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Synthesis and Uptake of the Compatible Solutes Ectoine and 5-Hydroxyectoine by *Streptomyces coelicolor* A3(2) in Response to Salt and Heat Stresses[▽]

Jan Bursy,[1] Anne U. Kuhlmann,[1] Marco Pittelkow,[1] Holger Hartmann,[1] Mohamed Jebbar,[2]† Antonio J. Pierik,[1] and Erhard Bremer[1]*

Laboratory for Microbiology, Department of Biology, Philipps University Marburg, Karl-von-Frisch Str. 8, D-35032 Marburg, Germany,[1] and Département Osmoregulation chez les Bactéries, Université de Rennes 1, UMR-CNRS 6026, Rennes, France[2]

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***Streptomyces coelicolor* A3(2) synthesizes ectoine and 5-hydroxyectoine upon the imposition of either salt (0.5 M NaCl) or heat stress (39°C). The cells produced the highest cellular levels of these compatible solutes when both stress conditions were simultaneously imposed. Protection against either severe salt (1.2 M NaCl) or heat stress (39°C) or a combination of both environmental cues could be accomplished by adding low concentrations (1 mM) of either ectoine or 5-hydroxyectoine to *S. coelicolor* A3(2) cultures. The best salt and heat stress protection was observed when a mixture of ectoine and 5-hydroxyectoine (0.5 mM each) was provided to the growth medium. Transport assays with radiolabeled ectoine demonstrated that uptake was triggered by either salt or heat stress. The most effective transport and accumulation of [^{14}C]ectoine by *S. coelicolor* A3(2) were achieved when both environmental cues were simultaneously applied. Our results demonstrate that the accumulation of the compatible solutes ectoine and 5-hydroxyectoine allows *S. coelicolor* A3(2) to fend off the detrimental effects of both high salinity and high temperature on cell physiology. We also characterized the enzyme (EctD) required for the synthesis of 5-hydroxyectoine from ectoine, a hydroxylase of the superfamily of the non-heme-containing iron(II)- and 2-oxoglutarate-dependent dioxygenases (EC 1.14.11). The gene cluster (*ectABCD*) encoding the enzymes for ectoine and 5-hydroxyectoine biosynthesis can be found in the genome of *S. coelicolor* A3(2), *Streptomyces avermitilis*, *Streptomyces griseus*, *Streptomyces scabiei*, and *Streptomyces chrysomallus*, suggesting that these compatible solutes play an important role as stress protectants in the genus *Streptomyces*.**

To counteract the deleterious effects of high salinity on cell physiology and loss of cell water, many microorganisms accumulate a particular class of highly soluble, low-molecular-weight organic compounds, the so-called compatible solutes. These organic osmolytes are congruous with cellular functions and can be amassed by the cell to exceedingly high levels either through synthesis or uptake from the environment (12, 26, 44, 78, 79). As a result, water reenters the cell, an appropriate hydration level of the cytoplasm is achieved, turgor is readjusted, and finally cell growth can proceed under osmotically unfavorable conditions (70).

In addition to their role in the adaptation of microorganisms to high-osmolality habitats, compatible solutes also have protein-stabilizing properties (3) that support the correct folding of polypeptides under denaturing conditions both in vitro (4, 9, 18, 53, 54, 69) and in vivo (5, 10, 22, 34). These properties most likely result from unfavorable interactions of compatible solutes with the protein backbone (7, 75) and the concomitant preferential exclusion of these compounds from the immediate hydration shell of proteins (3). The stabilizing properties of compatible solutes probably also contribute to their function as microbial stress protectants against heat stress (8, 16, 19, 24, 25, 31, 33, 56, 67) and chill stress (2, 13, 42, 48, 50). Since compatible solutes function as protein stabilizers under various types of stress conditions (47), they are also sometimes referred to in the literature as chemical chaperones (22, 24). Compatible solutes also interact in various ways with nucleic acids (52, 74) and can influence protein-DNA interactions (66).

One of the most widely produced compatible solutes in the domain of the *Bacteria* are the tetrahydropyrimidine ectoine [(*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] and its hydroxylated derivative, 5-hydroxyectoine [(*S*,*S*)-2-methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] (15, 23, 26). In all ectoine-producing *Bacteria* analyzed so far, ectoine biosynthesis is strongly enhanced under high-osmolality growth conditions (15, 17, 23, 26, 27, 49, 50, 71, 73). Molecular analysis of ectoine biosynthesis in various gram-positive and gram-negative *Bacteria* has shown that the ectoine biosynthetic enzymes are encoded by an evolutionarily highly conserved gene cluster, *ectABC* (20, 28, 49, 50, 55, 64, 71, 73). The disruption of the *ectABC* genes in various microorganisms results in a defect in ectoine synthesis and a concomitant salt-sensitive growth phenotype (21, 28, 64, 72, 80). These findings highlight the importance of ectoine biosynthesis for microbial adaptation to high-salinity environments.

Ectoine biosynthesis is mediated by a three-step enzymatic reaction that converts the precursor L-aspartate-β-semialde-

* Corresponding author. Mailing address: Laboratory for Microbiology, Department of Biology, Philipps University Marburg, Karl-von-Frisch Str. 8, D-35032 Marburg, Germany. Phone: (49)-6421-2821529. Fax: (49)-6421-2828979. E-mail: bremer@staff.uni-marburg.de.

† Present address: Laboratory of Extreme Environments, Microbiology, University of West Brittany (Brest), European Institute of Marine Studies, Technopôle Brest-Iroise, F-29280 Plouzané, France.

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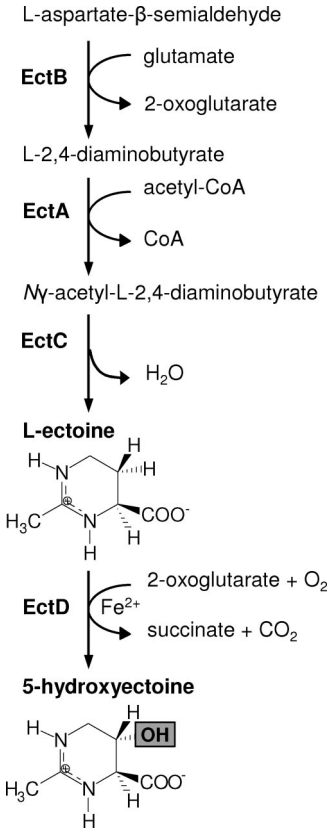

FIG. 1. Pathway for the biosynthesis of ectoine and 5-hydroxyectoine. The biosynthetic route for the production of ectoine is shown; it is taken from the proposals made by Louis and Galinski (55) and Ono et al. (61). The enzymes responsible for ectoine biosynthesis are L-2,4-diaminobutyrate transaminase (EctB), L-2,4-diaminobutyrate acetyltransferase (EctA), and ectoine synthase (EctC). The enzymatic conversion of ectoine into 5-hydroxyectoine by the EctD protein (ectoine hydroxylase) is based on results obtained by Bursy et al. (15).

hyde, an intermediate in amino acid metabolism, into ectoine (Fig. 1) (26, 55, 63, 71). The ectoine biosynthetic enzymes (EctABC) from *Halomonas elongata* have been purified and characterized biochemically (61). Some ectoine producers also synthesize a derivative of ectoine, 5-hydroxyectoine (36). Its production was first discovered in *Streptomyces parvulus* (37) and has since been shown to occur in a large number of *Bacteria* (15, 23, 26). Like ectoine, 5-hydroxyectoine serves as a compatible solute in vivo and it exhibits protein-stabilizing properties in vitro (9, 40, 41, 47, 54, 57, 58). The formation of 5-hydroxyectoine occurs through direct hydroxylation of ectoine (15) via the evolutionarily conserved enzyme ectoine hydroxylase (EctD) (15, 27, 65). This enzyme is a member of the non-heme-containing, iron(II)- and 2-oxoglutarate-dependent dioxygenase superfamily (EC 1.14.11) (15, 30).

