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# New insights into hydroxyectoine synthesis and its transcriptional regulation in the broad-salt growing halophilic bacterium *Chromohalobacter salexigens*

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## Summary

**Elucidating the mechanisms controlling the synthesis of hydroxyectoine is important to design novel genetic engineering strategies for optimizing the production of this biotechnologically relevant compatible solute. The genome of the halophilic bacterium *Chromohalobacter salexigens* carries two ectoine hydroxylase genes, namely *ectD* and *ectE*, whose encoded proteins share the characteristic consensus motif of ectoine hydroxylases but showed only a 51.9% identity between them. In this work, we have shown that *ectE* encodes a secondary functional ectoine hydroxylase and that the hydroxyectoine synthesis mediated by this enzyme contributes to *C. salexigens* thermoprotection. The evolutionary pattern of EctD and EctE and related proteins suggests that they may have arisen from duplication of an ancestral gene preceding the directional divergence that gave origin to the orders Oceanospirillales and Alteromonadales. Osmoregulated expression of *ectD* at exponential phase, as well as the thermoregulated expression of *ectD* at the stationary phase, seemed to be dependent on the general stress factor RpoS. In contrast, expression of *ectE* was always RpoS-dependent regardless of the growth phase and osmotic or heat stress conditions tested. The data presented here suggest that the AraC-GlxA-like EctZ transcriptional regulator, whose encoding gene lies upstream of *ectD*, plays a dual function under exponential growth as both a transcriptional activator of osmoregulated *ectD* expression and a repressor of *ectE* transcription, privileging the synthesis of the main ectoine hydroxylase EctD. Inactivation of *ectZ* resulted in a higher amount of the total ectoines pool at the expenses of a higher accumulation of ectoine, with maintenance of the hydroxyectoine levels. In addition to the transcriptional control, our results suggest a strong post-transcriptional regulation of hydroxyectoine synthesis. Data on the accumulation of ectoine and hydroxyectoine in *rpoS* and *ectZ* strains pave the way for using these genetic backgrounds for metabolic engineering for hydroxyectoine production.**

## Introduction

Ectoine and its derivative 5-hydroxyectoine (named hereafter hydroxyectoine) are members of a selected group of organic osmolytes, called compatible solutes, which are the most widely used in halophilic and halotolerant microorganisms to cope with environmental high osmotic stress (Galinski, 1995). Because these molecules are small soluble organic compounds, they can prevent water loss and promote water re-entry into the cells (da Costa *et al*., 1998). Ectoine was first discovered in the extremely halophilic phototrophic sulfobacterium *Ectothiorhodospira halochloris* (Galinski *et al*., 1985), whereas hydroxyectoine was originally discovered in the actinomycin D producer *Streptomyces parvulus* (Inbar and Lapidot, 1988). Currently, it is known that the genes for the synthesis of ectoine and hydroxyectoine are widespread in members of *Bacteria* and *Archaea* (Widderich *et al*., 2014).

The biosynthetic pathway of ectoine has been thoroughly studied, and three genes, *ectABC*, are found to be involved in the process (Louis and Galinski, 1997; Czech *et al*., 2018a). Specifically, l-aspartate-β-semialdehyde is catalysed into ectoine through a three-step enzymatic reaction, employing l-2,4-diaminobutyric acid transaminase (codified by *ectB*), *N*-γ-acetyltransferase (codified by *ectA*) and ectoine synthase (codified by *ectC*). Some bacteria harbour the *ectD* gene encoding the ectoine hydroxylase, the enzyme that synthesizes 5-hydroxyectoine from ectoine. Genome sequence analysis of hydroxyectoine producers has demonstrate that the *ectD* gene is not always included in the *ectABC*

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gene cluster, but it can be located somewhere else in the genome. In addition, some bacteria could harbour two or more copies of the ectoine hydroxylase gene within their genome (Czech *et al*., 2018a).

Osmotic stress triggers bacterial accumulation of both ectoine and hydroxyectoine. In addition, heat or cold stress can affect ectoines accumulation, and besides osmolytes ectoines are also considered as thermolytes. Thus, whereas an elevated temperature generally induces accumulation of hydroxyectoine (Malin and Lapidot, 1996; Garcia-Estepa *et al*., 2006; Bursy *et al*., 2008), the accumulation of ectoine could be induced by a decrease of temperature (Kuhlmann *et al*., 2008). On the other hand, hydroxyectoine accumulation normally occurs when cells enter stationary phase, indicating that this solute possesses stress-relieving properties that allow the cell to better cope with the multitude of challenges imposed by this growth phase (Czech *et al*., 2018a). Besides this role in alleviating different stresses *in vivo*, ectoines also serve as stabilizers of macromolecules and even whole cells (Pastor *et al*., 2010; Bissoyi *et al*., 2014; Schröter *et al*., 2017). This preserving function, together with the anti-aggregating and anti-inflammatory effects, promoted substantial interest to explore for a variety of biotechnological applications and potential medical uses of ectoines (Pastor *et al*., 2010; Marini *et al*., 2014; Srinivasan *et al*., 2014; Unfried *et al*., 2016; Nayak *et al*., 2020). Despite their closely related chemical structures, hydroxyectoine often possesses additional protecting effect and function-preserving properties than its precursor ectoine, especially against thermal stress, desiccation, or heavy metal- or low pH-induced stress conditions (Tanne *et al*., 2014; Moritz *et al*., 2015).

Hydroxyectoine is currently produced biotechnologically on an industrial scale using the natural producer *Halomonas elongata* and the 'bacterial milking' process, followed by chromatographic separation from ectoine, which is generally co-accumulated in the cell. In this process, high-level synthesis of ectoines is triggered by growing the cells in high-salinity media, followed by a strong osmotic downshock, producing the release of ectoines to the medium through mechanosensitive channels (Kunte *et al*., 2014). Despite of the biotechnological importance of hydroxyectoine, there have been few attempts of optimizing its production using bacterial systems (natural producers or heterologous production), mostly trying to avoid or minimize ectoine co-production (Seip *et al*., 2011; Czech *et al*., 2016). Alternative strategies to strain genetic engineering have focussed in optimizing the fermentation strategies, by adding factors to the medium (i.e. iron and α-ketoglutarate) that could especifically influence the enzymatic reaction towards hydroxyectoine production (Chen *et al*., 2019).

*Chromohalobacter salexigens* is a moderately halophilic bacterium which displays a remarkable salinity growth range (Arahal *et al*., 2001). This extremophilic microorganism has been extensively used in recent years to study the bacterial osmoadaptation process (Vargas *et al*., 2008; Pastor *et al*., 2010). In addition, *C. salexigens* has been proposed as an alternative natural producer of ectoine and hydroxyectoine (Fallet *et al*., 2010; Rodríguez-Moya *et al*., 2013). In this bacterium, accumulation of hydroxyectoine is upregulated by salinity and temperature and is maximal at 45°C and 2.5 M NaCl. Nevertheless, accumulation of ectoine is upregulated by salinity and downregulated by temperature, and it reaches its maximum at 37°C and 2.5 M NaCl. In addition, the levels of ectoine and hydroxyectoine in *C. salexigens* are maximal during the stationary phase of growth (Garcia-Estepa *et al*., 2006). In a first attempt to optimize hydroxiectoine production in this bacterium, genetically engineered strains were constructed that overproduce hydroxyectoine at low salinity, in a temperature-independent manner. Hydroxyectoine production was further improved by increasing the copies of *ectD* in a plasmid-based system and using a *C. salexigens* genetic background unable to synthesize ectoine (Rodríguez-Moya *et al*., 2013). Nonetheless, more knowledge regarding the synthesis and regulation of this compatible solute is needed to improve hydroxyectoine production.

As in other microorganisms, the genes *ectABC* are responsible for ectoine synthesis in *C. salexigens* (Cánovas *et al*., 1998), and ectoine hydroxylation is the main route for hydroxyectoine synthesis (Garcia-Estepa *et al*., 2006). Inspection of the *C. salexigens* genome allowed the identification of two genes encoding putative ectoine hydroxylases: *Csal_0542* and *Csal_3003*. In a previous work, we showed that the *Csal_0542*-encoded protein (EctD) was the principal enzyme responsible for ectoine hydroxylation (Garcia-Estepa *et al*., 2006). In this work, we demonstrate that the *Csal_3003*-encoded product (EctE) is a secondary ectoine hydroxylase that also contributes to *C. salexigens* hydroxyectoine accumulation and is therefore involved in thermoprotection. A phylogenetic and evolutionary origin analysis of EctD-like and EctE-like proteins was also performed. In addition, we have investigated the expression of the two ectoine hydroxylase genes under salinity and temperature stress, as a function of the growth phase. We also found that both the general stress sigma factor RpoS ($\sigma^S$) and the specific regulator EctZ contribute to the transcriptional control of ectoine hydroxylation but in a different manner, depending on the ectoine hydroxylase gene (*ectD* or *ectE*), the stress imposed, and the growth phase.

## Results

### *EctE is a secondary ectoine hydroxylase that contributes to* C. salexigens *thermoprotection*

The ectoine hydroxylase (EctD) belongs to the superfamily of Fe(II)- and 2-oxoglutarate-dependent dioxygenases, a group of versatile biocatalysts involved in various oxygenation/hydroxylation reactions (Reuter *et al*., 2010; Höppner *et al*., 2014). In a previous work (Garcia-Estepa *et al*., 2006), we constructed a *C. salexigens ectD* mutant (CHR136) and demonstrated that EctD is the main ectoine hydroxylase, responsible for hydroxyectoine synthesis from ectoine, in this halophile. In addition to *ectD* (*Csal_0542*), the *C. salexigens* genome carries the homologous gene *Csal_3003*, predicted to have an ectoine hydroxylase function. The product of *Csal_3003* was named EctE.

The genes *ectD* and *ectE* were very distant from each other within the *C. salexigens* genome and showed a very different genomic context (Fig. 1). None of them laid adjacent to the ectoine-encoding *ectABC* genes. Within the same strand, the *ectD* gene was preceded by *Csal_0541,* encoding a putative transcriptional regulator of the AraC-GlxA family (latter characterized in this paper as a regulator of hydroxyectoine synthesis, EctZ), and followed by *Csal_0543,* encoding a putative 2-keto-4 pentenoate hydratase (MhpD) that showed well-conserved domains of enzymes involved in the degradation of aromatic compounds. Upstream of *ectZ* in the complementary strand, the gene *csal_0540,* encoding a putative butyrobetaine hydroxylase-like, was found (Fig. 1A). The *in silico* analysis of the large intergenic region between *ectZ* and *ectD* (205 bp) revealed a putative Rho-independent transcriptional terminator downstream of *ectZ,* together with two putative promoters upstream of *ectD* (*ectD*p) showing −10 and −35 sequences resembling those recognized by RpoS/RpoD ($\sigma^S/\sigma^{70}$) and RpoH ($\sigma^{32}$) promoters (Fig. S1).

