Complex regulation of the synthesis of the compatible solute ectoine in the halophilic bacterium Chromohalobacter salexigens DSM 3043T

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The synthesis of the compatible solute ectoine, mediated by the ectABC gene products, is the main mechanism used by the halophilic bacterium Chromohalobacter salexigens to cope with osmotic stress. Evidence was found that this process is regulated at the transcriptional level. S1 protection analyses performed with RNA extracted from cells grown in minimal medium at low (0–75 M NaCl) or high (2–5 M NaCl) osmolarity suggested the existence of four promoters upstream of ectA. Two of these (PectA1 and PectA2) might be recognized by the main vegetative sigma factor σ70, and one (PectA3) might be dependent on the general stress sigma factor σS. The S1 protection assays suggest that PectA1 and PectA3 may be osmoregulated promoters. In addition, an internal promoter showing sequences homologous to promoters dependent on the heat-shock sigma factor σ32 was found upstream of ectB. Transcription from PectA in C. salexigens followed a pattern typical of σS-dependent promoters, and was reduced by 50% in an E. coli rpoS background. These data strongly suggest the involvement of the general stress sigma factor σS in ectABC transcription in C. salexigens. Expression of PectA–lacZ and PectB–lacZ transcriptional fusions was very high at low salinity, suggesting that ectABC may be partially constitutive. Both transcriptional fusions were induced during continuous growth at high temperature and their expression was reduced in cells grown in the presence of osmoprotectants (ectoine or glycine betaine) or the DNA gyrase inhibitor nalidixic acid. Moreover, PectA–lacZ expression was negatively modulated in cells grown with an excess of iron (FeCl3). Measurement of ectoine levels in the presence of glycine betaine at different NaCl concentrations suggests that an additional post-transcriptional control may occur as well.

INTRODUCTION

Prokaryotic osmoadaptation is a highly complex and integrated network of physiological and genetic mechanisms by which cells are able to sense the fluctuations in the water environment and react to them. This is achieved through a number of as yet poorly understood signal transduction pathways leading to the activation or repression of osmo-regulated systems (Bremer & Krämer, 2000; Csonka & Epstein, 1996; Poolman & Glaasker, 1998; Wood et al., 2001). With the exception of the Halobacteraeaceae, the Halanaerobiales, and the recently discovered bacterium Salinibacter rubrum (Antón et al., 2000), which respond to osmotic stress by the so-called ‘salt-in’ strategy and accumulate large amounts of inorganic ions in their cytoplasm, in prokaryotes, the long-term specific response to hyperosmotic conditions involves the transport and/or biosynthesis of small organic compounds, the compatible solutes, which reach high cytoplasmic levels without interfering with cellular metabolism. This ‘salt-out’ strategy is more flexible and versatile, as judged by the fact that it is used not only by the vast majority of bacteria and archaea, but also by fungal, plant, and animal – including human – cells (Da Costa et al., 1998; Galinski & Trüper, 1994; Ventosa et al., 1998).
Usually, halotolerant (i.e. tolerating but not requiring salt) or halophilic (i.e. requiring salt and also growing at higher salinities) micro-organisms are able to synthesize de novo one or a few compatible solutes. One of the most widespread compatible solutes is ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid). First discovered in the phototrophic sulfur bacterium *Ectothiorhodospira halochloris* (Galinski et al., 1985), ectoine has been found to be synthesized and accumulated by halophilic/halotolerant representatives of bacteria belonging to three major phylogenetic branches: the actinobacteria (i.e. genera *Actinopolyspora*, *Brevibacterium*, *Nocardiosis* and *Streptomyces*) the firmicutes (i.e. genera *Bacillus*, *Salibacillus*, *Virgibacillus*, *Halobacillus*, *Marinococcus* and *Sporosarcina*), and the proteobacteria (i.e. genera *Chromohalobacter*, *Ectothiorhodospira*, *Halomonas*, *Methylbacterium*, *Pseudomonas*, *Rhodovulum* and *Salinisvibrio*) (Da Costa et al., 1998; Galinski; 1995; Galinski & Trüper, 1994; Kuhlmann & Bremer, 2002; Severin et al., 1992; Ventosa et al., 1998). With the known exception of the β-proteobacteria *Rhodovulum sulfidophilum* (formerly *Rhodobacter sulfidophilum*; Hiraishi & Ueda, 1994) and *Rhodovibrio salinarum* (formerly *Rhodospirillum salinarum*; Nissen & Dundas, 1984) all ectoine-producing proteobacteria found so far belong to the gamma division of this bacterial lineage. Ectoine has not been reported, however, in cyanobacteria, archaea or eukaryotes.

