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ORIGINAL PAPER

Diversity and phylogeny of the ectoine biosynthesis genes in aerobic, moderately halophilic methylotrophic bacteria

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Abstract The genes of ectoine biosynthesis pathway were identified in six species of aerobic, slightly halophilic bacteria utilizing methane, methanol or methylamine. Two types of ectoine gene cluster organization were revealed in the methylotrophs. The gene cluster *ectABC* coding for diaminobutyric acid (DABA) acetyltransferase (EctA), DABA aminotransferase (EctB) and ectoine synthase (EctC) was found in methanotrophs *Methylobacter marinus* 7C and *Methylomicrobium kenyense* AMO1^T. In methanotroph *Methylomicrobium alcaliphilum* ML1, methanol-utilizers *Methylophaga thalassica* 33146^T, *Methylophaga alcalica* M8 and methylamine-utilizer *Methylarcula marina* h1^T, the genes forming the *ectABC–ask* operon are preceded by *ectR*, encoding a putative transcriptional regulatory protein EctR. Phylogenetic relationships of the Ect proteins do not correlate with phylogenetic affiliation of the strains, thus implying that the ability of methylotrophs to produce ectoine is most likely the result of a horizontal transfer event.

Keywords Methylotrophic bacteria · Methanotrophs · Compatible solutes · Ectoine biosynthesis genes

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Introduction

As known, microorganisms living in fluctuating salinity environments have developed several strategies to survive during osmotic up- and downshifts. The majority of moderately halophilic and halotolerant bacteria respond to an external salinity increase by accumulation of low-molecular weight organic compounds, so-called compatible solutes. Even if accumulated in exceedingly high concentrations (up to several moles per liter), compatible solutes do not interfere with cellular metabolism. Different classes of compatible solutes, such as amino acids, ectoines, betaines, sugars, and polyols, have been described. The cyclic imino acid ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) serves as a compatible solute in a variety of moderately halophilic and halotolerant bacteria (Galinski et al. 1985; Severin et al. 1992; Galinski 1995; Ventosa et al. 1998; Kempf and Bremer 1998; Grant 2004; Oren 2002, 2008). Ectoine is widely used in biotechnology, cosmetics and medicine as a multifunctional bioprotectant (Lippert and Galinski 1992; Buenger 1999; Göller and Galinski 1999; Graf et al. 2008; Knap et al. 1999). Detailed study on organization/regulation of the ectoine biosynthetic pathway in various bacterial producers is an active research area. Ectoine biosynthesis represents a branch in the pathway for synthesis of the aspartate family amino acids and includes three additional special enzymes:

diaminobutyric acid (DABA) acetyltransferase (EctA, EC 2.3.1.178), DABA aminotransferase (EctB, EC 2.6.1.76) and ectoine synthase (EctC, EC 4.2.1.108) (Peters et al. 1990; Ono et al. 1999). The genes coding for these enzymes are usually located in the gene clusters *ectABC* or *ectABC–ask* (Louis and Galinski 1997; Kuhlmann and Bremer 2002; Vargas et al. 2008; Schwibbert et al. 2010; Lo et al. 2009).

Aerobic methylotrophic bacteria (methylotrophs, also known as C_1-utilizers) are able to use single carbon compounds such as methane, methanol or methylamine as a sole source of carbon and energy (Anthony 1982; Hanson and Hanson 1996). Methylotrophic bacteria were found by us in a variety of aquatic and terrestrial habitats, including saline ecosystems (Lidstrom 2006; Trotsenko and Khmelenina 2002; Kalyuzhnaya et al. 2008). Recently, we have shown that the ability to synthesize ectoine plays a key role in osmoadaptation of aerobic methylotrophic bacteria (Doronina et al. 1998, 2003a, b; Khmelenina et al. 1999, 2010). However, the biochemistry and genetics of ectoine biosynthesis were characterized only in halotolerant obligate methanotroph *Methylomicrobium alcaliphilum* 20Z (synonym *Methylobacter alcaliphilus*) (Reshetnikov et al. 2005, 2006; Mustakhimov et al. 2010). In this study, the nucleotide sequences of the ectoine biosynthetic genes were determined in six species of methylotrophic bacteria isolated from various saline habitats. We found that organization of the *ect* genes in either the *ectABC* or *ectR–ectABC–ask* gene cluster well correlated with the salt tolerance of the strains. Our results indicate that in methylotrophic species, the ability to synthesize ectoine was most likely acquired via lateral gene transfer.

Materials and methods

Bacterial strains and culture conditions

Type cultures *Methylomicrobium kenyense* AMO1 (NCI MB 13566^T = VKM B-2464^T), *Methylophaga thalassica* (ATCC 33146^T = NCIMB 2163^T = VKM B-2057^T) and *Methylarcula marina* $h1^T$ (=VKM B-2159) were obtained from the All-Russian Collection of Microorganisms (VKM, Russia). Two aerobic methanotrophs *Methylomicrobium alcaliphilum* ML1 and *Methylobacter marinus* 7C were isolated from an alkaline lake Mono Lake (USA) and a hypersaline lake Burlinskoe (Kulunda Steppe of Russia), respectively, and taxonomically described recently (Khmelenina et al. 2010). The methanol-utilizer *Methylophaga alcalica* M8 isolated from Buryatyan soda lake (Russia) shared high homology (99.9% of the 16S rRNA sequence identity and 87% DNA–DNA hybridization with the type strain of this species *M. alcalica* M39 (ATCC BAA-297^T) (Doronina N.V., personal communication). The methanotrophic bacteria (strains ML1, 7C and AMO1) were grown under methane/air atmosphere (1:1) in a liquid nitrate mineral salt medium at 30°C as earlier described (Khmelenina et al. 1999, 2010; Sorokin et al. 2000). The methanol-utilizers *M. thalassica* ATCC 33146 and *M. alcalica* M8 were grown in nitrate mineral salt medium containing 0.5% (v/v) methanol and 10 μg l^{-1} vitamin B_{12} as described by Doronina et al. (2003a). *M. marina* $h1^T$ was grown in mineral salt medium supplemented with 0.5% (w/v) methylamine (Doronina et al. 2000). For growth of the alkaliphiles *M. alcaliphilum* ML1, *M. kenyense* AMO1 and *M. alcalica* M8, the pH of the medium was adjusted to 9.0 with $NaHCO_3/Na_2CO_3$ buffer (final concentration of 0.05 M). All media were supplemented with appropriate NaCl concentrations. *E. coli* TOP10 (Invitrogen, USA) was grown at 37°C in Luria-Bertani medium supplemented with ampicillin (100 μg/ml).

