

# Complex regulation of the synthesis of the compatible solute ectoine in the halophilic bacterium *Chromohalobacter salexigens* DSM 3043<sup>T</sup>

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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  $\sigma^{70}$ , and one (*PectA3*) might be dependent on the general stress sigma factor  $\sigma^S$ . 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  $\sigma^{32}$  was found upstream of *ectB*. Transcription from *PectA* in *C. salexigens* followed a pattern typical of  $\sigma^S$ -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  $\sigma^S$  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 a partially constitutive system. 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 (FeCl<sub>3</sub>). 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.

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## 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 osmoregulated systems (Bremer & Krämer, 2000; Csonka & Epstein, 1996; Poolman & Glaasker, 1998; Wood *et al.*, 2001). With the exception of the *Halobacteriaceae*, 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 *Actinopoly-spora*, *Brevibacterium*, *Nocardiopsis* and *Streptomyces*) the firmicutes (i.e. genera *Bacillus*, *Salibacillus*, *Virgibacillus*, *Halobacillus*, *Marinococcus* and *Sporosarcina*), and the proteobacteria (i.e. genera *Chromohalobacter*, *Ectothiorhodospira*, *Halomonas*, *Methylobacter*, *Pseudomonas*, *Rhodovulum* and *Salinivibrio*) (Da Costa *et al.*, 1998; Galinski, 1995; Galinski & Trüper, 1994; Kuhlmann & Bremer, 2002; Severin *et al.*, 1992; Ventosa *et al.*, 1998; Wohlfarth *et al.*, 1990). With the known exception of the  $\alpha$ -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 subdivision of this bacterial lineage. Ectoine has not been reported, however, in cyanobacteria, archaea or eukaryotes.

Among the  $\gamma$ -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<sup>+</sup> and Cl<sup>-</sup> 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 *N*<sup>5</sup>-acetyldiaminobutyric acid, and *ectC* specifies ectoine synthase, which catalyses the cyclic

condensation of *N*<sup>5</sup>-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* (Göller *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 upshock (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<sup>T</sup> (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

Strain/plasmid	Description	Reference or source
<b><i>C. salexigens</i></b>		
DSM 3043 <sup>T</sup>	Wild-type, formerly <i>Halomonas elongata</i> DSM 3043 (strain 1H11)	Vreeland <i>et al.</i> (1980); Arahal <i>et al.</i> (2001)
CHR61	Spontaneous Rf <sup>r</sup> mutant of DSM 3043	Cánovas <i>et al.</i> (1997)
<b><i>E. coli</i></b>		
DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> ; host for DNA manipulations	Hanahan (1983)
MC4100	F <sup>-</sup> <i>araD139</i> $\Delta$ <i>lacU169 rpsL150 relA1 flb-5301 deoC1 ptsF25</i>	Casabadan (1976)
JMH0039	MC4100 <i>rpoS::Tn10</i>	Corona-Izquierdo & Membrillo-Hernández (2002)
CHR112	Spontaneous Rf <sup>r</sup> mutant of MC4100	This study
CHR113	Spontaneous Rf <sup>r</sup> mutant of JMH0039	This study
<b>Plasmids</b>		
pKS(-)	Cloning vector, Ap <sup>r</sup>	Stratagene
pMP220	Promoter probe vector, <i>lacZ</i> , IncP, Tc <sup>r</sup>	Spaink <i>et al.</i> (1987)
PRK600	Helper plasmid, Cm <sup>r</sup>	Kessler <i>et al.</i> (1992)
pME2	2.8 kb <i>EcoRI</i> fragment from pDE9 (containing <i>ectABC</i> ) cloned in pKS(-)	Cánovas <i>et al.</i> (1998a)
pME7	296 bp region from pME2, containing the promoter region upstream of <i>ectA</i> ( <i>PectA1</i> to <i>PectA4</i> ), cloned in pMP220 as an <i>EcoRI</i> - <i>PstI</i> fragment	This study
pME8	431 bp region from pME2, containing the promoter <i>PectB</i> , cloned in pMP220 as a <i>KpnI</i> - <i>PstI</i> fragment	This study
pME9	662 bp region from pME2, containing 395 bp upstream of <i>ectC</i> , cloned into pMP220 as a <i>XbaI</i> - <i>PstI</i> fragment	This study

