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Osmotically Induced Synthesis of the Compatible Solute Hydroxyectoine Is Mediated by an Evolutionarily Conserved Ectoine Hydroxylase*[S]

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By using natural abundance ^{13}C NMR spectroscopy, we investigated the types of compatible solutes synthesized in a variety of *Bacilli* under high salinity growth conditions. Glutamate, proline, and ectoine were the dominant compatible solutes synthesized by the various *Bacillus* species. The majority of the inspected *Bacilli* produced the tetrahydropyrimidine ectoine in response to high salinity stress, and a subset of these also synthesized a hydroxylation derivative of ectoine, 5-hydroxyectoine. In *Salibacillus salexigens*, a representative of the ectoine- and 5-hydroxyectoine-producing species, ectoine production was linearly correlated with the salinity of the growth medium and dependent on an *ectABC* biosynthetic operon. The formation of 5-hydroxyectoine was primarily a stationary growth phase phenomenon. The enzyme responsible for ectoine hydroxylation (EctD) was purified from *S. salexigens* to apparent homogeneity. The EctD protein was shown *in vitro* to directly hydroxylate ectoine in a reaction dependent on iron(II), molecular oxygen, and 2-oxoglutarate. We identified the structural gene (*ectD*) for the ectoine hydroxylase in *S. salexigens*. Northern blot analysis showed that the transcript levels of the *ectABC* and *ectD* genes increased as a function of salinity. Many EctD-related proteins can be found in data base searches in various *Bacteria*. Each of these bacterial species also contains an *ectABC* ectoine biosynthetic gene cluster, suggesting that 5-hydroxyectoine biosynthesis strictly depends on the prior synthesis of ectoine. Our data base searches and the biochemical characterization of the EctD protein from *S. salexigens* suggest that the EctD-related ectoine hydroxylases are members of a new subfamily within the non-heme-containing, iron(II)- and 2-oxoglutarate-dependent dioxygenase superfamily (EC 1.14.11).

To avoid excessive water efflux, plasmolysis, molecular crowding of the cytoplasm, and cessation of growth in high salinity environments (1, 2), many *Bacteria* (3–5) and *Archaea* (6, 7) amass large amounts of a particular class of highly soluble organic osmolytes, the so-called compatible solutes (4, 8–10). Important examples of compatible solutes within the domain of the *Bacteria* are the imino acid proline, the disaccharide trehalose, the trimethylammonium compound glycine betaine, and the tetrahydropyrimidine ectoine (4, 10, 11).

Ectoine ((*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) (Fig. 1) was originally found as a compatible solute in the extremely halophilic phototrophic purple sulfur bacterium *Ectothiorhodospira halochloris* (12). Its discovery was soon followed by the description of its hydroxylated derivative, 5-hydroxyectoine ((*S*,*S*)-2-methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) (Fig. 1) in the Gram-positive soil bacterium *Streptomyces parvulus* (13). Ectoine was initially viewed as a rather uncommon compatible solute, but it is now known to be widely produced in response to high salinity within a taxonomically and physiologically diverse set of species within the domain of the *Bacteria* (4, 10, 14, 15).

Molecular analysis of the ectoine biosynthetic genes in various Gram-negative and Gram-positive *Bacteria* has shown that the ectoine biosynthetic enzymes are encoded by an evolutionarily conserved gene cluster, *ectABC* (14–16). Disruption of these genes in *Halomonas elongata*, *Chromohalobacter salexigens*, or *Vibrio cholerae* results in the loss of the ectoine biosynthetic capacity (17–19). Ectoine biosynthesis is mediated by a three-step enzymatic reaction (16, 20) that converts the precursor L-aspartate-β-semialdehyde, an intermediate in amino acid metabolism, into ectoine; L-2,4-diaminobutyrate and N^{γ}-acetyl-L2,4-diaminobutyrate are the intermediates in this process (Fig. 1). The ectoine biosynthetic enzymes (EctABC) from *H. elongata* have been purified and characterized biochemically (21).

The ability to synthesize ectoine is widespread within the domain of the *Bacteria* (15), but only a subset of the ectoine producers also synthesizes the hydroxylation derivative, 5-hydroxyectoine (4, 14). Like ectoine, 5-hydroxyectoine serves as a compatible solute *in vivo*, functions as an osmoprotectant, and exhibits protein stabilizing properties *in vitro* (22–25). The ability of a given microorganism to synthesize 5-hydroxyectoine invariably depends on its ability to produce ectoine, suggesting that 5-hydroxyectoine formation occurs either directly from ectoine (26, 27) or from one of its biosynthetic intermediates (17). The most straightforward route to produce 5-hydroxyectoine would be the direct hydroxylation of ectoine via a

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures" and additional references.

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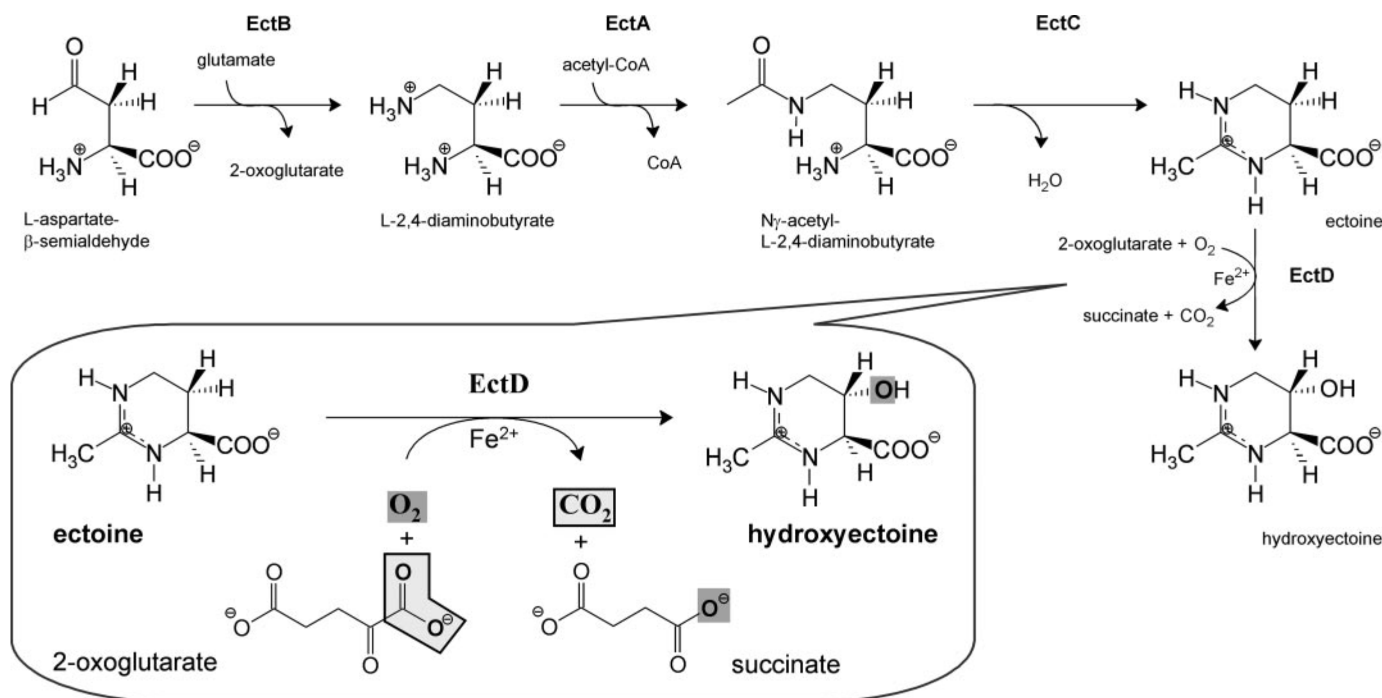