Analysis of intracellular ectoine content

The extraction of intracellular solutes from freeze-dried cells with methanol was done as described by Khmelenina et al. (1999). Ectoine content in the cell extracts was measured by high-performance liquid chromatography (HPLC) (Eshinimaev et al. 2007).

Identification of ectoine biosynthesis genes

Genomic DNAs from methylotrophs were prepared as earlier described (Kalyuzhnaya et al. 1999). Previously described degenerate primer sets Tra1/Tra4 and Tra3/C_R were used for amplification of *ectB* and *ectC*, respectively, from chromosomal DNA of *M. alcalica* M8 (Reshetnikov et al. 2006). Assuming that in *M. alcalica* M8, the ectoine biosynthesis genes are organized in a cluster as *ectABC–ask*, the new degenerate primer AspV for the region 792–813 bp from the *ask* start codon was designed on the basis of conserved regions of the *ect* and *ask* genes from *M. alcaliphilum* 20Z and *Vibrio cholerae*. The *ectA–ectB* region of the gene cluster was amplified by using an inverse PCR (IPCR). Samples of *M. alcalica* DNA were digested by *Acc*65I or *Mph*1103I and self-ligated to generate circular DNA molecules. The latter were used in IPCRs with M8Y/M8X and M8askR/M8askF primer sets and the resulting fragments were sequenced.

Degenerate primers Tra3 and C_R were used to amplify fragments containing the 3′ end of *ectB* and 5′ end of *ectC* genes from DNA samples of five other strains of methylotrophic bacteria. Based on the resultant sequences of the PCR products, the following homologous primers were

designed: MTZ for *M. thalassica* ATCC 33146, RTect for *M. alcaliphilum* ML1, AMOR for *M. kenyense* AMO1 and 7CF/7CR for *Mb. marinus* 7C (Table 1). In addition, a new degenerate primer ATZ was designed based on EctA sequences of *M. alcalica* M8 and *M. alcaliphilum* 20Z.

The ATZ and MTZ primer set was used to obtain a PCR fragment containing the 3′ end of *ectA* and 5′ end of *ectB* from *M. thalassica* ATCC 33146. For further characterization of the *ect* genes, *M. thalassica* DNA was digested by *Acc*65I and the restriction fragments were ligated. Using IPCR with MTF or MTRev primers (Table 1), a fragment of 1400 bp was obtained and sequenced. For deciphering of the locus upstream *ectA*, DNA was digested by *Vsp*I and the restriction fragments were ligated. Circular DNA molecules were used in IPCRs with primer sets MTr1/MTr2 and the resulting fragments were sequenced. To decipher the 3′ end of *ectC* and 5′ end of *ask*, the primers MTX and AspV were used for PCR. The newly generated sequence information was used to design primers MT and MTF (Table 1) for IPCR with the self-ligated *Mph*1103I-fragment. Additional IPCR with MTXR/MTXF primers and self-ligated *Cla*I fragments was performed, thus resulting in the identification of the 3′ end of the *ask* gene.

Primers ATZ and AMOR were used for amplification of the *ectA–ectB* fragment from *M. kenyense* AMO1 (Fig. S1, E). An amplicon of ~1280 bp containing the 3′-region of *ectA*