On the other hand, the putative ectoine hydroxylase *ectE* gene (*Csal_3003*) was the last of five genes in the same strand encoding solute binding (*Csal_2999*, *Csal_3000*, *Csal_3001*) and ABC transporter (*Csal_3002*) proteins (Fig. 1B). Downstream of *ectE*, in the opposite direction, the gene *Csal_3004,* encoding a putative membrane protein involved in aromatic hydrocarbon degradation, was found. Two putative RpoD

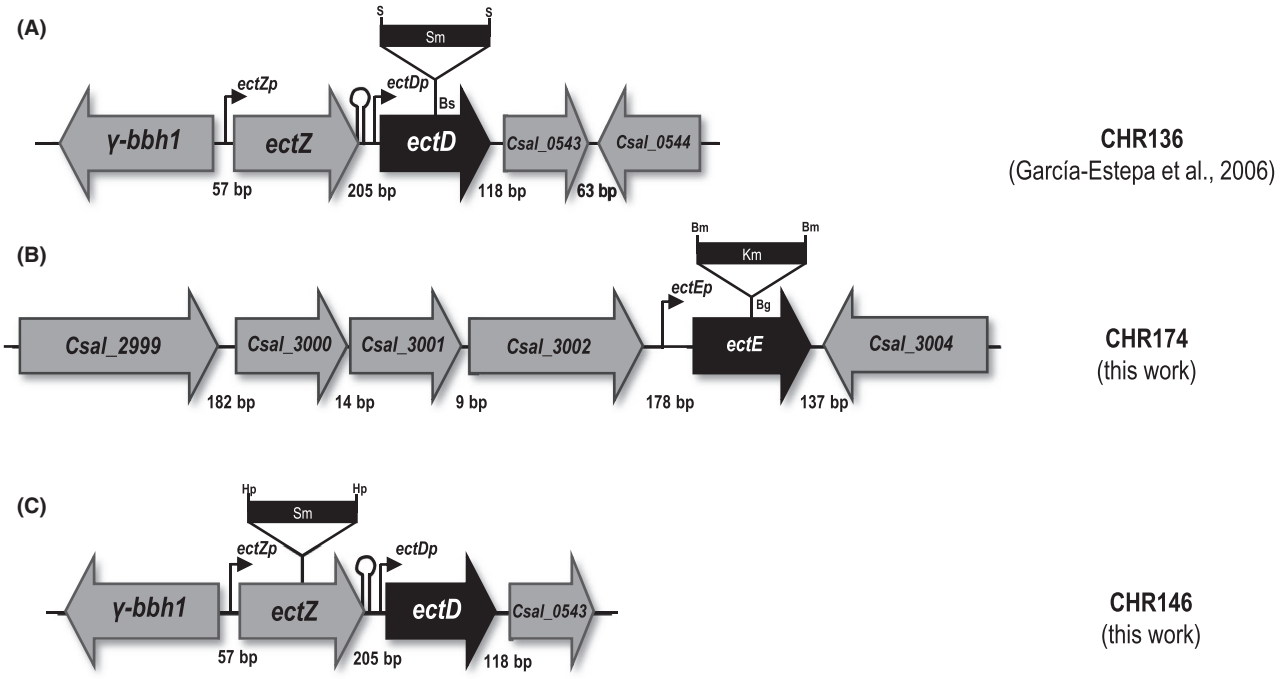


**Fig. 1.** Genetic organization of the *C. salexigens* hydroxyectoine synthesis genes and strategies of gene disruption.
A. Genetic context of the *ectZ* and *ectD* genes and insertional inactivation of *ectD* in strain CHR136.
B. Genetic context of the *ectE* gene and insertional inactivation of *ectE* in strain CHR174. The double mutant *s*train CHR175 (*ectDectE*) resulted from transferring the *ectE* mutation to the *ectD*-deficient strain CHR136.
C. Genetic context of the *ectZ* and *ectD* genes and insertional inactivation of *ectZ* in strain CHR146. Predicted promoters are indicated by angled arrows. The Rho-independent transcriptional terminator downstream of *ectZ* is indicated by a loop. Restriction enzymes: Bm (BamHI); Bg (BglII); Bs (Bsp119I); Hp (HapI); S (SmaI). The antibiotic resistance cassettes used to generate insertional mutants are indicated by rectangles. The *ectE* gene was inactivated by the insertion of an *aac4* cassette, which carries a resistance gene for kanamycin into a unique BglII site. Km: Kanamycin; Sm: Streptomycin. Ectoine hydroxylases codifying-genes are coloured in black.

($\sigma^{70}$) and RpoH ($\sigma^{32}$) promoters were predicted in the relatively large intergenic region (178 bp) between *ectE* and the preceding gene upstream of *ectE,* suggesting that *ectE* might constitute and independent transcriptional unit (Fig. 1B; Fig. S1).

EctE only showed a 51.9% identity to EctD at the amino acid level, and the predicted pI value for EctE (4.85) differed from that of EctD (5.93). Additionally, the predicted Tm index, a parameter that indicates the thermostability of proteins, was lower from EctE (0.566) than for EctD (0.932). Despite these differences, an alignment of EctE with EctD and other ectoine hydroxylases already characterized revealed that EctE presented the conserved residues involved in the binding of the iron catalyst and 2-oxoglutarate, as well as those interacting with the hydroxyectoine molecule, and the string of 17 amino acids that is considered as the consensus sequence of ectoine hydroxylases (Höppner *et al*., 2014) (Fig. S2). These findings suggested that *ectE* could encode a second *C. salexigens* ectoine hydroxylase.

To check the contribution of the EctE-derived hydroxyectoine to *C. salexigens* total hydroxyectoine pool under osmotic and temperature stress, we inserted a kanamycin resistance cassette within the *ectE* gene to construct single *ectE* (CHR174) and double *ectDectE* (CHR175) mutants (Fig. 1B). The ectoine/hydroxyectoine intracellular accumulation pattern of the *ectE* strains grown at late exponential phase was compared to those of the wild-type and the single *ectD* mutant (CHR136) (Fig. 2A). The ectoine content of single *ectE* or *ectD* mutants was similar to that of the wild type at any condition tested, whereas that of the double *ectDectE* strain was slightly lower at high salinity. At high salinity, the hydroxyectoine level of the *ectD* mutant was reduced to 56.1 $\pm$ 20.11% of the wild-type level, whereas that of the *ectE* strain was not significatively lower. However, the double *ectDectE* mutant did not accumulate hydroxyectoine, suggesting that at high salinity the combined action of the two ectoine hydroxylases is responsible for the total hydroxyectoine pool. On the other hand, at high salinity plus high temperature, the hydroxyectoine content of the *ectE* strain was reduced to 37.6 $\pm$ 0.89% of the wild-type level, showing the involvement of EctE in hydroxyectoine synthesis under these conditions. Growth of the *ectD* and *ectDectE* strains was severely impaired at high temperature, and cultures did not reach enough biomass as to measure their intracellular solute pool (see below).

To investigate the contribution of the EctE-derived hydroxyectoine to osmo- and/or thermoprotection of *C. salexigens,* the growth of the *ectE*-deficient strains was compared to that of the wild-type and the single *ectD* mutant under osmotic and heat stress. At high salinity, all mutant strains followed a growth pattern similar to that of the wild type (Fig. 2B). As previously reported (Garcia-Estepa *et al*., 2006), growth of the single *ectD* mutant was severely impaired at high salinity plus high temperature. When compared to the wild-type strain, the single *ectE* mutant also showed a thermosensitive phenotype, but its growth was not as affected as that of the *ectD*-deficient strain (Fig. 2C). When compared to the single *ectD* strain, the growth of the double *ectDectE* mutant was even more impaired.

All together, these findings indicated that EctE is a functional ectoine hydroxylase in *C. salexigens*. The differences observed in growth at high temperature also suggest that the *ectE* gene product contributes to *C. salexigens* thermoprotection, through to a lower extend than EctD.

### *Phylogenetic analysis of EctD-like and EctE-like ectoine hydroxylases*

First, the distribution of EctE-like or EctD-like proteins within proteobacteria was investigated. For this purpose, two independent BLAST searches were carried out using *C. salexigens* EctD or EctE protein sequences as queries. Hits ranging between 99 and 47% identity (around 2000 sequences from both analysis) were further analysed and those from unknown species were discarded. This analysis identified more that 500 species harbouring at least one ectoine hydroxylase (EctD-like or EctE-like) in *C. salexigens* genome. Approximately 5% of the Gammaproteobacteria, most of them belonging to the *Halomonas* genus, harboured more than one ectoine hydroxylase-codifying gene. Second, due to the low similarity between *C. salexigens* EctD and EctE protein sequences, we investigated their phylogenetic relationships and evolutionary origin, especially within gammaproteobacteria, focussing on those that harbour more than one enzyme. To this end, a phylogenetic analysis was conducted with 152 proteins from the previous analysis, including *C. salexigens* EctD and EctE, the majority of the gammaproteobacteria species harbouring more than one ortholog to *C. salexigens* EctD or EctE, and most of the sequences of ectoine hydroxylases previously characterized (bona fide ectoine hydroxylases).

As shown in Fig. 3, the resulting phylogenetic tree showed five main branches. The *C. salexigens* EctD and EctE proteins were distributed in two separate branches including ectoine hydroxylases from Gammaproteobacteria belonging to the halophilic and halotolerant representatives of the Oceanospirillales (Halomonadaceae and Alcanivoracaceae families; genera *Chromohalobacter*, *Halomonas*, *Cobetia, Salinicola* and *Alcalinivorax*) and the Alteromonadales (Alteromonadaceae family; genera *Marinobacter). C. salexigens* EctD was tightly

