JOURNAL OF BACTERIOLOGY, Sept. 2011, p. 4456–4468 Vol. 193, No. 17
0021-9193/11/$12.00 doi:10.1128/JB.00345-11

A Specialized Aspartokinase Enhances the Biosynthesis of the Osmoprotectants Ectoine and Hydroxyectoine in *Pseudomonas stutzeri* A1501▽†

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Received 11 March 2011/Accepted 18 June 2011

The compatible solutes ectoine and hydroxyectoine are widely produced by bacteria as protectants against osmotic and temperature stress. L-Aspartate-beta-semialdehyde is used as the precursor molecule for ectoine/hydroxyectoine biosynthesis that is catalyzed by the EctABCD enzymes. L-Aspartate-beta-semialdehyde is a central intermediate in different biosynthetic pathways and is produced from L-aspartate by aspartokinase (Ask) and aspartate-semialdehyde-dehydrogenase (Asd). Ask activity is typically stringently regulated by allosteric control to avoid gratuitous synthesis of aspartylphosphate. Many organisms have evolved multiple forms of aspartokinase, and feedback regulation of these specialized Ask enzymes is often adapted to the cognate biochemical pathways. The ectoine/hydroxyectoine biosynthetic genes (*ectABCD*) are followed in a considerable number of microorganisms by an *ask* gene (*ask_ect*), suggesting that Ask_Ect is a specialized enzyme for this osmoadaptive biosynthetic pathway. However, none of these Ask_Ect enzymes have been functionally characterized. *Pseudomonas stutzeri* A1501 synthesizes both ectoine and hydroxyectoine in response to increased salinity, and it possesses two Ask enzymes: Ask_Lys and Ask_Ect. We purified both Ask enzymes and found significant differences with regard to their allosteric control: Ask_LysC was inhibited by threonine and in a concerted fashion by threonine and lysine, whereas Ask_Ect showed inhibition only by threonine. The *ectABCD_ask* genes from *P. stutzeri* A1501 were cloned and functionally expressed in *Escherichia coli*, and this led to osmostress protection. An *E. coli* strain carrying the plasmid-based *ectABCD_ask* gene cluster produced significantly more ectoine/hydroxyectoine than a strain expressing the *ectABCD* gene cluster alone. This finding suggests a specialized role for Ask_Ect in ectoine/hydroxyectoine biosynthesis.

The growth arrest of microbial cells observed under severe high-osmolarity conditions is correlated with the reduction in the amount of free cytoplasmic water (12) and the reduction or collapse of turgor (7, 65). To promote water reentry and retention under high-osmolarity growth conditions and to balance turgor, many microorganisms amass a selected class of organic osmolytes, the compatible solutes (31, 66). Microbial cells accumulate compatible solutes through either synthesis or uptake from environmental resources (7, 65, 69). They can employ them not only as effective osmostress protectants, but also as protectants against heat (8, 10, 13, 17, 26) and cold (2, 25, 33) stress.

The tetrahydropyrimidines ectoine and 5-hydroxyectoine (Fig. 1) are an important class of compatible solutes (9, 45, 50). Biosynthesis of ectoine is catalyzed by L-2,4-diaminobutyric acid transaminase (EctB), *N*-γ-acetyltransferase (EctA), and ectoine synthase (EctC) (40, 44). A subset of the ectoine producers hydroxylate ectoine to 5-hydroxyectoine (9, 17, 48) in a stereospecific fashion using the ectoine hydroxylase EctD (8, 9, 50). The structural genes (*ectABC*) for the ectoine biosynthetic enzymes are typically organized as a gene cluster whose transcription can be induced by increased osmolarity of the growth medium (9, 11, 32, 33, 40, 43). In some microorganisms (e.g., in several bacilli) the *ectABC* genes are transcribed as an operon (8, 32, 33), but more complex patterns of *ect* transcription have been reported for *Chromohalobacter salexigens* (17) and *Halomonas elongata* (57). The *ectABC* operon might also contain the structural gene (*ectD*) for the ectoine hydroxylase (8, 48), but this gene can often also be found in a genomic location unlinked from the ectoine biosynthetic gene cluster (9, 17). Database searches by different groups have shown that the ability to synthesize ectoine and hydroxyectoine is widespread in the microbial world (9, 39, 45, 50, 62). Our updated database searches of microbial genomes that are completely sequenced (currently more then 1,400 entries) using the ectoine synthase EctC as a search template show that 220 microorganisms are likely ectoine producers, and about a third of these are predicted to synthesize hydroxyectoine as well (M. Pittelkow and E. Bremer, unpublished data).

Ectoine and hydroxyectoine have attracted considerable biotechnological attention, since these compatible solutes possess excellent stabilizing properties for proteins, cell membranes, nucleic acids, and even entire cells (19, 23, 34, 38, 45). They have therefore found versatile uses as *in vivo* protein-folding catalysts, as *in vitro* protein stabilizers, as PCR enhancers, as cytoprotectants, and, foremost, as skin care products in cos-

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† Supplemental material for this article may be found at http://jb.asm.org/.

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▽ Published ahead of print on 1 July 2011.