Among the γ-proteobacteria, most members of the *Halomonadaceae* (Halomonas and Chromohalobacter) are moderate halophiles that display one of the broadest salinity ranges found in prokaryotes (Ventosa et al., 1998). *Chromohalobacter salexigens* 3043 (formerly Halomonas elongata DSM 3043; Arahal et al., 2001) is able to grow from 0-15 M to 4·3 M NaCl in complex medium, and from 0-5 M to 3 M NaCl in minimal medium (Arahal et al., 2001; Cánovas et al., 1996). Recently, O’Connor & Csonka (2003) characterized the ion requirements of *C. salexigens* and made the unexpected finding that while this organism needs moderate concentrations of Na⁺ and Cl⁻ ions, its growth rate was stimulated by a number of other salts, indicating that *C. salexigens* requires a combination of NaCl and high ionic strength for optimal growth. Osmoadaptation in *C. salexigens* is mainly achieved by de novo synthesis of ectoine and its hydroxy derivative, hydroxyectoine (Cánovas et al., 1997). In addition, when they are provided externally, *C. salexigens* accumulates osmoprotectants such as glycine betaine, which is taken up from the medium or synthesized from choline (Cánovas et al., 1996, 1998b). In a previous work, we isolated and characterized the *C. salexigens* ectABC genes, encoding the main route for ectoine synthesis in this micro-organism. The *ectB* gene encodes the enzyme diaminobutyric acid transaminase, which catalyses the conversion of aspartate semialdehyde into diaminobutyric acid; *ectA* encodes diaminobutyric acid acetyltransferase, responsible for the acetylation of diaminobutyric acid to N⁵-acetyldiaminobutyric acid, and *ectC* specifies ectoine synthase, which catalyses the cyclization of N⁵-acetyldiaminobutyric acid into ectoine (Cánovas et al., 1998a). The *C. salexigens* EctA, EctB and EctC proteins show the same organization as well as a significant degree of sequence identity to the enzymes of ectoine synthesis in *Halomonas elongata* (Gölker et al., 1998), *Marinococcus halophilus* (Louis & Galinski, 1997) and *Sporosarcina pasteurii* (Kuhlmann & Bremer, 2002). Thus, it seems that the ectoine biosynthetic route, which was first elucidated at the biochemical level in the closely related *H. elongata* (Ono et al., 1999; Peters et al., 1990), is evolutionarily well conserved in all ectoine-producing bacteria characterized so far.

Knowledge on how ectoine synthesis is regulated is important for several reasons. This compatible solute has biotechnological applications as a stabilizing agent for enzymes, DNA and whole cells (Galinski, 1995; Ventosa et al., 1998). Therefore, the dissection of the region involved in the transcriptional control of ectoine synthesis, and the elucidation of post-transcriptional control mechanisms, are necessary to generate modified strains improved in ectoine production for prospective industrial use. In addition, the synthesis of this compatible solute is widespread in the bacterial world as a means to cope with changes in external osmolarity. In the absence of external osmoprotectants, *C. salexigens* synthesizes ectoine as its main osmoadaptation mechanism. Because *C. salexigens* can grow in a very broad salinity range, it must be able to rapidly and finely adjust its cytoplasmic solute concentration in response to variable osmotic stress. In order to achieve this, this micro-organism must presumably have a powerful regulatory mechanism controlling the synthesis of ectoine. Within the *Halomonadaceae*, the transcriptional regulation of ectoine synthesis has not been investigated. In *Halomonas elongata*, Kraegeloh & Kunte (2002) reported that, under a moderate upshift (from 0·5 M to 1 M NaCl), cells treated with chloramphenicol synthesized ectoine, suggesting that, at least under these experimental conditions, ectoine synthesis is mainly regulated at the level of enzyme activity. However, there is an important difference in the salinity requirements of *Halomonas elongata* and *C. salexigens* (Cánovas et al., 1996; Vreeland & Martin, 1980) and also a different pattern of ectoine accumulation in the presence of betaine (Cánovas et al., 1996; Wohlfarth et al., 1990), suggesting that regulation of the ectoine pathway might be different in these two micro-organisms. In this study we investigated the long-term regulation of ectoine synthesis in *C. salexigens*, with emphasis on the transcriptional regulation.

**METHODS**

**Bacterial strains, media and culture conditions.** Bacterial strains and plasmids used in this work are listed in Table 1. *Chromohalobacter salexigens* DSM 3043 (formerly *Halomonas elongata* DSM 3043) and derivatives were routinely grown in SW-10 medium, containing 10 % (w/v) total salts and 0·5 % (w/v) yeast extract (Nieto et al., 1989). Matings were done on SW-2 medium (similar to SW-10 but containing 2 %, w/v, total salts; Vargas et al.,...
Table 1. Bacterial strains and plasmids