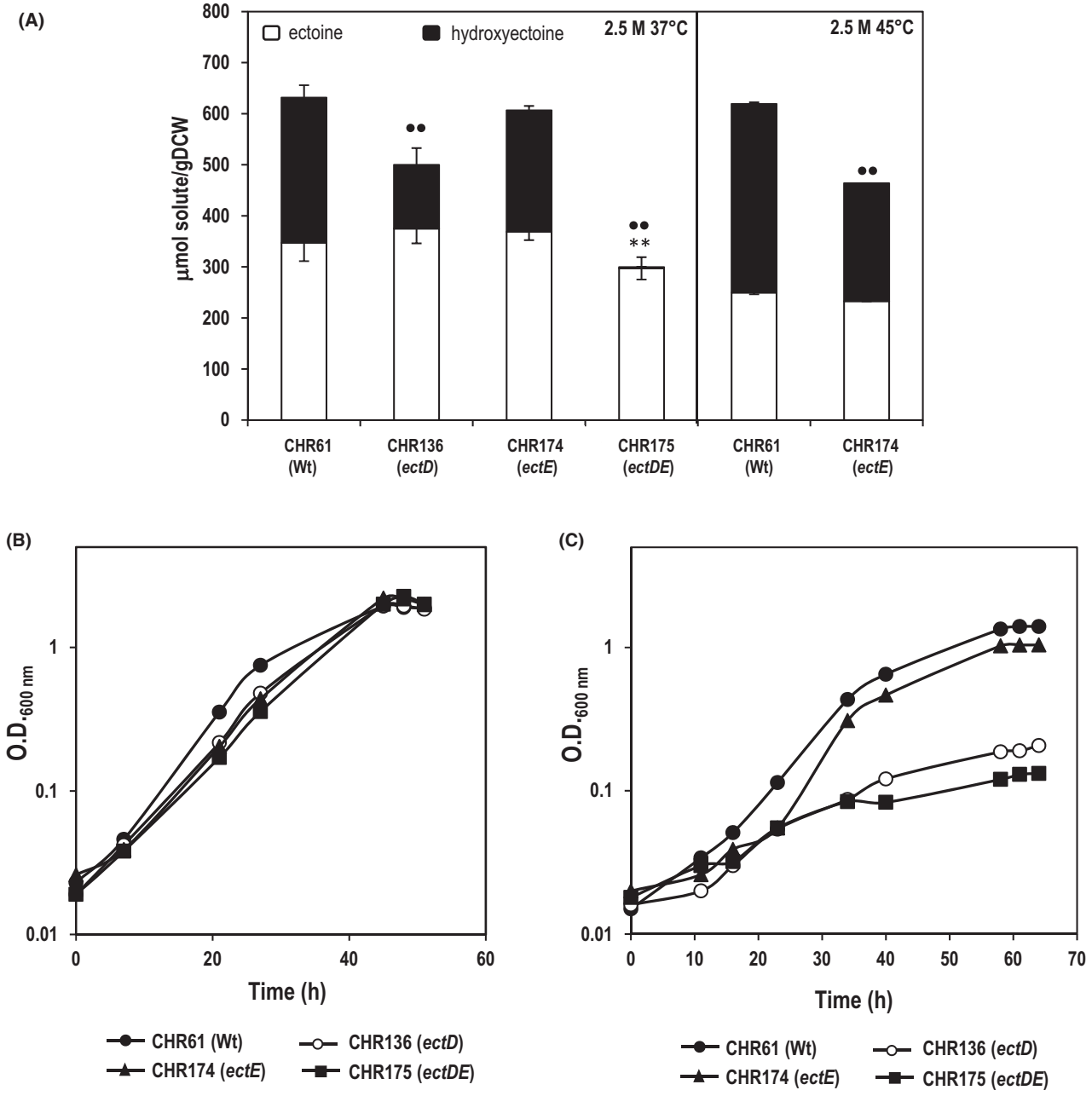


**Fig. 2.** EctE is a secondary ectoine hydroxylase and contributes to *C. salexigens* thermoprotection.
A. Cytoplasmic content of ectoines of *C. salexigens* wild-type and mutants affected in the ectoine hydroxylases EcD and EctE under osmotic and heat stress. Ectoines were quantified by LC-MS at late exponential phase. Values are the averages and standard deviations of three replicates for each condition in two independent experiments. Significant differences according to Student's *t*-test, (*P*-value ≤0.05) and% solute content variation (≥ ±15%) between the wild-type and mutant strains are shown by asterisks (ectoine) or spots (hydroxyectoine).
B. and (C) growth of *C. salexigens* wild-type and *ectD, ectE* and *ectDE* mutants under osmotic and heat stress. Growth was monitored in M63 minimal medium at high salinity (2.5 M NaCl at 37°C) (B) or at high salinity plus temperature (2.5 M at 45°C) (C).

associated with its orthologs in *C. israelensis* and *Salinicola socius* and closely clustered with the characterized *H. elongata* EctD protein, as well as putative ectoine hydroxylases from other species of *Halomonas*, *Cobetia* and *Alcalinivorax*. In turn, *C. salexigens* EctE clustered with EctE-like enzymes from *C. israelensis* and *Halomonas*, as well as ectoine hydroxylases from *Marinobacter* (some of them with 2 or more copies present in this second branch), and other related genera. Interestingly, *Marinobacter* only contained EctE-like, but not EctD-like, proteins. Remarkably, among those Gammaproteobacteria harbouring two or more ectoine


**Fig. 3.** Phylogenetic analysis of EctD-like and EctE-like ectoine hydroxylases. The evolutionary history was inferred using the Neighbour-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 152 amino acid sequences. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA6. Characterized ectoine hydroxylases are indicated by a star. EctE and EctD from *C. salexigens* are indicated by a red circle and red letters. Gammaproteobacteria that harbour one copy of EctE-like and one copy of EctD-like ectoine hydrolyases are indicated by a dark green and orange circle respectively; those that harbour two copies of EctE-like protein are indicated by light green circle; those that harbour three copies of EctD-like and/or EctE-like ectoine hydroxylases are indicated by a light blue circle. The rest of bacteria that do not belong to Gammaproteobacteria phylum and harbour two or more copies of ectoine hydroxylases are indicated by black circles.

hydroxylases (mostly from the genus *Halomonas*), most of them possesses at least one EctD-like enzyme and one EctE-like enzyme, as each protein was clearly grouped into separate branches (Fig. 3).

The third phylogenetic branch grouped ectoine hydroxylases from Alpha-, Beta- and Gammaproteobacteria, including the characterized proteins from *P. stutzeri and S. alaskensis,* whereas the fourth and fifth branches

clustered ectoine hydroxylases from Actinobacteria and Firmicutes (i.e. characterized proteins from *Virgibacillus salexigens*, *Paenibacillus lautus*). Within the same cluster, some species contained additional copy(es) of an ectoine hydroxylase, a situation compatible with more recent gene duplication events that occurred during the speciation process (Fig. 3).

### *Influence of salinity and temperature on the expression of the* C. salexigens *ectoine hydroxylase genes* ectD *and* ectE *at different growth phases*

In *C. salexigens*, accumulation of hydroxyectoine is maximal during stationary phase and triggered by osmotic and temperature stress (Garcia-Estepa *et al*., 2006). To determine whether this accumulation pattern is controlled (at least in part) at the transcriptional level, we used real-time PCR to measure the relative expression levels of the *ectD* and *ectE* genes in the wild type in response to osmotic and/or temperature stress, as a function of the growth phase (Fig. 4).

The relative expression of the *ectD* gene was higher than that of *ectE* at most of the studied conditions. This finding agrees with the role of EctD as the main ectoine hydroxylase in *C. salexigens*. At the exponential phase of growth, and compared to cells grown at low salinity, the expression of *ectE* and *ectD* was induced by 2.3-fold and 27.9-fold, respectively, by osmotic stress. In contrast, the expression levels of *ectD* and *ectE* at high salinity were not affected by temperature (Fig. 4A). The pattern of *ectD* expression in response to salinity and temperature in exponentially grown cells agrees with our previous transcriptomic studies on *C. salexigens* adaptative mechanisms (Salvador *et al*., 2018). At the stationary phase, expression of *ectD* and *ectE* was not induced by salinity. In contrast, expression of *ectD,* but not of *ectE*, was strongly induced (30.6-fold) by temperature at high salinity (Fig. 4B).

The above results suggest that the transcriptional response of hydroxyectoine synthesis to osmotic and temperature stress is different depending on growth phase. Thus, at the exponential phase, both *ectD* and *ectE* genes were osmoregulated, but not thermoregulated. In contrast, at the stationary phase, none of the genes appeared to be osmoregulated, whereas *ectD* (but not *ectE*) was strongly thermoregulated at high salinity.

### *The general stress response regulator RpoS contributes to the growth-phase-dependent expression of* ectD *and* ectE *under osmotic and heat stress*

In Gammaproteobacteria, the sigma factor RpoS ($\sigma^S$) regulates the general stress response, coordinating an immediate response to stress as well as long-term adaptation to many different stresses, such as starvation,

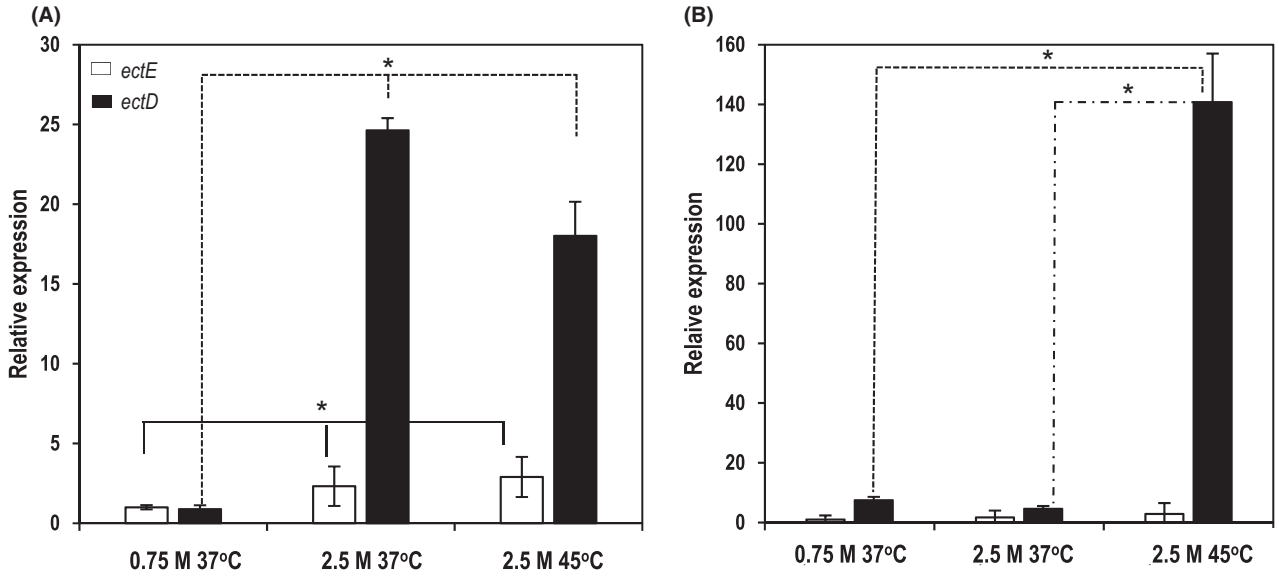


**Fig. 4.** Influence of salinity and temperature on the expression of the *C. salexigens* ectoine hydroxylase genes *ectD* and *ectE* at different growth phases. Relative expression of *ectE* and *ectD* was determined in the wild-type strain grown at low salinity (0.75 M NaCl, 37°C), high salinity (2.5 M NaCl, 37°C) and high salinity and temperature (2.5 M NaCl, 45°C) at exponential (A) and stationary phase (B). Data are expressed in relative units and were estimated by the $2^{-\Delta\Delta CT}$ method using the 16S rRNA gene as an internal control to normalize expression in each sample. Values are the averages and standard deviations of three replicates for each condition in two independent experiments According to Student's *t*-test and relative expression, significant differences (*P*-value $\leq$0.05 and $\geq \pm$ twofold) in each specific condition were shown by an asterisk.