FIGURE 1. **Pathway for the biosynthesis of ectoine and 5-hydroxyectoine.** The biosynthetic route for the formation of the compatible solute ectoine from L-aspartate-β-semialdehyde is shown. This pathway is based on proposals made by Louis and Galinski (16) and Ono *et al.* (21). The enzymes responsible for ectoine biosynthesis are the L-2,4-diaminobutyrat transaminase (EctB), the L-2,4-diaminobutyrat acetyltransferase (EctA), and the ectoine synthase (EctC). The suggested pathway for the enzymatic conversion of ectoine into 5-hydroxyectoine by the ectoine hydroxylase (EctD protein) from *S. salexigens* is based on the data presented in this study. The proposed enzymatic reaction scheme for the EctD ectoine hydroxylase is shown in the enlarged part of the figure.

substrate-specific hydroxylase (Fig. 1). Here we report the biochemical characterization of such an enzyme from the moderate halophile *Salibacillus salexigens*.

EXPERIMENTAL PROCEDURES

Chemicals—Ectoine ((*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) and 5-hydroxyectoine (*S,S*)-2-methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) were purchased from Biomol (Hamburg, Germany). L-Proline, 3-hydroxy-DL-proline, and 4-hydroxy-L-proline were obtained from Sigma-Aldrich. Two synthetic ectoine derivatives, homoectoine ((*S*)-4,5,6,7-tetrahydro-2-methyl-1H-(1, 3)-diazepine-4-carboxylic acid) (28) and DHMICA ((*RS*)-4,5-dihydro-2-methyl-imidazole-4-carboxylic acid) were kindly provided by E. A. Galinski (University of Bonn, Bonn, Germany). Anhydrotetracycline hydrochloride and *d*-desthiobiotin were purchased from IBA (Göttingen, Germany).

High Pressure Liquid Chromatography Analysis of Ectoine and 5-Hydroxyectoine from Cell Extracts—Cultures (80 ml) of *S. salexigens* (DSM 11483^T) were grown in a shaking water bath (220 rpm) in mineral salt-based medium (29) (for details, see supplemental material) with the indicated NaCl concentrations until the cultures reached an A_{578} of approximately 1. The cells were harvested by centrifugation (10 min; 4 °C; 2800 × *g*) and lyophilized, the dry weight of the cells was determined, and the cells were then extracted using a modified Bligh and Dyer technique (30). The ectoine and 5-hydroxyectoine content of the cells was measured by HPLC[2] analysis using a GROM-SIL 100 Amino-1PR, 125 mm by 4 mm (3 μm) column (GROM, Rottenburg-Hailfingen, Germany) as described by Kuhlmann and Bremer (14). Quantification of ectoine and 5-hydroxyectoine was performed with the ChromStar 6 software (SCPA, Stuhr, Germany) using commercially available ectoine and 5-hydroxyectoine as reference standards.

Enzyme Assay—Ectoine hydroxylase activity in cell extracts of *S. salexigens* and after purification of the EctD protein was assayed by measuring the conversion from ectoine to 5-hydroxyectoine by HPLC analysis. One unit of ectoine hydroxylase activity is defined as the conversion of 1 μmol of ectoine to 1 μmol of 5-hydroxyectoine permin. During EctD purification, ectoine hydroxylase activity was assayed in a 30-μl reaction mixture that contained 10 mM TES buffer, pH 7.5, 10 mM 2-oxoglutarate, 6 mM (*S*)-ectoine, 1 mM $FeSO_4 \cdot 7H_2O$, 1.3 kilounits beef liver catalase (Roche Applied Science) and maximally 20 μl of the various protein fractions. Each reaction mixture was incubated at 32 °C for 20 min in a thermomixer (Eppendorf, Hamburg, Germany) with vigorous shaking to efficiently aerate the reaction mixture because EctD is an O_2-dependent enzyme. The ectoine hydroxylase reaction was stopped by adding 30 μl of 100% acetonitrile (J. T. Baker, Deventer, The Netherlands) to the reaction mixture and subsequent immediate centrifugation (10 min, 4 °C; 32000 × *g*). Twenty microliters of the supernatant of these reaction mixtures were than loaded onto a GROM-SIL 100 Amino-1PR column (125 mm by 4 mm; 3-μm particle size (GROM, Rottenburg-Hailfingen, Germany), and ectoine and 5-hydroxyectoine were monitored by their absorbance at 210 nm by using a UV-visible detector (LINEAR UVIS 205; SYKAM, Fürstenfeldbruck, Germany).

[2] The abbreviations used are: HPLC, high pressure liquid chromatography; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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TABLE 1
^{13}C NMR determination of the organic osmolytes synthesized by *Bacilli* strains and strains from related genera

The strains were grown in Spizizen's minimal medium (SMM) with glucose as a unique source of carbon and energy and supplemented with trace elements, vitamins, and amino acids when necessary. For alcaliphilic strains, the medium was supplemented with 100 mM sodium sesquicarbonate.

