Identification and Characterization of EctR1, a New Transcriptional Regulator of the Ectoine Biosynthesis Genes in the Halotolerant Methanotroph *Methylomicrobium* *alcaliphilum* 20Z\(^\dagger\)

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Received 25 April 2009/Accepted 1 November 2009

Genes encoding key enzymes of the ectoine biosynthesis pathway in the halotolerant obligate methanotroph *Methylomicrobium alcaliphilum* 20Z have been shown to be organized into an ectABC-ask operon. Transcription of the *ect* operon is initiated from two promoters, *ectAp1* and *ectAp2* (ectAp-p\(2\)), similar to the \(\sigma^{70}\)-dependent promoters of *Escherichia coli*. Upstream of the gene cluster, an open reading frame (*ectR1*) encoding a MarR-like transcriptional regulator was identified. Investigation of the influence of EctR1 on the activity of the *ectAp-p\(1\)* promoters in wild-type *M. alcaliphilum* 20Z and *ectR1* mutant strains suggested that EctR1 is a negative regulator of the *ectABC-ask* operon. Purified recombinant EctR1-His\(_6\) specifically binds as a homodimer to the putative −10 motif of the *ectAp1* promoter. The EctR1 binding site contains a pseudopalindromic sequence (TATTTAGT-GT-ACTATATA) composed of 8-bp half-sites separated by 2 bp. Transcription of the *ectR1* gene is initiated from a single \(\sigma^{70}\)-like promoter. The location of the EctR1 binding site between the transcriptional and translational start sites of the *ectR1* gene suggests that EctR1 may regulate its own expression. The data presented suggest that in *Methylomicrobium alcaliphilum* 20Z, EctR1-mediated control of the transcription of the *ect* genes is not the single mechanism for the regulation of ectoine biosynthesis.

Mineralization of small saline and soda lakes can vary significantly depending on the season and weather conditions. The ability to rapidly adjust intracellular concentrations of key osmolytes (also known as compatible solutes) to changes in external salinity is an important property of microorganisms inhabiting these biotopes (8, 18, 34). Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrindine carboxylic acid) was found to be a major compatible solute in many halophilic or halotolerant bacteria isolated from alkaline, moderately hypersaline environments (14). This organic solute can be synthesized de novo or taken up from the environment when available (15, 18). The biochemistry and genetics of ectoine synthesis have been described for several bacteria (15, 28, 29, 32). However, little is known about the transcriptional regulation of the ectoine biosynthetic pathway. Comprehensive analysis of the ectoine gene cluster *ectABC* in *Chromohalobacter salexigens* showed four putative transcription initiation sites upstream of the *ectA* start codon. Two \(\sigma^{70}\)-dependent, one \(\sigma^{5}\)-dependent, and one \(\sigma^{32}\)-dependent promoter were identified and shown to be involved in *ectABC* transcription in this bacterium (6). Transcription of the *ectA*, *ectB*, and *ectC* genes from *Marinococcus halophilus* was initiated from three individual \(\sigma^{70}\)/\(\sigma^{5}\)-dependent promoter sequences located upstream of each gene (3). In *Bacillus pasteurii*, the *ectABC* genes are organized in a single operon preceded by a typical \(\sigma^{32}\)-dependent promoter region (21).

The halotolerant obligate methanotroph *Methylomicrobium alcaliphilum* 20Z is capable of growth at a salinity as high as 2 M NaCl (19). It was demonstrated that in response to the elevated salinity of the growth medium, *M. alcaliphilum* cells accumulate ectoine as a major osmoprotective compound (20). The ectoine biosynthesis pathway in *M. alcaliphilum* 20Z is similar to the pathway employed by halophilic/halotolerant heterotrophs and involves three specific enzymes: diaminobutyric acid (DABA) aminotransferase (EctB), DABA acetyltransferase (EctA), and ectoine synthase (EctC) (7, 21, 24, 30, 32, 49). In *M. alcaliphilum* 20Z, the ectoine biosynthetic genes were shown to be organized in the *ectABC-ask* operon containing the additional ask gene, encoding aspartokinase (32). Here we describe the transcriptional organization of the ectoine biosynthetic genes in *M. alcaliphilum* 20Z. We identify a new MarR-like transcriptional regulator (EctR1) and show that EctR1 represses the expression of the *ectABC-ask* operon from the *ectAp1* promoter by binding at the putative −10 sequence. These results demonstrate the presence of a new, previously uncharacterized regulatory system for ectoine biosynthesis in the salt-tolerant methanotroph.