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 ( $\mu$ g ml<sup>-1</sup>): ampicillin (Ap), 150; tetracycline (Tc), 15; chloramphenicol (Cm), 25; rifampicin (Rf), 25. When appropriate, the following compounds were added to the media (final concentration): X-Gal (Sigma, 40  $\mu$ g ml<sup>-1</sup>), ectoine (Bitop, 1 mM), glycine betaine (Sigma, 1 mM), nalidixic acid (1  $\mu$ M) and FeCl<sub>3</sub> (50  $\mu$ M). Growth was monitored as OD<sub>600</sub> with a Perkin Elmer 551S 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'-CGCTCTAGAACTAGTGGAT-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'-GTTCTCGGCTGCAGGCGTCAT-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'-CGGTACCCCTATCTGCTGCT-3') and MB2 (5'-GAATCTGGGTCTGCAGAGAT-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'-CCGTGTGGTCTAGAATCGCT-3') and MC4 (5'-TGCTGATCCTGCAGGTACAT-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  $\mu$ g total RNA and an excess of a <sup>32</sup>P-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* start codon). The primers used to generate the probes were MS-A2 (5'-TCGTCGGTTCGTGGGCTTAC-3'), MS-B1 (5'-AACGG-TAGGAAAAGAACG-3') and MS-C1 (5'-TGCGGGTGTATGTTGA-ACGAG-3') for mapping the 5'-end of putative transcripts starting at *ectA*, *ectB* and *ectC*, respectively.

**Extraction of intracellular solutes and  $^{13}\text{C}$ -NMR spectroscopy.** Cytoplasmic solutes were extracted essentially as described by Cánovas *et al.* (1997), but with the following modifications. *C. salexigens* was grown in 200 ml minimal medium M63 until late-exponential phase ( $\text{OD}_{600}$  1–1.2). Cells were collected by centrifugation and washed with the same medium with no carbon source. To extract the cytoplasmic compatible solutes, pellets were resuspended in 10 ml double-distilled water and, after 10 min incubation at room temperature, cell debris was removed by centrifugation. Supernatants were extracted twice with chloroform and freeze-dried. Cell extracts were resuspended in 0.5 ml  $\text{D}_2\text{O}$ . Natural  $^{13}\text{C}$ -NMR abundance spectra were recorded on a Bruker ac200 spectrometer at 50 MHz with a probe temperature of 20–22 °C. Signals were identified by comparison with spectra of pure compounds or with spectra previously described (Cánovas *et al.*, 1997, 1999).

**Assay for  $\beta$ -galactosidase activity.**  $\beta$ -Galactosidase activity was measured basically as described by Miller (1992). Overnight cultures of  $\text{Lac}^-$  *E. coli* cells (in LB with Tc), or *C. salexigens* DSM 3043 (lacks intrinsic  $\beta$ -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  $\beta$ -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  $\beta$ -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 (Cánovas *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 (GTGCCG) and –10 (TATAAT) sequences of putative promoter *PectA2* were very similar to the consensus sequences of  $\sigma^{70}$  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  $\sigma^{70}$ . In fact, *PectA2* had been previously detected by computer-assisted analysis of the region upstream of *ectA* and suggested to be the *ectABC* promoter (Cánovas *et al.*, 1998b). The sequence of the –35 (TCGATA) and –10 (TACATA) regions of putative promoter *PectA1* also matched the consensus recognition sequence for  $\sigma^{70}$  at a number of positions, with a 17 bp spacing between the –10 and –35

