JOURNAL OF BACTERIOLOGY, Jan. 2010, p. 410–417 Vol. 192, No. 2
0021-9193/10/$12.00 doi:10.1128/JB.00553-09

Identification and Characterization of EctR1, a New Transcriptional Regulator of the Ectoine Biosynthesis Genes in the Halotolerant Methanotroph *Methylomicrobium alcaliphilum* 20Z▽†

Ildar I. Mustakhimov,[1] Alexander S. Reshetnikov,[1] Anatoly S. Glukhov,[2] Valentina N. Khmelenina,[1] Marina G. Kalyuzhnaya,[3]* and Yuri A. Trotsenko[1]

G. K. Skryabin Institute of Biochemistry and Physiology of Microorganisms[1] *and Institute of Protein Research,*[2] *Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russia, and Department of Microbiology, University of Washington, Seattle, Washington*[3]

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, $ectAp_1$ and $ectAp_2$ ($ectAp_1p_2$), similar to the σ^{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 $ectAp_1p_2$ 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 $ectAp_1$ 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 σ^{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-pyrimidine 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 σ^{70}-dependent, one σ^{S}-dependent, and one σ^{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 σ^{70}/σ^{A}-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 σ^{A}-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 $ectAp_1$ 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.

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-

* Corresponding author. Mailing address: Department of Microbiology, University of Washington, Seattle, WA 98195-1750. Phone: (206) 543-0355. Fax: (206) 616-5721. E-mail: mkalyuzh@u.washington.edu.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 6 November 2009.

dium (2). The following antibiotic concentrations were used: tetracycline (Tet), 12.5 μg ml^{-1}; kanamycin (Kan), 100 μg ml^{-1}; ampicillin (Amp), 100 μg ml^{-1}.

Identification of the *ectR1* gene. Inverse PCR was used to amplify the upstream region of the *ectABC-ask* operon. Approximately 200 ng of the *M. alcaliphilum* DNA was digested with BstACI, and the resulting fragments were self-ligated overnight at 16°C in 100 μl of reaction mixture with 2 U T4 DNA ligase, followed by precipitation in ethanol. An aliquot of ligation products was used as a template in PCR with primers 20PROr and TraX (see Table S2 in the supplemental material), designed on the basis of previously identified sequences of the ectoine biosynthesis genes in *M. alcaliphilum* 20Z (GenBank accession no. DQ016501). The resulting PCR product of ~1 kb was sequenced using the same primers.

Construction of an *ectR1* mutant. Genomic fragments containing the *ectR1* upstream (724-bp) and downstream (607-bp) flanks were PCR amplified, cloned into pCR2.1 (Invitrogen), excised by AatII/KpnI (*ectR1* upstream flank) or SacI/SacII (*ectR1* downstream flank), and then subcloned into pCM184 (26) using appropriate restriction sites (see Table S2 in the supplemental material). The resulting plasmid (pEBP01) was transformed into *E. coli* S17-1, and the resulting donor strain was mated with wild-type *M. alcaliphilum* in a biparental mating. Biparental matings were performed at 30°C for 48 h on mating medium (MM), consisting of mineral salt medium (as described in reference 17, except that the carbonate salts were removed and the pH was adjusted to 7.6) supplemented with 10% nutrient broth (BD Difco) and methanol (5 mM) and containing 0.3% NaCl. Cells were then washed with sterile medium and plated on selective medium, consisting of alkaline mineral medium (pH 9.5) containing 3% NaCl and supplemented with methanol (50 mM) and, if needed, Kan (100 μg ml^{-1}). High pH and high salinity were used for *E. coli* counterselection. The Kan^r recombinants were selected on methanol plates and checked for resistance to Tet (25 μg ml^{-1}). Tet-sensitive (Tet^s) recombinants were chosen as possible double-crossover recombinants. Double-crossover mutants were further verified by diagnostic PCR with primers specific to the insertion sites.

Construction of an *ect*-promoter fusion. The 352-bp fragment containing the *ectR1-ectA* intergenic region was amplified by PCR (the primers are shown in Table S2) and cloned into the pCR2.1 vector. The fragments were subsequently excised by PstI and BamHI and then cloned into the pTSGex promoter probe vector (42), resulting in a construct (pEBP1) containing the respective DNA fragments upstream of the promoterless reporter gene *gfp*. The resulting constructs were transformed into *E. coli* S17-1 (39) and then transferred into *M. alcaliphilum* 20Z and the EBPR01 mutant via conjugation using plasmid pRK2073 as a helper (11). After 48 h of incubation on MM, cells were plated on selective medium, consisting of alkaline mineral medium (pH 9.5) containing 3% NaCl and supplemented with methanol (25 mM), Tet (25 μg ml^{-1}), and, if needed, Kan (100 μg ml^{-1}).

Fluorescence measurements. Validation of the *ectA*p_1p_2-*gfp* reporter system and fluorimetry-based gene expression analysis were performed on wild-type and mutant strains of *M. alcaliphilum* grown on alkaline mineral medium (19) supplemented with methanol (25 mM), Tet (25 μg ml^{-1}), and 1, 3, or 6% NaCl to mid-exponential phase (optical density at 600 nm [OD_{600}], 0.5 to 0.6). After growth, the cultures were centrifuged at 4,500 × *g* for 15 min at 21°C. Cells were washed once with the corresponding medium without Tet and were resuspended in the same medium at an OD_{600} of approximately 0.4 to 0.5. Fluorescence measurements were carried out with a Shimadzu RF-5301PC fluorimeter as described previously (42). Green fluorescent protein (GFP) UV excitation was conducted at 405 nm, and emissions were monitored at 509 nm. Promoter activities were calculated as previously described (27) by plotting fluorescence versus OD_{600}.