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<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td><strong>C. salexigens</strong></td>
<td>Wild-type, formerly Halomonas elongata DSM 3043 (strain 1H11)</td>
<td>Vreeland et al. (1980); Arahal et al. (2001)</td>
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<tr>
<td>DSM 3043&lt;sup&gt;T&lt;/sup&gt;</td>
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<tr>
<td>CHR61</td>
<td>Spontaneous Rif&lt;sup&gt;R&lt;/sup&gt; mutant of DSM 3043</td>
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<td><strong>E. coli</strong></td>
<td></td>
<td>Cánovas et al. (1997)</td>
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<tr>
<td>DH5&lt;sup&gt;x&lt;/sup&gt;</td>
<td>supE&lt;sup&gt;44&lt;/sup&gt; ΔlacU169(Δ80lacZ, ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1; host for DNA manipulations</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>MC4100</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; araD139 lacU169 rpsL150 relA1 flb-5301 deoC1 ptsF25</td>
<td>Casabdan (1976)</td>
</tr>
<tr>
<td>JMH0039</td>
<td>MC4100 rpoS::Tn10</td>
<td>Corona-Izquierdo &amp; Membrillo-Hernández (2002)</td>
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<tr>
<td>CHR112</td>
<td>Spontaneous Rif&lt;sup&gt;R&lt;/sup&gt; mutant of MC4100</td>
<td>This study</td>
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<tr>
<td>CHR113</td>
<td>Spontaneous Rif&lt;sup&gt;R&lt;/sup&gt; mutant of JMH0039</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pKS(−)</td>
<td>Cloning vector, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pMP220</td>
<td>Promoter probe vector, lacZ, IncP, Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Spink et al. (1987)</td>
</tr>
<tr>
<td>PRK600</td>
<td>Helper plasmid, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Kessler et al. (1992)</td>
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<td>pME2</td>
<td>2.8 kb EcoRI fragment from pDE9 (containing ectABC) cloned in pKS(−)</td>
<td>Cánovas et al. (1998a)</td>
</tr>
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<td>PMP220</td>
<td></td>
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<td>pME7</td>
<td>296 bp region from pME2, containing the promoter region upstream of ectA (PectA1 to PectA4), cloned in pMP220 as an EcoRI–PstI fragment</td>
<td>This study</td>
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<tr>
<td>pME8</td>
<td>431 bp region from pME2, containing the promoter PectR, cloned in pMP220 as a KpnI–PstI fragment</td>
<td>This study</td>
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<tr>
<td>pME9</td>
<td>662 bp region from pME2, containing 395 bp upstream of ectC, cloned into pMP220 as a Xbal–PstI fragment</td>
<td>This study</td>
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1997). *Escherichia coli* was grown aerobically in complex LB medium (Miller, 1992). M63 (Csonka, 1982), containing 20 mM glucose as the sole carbon source, was used as minimal medium for *C. salexigens* and *E. coli*. The osmotic strength of M63 was increased by the addition of 0–2.5 M final concentrations of NaCl. Although *C. salexigens* can grow in M63 with 0.5 M NaCl, growth at this salinity is extremely slow and cells take a very long time to reach exponential phase. Therefore we used M63 with 0.75 M NaCl as standard medium for low salt concentration in all experiments. The pH of all media was adjusted to 7.2 with KOH. Solid media contained 20 g·L<sup>−1</sup> of Bacto agar (Difco). Liquid cultures were incubated at 37 °C in an orbital shaker at 200 r.p.m. When used, filter-sterilized antibiotics were added to LB or SW-2 media at the following final concentrations (μg·ml<sup>−1</sup>): ampicillin (Ap), 150; tetracycline (Tc), 15; chloramphenicol (Cm), 25; rifampicin (Rif), 25. When appropriate, the following compounds were added to the media (final concentration): X-Gal (Sigma, 40 μg·ml<sup>−1</sup>), coline (Bitop, 1 nM), glycin betaine (Sigma, 1 mM), nalidixic acid (1 μM) and FeCl<sub>3</sub> (50 μM). Growth was monitored as OD<sub>600</sub> with a Perkin Elmer 5515 UV/Vis spectrophotometer.

**Conjugal transfer of plasmids.** Plasmids were transferred by conjugation between different strains of *E. coli* and from *E. coli* to *C. salexigens* by triparental matings on SW-2 medium, using pRK600 as helper plasmid and spontaneous rifampicin-resistant mutants of the recipient strains as described by Vargas et al. (1997).

**DNA manipulation and plasmid construction.** Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (Sambrook & Russell, 2001). Restriction enzyme digestion and ligations were performed as recommended by the manufacturer (Promega). DNA regions upstream of each *ect* gene were obtained by PCR amplification by using as a template plasmid pME2, which contains the *C. salexigens ectABC* region (GenBank/EMBL accession no. AJ11103), *Pfu* DNA polymerase (Stratagene), and specific oligonucleotides that in some cases were modified to include appropriate restriction sites (modifications are shown underlined). A 296 bp region containing the promoter region located upstream of *ectA* was amplified by using the oligonucleotide pair MA1 (5′-CGTCTCTAGAATTCTGTGGAT-3′; this sequence belongs to the vector polylinker located upstream of the *EcoRI* site defining the 5′-end of the *ectABC* region cloned in pME2,) and MA2 (5′-GTTCTGCTGCAGGCGTCAT-3′), digested with *EcoRI*–*PstI* (resulting in a 288 bp insert) and cloned into pMP220 to give plasmid pME7. A 442 bp region containing the promoter located upstream of *ectB* was synthesized by using the oligonucleotides MB1 (5′-CGTACCCTTATCTGCTGCTGCT-3′) and MB2 (5′-GAATCTGGGTCTGCGAGATGAT-3′), digested with KpnI–PstI (resulting in a 431 bp insert) and inserted into pMP220 to generate pME8. A 678 bp region was amplified by using the oligonucleotides MC3 (5′-CGGTGTCGTTAGATCAGC-3′) and MC4 (5′-TGCTGACCCGTGCGACGT-3′), digested with XbaI–PstI (resulting in a 662 bp insert containing 395 bp upstream of *ectC*) and cloned into pMP220. The plasmid constructed in this way was named pME9.

**S1 nuclease analyses of mRNAs.** *C. salexigens* cells were grown in minimal medium M63 to OD<sub>600</sub> 0.5, and total RNA was isolated as previously described (Monsalve et al., 1995). S1 nuclease reactions were performed as described by Ausubel et al. (1989), using 25 μg total RNA and an excess of a 32P-end-labelled single-stranded DNA (ssDNA) hybridizing to the 5′ region of the mRNA. The ssDNA probes were generated by linear PCR, using as a substrate the plasmid pME2, which contains a 3 kb *EcoRI* fragment including the *ectABC* region. Prior to the linear amplification reaction, this plasmid was digested with *EcoRI* for *ectA* S1 assay (the *EcoRI* recognition sequence is located 275 bp upstream of *ectA* start codon), *XmnI* for *ectB* S1 assay (the *XmnI* recognition sequence is located 629 bp upstream of *ectB* start codon) or *HindIII* for *ectC* S1 assay (the *HindIII* recognition sequence is located 378 bp upstream of *ectC*).
ectA start codon). The primers used to generate the probes were MS-A2 (5′-TGTCGCGTGGCTGCGTACTAC-3′), MS-B1 (5′-AACGGTTGAAAGAAGC-3′) and MS-C1 (5′-TGCGGGTGATTGTGACGGAG-3′) for mapping the 5′-end of putative transcripts starting at ectA, ectB and ectC, respectively.