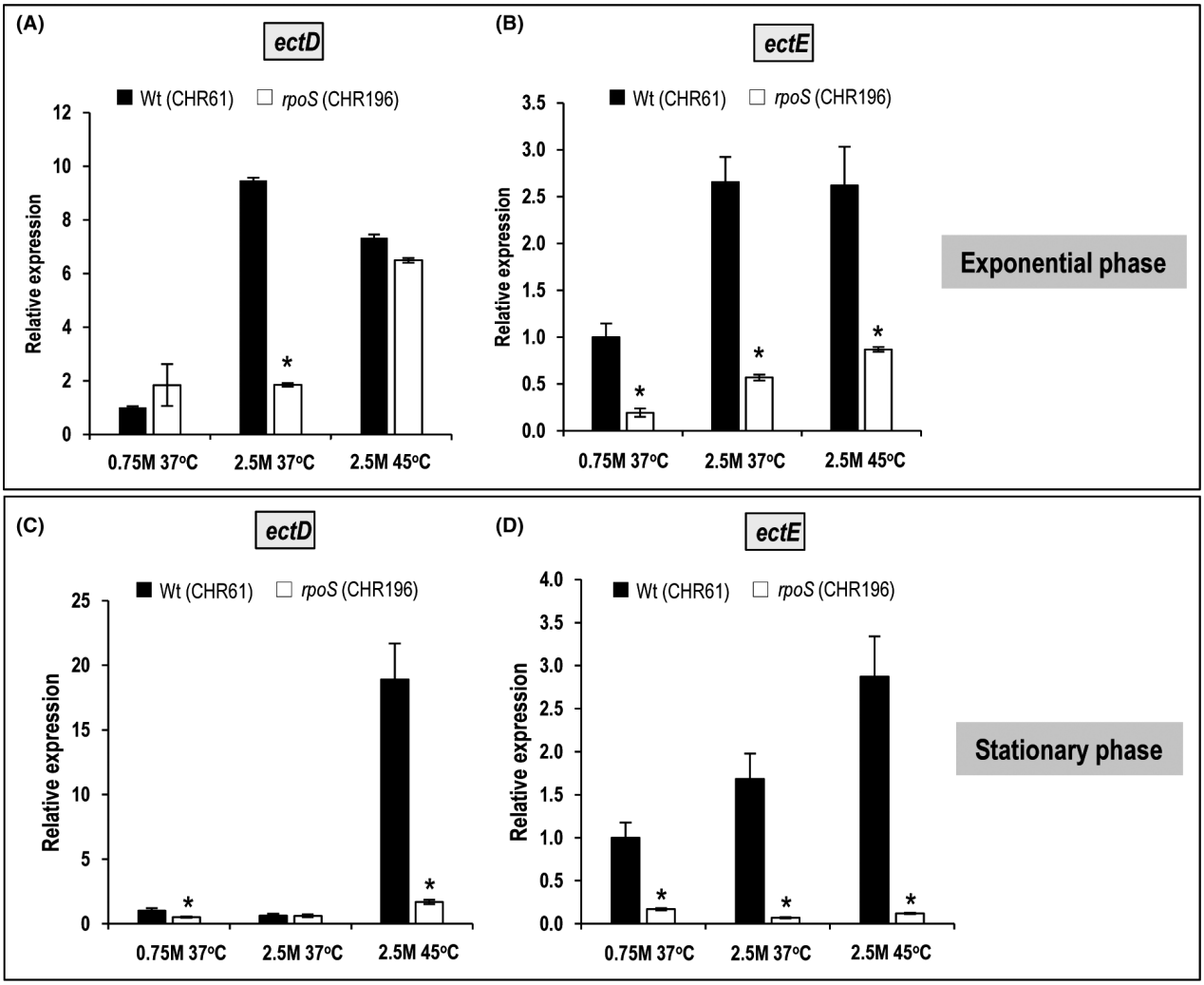


**Fig. 5.** Role of the general stress regulator RpoS in the expression of the *C. salexigens* ectoine hydroxylase genes *ectD* and *ectE*. Relative transcriptional levels of *ectD* and *ectE* were determined by qPCR in *C. salexigens* wild-type and *rpoS* strains at low salinity (0.75 M NaCl, 37°C), high salinity (2.5 M NaCl, 37°C) and high salinity plus high temperature (2.5 M NaCl, 45°C) at exponential (A, B) and stationary (C, D) phase. Data are expressed as relative units and were calculated by the $2^{-\Delta\Delta Ct}$ method considering the 16S rRNA gene as an internal control to normalize expression in each sample. Levels of expression were normalized to the wild-type ectoine hydroxylase gene (*ectD* or *ectE*) mRNA levels at 0.75 M 37°C, as it was the lowest expression value in the wild type. Values are the averages and standard deviations of three replicates for each condition in two independent experiments. According to Student's t-test and relative expression, significant differences (*P*-value ≤0.001 and ≥ ± twofold) compared with the wild type in each condition were shown by an asterisk.

osmotic stress, high or low temperature, acidic pH or oxidative agents, among others (Battesti *et al.*, 2011). In a previous work, we suggested that during exponential growth, RpoS does not trigger the early transcriptional response of *ectD* to an osmotic or heat upshock. However, it contributed to *ectD* expression during the responses to these upshocks (Salvador *et al.*, 2015).

To determine whether RpoS is involved in the expression of the ectoine hydroxylase genes *ectD* and *ectE* during *C. salexigens* long-term osmo- and thermoadaptation, we measured the relative expression of *ectD* and *ectE* in the wild-type and the *rpoS* mutant strain CHR196 previously characterized in our laboratory (Salvador *et al.*, 2015). As induction of the *ectD* and *ectE* gene expression by salinity and temperature is different depending on growth phase (see Fig. 4), samples were taken in the mid-late exponential and late stationary phases of growth (Fig. 5).

At the exponential phase, the expression of the *ectD* gene in the *rpoS* background decreased 5.1-fold at high salinity, compared to the wild type (Fig. 5A), whereas no differences were found in the rest of the conditions tested. At stationary phase, *ectD* expression in the *rpoS* background was twofold and 11.25-fold lower than that of the wild type at low salinity and at high temperature, respectively, whereas no differences were found at high

salinity with 37°C (Fig. 5C). Interestingly, in both exponential and stationary phases, the *ectE* gene expression was remarkably lower in the *rpoS* mutant, compared to the wild type, at any condition tested (at exponential phase: 5.17-fold, 4.67-fold and threefold, at low salinity, high salinity and high salinity plus high temperature respectively; at the stationary phase: 5.95-fold, 24-fold and 24.1-fold at low salinity, high salinity and high salinity plus high temperature respectively) (Fig. 5B and D).

All together, the above findings suggest that the osmoregulated expression of *ectD* at exponential phase, as well as the thermoregulated expression of *ectD* at the stationary phase, seemed to be RpoS-dependent. In contrast, expression of *ectE* was RpoS-dependent regardless of the growth phase and osmotic or heat stress conditions tested.

We also investigated the differences in the ectoine and hydroxyectoine accumulation pattern of wild-type and the *rpoS* mutant in response to osmotic and heat stress, in the exponential and stationary phases of growth. At the exponential phase, the only significative difference was observed at low salinity, where the lack of *rpoS* gave rise to an increment of ectoine accumulation of 15.9% ± 6 (Fig. 6A). At the stationary phase, a 21.73% ± 12.32 increment of hydroxyectoine was observed in the *rpoS* mutant under osmotic stress, which seemed to occur at the expenses of the ectoine pool. Remarkably, a 54% ± 7.1 increment of ectoine accumulation was observed in the *rpoS* mutant grown under osmotic and heat stress (Fig. 6B).

### *The transcriptional regulator EctZ contributes to the osmoregulated expression of* ectD *at the exponential growth phase*

The gene *Csal_0541,* encoding a putative transcriptional regulator of the AraC-GlxA family, lays upstream of *ectD* within the *C. salexigens* genome (Fig. 1A). To determine whether its encoded protein was involved in the transcriptional regulation of hydroxyectoine synthesis, we constructed a *Csal_541* mutant (CHR146) by insertional mutagenesis (Fig. 1C). The relative expression of *ectD* and *ectE* at the exponential and stationary growth phases was measured in the *Csal_541* mutant and wild-type strains under osmotic and heat stress (Fig. 7).

Compared to the wild type, expression of the *ectD* gene in the *Csal_0541* background at the exponential phase was 7.15-fold lower under osmotic stress (Fig. 7A), whereas at stationary growth phase, *ectD* expression was 2.3-fold lower at low salinity (Fig. 7C). No substantial differences were found in the rest of the conditions tested. Regarding *ectE*, mutation of *Csal_0541* led to increased expression at the exponential growth phase (4.3-fold) in cells grown at low salinity or high salinity plus high temperature (2.2-fold), but *ectE* expression was not affected under osmotic stress (Fig. 7B). No significative differences were observed in the *ectE* transcriptional expression pattern of wild-type and *Csal_0541* strains at the stationary growth phase (Fig. 7D).

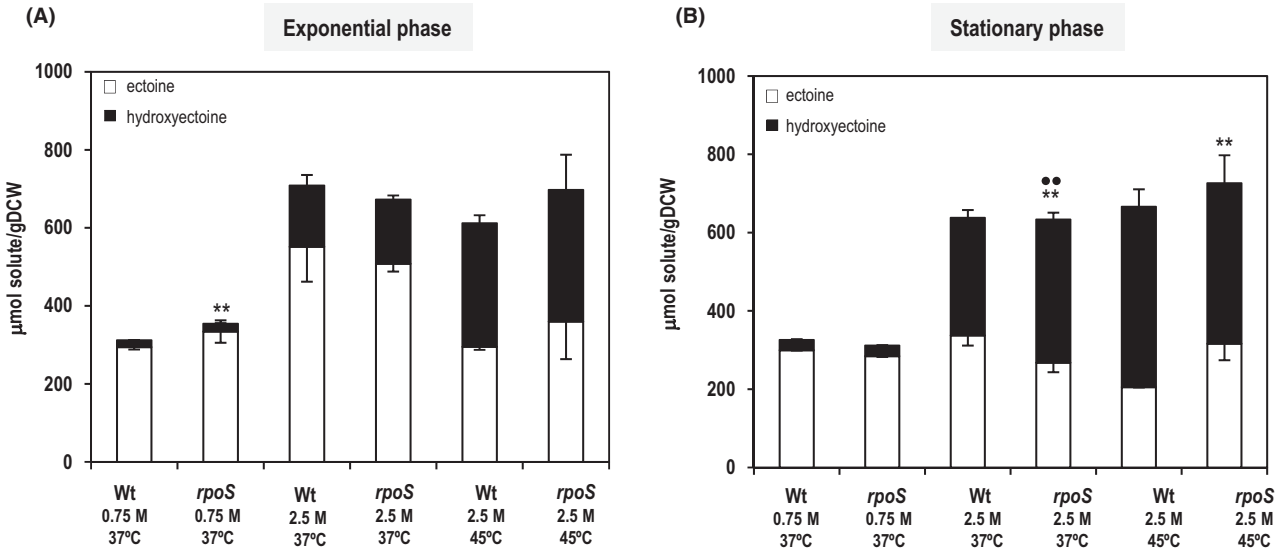


**Fig. 6.** Ectoine(s) content of *C. salexigens* Wt and *rpoS* mutant strains in response to osmotic and heat stress at different growth phases. Strains were grown at low salinity (0.75 M NaCl at 37°C), high salinity (2.5 M NaCl at 37°C) and high salinity and temperature (2.5 M NaCl at 45°C). Cytoplasmic accumulation of ectoine and hydroxyectoine was determined by LC-MS at exponential (A) and stationary phase (B). The values are the averages and standard deviations of three replicates for each condition in two independent experiments. According to Student's *t*-test and % solute content variation, significant differences (*P*-value ≤0.05 and ≥ ± 15%) compared with the wild type were shown by two asterisks (ectoine) or spots (hydroxyectoine).