Strain	DSM number	Medium (NaCl molarity)	Glutamate	Proline	Ectoine	Hydroxyectoine	Others
Aneurinibacillus aneurinilyticus[a]	DSM 5562^T	SMM (0.5 M)	+	−	−	−	
Bacillus cereus[a]	DSM 31^T	SMM (0.5 M)	+	−	−	−	
Bacillus circulans[a]	DSM 11^T	SMM (0.5 M)	+	−	−	−	
Bacillus thuringiensis[a]	DSM 2046^T	SMM (0.5 M)	+	−	−	−	
Sporosarcina globispora	DSM 4^T	SMM (0.2 M)	+	−	−	−	
Paenibacillus polymyxa[a]	DSM 36^T	SMM (0.5 M)	+	−	−	−	trehalose
Bacillus amyloliquefaciens	FZB 42	SMM (1.0 M)	+	+	−	−	
Bacillus licheniformis[a]	DSM 13^T	SMM (1.0 M)	+	+	−	−	
Bacillus megaterium[a]	DSM 32^T	SMM (1.0 M)	+	+	−	−	
B. subtilis[a]	JH642	SMM (1.0 M)	+	+	−	−	
Bacillus vallismortis	DSM 11031^T	SMM (1.8 M)	+	+	−	−	
Bacillus agaradhaerens	DSM 8721^T	SMM (1.3 M)	+	−	+	−	β-glutamate
Bacillus alcalophilus[a]	DSM 485^T	SMM (1.0 M)	+	−	+	−	
Bacillus halodurans	DSM 497^T	SMM (1.6 M)	+	−	+	−	β-glutamate
Bacillus pseudalcaliphilus	DSM 8725^T	SMM (1.0 M)	+	−	+	−	
Sporosarcina psychrophila[a]	DSM 3^T	SMM (1.0 M)	+	−	+	−	
Sporosarcina pasteurii[a,b]	DSM 33^T	SMM (1.0 M)	+	−	+	−	
Bacillus clarkii	DSM 8720^T	SMM (1.8 M)	+	−	+	+	β-glutamate
Gracilibacillus halotolerans	DSM 11805^T	SMM (2.0 M)	+	−	+	+	
Salibacillus marismortui	DSM 12325^T	SMM (1.8 M)	+	−	+	+	
Salibacillus salexigens[a,c]	DSM 11483^T	MM (3.4 M)	+	−	+	+	
Bacillus mojavensis	DSM 9205^T	SMM (1.5 M)	+	+	+	−	
Bacillus pseudofirmus	DSM 8715^T	SMM (1.6 M)	+	+	+	−	alanine
Halobacillus halophilus[d]	DSM 2266^T	802a (2.0 M)	+	+	+	−	*N*-ε-acetyl lysine
Halobacillus trueperi[d]	DSM 10404^T	802a (2.5 M)	+	+	+	−	*N*-ε-acetyl lysine
Virgibacillus pantothenticus[a]	DSM 26^T	SMM (1.0 M)	+	+	+	−	

[a] Compatible solute production of these strains were published by Kuhlmann and Bremer (14).
[b] *S. pasteurii* was grown in SMM supplemented with 2% urea.
[c] *S. salexigens* was grown in a mineral salt-based medium (MM) supplemented with glucose, casamino acids, and vitamins.
[d] *H. halophilus* and *H. trueperi* were grown in a minimal medium with glucose as source of carbon and energy and supplemented with trace elements, vitamins, and amino acids.

The solute employed for ectoine and 5-hydroxyectoine separation was 80% (v/v) acetonitrile. Chromatography was carried out isocratically at a flow rate of 1 ml/min at 20 °C (14). The retention times of ectoine and 5-hydroxyectoine were determined by using commercially available ectoine and 5-hydroxyectoine samples.

After purification of the ectoine hydroxylase, the properties of the purified enzyme were determined by using the assay conditions described above with 2 milliunits of the purified enzyme. 5-Hydroxyectoine was produced from ectoine linearly under these conditions for up to 10 min. Kinetic parameters were calculated from velocity data of up to 5-min reactions at various substrate concentrations ranging from 0.5 to 20 mM for (*S*)-ectoine and from 0.1 to 15 mM for 2-oxoglutarate.

Accession Numbers—The DNA sequence of the *ectABC* and *ectD* regions from *S. salexigens* were deposited in GenBank™ under accession numbers AY935521 and AY935522, respectively.

RESULTS AND DISCUSSION

De Novo Synthesis of Compatible Solutes within the Genus Bacillus and Closely Related Taxa—We used natural abundance ^{13}C NMR (14, 31–34) to evaluate the spectrum of compatible solutes synthesized *de novo* in osmotically stressed cultures of thirteen different *Bacilli* or closely related taxa. For these experiments, the various microorganisms were grown in chemically defined minimal media lacking components of rich media (*e.g.* yeast extract and peptone), thereby avoiding the accumulation of preformed osmoprotectants (*e.g.* glycine betaine and proline) from exogenous sources through transport processes by the microbial cells (35). For each of the 13 different *Bacilli* analyzed, we evaluated the spectrum of compatible solutes synthesized in high salinity cultures grown either to mid-exponential or to stationary growth phase. We then compared these data with results from ^{13}C NMR experiments conducted with cultures grown in the absence of high concentrations of NaCl. In each of the osmotically nonstressed cultures, we detected primarily resonances corresponding to glutamate, fully consistent with previous reports that this amino acid constitutes the dominant amino acid in the cytoplasmic organic solute pools of *Bacillus subtilis* (34) and *Sporosarcina pasteurii* (14). Because the salinity of the growth medium was raised with NaCl, resonances of proline, ectoine, and 5-hydroxyectoine were detected in addition to those of glutamate. Resonances corresponding to 5-hydroxyectoine were found only in cultures grown at high salinity to stationary phase. The data from these ^{13}C NMR experiments are summarized in Table 1, where they are compiled together with the data of a previous study conducted by Kuhlmann and Bremer (14) on the synthesis of compatible solutes produced in 13 additional osmotically stressed *Bacilli*. Hence, data on the *de novo* compatible solute synthesis in 26 different *Bacilli* are now available (Table 1), thereby providing a solid overview of the dominant compatible solutes synthesized within the genus *Bacillus* and closely related species.

The evaluation of the pattern of compatible solute synthesis under high salinity growth conditions by these 26 *Bacilli* (Table 1) revealed the existence of three major groups: (i) species that produce only glutamate (6 of 26); (ii) species that produce glutamate and proline (5 of 26); and (iii) the majority (15 of 26) of the investigated species, which produced glutamate and ectoine. Hence, the ability to synthesize the compatible solute ectoine in response to high salinity stress is widespread within the genus *Bacillus* and closely related taxa.

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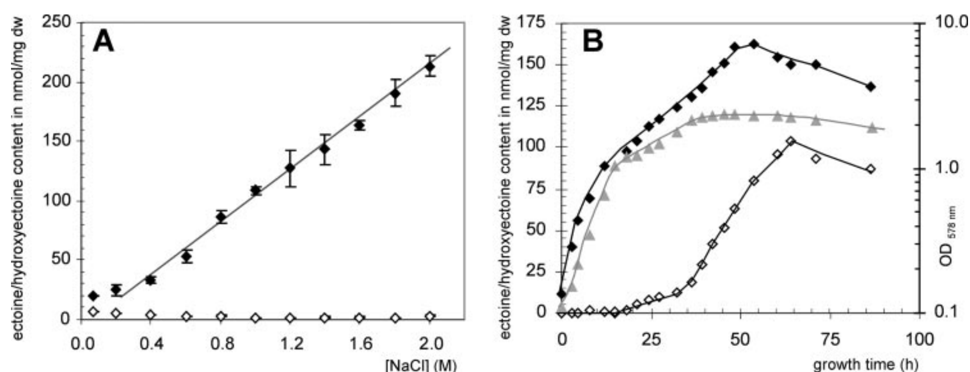

FIGURE 2. **HPLC determination of ectoine and 5-hydroxyectoine content in salt-stressed *S. salexigens* cells.** Cells of *S. salexigens* were grown at 37 °C in a mineral salt-based medium supplemented with casamino acids, various vitamins, and glucose as the carbon source. Ectoine and 5-hydroxyectoine content of the cells was quantitated by HPLC analysis. *A*, cultures of *S. salexigens* were grown in minimal medium with various salt concentrations. When the cultures reached the mid-exponential growth phase (A_{578} = ~1), they were harvested by centrifugation and lyophilized. The soluble compounds were then extracted, and the ectoine and 5-hydroxyectoine content of the cells was determined by HPLC analysis. *Closed diamonds*, ectoine; *open diamonds*, 5-hydroxyectoine. *B*, a culture of *S. salexigens* was grown in mineral salt-based medium with 3.2 M NaCl, and the growth of the culture was monitored by measuring the A_{578} (*gray triangles*). The ectoine (*closed diamonds*) and 5-hydroxyectoine (*open diamonds*) content of the cells was quantitated by HPLC analysis.