Enzymatic assays. Wild-type or mutant strains of *M. alcaliphilum* were grown to mid-exponential phase (OD_{600}, 0.5 to 0.6) in alkaline mineral medium (14) containing 1%, 3%, or 6% NaCl and supplemented with methanol (25 mM). Cells were collected by centrifugation at 4,500 × *g* for 15 min at 4°C. Extracts were prepared, and diaminobutyric acid acetyltransferase activity was measured, as previously described (32).

RNA isolation and primer extension assay. To determine the first transcribed nucleotides for the *ectABC-ask* operon, primer extension assays were performed using RNA isolated from salt-stressed cells. *M. alcaliphilum* 20Z was grown to an OD_{600} of 0.4 in the presence of 1% (wt/vol) NaCl. Expression of the *ectABC-ask* operon was induced by addition of NaCl up to a final concentration of 6% (wt/vol) followed by incubation for 1 h. RNA was isolated as described previously (32). RNA (10 μg) was mixed with deoxynucleoside triphosphates (1 mmol) and 2 pmol of ^{32}P-end-labeled primer ST2 (see Table S2 in the supplemental material), which anneals to the *ectA* gene from position +6 to position +30, in a total volume of 12 μl. The mixture was heated for 5 min at 70°C and then quickly chilled on ice. Actinomycin D (final concentration, 1 μg/ml), 4 μl (5×) reaction buffer for RevertAid H Minus Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas, Lithuania), and 40 U of RiboLock RNase inhibitor (Fermentas) were subsequently added. The mixture was incubated at 50°C for 1 min before the addition of 200 U of RevertAid H Minus M-MuLV reverse transcriptase (Fermentas). The primer extension reaction was conducted at 43°C for 60 min and was stopped by enzyme inactivation at 70°C for 15 min. The primer extension products were precipitated by ethanol. The same primer (ST2) was used to produce sequence ladders by using the *fmol* DNA cycle sequencing system (Promega) in accordance with the manufacturer's instructions. Primer extension products and sequence ladders were separated on a 6% urea-polyacrylamide sequencing gel. To determine the transcription start site for the *ectR1* gene, a primer extension reaction was carried out as described above using RNA obtained from cells grown with 1% (wt/vol) NaCl and ^{32}P-end-labeled primer TSR1 (see Table S2 in the supplemental material). Dried sequencing gels were visualized using a Cyclone Storage Phosphor system (Packard Instruments Co.).

The ability of the *E. coli* transcriptional system to recognize the promoter sequence of the *ect* operon of *M. alcaliphilum* 20Z was demonstrated by primer extension analysis. The primer extension reaction was performed using RNA extracted from *E. coli* XL1-Blue cells transformed with recombinant plasmid pHSG*ectABC*, carrying the nucleotide sequence from bp 301 upstream of the *ectA* gene to bp 173 downstream of the *ectC* gene, and grown in M9 minimal medium with 4% NaCl and glucose (32, 38).

Northern blotting. Northern blot analyses of the *ectR1* gene and *ectABC-ask* operon transcripts were performed as described previously (36). RNA was isolated from *M. alcaliphilum* cells grown either with 1% or 6% NaCl or with salt stressed cells. Total RNA was separated on a formaldehyde-containing agarose gel and was transferred to a positively charged nylon membrane (Hybond-N$^+$; Amersham Biosciences) using a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instruction. Hybridization was performed under high-stringency conditions (50°C, 50% formamide) with gene-specific ^{32}P-labeled probes for the *ectR1* and *ask* genes, which had been prepared by PCR using [α-^{32}P]dATP with oligonucleotide pairs ectR1C–20PROr and AspIF–RPHy (see Table S2 in the supplemental material), respectively. Double-stranded DNA probes were denatured at 100°C for 5 min before hybridization.

Heterologous expression of EctR1 and purification of the recombinant protein. The *ectR1* gene was amplified by PCR from chromosomal *M. alcaliphilum* 20Z DNA by using *Pfu* DNA polymerase (Fermentas, Lithuania) and primers ectR1N and ectR1C (see Table S2 in the supplemental material). The amplified fragment (556 bp) was digested by NdeI and HindIII and was ligated into the pET-22b(+) vector (Novagen), resulting in the pET*ectR1-his* construct.

E. coli BL21(DE3) cells transformed with pET*ectR1-his* were grown overnight in 2 ml of LB medium containing ampicillin (100 μg/ml). The overnight culture was placed in 200 ml of fresh LB medium with ampicillin and was grown at 37°C to an OD_{600} of 0.3 to 0.5. Protein expression was then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h of incubation, cells were harvested by centrifugation and disrupted by sonication in 5 ml lysis buffer (100 mM Tris-HCl [pH 8.0], 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) in an MSE (United Kingdom) sonifier. Cell debris was removed by centrifugation at 15,000 × *g* and 4°C for 30 min. EctR1-His_6 was purified with a QIAexpress nickel-nitrilotriacetic 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_6 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 *ect* promoter region, was amplified by PCR using primer RT20Z and ^{32}P-end-labeled primer F1. 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], 10 mM $MgCl_2$, 200 mM KCl) with increasing amounts of pure EctR1-His_6 (from 1 to 320 pmol) and 0.5 μg of sheared 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 1× Tris-borate-EDTA buffer (36).