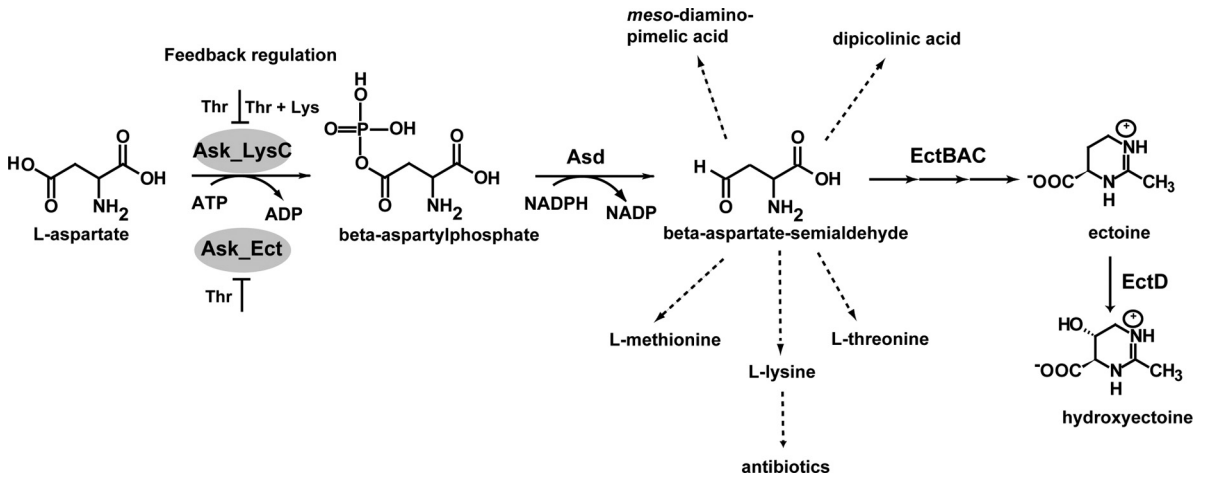

FIG. 1. Formation of aspartate-beta-semialdehyde and biosynthetic pathways derived from this metabolite. The scheme illustrates the key position of aspartate-beta-semialdehyde as a metabolic hub in microorganisms. The biosynthetic routes radiating from aspartate-beta-semialdehyde represent a composite sketch and do not indicate that all of the biosynthetic pathways shown occur simultaneously in a given microorganism. The chemical structures of ectoine and hydroxyectoine are shown, but the details of their biosynthesis are omitted (9, 40, 44).

metics (19, 38, 45). There is also increasing interest in using ectoine and hydroxyectoine for various medical applications (21, 61, 64). Ectoine and hydroxyectoine are currently produced on an industrial scale by high-density fermentation of salt-tolerant bacteria; the release of these solutes from the producer cells is accomplished by an osmotic down shock, a process known as "bacterial milking" (38, 54).

The precursor for ectoine synthesis (Fig. 1), L-aspartate-beta-semialdehyde, is a central metabolic hub in microorganisms from which a branched network of various biosynthetic pathways diverges (39). This precursor molecule is used not only for ectoine production, but also for the synthesis of the aspartate family of amino acids (Met, Thr, and Lys), dipicolate present in the spores of Gram-positive bacteria, and *meso*-diaminopimelic acid employed for peptidoglycan biosynthesis and for lysine-derived antibiotics in various streptomycetes (Fig. 1) (14, 39, 46). L-Aspartate-beta-semialdehyde is synthesized through the sequential enzymatic reactions of aspartokinase (Ask) and aspartate-semialdehyde-dehydrogenase (Asd). Ask synthesizes aspartyl-beta-phosphate though the ATP-dependent phosphorylation of L-aspartate, which is then in turn reduced to L-aspartate-beta-semialdehyde by Asd in an NADPH-dependent reaction (Fig. 1). To avoid wasteful production of the energy-rich intermediate aspartyl-beta-phosphate (68), the aspartokinase activity is often regulated by feedback inhibition (14, 27, 39, 46), and its structural gene is also often subjected to transcriptional regulation (14, 46, 47). To adapt the functioning of aspartokinases to the demands of specific biosynthetic pathways, many microorganisms have evolved multiple forms of aspartokinase whose enzyme activities are often differently regulated by allosteric control and whose structural genes are also frequently regulated differently (14, 39, 46, 47).

Aspartokinases can be grouped into two subdivisions. The beta subgroup is characterized by the presence of an internal translational start site that leads to the synthesis of an approximately 18-kDa C-terminal segment of the Ask protein that functions as a regulatory subunit of the multimeric enzyme (14, 39, 46). Members of the alpha subgroup lack this internal translational start site and possess an ~50-amino-acid-long indel region that is not present in members of the beta subgroup (39). Both Ask types have an N-terminal aspartokinase domain that comprises those amino acids involved in binding of the substrates L-aspartate and ATP and residues involved in catalytic activity. The C terminus contains two closely spaced ACT domains that are involved in allosteric control of enzyme activity (20, 39). Aspartokinases are of considerable biotechnological interest, since their allosteric control often constitutes a bottleneck for the production of commercially interesting products, e.g., L-lysine and antibiotics (24, 27). Mutants that abolish allosteric feedback control have been selected and employed for industrial-scale production processes to open this bottleneck (22, 55).

Reshetnikov et al. reported that the osmotically inducible *ectABC* ectoine biosynthetic gene cluster of *Methylomicrobium alcaliphilum* strain 20Z is cotranscribed with an *ask* gene (49). This finding hints that this particular *ask* gene (*ask_ect*) encodes an aspartokinase that could play a specialized role in the biosynthesis of the compatible solute ectoine under osmotic stress conditions. Subsequently, it was observed that a considerable number of ectoine/hydroxyectoine biosynthetic gene clusters are actually followed by an *ask* gene (39, 45, 57, 62). A comprehensive cohesion group analysis reported by Lo et al. (39) showed that the encoded proteins form a distinct subcluster (cohesion group 02 [CG-02]) within the Ask superfamily. However, none of these ectoine-associated Ask enzymes have so far been biochemically characterized, and nothing is known about their influence on the level of ectoine/hydroxyectoine production in osmotically stressed bacterial cells.

We exploited the fact that in the genome of the plant root-associated bacterium *Pseudomonas stutzeri* A1501 (67) two aspartokinase genes are present: *ask_ect* and *ask_lysC*, with the *ask_ect* gene linked to an *ectABCD* ectoine/hydroxyectoine biosynthetic gene cluster. Our biochemical analysis of these two Ask enzymes shows that the Ask_Ect enzyme possesses regulatory features different from those of the Ask_LysC enzyme.

Furthermore, heterologous expression of the *ectABCD_ask_ect* gene cluster from *P. stutzeri* A1501 in *Escherichia coli* revealed a significant influence of the *ask_ect* gene on the level of ectoine/hydroxyectoine production.

MATERIALS AND METHODS

Chemicals. ATP was purchased from Gerbu Biochemicals (Gaiberg, Germany); L-aspartate, L-aspartyl-beta-hydroxamate, and hydroxylamine were obtained from Sigma-Aldrich (Steinheim, Germany). Anhydrotetracycline-hydrochloride, desthiobiotin, and the streptavidin-Tactin Superflow chromatography material were purchased from IBA (Göttingen, Germany); ectoine and hydroxyectoine were kind gifts from G. Lentzen (Bitop AG, Witten, Germany). The enzymes trehalase, horseradish peroxidase, and glucose oxidase and the substrate *o*-toluidine were purchased from Sigma-Aldrich (Steinheim, Germany).