\(\dagger\) Supplemental material for this article may be found at http://jb.asm.org/.

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† Published ahead of print on 6 November 2009.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *M. alcaliphilum* and *Escherichia coli* strains, plasmids, and primers used in this study are listed in Tables S1 and S2 in the supplemental material. *M. alcaliphilum* strains were grown at 30°C under a methane-air atmosphere (1:1) or in the presence of 0.5% (vol/vol) methanol in a mineral salt medium containing 1%, 3%, or 6% NaCl (17, 19). *Escherichia coli* strains were routinely cultivated at 37°C in Luria-Bertani me-
buffer for RevertAid H Minus Moloney murine leukemia virus (M-MuLV) as previously described (32).

washed once with the corresponding medium without Tet and were resuspended in selective medium, consisting of alkaline mineral medium (pH 9.5) containing 3% NaCl. Kan (100 μg/ml) was used as an inhibitor (Fermentas) were subsequently added. The mixture was incubated at 50°C for 1 min before the addition of 200 μg of sonicated herring sperm DNA (Sigma). For the competition assay, unlabeled DNA was added to the reaction mixture after incubation with the labeled fragment. Samples were analyzed by native gel (6% polyacrylamide) electrophoresis in 1x Tris-borate-EDTA buffer (36). EctR1-His was purified with a QIAexpress nickel-nitri-ltriacetic acid Fast Start kit (Qiagen) according to the manufacturer’s protocol. Fractions were collected and analyzed by SDS-PAGE followed by Coomassie blue staining. The fractions with the majority of EctR1-His, were combined and dialyzed overnight at 4°C against 100 mM Tris-HCl (pH 8.0) buffer containing 400 mM NaCl and were then stored at 4°C. Protein concentrations were determined by the Lowry assay (37).

Electrophoretic mobility shift assay (EMSA). A DNA fragment of 213 bp, corresponding to the promoter region, was amplified by PCR using primer RT20Z and 32P-end-labeled primer Fr. One picomole of the labeled DNA fragment (10,000 cpm) was incubated for 15 min at room temperature in 20 μL of binding buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) in the presence of 400 mM NaCl and were then stored at 4°C. Protein concen-
trations were determined by the Lowry assay (37).
FIG. 1. Multiple sequence alignment of \textit{M. alcaliphilum} 20Z. EctR1 and representative MarR family members: SlyA, a regulator of virulence factors from \textit{Enterococcus faecalis} (NCBI accession no. 1LJ9_A) (41); OhrR, a repressor of an organic hydroperoxide resistance determinant from \textit{Bacillus subtilis} (13); MarR, a regulator of multiple antibiotic resistance from \textit{E. coli} (P27245) (1); MexR, a multidrug efflux system transcriptional repressor from \textit{Pseudomonas aeruginosa} (AAO40238) (22); and HucR, a repressor of the uricase genes from \textit{Deinococcus radiodurans} (2FBK_A) (47).

The alignment was generated using Clustal X, and the amino acid numbering was based on the HucR sequence. Number signs (#) above the sequence indicate residues identified from the MarR crystal structure as forming the hydrophobic core of the monomeric DNA binding domain (1). The HTH DNA-binding motif and flanking “wing 1” region identified from the MarR crystal structure are indicated below the alignment. Shaded and open bars represent alpha-helices and beta-sheets, respectively.