DNase I footprinting. A DNA fragment containing the promoter regions of the *ectR1* gene and the *ectABC-ask* operon was amplified using a combination of an unlabeled primer (F1) and primer RT20Z labeled with T4 polynucleotide kinase and [γ-^{32}P]dATP. The PCR product was purified from a 6% native polyacrylamide gel by the "crush-and-soak" method (36). Labeled PCR products (about 2 pmol) were incubated at room temperature with 4 μg of purified EctR1-His_6 in a 30-μl reaction mixture containing binding buffer (see above), 20 μg/ml bovine serum albumin (BSA), and 0.25 μg of sonicated herring sperm

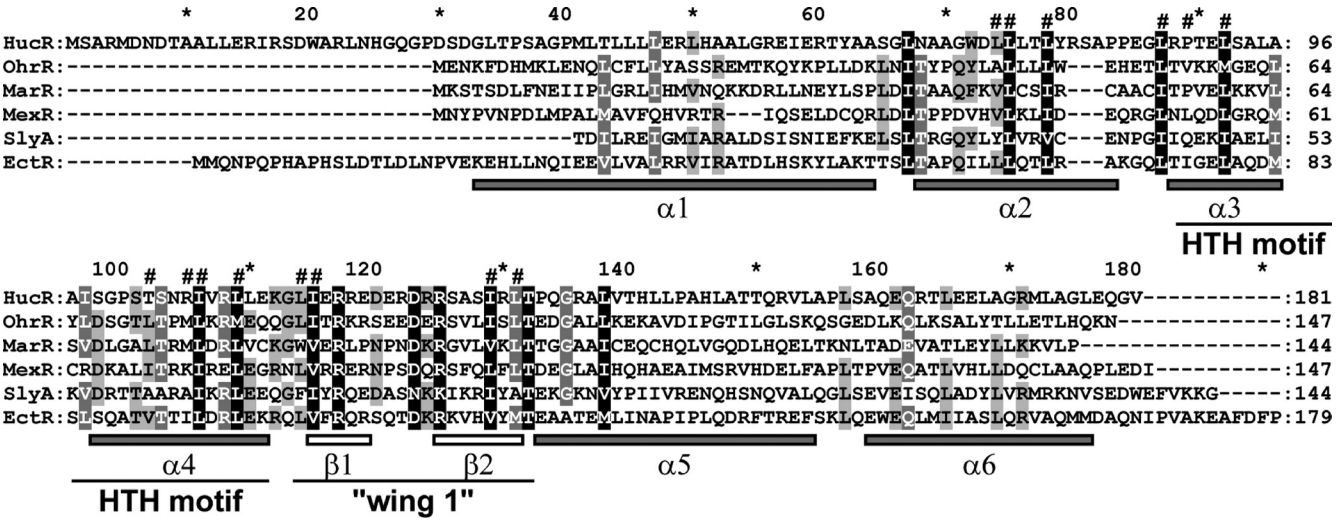

FIG. 1. Multiple sequence alignment of *M. alcaliphilum* 20Z EctR1 and representative MarR family members: SlyA, a regulator of virulence factors from *Enterococcus faecalis* (NCBI accession no. 1LJ9_A) (41); OhrR, a repressor of an organic hydroperoxide resistance determinant from *Bacillus subtilis* (13); MarR, a regulator of multiple antibiotic resistance from *E. coli* (P27245) (1); MexR, a multidrug efflux system transcriptional repressor from *Pseudomonas aeruginosa* (AAO40258) (22); and HucR, a repressor of the uricase genes from *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.

DNA. After 20 min of incubation, 0.1 mM $CaCl_2$ and 0.8 U DNase I (Fermentas, Lithuania) were added. The DNase I digestion reaction proceeded for 1 min at room temperature and was terminated by the addition of 100 μl of stop solution (192 mM sodium acetate, 50 mM EDTA, 0.14% SDS, 1 μg glycogen). DNA fragments were extracted by phenol-chloroform and precipitated with ethanol. Samples were separated alongside Maxam-Gilbert (G+A) chemical sequencing ladders (36) by 6% polyacrylamide–8 M urea gel electrophoresis.

Size exclusion chromatography. Gel filtration chromatography of recombinant EctR1 was performed on an Ultrogel AcA 44 column (1.5 cm by 60 cm; LKB, Sweden) equilibrated with 20 mM Tris-HCl (pH 8.0) containing 200 mM KCl and 10 mM $MgCl_2$ at a flow rate of 0.2 ml/min. For analysis of the EctR1-His_6-DNA complexes, 80 pmol of EctR1-His_6 was incubated for 20 min at room temperature with 1 pmol of the ^{32}P-end-labeled double-stranded oligonucleotide RBS, obtained by annealing complementary primers RBS1 and RBS2 (see Table S2 in the supplemental material). Gel filtration chromatography of the EctR1-His_6-DNA complexes was performed as indicated. Radioactive fractions were analyzed by SDS-PAGE and native PAGE to verify the presence of EctR1-His_6 and EctR1-His_6-DNA complexes, respectively. The apparent molecular masses of EctR1-His_6 and EctR1-His_6-DNA complexes were estimated using the partition coefficient K_{av}, calculated as $(V_e - V_0)/(V_t - V_0)$ versus the log of the molecular masses of the standard proteins (RNase A, 13.7 kDa; chymotrypsinogen A, 25 kDa; DNase, 31 kDa; ovalbumin, 45 kDa; BSA, 66 kDa). V_0 and V_e are the void and elution volumes, respectively, and V_t is the total column volume.