Here we have studied the effects of ectoine and 5-hydroxyectoine on the physiology of the soil-living actinomycete *Streptomyces coelicolor* A3(2). Our data show that both the synthesis and uptake of these two compatible solutes can be triggered by high salinity and high growth temperature, thereby conferring a considerable degree of salt and heat stress protection to the cells. We also have purified and biochemically characterized the 5-hydroxyectoine forming enzyme EctD from *S. coelicolor* A3(2).

MATERIALS AND METHODS

Chemicals. Ectoine and 5-hydroxyectoine were purchased from Biomol (Hamburg, Germany) or were a kind gift from Bitop AG (Witten, Germany). L-Proline, 3-hydroxy-DL-proline, and 4-hydroxy-L-proline were obtained from Sigma-Aldrich (Munich, Germany). [^{14}C]ectoine was biologically prepared from L-[U-^{14}C]glutamic acid supplied to *Brevibacterium linens* ATCC 9175 cells grown in minimal medium containing 1 M NaCl. Radiolabeled ectoine was extracted from these cells and purified by paper chromatography as previously described by Jebbar et al. (38). Ectoine specific radioactivity was determined from the radioactivity measured with a liquid scintillation counter, and the ectoine concentration was determined from the A_{220} (38).

Bacteria and growth conditions. The *Streptomyces coelicolor* A3(2) strain used throughout this study was kindly provided by W. Wohlleben (University of Tübingen, Germany). *S. coelicolor* A3(2) was maintained and propagated on LB agar plates incubated at 28°C. *S. coelicolor* A3(2) cultures were grown in a chemically defined minimal medium (SMM without polyethylene glycol 6000) with glucose as the carbon source (45) in a shaking water bath set at 220 rpm with the indicated NaCl concentrations and growth temperatures. To avoid mycelial growth of *S. coelicolor* A3(2), approximately 50 glass beads (diameter of 3 mm; Roth, Karlsruhe, Germany) were added to the culture. To allow efficient production of the ectoine hydroxylase, *S. coelicolor* A3(2) was cultivated in 2 liters of SMM with 0.5 M NaCl at 39°C without glass beads in a 5-liter flask that was shaken with 220 rpm. The cells were harvested by centrifugation (4°C, 6,000 × *g*) and stored at −20°C until further use for the purification of the ectoine hydroxylase.

HPLC analysis of ectoine and 5-hydroxyectoine from cell extracts. Cultures (80 ml) of *S. coelicolor* A3(2) were grown in a shaking water bath set at 220 rpm in minimal medium (SMM) with the indicated NaCl concentrations and growth temperatures until the cultures reached an optical density at 578 nm (OD_{578}) of approximately 1. The cells were harvested by centrifugation (4°C, 2,800 × *g*) and lyophilized, the dry weight of the cells was determined, and the cells were then extracted using a modified version of the technique of Bligh and Dyer (51). Ectoine and 5-hydroxyectoine contents of the samples were measured by high-performance liquid chromatography (HPLC) analysis as detailed by Kuhlmann and Bremer (49). Quantification of ectoine and 5-hydroxyectoine was performed with the ChromStar 6 software (SCPA, Stuhr, Germany) using commercially available ectoine and 5-hydroxyectoine samples as reference standards.

Uptake assays with [^{14}C]ectoine. Cells of *S. coelicolor* A3(2) were grown in SMM to mid-log growth phase (OD_{578} of about 0.5) under the indicated temperatures and salinity conditions. Samples of 2 ml were taken, and ^{14}C-ectoine (4.22 MBq mmol^{-1}) was added to the cells at a concentration of 19 μM; the Eppendorf tube was vigorously shaken to provide enough aeration to the cells. For the transport assays, 0.3-ml samples were taken at different time intervals, and the cells were then collected by filtration through 0.45-μm-pore-size filters (Schleicher and Schuell GmbH, Dassel, Germany). The cells were washed with 20 ml isotonic SMM, and the radioactivity retained on the filters was determined by liquid scintillation counting. For the determination of the pool size of [^{14}C]ectoine in *S. coelicolor* A3(2), 20-ml cell cultures were propagated in SMM in 100-ml Erlenmeyer flasks in a shaking water bath set at 220 rpm and the appropriate growth temperature; the cultures contained approximately 15 glass beads to prevent mycelium formation of *S. coelicolor* A3(2) cells. [^{14}C]ectoine (4.22 MBq mmol^{-1}) was added to the cultures at a concentration of 19 μM, and the cultures were propagated in a shaking water bath for 1 h. Samples (0.3 ml) were taken, and the [^{14}C]ectoine accumulated by the cells was measured as described above.

Ectoine hydroxylase activity assay. Ectoine hydroxylase activity in cell extracts and after purification of EctD was assayed by measuring the conversion of ectoine to 5-hydroxyectoine by HPLC analysis as detailed by Kuhlmann and Bremer (49) and by Bursy et al. (15). The purified ectoine hydroxylase was assayed as detailed by Bursy et al. (15), except that catalase was omitted from the reaction mixture. One unit of ectoine hydroxylase activity is defined as the conversion of 1 μmol of ectoine to 1 μmol of 5-hydroxyectoine per minute (15).

Detection of L-proline and its hydroxylation derivatives by HPLC analysis. To test whether L-proline can serve as a substrate for the purified ectoine hydroxylase from *S. coelicolor* A3(2), L-proline was used instead of ectoine in the activity assay as described above. Proline and its hydroxylated derivatives were analyzed by HPLC analyses after modification with 9-fluorenylmethyl chloroformate (FMOC) (51) as detailed by Bursy et al. (15). L-Proline, 3-hydroxy-DL-proline, and 4-hydroxy-L-proline were used as reference standards for the HPLC analysis.

