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Role of EctR as Transcriptional Regulator of Ectoine Biosynthesis Genes in *Methylophaga thalassica*

I. I. Mustakhimov, A. S. Reshetnikov, D. N. Fedorov, V. N. Khmelenina*, and Y. A. Trotsenko

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, 142290 Pushchino, Moscow Region, Russia; fax: (495) 956-3370; E-mail: khmelenina@rambler.ru

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Abstract—In the halophilic aerobic methylotrophic bacterium *Methylophaga thalassica*, the genes encoding the enzymes for biosynthesis of the osmoprotectant ectoine were shown to be located in operon *ectABC-ask*. Transcription of the *ect*-operon was started from the two promoters homologous to the σ^{70}-dependent promoter of *Escherichia coli* and regulated by protein EctR, whose encoding gene, *ectR*, is transcribed from three promoters. Genes homologous to *ectR* of methylotrophs were found in clusters of ectoine biosynthesis genes in some non-methylotrophic halophilic bacteria. EctR proteins of methylotrophic and heterotrophic halophiles belong to the MarR-family of transcriptional regulators but form a separate branch on the phylogenetic tree of the MarR proteins.

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Key words: methylotrophic bacterium, osmoprotectant, ectoine genes, transcriptional regulation

Aerobic methylotrophic bacteria utilizing methane, methanol, or methylated amines during growth at high salinity accumulate intracellularly the cyclic imino acid ectoine as an osmoprotective compound [1, 2]. These bacteria synthesize ectoine from aspartate by involving specific enzymes: diaminobutyric acid (DABA) acetyltransferase (EctA), DABA aminotransferase (EctB), and ectoine synthase (EctC) [3]. Analogously to most halophiles, in the methylotrophs studied these enzymes are encoded by genes organized in the cluster *ectABC* or *ectABC-ask*, the latter additionally coded for aspartokinase isozyme [4, 5]. The cluster *ectABC-ask* of methylotrophic bacteria is preceded by a gene encoding a homodimeric 20 kDa protein with ~20% identity to the transcriptional regulators of the MarR-family [4-8]. Earlier, we found evidence that in methanotroph *Methylomicrobium alcaliphilum* 20Z and methanol-utilizing bacterium *Methylophaga alcalica* the protein EctR repressed transcription of the ectoine operon [7, 8]. However, the function of EctR in other methylotrophic and heterotrophic halophilic bacteria was not investigated. In the methylobacterium *Methylophaga thalassica* upstream of the *ectA* gene an open reading frame (ORF) was found whose product showed ~60% identity with EctR from *Mm. alcaliphilum* 20Z and *M. alcalica* [5].

The goal of this work was to elucidate the function of the putative EctR protein in *M. thalassica*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Methylophaga thalassica* (VKMB-B 2057^T = ATCC 33146^T = NCMB 2163^T) was grown in mineral salt medium "K" containing (g/liter): KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.002, and 10 or 60 g/liter NaCl. The pH of the medium was 7.4. The bacterium was cultivated in 750 ml flasks containing 200 ml of the liquid medium supplemented before inoculation by 0.5% (v/v) methanol and 10 μg/liter vitamin B_{12}. *Methylomicrobium alcaliphilum* 20Z (VKM B-2133^T = NCIMB-14124^T) was grown as earlier indicated [2].

Escherichia coli XL1-Blue and BL21(DE3) were grown at 37°C in liquid or agarized (1.5% Difco agar (USA)) Luria–Bertani (LB) media supplemented when necessary with ampicillin (100 μg/ml).

General DNA manipulations. Chromosomal DNA from *M. thalassica* was prepared as earlier described [9]. Isolation of plasmids, ligation, endonuclease restriction, agarose gel electrophoresis, and transformation of *E. coli* cells were performed by standard methods [10]. The

Abbreviations: DABA, diamino butyric acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria–Bertani medium; ORF, open reading frame.

* To whom correspondence should be addressed.

restriction enzymes T4-DNA ligase, Taq and Pfu DNA-polymerases, and mixtures of dNTPs were obtained from Fermentas (Lithuania).