General DNA manipulations. Chromosomal DNA from *M. alcaliphilum* 20Z was prepared as described previously (17). Isolation of plasmid DNA from *E. coli* and transformations were carried out by standard techniques as described previously (36). Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and *Taq* and *Pfu* DNA polymerases were obtained from NBI Fermentas (Lithuania) and were used according to the manufacturer's instructions. DNA fragments were purified using "Wizard SV Gel and PCR Clean-Up System" columns (Promega) according to the manufacturer's protocol. Nucleotide sequences were determined by using an automatic ABI Prism DNA sequencer, model 310 (Perkin-Elmer). DNA and peptide sequence homologies were analyzed using BLAST (http://au.expasy.org/sprot/). Sequence alignment was performed using Vector NTI, version 9 (Invitrogen). The secondary structure of EctR1 was predicted using the PSIPRED Protein Structure Prediction server (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html). Multiple alignments were done using the Clustal X program (45).

RESULTS

Identification of a new gene upstream of the *ectABC-ask* operon in *M. alcaliphilum* 20Z. In *M. alcaliphilum* 20Z, the ectoine biosynthesis genes are organized in the *ectABC-ask* operon (32). A partial sequence of an additional open reading frame (*orf1*), in reverse orientation relative to the *ectABC-ask* cluster, was identified upstream of the *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 *E. coli* (10, 13, 23, 25, 41, 43, 44), PecS (18.6%) from *Erwinia chrysanthemi* strain 3937 (33), MexR (17.8%) from *Pseudomonas aeruginosa* (12, 31, 40), and SlyA (15.6%) from *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 *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 *ectABC-ask* and *ectR1* transcription units and the effect of medium salinity on their expression. To determine the sizes and expression levels of the *ect* operon and

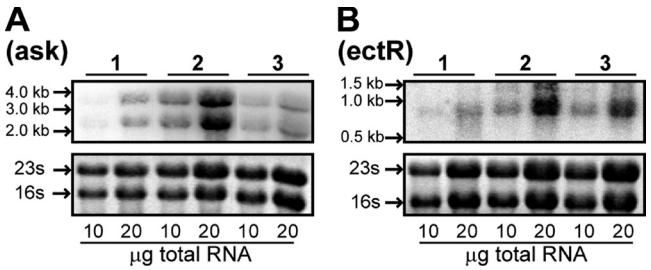

FIG. 2. Northern blot analysis of *ectABC-ask* operon and *ectR1* gene mRNAs. Total RNA was prepared either from *M. alcaliphilum* 20Z cells grown with 1% NaCl (lanes 1) or 6% NaCl (lanes 3) or with cells exposed for 1 h to an osmotic upshift from 1% NaCl to a final salt concentration of 6% NaCl (lanes 2). Increasing amounts of total RNA were resolved on a denaturing agarose gel, blotted onto a Hybond-N^+ membrane, and hybridized with [α^{32}P]dATP-labeled *ask* (A) or *ectR1* (B) gene-specific probes. RNA ladders are indicated by arrows. rRNA (lower panels) was detected by ethidium bromide staining.

ectR1 gene transcripts, we used Northern hybridization with probes corresponding to the 3′-proximal sequences of the *ask* and *ectR1* 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 OD_{600} 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 *ectR1* 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 *ectR1* 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 *ectR1* 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 effect 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 L-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 *ectR1* gene. To map the transcriptional start sites of the *ectABC-ask* operon and the *ectR1* 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 *ectA* gene. This observation suggested that the *ect* operon is transcribed from two promoters, $ectAp_1$ and $ectAp_2$ (Fig. 3D). The potential $ectAp_1$ promoter elements, TACTAT for the −10 and TGGACA for the −35 site with 16-bp spacing, match well with the consensus sequences (TATAAT and TTGACA) for a promoter recognized by the primary vegetative sigma factor, σ^{70} of *E. coli* (16). Moreover, $ectAp_1$ displayed a TG motif as an upstream extension of the −10 box (35). The putative −10 sequence (TAAAAA) of $ectAp_2$ showed identity to the σ^{70}-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 *ectR1* gene was assigned to a position 250 bp upstream of the ATG translational initiation site. The sequence of the putative −10 box (TCGAAT) exhibited similarity with the −10 consensus sequence of the *E. coli* σ^{70}-recognized promoter, while the putative −35 site of the *ectR1p* 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 pHSG:*ectABC*, 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 $ectAp_1$ and $ectAp_2$ promoters are recognized by transcriptional systems in both strains *M. alcaliphilum* 20Z and *E. coli* XL1-Blue.