Purification of the ectoine hydroxylase. All operations were carried out at 4°C unless otherwise stated. Column chromatography was performed with a fast-performance liquid chromatography system (Amersham Biosciences, Freiburg, Germany). Approximately 20 g (wet weight) of frozen cells was resuspended in 40 ml of buffer A: 100 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.5], 2 mM dithiothreitol, 0.5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.4 mM EDTA, and 5% (vol/vol) glycerol. The cells were disrupted by passing them four times through a French pressure cell (16,000 lb/in^2). After removal of cell debris by ultracentrifugation (1 h, 95,000 × *g*), the supernatant was extensively dialyzed at 4°C against 5 liters of 25 mM TES (pH 7.5) (buffer B) to completely remove the endogenously produced ectoine and 5-hydroxyectoine in *S. coelicolor* A3(2) cells grown in SMM with 0.5 M NaCl at 39°C since the endogenously produced ectoine and 5-hydroxyectoine would interfere with the HPLC-based activity assay of the ectoine hydroxylase. The cell extract was filtered with a 0.45-μm sterile filter and loaded onto a Source 15Q column (bed volume, 22 ml; Amersham Biosciences, Freiburg, Germany). This column was equilibrated with buffer B. The sample-loaded column was washed with 70 ml of buffer B, and the proteins were eluted from the column by applying a linear NaCl gradient (0 to 0.4 M NaCl in buffer B). The ectoine hydroxylase eluted from the Source 15Q column at approximately 150 mM NaCl. The pooled protein fractions exhibiting ectoine hydroxylase activity were concentrated by ultrafiltration (10-kDa cutoff [Centricon YM-10; Millipore GmbH, Germany]) and loaded onto a HiLoad 16/60 Superdex 75 prep-grade column (Amersham Biosciences, Freiburg, Germany). This gel filtration column was equilibrated with buffer B containing 150 mM NaCl at a temperature of 25°C. Fractions with ectoine hydroxylase activity were eluted with buffer B containing 150 mM NaCl, after 55 ml had passed through the column; this corresponds approximately to a molecular mass of 35 kDa. The pooled active fractions were concentrated (Centricon YM-10), desalted with buffer C (3 mM potassium phosphate buffer [pH 7.4]), and loaded onto a ceramic hydroxyapatite column (Macro-Prep type I, 20 μm; Bio-Rad Laboratories, Munich, Germany) that had been equilibrated with buffer C. The EctD enzyme did not bind to this column material and was recovered from the flowthrough fractions. The purified enzyme was stored at −80°C in buffer B with 150 mM NaCl after shock freezing in liquid nitrogen.

Purity of the EctD protein was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in a Mini-PROTEAN 3 apparatus (Bio-Rad Laboratories, Munich, Germany) on a 12.5% polyacrylamide gel, and proteins were visualized by staining with Coomassie brilliant blue. Protein concentrations were measured by the method of Bradford (11) with the Bio-Rad Protein assay (Bio-Rad Laboratories, Munich, Germany), using bovine serum albumin as a reference standard. The concentration of the pure EctD protein was calculated by measuring its A_{280}, taking into account the molecular mass and the extinction coefficient derived from the amino acid composition of the EctD protein.

Amino acid sequencing of EctD. For the determination of the amino-terminal end of the purified ectoine hydroxylase, the EctD protein was separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Millipore, Schwalbach, Germany). After the protein was stained with Coomassie brilliant blue, the band was cut out of the membrane and subjected to sequential protein sequencing cycles by Edman degradation. Protein sequencing was performed by the Universitätsklinikum Münster, AG Proteinanalytik (G. Mersmann).

Determination of the molecular mass of EctD by matrix-assisted laser desorption ionization–time of flight mass spectrometry. One-microliter samples of various dilutions of the purified EctD enzyme were mixed on a gold-plated target with 1 μl of a saturated solution of sinapinic acid in 0.1% trifluoroacetic acid–67% acetonitrile and dried under air. The samples were analyzed using a Voyager-DE/RP matrix-assisted laser desorption ionization–time of flight mass spectrometer in the linear mode.

Determination of the relative molecular mass of EctD by gel filtration chromatography. The apparent molecular mass of the purified ectoine hydroxylase was determined by gel filtration chromatography on a HiLoad 16/60 Superdex 75 prep-grade column (Amersham Biosciences, Freiburg, Germany) equilibrated with buffer B containing 150 mM NaCl at 25°C. Aprotinin from bovine lung (6.5 kDa), cytochrome *c* from horse heart (12.4 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), albumin from bovine serum (66.2 kDa), and dextran blue (2,000 kDa) were used as molecular mass marker standards (Sigma-Aldrich, Munich, Germany).

Computer analysis of DNA and protein sequences. DNA and protein sequences were analyzed and assembled with the Vector NTI 8.0 software (Invitrogen Bioinformatics, Karlsruhe, Germany). Searches for proteins related to the EctABC and EctD enzymes from *S. coelicolor* A3(2) were performed with the BLAST program at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/BLAST) (1). Additional amino acid sequence comparisons were carried out through the web site provided by the Sanger Institute (www.sanger.ac.uk/Projects/S_scabies). Protein sequences were aligned with the ClustalW algorithm (76) provided with the Vector NTI software using default values.

RESULTS

Ectoine and 5-hydroxyectoine biosynthesis is triggered in response to high salinity and high growth temperature in *S. coelicolor* A3(2). The *S. coelicolor* A3(2) genome sequence (6) contains an ectoine biosynthetic gene cluster (*ectABC*) (GeneID 1097298, 1097299, and 1097300). The *ectABC* genes are followed in the same transcriptional direction by a gene (GeneID 1097301) whose deduced amino acid sequence is homologous to functionally characterized ectoine hydroxylases (EctD) from *Streptomyces chrysomallus* (65) (84% amino acid identity), *Chromohalobacter salexigens* (27) (48% amino acid identity) and *Salibacillus salexigens* (15) (48% amino acid identity). The presence of the *ectABCD* gene cluster in the genome of *S. coelicolor* A3(2) suggested that this soil-living actinomycete may be able to synthesize both of the compatible solutes ectoine and 5-hydroxyectoine.

We monitored ectoine and 5-hydroxyectoine production by HPLC in cultures of *S. coelicolor* A3(2) that were grown in a minimal medium (SMM) with glucose as the carbon source at the optimal growth temperature of 28°C and in a minimal medium whose salinity had been modestly raised by the addition of 0.5 M NaCl. No ectoine or 5-hydroxyectoine was detectable by cells grown in SMM in the absence of added salt, but considerable amounts of both ectoine and 5-hydroxyectoine were synthesized by *S. coelicolor* A3(2) under the high-salinity growth conditions (Fig. 2B). Ectoine biosynthesis preceded 5-hydroxyectoine production, and eventually the 5-hydroxyectoine content of the cells superseded that of ectoine (Fig. 2B).

Previously, ectoine and 5-hydroxyectoine formation was observed in *Streptomyces griseus* when the growth temperature was suddenly shifted from 28°C to 39°C (56). To test whether the ectoine and 5-hydroxyectoine contents of the cells would also increase in *S. coelicolor* A3(2) continuously grown at high growth temperature in the absence of salt stress, we propagated this strain in SMM at 39°C. We found that ectoine and 5-hydroxyectoine were both produced in the heat-stressed cells (Fig. 2C), but they contained primarily 5-hydroxyectoine. The overall ectoine and 5-hydroxyectoine contents of the heat-stressed cells (Fig. 2C) were considerably lower then those of the *S. coelicolor* A3(2) cells grown at 28°C with 0.5 M NaCl (Fig. 2B). The highest intracellular levels of ectoine and 5-hydroxyectoine were detected when *S. coelicolor* A3(2) was grown simultaneously under salt (0.5 M NaCl) and heat stress (39°C) (Fig. 2D), suggesting that both environmental cues act together to trigger enhanced ectoine and 5-hydroxyectoine production.