**Extraction of intracellular solutes and 13C-NMR spectroscopy.** Cytoplasmic solutes were extracted essentially as described by Cañovas et al. (1997), but with the following modifications. C. salexigens was grown in 200 ml minimal medium M63 until late-exponential phase. Cells were washed with buffer Z (without NaCl) or with spectra previously described (Cañovas et al., 1998). Signals were identified by comparison with spectra of pure compounds. Extraction of intracellular solutes and 13C-NMR spectroscopy.

**Assay for β-galactosidase activity.** β-Galactosidase activity was measured basically as described by Miller (1992). Overnight cultures of Lac− E. coli cells (in LB with Tc), or C. salexigens DSM 3043 (lacks intrinsic β-galactosidase activity) cells (in SW-2 with Tc), harbouring each lacZ fusion or the plasmid vector pMP220, were diluted (1:100) in fresh minimal medium M63. Cultures were grown at 37 or 40°C and 0.8 ml aliquots were taken at different time intervals. Cells were washed with buffer Z (without β-mercaptoethanol) to remove salts. Absorbance at 420 nm was determined with a Perkin Elmer 551S UV/Vis spectrophotometer. All assays were performed three times from two parallel cultures, and the standard deviation was calculated. The residual β-galactosidase activity derived from the plasmid vector pMP220 was subtracted in all determinations.

**RESULTS**

The ectABC genes are transcribed from multiple promoters

The tight spacing between the C. salexigens ectA and ectB (26 bp), and ectB and ectC (119 bp) genes, together with the existence of a putative rho-independent transcriptional terminator downstream of ectC, suggested that the ectABC gene cluster is transcribed as an operon (Cañovas et al., 1998a). Since a preliminary Northern analysis did not yield consistent results (data not shown), we used S1 protection assays to investigate the transcriptional organization of the ectABC genes in C. salexigens. Total RNA was isolated from C. salexigens grown in minimal medium M63 with low (0.75 M NaCl) or high (2.5 M NaCl) salinity, in the presence or absence of 1 mM glycine betaine, and transcription start sites for the each ectA, ectB and ectC genes were determined by S1 nuclease mapping. Four putative transcription initiation sites were found, at 44, 96, 134 and 149 bp upstream of the ectA start codon (Fig. 1). This finding suggested the existence of four putative PectA promoters (PectA1 to PectA4). The same transcription start pattern was found by primer extension analysis (not shown), indicating that the signals were not an artefact of the S1 mapping. In addition, a fifth possible transcription start was detected 25 bp upstream of the ectB coding sequence, within the last codon of ectA (Fig. 1). This might correspond to another promoter, PectB. In contrast, no transcription initiation site was found upstream of ectC with the S1 protection assay (not shown). The intensity of the S1-protected products corresponding to putative promoters PectA1, PectA3 and PectA4 was clearly stronger at high salinity, indicating that these promoters may be osmoregulated (Fig. 1a). In addition, inhibition of transcription from putative promoters PectA1, PectA2 and PectA3 was detected at low salt in the presence of glycine betaine (Fig. 1a), suggesting that this compatible solute negatively modulates the transcription of the ect genes under low-salinity conditions.

**Analysis of the −10 and −35 promoter sequences**

Inspection of the regions upstream of the detected transcription initiation sites revealed that the −35 (GTTGCCG) and −10 (TATAAT) sequences of putative promoter PectA2 were very similar to the consensus sequences of σ70-dependent promoters (TTGACA-16–18 bp-TATAAT; Wösten, 1998) (Fig. 1b). The −10 region was identical to the consensus, whereas only 3 of the 6 nt of the −35 region matched the consensus. The two regions of PectA2 are separated by 17 bp, similar to the spacing in E. coli. These data suggest that PectA2 is a true promoter, which may be recognized by the C. salexigens vegetative sigma factor σ70. In fact, PectA2 had been previously detected by computer-assisted analysis of the region upstream of ectA and suggested to be the ectABC promoter (Cañovas et al., 1998b). The sequence of the −35 (TCGATA) and −10 (TATAAT) regions of putative promoter PectA1 also matched the consensus recognition sequence for σ70 at a number of positions, with a 17 bp spacing between the −10 and −35 promoters.
regions. The $-10$ region of PectA3 (CTAGACT) showed a very good match with the consensus $-10$ sequence of $\sigma^\text{s}$-dependent promoters (CTATACT; Wösten, 1998) (Fig. 1b). Cytosine at the $-13$ position (in bold) in the ‘extended $-10$ region’, present in the $-10$ sequence of PectA3, is specific for $\sigma^\text{s}$-dependent promoters (Hengge-Aronis, 2002a). The $-35$ sequence of PectA3 (GTCATG) was not well conserved, but this is in agreement with the general observation that the $-35$ elements of $\sigma^\text{s}$-dependent promoters tend to be more degenerate than those of the $\sigma^\text{70}$-dependent promoters (Hengge-Aronis, 2002a). Sequences resembling the consensus of promoters recognized by the heat-shock factor
The three enzymes involved are L-diaminobutyric acid transaminase (EctB), L-diaminobutyric acid acetyl transferase (EctA) and ectoine synthase (EctC). (b) Genetic organization of the ectoine synthesis genes and location of the putative transcription start sites upstream of the genes ectA (PectA1 to PectA4) and ectB (PectB). (c) Transcriptional fusions of the regions upstream of ectA (pME7), ectB (pME8) and ectC (pME9) with the E. coli reporter gene lacZ. β-Galactosidase activity of E. coli DH5× and C. salexigens transconjugants carrying these constructs on M63 plates with X-Gal is shown on the right.