The 15 ectoine producers can be further subgrouped into three different classes: (i) those species that produce ectoine alone (6 of 15); (ii) those species that produce ectoine and 5-hydroxyectoine (4 of 15); and (iii) those species that produce both ectoine and proline (5 of 15). Osmoregulatory ectoine production is therefore a widespread trait within the genus *Bacillus* and taxonomically closely related species.

Finely Tuned and Osmotically Controlled Ectoine and 5-Hydroxyectoine Biosynthesis in S. salexigens—The main focus of this work is the elucidation of 5-hydroxyectoine formation in osmotically stressed *Bacilli*. We therefore chose *S. salexigens* as a representative of the four ectoine and 5-hydroxyectoine producers discovered thus far within in the *Bacilli* (Table 1). *S. salexigens* (29) is a moderate halophile and soil bacterium that can grow over a wide range of salinities (between 0.1 and 3.4 M NaCl). This species has recently been reclassified, and the new name *Virgibacillus salexigens* has been suggested (36).

To analyze the correlation between the salinity of the growth medium and the level of ectoine and 5-hydroxyectoine production in *S. salexigens*, we grew this strain in minimal media of different salinities (0.1–2.0 M NaCl). We then quantitated the produced ectoine and 5-hydroxyectoine by HPLC analysis when the cultures had reached approximately the same optical density (A_{578} = ~1). We found an essentially linear relationship between the ectoine content of the cells and the salinity of the growth medium over a wide range of osmotic conditions in these exponentially growing *S. salexigens* cultures (Fig. 2*A*). It is thus apparent that the cells sensitively adjust their ectoine content to the prevalent salinity of the growth medium to maintain a physiological appropriate level of cell water and consequently turgor.

Although the exponentially growing cultures of *S. salexigens* contained a considerable amount of ectoine when the cells were stressed with various concentrations of NaCl, we found that the amount of 5-hydroxyectoine was negligible in these cultures (Fig. 2*A*).

Our ^{13}C NMR experiments with *S. salexigens* revealed that 5-hydroxyectoine production in osmotically challenged cells occurred primarily when the cultures entered the stationary growth phase. To investigate this in greater detail, we grew *S. salexigens* in a minimal medium with 3.2 M NaCl and monitored ectoine and 5-hydroxyectoine production along the growth curve of the culture by HPLC analysis. Increased ectoine biosynthesis occurred as soon as the cells started to grow in this high osmolality medium, but there was no immediate 5-hydroxyectoine production. Appreciable amounts of 5-hydroxyectoine were made only when cell growth slowed and the culture entered stationary growth phase. Production of this compatible solute reached its maximum when the cells had stopped their growth (Fig. 2*B*). In these stationary growth phase cultures, we always found a mixture of both ectoine and 5-hydroxyectoine (Fig. 2*B*), indicating that the cell converted only part of the *de novo* synthesized ectoine into the hydroxylation product.

We examined a second ectoine and 5-hydroxyectoine producer, *Bacillus clarkii* (Table 1), for the pattern of ectoine and 5-hydroxyectoine synthesis in osmotically challenged exponential phase and stationary phase cultures. *B. clarkii* showed the same pattern of ectoine and 5-hydroxyectoine production as that described in Fig. 2 for *S. salexigens*.[3] The other two 5-hydroxyectoine producers, *Gracilibacillus halotolerans* and *Salibacillus marismortui*, were not investigated for the pattern of ectoine and 5-hydroxyectoine production.

Purification and Characterization of the Ectoine Hydroxylase—We took a biochemical approach to characterize the ectoine hydroxylase from *S. salexigens*. Kuhlmann and Bremer (14) have previously noted that the *ectABC* gene cluster in *Streptomyces coelicolor* A3 (2) (37) is immediately followed by a gene that encodes a protein with limited sequence identity (27%) to a L-proline 4-hydroxylase from *Dactylosporangium* sp (38). This observation suggested to us that the function of this gene product from *S. coelicolor* A3 (2) might have been incorrectly annotated during the genome sequencing project and may actually serve as an ectoine hydroxylase.

We developed an enzymatic assay for ectoine hydroxylase activity that used assay conditions similar to those employed for the biochemically characterized L-proline 4-hydroxylase from *Dactylosporangium* sp. strain RH1 (38), a L-proline 3-hydroxylase from the *Streptomyces* sp. strain TH1 (39), and a L-proline 4-hydroxylase from the *Streptomyces griseoviridus* strain P8648 (40). Each of these L-proline hydroxylases are members of the non-heme-containing, iron(II)- and 2-oxoglutarate-dependent dioxygenase superfamily (EC 1.14.11.2) (41–43). This ectoine hydroxylase activity assay enabled us to detect ectoine hydrox-

[3] N. Pica and E. Bremer, unpublished results.

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ylase enzyme activity in total cell extracts prepared from stationary growth phase cultures of *S. salexigens* propagated in a minimal medium with 3.2 M NaCl (Table 2), growth conditions that allow the effective formation of 5-hydroxyectoine (Fig. 2*B*).

Using this ectoine hydroxylation assay, we purified the ectoine hydroxylase from *S. salexigens* to apparent homogeneity (Fig. 3*A*) via several chromatographic steps. Data from a typical purification of the *S. salexigens* ectoine hydroxylase are summarized in Table 2. The purified protein effectively converted ectoine into 5-hydroxyectoine in a time-dependent fashion (Fig. 3*B*). In the following, we refer to this ectoine hydroxylase from *S. salexigens* as the EctD protein.

Properties of the Purified Ectoine Hydroxylase—The ectoine hydroxylase from *S. salexigens* migrates on a SDS-12.5% polyacrylamide gel as a polypeptide with an apparent molecular mass of 34 kDa (Fig. 3*A*). It behaves in a gel filtration chromatography step on Superdex 75 as a protein species with an approximately molecular mass of 36 kDa, indicating that the EctD protein from *S. salexigens* is a monomer. The precise molecular mass of the purified EctD protein was determined by mass spectrometry to be 34.4 kDa. The NH_2-terminal end of the purified EctD protein was determined by sequential Edman degradation, and the following amino acid sequence was found: NH_2-M-E-D-L-Y-P-S-R-Q-N-N-Q-P-K-I.