**Fig. 1.** Analysis of the *C. salexigens* promoter regions located upstream of *ectA* and *ectB*. (a) Identification of the transcription start sites by S1 protection assays. Total RNA was prepared from *C. salexigens* cells grown in minimal medium M63 at low (0.75 M NaCl) and high (2.5 M NaCl) salinity, in the presence (+) or absence (–) of 1 mM betaine, using equal amounts of RNA for each sample. Lane M, DNA size ladder. (b) Nucleotide sequence of the *ectA* and *ectB* promoter regions. The *EcoRI* site flanking the 5'-end of the *ectABC* region cloned in plasmid pME2 is framed. The asterisk downstream of *EcoRI* denotes the stop codon of the gen *gor* (encoding a putative glutathione reductase), which precedes the *ectABC* region within the *C. salexigens* chromosome; the inverted repeat IR2 most probably corresponds to a rho-independent transcriptional terminator located between *gor* and *ectABC* (unpublished results). The transcription start sites (+ 1), –10 and –35 regions for each putative promoter are indicated. The ribosome-binding sites (RBS) are underlined, and the *ectA* and *ectB* start codons are shown in bold. The partial amino acid sequence of EctA is shown in one-letter code. The sequences corresponding to the oligonucleotides used for the S1 assays (MS-A2 and MS-B1) are underlined with arrows.



$\sigma^{32}$  (CTTGAAA-11–16 bp-CCCATnT; Wösten, 1998) could be detected upstream from the putative *PectB* promoter (CCGGAAA-16 bp-CCCGTTC). The length of the spacer was 16 nt, which is in agreement with the  $\sigma^{32}$  promoter consensus sequence. Finally, the -10 and -35 regions of putative *PectA4* did not show similarity to promoter sequences recognized by any known sigma factor.

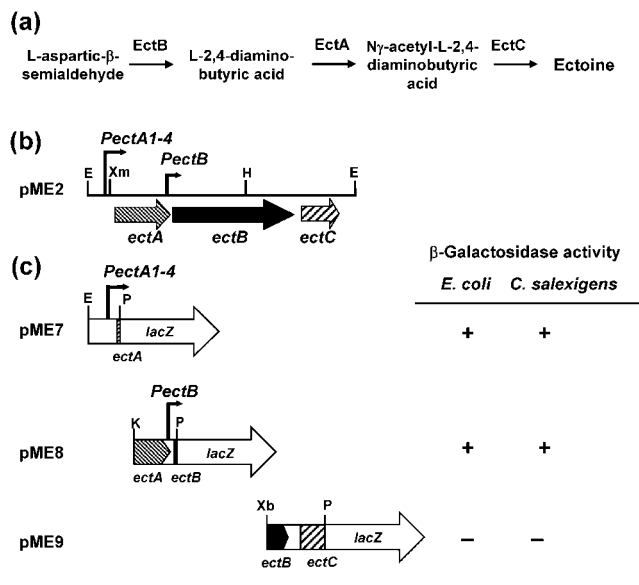
### Construction of *PectA-lacZ* and *PectB-lacZ* transcriptional fusions

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 $\alpha$  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  $\beta$ -galactosidase substrate X-Gal (Fig. 2c). Whereas colonies of *E. coli* and *C. salexigens* carrying plasmids pME7 and pME8 showed a Lac<sup>+</sup> phenotype, transconjugants harbouring pME9 were Lac<sup>-</sup>. 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).

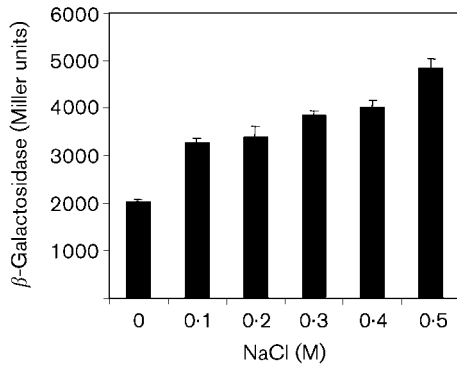
### Influence of salinity on *PectA* expression

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  $\beta$ -galactosidase activity in *C. salexigens*, as described in Methods. Unexpectedly, we were not able to make reliable measurements of  $\beta$ -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.



**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*.  $\beta$ -Galactosidase activity of *E. coli* DH5 $\alpha$  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, *PstI*; Xb, *XbaI*; Xm, *XmnI*.

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).