The primers were designed using the program VectorNTI®Advance v.9.0. Oligonucleotides were synthesized by Syntol (Russia). DNA sequencing was performed by Genotekh (Russia).

Purification of recombinant protein EctR. Gene *ectR* was amplified from the genomic DNA of *M. thalassica* by using the following primers: TTCATATGAGTAAAACTATCGGTTTAAATT (containing the site for *Nde*I) and TTCTCGAGGCTTTTGTCTTTTTCGTC (containing the site for *Xho*I).

The PCR fragment was treated by the restrictases and cloned into vector pET-22b(+) (Novagen, USA). Then the cells of *E. coli* BL21(DE3) were transformed by the recombinant plasmid ETectR-MT. Protein expression in *E. coli* BL21(DE3) cells was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at final concentration of 1 mM. The recombinant protein was purified by affinity chromatography on a column with Ni^{2+}-NTA (Qiagen, Germany) according to the manufacturer's instructions. The protein in the selected fractions (0.5 ml) was identified by electrophoresis according the Laemmli method [11]. The fractions containing the desired protein were dialyzed against 200 mM KCl in 50 mM Tris-HCl buffer (pH 8.0). The protein concentrations were determined by a modified Lowry method [12] using BSA as a standard.

RNA isolation and Northern blotting. RNA was isolated from *M. thalassica* cells in the logarithmic growth phase (A_{600} = 0.4-0.5) as earlier described [13]. The total RNA was isolated from the cells grown in the presence of 1 or 6% NaCl, as well as from cells pre-grown in the presence of 1% NaCl and exposed to an osmotic stress by addition of NaCl up to final concentration 6% (w/v). RNA was separated by electrophoresis in 1.2% agarose gel [10] and transferred to a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Sweden) using a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad, USA) according to the manufacturer's instructions. Hybridization was carried out at 50°C in the presence of 50% formamide with ^{32}P-labeled probes specific to the *ectR* or *ask* genes. The probes were prepared by PCR using [α-^{32}P]dATP and primers TTCATATGAGTAAAACTATCGGTTTAAATT//GGCTCATTTCATTTGCAAGTTCACC (specific to *ectR*) and ACCCACTATCTCTCTGCCAATCTCAA//ATATCAACCTGACGCATGGACTG (specific to *ask*). Visualization was performed by using the Cyclone Storage Phosphor System (Packard Instruments Co., USA).

Determination of transcriptional start sites for the *ectABC-ask* operon and gene *ectR*. The transcriptional start sites for the *ectABC-ask* genes were determined with primer AAGCTTTTTCAGGTTTATTGTTAGTCATTA complementary to *ectA* and the total RNA prepared from cells grown in the presence of 6% NaCl. The transcriptional start sites for the *ectR* gene were determined with primer GGTGATCCTTCTGTTCATCAGTCAG complementary to the gene *ectR* and RNA from cells grown at 1% NaCl. The ^{32}P-labeling of primers was carried out by using T4 polynucleotide kinase (Fermentas). The reaction of reverse transcription by the reverse transcriptase RevertAid™ M-MuLV (Fermentas) was performed according to the manufacturer's instructions. The reaction products were precipitated by ethanol and repeatedly precipitated from 0.3 M CH_3COONa (pH 5.0) and then dissolved in buffer for plotting (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylol cyanole). The PCR fragments were sequenced from the same labeled primers by using the "*fmol*® DNA Sequencing System" (Promega, USA). The products of both reverse transcriptase reaction and sequencing reactions were simultaneously separated by electrophoresis in denaturing 6% polyacrylamide gel with subsequent radioautography.