Without the addition of the co-substrate 2-oxoglutarate, there was no hydroxylation of ectoine by the purified EctD protein. Likewise, molecular oxygen was absolutely required for the *in vitro* hydroxylation reaction, because the enzyme was completely inactive under anoxic assay conditions. The enzyme activity of EctD was stimulated by $FeSO_4$ up to a concentration of 1 mM, whereas higher $FeSO_4$ concentrations inhibited the ectoine hydroxylase enzyme activity of EctD. For enzymes of the superfamily of the non-heme-containing and iron(II)- and 2-oxoglutarate-dependent dioxygenases (EC 1.14.11), the addition of ascorbate and catalase is sometimes used to increase the enzymatic activity of these types of enzymes (40, 41). The addition of 1.3 kilounits catalase to the enzyme assay enhanced the hydroxylation activity of EctD by ~5-fold. In contrast, the addition of ascorbate inhibited the enzyme activity of EctD by ~70% when ascorbate was present in a concentration of 5 mM in the *in vitro* hydroxylation assay. The EctD enzyme activity of the purified ectoine hydroxylase had a pH optimum of 7.5 and a temperature optimum at 32 °C. Members of the superfamily of the non-heme-containing, iron(II)- and 2-oxoglutarate-dependent dioxygenases (EC 1.14.11) generally contain a mononuclear iron center (39, 41, 44). Consistent with our assignment of the EctD protein to this superfamily, we found that the purified EctD protein from *S. salexigens* contained 0.6 mol of iron/mol of protein. Taken together, these findings support the view that the *S. salexigens* EctD protein is actually an iron-containing enzyme with a mononuclear iron(II) center.

The kinetic parameters for the ectoine hydroxylase from *S. salexigens* were as follows: the K_m for the substrate ectoine was 3.5 ± 0.2 mM, and the K_m for the co-substrate 2-oxoglutarate was 5.2 ± 0.2 mM. The V_{max} of the EctD enzyme was 13.8 ± 0.1 units mg^{-1} protein.

We suggest a reaction mechanism for the EctD ectoine hydroxylase from *S. salexigens* (Fig. 1) in which the co-substrate 2-oxoglutarate is stoichiometrically decarboxylated during the hydroxylation of the substrate ectoine. CO_2 is thereby liberated from 2-oxoglutarate to form succinate. During the hydroxylation reaction, one atom of the atmospheric oxygen molecule is incorporated into succinate, whereas the other atom is incorporated into the hydroxygroup formed on ectoine (Fig. 1).

The EctD enzyme requires molecular oxygen, Fe^{2+}, and 2-oxoglutarate for its activity *in vitro* (Fig. 3*B*). EctD is a member of the non-heme-containing, iron(II)- and 2-oxoglutarate-dependent dioxygenases (41–44), an enzyme superfamily (EC 1.14.11) that carries out a diverse set of enzymatic reactions including hydroxylations, desaturations, and oxidative ring closures/rearrangements. The enzymatic reactions of members of this family are usually coupled to the oxidative decarboxylation of 2-oxoglutarate to succinate (41, 44), and this is also true for the EctD-mediated hydroxylation of ectoine (Fig. 1).

The conditions used by us for assaying the EctD-mediated ectoine hydroxylase activity are similar to those used previously to determine the enzyme activity of various pro-

TABLE 2

Purification of the ectoine hydroxylase from *S. salexigens*

The enzyme was prepared from 15 g of wet cells grown in mineral salt-based medium with 3.2 M NaCl at 37 °C.

Purification step	Total activity	Protein	Specific activity	Yield
	units	*mg*	*units · (mg protein)$^{-1}$*	*%*
Cell-free extract	54.3	702.0	0.08	100
Source 15 Q	41.1	35.7	1.15	76
Ceramic hydroxylapatite	33.8	11.4	2.97	62
Source 15 Phe	17.1	8.5	2.02	32
Mono Q	12.5	5.2	2.4	23
Superdex 75 pg	11	3.6	3.06	20

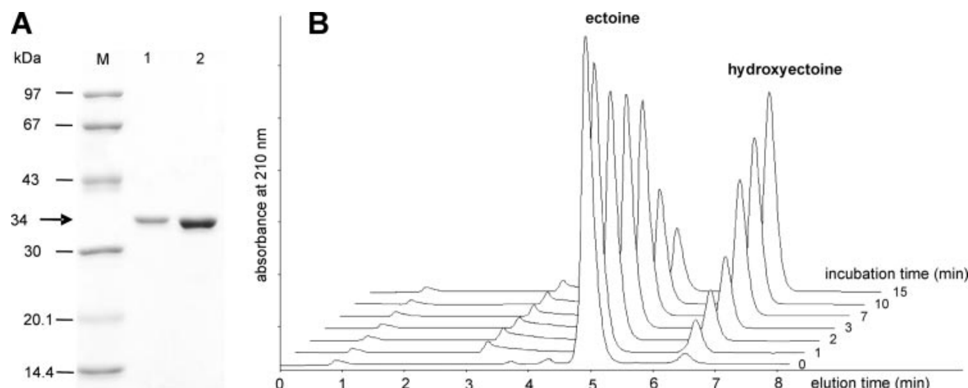

FIGURE 3. **Purification of the EctD ectoine hydroxylase from *S. salexigens* and *in vitro* conversion of ectoine into 5-hydroxyectoine by the purified EctD protein.** *A*, the ectoine hydroxylase (EctD) from *S. salexigens* was purified through a series of chromatographic steps to apparent homogeneity and electrophoretically separated on an SDS-polyacrylamide gel (12.5% polyacrylamide). The proteins were stained with Coomassie Brilliant Blue. *Lane 1*, 0.6 μg of EctD protein was loaded onto the gel; *lane 2*, 2 μg of EctD protein was loaded onto the gel. *B*, chromatograms from the HPLC analysis of the reaction mixture of the *in vitro* conversion of ectoine to 5-hydroxyectoine by the purified EctD enzyme from *S. salexigens*. The retention times of ectoine and 5-hydroxyectoine were determined by using commercially available samples of these compounds.