Table 1 Primers used in the study

Primers	Gene targeted	Sequences (5′–3′)
Tra1	*ectB*	CCCTIAA(T/C)TA(T/C)GGICA(T/C)AA(C/T)
Tra3	*ectB*	ACCGG(T/C)ACITT(C/T)TT(C/T)AGITT(C/T)GA
Tra4	*ectB*	CG(G/A)AAIGTGCC(A/G)TT(G/A)TG(T/C)TCNCC
C_R	*ectC*	GGIGG(A/G)TT(A/G)AANAC(A/G)CA
ATZ	*ectA*	TT(C/T)GT(G/T/C)TGGCA(A/G)GTIGCNGT
AspV	*Ask*	TG(A/G)TCIGCNAC(A/G)TC(A/G)(A/T)A(A/G)TT
C20		CTCTCCCTTCTCGAATCGTAA
BC		GGAGCAAGCCATTAAAGAGGTCTG
RTectC	*ectC*	ATAGCCCATACCGTCATCCTTC
AtfX	*ectA*	GCAAAATGGCGTGTCTTATCAA
AMOFor	*ectA*	CGAAATGCTGGTTAAGATTGGTCCGT
AMOA	*ectA*	ATTGGGCGGATTGATCGTAGTTTC
AMOR	*ectB*	ATTTGATCGGCGAAACGGTTAT
AMOZ	*ectB*	CTTGAGGCTTGCCTCGGCTATC
AMOF	*ectC*	CGACGATTTATCAAGGTGCGGA
MTRev	*ectB*	GCGAGGAAAAGAACGAGCGTAAGAAC
MTFor	*ectB*	GGGGCTGGTTTCATTGAATTATGGTC
MTZ	*ectB*	ACCAGTACGACCACAACCCGCC
MTX	*ectC*	AAATATGCCATCACGCCAGGTAC
MTR	*ask*	ATCGCTATGTGCATAACCGGTGAC
MTF	*Ask*	ACATTTGACCGTGGTTACAGTGAA
MTr1	*ectA*	CATAAGAGGCGAGATAGTCAGC
MTr2	*ectA*	CGTGTGCATAGGTTTCATCTTC
M8Y	*ectB*	ATCCAAATTGCGTGGCTTCAGA
M8X	*ectB*	TGCATGGCTCTACCCGTATTCCT
M8askR	*ask*	TCGCCACTTTCTGCTGATTGATTTC
M8askF	*ask*	AAACGGTCGCGGCATTGGCAGA
H1-A	*ectA*	TT(C/T)GTITGGCA(A/G)GTNGC
h1 V	*Ask*	ACCAT(A/G)TC(C/T)TG(C/T)TC(A/G)AA
h1B	*ectB*	GCATAAGAAGTCCTTTCGCACC
H1-ask	*ask*	TGTCGCCGATCAGCTTTCCAAC
h1R	*ectB*	CAATACCCATGCCTTCGTCACC
h1F	*ectC*	CTTGTGATCGAGACTTCGGGCAGC
7CF	*ectC*	CGATCAAGGCGGGCACGGTCTAT
7CR	*ectB*	TCGACAATGGCCTGCAGACTT

N = A, G, C or T; I = inosin

and 5′-region of *ectB* was sequenced. Two IPCR rounds were employed: (1) circular DNA molecules obtained after digestion of DNA with *Mun*I, and IPCR with homologous primers AMOZ (corresponding to *ectC*) and AMOF (corresponding to *ectB*) allowed sequencing the 3′ end of *ectC* and its flanking sequence, (2) IPCR with homologous primers AMOA and AMOF or by using template circular DNA obtained after digestion by *Acl*I. DNA fragments of, respectively, 1200 bp (3′-end of *ectC*) and 800 bp (5′-end of *ectA*) were sequenced.

Vectorette PCR was used for identification of nucleotide sequences of *ect* genes as described by Ko et al. (2003). Samples of *M. marinus* 7C genomic DNA were digested by *Eco*RI. After annealing and ligation of a double strand vectorette unit (vect 57TTAA which complemented vect 53) to DNA digested by *Eco*RI, a vectorette PCR amplification with primer 7CF was performed (Table 1). For the second vectorette PCR, genomic DNA was digested by *Acl* I. After annealing and ligation of vectorette unit (vect 57CG, which also complemented vect 53), 7CR was used in a second round of PCR amplification. The generated fragments were sequenced and assembled as described below.

An *ectA–ectB* fragment from *M. alcaliphilum* ML1 DNA was amplified using the ATZ/RTect primers set. To obtain a complete sequence of the *ectA* gene, IPCR was performed with circular DNA fragments generated by *Mph*11031 digestion and self-ligation, and the primer pair AtfX and BC (corresponding to *ectB*). To obtain sequence information for the 3′ end of the *ectC* and 5′ end of the *ask* genes, PCR with BC and AspV was carried out. In addition, a vectorette PCR with AtfX primer and DNA fragments generated by *Hin*dIII digestion was performed and the resulting fragments were sequenced (Fig. S1).

For identification of *ect* genes in *M. marina* h1, two PCRs with degenerate and complementary primers (h1-A/h1R, and h1V/h1F) were carried out. Two vectorette PCRs with *Eco*RI-digested DNA samples and the specific primers hl-B and hl-ask were performed (Table 1). The generated fragments were sequenced and assembled.

DNA manipulation and sequence analysis

Plasmid DNA isolation, restriction, digestion, DNA ligation, PCR amplification, agarose gel electrophoresis and other routine DNA manipulations were carried out according to Sambrook and Russell (2001). Restriction endonucleases were purchased from Fermentas (Lithuania). The PCR products were purified by using "Wizard SV Gel and PCR Clean-Up System" columns (Promega, USA) and cloned into the *Eco*RV site of pZErO2.0 (Invitrogen, USA). DNA sequencing of the cloned regions was performed by using an ABI Prism 310 DNA sequencer (Perkin Elmer, USA) and following the supplier's instruction. DNA and protein sequences were assembled and analyzed with the Lasergene software package (DNASTAR). Searches for homologies were performed at NCBI (http://www.ncbi.nlm.nih.gov/) using the PSI-BLAST program. Protein sequences were aligned with the CLUSTAL X software program (Thompson et al. 1997). Minor corrections in alignment of sequences were also made manually. All positions containing gaps and missing data were eliminated using Complete Deletion algorithm. The resulting sequences included 628 positions per concatenated EctABC sequences.

Phylogenetic analyses were conducted in MEGA v.4.0 (Tamura et al. 2007) using the maximum-parsimony method (Close-Neighbor-Interchange algorithm). The branch lengths were calculated using the average pathway method and represented in the number of changes over the whole sequence.

Accession numbers

The nucleotide sequences of *M. alcaliphilum* ML1, *M. kenyense* AMO1^T, *M. marinus* 7C, *M. alcalica* M8, *M. thalassica* ATCC 33146^T and *M. marina* h1^T were deposited in the GenBank under the accession numbers EU523735, DQ238213, EU523734, EU315063, EU315062 and GU249592, respectively.