Analysis of complexes EctR–DNA. Formation of the complexes of EctR from *M. thalassica* with the DNA fragment containing the promoter region of the *ect*-operon from *Mm. alcaliphilum* 20Z was studied by electrophoretic mobility shift assay. DNA fragment was amplified by PCR using primers F1 and RT20Z [8], and the PCR product was electrophoresed in 6% polyacrylamide gel. The gel strip possessing the DNA fragment of desired size was cut out. The gel was ground, and the DNA was eluted by 1 M NaCl. The DNA was precipitated by ethanol and dissolved in 200 μl 0.2 M NaCl. For purification, the DNA fragment was placed on a DEAE-cellulose column (Whatman, UK) equilibrated by 0.2 M NaCl, washed by the same solution, and eluted by 1 M NaCl. The eluted DNA was sedimented by ethanol in the presence of 20 μg glycogen (Fermentas) and 0.3 M CH_3COOK, dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The EctR–DNA complexes were formed at 37°C in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 200 mM KCl in the presence of 20 μg/ml of synthetic polymer poly[dI-dC] as a competitive DNA. The complexes were analyzed by electrophoresis in 6% polyacrylamide gel. The gels were dried and visualized using the Cyclone Storage Phosphor System.

Sequence analysis. DNA and protein sequences were comparatively analyzed using the PSI-BLAST program accessible from the server (http://www.ncbi.nlm.nih.gov). The amino acid sequences of proteins were aligned using computer program CLUSTALX (version 1.8) [14]. Phylogenetic analysis was done by using MEGA 4 and model Neighbor-Joining [15].

RESULTS

Northern blot analysis of ectoine biosynthesis genes. Earlier, in the moderately halophilic methylotrophic bac-

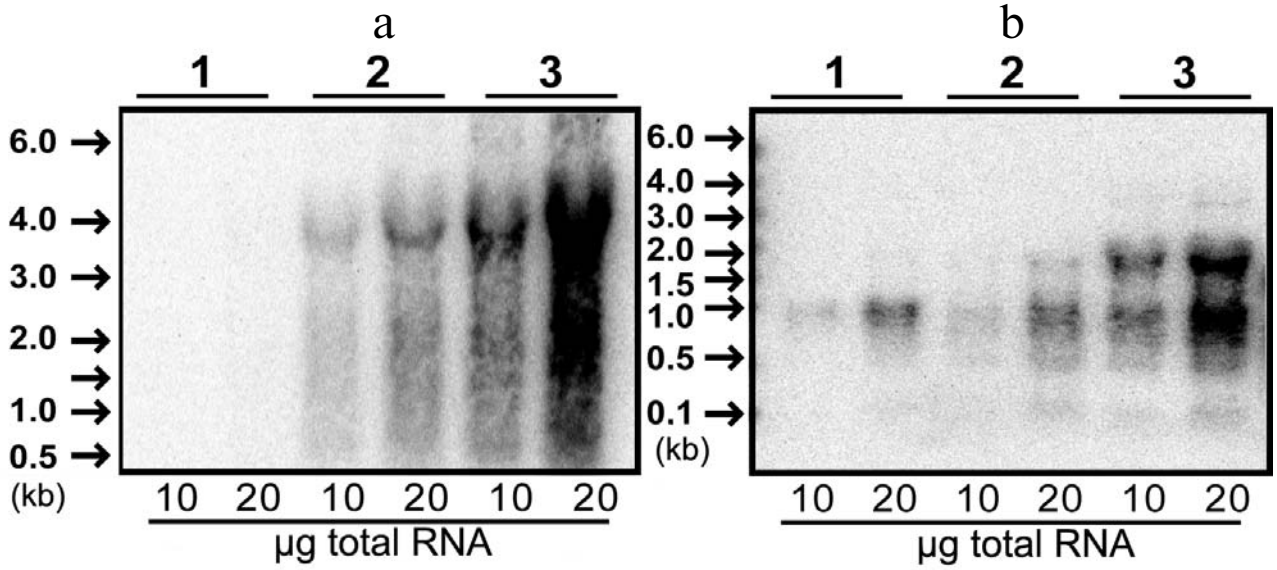

Fig. 1. Northern blot analysis of transcription of the *ectABC-ask* operon (a) and *ectR* gene (b) in *M. thalassica*. Cells were grown at 1% NaCl (*1*), 6% NaCl (*2*), or grown at 1% NaCl and exposed to osmotic up-shift (*3*).