RESULTS

Identification of a new gene upstream of the \textit{ectABC}-ask operon in \textit{M. alcaliphilum} 20Z. In \textit{M. alcaliphilum} 20Z, the ectoine biosynthesis genes are organized in the \textit{ectABC}-ask operon (32). A partial sequence of an additional open reading frame (orf1), in reverse orientation relative to the \textit{ectABC}-ask cluster, was identified upstream of the \textit{ectA} gene. The whole sequence of orf1 was determined by using inverse PCR. A BLAST search suggested that the orf1 region encodes a putative MarR-type transcriptional regulator. Multiple sequence alignments of the predicted protein with characterized MarR regulators indicated that Orf1 has the highest identities with OhrR (20.5%), MarR (15%), and EmrR (19.6%) from \textit{E. coli} (10, 13, 23, 25, 41, 43, 44), PecS (18.6%) from \textit{Erwinia chrysanthemi} strain 3937 (33), MexR (17.8%) from \textit{Pseudomonas aeruginosa} (12, 31, 40), and SlyA (15.6%) from \textit{Salmonella enterica} serovar Typhimurium (41, 48). Downstream of the orf1 stop codon (38 bp), a stem-loop-like structure that might function as a rho-independent terminator (with a calculated free energy of $-12.4$ kcal/mol) was found. In view of the opposite orientation of orf1 with respect to the \textit{ectABC}-ask operon and the sequence homology of the gene product with MarR family regulators, orf1 was tentatively designated ectR1 (for “ectoine biosynthesis regulator”). The predicted EctR1 protein consists of 180 amino acids and has a molecular mass of 20.6 kDa and a pi of 7.17. An amino acid sequence similar to that of the conserved DNA binding domain (a winged helix-turn-helix [HTH] motif) of the MarR family of transcriptional regulators was identified in the central part of putative EctR1 (Fig. 1).

Identification of the \textit{ectABC}-ask and \textit{ectR1} transcription units and the effect of medium salinity on their expression. To determine the sizes and expression levels of the \textit{ect} operon and...
ectRI gene transcripts, we used Northern hybridization with probes corresponding to the 3′-proximal sequences of the ask and ectRI genes. For this purpose, RNA was extracted from M. alcaliphilum 20Z cells grown in mineral medium with either 1% NaCl, 6% NaCl, or salt-stressed cells. Cells defined as “salt-stressed cells” were grown with 1% NaCl to an O.D. of 0.5 to 0.6 and were then incubated for 1 h at a higher salinity (6% NaCl). With the probe corresponding to the ask gene, two radiolabeled bands at about 4 and 2.3 kb were detected (Fig. 2A). The length of the 4-kb transcript was sufficient to carry the ectA, ectB, ectC, and ask genes on a polycistronic message. The presence of a 2.3-kb RNA band indicated that the ectC and ask genes (1,908-bp fragment) might be cotranscribed as an additional transcriptional unit from the promoter region located upstream of the ectC gene.

For the ectRI probe, a single hybridization band was found under all growth conditions tested. The molecular size of the transcript (Fig. 2B) was calculated to be approximately 0.8 kb, which was larger than the expected length of a monocistronic transcript of the ectRI gene (543 bp).

The abundance of the ectABC-ask transcripts was 30-fold higher in salt-stressed cells than in cells grown at 1% NaCl (Fig. 2). In cells of M. alcaliphilum 20Z grown at 6% NaCl, the level of ectABC-ask mRNA increased about 13-fold. The levels of transcription of ectRI in salt-stressed cells and in cells grown at 6% NaCl also increased (5- and 3-fold, respectively).