Fig. 2. (a) The ectoine biosynthetic pathway in C. salexigens. The three enzymes involved are L-diaminobutyric acid transaminase (EctB), L-diaminobutyric acid acetyl transferase (EctA) and ectoine synthase (EctC). (b) Genetic organization of the ectoine synthesis genes and location of the putative transcription start sites upstream of the genes ectA (PectA1 to PectA4) and ectB (PectB). (c) Transcriptional fusions of the regions upstream of ectA (pME7), ectB (pME8) and ectC (pME9) with the E. coli reporter gene lacZ. β-Galactosidase activity of E. coli DH5× and C. salexigens transconjugants carrying these constructs on M63 plates with X-Gal is shown on the right. Restriction sites: E, EcoRI; H, HindIII, K, KpnI; P, Psfl; Xb, Xbal, Xm, XmnI.

**Construction of PectA–lacZ and PectB–lacZ transcriptional fusions**

To further characterize the expression of the ectABC genes, transcriptional fusions of the promoter regions PectA (containing putative promoters PectA1 to PectA4) and PectB (containing putative promoter PectA5) were constructed to the E. coli reporter gene lacZ (Fig. 2). A DNA region of 288 bp spanning positions -231 to +56 relative to the PectA1 transcription start site was placed upstream of the promoterless lacZ gene in the low-copy vector pMP220, yielding the plasmid pME7. Similarly, a 431 bp fragment spanning positions -400 to +30 relative to the PectB transcription start site was cloned in pMP220 to give the plasmid pME8. To confirm the absence of a monocistronic ectC transcript, a 662 bp region containing 395 bp upstream of ectC was also cloned in pMP220, giving the plasmid pME9. The constructs were transferred from E. coli DH5× to wild-type C. salexigens by conjugation, and expression of the three fusions in both host strains was preliminarily tested on plates of minimal medium M63 supplemented with the β-galactosidase substrate X-Gal (Fig. 2c). Whereas colonies of E. coli and C. salexigens carrying plasmids pME7 and pME8 showed a Lac + phenotype, transconjugants harbouring pME9 were Lac - . These results revealed that the ectABC genes are transcribed in E. coli and confirmed the existence of promoter(s) upstream of ectA (in pME7) and ectB (in pME8), but not upstream of ectC (in pME9).

**Influence of salinity on PectA expression**

We previously reported that accumulation of ectoine and hydroxyectoine in C. salexigens is osmoregulated (Cánovas et al., 1999). On the other hand, the S1 protection assay (Fig. 1a) showed that putative promoters PectA1, PectA3 and PectA4 may be under osmotic control, indicating that osmoregulation of ectoine synthesis could occur, at least partially, at the level of transcription. To more precisely monitor the transcription of the ectABC genes in response to increasing osmolarity, C. salexigens cells carrying the fusion PectA–lacZ (containing promoters PectA1 to PectA4) were grown in minimal medium M63 with increasing NaCl concentrations (from 0-75 M to 2-5 M) and expression of the PectA promoter region was determined by measuring β-galactosidase activity in C. salexigens, as described in Methods. Unexpectedly, we were not able to make reliable measurements of β-galactosidase activity in cells grown with >0-75 M NaCl, most probably due to salt-inactivation of the E. coli enzyme. These problems could not be overcome by performing additional washing steps with buffer Z before cell lysis, or by a number of modifications of the standard protocol, including spheroplast preparation to remove salt attached to the outer membrane (not shown). Thus, we concluded that the E. coli lacZ gene is not a suitable reporter for monitoring expression in C. salexigens of genes in media with a salt concentration higher than 0-75 M NaCl.

Because the ectABC genes were expressed in E. coli, we measured the response of the PectA promoter region to increasing osmolarity in this host (Fig. 3). Basal transcription levels in cells grown in minimal medium M63 to the stationary phase (see below) in the absence of NaCl were quite high (approximately 2000 Miller units). In addition, the activity of the PectA–lacZ fusion was linearly correlated with the osmotic strength of the growth medium, with a 2-5-fold increase in expression from 0 M to 0.5 M NaCl. These results indicate that, in E. coli, the PectA promoter region is not only expressed at a high basal level, but is also osmoregulated. In addition, the basal expression level of the PectA–lacZ fusion appeared to be as efficient in E. coli as in C. salexigens (see below).
Influence of the growth phase on PectA and PectB expression

Our previous observation that ectoine accumulation in C. salexigens was maximal at the stationary phase of growth (Cañovas et al., 1999) led us to investigate the influence of the growth stage on the transcription of the ect genes. C. salexigens cells carrying the transcriptional fusions PectA–lacZ (pME7) and PectB–lacZ (pME8) were grown in M63 supplemented with 0.75 M NaCl, and samples were taken at different time intervals to monitor β-galactosidase activity. Fig. 4(a) shows that expression of PectA–lacZ fusion was low (although detectable) in the early (approx. 200 Miller units at OD600 ≈ 0.5) and late (approx. 900 Miller units at OD600 ≈ 1.1) exponential phase, and increased considerably when cultures approached stationary phase, eventually reaching very high values (up to approx. 4500 Miller units) in stationary-phase cells. The expression of the PectB–lacZ transcriptional fusion followed a different transcription pattern (Fig. 4b). It was slightly higher than PectA expression during the early (approx. 1500 Miller units at OD600 ≈ 0.5) and late (approx. 2000 Miller units at OD600 ≈ 1.0) exponential phase of growth, but it did not increase suddenly when cells entered stationary phase (between 2000 and 2500 Miller units at OD600 ≈ 1.7–1.8). Although a peak of maximal activity was repeatedly detected during stationary phase, the expression was not maintained at a high level, as it was in the case of PectA–lacZ transcription.