terium *M. thalassica*, the genes coding for ectoine biosynthesis enzymes have been identified (GenBank No. EU315062) [5]. The possibility of co-transcription of the genes located in the *ectABC-ask* gene cluster was analyzed by Northern blotting with the total RNA isolated from cells grown in the presence of 1 or 6% NaCl as well as from cells treated by osmotic up-shock (see "Materials and Methods"). For hybridization, the probe specific to the 3′-end of the *ask* gene was used. Four genes were found to be transcribed as a single polycistronic mRNA (size of the transcript ~4000 bp). Such transcripts were found in the cells grown at 6% NaCl or osmotically stressed cells but was absent in cells grown at 1% NaCl (Fig. 1a).

Determination of the transcriptional start site for the *ect*-operon and analysis of EctR–DNA complexes. The primer extension assay was used for detection of the promoter region of the *ectABC-ask* operon. Two transcriptional start sites located 130 and 82 bp from the start codon of the gene *ectA* were found. Thus, transcription of the *ect*-operon in *M. thalassica* proceeded from two promoters, *ectP1* and *ectP2* (Fig. 2a). Putative −35 (TGGACA) and −10 (TACTAT) sequences of *ectP1* showed high similarity with the respective sequences of the σ^{70}-dependent promoter of *E. coli* (TTGACA - 16-18 bp - TATAAT) [16]. The presence of the TG motif in −14 position as an extension of the −10 sequence additionally implicated the similarity with σ^{70} [17]. In contrast, the putative −35 (CCGAAT) and −10 (TTTATT) boxes of the *ectP2* promoter displayed only low similarity with the σ^{70}-dependent promoter of *E. coli* (Fig. 2c), while the TG motif upstream of the −10 box was found.

Earlier we found that transcription of the operon *ectABC-ask* in *Mm. alcaliphilum* 20Z proceeded from the two promoters, *ectAp1* and *ectAp2*, but was repressed by the protein EctR whose binding site included the −10 sequence of the promoter *ectAp1* [8]. The binding site of EctR from *Mm. alcaliphilum* 20Z demonstrated full identity with the −10 sequence of the promoter *ectP1* from *M. thalassica* (Fig. 3). Therefore, it is likely that transcription of the ectoine biosynthesis genes in *M. thalassica* is also regulated by the repressor EctR.

The possibility that EctR from *M. thalassica* forms the complexes with the DNA containing the promoter region of the *ect*-operon from *Mm. alcaliphilum* 20Z was tested by an electrophoretic mobility shift assay. The homogenous preparation of the recombinant protein, EctRMT-His_6, was obtained by expression of *ectR* in cells of *E. coli* BL21(DE3). Electrophoretic mobility of the protein in SDS-PAGE corresponded to molecular mass ~23 kDa, which is in agreement with the predicted size deduced from the amino acid sequence (23.75 kDa). Then, by using PCR with ^{32}P-primers F1 and RT20Z, the labeled DNA fragment containing *ectAp1* promoter from *Mm. alcaliphilum* 20Z was obtained. Addition of EctR-His_6 from *M. thalassica* to the PCR fragment resulted in lowering of its electrophoretic mobility, including in the presence of heterologous DNA (Fig. 4). These results suggested the specific interaction between recombinant protein EctR-His_6 from *M. thalassica* and promoter *ectAp1* of *Mm. alcaliphilum* 20Z.

Phylogenetic analysis of EctR. Searches in the GenBank database of ORFs homologous to the *ectR* genes of methylotrophic bacteria revealed their presence in the genomes of the marine bacteria *Alcanivorax*