Bacterial strains. *E. coli* DH5α was used for routine cloning purposes. The *E. coli* strain BL21(DE3) (Novagen, Madison, WI) was employed for the heterologous production of the Ask_Ect and Ask_Lys recombinant proteins. The trehalose-negative *E. coli* strain FF4169 (*otsA1*::Tn*10*), used for the plasmid-based recombinant synthesis of ectoine and hydroxyectoine, was kindly provided by A. Strom (University of Tromso, Tromso, Norway), and its construction and properties have been described previously (18). The *P. stutzeri* strain A1501 (67) was kindly provided by C. Elmerich (Institute Pasteur, Paris, France).

Media and growth conditions. *P. stutzeri* A1501 and strains of *E. coli* were routinely propagated and maintained on Luria-Bertani (LB) agar plates (42) at 37°C. For the heterologous production of proteins, recombinant cells of *E. coli* were cultivated in defined minimal medium A (MMA) supplemented with 0.4% (wt/vol) Casamino Acids, 1 mM $MgSO_4$, 1 mg liter^{-1} thiamine, and 0.4% (wt/vol) glucose as a carbon source. Minimal medium 63 (MM63) (42) supplemented with 0.05 M or 0.3 M NaCl and 0.5% (wt/vol) glucose as a carbon source was used for the recombinant synthesis of ectoine and hydroxyectoine in *E. coli* strains carrying the *ectABCD_ask* gene cluster. Ampicillin (100 mg ml^{-1}), tetracycline (12.5 mg ml^{-1}), or kanamycin (50 mg ml^{-1}) was added to the growth medium to select for recombinant plasmids.

DNA isolation and recombinant DNA techniques. Chromosomal DNA of *P. stutzeri* A1502 was isolated according to the method of Marmur (41). Plasmid DNA was isolated using the GeneJet Plasmid Miniprep Kit (Fermentas, St. Leon-Rot, Germany). Restriction endonucleases and DNA ligase were purchased from Fermentas (St. Leon-Rot, Germany) and used under conditions recommended by the manufacturer. Chemically competent cells of *E. coli* were prepared and transformed as described previously (53). Customized oligonucleotides were synthesized by Sigma-Aldrich (Steinheim, Germany), and DNA sequencing of plasmid constructs was performed by Eurofins MWG Operon (Ebersberg, Germany).

Construction of plasmids. The *ask_ect* and *ask_lysC* genes were amplified from genomic DNA of *P. stutzeri* A1501 using Phusion DNA polymerase (Finnzymes, Espoo, Finland) and custom-synthesized DNA primers (see Table S1 in the supplemental material). Primer sequences were deduced from the available genome sequence of *P. stutzeri* A1501 (67) (Department of Energy [DOE] Joint Genome Institute [JGI] [http://img.jgi.doe.gov]). The translational start site of the *ask_ect* gene was revised by us from the published start site of the open reading frame YP_001170726 (67) 132 bp upstream of the annotated site, since sequence inspection of the translated gene revealed that the predicted N terminus was missing amino acids that are typically highly conserved among Ask proteins (39). The modified nucleotide sequence of the *ask_ect* gene that was used throughout this work, therefore, included 1,431 bp (209889 to 208459) instead of the originally annotated 1,299 bp (209757 to 208459) for this gene (67). The amplified *ask_ect* gene was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and used for construction of the *ask*_ect expression plasmid pMP42, applying Stargate combinatorial cloning technology (IBA, Göttingen, Germany). The gene was first introduced into a pENTRY vector and subsequently cloned into the expression plasmid pASG-IBA-3, which allows *ask_ect* gene expression under the control of the tetracycline-inducible *tet* promoter. For affinity purification of the recombinant Ask_Ect protein, the *ask_ect* gene was fused C-terminally to the plasmid-based streptavidin tag II peptide sequence. The *ask_lysC* PCR product (1.23 kbp) was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and subsequently cloned into the expression plasmid pASK-IBA-3 by standard cloning methods. The DNA of the amplified *ask_lysC* gene was digested with BsaI and ligated into the *tet* promoter-based expression plasmid pASK-IBA3 plus (IBA, Göttingen, Germany), which was linearized with the same restriction endonuclease, yielding the plasmid pMP-ask1. The expression plasmids pMP-ask1 (Ask_LysC) and pMP42 (Ask_Ect) were transformed into *E. coli* BL21(DE3). Plasmids pNST5 (*ectABCD*$^+$) and pNST6 (*ectABCD_ask_ect*$^+$) were constructed to analyze the influence of Ask_Ect on ectoine/hydroxyectoine synthesis in *E. coli*. Both *ect* gene clusters contain the native promoter upstream of the *ectA* gene and were amplified from genomic DNA of *P. stutzeri* A1501 in a hot-start PCR using Phusion DNA Polymerase (Finnzymes, Espoo, Finland) and custom synthesized primers (see Table S1 in the supplemental material). The resulting 5.1-kb (*ectABCD_ask_ect*) and 3.5-kb (*ectABCD*) PCR products were purified (QIAquick PCR Purification Kit; Qiagen, Hilden, Germany), digested with SpeI and KpnI, and subsequently ligated into plasmid pBluescript SK(−) that had been linearized with the same restriction endonucleases. The resulting hybrid plasmids, pNST5 (*ectABCD*$^+$) and pNST6 (*ectABCD_ask_ect*$^+$), were used for the transformation of the trehalose-negative strain *E. coli* FF4169 (*otsA1*::Tn*10*) (18) to study ectoine and hydroxyectoine synthesis in a recombinant system.