Efficient transcription from the PectA promoter region requires a functional σS

The transcriptional pattern of PectA–lacZ fusion correlated with that of stationary-phase σS-dependent promoters (low expression in exponential phase and high in stationary phase). Considering the high homology of putative promoter PectA3 of the PectA promoter region with promoters recognized by σS, it seemed likely that transcription of the ectABC genes from the PectA promoter may be (at least partially) dependent on this sigma factor. The C. salexigens gene encoding σS has not been identified and therefore a σS-deficient mutant (rpoS) of C. salexigens was not available. To determine whether the expression of the PectA promoter in E. coli requires the participation of σS-RNA polymerase, the PectA–lacZ fusion was introduced by conjugation to the isogenic E. coli strains MC4100 (wild-type for rpoS) and JMH0039 (rpoS deficient). As previously noted for the activity of E. coli strain DH5x carrying the fusion PectA–lacZ (Fig. 3), expression of PectA in E. coli strain MC4100 seemed to be as efficient (or even more efficient) as in C. salexigens (see Figs 4a and 5a). The pattern of PectA expression in E. coli MC4100 was very similar to that found in C. salexigens, with higher expression in stationary phase than in exponentially growing cells (Fig. 5a).
contrast, the higher activity in stationary phase was not observed in the rpoS mutant, in which expression was approximately 50% less than in the wild-type strain at the same optical densities (Fig. 5b). These results indicate that efficient expression of the PectA promoter region in E. coli (and probably in C. salexigens) greatly depends on the sigma factor sS. However, the fact that about 50% of PectA promoter activity can be found in the rpoS mutant strain suggests the involvement of additional factor(s) in the expression of the ectABC genes.

Influence of temperature, external osmoprotectants, excess of iron, and nalidixic acid, on PectA and PectB expression

We tested whether, in addition to salt stress and phase of growth, other environmental conditions such as high temperature, the presence of external osmoprotectants, or an excess of iron, could modulate the expression of the ectABC genes. The DNA gyrase inhibitor nalidixic acid was also tested, to check the possible influence of DNA topology on the transcriptional control of ectoine synthesis. In these experiments (Fig. 6), the basal expression levels of the PectA–lacZ and PectB–lacZ transcriptional fusions were those corresponding to the β-galactosidase activity of C. salexigens cells (carrying the fusions) grown in M63 with 0.75 M NaCl at 37 °C to stationary phase (OD600 approx. 1.7). The steady-state expression level of the PectA–lacZ fusion increased by approximately 1.4-fold when the growth temperature was increased from 37 °C to 40 °C. The temperature-dependent induction of transcription was more marked in the case of the PectB–lacZ fusion, with a 3.6-fold increase of expression at 40 °C. This finding is in agreement with the similarity of promoter PectB to σ32-dependent heat-shock promoters. The addition of 1 mM glycine betaine to the growth medium reduced PectA and PectB expression by 1.6- and 2.3-fold, respectively, but did not abolish it. Similarly, 1 mM ectoine also reduced the steady-state levels for each of the Pect–lacZ fusions, although not as much as glycine betaine. Interestingly, the addition of an excess of iron (as FeCl3) to the growth medium led to a 1.8-fold reduction of basal expression of the PectA–lacZ fusion, but did not affect the expression of the PectB–lacZ fusion. Finally, the addition of nalidixic acid resulted in 1.5- and 1.7-fold reduction of PectA and PectB expression, respectively. This result suggests that changes in DNA topology may affect transcriptional regulation of ect genes.

Post-transcriptional regulation of ectoine accumulation by glycine betaine

Although externally added glycine betaine negatively modulates the expression of PectA and PectB, their expression is not completely repressed by glycine betaine at low (0.75 M
NaCl) or at high (2-5 M NaCl) osmolarity (see Figs 1a and 6). However, we have previously shown that when C. salexigens was grown at the optimal salinity (2 M NaCl) in the presence of choline (the precursor of glycine betaine), only glycine betaine, but no ectoine or hydroxyectoine, was detected by $^{13}$C-NMR (Cánovas et al., 1996). These data suggested that, in addition to the transcriptional control of ectoine synthesis by glycine betaine, a post-transcriptional control mechanism might operate in C. salexigens. To further investigate this at salinities different from the optimal, $^{13}$C-NMR was used to analyse the cytoplasmic content of C. salexigens grown in M63 with low (0-75 M NaCl) or high (2-5 M NaCl) osmolarity in the absence or presence of 1 mM glycine betaine (Fig. 7). The spectra of C. salexigens grown in the absence of glycine betaine at low (Fig. 7a) or high salinity (Fig. 7c) were very similar. In both cases, cells accumulated ectoine and hydroxyectoine as the main cytoplasmic solutes, and also minor amounts of glutamate. Glucosylglycerate, a solute previously reported to be present in C. salexigens grown in M63 supplemented with 0-5 to 1-5 M NaCl (Cánovas et al., 1999), was found in the spectrum of cells grown at 0-75 M NaCl, but could not be detected in cells grown at high salinity. The synthesis of ectoine and its derivative hydroxyectoine was completely repressed by the presence of 1 mM glycine betaine in M63 supplemented with 0-75 M NaCl (Fig. 7b). Under these conditions, the spectrum contained only resonances that were assigned to glycine betaine and glutamate. In cells grown at high salinity with glycine betaine (Fig. 7c), the situation was slightly different. Although glycine betaine was still the predominant compatible solute accumulated, in this case we could detect some signals corresponding to ectoine, indicating that the inhibition of ectoine synthesis by glycine betaine was partial.