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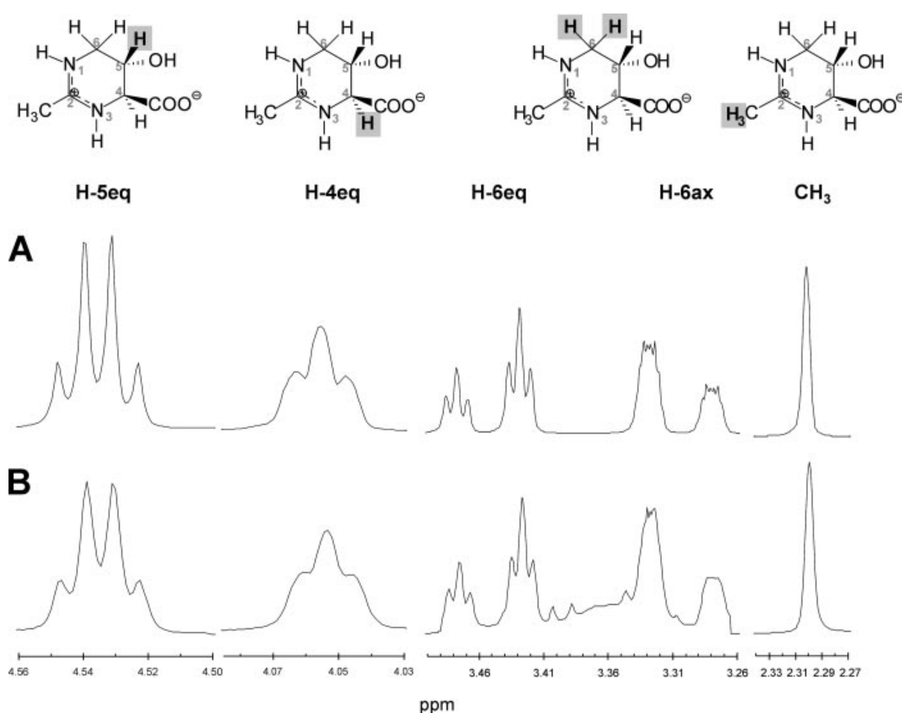

FIGURE 4. **One-dimensional ^{1}H NMR spectra of *in vitro* synthesized 5-hydroxyectoine by EctD.** *A*, commercially available 5-hydroxyectoine was analyzed by 300 MHz ^{1}H NMR spectroscopy. *B*, 5-hydroxyectoine was synthesized *in vitro* from ectoine by the purified ectoine hydroxylase (EctD) from *S. salexigens*, and the reaction product was analyzed by 300 MHz ^{1}H NMR spectroscopy. Above the spectra, the structural formula of 5-hydroxyectoine is shown and the hydrogens responsible for the recorded signals are highlighted.

line hydroxylases (38–40). To make certain that we had not isolated a proline hydroxylase from *S. salexigens* that also exhibited ectoine hydroxylase activity, we tested whether the purified EctD protein would use L-proline as a substrate and convert it to a hydroxylated product. No EctD-mediated hydroxylation of L-proline was detected using a sensitive HPLC assay with either 3- or 4-hydroxy proline as reference standards for the HPLC analysis.

Recently, two synthetic ectoine derivatives, homoectoine ((*S*)-4,5,6,7-tetrahydro-2-methyl-1H-(1,3)-diazepine-4-carboxylic acid) and DHMICA ((*RS*)-4,5-dihydro-2-methyl-imidazole-4-carboxylic acid), have been described that function as effective PCR enhancers (28). We also tested whether these compounds would serve as substrates for an EctD-mediated hydroxylation reaction, but this was not the case. This finding indicates that the *S. salexigens* EctD protein is rather substrate-specific and does not hydroxylate ectoine derivatives with either a five-membered ring structure (DHMICA) or a seven-membered ring structure (homoectoine); ectoine itself is a six-membered ring structure (Fig. 1).

One-dimensional ^{1}H NMR Spectroscopy of the EctD Enzymatic Product—5-Hydroxyectoine produced *in vivo* by *S. parvulus* is known to have the (4*S*,5*S*) stereochemical configuration (45). To test whether the 5-hydroxyectoine produced by the purified ectoine hydroxylase from *S. salexigens* yielded 5-hydroxyectoine in the same stereochemical configuration, we incubated (*S*)-ectoine with the EctD protein *in vitro* until the substrate was completely converted. The stereochemical configuration of the *de novo* synthesized 5-hydroxyectoine was then analyzed by 300 MHz ^{1}H NMR spectroscopy. The resulting NMR spectrum of the *in vitro* synthesized 5-hydroxyectoine was compared with an ^{1}H NMR spectrum prepared from commercially available 5-hydroxyectoine that was isolated by bacterial milking (46) from the *Marinococcus* sp. strain M52. Both ^{1}H NMR spectra were identical with respect to chemical shifts and ^{1}H-^{1}H coupling-patterns (Fig. 4). It thus can be inferred from this experiment that the stereochemical positioning of the hydroxyl-group into (*S*)-ectoine by the purified *S. salexigens* EctD protein is the same as that observed *in vivo* for 5-hydroxyectoine isolated from *S. parvulus* (45) and *Marinococcus* sp. strain M52.

Cloning of the Ectoine Biosynthetic Genes from S. salexigens—Genes for enzymes that operate in the same biosynthetic pathway often cluster together in bacterial genomes. We speculated that the gene encoding the ectoine hydroxylase from *S. salexigens* might be positioned in the vicinity of the ectoine biosynthetic genes (*ectABC*), and we set out to clone and sequence the *ectABC* gene cluster from *S. salexigens*. In total, the DNA sequence of a 2893-bp fragment covering the entire *ectABC* gene cluster was established and deposited in GenBank™ under accession number AY935521. The *ectABC* genes from various microorganisms have already been characterized in considerable detail at the molecular level (14–16, 18, 19, 47, 48), and the EctABC proteins from *H. elongata* have been studied biochemically (21). The features of the EctABC proteins from *S. salexigens* closely correspond to those from other microorganisms and are therefore not further discussed here.

Cloning of the Structural Gene for the Ectoine Hydroxylase from S. salexigens—We inspected the 5′ and 3′ regions of the sequenced *ectABC* gene cluster from *S. salexigens* for the presence of an open reading frame (or an incomplete reading frame), which potentially could encode a hydroxylase. However, neither region could encode such a protein. Hence, our initial hypothesis that the structural gene for the ectoine hydroxylase from *S. salexigens* might be located in the vicinity of the *ectABC* ectoine biosynthetic genes proved to be incorrect.

To clone the structural gene for the ectoine hydroxylase from *S. salexigens*, we took advantage of the genome sequences of the *Mycobacterium smegmatis* strain MC2 155 (NC 008596), *S. coelicolor* A3 (2) (NC 003888), and *Bordetella parapertussis* (NC 002928). Each of these microbial species contains an *ectABC* gene cluster, which is immediately followed at the 3′ end by a gene currently annotated in the data bases as a potential proline hydroxylase. These observations raised the possibility that the function of the mentioned genes have been incorrectly

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annotated in the various genome projects and actually operate as ectoine hydroxylases. Using a PCR approach with heterologous primers derived from the deduced amino acid sequence of the various potential ectoine hydroxylases, we initially recovered a 270-bp DNA fragment that encoded part of a protein with high sequence identity to the putative ectoine hydroxylases from *M. smegmatis*, *S. coelicolor* A3 (2), and *B. parapertussis*. This DNA fragment was then used as a hybridization probe to recover a 4.1-kb EcoRI restriction fragment from a λZAP-Express gene library. The recombinant λ phage was converted to a plasmid, and a 1977-bp DNA segment was sequenced (AY935522). Inspection of the DNA sequence (Fig. 5) revealed a 900-bp open reading frame that encodes a protein with a calculated molecular mass of 34.4 kDa (300 amino acids). This molecular mass matches exactly that of the purified ectoine hydroxylase from *S. salexigens* determined by us by mass spectrometry. Furthermore, the predicted 15 NH_2-terminal amino acids, as deduced from the DNA sequence of the cloned gene, perfectly match those that were determined experimentally for the NH_2 terminus of the purified EctD ectoine hydroxylase from *S. salexigens*.