Results

Salt tolerance and intracellular ectoine content in methylotrophic bacteria

All the methylotrophic bacteria used herewith were isolated from saline ecosystems. Methanol-utilizers *M. alcalica* M8, *M. thalassica* ATCC 33146 and methylamine-utilizer *M. marina* h1 showed optimal growth at approximately 0.5 and 1 M salt (Doronina et al. 1998, 2003a). Methanotroph *M. alcaliphilum* ML1 grew well in the range 0.05 and 1.7 M NaCl and optimally at 0.5 M salt. These cultures could be described as slightly halophilic bacteria in accordance with classification by Imhoff (1993). *M. marinus* 7C and *M. kenyense* AMO1 were also salt dependent, since these methanotrophs could not grow at all in mineral medium unless it contained 0.2 M NaCl (Khmelenina et al. 2010; Sorokin et al. 2000).

For some cultures, the ability to produce ectoine has been previously demonstrated (Doronina et al. 1998, 2003a). As shown, *M. alcalica* M8 and *M. marina* h1 compensated for the salinity of growth medium by accumulation of ectoine up to 235 mg per g of dried cell weight (DCW) at 9% NaCl. We also tested the ability of the other

strains of methylotrophic bacteria to produce ectoine in response to increased external osmolarity. They were grown in defined mineral medium at different salinities. When cultures reached $OD_{600} = 1$, cell samples were collected and intracellular ectoine content was measured by HPLC. In all cultures tested, the intracellular pool of ectoine increased with elevated salinity. In methanotroph *M. alcaliphilum* ML1 and methanol-utilizer *M. thalassica* ATCC 33146 grown at 9% NaCl, the intracellular concentrations of ectoine reached 230 ± 20 and 200 ± 15 mg per g of DCW, respectively. *M. marinus* 7C and *M. kenyense* AMO1 grown at the upper limit of salinity (4 and 5% NaCl) accumulated ectoine to 60 and 70 mg per g of DCW. Remarkably, hydroxyectoine was not detected in all species studied.

Organization of the ectoine biosynthesis genes in methylotrophic bacteria

The 3′-end of *ectB* and 5′-end of *ectC* gene fragments were successfully amplified from DNA samples of the methylotrophic cultures. The newly obtained sequences were then used to design the homologous primers for IPCR or vectorette PCR as described in "Materials and methods". The resultant products were sequenced and assembled. DNA fragments from *M. alcaliphilum* ML1 (4.3 kb), *M. alcalica* M8 (4.8 kb), *M. thalassica* ATCC 33146 (6.3 kb) and *M. marina* h1 (5.1 kb) contained four open reading frames (ORFs). Three of the ORFs encoded proteins with high amino acid sequence identities to the ectoine biosynthesis enzymes, DABA acetyltransferase (EctA), DABA aminotransferase (EctB) and ectoine synthase (EctC) (Fig. S1). The product of the fourth ORF located downstream of *ectC* showed sequence identity to an aspartokinase gene from *V. cholerae* (43–48% AA sequence identity), and the ORF was designated as *ask*. The lengths and molecular masses of these predicted polypeptides were similar among the methylotrophic strains studied. The intergenic regions between *ectA* stop codon and *ectB* start codon comprised 42, 54, 60 and 55 bp in *M. alcaliphilum* ML1, *M. alcalica* M8, *M. thalassica* ATCC 33146 and *M. marina* h1, respectively. A similar 40–131 bp gap was observed between *ectB* and *ectC* in three former species, whereas in *M. marina* h1, *ectB, ectC* and *ask* genes were tightly linked, with only 7 and 2 bp between the genes, respectively. The overall organization of the *ect* genes in *M. alcaliphilum* ML1, *M. alcalica* M8, *M. thalassica* ATCC 33146 and *M. marina* h1 implied that the four genes might form an *ectABC–ask* operon.

As shown, the *ectR* gene, encoding a transcriptional regulatory protein of the MarR family, is located upstream of the *ect* operon in *M. alcalica* M8 (Mustakhimov et al. 2009). The EctR-like gene oriented in the opposite direction was also identified upstream of the *ectABC–ask* cluster in *M. alcaliphilum* ML1, *M. thalassica* ATCC 33146 and *M. marina* h1. The EctRs showed a significant homology to the EctR1 protein from *M. alcaliphilum* 20Z (99, 60 and 54% AA identity for *M. alcaliphilum* ML1, *M. thalassica* ATCC 33146 and *M. marina* h1, respectively).

A genomic fragment (3.5 kb) of *M. kenyense* AMO1 contained only three ORFs corresponding to *ectA, ectB* and *ectC*. The intergenic region between *ectA* and *ectB* consisted of 47 bp and that between *ectB* and *ectC* was 170 bp. No additional ORF was found within 600 bp downstream of the *ect*C gene and ~300 bp upstream of *ectA* gene.

The sequenced 4.25-kb DNA fragment from *M. marinus* 7C included tightly linked *ectABC* genes without intergenic regions (Fig. S1). An additional ORF4 (1371 bp) was detected in the DNA locus 81 bp downstream of *ect*C. The product of this Orf4 (49 kDa) was similar to the transport proteins belonging to Na^+/solute symporter (SSS) and PutP families (Jung 2001) of *Mariprofundus ferrooxydans* PV-1 ZP_01451115 (56% identity of amino acid sequences), *Nitrosococcus oceani* ATCC 19707 YP_343605 (46%) and *Hyphomonas neptunium* ATCC 15444 YP_760353 (43%). No coding sequence in the DNA locus ~300 bp upstream of *ectA* gene in *M. kenyense* AMO1 and *M. marinus* 7C was found.