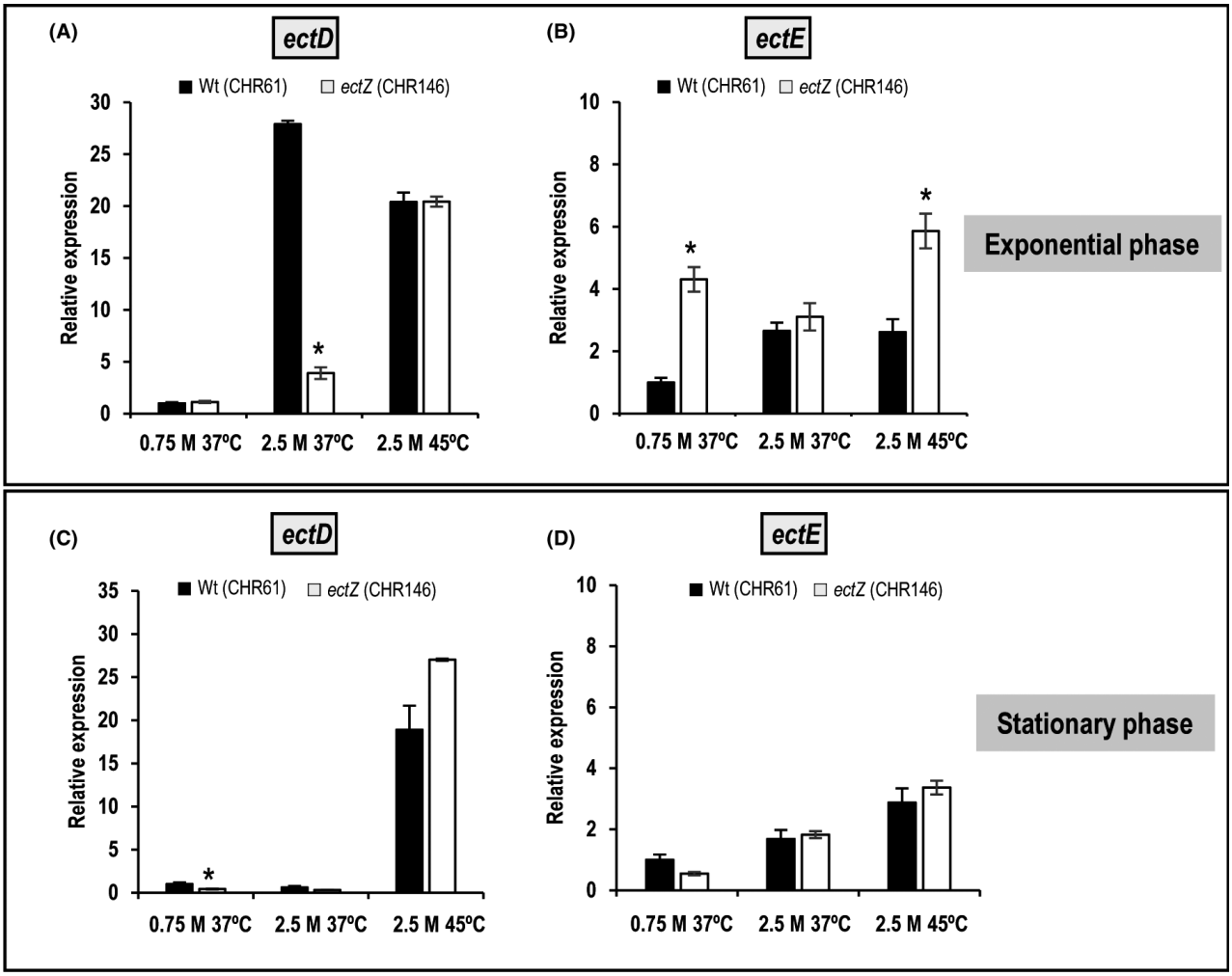


**Fig. 7.** Role of the regulator EctZ in the expression of the *C. salexigens* ectoine hydroxylase genes *ectD* and *ectE*. Relative transcriptional levels of *ectD* and *ectE* were determined by qPCR in *C. salexigens* wild-type and *ectZ* strains at low salinity (0.75 M NaCl, 37°C), high salinity (2.5 M NaCl, 37°C) and high salinity plus high temperature (2.5 M NaCl, 45°C) at exponential (A, B) and stationary (C, D) phase. Data are expressed as relative units and were calculated by the $2^{-\Delta\Delta Ct}$ method considering the 16S rRNA gene as an internal control to normalize expression in each sample. Levels of expression were normalized to the wild-type ectoine hydroxylase gene (*ectD* or *ectE*) mRNA levels at 0.75 M 37°C, as it was the lowest expression value in the wild type. Values are the averages and standard deviations of three replicates for each condition in two independent experiments. According to Student's *t*-test and relative expression, significant differences (*P*-value ≤0.001 and ± ≥twofold), compared with the wild type in each condition, were shown by an asterisk.

The above findings suggest that, at the exponential growth phase, the product of *Csal_0541* gene (named hereafter as EctZ) is involved in the osmoregulated expression of *ectD*. Additionally, it might function as a repressor of *ectE*, mainly at low salinity and high temperature. They also suggest that during the stationary phase of growth, EctZ is not involved in the expression of either *ectD* or *EctE*.

### *Inactivation of EctZ leads to a higher ectoines accumulation at the expenses of the ectoine pool*

Next, we investigated the effect of the *ectZ* inactivation on *C. salexigens* growth in response to osmotic and heat stress, as well as on the accumulation pattern of ectoine and hydroxyectoine. As shown in Fig. 8A, no differences in growth of the wild-type and *ectZ* strains were observed at any of the studied conditions.

In general, the amount of hydroxyectoine accumulated by the *ectZ* mutant did not change at any condition tested, with respect to the wild type (Fig. 8B and C). Remarkably, inactivation of *ectZ* led to a higher accumulation of ectoine, the precursor of hydroxyectoine, in the mutant (from 18 to 57.6%), with respect to the wild type, at most of the conditions tested (Fig. 8B and C). The highest differences were observed under heat stress, in both exponential (57.6 ± 10.8%) and stationary (52 ± 8.3%) phases.

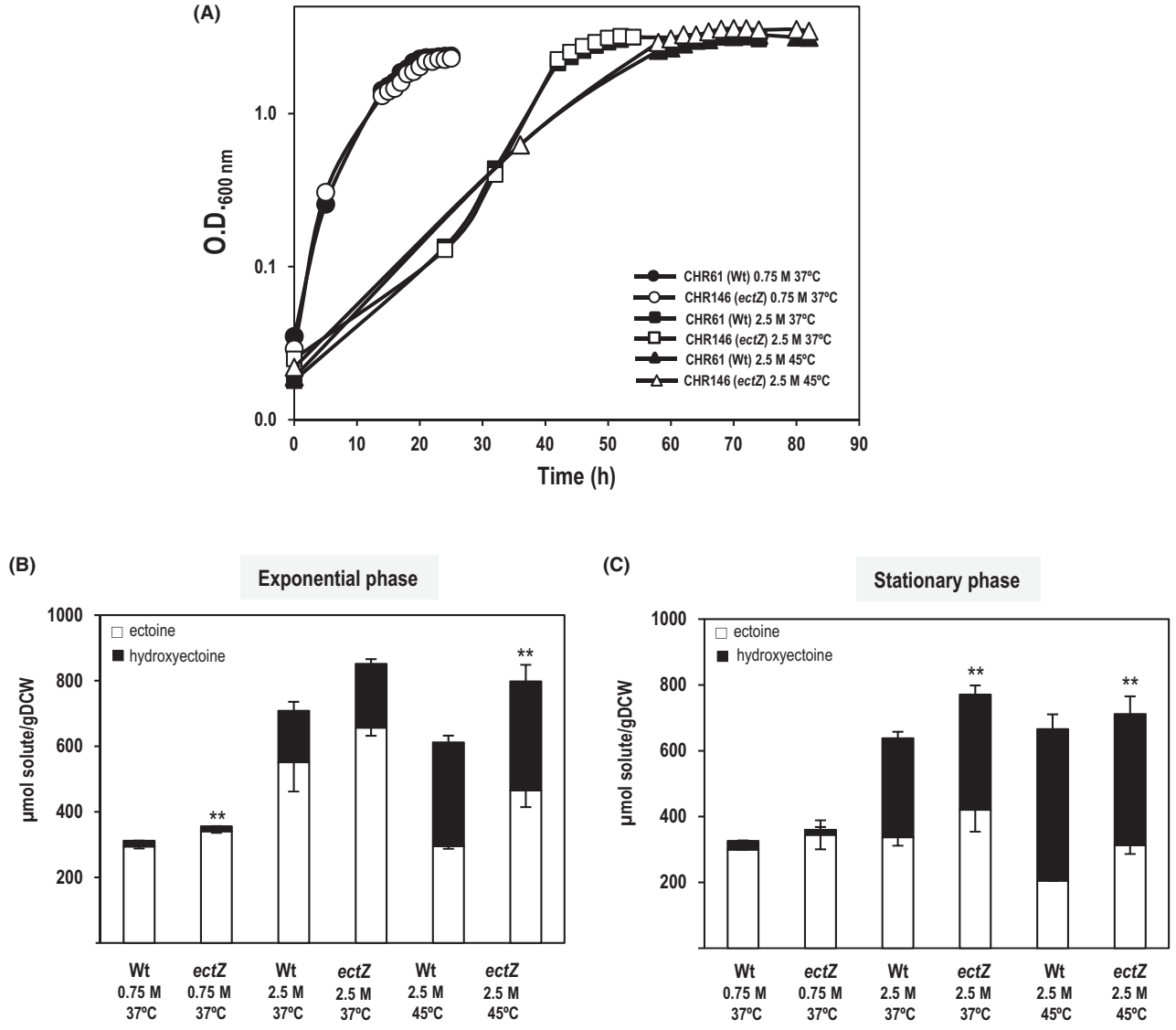


**Fig. 8.** Growth and ectoine(s) content at different growth phases of *C. salexigens* Wt and *ectZ* mutant strains in response to osmotic and heat stress.
A. Strains were grown at low salinity (0.75 M NaCl at 37°C), high salinity (2.5 M NaCl at 37°C) and high salinity plus temperature (2.5 M NaCl at 45°C). Cytoplasmic accumulation of ectoine and hydroxyectoine was determined by LC-MS at exponential (B) and stationary phase (C) at 0.75 M NaCl at 37°C, 2.5 M NaCl at 37°C and 2.5 M NaCl at 45°C. The values are the averages and standard deviations of three replicates for each condition in two independent experiments. According to Student's *t*-test and % solute content variation, significant differences (*P*-value $\leq 0.05$ and $\geq \pm$ 15%) compared with the wild type were shown by two asterisks (ectoine) or spots (hydroxyectoine).

In summary, inactivation of *ectZ* mainly resulted in a higher amount of the total ectoines pool at the expenses of a higher accumulation of ectoine, with maintenance of the hydroxyectoine levels.

### *EctZ, a transcriptional regulator of the AraC-GlxA-family specific of moderately halophilic bacteria*

Blast searches showed that the EctZ protein was present in a limited number of bacteria, with an identity ranging from 55.4 to 99.4% to the *C. salexigens* EctZ protein. Interestingly, all of them were moderately halophilic Gammaproteobacteria and most species (29) belonged to *Halomonas* (70.0%) (Fig. 9A). The rest of species corresponded to other genera of the *Halomonadaceae* family such as *Cobetia* (2), *Chromohalobacter* (3), *Kushneria* (1), *Pistricoccus* (1) and *Salinicola* (4) and of the *Saccharospirillaceae* family such as *Saccharospirillum* (1). The only exception was the EctZ-like protein from the Firmicute *Virgibacillus halodenitrificans*, which showed 90.80% of identity to the *C. salexigens* EctZ. Inspection of the *ectZ* genomic context in some of these