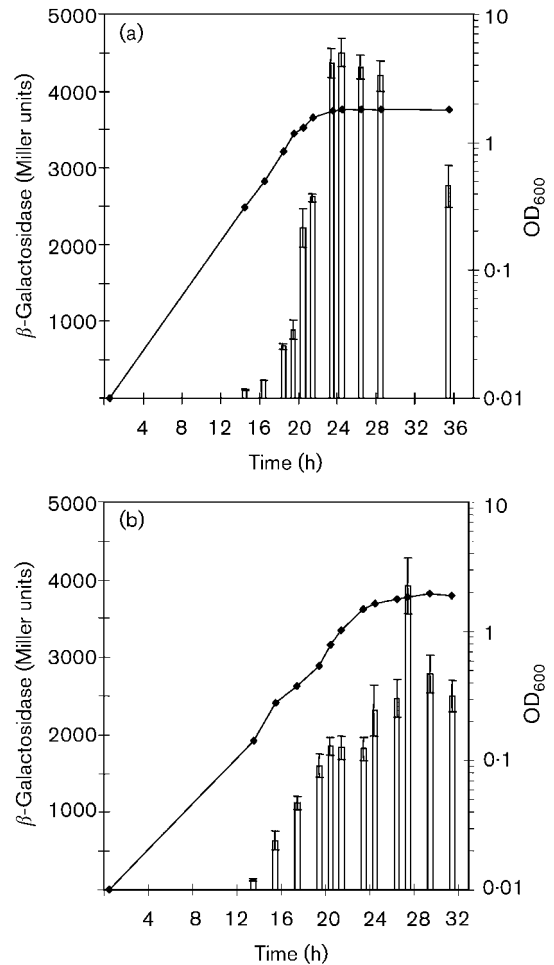
**Fig. 3.** Osmoregulation of the *PectA* promoter region in *E. coli*. *E. coli* DH5 $\alpha$  cells carrying the plasmid pME7 (*PectA-lacZ*) were grown in minimal medium M63 with NaCl concentrations ranging from 0 to 0.5 M. Samples were taken at the early stationary phase of growth (OD<sub>600</sub> approx. 1.7) and  $\beta$ -galactosidase activity was measured as described in Methods.

### 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 (Cánovas *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  $\beta$ -galactosidase activity. Fig. 4(a) shows that expression of *PectA-lacZ* fusion was low (although detectable) in the early (approx. 200 Miller units at OD<sub>600</sub> 0.5) and late (approx. 900 Miller units at OD<sub>600</sub> 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 OD<sub>600</sub> 0.5) and late (approx. 2000 Miller units at OD<sub>600</sub> 1.0) exponential phase of growth, but it did not increase suddenly when cells entered stationary phase (between 2000 and 2500 Miller units at OD<sub>600</sub> 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.

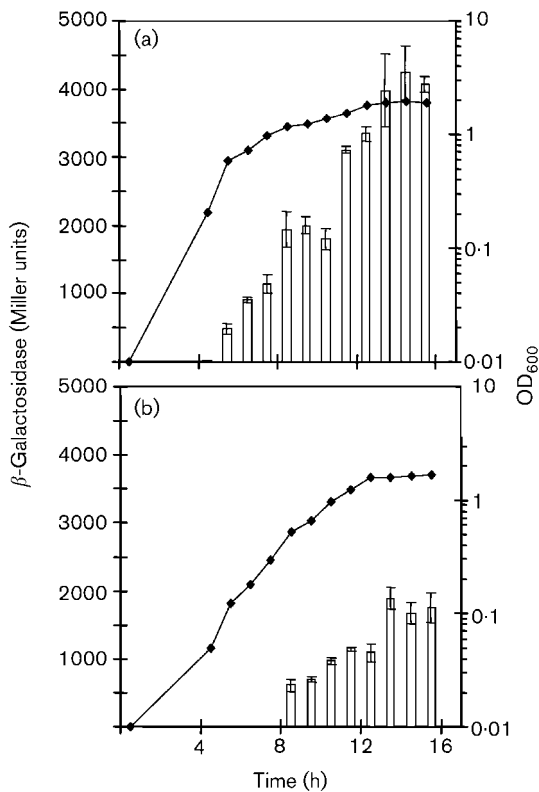
### Efficient transcription from the *PectA* promoter region requires a functional $\sigma^S$

The transcriptional pattern of *PectA-lacZ* fusion correlated with that of stationary-phase  $\sigma^S$ -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  $\sigma^S$ , it seemed likely that transcription of the



**Fig. 4.** Influence of the growth phase on *PectA* and *PectB* expression. *C. salexigens* cells carrying the plasmids pME7 (*PectA-lacZ*) (a) and pME8 (*PectB-lacZ*) (b) were grown in minimal medium M63 supplemented with 0.75 M NaCl. Samples were taken at different time intervals and the OD<sub>600</sub> (●) and  $\beta$ -galactosidase activity (histogram bars) were measured as described in Methods.