Hence, two main types of organization of genes for ectoine biosynthesis (*ectABC* or *ectR–ectABC–ask* cluster) were found in the methylotrophs studied. Interestingly, differences in organization of the *ect* genes clearly correlated with salt tolerance of the methylotrophs. A three-gene cluster, *ectABC,* was identified in two methanotrophs, the neutrophilic *M. marinus* 7C and alkaliphilic *M. kenyense* AMO1, and both were capable of growth at salinity no more than 5% NaCl. Remarkably, methylotrophic species that were able to grow at higher salinity (up to 10% NaCl) possess a gene cluster *ectR–ectABC–ask*.

Phylogenetic analysis of ectoine biosynthesis genes

The amino acid sequences of EctA, EctB and EctC of the methylotrophs studied were individually compared to each other (Table S1). The highest homologies were found between Ect proteins from methanotrophs *M. alcaliphilum* ML1, *M. kenyense* AMO1 and methanol-utilizers, *M. alcalica* M8 and *M. thalassica* ATCC 33146. However, only low identities of EctABC of *M. marinus* 7C and *M. marina* h1 with those of the above-mentioned bacterial species were found, with EctA being the most divergent protein (36–71% identity, Table S1).

The NCBI database searches using the BLAST network service revealed *ectABC* gene clusters in complete genome sequences of a wide variety of halophilic and halotolerant bacteria. From them, 30 species of gram-

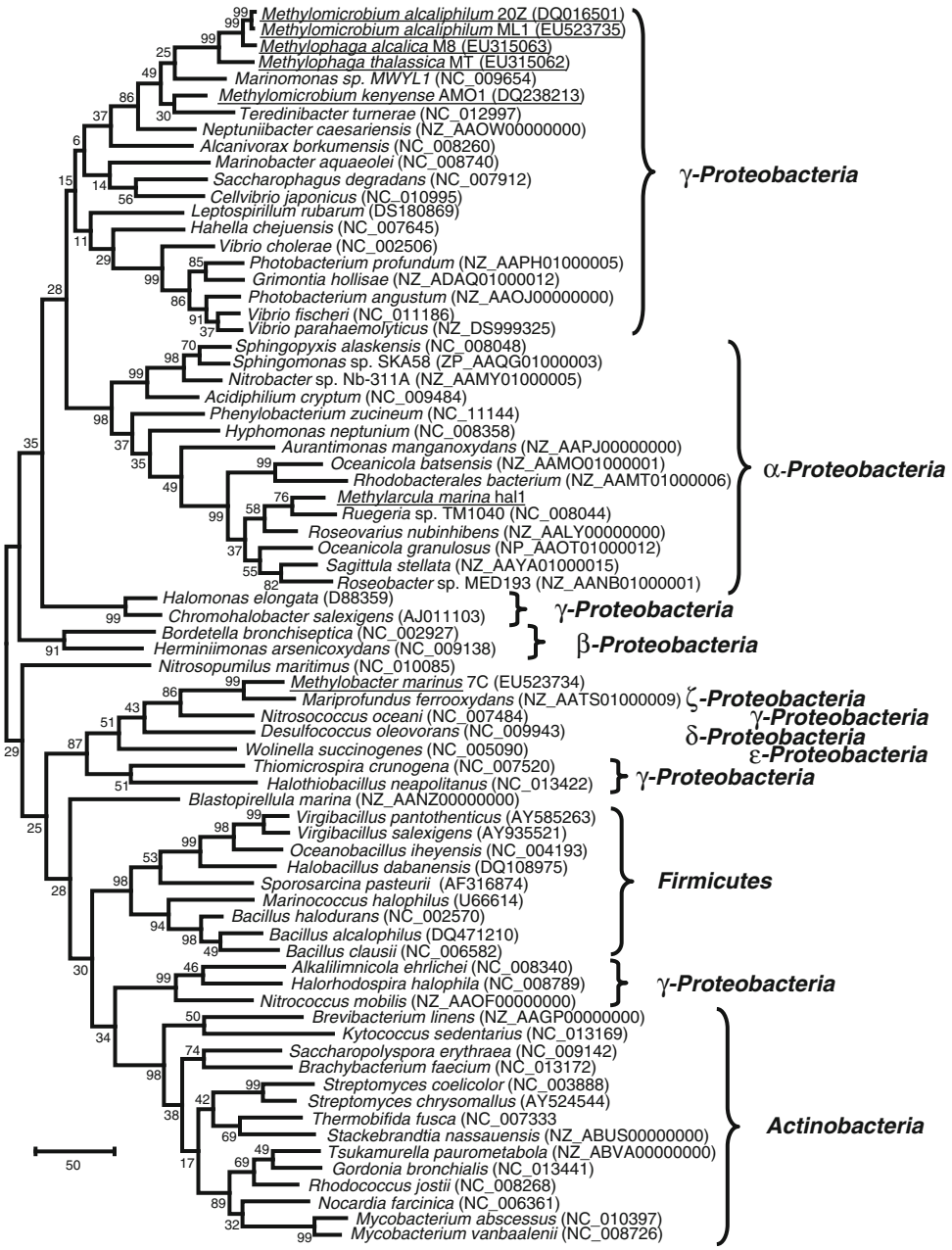

Fig. 1 Maximum-parsimony phylogenetic tree based on deduced amino acid sequences from *ectABC* genes of methylotrophs (*underlined*) and those from other bacteria possessing *ect* genes. The bootstrap consensus tree is based on 1,000 replicates (Felsenstein 1985). *Branches* corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The accession numbers of the respective peptides are given in *brackets*

negative bacteria additionally contained *ask* immediately downstream of *ectABC*, while all sequenced gram-positive halotolerant/halophilic bacteria possessed only the three-gene cluster.