Salt-stressed cells of *S. coelicolor* A3(2) contained a mixture of ectoine and 5-hydroxyectoine, with 5-hydroxyectoine dominating the ectoine/5-hydroxyectoine solute pool in stationary-phase cultures (Fig. 2B and 2D). A preferential production of 5-hydroxyectoine in the stationary phase has also been observed in the moderate halophile *S. salexigens* (15). One might thus speculate that 5-hydroxyectoine serves a special protect-

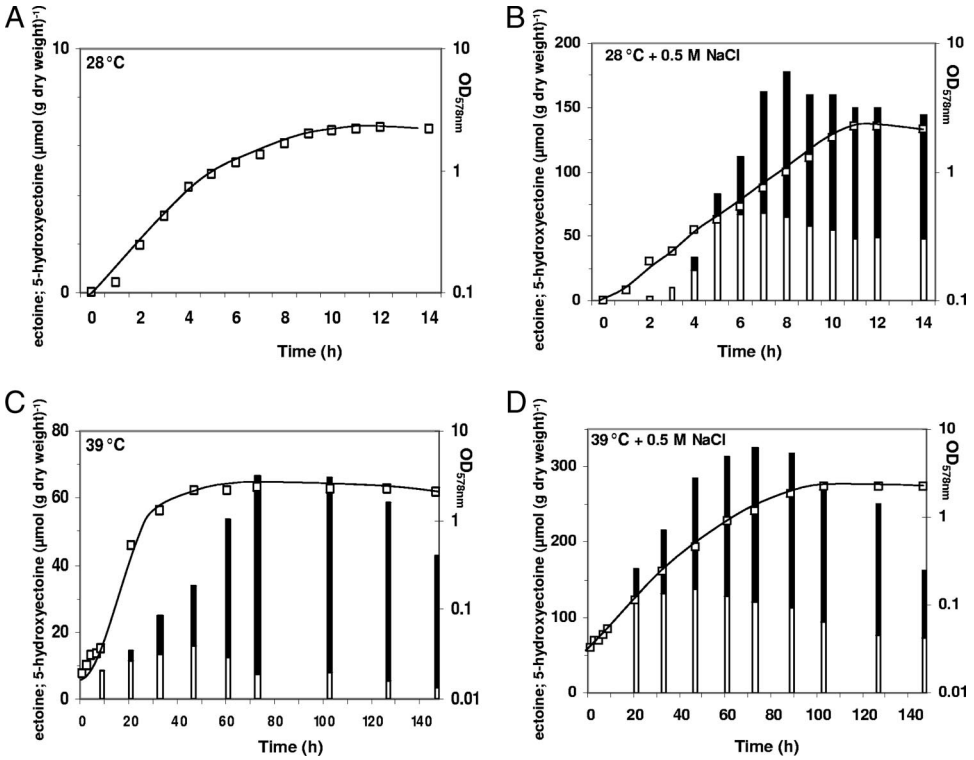

FIG. 2. Synthesis of ectoine and 5-hydroxyectoine in *S. coelicolor* A3(2) in response to high salinity and high growth temperature. *S. coelicolor* A3(2) was grown in a chemically defined minimal medium (SMM) with the indicated salinities and growth temperatures. Growth of the *S. coelicolor* A3(2) cultures was monitored by measuring the OD_{578} (open squares). The ectoine and 5-hydroxyectoine contents of the cells were determined by HPLC analysis. Intracellular ectoine content is indicated by white bars, and intracellular 5-hydroxyectoine content is shown by black bars. The data shown are the means of two independent measurements of two separately grown cultures.

ing function in those cells whose growth has slowed down or ceased entirely. This physiological function of 5-hydroxyectoine might not necessarily be connected with its role in the adaptation to unfavorable osmotic conditions. Heat-stressed *S. coelicolor* A3(2) cells contained primarily 5-hydroxyectoine (Fig. 2C), implying that the hydroxylated derivative of ectoine is the physiologically better heat stress protectant.

The inspection of the genetic organization of the *ectABCD* gene cluster in the genome of *S. coelicolor* A3(2) (6) suggests that these four genes are cotranscribed as an operon. Biosynthesis of 5-hydroxyectoine by the EctD hydroxylase requires the prior production of ectoine via the sequential enzymatic reactions of the EctBAC enzymes (Fig. 1) (15). The preferential accumulation of 5-hydroxyectoine in either heat-stressed or stationary-phase cultures (Fig. 2) suggests that either posttranscriptional (e.g., preferential stabilization of the *ectD* portion of the *ectABCD* mRNA) or posttranslational (e.g., enhanced stability of the EctD enzyme in comparison to the EctABC proteins) effects might operate in *S. coelicolor* A3(2) that eventually lead to 5-hydroxyectoine pools exceeding those of ectoine. In addition, the intracellular level of 2-oxoglutarate, a cosubstrate of the EctD enzyme (15), could modulate the extent of hydroxylation of preexisting ectoine in either heat-stressed or stationary-phase cultures.

There was a drop in the intracellular pool of ectoine and 5-hydroxyectoine in the late stationary growth phase of *S. coelicolor* A3(2) cultures that underwent prolonged propagation at 39°C and 0.5 M NaCl (Fig. 2D); we currently have no adequate physiological explanation for this observation. However, we have previously observed such a phenomenon when we analyzed the ectoine and 5-hydroxyectoine contents of cells from the moderate halophile *S. salexigens* (15).

Exogenously provided ectoine and 5-hydroxyectoine extend the upper growth limit of *S. coelicolor* A3(2) under high-salinity growth conditions. Ectoine and 5-hydroxyectoine function as osmoprotectants, and an exogenous supply of low concentrations (1 mM) of either ectoine or 5-hydroxyectoine often offsets the detrimental effects of high salinity on cell growth (29, 38, 39, 62, 77). To test if a supply of either ectoine or 5-hydoxyectoine would have a beneficial effect on the proliferation of cells of *S. coelicolor* A3(2) confronted with high salinity, we grew this strain in a minimal medium at various NaCl concentrations in the absence or presence of either ectoine or 5-hydroxyectoine for 24 h and then determined the OD of the cultures (Fig. 3). In the absence of a compatible solute, the growth of *S. coelicolor* A3(2) was not significantly affected up to a concentration of 0.6 M NaCl. However, a further increase in the salinity of the growth medium resulted in a successive decline in cell growth and the addition of 1.4 M NaCl completely inhibited proliferation of *S. coelicolor* A3(2) (Fig. 3). The addition of either 1 mM ectoine or 1 mM 5-hydroxyectoine to the high-salinity-grown cultures had a strong osmoprotective effect and allowed *S. coelicolor* A3(2) to grow under

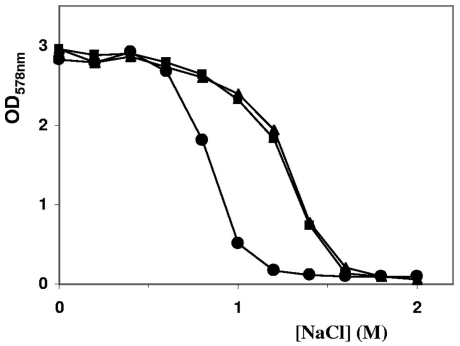

FIG. 3. Ectoine and 5-hydroxyectoine protect *S. coelicolor* A3(2) against the growth-inhibiting effect of high salinity. *S. coelicolor* A3(2) was grown at 28°C in a chemically defined minimal medium (SMM) with the indicated salinities. The various cultures were inoculated (OD_{578} of 0.1) with an overnight culture of *S. coelicolor* A3(2) grown in SMM in the absence of additional NaCl. The cultures were then cultivated for 24 h in the absence (closed circles) or presence of 1 mM ectoine (closed squares) or 1 mM 5-hydroxyectoine (closed triangles).

saline conditions that were completely inhibitory for the cells propagated in the absence of these compatible solutes (Fig. 3).