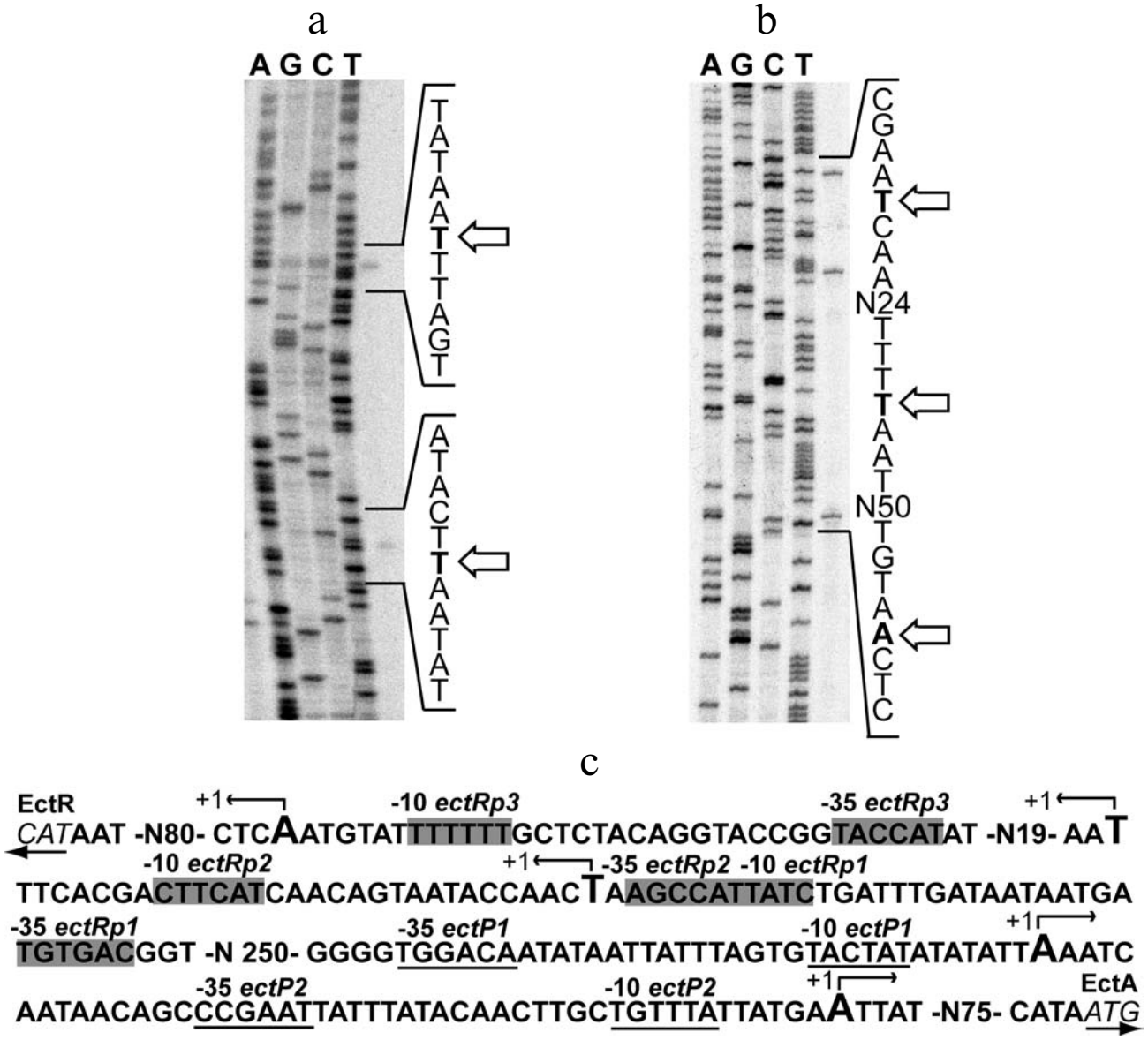

Fig. 2. Determination of transcriptional start sites for the *ectABC-ask* operon and *ectR* gene. Electrophoresis of products of reverse transcription performed with mRNA of the *ect*-operon (a) or the *ectR* gene (b). White arrows show +1 nucleotides. c) Nucleotide sequence of intergenic region. Putative promoter elements (−10 and −35 boxes) for *ectP1* and *ectP2* promoters of the *ectABC-ask* operon are underlined. The respective sequences for promoter elements of the *ectR* gene have shaded gray background. Black arrows show +1 nucleotide.