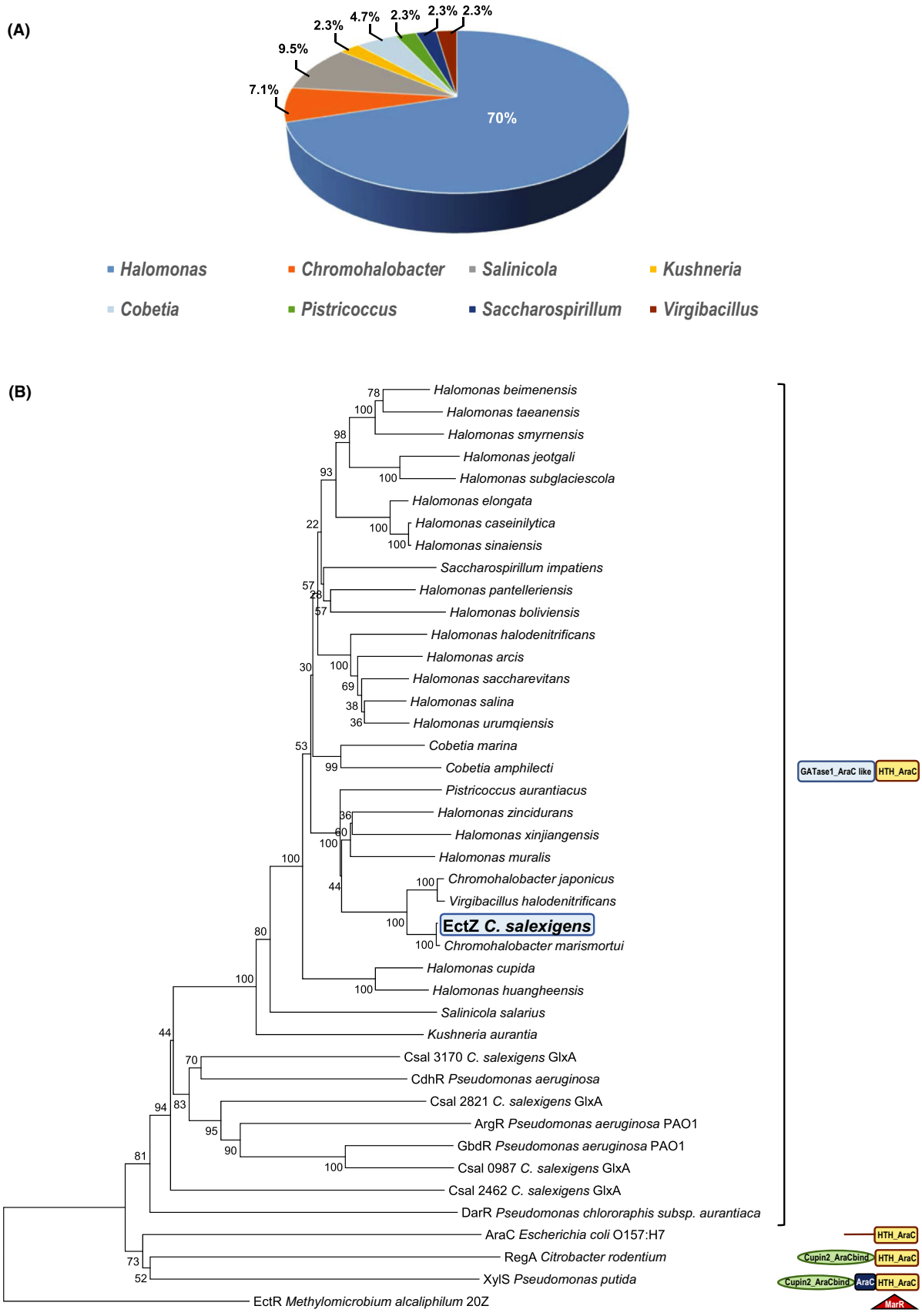

**Fig. 9.** *In silico* analysis of EctZ protein.
A. Distribution of EctZ protein in bacteria. Blast searches were performed and showed that EctZ protein is present in a limited number of bacteria, only in 42 species. Percentages of distribution among the genera where EctZ is present are shown.
B. Phylogenetic analysis of EctZ protein. Neighbour-Joining tree based on proteins with a common AraC-like domain is showed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 42 amino acid sequences. All ambiguous positions were removed for each sequence pair. Bootstrap probabilities (as percentage) were determined from 1000 resamplings and are shown next to branches. Domain architecture of each group is represented at the side of the tree and is based on the graphical output of the NCBI's conserved domain database (CDD web interface) with modifications.

species revealed that the organization of the upstream region of *ectZ* was highly conserved among all of them, with the exception of *Cobetia* (Fig. S3). However, the genetic organization of the region downstream of *ectZ* was not conserved. As a matter of fact, *C. salexigens* genome was the unique, among all the analysed genomes, where *ectD* gene was located downstream of *ectZ*.

Analysis of conserved domains revealed that the *C. salexigens* EctZ protein is an AraC-type transcriptional regulator presenting a GlxA domain, which consists of an N-terminal Type 1 glutamine amidotransferase (GATase1)-like motif and the AraC-type DNA-binding HTH motif. This domain is conserved in a subgroup of AraC-family proteins including, for instance, the *Pseudomonas aeruginosa* ArgR regulator, which controls the expression of certain genes involved in arginine biosynthesis and catabolism (Chou *et al*., 2010).

To check the evolutionary relationship between EctZ-like proteins and other AraC-like proteins, a phylogenetic tree was constructed including all EctZ-like proteins found so far, other GlxA-like proteins present in the *C. salexigens* genome, and other GlxA or AraC-type characterized proteins such as GbdR, CdhR and ArgR from *P. aeruginosa* (Wargo *et al*., 2008; Chou *et al*., 2010; Meadows and Wargo, 2018), XylS from *P. putida* (Gallegos *et al*., 1997b,1997a), DarR from *P. chlororaphis* (Calderón *et al*., 2014), RegA from *Citrobacter rodentius* (Hart *et al*., 2008) or AraC from *E. coli* (Sheppard and Englesberg, 1967). The MarR-type regulator EctR from the halotolerant obligate methanotroph *Methylomicrobium alcaliphilum*, which negatively regulates the transcription of ectoine synthesis genes (Mustakhimov *et al*., 2010), was also included for comparative purposes.

As shown in Fig. 9B, two clearly separated branches, including all orthologs to *C. salexigens* EctZ and the rest of GlxA-like proteins, respectively, were observed. They were evolutionarily distant from other AraC regulators not containing the amidase motif, and the MarR repressor EctR. In the EctZ-like branch, *C. salexignes* EctZ clustered with ortholog proteins from *C. marismortui*, *C. japonicus, V. halodenitrificans* and *Halomonas*. This arrangement suggested that the protein from *V. halodenitrificans* might have been acquired by a horizontal transfer genetic (HTG) event. The rest of the GlxA-type proteins from *C. salexigens* were included in the second branch. Csal_3170, Csal_0987 and Csal_2821 were close to the characterized CdhR, GbdR and ArgR proteins from *P. aeruginosa*, respectively, whereas Csal_2462 was evolutionary distant from the former ones. However, they did not show a clear phylogenetic relationship that indicate that they were orthologous proteins (Fig. 9B). The above findings suggest that the EctZ-type proteins, although phylogenetically related to GlxA-type proteins, might constitute a different subtype of AraC-like regulators, probably with a specific function related to osmoadaptation.

## Discussion

The genome of the halophilic Gammaproteobacterium *C. salexigens* carries two ectoine hydroxylase genes, namely *ectD* and *ectE*, whose encoded proteins share the characteristic consensus motif of ectoine hydroxylases. Although the *ectD*-encoded ectoine hydroxylase is the main enzyme responsible for hydroxyectoine synthesis in *C. salexigens*, an *ectD* mutation did not totally suppress hydroxyectoine accumulation in this microorganism (Garcia-Estepa *et al*., 2006), suggesting that (at least in part) the *ectD* homolog could contribute to hydroxyectoine synthesis. In this work, we have shown that *ectE* encodes a secondary functional ectoine hydroxylase and that the hydroxyectoine synthesis mediated by this enzyme also contributes to thermoprotection in *C. salexigens*.

Interestingly, the two *C. salexigens* ectoine hydroxylases, and their corresponding orthologs in *Halomonas* and other *Chromohalobacter* species, among other Gammaproteobacteria, were phylogenetically distant, separated in two clusters of related representatives. This evolutionary pattern suggests that they may have arisen from duplication of an ancestral gene preceding the directional divergence that gave origin to the orders Oceanospirillales and Alteromonadales. In this sense, *ectD* and *ectE* may be considered as 'out-paralogs', according to the definition by Koonin (2005). Our phylogenetic analysis also suggests that the *ectD* or *ectE* copies were lost in some of the lineages and that additional duplication events occurred during the speciation process. Some interesting examples are the type strain

of *H. elongata*, which only carries the EctD-like protein, or some *Marinobacter* species carrying two or three copies of EctE-like.

Differences in their pI values suggest that *C. salexigens* EctD and EctE could differ in their ability to function at different ionic strengths, an interpretation that needs experimental support. In addition, predictions based on their Tm index suggest that EctD might be more thermoresistant than EctE, a hypothesis compatible with the predominant role of EctD in *C. salexigens* thermoprotection, and the strong thermoregulation of *ectD* found at stationary phase of growth. In addition, the different regulation patterns in response to growth phase, salinity and temperature, as well as the differences found in their transcriptional control by general (RpoS) and specific (EctZ) regulators, suggest that *C. salexigens ectD* and *ectE* might be also considered as 'ecoparalogs'. This was an evolutionary term proposed by Sánchez-Perez *et al*. (2008) for genes that perform the same cellular function under different ecological conditions. This genetic redundancy of ectoine hydroxylases could confer a certain degree of robustness to *C. salexigens* and other related microorganisms that live in fluctuating temperature and salinity environments, to better adapt to these environmental changes.

In *C. salexigens*, accumulation of hydroxyectoine is maximal during stationary growth phase and upregulated by osmotic and temperature stress, regardless of the growth phase (Garcia-Estepa *et al*., 2006; this work). The balance between ectoine and hydroxyectoine shifts towards the latter in the stationary phase (Fallet *et al*., 2010). This could be explained for the better protective properties of hydroxyectoine to cope with stationary-phase-related stresses such alterations of pH or oxidative damage (Andersson *et al*., 2000; Moritz *et al*., 2015). Stationary-phase-dependent hydroxyectoine production was also reported in other microorganisms such as *Salibacillus salexigens* (Bursy *et al*., 2007), *Virgibacillus halodenitrificans* (Tao *et al*., 2016) and *Streptomyces coelicolor* (Bursy *et al*., 2008).

Accumulation of hydroxyectoine in response to temperature in exponentially grown *C. salexigens* wild-type cells seemed to be primarily regulated at the post-transcriptional level, as osmoregulated, but not thermoregulated, expression of *C. salexigens ectD* and *ectE* was found at exponential growth phase (Salvador *et al*., 2018; this work). Post-transcriptional control at high salinity plus temperature in exponential growth phase has also been suggested for the synthesis of trehalose, a secondary solute involved in thermoprotection of *C. salexigens* (Reina-Bueno *et al*., 2012). Interestingly, at stationary growth phase, the transcriptional vs post-transcriptional control of hydroxyectoine synthesis pattern in response to osmotic and heat stress was the opposite to that of the exponential phase. In these conditions, *C. salexigens ectD* and *ectE* were not osmoregulated, suggesting that accumulation of hydroxyectoine in response to salinity in the stationary phase is primarily regulated at the post-transcriptional level. On the other hand, the strong thermoregulation of *ectD* may account (at least in part) for the accumulation of hydroxyectoine in response to heat stress found in this phase of growth. Despite the EctE-derived hydroxyectoine contributed to *C. salexigens* thermoprotection at stationary growth phase, *ectE* expression was not thermoregulated.

Even though there is a considerable number of studies related to structural and biochemical aspects of the bacterial ectoine hydroxylases (Reuter *et al*., 2010; Widderich *et al*., 2014; Czech *et al*., 2018a), little is known about the transcriptional control of hydroxyectoine synthesis. In fact, studies on transcriptional regulation of ectoines synthesis have been primarily focussed on the *ectABC* gene cluster encoding the synthesis of ectoine.

Regarding the RNA polymerase sigma factor subunit required for transcription initiation, it was proposed that the osmotic induction of ectoine synthesis is dependent of *ectABC* expression mediated by the general stress factors RpoS ($\sigma^S$) or SigB. This induction could be in addition, or alternatively, dependent on the housekeeping sigma factors RpoD ($\sigma^{70}$)/SigA and the sigma factor RpoN ($\sigma^{54}$) (reviewed in Czech *et al*., 2018a). In *C. salexigens*, we reported that the *ectABC* genes are controlled through a complex osmoregulated promoter region located upstream of *ectA* and that RpoS is involved in the long-term control of *ectABC* transcription (Calderón *et al*., 2004). Subsequent experiments with a *rpoS* mutant showed that RpoS also contributed to the expression of *ectA* and *ectD* after osmotic or thermal upshocks. However, RpoS did not seem to be the main regulator triggering the immediate transcriptional response of ectoines synthesis to osmotic or heat stress (Salvador *et al*., 2015).

