°C on a K medium with 0.3% (wt/vol) methylamine [5]. Ectoine was extracted from the biomass and analyzed as previously reported [4]. The activity of DAB acetyltransferase was determined according to the published techniques [2, 3].

To identify the genes coding for the specific enzymes of ectoine synthesis in *M. marina*, a PCR-based approach was used. At the first stage, PCR was performed with degenerate primers Tra3 and C_R [2]. On the basis of the sequence obtained, the homologous primers halR (5'-CAATACCCATGCCTTCGTCACC-3') and halF (5'-CTTGTGATCGAGACTTCGGGCAGC-3') were synthesized and used in combination with the degenerate primers HalA (5'-TT(C/T)GTITGGCA(A/G)GTNGC-3') and HalV (5'-GCATAAGAAGTCCTTTCGCACC-3') in two additional PCRs (figure).

To identify the 5'-end of the *ectA* gene and the 3'-end of the *ask* gene, two vectorette PCRs [6] were carried out using *Eco*RI-treated genome DNA as a template and the specific primers halB (5'-GCATAAGAAGTCCTTTCGCACC-3') and Hal-ask (5'-TGTCGCCGATCAGCTTTCCAAC-3').

In the resulting sequence of a 5.26-kbp DNA fragment, five open reading frames were revealed. Four of them were oriented in the same direction and encoded proteins exhibiting similarity to the proteins EctA, EctB, EctC, and Ask of *Methylomicrobium alcaliphilum* 20Z (36, 52, 49, and 50% homology, respectively) [2]. The distances between the assumed genes *ectA* and *ectB, ectB* and *ectC*, *ectC* and *ask* were 55, 7, and 2 bp, respectively, evidencing their probable organization into a single operon *ectABC-ask*. The product of the fifth gene, oriented in the opposite direction and situated 175 bp away from the start codon of gene *ectA*, exhibited similarity (54% identity) with the transcription regulator EctR of *Mm. alcaliphilum* 20Z [3].

Analysis of the genomes deposited in GenBank revealed the presence of ectoine synthesis genes in



Scheme of organization and strategy of sequencing of ectoine biosynthesis genes in *Methylarcula marina*. The nucleotide sequence of the *ectABC-ask* cluster of *Methylarcula marina* defined in the present study was deposited in GenBank under accession no. CU249592.

200 bacterial and 1 archaeal species. Phylogenetic analysis of the translated amino acid sequences of the discovered genes demonstrated the highest homology level between the Ect protein families of *M. marina* and those of other alphaproteobacterial representatives, while the similarity with the homologous proteins of the *Gammaproteobacteria*, including methano- and methylotrophic ones, was more distant. Apart from several exceptions, Ect proteins of *Gamma-* and *Alphaproteobacteria* form separate clusters on the phylogenetic trees. This may indicate ancient acquisition of the ectoine biosynthesis genes and prolonged evolution within the relevant phyla.

A homogeneous preparation of DAB acetyltransferase with a 6-His tag at the C-terminus was obtained by cloning of the *M. marina ectA* gene, expression in *Escherichia coli*, and purification of the recombinant protein by nickel–affinity chromatography according to an earlier described scheme [3, 7]. EctA, a 40-kDa homodimer, exhibits maximum activity at pH 7.5–8.0 and 15°C and is stable only in the presence of NaCl (0.1 M). Thus, DAB acetyltransferase of *M. marina* differs by a number of parameters from the enzymes of *Mm. alcaliphilum* 20Z, *Methylophaga alcalica*, and *Methylophaga thalassica*, with the maximum activity at 20, 35, and 30°C and pH 9.5, 9.0, and 8.2, respectively. In particular, the low-temperature optimum of EctA is to be taken into account in the optimization of the growth conditions of this potential ectoine producer in methylamine-containing industrial wastewaters.

To conclude, ectoine biosynthesis genes of an alphaproteobacterium *M. marina* were discovered to be organized into a cluster *ectABC-ask* preceded by the *ectR* gene of a potential transcription regulator, similar to other methylotrophs synthesizing ectoine with high efficiency (figure). The obtained results allow to improve our knowledge on the organization and regulation of ectoine biosynthesis in methylotrophic bacteria of various phylogenetic groups and offer new opportunities in further studies on the efficient use of producers of this polyfunctional bioprotectant.

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