The phylogenetic analysis of concatenated polypeptides representing EctABC sequences from taxonomically diverse groups of halophilic/tolerant bacteria is presented in Fig. 1. Similar topologies were observed for the individual EctA-, EctB- and EctC-based phylogenetic trees (data not shown). The Ect proteins of *M. alcaliphilum* ML1, *M. alcalica* M8, *M. thalassica* ATCC 33146 and *M. kenyense* AMO1 branched together with the other representatives of *Gammaproteobacteria*. The nearest neighbors for "methylotrophic" Ect proteins were those of *Marinomonas* sp. MWYL1 and *Teredinibacter turnerae*. However, Ect proteins from the gammaproteobacterial methanotroph *M. marinus* 7C were most closely related to the proteins of zetaproteobacterial *Marinoprofundus ferrooxidans* (55.3, 80.7 and 78.5% identities of translated amino acid sequences of EctA, EctB and EctC, respectively). In contrast to *M. marinus* 7C, which contained the cluster *ectABC*, the *ect* genes in *M. ferrooxidans* most likely comprised the four-gene cluster *ectABC–ask*. Ect proteins from alphaproteobacterial *M. marina* h1 clustered together with other members of *Alphaproteobacteria* and shared the highest identity with the enzymes from *Ruegeria*

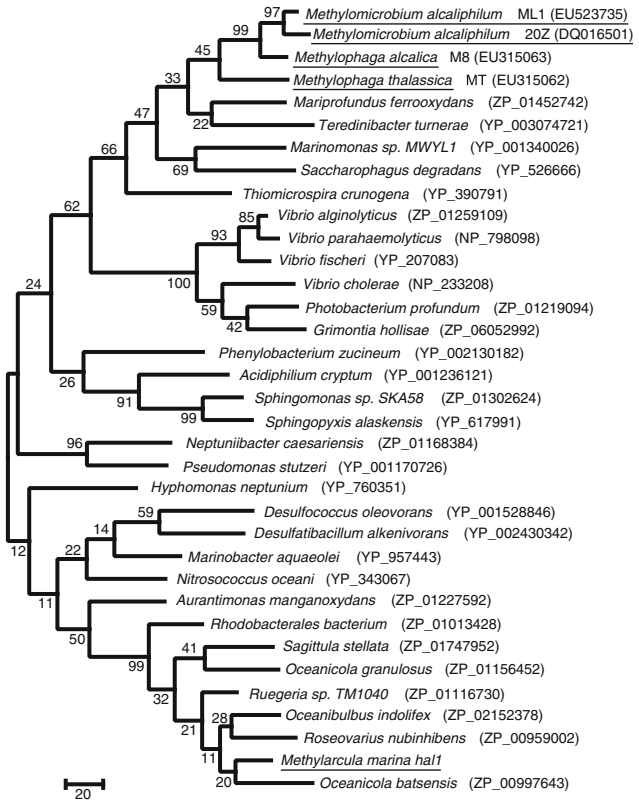

Fig. 2 Phylogenetic tree based on deduced amino acid sequences of *ask* genes located in ectoine gene clusters of methylotrophs (*underlined*) and those from other bacteria possessing *ask* genes downstream *ectABC*

sp. TM 1040 (~52%) and *Roseovarius nubinhibens* (~51%) (Fig. 1).

Figure 2 shows the phylogenetic relationships among homologs of aspartokinase that are co-located with the *ect* genes on the chromosome and presumably involved in ectoine biosynthesis. Sequences of the *ask* genes that are part of *ect* operons in 30 halophilic bacteria were also included in this analysis. Aspartokinases from *M. alcaliphilum* 20Z, *M. alcaliphilum* ML1, *M. alcalica* M8 and *M. thalassica* ATCC 33146 comprise the separate branch on the phylogenetic tree, being most closely related to the protein of *M. ferrooxidans* (94.8, 76.7, 66.9, 57.9% AA identities, respectively).

The homologs of the novel negative transcriptional regulator EctR were identified in 20 genomes of sequenced halophilic bacterial species, including methylotroph *Methylophaga thiooxydans* DMS010. In these cultures, the *ectR*-like gene located immediately upstream of the ectoine gene cluster. Figure 3 shows the phylogenetic relationships among the newly identified EctR homologs and described regulatory proteins of the MarR family. Putative EctR proteins from *M. alcaliphilum* ML1, *M. alcalica* M8, *M. thalassica* ATCC 33146 and *M. marina* h1 showed high sequence identities (54–99%) to EctR1 from *M. alcaliphilum* 20Z (Mustakhimov et al. 2010). EctRs from other halophilic bacteria shared 35.5–55.1% identity with that of strain 20Z. However, all EctR-like proteins shared relatively low identity (≤20%) with other Mar-like transcriptional regulators.

Discussion

In this study, we first identified and compared genetic elements involved in ectoine biosynthesis by slightly halophilic gamma- or alphaproteobacterial methylotrophs. These methano- and methylotrophs were isolated from distantly located saline ecosystems: *M. marinus* 7C (hypersaline lake in Kulunda Steppe, Russia), *M. kenyense* AMO1 (soda lake, Kenya), *M. alcaliphilum* ML1 (Mono Lake, USA), *M. alcalica* M8 (soda lake, Buryatia), *M. thalassica* ATCC 33146 and *M. marina* h1 (Black Sea) (Khmelenina et al. 2010; Sorokin et al. 2000; Doronina et al. 1998, 2000, 2003a). The cultures differed in their osmotolerance and accumulated ectoine as a major osmoprotectant (Doronina et al. 1998, 2000, 2003a; Khmelenina et al. 2010). In these bacteria, ectoine biosynthesis involves DABA acetyltransferase, DABA aminotransferase and ectoine synthase encoded by *ectABC* genes. The salt-dependent methanotrophs, alkaliphilic *M. kenyense* AMO1 and neutrophilic *M. marinus* 7C possess the triad *ectABC*, but in the remainder methylotrophs ectoine biosynthesis genes clustered with a putative aspartokinase and negative transcriptional regulator EctR (*ectR–ectABC–ask*). The presence of the *ask* gene in ectone gene cluster correlated with the enhanced halotolerance of the methylotrophs. The aspartokinase converting aspartate into *β*-aspartyl phosphate represents the starting point of the pathway for biosynthesis of both aspartate family amino acids (threonine, methionine, lysine) and ectoine. The presence of the 'osmotically controlled' aspartokinase would allow ectoine biosynthesis to be independent from the complex regulatory mechanisms that normally control pathways for aspartate family amino acid biosynthesis and might provide an advantage at higher osmolarity.