Both the *ectD* and *etcE* promoter regions contain consensus sequences that might be recognized by RpoS/RpoD and RpoH. In this work, we found that, during long-term adaptation, the transcriptional activation of the primary ectoine hydroxylase gene *ectD* in response to osmotic and heat stress (observed at exponential and stationary phases of growth respectively) was mainly RpoS-dependent. Furthermore, expression of the secondary ectoine hydroxylase gene *ectE* was always RpoS-dependent, regardless of growth phase or stress conditions. From these results, we conclude that the general stress factor RpoS is the main sigma factor controlling the long-term transcriptional induction of *C. salexigens* ectoine hydroxylase genes *ectD* (in a growth-phase-dependent way) and *ectE,* to osmotic and heat stress. However, as *ectD* and *ectE* expression was not

completely abolished in the *rpoS* background, there might be additional sigma factors contributing to their transcriptional initiation under these conditions. Putative candidates are the vegetative sigma factor RpoD, or the heat stress factor RpoH. Repeated attempts to construct a *C. salexigens rpoH* mutant were unsuccessful, hampering the measurement of *ectD* and *ectE* expression in a *rpoH* background.

Altogether, our findings suggest a growth-phase- and stress-dependent complex level of transcriptional and post-transcriptional control of hydroxyectoine synthesis in *C. salexigens*. This multi-level control should not be seen as unusual, as in its natural habitat *C. salexigens* cells need to rapidly and reversibly react to changing osmotic and temperature conditions.

Concerning other transcription factors involved in the control of ectoines synthesis, none of the so far reported transcriptional regulators present in other microorganisms are found in *C. salexigens* genome. These include GlnR, a major regulator for nitrogen metabolism in many actinomycetes, which functions as a repressor of the *ectABCD* operon in *Streptomyces coelicolor* (Shao *et al*., 2015), and the MarR-type regulator EctR, from halophilic and halotolerant methylotrophic bacteria (Mustakhimov *et al*., 2010; Mustakhimov *et al*., 2012), and CosR from *Vibrionaceae* (Shikuma *et al*., 2013; Gregory *et al*., 2020), which function as negative regulators of the *ectABC-ask* operon. Whereas the above regulators function as repressors, in a previous work we showed that the ferric iron uptake regulator Fur mediated the osmoregulated expression of the *C. salexigens ectABC* genes, linking the salt stress response with iron homeostasis. Fur boxes were found in the *ectABC* promoter, suggesting that Fur directly interacts with DNA in this region (Argandoña *et al*., 2010).

The data presented here suggest that EctZ is involved in the transcriptional control of hydroxyectoine synthesis in *C. salexigens*. EctZ belonged to a subtype of the AraC transcription regulators phylogenetically related to GlxA-type proteins. Most characterized GlxA-type regulators are involved in the control of amino acid catabolic pathways or related quaternary compounds, as arginine, L-lysine, carnitine or glycine-betaine catabolism. However, EctZ-like proteins were phylogenetically distant from other GlxA-type regulators, suggesting that they might constitute a different subtype of AraC-like regulators, with a specific function related to osmo- and thermoadaptation.

Whereas the majority of AraC regulators are transcriptional activators, the AraC subfamily members concerned with stress response, such as MarA, SoxS and Rob, may also act as repressors (Martin and Rosner, 2001). Performing this dual regulatory role seems to be the case for EctZ, which at exponential growth phase functioned both as an activator of the *ectD* expression at high salinity and a repressor of the *ectE* expression at low salinity and high temperature, favouring the expression of the main ectoine hydroxylase under osmotic stress. Many AraC-like activators directly contact the sigma factor at the –35 promoter element (Davis *et al*., 2017). Determining the interaction of EctZ with the sigma factor(s) and/or the *ectD* and *ectE* promoter regions deserves further experimental work. In addition, *ectD* and *ectE* promoter engineering could lead to improved hydroxyectoine production. This strategy has been successfully used to promoter driving the osmotically induced transcription of the *Pseudomonas stutzeri ectABCD-ask*_ect operon in *E. coli*, which resulted in the production and efficient secretion of ectoines into the growth medium (Czech *et al*., 2018b).

Interestingly, lack of RpoS did not influence the *C. salexigens* ectoines content at the exponential growth phase, apart from a higher ectoine content at low salinity conditions. This agrees with that previously observed by Salvador *et al*. (2015). In addition, at the stationary growth phase, the *rpoS* strain accumulated more hydroxyectoine and more ectoine under osmotic- and heat stress, respectively, than the wild-type strain. On the other hand, the loss of the EctZ regulator provoked an increased accumulation of total pool of ectoines, at the expenses of an increment of the ectoine pool, whereas the hydroxyectoine`s one remained unchanged.

In general, the above accumulation pattern did not always correlate with the *ectD* or *ectE* expression patterns in the *rpoS* and *ectZ* strains. There are several (non-exclusive) explanations for this. First, it might be that these regulators are influencing the expression of other genes involved directly or indirectly in the metabolism of ectoines in *C. salexigens* (i.e. catabolism or synthesis of ectoines). Second, low levels of mRNA could be highly translated and consequently high levels of protein could be produced. Third, even if both low levels of mRNA and protein would be produced, a later activation of the enzyme could occur by post-translational modifications or allosteric regulation. In addition to the importance of elucidating the contribution of these transcriptional and post-transcriptional mechanisms in the control of hydroxyectoine synthesis in *C. salexigens*, the *rpoS* and *ectZ* genetic backgrounds offer interesting possibilities to further engineering strains improved in the synthesis of ectoine and hydroxyectoine. For instance, as ectoines synthesis burdens central metabolism pathways (Pastor *et al*., 2013), the high-quality genome-scale metabolic model of *C. salexigens* (Piubeli *et al*., 2018) can provide a very useful tool for metabolic engineering, guiding the model-driven design of *rpoS* and *ectZ* strains for ectoines overproduction.

## Experimental procedures

### *Bacterial strains, plasmids and growth conditions*

The bacterial strains and plasmids used in this study are described in Table 1. Strain CHR61, a spontaneous $Rf^r$ mutant of *C. salexigens* DSM $3043^T$, was used as the wild-type strain. CHR61 displays wild-type growth at all conditions tested. *C. salexigens* strains were routinely grown in complex SWYE-2 medium, containing 2% (w/v) total salts (Nieto *et al.*, 1987). Medium M63 (Csonka, 1982), which contains 20 mM glucose as the sole carbon source, was used as minimal medium for *C. salexigens*. The osmotic strength of M63 was increased by the addition of a 0.75 to 2.5 M final concentration of NaCl. *Escherichia coli* was grown aerobically in complex Luria-Bertani (LB) or SWYE-2 media. The pH of all media was adjusted to 7.2 with KOH. Solid media contained 20 g of Bacto agar per litre (Difco). Otherwise stated, cultures were incubated at 37 or 45°C in an orbital shaker at 220 rpm. Growth was monitored as the optical density of the culture at 600 nm ($O.D._{600}$) with a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer.

### *DNA techniques*

Plasmid DNA was isolated from *E. coli* with Wizard® *Plus* SV Miniprep kit (Promega, Madison, WI, USA), and genomic DNA was isolated with Quantum Prep Aquapure Genomic DNA kit (Biorad, Hercules, CA, USA) and ISOLATE II genomic DNA kit (Bioline, Memphis, TN, USA). Restriction enzymes and T4DNA ligase were provided by Promega. Digestion with restriction enzymes, analysis by agarose gel electrophoresis, isolation of fragments, ligation, transformation and PCR were done according to standard procedures (Sambrook and Russell, 2001) or to the manufacturer's recommendations. High fidelity PCR (for cloning from genomic DNA and site-directed mutagenesis) was performed with Pfu Turbo

**Table 1.** Strains and plasmids used in this study.

| Strain or plasmid | Relevant genotype and/or description | Source of reference |
|---|---|---|
| *C. salexigens* strains | | |
| CHR61 | Spontaneous $Rf^r$ mutant of *C. salexigens* DSM $3043^T$ | Cánovas *et al.* (1997) |
| CHR136 | CHR61 *ectD*::Ω, $Rf^r$ $Sm^r$ | Garcia-Estepa *et al.* (2006) |
| CHR174 | CHR61 *ectE*::Km, $Rf^r$ $Km^r$ | This study |
| CHR175 | CHR61 *ectD*:: Ω *ectE*::Km, deficient in hydroxyectoine synthesis; $Rf^r$ $Km^r$ $Sm^r$ | This study |
| CHR146 | CHR61 *ectZ*:: Ω , $Rf^r$ $Sm^r$ | This study |
| CHR196 | CHR61 *rpoS*:: Ω , $Rf^r$ $Sm^r$ | Salvador *et al.* (2015) |
| *E. coli* strains | | |
| DH5α | *supE44 D(lac)U169* φ80d*lacZDM15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*; host for DNA manipulation | Invitrogen |
| GM242 | *dam*-3 *rec*Alsin-2 *thr*-1 *leuB* 6*pro*A2 *his4 met*B1 *lac*Y1 *gal*K1 *ara*-14 *tsk*-33 *phi*-l *deo*B6 *sup*E44 *rps*L260 | McGraw and Marinus (1980) |
| Plasmids | | |
| pKS(-) Bluescript | cloning vector; $Ap^r$ | Stratagene |
| pUC4K | pUC4 and Tn903 derivative plasmid carrying Kanamycin resistance cassette; $Ap^r$ $Km^r$ $Neo^r$ $Gn^r$ | Pharmacia, Healthcare |
| pRK600 | Helper plasmid: $Cm^r$ *tra* | Kessler *et al.* (1992) |
| pJQ200-SK | Suicide vector; $Gm^r$ *mob sac* | Quandt and Hynes (1993) |
| pMectE1 | 3 kb HindIII fragment amplified by PCR (containing *Csal_3002*, *ectE, Csal_3004*) cloned into pKS in HindIII; $Ap^r$ | This study |
| pMectE3 | pMectR1 derivative with a BglII restriction site 548 bp downstream of *ectE* gene start codon originated by PCR-directed mutagenesis | This study |
| pMectE4 | pMectE1 derivative with a BclI restriction site 950 bp downstream of *ectE* stop codon originated by PCR-directed mutagenesis | This study |
| pMEctE5 | pMectE4 derivative isolated from the *E. coli dam* strain GM242 | This study |
| pMectE6 | pMectE5 derivative with Kanamycin cassette inserted within *ectE* gene; $Ap^r$ $Km^r$ | This study |
| pMectE7 | pMectE6 derivative isolated from the *E. coli dam* strain GM242 | This study |
| pMectE8 | pJQ200-SK derivative with a 4.3 kb XbaI-BclI fragment from pMectE7 cloned into XbaI-BamHI sites; $Km^r$ $Gm^r$ | This study |
| pRectZ1 | 3.3 kb fragment amplified by PCR (containing *ectZ, ectD, Csal_0543*) cloned into pKS in *Sfr*I; $Ap^r$ | This study |
| pRectZ2 | pRectZ1 derivative with a HpaI restriction site 44 bp downstream of *ectE* start codon originated by PCR-directed mutagenesis | This study |
| pRectZ4 | pRectZ2 derivative with Streptomycin cassette inserted within *ectZ* gene; $Ap^r$ $Sm^r$ | This study |
| pRectZ5 | pJQ200-SK derivative with a 4.7 kb PstI-NotI fragment from pRectZ4 cloned into PstI-NotI sites; $Sm^r$ $Gm^r$ | This study |

DNA polymerase (Stratagene, La Jolla, CA, USA). Introduction of new restriction sites into cloned fragments was performed by site-directed mutagenesis using the QuickChange®IIXL kit (Stratagene). All primers used for cloning, site-directed mutagenesis and real-time PCR were purchased from MWG (Germany) and are listed in Table S1 of supplemental material. DNA sequencing was performed by NewBioTechniques (Seville, Spain).