borkumensis, *Reinekea* sp., *Oceanospirillum* sp., the pathogenic *Bordetella bronchiseptica*, *Bordetella parapertussis*, the soil bacterium *Nitrobacter* sp., and other halophiles (Fig. 5). In most of these bacteria, these ORFs (designed as ORF_{PTR}) are located directly upstream of the ectoine biosynthesis genes in divergent direction. Such location is characteristic for the genes encoding the MarR-family regulatory proteins [18-22]. Analysis of deduced amino acid sequences of ORF_{PTR} showed that these polypeptides have domain structure similar to that of EctR in methylotrophs and to the MarR-family proteins. However, EctR-like proteins form a separate cluster on the phylogenetic tree of the MarR proteins (Fig. 5).

Analysis of the translated amino acid sequences of putative EctR of methylotrophs by using PsiPred program (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html) showed the presence of a DNA-binding domain that possesses a helix–turn–helix (HTH) motif and flanking "wing 1" region analogous to the respective motifs in the MarR-family of transcriptional regulators (IPR000835 and IPR011991; InterPro) [8, 23, 24].

Determination of the transcriptional start sites and transcription level of the *ectR* gene. The primer extension

Fig. 3. Sequence alignment of promoter regions of the ectoine biosynthesis genes from halophilic bacteria. 20Z, *Mm. alcaliphilum* 20Z; M8, *M. alcalica*; MT, *M. thalassica*; Ob. s., *Oceanobacter* sp., Re. s., *Reinekea* sp. Identical nucleotides are located on black ground. Numeration of nucleotides is given against to the first nucleotide of gene *ectA*.

assay revealed that transcription of the *ectR* gene in *M. thalassica* is started from A, T, and T mapped, respectively, at positions 176, 145, and 89 bp upstream of the *ectR* gene (Fig. 2b). The potential −10 and −35 *ectRp1* promoter elements (GATAAT and GTCACA, respectively) match well with the consensus sequences of σ^{70}-dependent promoter of *E. coli* [16]. Moreover, the *ectRp1* displayed a TG motif in position −14 as an upstream extension of the −10 box, additionally implicating the similarity of *ectRp1* with σ^{70}-dependent promoter [17]. In contrast, the potential −35 and −10 sequences of promoters *ectRp2* (ATGGCT and ATGAAG) and *ectRp3* (ATGGTA and AAAAAA) are degenerative and have no similarity with σ^{70} (Fig. 2c).

Northern blot analysis with the gene-specific probe to the 3′-end of *ectR* was used to determine the size and level of this gene transcript. The gene *ectR* was found to be transcribed as a monocistronic RNA (size of the transcript ~900 bp) at all growth conditions tested. The amount of *ectR* mRNA was practically the same in cells grown at 1 or 6% NaCl but increased in cells subjected to the osmotic up-shock (Fig. 1b).

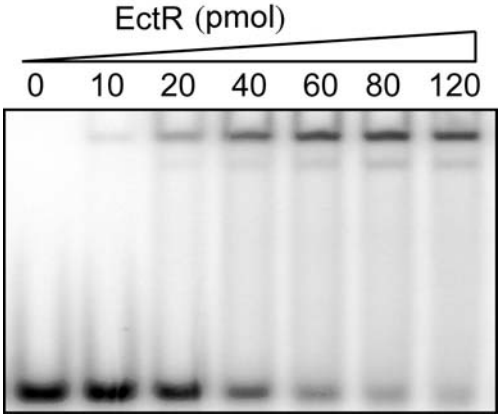

Fig. 4. Analysis of the EctR–DNA complexes by electrophoretic mobility shift assay. To form DNA–protein complexes, various amounts (0-120 pmol) of EctR-His_6 were mixed with 1 pmol of the DNA fragment in the presence of 20 μg/ml of poly[dI-dC].