Exogenously provided ectoine and 5-hydroxyectoine confer both thermoprotection and osmoprotection to *S. coelicolor* A3(2). To monitor the beneficial effects of ectoine and 5-hydroxyectoine on the growth of salt-stressed *S. coelicolor* A3(2) cells in greater detail, we grew this strain in a minimal medium with 1.2 M NaCl at its optimal growth temperature (28°C) in the presence of either ectoine (1 mM), 5-hydroxyectoine (1 mM), or a mixture of both compounds (0.5 mM each). As expected from the data documented in Fig. 3, the addition of 1.2 M NaCl to the medium strongly impaired the growth of *S. coelicolor* A3(2). Both ectoine and 5-hydroxyectoine greatly improved cell growth, and the equimolar mixture (0.5 mM each) of both ectoines turned out to be the best osmoprotectant (Fig. 4A).

S. coelicolor A3(2) synthesizes both ectoine and 5-hydroxyectoine when it is propagated at high growth temperature (39°C), suggesting that the production of these compatible solutes serves a thermoprotective function. We found that an exogenous supply of either ectoine (1 mM) or 5-hydroxyectoine (1 mM) conferred effective thermoprotection to *S. coelicolor* A3(2) cells (Fig. 4B). As observed for the salt-stressed cells (Fig. 4A), the most efficient stress protection was achieved when an equimolar mixture of ectoine and 5-hydroxyectoine was provided to the heat-stressed cells (Fig. 4B).

We then combined both salt stress (1.2 M NaCl) and heat stress (39°C) and monitored the influence of the ectoines on cell growth. In the absence of a compatible solute, *S. coelicolor* A3(2) was unable to grow at all under these stressful conditions, but both ectoine and 5-hydroxyectoine had a strong stress protective effect and efficiently rescued cell growth. Again, the most efficient stress protection was afforded by the equimolar mixture of both ectoines (Fig. 4C).

Addition of the equimolar mixture of the ectoines to the medium provided the best salt stress (Fig. 3A) and heat stress (Fig. 3B) protection to *S. coelicolor* A3(2) cultures and in particular against the combined detrimental effects of both types of stress (Fig. 3C). We currently have no adequate physiological or biophysical explanation why the stress-protective effects of a mixture of the ectoines exceed that of either of the compatible solutes alone (Fig. 3).

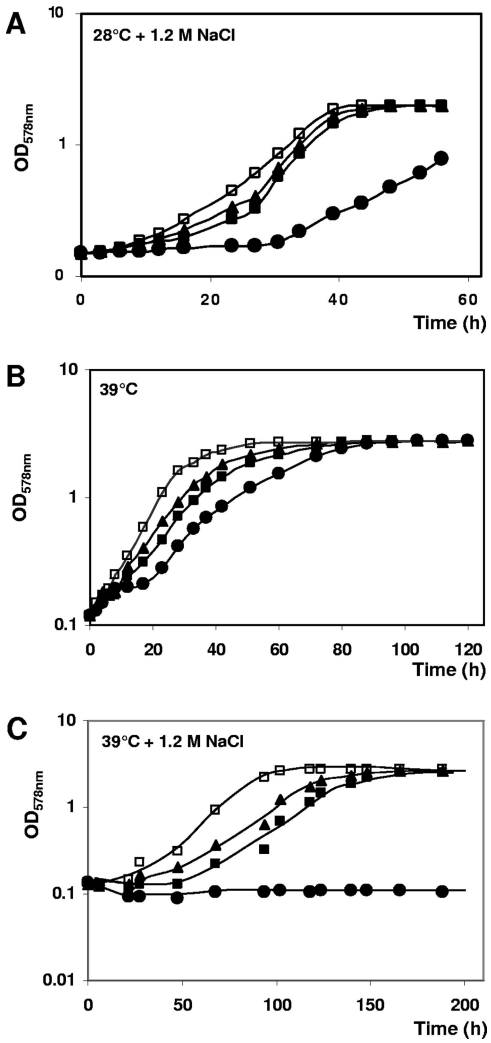

FIG. 4. Salt and heat protection of *S. coelicolor* A3(2) by exogenously provided ectoine and 5-hydroxyectoine. The cultures were inoculated to an OD_{578} of 0.1 with an overnight culture of *S. coelicolor* A3(2) in SMM at 28°C, and the cells were then grown in 25 ml of SMM in a 100-ml Erlenmeyer flask with glass beads on an orbital shaker. (A) *S. coelicolor* A3(2) cultures were grown in SMM with 1.2 M NaCl at 28°C in the absence of added compatible solutes (closed circles) or in the presence of either 1 mM ectoine (closed squares), 1 mM 5-hydroxyectoine (closed triangles), or a mixture of 0.5 mM ectoine and 0.5 mM 5-hydroxyectoine (open squares). (B) *S. coelicolor* A3(2) cultures were grown in SMM at 39°C in the absence of added compatible solutes (closed circles) or in the presence of either 1 mM ectoine (closed squares), 1 mM 5-hydroxyectoine (closed triangles), or a mixture of 0.5 mM ectoine and 0.5 mM 5-hydroxyectoine (open squares). (C) *S. coelicolor* A3(2) cultures were grown in SMM at 39°C with 1.2 M NaCl in the absence of added compatible solutes (closed circles) or in the presence of either 1 mM ectoine (closed squares), 1 mM 5-hydroxyectoine (closed triangles), or a mixture of 0.5 mM ectoine and 0.5 mM 5-hydroxyectoine (open squares).

High salinity and high growth temperature trigger ectoine uptake in *S. coelicolor* A3(2). The osmoprotective and heat stress protective effects of compatible solutes added to the