Most halophilic/halotolerant heterotrophic bacteria prefer the accumulation of exogenous osmoprotectants to de novo synthesis. In general, the addition of an osmoprotectant (or its precursor) to the growth medium represses the biosynthesis of an endogenous solute (8, 18, 34). We examined the effects of exogenously provided organic solutes on the expression of the ectABC-ask operon by M. alcaliphilum 20Z during an osmotic upshift. Cultures grown at 1% NaCl were supplemented with ectoine (1 mM) or glycine betaine (1 mM) and were stressed by salt addition up to 6% NaCl. The activity of DABA acetyltransferase, an indicator of the induction of the ectoine biosynthesis pathway, was monitored after 1 and 3 h of incubation at high salinity. Cells incubated either alone or with added osmoprotectants demonstrated similar rates of 1-diaminobutyric acid conversion (see Table S3 in the supplemental material). Thus, the addition of exogenous solutes has no noticeable effect on the induction of the ectoine biosynthesis pathway in M. alcaliphilum 20Z.

Identification of transcriptional start sites of the ectABC-ask operon and the ectRI gene. To map the transcriptional start sites of the ectABC-ask operon and the ectRI gene, primer extension reactions were conducted with total-RNA samples isolated from salt-stressed cells. Two transcripts were detected (Fig. 3A). The start sites of the transcripts were mapped at bp 118 and bp 69 upstream of the ectRI gene. This observation suggested that the ect operon is transcribed from two promoters, ectAp1 and ectAp2 (Fig. 3D). The potential ectAp1 promoter elements, TACTAT for the −10 and TGGACA for the −35 site with 16-bp spacing, match well with the consensus sequences (TATAAT and TGGACA) for a promoter recognized by the primary vegetative sigma factor, σ70 of E. coli (16). Moreover, ectAp1 displayed a TG motif as an upstream extension of the −10 box (35). The putative −10 sequence (TAAAAA) of ectAp2 showed identity to the σ70-dependent consensus for E. coli, while the putative −35 sequence (CAGAAT) matched two of the six nucleotides (Fig. 3D).

The transcriptional start site of the ectRI gene was assigned to a position 250 bp upstream of the ATG translational initiation site. The sequence of the putative −10 box (TCTGAA) exhibited similarity with the −10 consensus sequence of the E. coli σ70-recognized promoter, while the putative −35 site of the ectRip promoter does not resemble that of E. coli (Fig. 3B and D).

The primer extension analysis was also performed by using total RNA isolated from E. coli cells containing plasmid pHSGectABC, carrying the sequence of the ectABC genes and their promoter region. Two transcriptional start sites identical to those obtained from M. alcaliphilum 20Z were revealed (Fig. 3C). Hence, the ectAp1 and ectAp2 promoters are recognized by transcriptional systems in both strains M. alcaliphilum 20Z and E. coli XL-1-Blue.

Null mutations in the ectRI gene result in overexpression of the ectABC-ask operon. In order to elucidate the function of EctR1 in the regulation of the ect operon, we constructed an ectRI gene insertion mutant via allelic exchange. The growth rate of the ectRI knockout strain (designated EBP01) was similar to that of the wild type at 1, 3, and 6% NaCl, thus indicating that EctR1 is most likely not essential for activation of ect operon transcription in response to elevated salinity.

We further tested the expression of the ectoine biosynthesis genes in the ectRI mutant via transcriptional fusions to a promoterless reporter gene, gfp. The ectA promoter region (352 bp) was amplified by PCR and cloned into the pTSGEx vector. The plasmid carrying the ectAp1,-gfp fusion was introduced into both wild-type M. alcaliphilum 20Z and EBP01, and the resulting strains were assayed for gfp expression. At all salt concentrations tested, the activities of the ectAp1 promoter region were 2- to 3-fold higher in the strain lacking ectRI than in the wild type (Table 1). The activities of DABA acetyltransferase, a key enzyme of the ectoine biosynthesis pathway, followed the same pattern: they were 2- to 6-fold higher in strain EBP01 (Table 1). Thus, the mutation in the ectRI gene resulted in derepression of the ect operon. The data indicated that, like most MarR family proteins, EctR1 acts as a negative transcriptional regulator. However, the expression of the ect...
operon was activated in response to high osmolarity of the growth medium in the mutant strain, thus indicating that EctR1 is not the sole regulator of the system.