*ectABC* genes from the *PectA* promoter may be (at least partially) dependent on this sigma factor. The *C. salexigens* gene encoding  $\sigma^S$  has not been identified and therefore a  $\sigma^S$ -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  $\sigma^S$ -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 DH5 $\alpha$  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). In

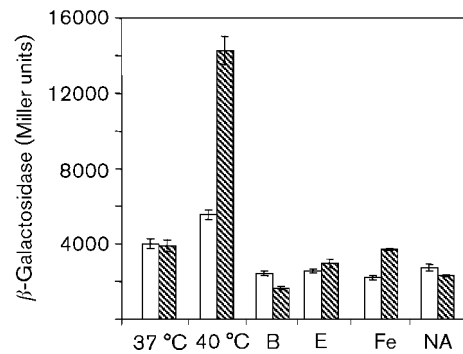


**Fig. 5.** Efficient transcription from the *PectA* promoter in *E. coli* requires a functional  $\sigma^S$ . Cells of *E. coli* CHR112 (spontaneous Rif<sup>r</sup> mutant of MC4100; a) and CHR113 (spontaneous Rif<sup>r</sup> mutant of *rpoS* strain JMH0039; b) carrying plasmid pME7 (*PectA-lacZ*) were grown in minimal medium M63 supplemented with 0.25 M NaCl. Samples were taken at different time intervals and the OD<sub>600</sub> (●) and  $\beta$ -galactosidase activity (histogram bars) were measured as described in Methods.

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  $\sigma^S$ . 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



**Fig. 6.** Influence of various environmental factors and nalidixic acid on *PectA* and *PectB* expression. *C. salexigens* cells harbouring the plasmids pME7 (*PectA-lacZ*, white bars) and pME8 (*PectB-lacZ*, hatched bars) were grown in minimal medium M63 supplemented with 0.75 M NaCl and incubated at 37, 40 and 37 °C in the presence of 1 mM betaine (B), 1 mM ectoine (E), 50 mM FeCl<sub>3</sub> (Fe) or 1  $\mu$ M nalidixic acid (NA). Samples were taken at the early stationary phase of growth (OD<sub>600</sub> approx. 1.7) and  $\beta$ -galactosidase activity was measured as described in Methods.

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  $\beta$ -galactosidase activity of *C. salexigens* cells (carrying the fusions) grown in M63 with 0.75 M NaCl at 37 °C to stationary phase (OD<sub>600</sub> 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  $\sigma^{32}$ -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 FeCl<sub>3</sub>) 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