### *Construction of* C. salexigens ectE*,* ectDE *and* ectZ *mutant strains*

To construct the *ectE* mutant strains, a 3040 bp DNA fragment, which contained the *ectE* gene (*Csal_3003*) and part of the sequence of *Csal_3002* and *Csal_3004* genes, was amplified with PfuTurbo (Stratagene) using *C. salexigens* genomic DNA as template and the primer pair *ectE-fw*/*ectE-rv*. The sequences of both primers included a HindIII site in 5'. The 3-kb product was digested with HindIII and inserted into HindIII-digested pKS(-) Bluescript plasmid, resulting in plasmid pMectE1. To generate *ectE* mutants, a BglII site was generated at 548 bp downstream of the *ectE* start codon by site-directed mutagenesis using pMectE1 as a template and the primer pair *ectE-Bgl-fw*/*ectE-Bgl-rv*. The resulting plasmid, pMectE3, was subjected to a new site-directed mutagenesis using the primer pair *ectE-Bcl-fw*/*ectE-Bcl-rv* to generate a BclI site 950 bp downstream of *ectE* stop codon to give the pMectE4 plasmid. As BclI is sensitive to Dam methylation, pMectE4 was transformed in the *E. coli dam* strain GM242. The transformed plasmid was isolated (pMectE5) and digested with BclI to check the generation of the BclI restriction site. A 1.3-kb BamHI fragment from pUC4K, containing a $Km^R$ cassette, was inserted into BglII-digested pMectE4 plasmid to yield plasmid pMectE6. This plasmid was transformed in *E. coli* GM242 and isolated as pMectE7. Finally, a 4.3-kb XbaI-BclI fragment from pMectE7 was ligated to pJQ200SK digested with XbaI and BamHI resulting in plasmid pMectE8, which was transferred to *C. salexigens* wild-type strain and to the *ectD*-deficient strain CHR136. The resulting double cross-over mutants CHR174 (*ectE*) and CHR175 (*ectDectE*), respectively, were selected as $Km^rGm^s$ colonies on SWYE-2 plates containing 10% sucrose and confirmed by amplification of the mutated DNA with the primer pair *ectE-kan-fw*/*ectE-kan-rv* and subsequent sequencing (Fig. 1B).

The *ectZ* gene was amplified from *C. salexigens* genomic DNA by PCR using the primer pair *ectZ-fw*/*ectZ-rv*. The resulting 3.3-kb blunt-end fragment (containing *ectZ*, *ectD* and *Csal_0543*) was ligated to SfrI-digested pKS (-) Bluescript plasmid to give plasmid pRectZ1. To inactivate the *ectZ* gene, an HpaI site was generated at 44 bp downstream of the *ectZ* start codon by site-directed mutagenesis using pRectZ1 as a template and the primer pair *ectZ-Hpa-fw*/*ectZ-Hpa-rv*. The resulting plasmid (pRectZ2) was digested with HpaI and ligated to a 2-kb SmaI fragment from pHP45-Ω (Prentki and Krisch, 1984), containing the Ω interposon for insertional mutagenesis ($Sm^R/Spc^R$), to give plasmid pRectZ4. To recombine the *ectZ* mutation into the *C. salexigens* chromosome, a 4.7-kb PstI-NotI fragment from pRectZ4 was cloned into the suicide vector pJQ200SK digested with PstI and NotI to give plasmid pRectZ5, which was mobilized into *C. salexigens* wild-type strain by triparental mating. Mutant CHR146 (*ectZ*::Ω), resulting from a double homologous recombination event, was identified as a $Sm^rGm^s$ colony on SWYE-2 plates containing 10% sucrose and confirmed by amplification of the mutated DNA with the pair primer *ectZ-Ω-fw/ectZ-Ω-rv* and subsequent sequencing (Fig. 1C).

### *Quantification of intracellular ectoine and hydroxyectoine by LC-MS*

*C. salexigens* wild-type strain CHR61 and mutant strains CHR136 (*ectD*), CHR174 (*ectE*), CHR175 (*ectDectE*), CHR146 (*ectZ*) and CHR196 (*rpoS*) (Salvador *et al*., 2015) were grown in M63 with 0.75 M NaCl at 37°C and with 2.5 M NaCl at 37 and 45°C. Cells cultured until mid-late exponential phase and late stationary phase were collected by centrifugation and washed with the same medium without any carbon source. Solutes were extracted from 1 ml of culture using a modified Bligh and Dyer (1959) technique as described by Kraegeloh and Kunte (2002). Cell pellets from 200 ml cultures were resuspended in 10 ml of extraction mixture (methanol: chloroform:water; 10:5:4), and intracellular solutes were extracted by gently shaking for 30 min at 37°C. The cell debris was removed by centrifugation, and supernatants were extracted once with chloroform:water (1:1) and freeze-dried. The solids were dissolved in $D_2O$ (0.6 ml). Ectoine and hydroxyectoine were quantified by high-performance liquid chromatography as described by Argandoña *et al*. (2010).

### *RNA isolation and real-time PCR*

Total RNA of cells from cultures of *C. salexigens* was extracted using High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. The absence of DNA contamination was checked by PCR using the 16S rRNA primers 16S-RT-fw and 16S-RT-rv (Argandoña *et al*., 2010). After isolation, purity and concentration were assessed in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific Waltham, MA, USA). RNA quality was evaluated by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyser (Agilent,

Santa Clara, CA, USA) using Agilent RNA 6000 Nano kit. Chips were prepared and loaded according to the manufacturer's instructions. High-quality RNA [rRNA ratio (23S/16S) $\approx$ 1.6, RNA integrity number $>$ 9.0 and A260/A280 ratio $>$ 2.0] was stored at $-80$°C until it was used.

cDNA was synthesized by using a Transcriptor first-strand cDNA synthesis kit (Roche, Basilea, Switzerland) according to the manufacturer's recommendations. Two micrograms of total RNA was denatured at 65°C for 10 min, and then random hexamers (60 μM), protector RNase inhibitor (20 U), reverse transcriptase (RT) (10 U) and reaction buffer were added to a 20 μl (final volume) mixture. The reaction mixture was incubated at 25°C for 10 min and then at 50°C for 60 min. Finally, the mixture was incubated for 5 min at 85°C to inactivate the reverse transcriptase. The cDNA synthesized was kept at $-20$°C until it was used.

Real-time PCR was performed as described by Argandoña *et al*. (2010) in 96-well plates using a LightCycler® 480 Real-Time PCR System (Roche) and a FastStart SYBR Green Master (Rox) (Roche). Primers used were *RT-ectD-fw*/*RT-ectD-rv* for *ectD* (Salvador *et al*., 2018) and *RT-ectE-fw*/*RT-ectE-rv* for *ectE*. Amplification data were analysed with the LightCycler® 480 Gene Scanning Software v1.5 (Roche). Transcript levels were calculated by the $2^{-\Delta\Delta CT}$ method using the mRNA levels of 16S rRNA gene as an endogenous control to normalize the data obtained within each sample.

### *Bioinformatic analysis*

Predictions of $\sigma^{70}$-dependent promoter sequences were done with the program 'Neural Network Promoter Prediction' (http://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001). Transcriptional terminators were predicted by running MFOLD at http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi (Zuker *et al*., 1999). Prediction of Tm index were carried out by Tmpredictor (http://tm.life.nthu.edu.tw/) (Ku *et al*., 2009). Theoretical isoelectric point of proteins was predicted on ExPASy server (https://web.expasy.org/compute_pi/) (Gasteiger *et al*., 2005). Searches for identities were performed at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) by using BLASTP program. Conserved domains within proteins were identified at NCBI's Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml. Absynte and SyntTax web servers were used for genomic context comparisons (Despalins *et al*., 2011; Oberto, 2013).

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura *et al*., 2013) Sequences were aligned with ClustalW (1.6) using a BLOSUM62 matrix and manually edited. The phylogenetic tree was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and were in the units of the number of amino acid differences per site. The robustness of the tree branches was assessed by performing bootstrap analysis of the Neighbour-Joining data based on 1000 resamplings (Felsenstein, 1985).

### *Nucleotide sequence accession numbers*

The *C. salexigens* genome sequence is available at GenBank under the accession NC_007963. NCBI Reference sequences for EctE (Csal_3003) and for EctZ (Csal_0542) proteins are WP_043558653.1 and WP_011505849.1 respectively.

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## Conflict of interest

None declared.

## Author contributions

MA and CV conceived and supervised the study. MA and MRB developed the mutant strains. MA, FP and MRB performed experimental phenotypic and expression analysis. MA performed the bioinformatics and phylogenetic analysis. MA, FP, MRB, JJN and CV were involved in the analysis and discussion of results. MA, FP and CV drafted the manuscript. All authors revised and approved the final manuscript.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1**. Nucleotide sequence of the *ectD* and *ectE* putative promoter regions. Coding sequences are in red. The stop codons of the genes upstream of *ectD* and *ectE* are denoted by an asterisk. The inverted repeated sequence (IR1) upstream of *ectZ* most probably corresponds to a rho-independent transcriptional terminator. −10 and −35 regions for each putative promoter are boxed and the nucleotides matching sigma factor consensus sequences are shown in bold an underlined. The transcription start sites (+ 1) are indicated by an arrow, and the ribosome-binding sites are indicated as RBS. The *ectD* and *ectE* start codons are indicated, and the partial amino acid sequences of *ectD* and *ectE* are shown in red one-letter code.

**Fig S2**. Alignement of the amino acid sequences of characterized ectoine hydroxylase proteins, and EctD and EctE from *Chromohalobacter salexigens*. The ectoine hydroxylases included in the alignement were from: *Acidiphilium cryptum* (AER00258), *Alkalilimnicola ehrlichii* (AER00257), *Halomonas elongata* (WP_013333764.1), *Paenibacillus lautus* (WP_113059475.1), *Pseudomonas stutzeri* (CBM40642.1), *Virgibacillus salexigens* (AAY29689), *Sphingopixis alaskensis* (WP_011543221.1), *Sphingobium japonicum* (WP_006964700), *Chromohalobacter salexigens* (EctD: CAJ77720; EctE: ABE60347). Residues involved in the binding of the iron catalyst are marked in red; those that mediate the binding of the 2-oxoglutarate co-substrate are labeled in green, and residues contacting the 5-hydroxyectoine

molecule are marked in blue. His and Asp residues, both involved in the binding of the iron catalyst and also of the 5-hydroxyectoine molecule, are marked with black dots. The string of the 17 amino acids that serves as the consensus sequence of ectoine hydroxylases (F-XWHSDFETWH-X-EDG-M/L-P) is labeled with a red line.

**Fig S3**. Comparison of the genomic context of *ectZ* in *C. salexigens* and in other gamma-proteobacteria genomes. Given scores are referred to normalized genomic context BLAST results, and they were obtained by SYNTTax (Oberto, 2013) and Absynte (Despalins *et al*, 2011) web-servers. Open reading frames are indicated by arrows. A consistent color code is assigned to matched proteins across genomes. Colored and white arrows represent conserved and non- conserved genes, respectively. *C. salexigens ectZ* and its orthologs genes are red framed. *ectD*: ectoine hydroxylase gene; γ_BBH: Gamma-butyrobetaine gene,2-oxoglutarate dioxygenase gene; *ilvG*: thiamine pyrophosphate enzyme-like gene with aTPP binding region.

**Table S1**. Primers used in this study.