Null mutations in the *ectR1* 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 *ectR1* gene insertion mutant via allelic exchange. The growth rate of the *ectR1* knockout strain (designated EBPR01) 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 *ectR1* 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 $ectAp_1p_2$-*gfp* fusion was introduced into both wild-type *M. alcaliphilum* 20Z and EBPR01, and the resulting strains were assayed for *gfp* expression. At all salt concentrations tested, the activities of the $ectAp_1p_2$ promoter region were 2- to 3-fold higher in the strain lacking *ectR1* 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 EBPR01 (Table 1). Thus, the mutation in the *ectR1* 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*

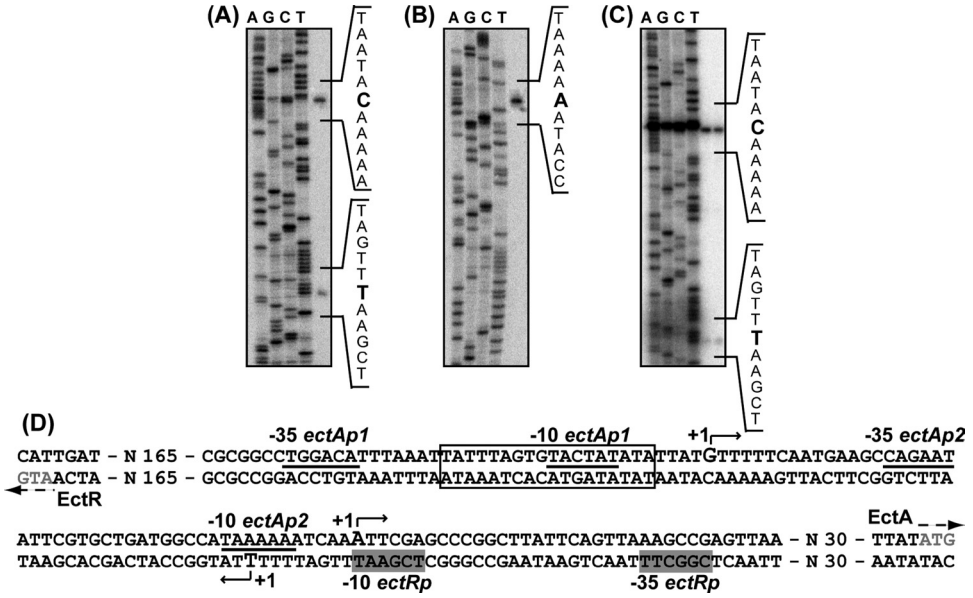

FIG. 3. Determination of transcriptional start sites for the *ectABC-ask* operon and the *ectR1* gene. Primer extension analyses were carried out with total RNA prepared from *M. alcaliphilum* 20Z cells (A and B) and *E. coli* cells (C) using primers complementary to the *ectA* (A and C) and *ectR1* (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 *ectR1-ectA* 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 $ectAp_1$ and $ectAp_2$ 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 *ectR1* genes are indicated by arrows. The inverted repeat of the EctR1 binding site (see below) is boxed.

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-His$_6$ specifically binds to DNA fragments containing the $ectAp_1$, $ectAp_2$, 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-His$_6$, 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-His$_6$ and 1 pmol of an end-labeled DNA fragment (213 bp) containing the $ectAp_1$, $ectAp_2$, and *ectR1p* promoters were used. Sixty picomoles of EctR1-His$_6$ 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.

TABLE 1. Activities of the $ectAp_1p_2$-*gfp* promoter fusion and diaminobutyric acid acetyltransferase in wild-type and *ectR1* mutant strains of *M. alcaliphilum*

Salinity of the growth medium (% NaCl)	Activity with the indicated strain			
	$ectAp_1p_2$-*gfp* (RFU OD_{600}^{-1} h^{-1})[a]		DABA-acetyltransferase (nmol min^{-1} mg of $protein^{-1}$)	
	Wild type	EBPR01	Wild type	EBPR01
1	22 ± 2.3	65 ± 9	5 ± 1.2	33 ± 5.3
3	40 ± 5	95 ± 15	57 ± 7	200 ± 23
6	130 ± 25	220 ± 22	83 ± 11	150 ± 18

[a] RFU, relative fluorescence units.

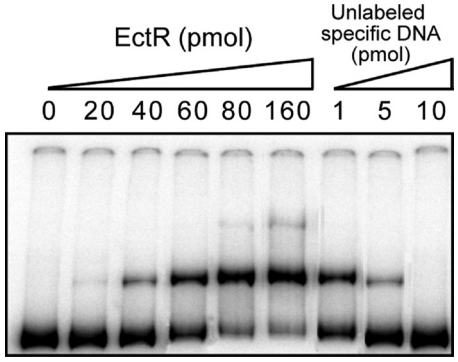

FIG. 4. Binding of recombinant EctR1 to the promoter regions of the *ectR1* gene and *ect* operon. EMSAs of the labeled DNA fragment (1 pmol) from nucleotide +231 to +18 relative to the translational start codon of *ectA* were performed with purified EctR1-His$_6$ in the presence of 0.5 μg sheared herring sperm DNA. The leftmost lane represents the labeled DNA probe alone. Increasing amounts of EctR1-His$_6$ (20, 40, 60, 80, and 160 pmol) were added to the probe. For the competition assay, an unlabeled specific DNA was added to the reaction mixture after incubation with the labeled fragment. Unlabeled DNA was added at 1-, 5-, and 10-molar-ratio excesses (three rightmost lanes) to a lane containing 80 pmol of the purified EctR1-His$_6$ protein.