Overproduction and purification of Ask proteins. Recombinant *E. coli* strains BL21(DE3)(pMP42) and BL21(DE3)(pMPask-1) were cultivated in 1 liter minimal medium A at 37°C in an aerial shaker set at 200 rpm until an absorbance (at 600 nm) of 0.5 to 0.7 of the culture was reached. Subsequently, expression of the recombinant *ask_ect* and *ask_lysC* genes was induced by the addition of 0.2 mg liter anhydrotetracycline^{-1} to the cultures; the cells were then grown for an additional 2 h. Cells were harvested by centrifugation (4°C; 500 × *g*; 20 min), washed twice in buffer W (100 mM Tris-HCl, pH 7.5, 150 mM NaCl), and stored at −70°C until further use. For the purification of the recombinant Ask proteins, the cells were disrupted by resuspending the frozen pellet in buffer W (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) and passing them four times through a chilled French press cell. After an ultracentrifugation step (4°C; 100,000 × *g*; 1 h), the cleared supernatant was applied on a 10-ml streptavidin-Tactin-Superflow column (IBA, Göttingen, Germany) attached to an Äkta fast protein liquid chromatography (FPLC) system (GE Healthcare, Freiburg, Germany); the column was preequilibrated with 10 column volumes of buffer W (set at a flow rate of 1.5 ml/min). To remove proteins that had bound nonspecifically to the affinity column, the streptavidin-Tactin-Superflow column was washed with 17 column volumes of buffer W (set at a flow rate of 2.5 ml/min). Elution of streptavidin-tagged fusion proteins was performed by addition of 3 column volumes of buffer E (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM desthiobiotin at a flow rate of 1.0 ml min^{-1}) and collecting the eluate in 3-ml fractions. The protein content of these fractions was determined using a NanoDrop spectrometer (Thermo Scientific, Wilmington, DE), and the purity of the Ask_Ect or Ask_LysC protein was analyzed by SDS gel electrophoresis. All Ask_Ect or Ask_LysC protein-containing fractions were pooled, and the proteins were concentrated by using Vivaspin 4 columns (exclusion size, 10 kDa; Vivascience, Hannover, Germany) with a simultaneous exchange of elution buffer W for buffer E. Purified Ask_Ect and Ask_LysC proteins were stored at 4°C for short intervals (up to 1 week) or at −70°C for longer periods with the addition of 20% (vol/vol) glycerol.

The N-terminal amino acid sequence of the Ask_LysC beta subunit was analyzed by Edman sequencing. A sample of 54 μg purified Ask_LysC protein was run on a 13% (vol/vol) polyacrylamide gel and subsequently blotted onto an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore, Schwalbach, Germany) as described by the manufacturer using a Bio-Rad (Munich, Germany) Trans-Blot Cell. The transferred proteins were visualized by staining with PhastGel Blue R (GE Healthcare, Freiburg, Germany), and the N terminus of the 18-kDa Ask_LysC beta subunit was then determined by Edman sequencing. Protein sequencing was carried out by the Central Analytical Facility of the Justus-Liebig-University, Gießen, Germany (G. Lochnit, Faculty of Medicine, Biochemical Institute).

Enzyme activity assay. Aspartokinase enzyme activity was assayed by the method of Black and Wright (6). The standard reaction mixture (total volume, 0.5 ml) contained 60 mM Tris-HCl buffer (pH 8), 0.1 M L-aspartate, 20 mM ATP, 20 mM $MgCl_2$, 0.1 M hydroxylamine, and 5 μg purified Ask protein. After 30 min of incubation at 30°C, the enzymatic reaction was stopped by addition of 0.5 ml acidified $FeCl_3$ solution (10% [wt/vol] $FeCl_3$, 3.3% [vol/vol] trichloroacetic acid, 0.7 N HCl), and the absorbance of the aspartyl-beta-hydroxamate-iron complex was determined at 540 nm in a spectrophotometer. The amount of the formed aspartyl-beta-hydroxamate was calculated from calibration curves of purchased aspartyl-beta-hydroxamate (Sigma-Aldrich, Steinheim, Germany).

The trehalose content of *P. stutzeri* 1501 cells grown in MM63 with either 50 mM or 700 mM NaCl was assayed enzymatically as described by Fernandez-Aunión et al. (16), using a method that involves the hydrolysis of trehalose by trehalase to two glucose molecules and the subsequent quantification of glucose by a coupled assay of glucose oxidase-peroxidase in the presence of *o*-toluidine. The oxidation of *o*-toluidine was monitored spectrophotometrically at 420 nm.

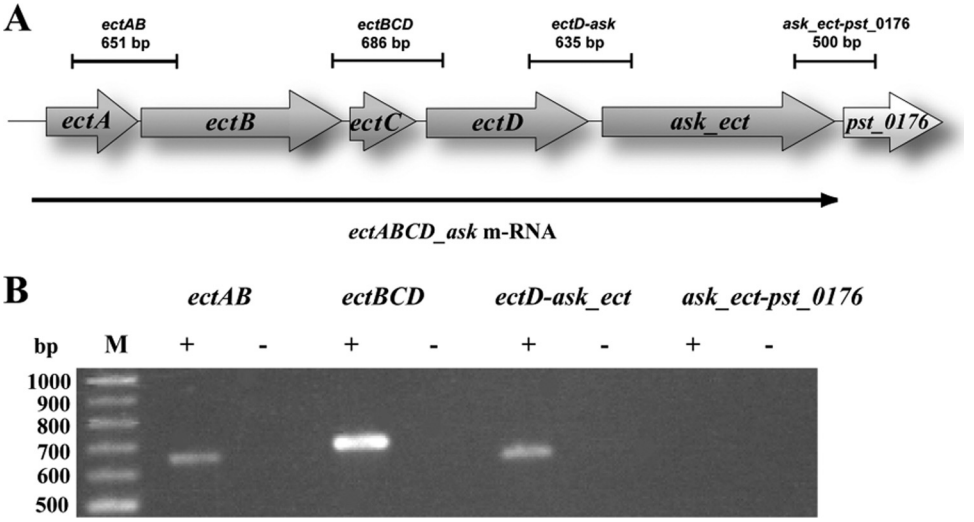

FIG. 2. Mapping of the transcriptional organization of the *ectABCD_ask_ect* gene cluster. (A) Overview of the genetic organization of the *ectABCD_ask_ect* genes in the genome of *P. stutzeri* A1501. The positions of the PCR-amplified segments of the *ectABCD-ask_ect* mRNA are indicated. (B) Transcriptional analysis of the putative *ectABCD_ask* operon by one-step RT-PCR. Total RNA was isolated from cells of *P. stutzeri* A1501 grown in MM63 containing 0.7 M NaCl. +, the reverse transcription reaction of the mRNA; −, PCR with an RNA sample as the template but no reverse transcriptase to ensure that the RNA used was not contaminated with DNA.