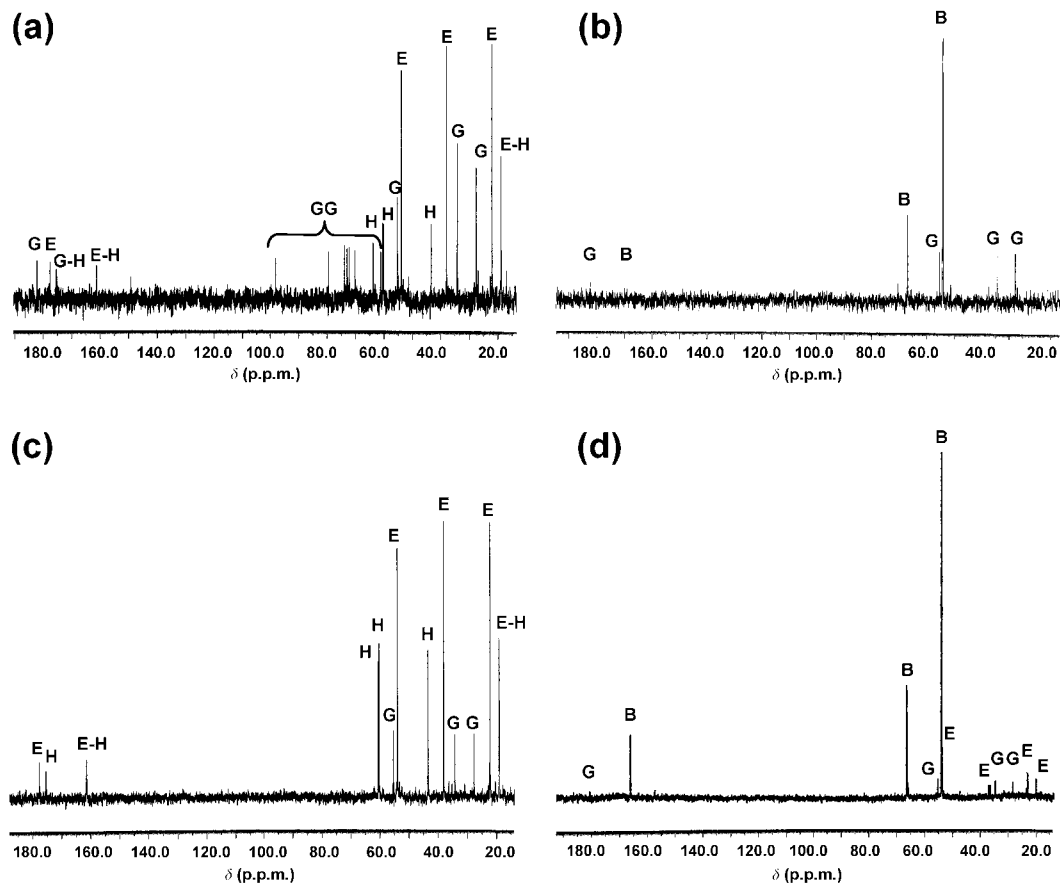
Although externally added glycine betaine negatively modulates the expression of *PectA* and *PectB*, their expression is not completely repressed by glycine betaine at low (0.75 M

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  $^{13}\text{C}$ -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,  $^{13}\text{C}$ -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



**Fig. 7.** Natural-abundance  $^{13}\text{C}$ -NMR spectra of major cytosolic solutes of *C. salexigens* grown in minimal medium M63 supplemented with 0.75 M NaCl (a), 0.75 M NaCl with 1 mM betaine (b), 2.5 M NaCl (c), or 2.5 M NaCl with 1 mM betaine (d). The signals were as follows: ectoine (E), hydroxyectoine (H), betaine (B), glutamate (G) and glucosylglycerate (GG).

*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  $\gamma$  subdivision of proteobacteria. In addition, the consensus sequences of *C. salexigens* *Pect* promoters closely resembled those of  $\sigma^{70}$ - (*PectA1* and *PectA2*),  $\sigma^S$ - (*PectA3*) and  $\sigma^{32}$ - (*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*  $\sigma^{70}$  and  $\sigma^S$ , and  $\sigma^{32}$  proteins, respectively. The *PectA-lacZ* fusion in *C. salexigens* and *E. coli* wild-type strains behaved as a typical  $\sigma^S$ -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  $\sigma^S$  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  $\sigma^{70}$ .