Using the predicted amino acid sequence of EctD as template for data base searches, we identified 46 proteins from a variety of both Gram-negative and Gram-positive *Bacteria* with a high degree (61–73%) of amino acid sequence identity to the EctD protein from *S. salexigens*. In 30 of the 46 *ectD*-containing microorganisms, the *ect*D gene is positioned immediately downstream of an *ectABC* gene cluster, whereas in 16 cases the *ectD* gene is not linked to the *ectABC* operon. However, these microorganisms contain *ectABC* gene clusters elsewhere in their genome. This situation is therefore identical to that occurring in *S. salexigens*.

The 46 putative ectoine hydroxylases are each of similar length (284–318 amino acids), and the amino acid sequence identity of these proteins with the EctD protein from *S. salexigens* extends over the entire length of the polypeptide chain. The various EctD proteins exhibit a limited amino acid sequence identity (26% to 32%) to the L-proline 4-hydroxylase from *Dactylosporangium* sp. strain RH1 (38). This latter protein is a member of the non-heme-containing, iron(II)- and 2-oxoglutarate-dependent dioxygenase superfamily (EC 1.14.11.2) (41–44), members of which contain a highly conserved iron-binding motif that is referred to in the literature as the 2-His-1-carboxylate facial triad (44). Such a putative iron-binding motif can also be found in the EctD protein from *S. salexigens*, and this motif is completely conserved within the amino acid sequence of the 46 EctD-related proteins. To document this fact, we show in Fig. 6 sequence alignment of an EctD protein segment from 14 ectoine hydroxylases that contains this putative iron-binding motif. This region also contains a block of 9 amino acid residues that is completely conserved within the 46 EctD related proteins currently deposited in the data base. These 9 amino acid residues (W-H-S-D-F-E-T-W-H) can serve as a signature sequence motif for ectoine hydroxylases because only these types of proteins are recovered in data base searches when this motif is used as a search template.

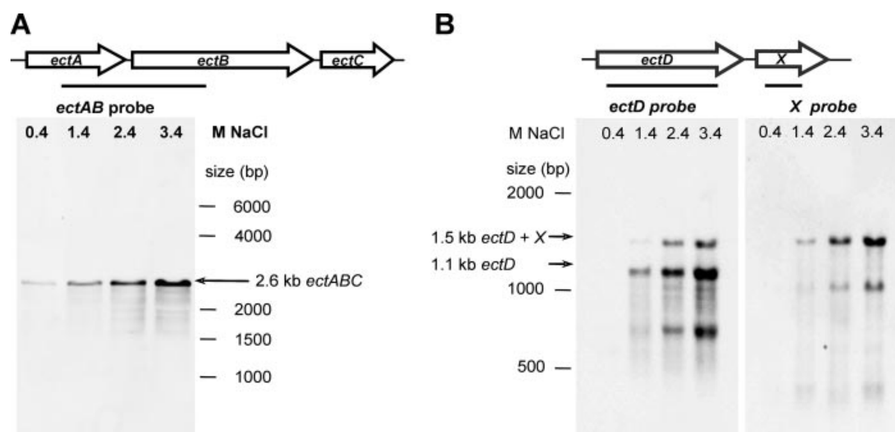

FIGURE 5. **Northern blot analysis of the osmotically controlled *S. salexigens ectABC* operon and the *ectD* region.** Shown is the genetic organization of the *ectABC* gene cluster (*A*) and the *ectD* region (*B*) in *S. salexigens*. The cells of *S. salexigens* were grown in minimal medium with various NaCl concentrations, and total RNA was isolated from these cells when the cultures reached an optical density (AD_{578}) of ~0.5–1. Equal amounts of the obtained RNA (5 μg) were electrophoretically separated on an 1% agarose gel, blotted onto a nylon membrane, and reacted with a digoxigenin-labeled single-stranded antisense RNA-probe that covered *ectAB* (*A*), *ectD* (*B*), or a DNA segment downstream of *ectD* (*B*).

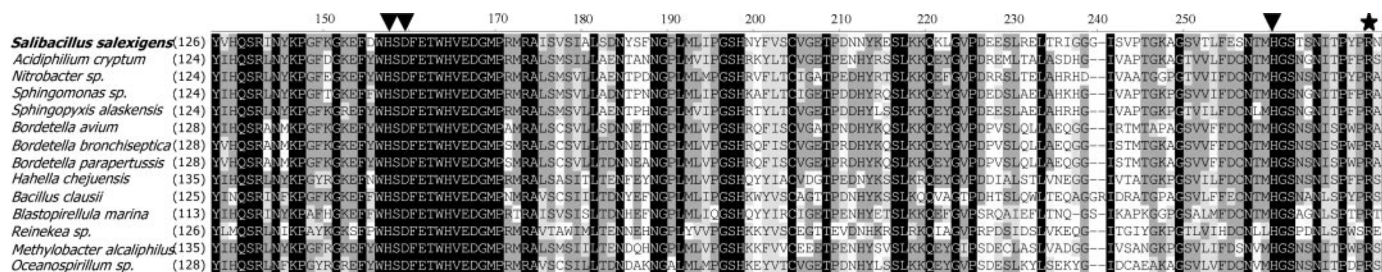

FIGURE 6. **Alignment of the EctD amino acid sequence from *S. salexigens* with various putative ectoine hydroxylases.** The amino acid sequence of the EctD protein from *S. salexigens* is aligned with putative ectoine hydroxylases from *Acidiphilium cryptum* (NZ AAOO01000006), *Bacillus clausii* (AP006627), *Blastopirellula marina* (NZ AANZ01000014), *Bordetella avium* (AM167904), *Bordetella bronchiseptica* (BX640446), *B. parapertussis* (NC 002928), *Nitrobacter* sp. Nb-311A (NZ_AAMY01000005), *Sphingomonas* sp. SKA58 (NZ AAQG01000003), *Sphingopyxis alaskensis* (NC 008048), *Hahella chejuensis* (NC 007645), *Reinekea* sp. MED297 (NZ AAOE01000022), *Methylobacter alcaliphilus* (DQ016501), and *Oceanospirillum* sp. MED92 (ZP 01167714). The iron-binding motif (H-*X*-D-X_{98-100}-H) conserved within the class of non-heme-containing, iron(II)- and 2-oxoglutarate-dependent dioxygenases is indicated by *black inverted triangles*. An arginine residue involved in the coordination of the co-substrate 2-oxoglutarate in the crystal structure of the TauD protein from *E. coli* (54) is indicated by a *star*.