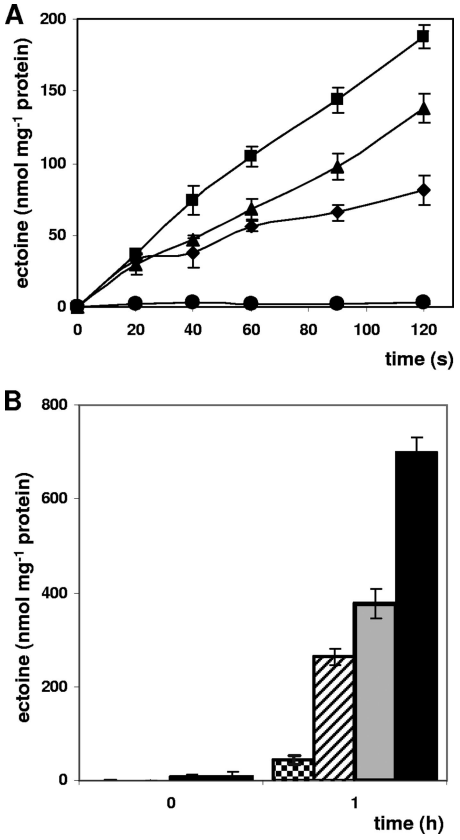

FIG. 5. Uptake and accumulation of [^{14}C]ectoine by *S. coelicolor* A3(2). (A) Cultures of *S. coelicolor* A3(2) were grown in SMM at 28°C (closed circles), at 28°C with 0.5 M NaCl (closed diamonds), at 39°C (closed triangles), and 39°C with 0.5 M NaCl (closed squares) to the mid-log growth phase (OD_{578} of 0.5 to 1). [^{14}C]ectoine was then added to the cultures to a substrate concentration of 19 μM, and uptake of the radiolabeled ectoine by the cells was monitored by scintillation counting. The data shown are the mean of at least two independent experiments. (B) Cultures of *S. coelicolor* A3(2) were grown in SMM under the conditions described in panel A. [^{14}C]ectoine was then added to the growth medium at a substrate concentration of 19 μM, and the accumulation of the radiolabeled substrate was monitored either immediately after the addition of [^{14}C]ectoine or after 1 h of incubation at the indicated conditions (dotted bars, growth in SMM at 28°C; hatched bars, growth in SMM at 28°C with 0.5 M NaCl; gray bars, growth in SMM at 39°C; black bars, growth in SMM at 39°C with 0.5 M NaCl). The data shown are the mean of at least two independent experiments.

growth medium depend on the uptake of these compounds into the cell via dedicated transport systems (33, 43). The fact that exogenously provided ectoine and 5-hydroxyectoine confer heat stress and salt stress protection to *S. coelicolor* A3(2) cultures (Fig. 4) implies that at least one ectoine/5-hydroxyectoine transport system is operating in *S. coelicolor* A3(2). To investigate this directly, we carried out transport assays with ^{14}C-labeled ectoine. The radiolabeled ectoine was added to mid-exponential growth-phase cultures (OD_{578} of 1) of *S. coelicolor* A3(2) at a substrate concentration of 19 μM, and the initial uptake of [^{14}C]ectoine by the cells was measured. No uptake of [^{14}C]ectoine was detectable in cells that were grown at the optimal growth temperature of 28°C in the absence of salt stress (Fig. 5A). A rise in the salinity (0.5 M NaCl) or growth temperature (39°C) of the cultures triggered [^{14}C]ectoine uptake (Fig. 5A). The highest level of [^{14}C]ectoine uptake occurred in *S. coelicolor* A3(2) cells that were grown under both high-salinity (0.5 M NaCl) and high-temperature (39°C) conditions (Fig. 5A).

We also monitored the accumulation of [^{14}C]ectoine by *S. coelicolor* A3(2) cells over a longer period (1 h). [^{14}C]ectoine was added at a substrate concentration of 19 μM to the cultures growing at either (i) 28°C, (ii) 28°C with 0.5 M NaCl, (iii) 39°C, or (iv) 39°C with 0.5 M NaCl. The amount of [^{14}C]ectoine accumulated by the cells was then determined after 1 h of growth by scintillation counting (Fig. 5B). Cells grown at 28°C did not accumulate significant levels of [^{14}C]ectoine. Increased salinity or an increase in growth temperature resulted in a substantial intracellular pool of [^{14}C]ectoine (Fig. 5B). The highest level of intracellular [^{14}C]ectoine was observed in *S. coelicolor* A3(2) cells that were grown under high-salinity and high-temperature conditions (Fig. 5B). Hence, the buildup of the intracellular [^{14}C]ectoine pool followed the pattern that was observed for the initial uptake characteristics of [^{14}C]ectoine (Fig. 5A and B).

Ectoine and 5-hydroxyectoine are closely related in chemical structure (Fig. 1), and it is thus likely that both compounds are taken up by the cells via the same transport system(s). The identity of the ectoine/5-hydroxyectoine transport system(s) operating in *S. coelicolor* A3(2) is currently unknown. It is also not clear whether increased [^{14}C]ectoine uptake at high salinity and high growth temperature is a consequence of increased gene expression, an effect on transporter activity, or a combination of both types of regulations.

Purification of the ectoine hydroxylase from *S. coelicolor* A3(2). The ectoine hydroxylase (EctD) from the moderate halophile *S. salexigens* is currently the only ectoine hydroxylase whose enzymatic properties have been biochemically characterized (15). We therefore set out to purify and biochemically characterize the ectoine hydroxylase (EctD) from *S. coelicolor* A3(2) as an additional representative of this family of enzymes.

Cell extracts were prepared from cells grown at 39°C in the presence of 0.5 M NaCl for the purification of the ectoine hydroxylase. Ectoine hydroxylase activity was readily detected in the crude cell extract (Table 1). We initially followed the purification scheme developed by Bursy et al. (15) for the purification of the ectoine hydroxylase from *S. salexigens*, but this did not yield a pure enzyme preparation for the EctD protein from *S. coelicolor* A3(2) (data not shown). We therefore modified the original purification scheme and purified the

TABLE 1. Summary of the steps used for purification of ectoine hydroxylase EctD from *S. coelicolor* A3(2)[a]

Purification step	Total activity (U)	Amt of protein (mg)	Sp act (U mg^{-1})	Yield (%)
Cell extract	1,546	644	2.4	100
Source 15Q column	1,142	56	20	74
Superdex column (75 pg)	519	23	22	34
Ceramic hydroxyapatite	12	0.6	20	0.8

[a] The EctD ectoine hydroxylase was purified from 20 g (wet weight) of cells of *S. coelicolor* A3(2) grown in SMM with 0.5 M NaCl at 39°C. One unit of ectoine hydroxylase activity is defined as the conversion of 1 μmol of ectoine to 1 μmol of 5-hydroxyectoine per minute (15).

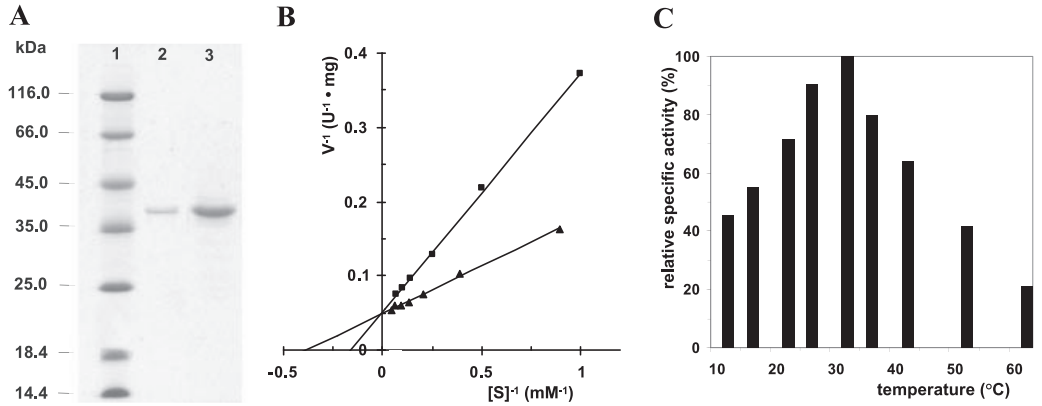

FIG. 6. Purification of the ectoine hydroxylase EctD and enzymatic properties of the purified enzyme. (A) SDS-PAGE of the purified ectoine hydroxylase (EctD) from *S. coelicolor* A3(2). Samples of the marker proteins (lane 1) and of EctD (lane 2, 0.5 μg protein; lane 3, 2 μg protein) were electrophoretically separated on an SDS–12.5% polyacrylamide gel, and the proteins were stained with Coomassie brilliant blue. (B) Double-reciprocal plot of the initial velocity of 5-hydroxyectoine formation (V) as a function of substrate concentration ([S]). For the determination of the kinetic parameters of EctD, the concentration of ectoine (closed triangles) or the 2-oxoglutarate cosubstrate (closed squares) was varied at an affixed concentration of 10 mM 2-oxoglutarate or ectoine, respectively. Kinetic parameters were obtained from the Michaelis-Menten equation. (C) Temperature dependence of the EctD activity. EctD activity was measured in samples incubated at the indicated temperatures. The values given are the mean of two independent measurements. The maximum corresponds to a specific activity of 20 U mg^{-1}.