Analysis of ectoine and hydroxyectoine production. The synthesis of ectoine and hydroxyectoine was analyzed in cells of *P. stutzeri* A1501 or in the recombinant *E. coli* FF4169 strain; cells were grown in MM63 containing 0.5% (wt/vol) glucose as a carbon source and 0.05 to 0.7 M NaCl. Cells of *P. stutzeri* A1501were harvested by centrifugation in the mid-exponential growth phase. The contents of ectoine and hydroxyectoine in the recombinant *E. coli* strains were determined after 16 h of incubation at 37°C. The cell pellets obtained were lyophilized, and 20 to 30 mg of the dried cell material was used for extraction and determination of the intracellular ectoine/hydroxyectoine content by isocratic high-performance liquid chromatography (HPLC) as previously described (32). The culture supernatant was analyzed for exported ectoine or hydroxyectoine by adding an equal volume of acetonitrile (100%) and applying a 20-μl aliquot to isocratic HPLC using a Grom Sil 100 Amino 1PR (125 mm by 4 mm; Grom Rottenburg-Hailfingen, Germany).

Isolation of RNA and transcription analysis of *ectABCD_ect_ask* by RT-PCR. Total RNA was isolated from cells of *P. stutzeri* A1501 by using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the instructions of the manufacturer. Since the eluted RNA was still contaminated with DNA, an additional DNase I digestion was performed for 1 h at room temperature using DNase I purchased from Roche (Mannheim, Germany). RNA samples were further purified using the RNeasy Kit (Qiagen, Hilden, Germany) as described in the manual and used for one-step reverse transcription (RT)-PCR. To analyze if the *ectABCD_ask* genes are transcribed as a unit, four intergenic regions of the putative operon were amplified from isolated RNA using the Qiagen One Step RT-PCR Kit and *ect_ask*-specific DNA primers (see Table S1 in the supplemental material). To recognize PCR products that resulted from DNA contamination in the RNA sample, a control was performed in which total RNA was added after the reverse transcription step. In addition, to exclude the possibility that the *ask_ect–pst-0176* product (see Fig. 3A) was simply missing because of a nonfunctional RT-PCR, the reaction was repeated and the *ectBCD* product was amplified as an internal control in the same reaction mixture. PCR on genomic DNA was successful under the RT-PCR conditions used.

Transcriptional regulation of the *ectABCD_ask* cluster as analyzed by dot blot analysis. A potential increase in the level of transcription of the *P. stutzeri* A1501 *ectABCD-ask_ect* gene cluster and of the *ask_lysC* gene in response to high salinity was assessed by dot blot analysis. RNA was prepared from *P. stutzeri* A1501 cultures grown in MM63 minimal medium containing either 50 mM or 700 mM NaCl by a modified acidic phenol procedure described by Völker et al. (63). Different concentrations of total RNA (1.6 μg to 50 ng) were dotted onto a nylon membrane (NytranN; Schleicher & Schuell, Dassel, Germany) using a dot blot apparatus. For the detection of *ect* and *ask* mRNAs, the dot-blotted total RNA from *P. stutzeri* A1501 was hybridized (at 68°C) to various digoxigenin-labeled single-stranded anti-sense RNA probes, and the membranes were then processed and developed according to the manual of the Dig Northern Starter Kit (Roche Diagnostic, Grenzach-Wyhlen, Germany). Digoxigenin-labeled antisense RNA probes were prepared by *in vitro* transcription using the Dig Northern Starter Kit with PCR templates containing the T7 RNA polymerase promoter sequence. The hybridization signals were detected using a Storm 860 fluorescence imager (Amersham Pharmacia Biotech).

Database searches for Ask_Ect-related proteins. Proteins that are homologous to the EctC protein from *Virgibacillus salexigens* (9) were searched via the Web server of the DOE JGI (http://www.jgi.doe.gov/) or that of the National Center for Biotechnology Information (http://www.ncbi,nlm.nih.gov/) using the BLAST algorithm (1). The genome context of finished and unfinished microbial genomes in the vicinity of *ectC*-type genes was assessed using the gene neighborhood tool (http://img.jgi.doegov/cgi-bin/pub/main.cgi) provided by the JGI Web server. Amino acid sequence alignments of proteins related to the *P. stutzeri* A1501 Ask_Ect and Ask_LysC proteins were performed with ClustalW (37).

The genome sequences of *P. stutzeri* A1501 were analyzed for the presence of homologous enzymes involved in synthesis of the compatible solutes trehalose and *N*-γ-acetlyglutaminyl glutamine 1-amide (NAGGN) (3, 35, 58). The following protein sequences were used as search queries for BlastP: trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase of *E. coli* (NP_416410 and NP_416411), trehalose synthase of *Streptomyces avermitilis* (NP_823979), alpha amylase of *Streptomyces coelicolor* (NP_630189), maltooligosyltrehalose trehalohydrolase of *Rhodopseudomonas palustris* (NP_948984), trehalose synthase of *Pyrococcus furiosus* (NP_579471), trehalose phosphorylase of *Propionibacterium acnes* (YP_055814), and glutamine amidotransferase and NGG acetyltransferase of *Pseudomonas aeruginosa* (NP_252149).

RESULTS

Ectoine and hydroxyectoine biosynthesis in *P. stutzeri* A1501. Inspection of the *P. stutzeri* A1501 genome sequence (67) revealed a gene cluster for the synthesis of ectoine and hydroxyectoine. This gene cluster comprises four genes encoding the enzymes for ectoine (*ectABC*) and hydroxyectoine (*ectD*) biosynthesis. These ectoine/hydroxyectoine biosynthetic genes are followed by a fifth gene (*pst_0177*) encoding a putative L-aspartokinase, which we refer to here as *ask_ect* (Ask_Ect) (Fig. 2A). The *ectABCD* genes encode proteins closely related to functionally characterized ectoine and hydroxyectoine biosynthetic enzymes (8, 9, 11, 17, 32, 33, 40, 57);

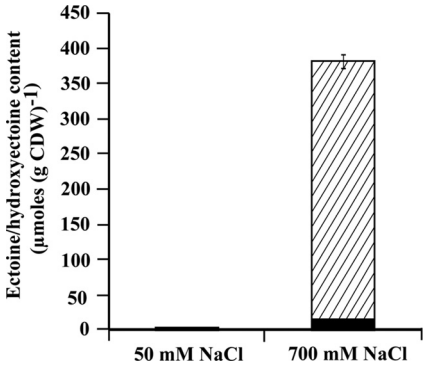

FIG. 3. Osmotic controlled synthesis of ectoine and hydroxyectoine in *P. stutzeri* A1501. Synthesis of ectoine (black bar) and hydroxyectoine (hatched bar) in *P. stutzeri* A1501 cells grown in MM63 with either low (50 mM NaCl) or high (700 mM NaCl) osmolarity was assessed by HPLC analysis. The cultures were grown to mid-exponential phase (A_{600} = 1). The error bar indicates the standard deviation.