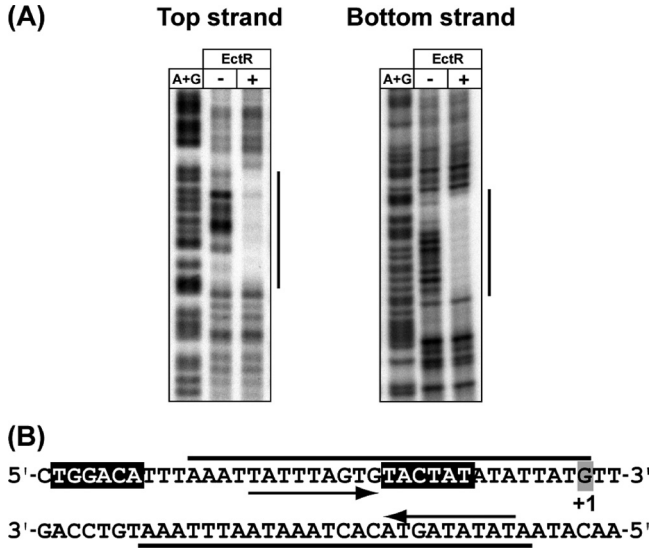

FIG. 5. EctR1-His$_6$ binding site at the *ectAp*$_1$ promoter region of the *ectABC-ask* operon. (A) The DNA probes, labeled at the 5′ end of either the top strand or the bottom strand, were incubated with purified EctR1-His$_6$ and treated with DNase I. The samples were run on a 6% polyacrylamide sequencing gel with a Maxam-Gilbert (A+G) sequencing reaction. (B) Nucleotide sequences of the *ectAp*$_1$ promoter region of the *ectABC-ask* operon. Regions protected by EctR1, as determined by the DNase I protection assay, are indicated by lines. Arrows represent the inverted-repeat sequences found in the EctR1 binding site. The transcription start site from the *ectAp*$_1$ promoter is shaded. Putative −10 and −35 sequences for the promoter are shown on a black background.

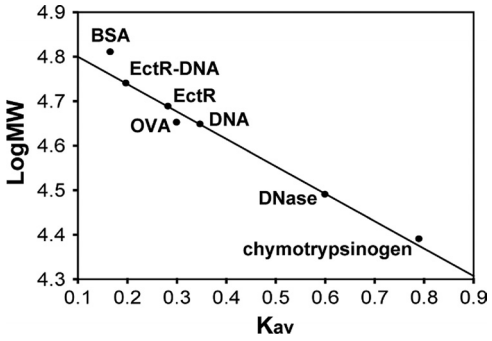

FIG. 6. Gel filtration analysis of the EctR1-His$_6$ and EctR1-His$_6$-DNA complexes. Gel filtration chromatography was performed on an Ultrogel AcA 44 column equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 200 mM KCl and 10 mM $MgCl_2$ at a flow rate of 0.2 ml/min. The apparent molecular masses were estimated using the log of the molecular masses of the standard proteins versus the coefficient K_{av}, calculated as $(V_e - V_0)/(V_t - V_0)$, where V_0 and V_e are the void and elution volumes, respectively, and V_t is the total column volume. Standard proteins: RNase A (13.7 kDa), chymotrypsinogen A (25 kDa), DNase (31 kDa), ovalbumin (OVA) (45 kDa), and BSA (66 kDa).

EctR1 specifically binds as a dimer to the *ectAp*$_1$ promoter. In order to locate the EctR1 binding site, DNase I footprinting assays were performed. As shown in Fig. 5, EctR1 protected an area (31 bp) covering positions from bp −30 to bp +1 relative to the transcription start site from *ectAp*$_1$. Hence, the protected region included the putative −10 motif of this promoter. Sequence analysis of the EctR1-protected region evidenced the imperfect inverted repeat (TATTTAGT-GT-ACTATATA) with 2 bp separating each half of the pseudopalindrome.

The oligomerization state of the EctR1 protein was analyzed by gel filtration. EctR1 was eluted as a 40-kDa protein, suggesting that it is most likely present as a homodimer in free solution (Fig. 6). A complex of EctR1 plus DNA was also eluted at the position corresponding to a homodimer, indicating that EctR1 binds to DNA as a dimer and that DNA binding does not induce changes in the oligomerization of the protein. This oligomerization state is consistent with the crystallographic and biochemical data for other MarR homologs that have been demonstrated to form homodimers (1, 4, 22, 46).

Overall, the data presented indicate that EctR1 binds at the *ectAp*$_1$ promoter region and thereby may repress the transcription of the *ectABC-ask* operon.

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 *ectAp*$_1$ 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 present in *M. alcaliphilum* 20Z may be absent in *E. coli*.

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 osmoresponse 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), EmrR (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.

EctR1 orthologs are present in other halophilic bacteria. In the ectoine- and 5-hydroxyectoine-producing species *Salibacillus salexigens*, in the DNA segment following the *ectD* (ectoine hydroxylase) gene, a partial reading frame whose deduced product showed similarity to MarR-type regulators was found (5). However, this reading frame, which is cotranscribed with *ectD*, was disrupted by two stop codons. Our analysis of the DNA fragment containing the ectoine biosynthetic genes (NCBI accession no. EU315063) in the methanol-utilizing bacterium *Methylophaga alcalica* showed the presence of an open reading frame with high homology to the *ectR1* gene of *M. alcaliphilum* 20Z (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. Between them, the open reading frames of an *Oceanospirillum* sp. (EAR60187), *Nitrosococcus oceani* (ABA57535), *Saccharophagus degradans* (ABD80450), a *Reinekea* sp. (ZP_01114878), and an *Oceanobacter* sp. (EAT11341) showed the highest identities of translated amino acid sequences with *M. alcaliphilum* EctR1 (35.5, 42.2, 45.6, 51.7, and 55.1%, respectively). These results indicate the presence of an EctR-mediated regulatory system controlling ectoine biosynthesis at the transcriptional level in diverse halophilic/halotolerant bacteria.