DISCUSSION

We showed that the genes *ectABC-ask* encoding the enzymes of biosynthesis of the compatible solute ectoine in *M. thalassica* are transcribed as one polycistronic RNA. This operon is preceded by the gene for the transcriptional repressor EctR. Similar organization of the ectoine biosynthesis genes was earlier found in methanotroph *Mm. alcaliphilum* 20Z and methylobacterium *M. alcalica* [7, 8].

Interestingly, transcription of the *ect*-operons in *M. thalassica* and *Mm. alcaliphilum* 20Z proceeds from two σ^{70}-dependent promoters, one of them being fully identical in the two bacteria. It was found that in other halophilic bacteria the promoters of *ect*-genes have homology to the σ^{70}-dependent promoter of *E. coli*. Thus, gram-negative *Chromohalobacter salexigens* has five putative promoters of (*PectA1-4*, *PectB*), two of which (*PectA1* and *PectA2*) showed homology with σ^{70}, the −10 sequence of *PectA2* being identical to the consensus sequence (TATAAT) [25]. Operons *ectABC* of gram-positive *Bacillus pasteurii*, *Marinococcus halophilus*, and *Salibacillus salexigens* are transcribed from the promoters homologous to the σ^{A}-dependent promoter of *Bacillus subtilis*, which is an analog of σ^{70}-dependent promoter of *E. coli* [26-28]. Besides the methylotrophs studied, homologs of the transcriptional repressors EctR are present in bacteria of other physiological groups possessing operon *ectABC-ask* (Fig. 5). The availability of EctR is characteristic of bacteria tolerant to the moderate salinity (up to 15% NaCl). In contrast, extremely halophilic bacteria growing at NaCl concentration above 20% (*Chromohalobacter salexigens*, *Bacillus pasteurii*, *Marinococcus halophilus*, and *Salibacillus salexigens*) have no homologs of the *ask* and *ectR* genes.

Along with similarity in the *ect*-gene organization in *Mm. alcaliphilum* 20Z and *M. thalassica*, differences in mechanisms of their transcriptional regulation were revealed. Particularly, in *Mm. alcaliphilum* 20Z the transcription of *ectABC-ask* proceeded at both low and high medium salinity [8]. Moreover, *Mm. alcaliphilum* 20Z synthesized osmoprotectant at medium salinity <1% NaCl [2]. In contrast, the respective mRNA was not