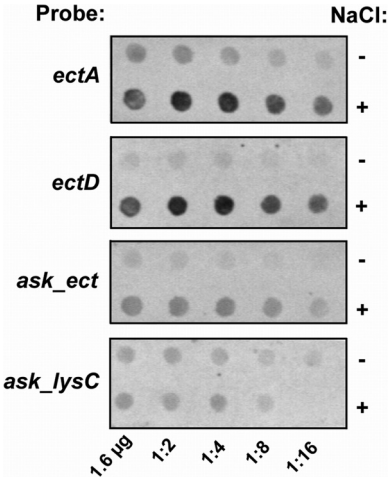

FIG. 4. Transcriptional analysis of the *ect* and *ask* genes of *P. stutzeri* A1501. Cultures of *P. stutzeri* A1501 were grown in MM63 containing either 50 mM (−) or 700 mM (+) NaCl. Total RNA was extracted from these cells, and a dilution series of RNA was subjected to dot blot analysis using *ectA*-, *ectD*-, *ask_ect*-, or *ask_lysC*-specific probes labeled with digoxigenin.

hence, *P. stutzeri A1501* was expected to produce ectoine and hydroxyectoine in response to osmotic stress.

To test the functional expression of the ectoine/hydroxyectoine biosynthetic gene cluster, we cultivated *P. stutzeri* A1501 in minimal medium (MM63) that contained either 0.05 M NaCl (low osmolarity) or 0.7 M NaCl (high osmolarity). Cells were harvested in the mid-exponential growth phase, the cells were lyophilized, the solute pool was extracted, and the intracellular content of ectoine and hydroxyectoine was determined by isocratic HPLC analysis. Only minor amounts of hydroxyectoine and ectoine (1.72 and 0.66 μmol/g cell dry weight [CDW]) were synthesized in cells cultivated at low osmolarity, but there was a strong increase of the hydroxyectoine (367.77 μmol/g CDW) and ectoine (15.69 μmol/g CDW) contents of the cells cultivated at high osmolarity, increases of approximately 215-fold and 24-fold in hydroxyectoine and ectoine levels, respectively (Fig. 3). The substantial increase in the hydroxyectoine and ectoine pool of osmotically stressed *P. stutzeri* A1501 cells is consistent with previous assessments of ectoine and hydroxyectoine production in response to osmotic stress in various microorganisms (8, 9, 32, 33, 58). However, the almost exclusive synthesis of hydroxyectoine by *P. stutzeri* A1501 is unusual, since most ectoine and hydroxyectoine producers typically contain a mixture of these two solutes with a considerable fraction of ectoine in exponential-phase cells (8, 9, 58). Our data, therefore, show that the *ectABCD* gene cluster of *P. stutzeri* A1501 is functional and allows the bacterium to amass considerable amounts of hydroxyectoine and moderate amounts of ectoine as osmostress protectants.

The type strain of *P. stutzeri*, DSM5190^T, is known to synthesize, in addition to large amounts of hydroxyectoine, minor quantities of trehalose and the peptide NAGGN in response to osmotic stress (58). Since these compatible solutes are also produced by many other *Pseudomonas* species (35), we assessed the presence of the corresponding structural genes in the genome sequence of *P. stutzeri* A1501 (67). We found three different putative biosynthetic pathways for the formation of trehalose (3): the trehalose-6-phosphate synthase/trehalose-6-phosphatase (TPS/TPP) pathway from UDP-glucose and glucose-6-phosphate, the trehalose synthase (TS) pathway from maltose, and the synthesis route of trehalose from maltooligsaccarides (TreY/TreZ). We assayed the trehalose content of *P. stutzeri* A1501 cells grown in MM63 containing either 50 mM (low osmolarity) or 700 mM (high osmolarity) NaCl and found increased amounts of trehalose in the high-osmolarity-stressed cells (data not shown), in accord with the data reported by Seip et al. (58) for the *P. stutzeri* DSM5190 type strain. Genes (*asnO* and *ngg*) encoding a glutamine amidotransferase (AsnO) and *N*-γ-acetylglutaminyl glutamine acetyltransferase (Ngg) for the synthesis of NAGGN (52) are also present in the genome sequence of *P. stutzeri* A1501, but we have not assessed the formation of this peptide in osmotically stressed cells.

Transcriptional analysis of the *ectABCD_ask_ect* gene cluster by RT-PCR. As noted above, a gene (*ask_ect*) encoding a putative L-aspartokinase follows the *ectABCD* gene cluster; the distance from the stop codon of *ectD* to the start codon of *ask_ect* is 152 bp. Northern blot analysis of the *ectABC* mRNA in several bacilli has shown that this gene cluster is transcribed as an osmotically inducible operon (9, 32, 33). Osmotic induction of the corresponding genes in *C. salexigens* (11) and in *H. elongata* (57) has also been observed, but the *ect* gene cluster of these bacteria exhibited a more complex pattern of transcription than that observed in bacilli.

The first transcriptional evaluation of an *ask_ect*-containing *ect* gene cluster was reported for the methylotrophic bacterium *M. alcaliphilum* 20Z. This analysis showed that the *ask_ect* gene was cotranscribed with the ectoine biosynthetic genes (49). To test for a similar genetic arrangement in *P. stutzeri* A1501, we carried out a one-step RT-PCR using total RNA isolated from cells of *P. stutzeri* grown in MM63 minimal medium containing 0.7 M NaCl, conditions which lead to strong hydroxyectoine/ectoine synthesis (Fig. 3) and under which the ectoine biosynthetic genes should therefore be actively transcribed (see below; Fig. 4). Successful amplification of intergenic regions of *ectAB*, *ectBCD*, and *ectD_ask_ect* (Fig. 2A)