**DISCUSSION**

In this work, we examined the long-term transcriptional regulation of ectoine synthesis in C. salexigens, and found preliminary indications of a great complexity of this process. According to our data, the C. salexigens ectABC genes can be expressed from two promoter regions, one located upstream of ectA, composed of four putative promoters (PectA1–4) and one internal promoter located upstream of
ectB (PectB). The reason why C. salexigens would need an accessory promoter upstream of ectB is unclear. The PectA–lacZ and PectB–lacZ fusions were expressed in E. coli (Figs 2, 3 and 5). Remarkably, PectA–lacZ was expressed at a high basal level in E. coli cells grown in minimal medium M63 without NaCl (Fig. 3). Both C. salexigens and E. coli belong to the γ subdivision of proteobacteria. In addition, the consensus sequences of C. salexigens Pect promoters closely resembled those of σ70, (PectA1 and PectA2), σ32, (PectA3) and σ32, (PectB) dependent promoters of E. coli. Therefore it seemed plausible that PectA and PectB expression in C. salexigens might be driven by sigma factors orthologous to the E. coli σ70 and σ32, and σ32 proteins, respectively. The PectA–lacZ fusion in C. salexigens and E. coli wild-type strains behaved as a typical σ-dependent promoter, showing a clear preferential expression during the stationary phase. In addition, expression was significantly lower in the rpoS strain JMH0039. These results suggest that although σ32 plays an important role in PectA expression, possibly through interaction with PectA3, it is not the sole sigma factor involved. This finding is consistent with the presence in the PectA promoter region of at least two additional putative promoters, PectA1 and PectA2, that probably depend on the primary factor σ70.

Kuhlmann & Bremer (2002) reported that the ectABC genes of Salibacillus pasteurii are expressed from a single osmo-regulated promoter. The −10 and −35 sequences of this promoter resemble the consensus sequences of promoters recognized by the main vegetative sigma factor σ70 (equivalent to σ70 of Gram-negative bacteria). This situation differs from what we have found upstream of ectA in C. salexigens. Nevertheless, it has been firmly established that σ70 controls the expression of several osmoregulated genes. Thus, the E. coli genes proP, encoding the proline uptake system (Xu & Johnson, 1997), and cfa, encoding the cycopropane fatty acid synthase (Wang & Cronan, 1994), are transcribed from two promoters, one depending on σ70, the other one being recognized by σ32. There are some osmoregulated genes whose transcription is entirely dependent on σ32. Some examples are the E. coli genes osxAB, involved in trehalose synthesis (Hengge-Aronis et al., 1991), and osmY, encoding a periplasmic protein of unknown function (Yim et al., 1994), among others. Thus, in E. coli and other enteric bacteria σ32 is not only involved in stationary-phase induction of transcription, but functions as the master regulator of the general stress response, coordinating an emergency reaction to stress as well as long-term adaptation to many different stresses including starvation, high osmolality, high or low temperature, acidic pH, or oxidative agents (Hengge-Aronis, 1999, 2000). Similarly, σ3, controlling the general stress response in Gram-positive bacteria, is partially responsible for the transcriptional control of some osmoregulated genes, such as opuE and opud from Bacillus subtilis, encoding the proline and glycine betaine uptake systems, respectively (Bremer & Krämer, 2000). In agreement with this, computer-assisted analysis of the Marinococcus halophilus DNA upstream of ectABC revealed the presence of a putative binding site for σ3 as the most promising candidate for transcription regulation in this bacillus-like micro-organism (Louis & Galinski, 1997). Very interestingly, a transcriptional fusion of this promoter region (referred to as ectUp) with the reporter gene gfp was not only expressed but also osmotically induced in E. coli (Bestvater & Galinski, 2002). The presence of sequences at the promoter region that might be potentially recognized by the E. coli sigma factors σ70 or σ3 may explain this heterologous expression.

As C. salexigens is a truly halophilic bacterium, unable to grow below 0.5 M NaCl, transcriptional analysis of the ectABC genes in C. salexigens could not be performed in the absence of salt. Since at 0.5 M NaCl the growth is extremely slow, 0.75 M NaCl was selected as the lowest salt concentration in this study. It is remarkable that, at this low salinity, expression of the PectA and PectB promoters was quite high (Fig. 4a), suggesting that ectABC may be a partially constitutive system, and cells accumulated high amounts of ectoine and hydroxyectoine (Fig. 7a), indicating that even at this salinity they are under osmotic stress. These findings agree with the fact that addition of osmo-protectants such as choline or glycine betaine stimulates growth of C. salexigens at all salinities, including 0.5 M NaCl (Cavanovas et al., 1996). In contrast, the halotolerant bacillus S. pasteurii (Kuhlmann & Bremer, 2002) produced ectoine in significant amounts only at elevated osmolalities. Fully consistent with this is the finding that the ectABC genes of S. pasteurii are not expressed at 0 M NaCl. Consequently, it has been suggested that ectoine synthesis in S. pasteurii must depend primarily on the stimulation of gene transcription under hypertonic conditions (Kuhlmann & Bremer, 2002).

The technical problems related to the failure of the E. coli β-galactosidase to function above 0.75 M NaCl prevented us from quantitatively monitoring the expression of the ectABC at higher salinities. Nevertheless, the S1 protection assay performed with RNA from cells grown at 2.5 M NaCl (Fig. 1a) clearly indicated that the putative PectA1 (σ70-dependent), PectA3 (σ32-dependent) and PectA4 might be osmoregulated promoters. These results suggest that transcriptional activation of ectoine synthesis plays a role in the long-term response to osmotic stress in C. salexigens. In agreement with this, a linear relationship was found between the expression of the PectA–lacZ fusion and increasing salinity in E. coli. This induction by hyperosmolality in E. coli may be attributed, at least in part, to an increased intracellular concentration of σ3. It has been demonstrated that high osmolality increases the rate of σ3 translation of already existing E. coli rpoS mRNA, and reduces σ3 proteolysis by the RssB recognition factor-ClpXP protease system (Hengge-Aronis, 2002b). However, the nature of the intracellular signals that are triggered by osmotic stress and their interactions with the post-transcriptional control mechanisms in E. coli are completely unknown at present.