EctR1-His6 specifically binds to DNA fragments containing the ectAp1, ectAp2, and ectR1p promoters. The coding region of ectR1 was cloned into the pET-22b(+) expression vector, and the resulting C-terminally His-tagged protein was overexpressed in E. coli. The recombinant protein, EctR1-His6, was purified to high homogeneity (data not shown). The molecular mass of EctR1 estimated by SDS-PAGE was about 23 kDa, which is in reasonable agreement with the predicted size (22.039 kDa) deduced from the amino acid sequence. To prove the specific binding of EctR1 to the promoter region of the ectABC-ask operon, an electrophoretic mobility shift assay (EMSA) was performed. A clear DNA shift was observed when 40 to 160 pmol of EctR1-His6 and 1 pmol of an end-labeled DNA fragment (213 bp) containing the ectAp1, ectAp2, and ectR1p promoters were used. Sixty picomoles of EctR1-His6 shifted about half of the DNA probe (Fig. 4). Incubation of EctR1 with unlabeled specific DNA fragments (at a 10-fold molar ratio) abolished the retardation of labeled DNA. These results suggested a specific interaction between EctR1 and the promoter regions of the ectABC-ask and ectR1 genes.

**FIG. 3.** Determination of transcriptional start sites for the ectABC-ask operon and the ectR1 gene. Primer extension analyses were carried out with total RNA prepared from M. alcaliphilum 20Z cells (A and B) and E. coli cells (C) using primers complementary to the ectA (A and C) and ectR1 (B) genes as indicated in Materials and Methods. Primer-extended products were separated by electrophoresis under denaturing conditions alongside the products of sequencing reactions with the same primers. (D) Sequence of the ectRI-ect4 intergenic region. Bent arrows indicate the transcriptional initiation sites of the ectABC-ask operon and the ectR1 gene. Putative promoter elements (−10 and −35 boxes) for the ectAp1 and ectAp2 promoters of the ectABC-ask operon are underlined. Putative −10 and −35 elements for the promoter of the ectR1 gene are shaded. The translational start codons for the ectA and ectRI genes are indicated by arrows. The inverted repeat of the EctR1 binding site (see below) is boxed.

**TABLE 1.** Activities of the ectAp1p2-gfp promoter fusion and diaminobutyric acid acetyltransferase in wild-type and ectR1 mutant strains of M. alcaliphilum. 

<table>
<thead>
<tr>
<th>Salinity of the growth medium (% NaCl)</th>
<th>Activity with the indicated strain</th>
<th>Activity with the indicated strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ecatp1p2-gfp (RFU OD600⁻¹ h⁻¹)</td>
<td>DABA-acetyltransferase (mmol min⁻¹ mg of protein⁻¹)</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>EBPR01</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22 ± 2.3</td>
<td>5 ± 1.2</td>
</tr>
<tr>
<td>3</td>
<td>40 ± 5</td>
<td>57 ± 7</td>
</tr>
<tr>
<td>6</td>
<td>130 ± 25</td>
<td>83 ± 11</td>
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<tr>
<td></td>
<td>Wild type</td>
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<td>1</td>
<td>65 ± 9</td>
<td>33 ± 5.3</td>
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<tr>
<td>3</td>
<td>95 ± 15</td>
<td>200 ± 23</td>
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<tr>
<td>6</td>
<td>220 ± 22</td>
<td>150 ± 18</td>
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| a RFU, relative fluorescence units.
**Ectoine Biosynthesis Regulation in M. Alcaliphilum 20Z**

**DISCUSSION**

We previously reported that in the halotolerant obligate methanotroph *M. Alcaliphilum* 20Z, genes encoding enzymes for biosynthesis of the compatible solute ectoine are organized into the *ectABC-ask* operon (32). The *ectABC-ask* operon is transcribed from two σ^70-like promoters. Similar σ^70 promoters drive the expression of ectoine gene clusters in a variety of halophilic species, such as *Chromohalobacter salexigens*, *Bacillus pasteurii*, and *Marinococcus halophilus* (6, 21, 24), and thus, such an organization of transcriptional machinery is not unique for the methanotrophic bacterium.