Kuhlmann & Bremer (2002) reported that the *ectABC* genes of *Salibacillus pasteurii* are expressed from a single osmoregulated promoter. The  $-10$  and  $-35$  sequences of this promoter resemble the consensus sequences of promoters recognized by the main vegetative sigma factor  $\sigma^A$  (equivalent to  $\sigma^{70}$  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  $\sigma^S$  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 cyclopropane fatty acid synthase (Wang & Cronan, 1994), are transcribed from two promoters, one depending on  $\sigma^{70}$ , the other one being recognized by  $\sigma^S$ . There are some osmoregulated genes whose transcription is entirely dependent on  $\sigma^S$ . Some examples are the *E. coli* genes *otsAB*, 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  $\sigma^S$  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 osmolarity, high or low temperature, acidic pH, or oxidative agents (Hengge-Aronis, 1999, 2000). Similarly,  $\sigma^B$ , 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  $\sigma^B$  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  $\sigma^{70}$  or  $\sigma^S$  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 osmoprotectants such as choline or glycine betaine stimulates growth of *C. salexigens* at all salinities, including 0.5 M NaCl (Cánovas *et al.*, 1996). In contrast, the halotolerant bacillus *S. pasteurii* (Kuhlmann & Bremer, 2002) produced ectoine in significant amounts only at elevated osmolarities. 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*  $\beta$ -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* ( $\sigma^{70}$ -dependent), *PectA3* ( $\sigma^S$ -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 hyperosmolarity in *E. coli* may be attributed, at least in part, to an increased intracellular concentration of  $\sigma^S$ . It has been demonstrated that high osmolarity increases the rate of  $\sigma^S$  translation of already existing *E. coli* *rpoS* mRNA, and reduces  $\sigma^S$  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 *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 *in vivo* as general stress protectants. Cánovas *et al.* (2001) reported that trehalose accumulation by *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 *in vitro* to function as powerful stabilizing agents of enzymes against a number of stresses, including thermal denaturation (Cánovas *et al.*, 1999; Lippert & Galinski, 1992). In *E. coli* cells exposed to high temperature,  $\sigma^S$  proteolysis is reduced (Hengge-Aronis, 2002b). Moreover, temperature-triggered translational induction, as well as stabilization of otherwise labile  $\sigma^{32}$ , occurs (Yura *et al.*, 2000). Although it is very tempting to speculate that induction of *PectA-lacZ* and *PectB-lacZ* transcription in *C. salexigens* cells grown at 40 °C might be attributed to an increased cellular level of sigma factors orthologous to *E. coli*  $\sigma^S$  and  $\sigma^{32}$  as a consequence of the heat stress, this needs experimental evidence.

As expected, expression of *PectA-lacZ* and *PectB-lacZ* fusions was reduced in the presence of the DNA gyrase inhibitor nalidixic acid. The S1 protection assays (Fig. 1a) suggest that when *C. salexigens* is grown at 0.75 M or 2.5 M NaCl, active  $\sigma^{70}$  and  $\sigma^S$  bind to the *PectA* promoter region. The -10 sequences of putative *PectA2* ( $\sigma^{70}$ -dependent) and *PectA3* ( $\sigma^S$ -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  $\sigma^S$ -RNA polymerase holoenzymes must compete for binding to these two promoters. Similar to the situation in *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 *ectABC* expression in response to environmental conditions.

The presence of external osmoprotectants (ectoine or glycine betaine) reduced the basal transcription level of *PectA-lacZ* and *PectB-lacZ* in *C. salexigens* (Figs 1 and 6). The same transcriptional response was found by Bestvater & Galinski (2002) for the *M. halophilus ectUp-gfp* fusion in *E. coli*. Likewise, relief of osmotic stress by glycine betaine resulted in a reduced expression of the *E. coli betA* and *betB*, and *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 on the transcription machinery. Instead, it may be that osmoprotectant accumulation attenuates the (unknown) activation signal for the former systems (Wood *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 post-transcriptional 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 *et al.*, 1999). For some osmoregulated systems, the general stress response and the specific osmostress response are linked through the general stress factors  $\sigma^S$  or  $\sigma^B$  that control systems such as trehalose synthesis in *E. coli* (Hengge-Aronis *et al.*, 1991), osmoprotectant uptake in *B. subtilis* (Bremer & Krämer, 2000), and possibly ectoine synthesis in *C. salexigens* (this study). In *C. salexigens*, the response to heat and osmotic stress might also overlap since *PectB* might be dependent on  $\sigma^{32}$ . Some osmoregulated systems, such as the *E. coli* and *C. salexigens betBA* genes for glycine betaine synthesis, are clustered with their transcriptional regulator (i.e. *betI*) within the chromosome (Lamark *et al.*, 1991; Cánovas *et al.*, 2000). However, no sequences encoding regulatory proteins are found upstream or downstream of the *C. salexigens ectABC* cluster (unpublished results). In conclusion, a number of as yet undetermined signal transduction pathways might operate in *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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