Temperature induction of PectA and PectB suggests that
ectoine might have a physiological role in thermoprotection of \textit{C. salexigens}, in addition to osmoprotection. This hypothesis, which has yet to be tested experimentally, correlates with the emerging evidence that some compatible solutes, such as the disaccharide trehalose, function \textit{in vivo} as general stress protectants. Cánovas \textit{et al.} (2001) reported that trehalose accumulation by \textit{Salmonella typhimurium} is thermoregulated, and found evidence that this disaccharide is crucial for growth at high temperature. In fact, ectoine and its derivative hydroxyectoine were shown \textit{in vitro} to function as powerful stabilizing agents of enzymes against a number of stresses, including thermal denaturation (Cánovas \textit{et al.}, 1999, Lippert & Galinski, 1992). In \textit{E. coli} cells exposed to high temperature, $\sigma^8$ proteolysis is reduced (Hengge-Aronis, 2002b). Moreover, temperature-triggered translational induction, as well as stabilization of otherwise labile $\sigma^32$, occurs (Yura \textit{et al.}, 2000). Although it is very tempting to speculate that induction of \textit{PectA}–\textit{lacZ} and \textit{PectB}–\textit{lacZ} transcription in \textit{C. salexigens} cells grown at 40 °C might be attributed to an increased cellular level of sigma factors orthologous to \textit{E. coli} $\sigma^8$ and $\sigma^32$ as a consequence of the heat stress, this needs experimental evidence.

As expected, expression of \textit{PectA}–\textit{lacZ} and \textit{PectB}–\textit{lacZ} fusions was reduced in the presence of the DNA gyrase inhibitor nalidixic acid. The SI protection assays (Fig. 1a) suggest that when \textit{C. salexigens} is grown at 0.75-7 M or 2.5 M NaCl, active $\sigma^{70}$ and $\sigma^8$ bind to the \textit{PectA} promoter region. The $\ominus$-10 sequences of putative \textit{PectA2} ($\sigma^{70}$-dependent) and \textit{PectA3} ($\sigma^8$-dependent) are separated by 30 nt, whereas the association RNA polymerase core-sigma factor is estimated to cover a DNA region of about 80 nt. Therefore, $\sigma^{70}$ and $\sigma^8$-RNA polymerase holoenzymes must compete for binding to these two promoters. Similar to the situation in \textit{E. coli} (Hengge-Aronis 1999, 2002a), local DNA topology, as well as the formation of specific nucleoprotein structures that involve additional regulatory factors and/or histone-like protein, might modulate \textit{ectABC} expression in response to environmental conditions.

The presence of external osmoprotectants (ectoine or glycine betaine) reduced the basal transcription level of \textit{PectA}–\textit{lacZ} and \textit{PectB}–\textit{lacZ} in \textit{C. salexigens} (Figs 1 and 6). The same transcriptomic response was found by Bestvater & Galinski (2002) for the \textit{M. halophilus} eucU–\textit{gfp} fusion in \textit{E. coli}. Likewise, relief of osmotic stress by glycine betaine resulted in a reduced expression of the $\textit{E. coli}$ \textit{betA} and \textit{betB}, and \textit{B. subtilis} opuE loci, encoding osmoregulated glycine betaine synthesis from choline, and proline transport, respectively (Eshoo, 1988; Spiegelhalter & Bremer, 1998). The decreased transcription of these osmoregulated genes upon addition of external osmoprotectants does not seem to be due to a direct effect of the solute in the transcription machinery. Instead, it may be that osmoprotectant accumulation attenuates the (unknown) activation signal for the former systems (Wood \textit{et al.}, 2001). Our data indicate that in cells continuously grown at low or high salinity, transcriptional control by glycine betaine cannot be the only mechanism responsible for the absence of cytoplasmic ectoine. This suggests the existence of a posttranscriptional control mechanism that might operate at the level of enzyme activity. However, alternative mechanisms such as an increased efflux of ectoine in the presence of betaine cannot be ruled out. This is in agreement with the general assumption that transport of compatible solutes is preferred over their synthesis, because the latter is energetically less favourable (Oren, 1999).

Bacterial response to environmental stresses is complex, and the involvement of multiple promoters and transcription factors belonging to different regulatory pathways is a rather common strategy that ensures an appropriate expression to the changing environment (Vicente \textit{et al.}, 1999). For some osmoregulated systems, the general stress response and the specific osmostress response are linked through the general stress factors $\sigma^8$ or $\sigma^8$ that control systems such as trehalose synthesis in \textit{E. coli} (Hengge-Aronis \textit{et al.}, 1991), osmoprotectant uptake in \textit{B. subtilis} (Bremer & Krämer, 2000), and possibly ectoine synthesis in \textit{C. salexigens} (this study). In \textit{C. salexigens}, the response to heat and osmotic stress might also overlap since \textit{PectB} might be dependent on $\sigma^8$. Some osmoregulated systems, such as the \textit{E. coli} and \textit{C. salexigens} \textit{betBA} genes for glycine betaine synthesis, are clustered with their transcriptional regulator (i.e. \textit{betI}) within the chromosome (Lamark \textit{et al.}, 1991; Cánovas \textit{et al.}, 2000). However, no sequences encoding regulatory proteins are found upstream or downstream of the \textit{C. salexigens} \textit{ectABC} cluster (unpublished results). In conclusion, a number of as yet undetermined signal transduction pathways might operate in \textit{C. salexigens} leading to osmoregulated ectoine synthesis. Identifying and characterizing these routes will be some of our main experimental aims in the near future.

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