A gene (designated *ectR1*) encoding a transcriptional regulator belonging to the MarR family was identified upstream of the *ectABC-ask* operon in *M. Alcaliphilum* 20Z. We showed that deletion of the *ectR1* gene results in derepression of the ectoine biosynthesis genes at different salt concentrations in the growth medium, thus indicating that *EctR1* acts as a negative regulator of the *ectABC-ask* operon. This finding was further supported by the DNase I footprinting assay data. We found that the EctR1 binding site overlaps with the putative −10 element of the *ectAp1* region of the *ect* operon, resulting in complete blocking of the promoter and thus suggesting steric inhibition of RNA polymerase recruitment. However, very low DNA binding activity (160 pmol of protein per 1 pmol of DNA) of the recombinant EctR1 preparation was detected. We suppose that an unknown mechanism (modification or metabolic signal interactions) may regulate the DNA binding ability of EctR1 depending on the medium osmolarity, but the respective systems for posttranslational modification that are involved (8, 9, 37) are absent in *M. Alcaliphilum* 20Z.

The salt-dependent activation of expression of the *ect* operon observed for the *ectR1*-impaired strain also indicated that *M. Alcaliphilum* 20Z may possess a complex regulatory system that involves multiple layers of responses. At present, we may only speculate that regulation of the *ect* genes involves a dynamic balance between repression by the EctR1 regulator and, most likely, activation mediated by a specific, yet unknown component(s). Characterization of the additional regulatory components of the transcriptional control system of the *ectABC-ask* operon is currently under way.

The MarR family includes a diverse group of regulators that can be classified into three general categories in accordance...
with their physiological functions: (i) regulation of response to environmental stress, (ii) regulation of virulence factors, and (iii) regulation of aromatic catabolic pathways (48). To our knowledge, EctR1 is the first example of a MarR-like regulator that controls osmoreponse genes. Like other members of the MarR family, EctR1 is located in an orientation opposite that of the controlled gene cluster, has a conservative winged helix-turn-helix motif, and binds to DNA as a homodimer. The EctR1 binding site contains two imperfect inverted repeats with 2 bp separating the two halves of the pseudopalindrome. The centers of the palindrome half-sites are separated by 10 bp, thus indicating that the positioning of each subunit of the EctR1 homodimer occurs on the same face of the DNA helix. This mode of EctR1-DNA binding is similar to that for other members of the MarR family proteins, such as HucR and MexR (12, 47), but different from that for E. coli MarR, which binds to DNA on different faces of the double helix (25).

The levels of both ectABC-ask and ectR1 transcription correlate with the salinity of the growth medium. Since the EctR1 binding site is located between the ectR1 transcription and translation start sites, EctR1 may repress its own expression via inhibition of the elongation process. Autoregulation has been observed for many transcriptional regulators, such as MarR (25), CinR (9), EmR (10, 23), and HucR (47). In particular, HucR represses the transcription of its own gene and that of genes involved in the catabolism of uric acid (47). This repression is relieved by the binding of uric acid to the repressor, reducing its DNA binding ability. In the case of M. alcaliphilum 20Z, it was demonstrated that cells grown in a medium without NaCl accumulate small concentrations of ectoine (20); thus, ectoine is always present in the cytoplasm and most likely does not alter the DNA binding ability of EctR1. Moreover, the addition of exogenous ectoine or glycine betaine to the growth medium did not affect the induction of enzymes involved in the ectoine biosynthesis pathway.


REFERENCES
ECTOINE BIOSYNTHESIS REGULATION IN M. ALCALIPHILUM 20Z