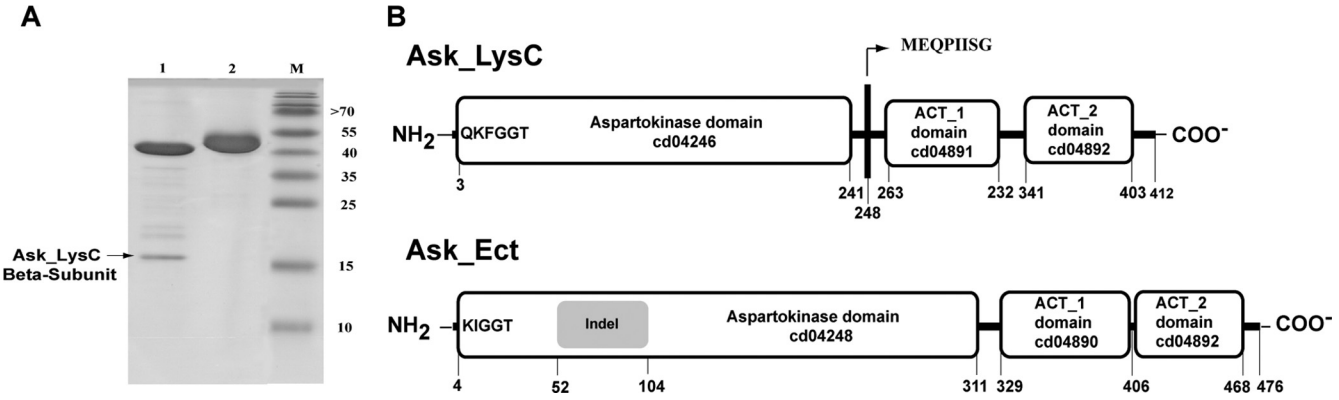

FIG. 5. Purification of the Ask_LysC and Ask_Ect proteins from *P. stutzeri* A1501 and schematic overview of their predicted domain structure. (A) SDS-PAGE of the purified Ask_LysC (lane 1) and Ask_Ect (lane 2) proteins; these proteins were purified by affinity chromatography using a streptavidin-tagII affinity matrix. The 18-kDa beta subunit of Ask_LysC is indicated by an arrow. (B) The predicted domain structures of Ask_LysC and Ask_Ect were analyzed by BLASTP at the NCBI Web server. The Ask signature sequence motif of Ask_LysC and the various motifs present in Ask_Ect are indicated; the positions of the aspartokinase domains and the ACT regulatory domains are shown schematically, and the numbers indicate their positions within the Ask polypeptides. The internal translational start site in the *ask_lysC* gene is marked by a bent arrow, and the resulting amino-terminal sequence of the beta subunit of the Ask_LysC protein is shown.

revealed that *ectABCD_ask_ect* genes are cotranscribed (Fig. 2B). In contrast, the transcription of the open reading frame *pst_0176*, which follows the stop codon of the *ask_ect* gene at a distance of 184 bp (Fig. 2A), is not part of the *ectABCD_ask_ect* gene cluster, since no RT-PCR product of the intergenic region between *ask_ect* and *pst_0176* could be amplified (Fig. 2B).

Osmotic induction of the *ectABCD_ask_ect* gene cluster. The transcription of the ectoine/hydroxyectoine biosynthetic genes is typically increased in high-salinity-stressed cells to foster enhanced ectoine/hydroxyectoine production to fulfill the role of these compounds as osmostress protectants (9, 11, 32, 33, 57). To test if this was also the case in *P. stutz*eri A1501, we isolated total RNA from cells cultivated in MM63 containing either 50 mM or 700 mM NaCl and analyzed the transcriptional levels of the *ect* and *ask_ect* genes by dot blot analysis using *ectA*-, *ectD*-, and *ask_ect*-specific single-stranded antisense RNA probes. The transcription of both the first (*ectA*) and the last (*ectD*) gene in the *ectABCD* gene cluster was strongly upregulated in the osmotically stressed cells (Fig. 4). Likewise, the transcription of the *ask_ect* gene was also increased in response to osmotic stress (Fig. 4), as expected from the cotranscription of this gene with the *ectABCD* genes (Fig. 2). In contrast, the transcriptional level of the second *ask* gene, *ask_lysC*, present in the *P. stutzeri* A1501 genome sequence was not increased in the cells grown under high salinity (Fig. 4).

Bioinformatic assessment of two Ask enzymes from *P. stutzeri* A1501. Inspection of the genome sequence of *P. stutzeri* A1501 (67) revealed genes encoding two aspartokinases, Ask_Ect (Pst_0177) and Ask_LysC (Pst_1370). The amino acid sequences of these two proteins are 25% identical, reflecting the membership of the two Ask proteins in different subgroups of the Ask superfamily; Ask_Ect belongs to the alpha subgroup, whereas Ask_LysC is a member of the beta subgroup (39). A schematic overview of the domain organization of Ask_LysC and Ask_Ect from *P. stutzeri* A1501 is shown in Fig. 5B, and an alignment of the amino acid sequence of the two enzymes is provided in Fig. S1 in the supplemental material.

The Ask_LysC protein comprises 412 amino acids (44.3 kDa; charge at pH 7, −11.64; pI, 5.1) and shows 46% identity to the well-characterized Ask of *Corynebacterium glutamicum* (29, 68). It exhibits the typical N-terminal cd04246 aspartokinase domain and possesses the characteristic N-terminal Q-K-F-G-G-T motif (39) (Fig. 5B; see Fig. S1 in the supplemental material). The *C. glutamicum* Ask protein is subject to concerted feedback inhibition by Thr and Lys, and mutants with this allosteric control abrogated have been characterized. There is considerable interest in the regulatory features of this enzyme, since it is a bottleneck for the biotechnological large-scale production of lysine (55, 68). Inspection of the amino acid sequence of the *P. stutzeri* A1501 Ask_LysC protein revealed that those amino acids that are involved in the allosteric control of the *C. glutamicum* Ask enzyme activity are conserved (see Fig. S2 in the supplemental material). Hence, one can expect that the *P. stutzeri* A1501 Ask_LysC enzyme will be inhibited through concerted feedback inhibition by Thr and Lys.