ectoine hydroxylase from *S. coelicolor* A3(2) to apparent homogeneity by sequential anion-exchange chromatography on Source 15Q and gel filtration on Superdex 75 and ceramic hydroxyapatite material (Table 1). Unfortunately, the last chromatographic step resulted in a substantial loss of ectoine hydroxylase activity (Table 1), but it effectively removed a number of contaminating proteins from the enzyme preparation. We obtained a highly pure ectoine hydroxylase as judged by SDS-PAGE on a 12.5% polyacrylamide gel (Fig. 6A). The purified protein migrated on the SDS-PAGE with an apparent electrophoretic mobility corresponding to a 38.5-kDa protein species.

We determined the NH_2-terminal end of the purified EctD protein (Fig. 6A) from *S. coelicolor* A3(2) by sequential Edman degradation. The following amino acid sequence was found: NH_2-(M)-T-T-T-T-T-N-V-T-D-(L). This amino acid sequence matches perfectly that of the amino-terminal end of the EctD protein as deduced from the recently revised annotation of the *ectD* coding region in the genome sequence of *S. coelicolor* A3(2) (6; see Swissprot entry Q93RV9). It should be noted, however, that an EctD protein from *S. coelicolor* A3(2) with an incorrectly predicted amino terminus is still listed in the NCBI database (NP_626134). The calculated molecular mass of the ectoine hydroxylase as predicted from the experimentally determined NH_2-terminal end and the nucleotide sequence of the *ectD* structural gene in the *S. coelicolor* A3(2) genome is 32.833 kDa. This value is in excellent agreement with the molecular mass of the purified EctD protein (32.7 kDa) that we determined by mass spectrometry. Thus the electrophoretic mobility (a 38.5-kDa protein species) of the purified EctD protein by 12.5% SDS-PAGE (Fig. 6A) does not reflect the actual molecular mass of the *S. coelicolor* A3(2) EctD protein.

The start codon of *ectD* in the *S. coelicolor* A3(2) genome is separated from the stop codon of *ectC* by only 6 bp, suggesting that *ectD* might be cotranscribed with the *ectABC* gene cluster. Database searches revealed putative *ectABCD* operons in the streptomycetes *S. chrysomallus* (65), *Streptomyces avermitilis* (35), *Streptomyces griseus* (60), and the plant pathogen *Streptomyces scabiei* (www.sanger.ac.uk/Projects/S_scabies). The putative ectoine hydroxylases encoded by the *ectD* genes of these *Streptomyces* species are all very closely related to the amino acid sequence of the EctD protein from *S. coelicolor* A3(2), with amino acid sequence identities ranging from 83% to 86% (data not shown).

Properties of the purified ectoine hydroxylase. Like the ectoine hydroxylase from *S. salexigens* (15), the EctD protein from *S. coelicolor* A3(2) was also found to be a monomeric enzyme as judged by gel filtration chromatography. Addition of $FeSO_4$ to the enzyme assay was absolutely required for ectoine hydroxylase activity. The enzyme activity of EctD was stimulated up to a concentration of 1 mM $FeSO_4$, whereas higher $FeSO_4$ concentrations reduced the ectoine hydroxylase enzyme activity (data not shown). Within the superfamily of non-heme-containing iron(II)- and 2-oxoglutarate-dependent dioxygenases, the addition of ascorbate and catalase sometimes leads to increased enzymatic activities (15, 30). However, ectoine hydroxylase activity of the purified EctD protein was neither enhanced nor inhibited by either catalase or ascorbate or both (data not shown). Oxygen and the cosubstrate 2-oxoglutarate were absolutely required for the EctD-mediated enzyme reaction (data not shown). The kinetic parameters for the ectoine hydroxylase from *S. coelicolor* A3(2) were as follows: the K_m for the substrate ectoine was 2.6 ± 0.2 mM, and the K_m for the cosubstrate 2-oxoglutarate was 6.2 ± 0.2 mM. The V_{max} of the EctD enzyme was 20 ± 1 U mg^{-1} protein (Fig. 6B). The enzyme had a pH optimum of 7.5 and was active in a broad temperature range with an optimum at 32°C (Fig. 6C). Consequently, there is no specific temperature activation of the purified EctD hydroxylase activity that could explain the preferential production of 5-hydroxyectoine found in *S. coelicolor* A3(2) cells grown at 39°C (Fig. 2C).

The EctD-type proteins are distantly related to L-proline 4-hydroxylases and L-proline 3-hydroxylases (15). These proline-hydroxylating enzymes also belong to the superfamily of

the non-heme-containing iron(II)- and 2-oxoglutarate-dependent dioxygenases and carry out enzymatic reactions similar to those of the ectoine hydroxylase (30). We therefore tested whether the *S. coelicolor* A3(2) EctD protein would use L-proline as its substrate and hydroxylate this amino acid. However, this was not the case (data not shown), demonstrating that the EctD enzyme from *S. coelicolor* A3(2) is a bona fide ectoine hydroxylase with no additional L-proline hydroxylase activity.

A comparison of the characteristics of the ectoine hydroxylase purified in this study from *S. coelicolor* A3(2) with those of the ectoine hydroxylase recently purified from the moderate halophile *S. salexigens* (15) revealed similar enzymatic properties with respect to pH [*S. salexigens*, 7.5; *S. coelicolor* A3(2), 7.5] and temperature profile [*S. salexigens*, 32°C; *S. coelicolor* A3(2), 32°C], affinity (K_m) to the substrate ectoine [*S. salexigens*, 3.5 mM; *S. coelicolor* A3(2), 2.6 mM], and the cosubstrate 2-oxoglutarate [*S. salexigens*, 5.2 mM; *S. coelicolor* A3(2), 6.2 mM], overall catalytic performance (V_{max}) [*S. salexigens*, 13.8 U mg^{-1}; *S. coelicolor* A3(2), 20 U mg^{-1}], and inability to hydroxylate L-proline. These findings suggest that EctD-type proteins from other microorganisms are likely to possess similar enzymatic characteristics. EctD-type proteins are closely related in amino acid sequence (15) and form a separate subgroup within the superfamily of the non-heme-containing iron(II)- and 2-oxoglutarate-dependent dioxygenases (EC 1.14.11) (30).