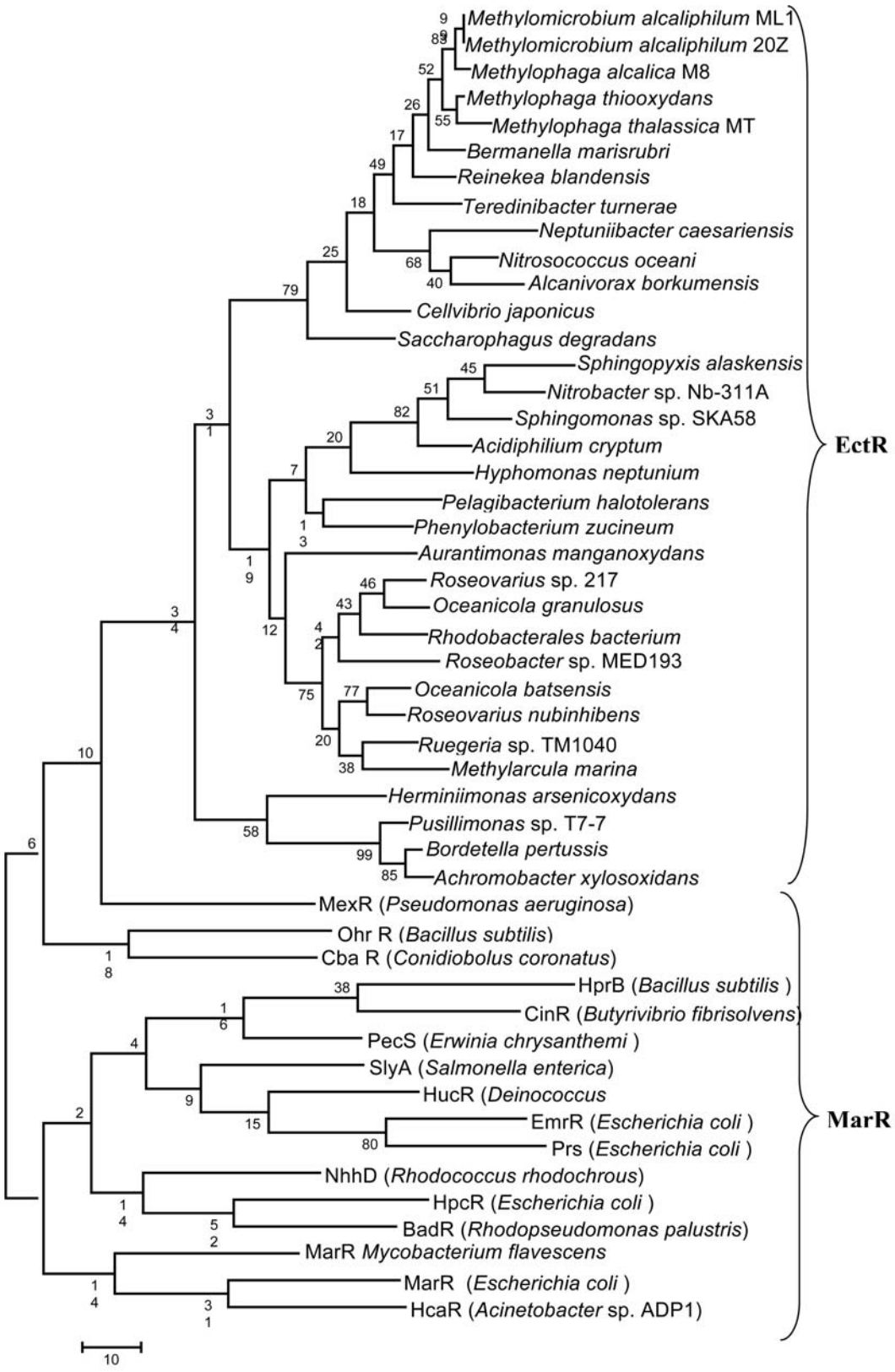

Fig. 5. Phylogenetic tree of putative transcriptional regulators EctR of halophilic bacteria and regulators of the MarR-family. The tree was designed on basis of the deduced amino acid sequences of the putative *ectR* genes found in genomes of halophilic bacteria and known MarR-family transcriptional regulators.

found in *M. thalassica* growing at 1% NaCl. These data correlate with the absence of ectoine in *M. thalassica* cells growing at 1-3% NaCl [1]. The absence of transcripts of ectoine biosynthesis genes in *M. thalassica* growing at low salinity may be related with the peculiarities of the EctR expression. So, EctR in *Mm. alcaliphilum* 20Z controls transcription of its own gene, *ectR*, since an EctR-binding site is located between *ectR* and the promoter of this gene. In contrast, no binding site for the repressor was found in the promoter region of the *ectR* gene of *M. thalassica*, in other words, EctR in this methylobacterium is not an autoregulatory protein. The *ectR* mRNA level increased in hyperosmotic shock in both methylotrophs.

The presence of the *ask* gene coding for the specific aspartokinase isozyme in *ect*-operons of *M. thalassica* and some other bacteria may result in intensification of aspartate flow-out into aspartyl phosphate, the precursor of other aspartate family amino acids and ectoine. During external salinity fluctuation, these halophiles need the fine regulation of the ratio of aspartate and its derivatives, and this may be provided at the level of gene transcription by an involvement of EctR.

Analysis of the genomes and *ect*-genes deposited in GenBank showed that along with methylotrophs, 28 halophilic species possess the EctR-like proteins. Based on these data, it seems logical to propose the similarity of mechanisms for transcriptional regulation of the *ect*-operons in these bacteria. Although the EctR-proteins of halophilic methylotrophs and heterotrophs belong to the MarR-family of transcriptional regulators, they comprise a specific subgroup EctR, forming on the phylogenetic tree a separate branch.

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