So far, the correlation between the upper limit of salinity and the presence of the *ask* gene in the *ect* operon was observed only for methylotrophic bacteria described in this work. Database searches of genomes of gram-positive halophiles sequenced so far did not reveal that the *ask* gene co-located with *ect* operon. Besides ectoine, other organic compatible solutes belonging to the glutamate family of amino acids, mostly proline and glutamine, contributed to osmotic balance at high external salinities in gram-positive bacteria (Kuhlmann and Bremer 2002; Roberts 2005; Saum and Müller 2008; Rajan et al. 2008). For example, the chloride-dependent *Halobacillus halophilus* produced

proline at high salinity of growth media (Saum and Müller 2008). It appears that osmoresponse of true halophiles synthesizing ectoine along with other nitrogen-containing osmoprotectants must include separate regulation of different branching pathways for amino acid biosyntheses to avoid the need for co-expression of *ask* and *ect* genes. *Photobacterium profundum* and *Vibrio cholerae* genomes contain two or three *ask* genes in addition to the *ect*-associated *ask*. Alternatively, in the chromosomes of three marine species, *Oceanobacter* sp. RED65, *Sphingomonas* sp. SKA58 and *Lentisphaera araneosa,* no *ask* gene outside the *ect* gene cluster was found. Since amplitude of the seasonal variation in salinity in the ocean is relatively small, we may speculate that constitutive expression of the *ect*- and the linked *ask* genes could provide sufficient activity of Ask enzyme for both osmoprotection and protein biosynthesis.

Hydroxyectoine is known to play an important role in adaptation to high salinity and temperature in a variety of salt-resistant species (Vargas et al. 2008). The enhanced salt tolerance of some gram-negative halophilic bacteria correlates with the occurrence of the *ectD* gene co-located with the ectoine gene cluster (Schwibbert et al. 2010). For example, *Halomonas elongata* DSM 2584 having *ectABCD* operon was capable of growth in the defined glucose–mineral medium at very high salinity as ectoine and hydroxyectoine counterbalance external osmotic pressure (Wohlfarth et al. 1990; Cánovas et al. 1998). Despite that *M. alcaliphilum* 20Z possessed an *ectR–ectABC–ask–ectD* gene cluster (Reshetnikov et al. 2006), transcriptomic studies demonstrated that *ectD* was not a part of the osmotically controlled *ect* operon (Mustakhimov et al. 2010). Consequently, no hydroxyectoine was detected in *M. alcaliphilum* 20Z cells grown at high salinity (Mustakhimov et al. 2010). These data suggested that either the *ectD* was 'silent' in the methanotroph, or the gene was expressed under stress conditions other than high salinity. Also, we failed to identify the *ectD* in the strain *M. alcaliphilum* ML1, a close relative of *M. alcaliphilum* 20Z. Although some methylotrophic strains might possess *ectD* in chromosome, nevertheless our biochemical data showed that none of them accumulated hydroxyectoine. Thus,