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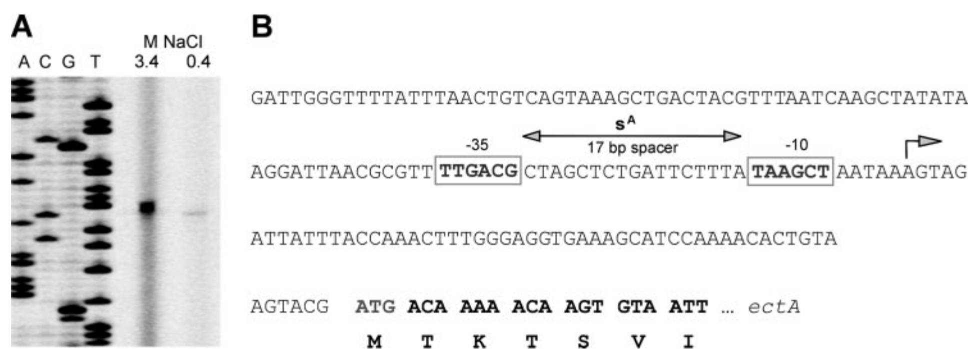

FIGURE 7. **Mapping of the transcriptional start site of the *ectABC* operon from *S. salexigens* by primer extension analysis.** *A*, cultures of *S. salexigens* were grown in minimal medium with either 0.4 M or 3.4 M NaCl until they reached the exponential growth phase (A_{578} = 0.5–1). Total RNA was isolated from these cultures, and 20 μg of this RNA was used for primer extension reactions with a DNA primer labeled with the infrared dye IRD-800; the DNA primer was located within the *ectA* coding region. The reaction products of the primer extension reaction were analyzed on an automatic DNA sequencer, and a DNA sequencing reaction was run along the primer extension products to pinpoint the exact start site of the *ectABC* mRNA transcript. *B*, nucleotide sequence of the *ectABC* promoter region. The transcription initiation site is indicated by an *arrow*, and the putative −10 and −35 sequences are *boxed*. The part of the coding sequence of *ectA* shown here is printed in *bold type*, and the encoded amino acids of EctA are shown beneath the DNA sequence.

Heterologous Expression of the ectD Gene in Escherichia coli Results in an Enzymatically Active Protein—To provide further evidence that the *ectD*-encoded protein from *S. salexigens* actually possesses ectoine hydroxylation activity, we cloned the *ectD* gene into the expression vector pASK-IBA3. In the resulting recombinant plasmid pBJ10, the *ectD* gene is positioned under the control of an anhydrotetracycline-inducible *tet* promoter, thereby allowing selective *ectD* gene expression in *E. coli*. *E. coli* is not capable of ectoine synthesis, but it can acquire this compatible solute from exogenous sources through the osmoregulated compatible solute transport systems ProU and ProP (49, 50). Plasmid pBJ10 was introduced into the *E. coli* strain DH5α by transformation, and the resulting strain was grown in a minimal medium with 250 mM NaCl that contained 5 mM ectoine. Expression of *ectD* was initiated by the addition of anhydrotetracycline to the culture, and after 1 h of further growth, the ectoine and 5-hydroxyectoine content of the cells was determined by HPLC analysis. Strain DH5α carrying the empty vector pASK-IBA3 had an ectoine pool of 213 nmol/mg dry weight, but as expected, there was no 5-hydroxyectoine detectable. In contrast, DH5α carrying the $ectD^+$ plasmid pBJ10 had an ectoine pool of 81 nmol/mg of dry weight and a 5-hydroxyectoine content of 138 nmol/mg of dry weight. Hence, 63% of the ectoine taken up by the *E. coli* cells was converted into 5-hydroxyectoine when the cells carried the *ectD* gene from *S. salexigens*. Taken together, these data provide compelling evidence that the *ectD*-encoded protein from *S. salexigens* actually possesses ectoine hydroxylase activity *in vivo*.

Transcription Levels of the ectABC Genes from S. salexigens Increase as a Function of Salinity—Ectoine biosynthesis in *S. salexigens* occurs in an osmotically controlled fashion (Fig. 2*A*). To test whether the osmotically stimulated ectoine biosynthesis was dependent on increased *ectABC* transcript level in high salinity-grown cells, we probed total RNA from *S. salexigens* cultures grown at various salinities with an *ectAB*-specific probe in a Northern blot experiment. As shown in Fig. 5*A*, the level of the *ectABC* transcript in *S. salexigens* was strongly increased when the salinity of the growth medium was raised. The detected *ectABC* transcript was 2.6 kb in size, thereby corresponding approximately to the calculated size (2232 bp) for the *ectABC* coding regions.

To identify the promoter of the *ectABC* operon, we mapped the *ectABC* promoter by primer extension analysis using total RNA from *S. salexigens* as a template for the primer extension reaction. A single transcription initiation site was detected 56 bp upstream of the *ectA* gene (Fig. 7*A*). In its vicinity there are −35 and −10 promoter sequences that correspond closely to promoter sequences typically recognized by the housekeeping sigma factor of *B. subtilis*, SigA (51) (Fig. 7*B*). From the inspection of the *S. salexigens ectABC* promoter sequence, it is not immediately obvious which DNA features make this promoter responsive to high salinity stress.

Transcription Levels of the ectD Gene from S. salexigens Increase as a Function of Salinity—To test whether the transcription of the ectoine hydroxylase structural gene *ectD* was increased when the cells were grown under high salinity, we probed total RNA from *S. salexigens* cultures grown at various salinities with an *ectD*-specific antisense RNA probe. We found that the amount of the *ectD* mRNA was strongly increased in high salinity grown cultures of *S. salexigens* (Fig. 5*B*). We detected a transcript with an approximate length of 1.5 kb (Fig. 5*B*). The size of the *ectD* coding region is 900 bp long; hence the *ectD* gene appeared to be co-transcribed with a DNA region located downstream of the *ectD* stop codon. This was confirmed by Northern blot analysis, using a hybridization probe derived from the DNA segment immediate following *ectD* (Fig. 5*B*).

Data base searches using the BLAST network service (52) revealed that the DNA region positioned downstream of *ectD* could encode a partial reading frame whose deduced gene product shows amino acid sequence similarity to MarR-type regulators of bacterial transcription (53). However, the reading frame for this putative regulatory protein was disrupted by two stop codons, and no start codon could be found. We do not have a satisfying explanation for the puzzling observation that the DNA segment positioned downstream of *ectD* is transcribed (Fig. 5*B*), but no appropriate open reading frame is detectable.

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