We have identified in recent database searches over 50 EctD-related proteins from various gram-negative and gram-positive *Bacteria* with amino acid sequence identities that range between 86% and 40% (data not shown). Many of these proteins are annotated as putative proline hydroxylases in the NCBI database. We believe that most of these annotations are incorrect and stem from the fact that EctD-type proteins exhibit a substantial degree of amino acid sequence relatedness to proline hydroxylases. These enzymes carry out enzymatic reactions similar to that of the ectoine hydroxylase, and both groups of enzymes are members of the same dioxygenase superfamily (15, 30). Similarly, some EctD-related proteins are annotated in the NCBI database as phytanoyl-coenzyme A (CoA) dioxygenases. The recent structural analysis of the EctD protein from *S. salexigens* (K. Reuter, J. Bursy, A. Heine, M. Pittelkow, and E. Bremer; unpublished results) revealed an overall fold of this protein similar to that of the human phytanoyl-CoA 2-hydroxylase, an enzyme that carries out the initial alpha-oxidation step in the degradation of phytenic acid in peroxisomes (59). This observation explains the probably incorrect annotation of EctD-related proteins as putative phytanoyl-CoA dioxygenases.

DISCUSSION

The ability of various *Streptomyces* species to tolerate considerable concentrations of NaCl is well known (46, 56), but the molecular and physiological processes that allow streptomycetes to withstand salt stress are little explored. The data presented in this communication address physiological aspects of the salt stress response of *S. coelicolor* A3(2) and demonstrate that this soil-living actinomycete belongs to the group of microorganisms that synthesize the compatible solutes ectoine and 5-hydroxyectoine upon exposure to high salinity (Fig. 2). There are reports in the literature that *Streptomyces clavuligerus*, *S. griseus*, *S. parvulus*, *Streptomyces peucetius*, and *Streptomyces antibioticus* are also capable of synthesizing ectoine and 5-hydroxyectoine under salt stress (56). Furthermore, ectoine and 5-hydroxyectoine biosynthetic genes have been identified in *S. chrysomallus* (65), and our database searches revealed that they are also present in the genome sequences of *Streptomyces avermitilis* (35), *S. griseus* (60), and the plant pathogen *S. scabiei* (www.sanger.ac.uk/Projects/S_scabies). Taken together, these findings suggest that both ectoine and 5-hydroxyectoine are rather common compatible solutes synthesized by members of the genus *Streptomyces* as a physiological stress response to increased salinity.

The function of compatible solutes as microbial heat and chill stress protectants does not seem to rely on a massive intracellular accumulation of these compounds. Both heat- and chill-stressed cells contained substantially smaller amounts of compatible solutes than osmotically stressed cells in various microorganisms (16, 33, 42, 50). This is also true for the heat stress-induced synthesis of ectoine and 5-hydroxyectoine in *S. coelicolor* A3(2) in comparison to osmotically stressed cells (Fig. 2). Our data suggest that ectoine and 5-hydroxyectoine synthesized by *S. coelicolor* A3(2) serve as both salt and heat stress protectants in this actinomycete. However, definitive proof of this hypothesis can only come from a physiological analysis of *S. coelicolor* A3(2) mutants with gene disruptions in the *ectABCD* gene cluster.

When both high salinity (1.2 M NaCl) and heat stress (39°C) were simultaneously applied (Fig. 4C), the growth of *S. coelicolor* A3(2) was entirely prevented (Fig. 4C). This observation implies that the intracellular levels of ectoine and 5-hydroxyectoine achieved via de novo synthesis under these circumstances are insufficient to provide adequate cellular protection. However, under such conditions, *S. coelicolor* A3(2) can still rely on the uptake of both ectoine and 5-hydroxyectoine from environmental sources for efficient salt and heat stress protection (Fig. 4C).

Ectoine and 5-hydroxyectoine are only synthesized by microorganisms (15, 23, 26, 78), and, hence, the only sources for these two compatible solutes within the soil habitat of *S. coelicolor* A3(2) must stem from either decomposing or osmotically down-shocked bacterial cells (32, 78). It is likely that the concentrations of both ectoines in the soil are very low and probably highly variable, but our transport assays with radiolabeled ectoine demonstrate that *S. coelicolor* A3(2) can scavenge these compounds from environmental sources and accumulate them even at very low (μM) external substrate concentrations (Fig. 5).

Ectoine and 5-hydroxyectoine are closely related in chemical structure (36) (Fig. 1), and both compatible solutes have protein-stabilizing properties (4, 5, 9, 18, 47, 54, 57, 58). However, not all ectoine producers also synthesize 5-hydroxyectoine (15, 26, 49, 50, 71, 73). The formation of the hydroxylated derivative of ectoine might provide additional advantages to a microbial cell. In vitro studies have shown that 5-hydroxyectoine often has protein-stabilizing properties superior to those of ectoine (9, 18, 54), and its capacity to provide desiccation tolerance to *Escherichia coli* and *Pseudomonas putida* was comparable to that of trehalose (57, 58). Likewise, substantial

differences exist between ectoine and 5-hydroxyectoine with respect to their effects on the melting temperature of double-stranded DNA: ectoine decreases whereas 5-hydroxyectoine increases the melting temperature of the DNA (74).

How might ectoine and 5-hydroxyectoine provide thermoprotection to *S. coelicolor* A3(2)? Both ectoine and 5-hydroxyectoine might directly help to maintain the proper three-dimensional structure of thermolabile proteins in *S. coelicolor* A3(2) in vivo. In vitro studies with various model enzymes support such a function of ectoine and 5-hydroxyectoine (4, 9, 47, 54). One should note, however, that 5-hydroxyectoine and ectoine can have very different thermostabilizing properties when tested with the same model enzyme (9), despite their close chemical relatedness (Fig. 1).

Another possible way to positively influence proper protein folding under heat stress conditions is through the effects of compatible solutes (e.g., glycine betaine) on the chaperone network of the cell (24, 25). Adaptation to heat stress in many microbial species depends on the synthesis of heat shock proteins, many of which are molecular chaperones that prevent protein aggregation, disassemble protein aggregates, and assist in protein refolding (14). Low physiological concentrations of glycine betaine activated the molecular chaperone system in *E. coli*, thereby promoting local folding of chaperone-bound polypeptides and allowing protein disaggregation under heat stress conditions (24, 25). Overproduction of the compatible solute proline in *E. coli* restored the viability of a mutant with a defect in the major heat shock chaperone DnaK at 42°C and significantly reduced the protein aggregation defect of the *dnaK* mutant strain (22).

The influence of compatible solutes on the structure of nucleic acids, in particular on double-stranded DNA (52) and protein-DNA interactions (66), is not well understood. The compatible solutes proline, glycine betaine, ectoine, and 5-hydroxyectoine have been shown to influence (either as destabilizers or as stabilizers) the melting temperature of the DNA helix (68, 74). In addition, ectoine served as a stabilizer of a higher-order nucleoprotein complex at the regulatory region of bacterial rRNA promoters (66). Hence, it is conceivable that the accumulation of ectoine and 5-hydroxyectoine under heat stress in *S. coelicolor* A3(2) could have profound effects on the local melting of DNA within promoter regions and thereby affect gene transcription or the productive interactions of regulatory proteins with their DNA target sequences.

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