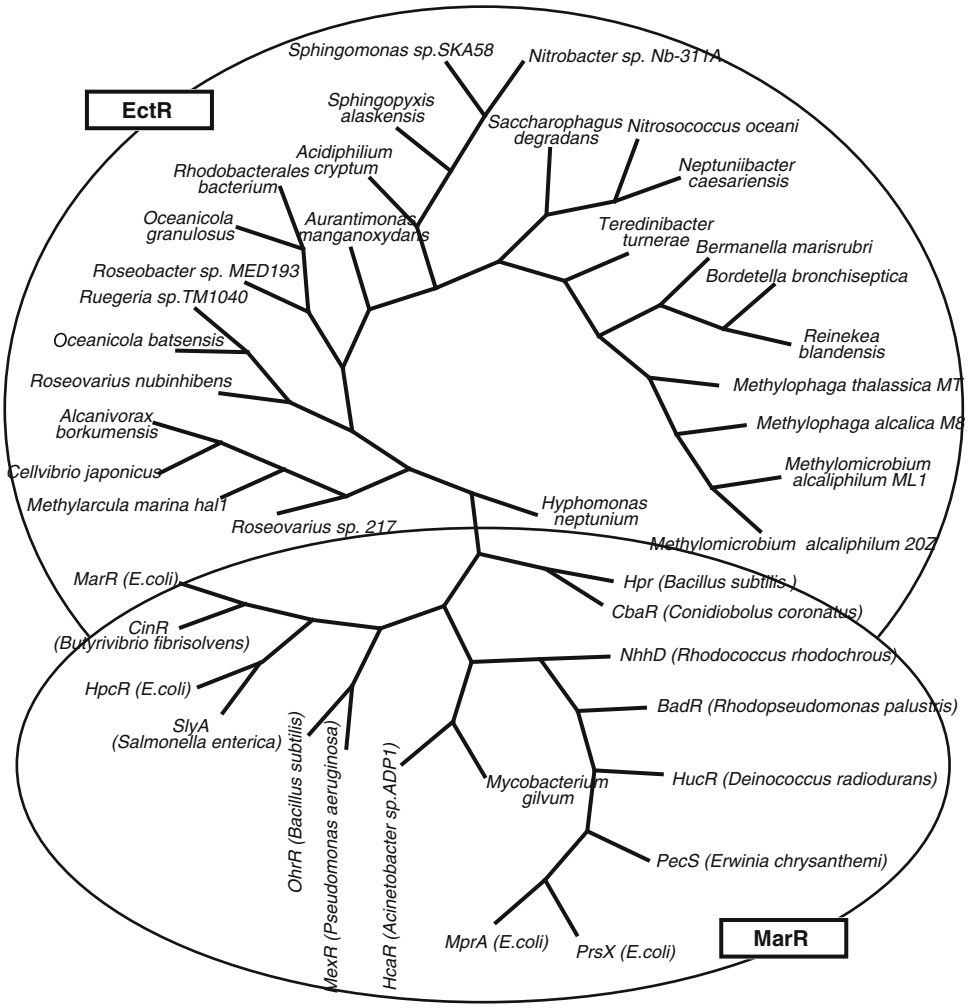

Fig. 3 Phylogenetic tree of putative transcriptional regulators EctR1 of halophilic bacteria and other regulators of the MarR family

hydroxyectoine could not provide the relatively high salt tolerance of the methylotrophs studied.

We believe that transcriptional regulatory mechanisms have an additional impact on bacterial halotolerance. The proteins belonging to a multiple antibiotic resistance regulator (MarR) family of transcriptional regulators are widely distributed across bacterial and archaeal domains. Recently, it has been shown that a MarR homolog (named EctR1) negatively controls expression of the ectoine biosysnthesis enzymes in *M. alcalica* M8 and *M. alcaliphilum* 20Z (Mustakhimov et al. 2009, 2010). Also, *ectR*-like sequences were identified upstream the *ect* genes in methanotroph *M. alcaliphilum* ML1 and methanol- and methylamine-utilizers *M. thalassica* ATCC 33146 and *M. marina* h1. Moreover, co-linkage of the *ectR*-like gene and *ect* operon was also demonstrated in 20 halophilic bacterial genomes from non-redundant databases (Fig. 3). Such a wide distribution and common organization of *ect* operon imply some functional links between these genes. Perhaps, the EctR homologs control ectoine biosynthesis at the transcriptional level in diverse heterotrophic halotolerant/halophilic bacteria. Anyway, gram-positive bacteria and true halophilic gram-negative ones (such as *Halomonas elongata, Halorhodospira halophila, Chromohalobacter salexigens*) do not contain an *ectR*-like gene upstream of the *ect* operon. Contrarily, all of the species presented on the phylogenetic tree for EctR are slightly or moderately halophilic. Moreover, most of them carry either *ectR–ectABC–ask* or *ectR–ectABCD* gene cluster (with the exceptions of *Cellvibrio japonicus, Alcanivorax borkumensis* and *Roseobacter sp.* MED193 not possessing either *ask* or *ectD* gene). Remarkably, in the slightly halophilic autotroph *Nitrobacter* sp. Nb-311A and heterotroph *Bordetella bronchiseptica*, the ectoine biosynthesis genes are organized in *ectR–ectABCD* cluster and this may additionally imply the EctR as a negative regulator of the *ect* genes expression.

Our phylogenetic analysis showed high sequence identities of the EctABC proteins from both methane- and methanol-utilizing bacteria belonging to the *Methylomicrobium* and *Methylophaga* genera. The proteins comprise a coherent group on the phylogenetic tree, being most closely related with EctABC of other representatives of *Gammaproteobacteria*. Two strains of the *M. alcaliphilum* species, ML1 and 20Z, shared very high similarity among Ect proteins (94.8, 98.2 and 98.5% identity for EctA, EctB and EctC, respectively). These strains were isolated from two soda lakes located in geographically remote areas (USA and Russia). Such conservation of *ect* genes may indicate that efficient osmoregulation is a key metabolic function in environmental niches with seasonally fluctuating salinity. Interestingly, amino acid sequences of the Ect proteins from the methanotroph *M. kenyense* AMO1 have higher sequence identity with those of methanol-utilizing bacteria than other methanotrophs. The sequences of EctABC from gammaproteobacterial *M. marinus* 7C also possessed very low homology to the ectoine biosynthesis enzymes from other methylotrophs, being closely related to EctABC from autotrophic zetaproteobacterial bacterium *Mariprofundus ferrooxydans*. As judged from database searches, homologs of the ectoine biosynthetic genes occurred in the autotrophic ammonia-oxidizing archaeon *Nitrosopumilus maritimus*. However, ectoine accumulation in this organism has not yet been examined. The EctABC proteins deduced from the genome of the *Nitrosopumilus maritimus* showed a considerable degree of the amino acid sequence identity (54 and 49% for EctB, 49.3 and 48.5% for EctC, 31.3 and 36.7% for EctA) to the corresponding and functionally characterized EctABC proteins from *M. alcaliphilum* 20Z (Reshetnikov et al. 2006) and *Marinococcus halophilus* (Louis and Galinski 1997).

Overall, a rather high homology of *ect* genes in prokaryotes of various taxonomic positions and physiological properties supported the hypothesis of evolutionary conservation of this biochemical pathway (Kuhlmann and Bremer 2002). Lateral transfers of *ect* genes can be considered as a possible evolutionary event that extended distribution of methylotrophic bacteria across saline ecosystems.

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