ACKNOWLEDGMENTS

We thank Tanya Ivashina and Marina Zakharova (G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, RAS, Pushchino, Moscow Region, Russia) for valuable suggestions and helpful discussions; Ekaterina Latypova, Linda M. Sauter, and Elizabeth C. Skovran for insightful revision of the manuscript; and Ján Kormanec (Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovak Republic) and Steven Lindow (Department of Plant and Microbial Biology, University of California) for providing plasmids.

This study was supported by grants from the Russian Foundation for Basic Research (07-04-01064, 07-04-12053-ofi-a), the Ministry of Education and Science (RF RNP 2.1.1/605, Russian State Contract 02.740.11.0296, CRDF Rub1-2946-PU-09), and the U.S. National Science Foundation (MCB-0842686).

REFERENCES

1. **Alekshun, M. N., S. B. Levy, T. R. Mealy, B. A. Seaton, and J. F. Head.** 2001. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 Å resolution. Nat. Struct. Biol. **8:**710–714.
2. **Atlas, R. M., and L. C. Parks.** 1997. Handbook of microbiological media, 2nd ed. CRC Press, Boca Raton, FL.
3. **Bestvater, T., P. Louis, and E. A. Galinski.** 2008. Heterologous ectoine production in *Escherichia coli*: by-passing the metabolic bottle-neck. Saline Systems **4:**12.
4. **Brooun, A., J. J. Tomashek, and K. Lewis.** 1999. Purification and ligand binding of EmrR, a regulator of a multidrug transporter. J. Bacteriol. **181:** 5131–5133.
5. **Bursy, J., A. J. Pierik, N. Pica, and E. Bremer.** 2007. Osmotically induced synthesis of the compatible solute hydroxyectoine is mediated by an evolutionarily conserved ectoine hydroxylase. J. Biol. Chem. **282:**31147–31155.
6. **Calderón, M. I., C. Vargas, F. Rojo, F. Iglesias-Guerra, L. N. Csonka, A. Ventosa, and J. J. Nieto.** 2004. Complex regulation of the synthesis of the compatible solute ectoine in the halophilic bacterium *Chromohalobacter salexigens* DSM 3043^T. Microbiology **150:**3051–3063.
7. **Cánovas, D., C. Vargas, M. I. Calderon, A. Ventosa, and J. J. Nieto.** 1998. Characterization of the genes for the biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. Syst. Appl. Microbiol. **21:**487–497.
8. **Ciulla, R. A., M. R. Diaz, B. F. Taylor, and M. F. Roberts.** 1997. Organic osmolytes in aerobic bacteria from Mono Lake, an alkaline, moderately hypersaline environment. Appl. Environ. Microbiol. **63:**220–226.
9. **Dalrymple, B. P., and Y. Swadling.** 1997. Expression of a *Butyrivibrio fibrisolvens* E14 gene (*cinB*) encoding an enzyme with cinnamoyl ester hydrolase activity is negatively regulated by the product of an adjacent gene (*cinR*). Microbiology **143:**1203–1210.
10. **del Castillo, I., J. M. Gomez, and F. Moreno.** 1990. *mprA*, an *Escherichia coli* gene that reduces growth-phase dependent synthesis of microcins B17 and C7 and blocks osmoinduction of *proU* when cloned on a high-copy-number plasmid. J. Bacteriol. **172:**437–445.
11. **Ditta, G., T. Schmidhauser, F. Yakobson, P. Lu, X. Liang, D. Finlay, D. Guiney, and D. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and monitoring gene expression. Plasmid **13:**149–153.
12. **Evans, K., L. Adewoye, and K. Poole.** 2001. MexR repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic region. J. Bacteriol. **183:** 807–812.
13. **Fuangthong, M., and J. D. Helmann.** 2002. The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine-sulfenic acid derivative. Proc. Natl. Acad. Sci. U. S. A. **99:**6690–6695.
14. **Galinski, E. A.** 1995. Osmoadaptation in bacteria. Adv. Microb. Physiol. **37:**272–328.
15. **Galinski, E. A., and H. G. Trüper.** 1994. Microbial behavior in salt-stressed ecosystems. FEMS Microbiol. Rev. **15:**95–108.
16. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2254.
17. **Kalyuzhnaya, M. G., V. N. Khmelenina, S. Kotelnikova, L. Holmquist, K. Pedersen, and Y. A. Trotsenko.** 1999. *Methylomonas scandinavica* sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden. Syst. Appl. Microbiol. **22:**565–572.
18. **Kempf, B., and E. Bremer.** 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolarity environments. Arch. Microbiol. **170:**319–330.
19. **Khmelenina, V. N., M. G. Kalyuzhnaya, N. G. Starostina, N. E. Suzina, and Y. A. Trotsenko.** 1997. Isolation and characterization of halotolerant alkaliphilic methanotrophic bacteria from Tuva soda lakes. Curr. Microbiol. **35:** 257–261.

20. **Khmelenina, V. N., M. G. Kalyuzhnaya, V. G. Sakharovsky, N. E. Suzina, Y. A. Trotsenko, and G. Gottschalk.** 1999. Osmoadaptation in halophilic and alkaliphilic methanotrophs. Arch. Microbiol. **172:**321–329.
21. **Kuhlmann, A. U., and E. Bremer.** 2002. Osmotically regulated synthesis of the compatible solute ectoine in *Bacillus pasteurii* and related *Bacillus* spp. Appl. Environ. Microbiol. **68:**772–783.
22. **Lim, D., K. Poole, and N. C. J. Strynadka.** 2002. Crystal structure of the *mexR* repressor of the *mexRAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*. J. Biol. Chem. **277:**29253–29259.
23. **Lomovskaya, O., K. Lewis, and A. Matin.** 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. J. Bacteriol. **177:**2328–2334.
24. **Louis, P., and E. A. Galinski.** 1997. Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*. Microbiology **143:**1141–1149.
25. **Martin, R. G., and J. L. Rosner.** 1995. Binding of purified multiple antibiotic resistance repressor protein (MarR) to *mar* operator sequences. Proc. Natl. Acad. Sci. U. S. A. **92:**5456–5460.
26. **Marx, C. J., and M. E. Lidstrom.** 2002. Broad-host-range cre-lox system for antibiotic marker recycling in gram-negative bacteria. Biotechniques **33:** 1062–1067.
27. **Miller, W. G., and S. E. Lindow.** 1997. An improved GFP cloning cassette designed for prokaryotic transcriptional fusions. Gene **191:**149–153.
28. **Mustakhimov, I. I., O. N. Rozova, A. S. Reshetnikov, V. N. Khmelenina, J. C. Murrell, and Y. A. Trotsenko.** 2008. Characterization of the recombinant diaminobutyric acid acetyltransferase from *Methylophaga thalassica* and *Methylophaga alcalica*. FEMS Microbiol. Lett. **283:**91–96.
29. **Ono, H., K. Sawada, N. Khunajakr, T. Toa, M. Yamamoto, M. Hiramoto, A. Shinmyo, M. Takano, and Y. Murooka.** 1999. Characterization of biosynthetic enzymes for ectoine as a compatible solute in a moderately halophilic eubacterium, *Halomonas elongata*. J. Bacteriol. **181:**91–99.
30. **Peters, P., E. A. Galinski, and H. G. Trüper.** 1990. The biosynthesis of ectoine. FEMS Microbiol. Lett. **71:**157–162.
31. **Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. E. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40:**2021–2028.
32. **Reshetnikov, A. S., V. N. Khmelenina, and Y. A. Trotsenko.** 2006. Characterization of the ectoine biosynthesis genes of haloalkalotolerant obligate methanotroph "Methylomicrobium alcaliphilum 20Z." Arch. Microbiol. **184:** 286–297.
33. **Reverchon, S., W. Nasser, and J. Robert-Baudouy.** 1994. *pecS*: a locus controlling pectinase, cellulase and blue pigment production in *Erwinia chrysanthemi*. Mol. Microbiol. **11:**1127–1139.
34. **Roberts, M. F.** 4 August 2005. Organic compatible solutes of halotolerant and halophilic microorganisms. Saline Systems **1:**5.
35. **Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse.** 1993. A third recognition element in bacterial promoters: DNA binding by the α-subunit of RNA polymerase. Science **262:**1407–1412.
36. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
37. **Schacterle, G. R., and R. L. Pollack.** 1973. A simplified method for the quantitative assay of small amounts of protein in biologic material. Anal. Biochem. **51:**654–655.
38. **Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, C. Thierbach, and A. Pühler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
39. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotechnology **1:**784–791.
40. **Srikumar, R., C. J. Paul, and K. Poole.** 2000. Influence of mutations in the *mexR* repressor gene on expression of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa*. J. Bacteriol. **182:**1410–1414.
41. **Stapleton, M. R., V. A. Norte, R. C. Read, and J. Green.** 2002. Interaction of the *Salmonella typhimurium* transcription and virulence factor SlyA with target DNA and identification of members of the SlyA regulon. J. Biol. Chem. **277:**17630–17637.
42. **Strovas, T. J., and M. E. Lidstrom.** 2009. Population heterogeneity in *Methylobacterium extorquens* AM1. Microbiology **155:**2040–2048.
43. **Sukchawalit, R., S. Loprasert, S. Atichartpongkul, and S. Mongkolsuk.** 2001. Complex regulation of the organic hydroperoxide resistance gene (*ohr*) from *Xanthomonas* involves OhrR, a novel organic peroxide-inducible negative regulator, and posttranscriptional modifications. J. Bacteriol. **183:**4405–4412.
44. **Sulavik, M. C., L. F. Gambino, and P. F. Miller.** 1995. The MarR repressor of the multiple antibiotic resistance (*mar*) operon in *Escherichia coli*: prototypic member of a family of bacterial regulatory proteins involved in sensing phenolic compounds. Mol. Med. **1:**436–446.
45. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmaugin, and D. G. Higgins.** 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
46. **Tropel, D., and J. R. van der Meer.** 2004. Bacterial transcriptional regulators for degradation pathways of aromatic compounds. Microbiol. Mol. Biol. Rev. **68:**474–500.
47. **Wilkinson, S. P., and A. Grove.** 2004. HucR, a novel uric acid-responsive member of the MarR family of transcriptional regulators from *Deinococcus radiodurans*. J. Biol. Chem. **279:**51442–51450.
48. **Wilkinson, S. P., and A. Grove.** 2006. Ligand-responsive transcriptional regulation by members of the MarR family of winged helix proteins. Curr. Issues Mol. Biol. **8:**51–62.
49. **Zhao, B., W. Lu, L. Yang, B. Zhang, L. Wang, and S. S. Yang.** 2006. Cloning and characterization of the genes for biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halobacillus dabanensis* D-8T. Curr. Microbiol. **53:**183–188.