The Ask_Ect protein of *P. stutzeri* A1501 comprises 476 amino acids (52.4 kDa; charge at pH 7, −14.70; pI, 5.5) and belongs to the recently defined cohesion group 02 that encompasses only Ask_type proteins associated with ectoine biosynthetic gene clusters (39). Members of this cohesion group carry a modification of the typical N-terminal Q-K-F/Y-G-G-T signature sequence motif; it is changed in Ask_Ect to a K-I-G-G-T motif (39). The Ask_Ect protein of *P. stutzeri* A1501 possesses this K-I-G-G-T motif (Fig. 4B; see Fig. S1 and S3 in the supplemental material). Ask enzymes possess a C-terminal regulatory domain that usually comprises tandem copies of sequence motifs referred to as ACT_1 and ACT_2 (20, 39). Both Ask_Ect and Ask_LysC possess such ACT regulatory domains (Fig. 5B). However, members of cohesion group 02 (all Ask_Ect-type enzymes) carry the ACT_1 cd04890 domain; Ask_LysC from *P. stutzeri* A1501 utilizes the cd04891 domain in this position (Fig. 5B). None of the Ask enzymes that are associated with ectoine/hydroxyectoine biosynthetic pathways have been enzymatically characterized, and therefore, nothing

TABLE 1. Apparent kinetic properties of Ask_LysC and Ask_Ect from *P. stutzeri* A1501

Property	Value	
	Ask_LysC	Ask_Ect
L-aspartate		
K_m (mM)	21.6 ± 2.6	29.7 ± 2.5
V_{max} (U mg^{-1})	5.1 ± 0.1	6.9 ± 0.2
ATP		
K_m (mM)	3.8 ± 0.4	1.3 ± 0.1
V_{max} (U mg^{-1})	5.9 ± 0.2	6.8 ± 0.2 U

TABLE 2. Allosteric control properties of Ask_LysC and Ask_Ect from *P. stutzeri* A1501

Amino acid or combination	IC_{50} (mM)	
	Ask_LysC	Ask_Ect
Thr	2.9	3.6
Thr + Lys	0.4	–[a]
Thr + 650 mM NaCl	0.7	18.7
Thr + 650 mM KCl	3.9	13.5

[a] The concerted inhibition of Ask_Ect by Thr and Lys was not analyzed, since our results showed no inhibitory effect of lysine alone or in combination with threonine (see Fig. S4 in the supplemental material).

is known about the regulatory properties of this class of enzymes. Notably, conserved residues typically involved in the allosteric control of Ask activity (39, 68) are not present in members of the ectoine/hydroxyectoine-linked Ask enzymes.

Purification of the Ask_LysC and Ask_Ect proteins. To study the biochemical and regulatory properties of the Ask_LysC and Ask_Ect enzymes, we heterologously expressed both *ask* genes of *P. stutzeri* A1501 as C-terminal streptavidin-tagged fusion proteins using the recombinant *E. coli* strain BL21(DE3) harboring the hybrid plasmid pMP41(*ask_ect*) or pMPask1(*ask_lysC*). The respective Ask_streptavidin-tagII proteins were purified by streptavidin-Tactin affinity chromatography and analyzed by SDS-polyacrylamide gel electrophoresis. The purified Ask_Ect protein was a single protein with an estimated subunit molecular mass of about 50 kDa (Fig. 5A), a value that is in agreement with the calculated molecular mass of 53.6 kDa of the Ask_Ect-streptavidin-tagII recombinant protein. Heterologous expression of *ask_lysC* resulted in synthesis of two proteins with estimated molecular masses of about 44 kDa and 18 kDa (Fig. 5A). The 44-kDa protein has a subunit molecular mass that is in good agreement with that calculated (45.4 kDa) for the full-length Ask_LysC-streptavidin-tagII recombinant protein. As discussed above, the Ask_LysC protein is a member of the beta subgroup of Ask enzymes that typically harbor an internal translational start site in their mRNAs, leading to the synthesis of an additional C-terminal regulatory beta subunit of about 18 kDa (39). To investigate whether the observed 18-kDa protein species (Fig. 5A) present in the Ask_LysC protein preparation might represent such a beta subunit, the purified 18-kDa protein was subjected to N-terminal Edman sequencing. The amino acid sequence NH_2-M-E-Q-P-I-I-S-G was found, which perfectly matched amino acids 248 to 255 of the 412-amino-acid Ask_LysC protein. The inferred internal translational start site of the *ask_lysC* gene from *P. stutzeri* A1501 matches that of other characterized internal start sites of beta subunits of *ask* genes encoding proteins belonging to the beta subgroup of this family of enzymes (39). The beta subunit of Ask_LysC thus comprises 156 amino acids (18.8 kDa with the attached streptavidin-tagII peptide) and possesses two regulatory ACT domains (Fig. 5B).

Allosteric control of Ask_LysC and Ask_Ect enzyme activity. Aspartokinase enzyme activity was assayed by the method described by Black and Wright (6). Using this assay, we found both the recombinantly produced Ask_LysC and Ask_Ect proteins (Fig. 5A) to be enzymatically active, with specific activities of 5.6 and 6.8 U mg protein^{-1}, respectively (Table 1). These enzymatic activities are comparable to those of other biochemically characterized microbial aspartokinases (22, 30, 60). The kinetic data for the substrates ATP and L-aspartate of both Ask_LysC and Ask_Ect from *P. stutzeri* A1501 are summarized in Table 1.

We first analyzed the inhibition pattern of Ask_LysC and Ask_Ect in response to various amino acids (Thr, Lys, Met, Ile, Ala, and Gly) and different amino acid combinations. The addition of either Ala, Gly, Ile, or Met to the *in vitro* assay mixtures had no significant influence on the enzyme activity of either Ask protein (see Fig. S4 in the supplemental material). However, the addition of Thr strongly inhibited the activity of either the Ask_LysC or the Ask_Ect enzyme. The enzyme activity of Ask_LysC was not inhibited by addition of Lys alone; in fact, it resulted in slightly enhanced activity. In contrast, the simultaneous addition of Lys and Thr to Ask_LysC led to very strong inhibition of the enzyme activity. The inhibitory effect of a combination of Thr plus Lys was appreciably stronger than that of Thr alone (see Fig. S4A in the supplemental material). Hence, the Ask_LysC enzyme from *P. stutzeri* A1501 is subjected to feedback inhibition by Thr and additional concerted feedback inhibition by Thr and Lys. The latter effect resembles the regulatory properties of the amino acid sequence-related LysC enzyme from *C. glutamicum* (29, 68). In contrast, Lys neither inhibited the Ask_Ect enzyme alone nor did it potentiate the inhibitory effect of Thr on the activity of the enzyme (see Fig. S4B in the supplemental material). Hence, the Ask_LysC and Ask_Ect enzymes from *P. stutzeri* A1501 exhibit distinctly different patterns of allosteric regulation.

These results were extended by a more detailed analysis of the regulatory properties of the Ask_LysC and Ask_Ect aspartokinases with different concentrations of Thr and Lys. Ask_LysC activity was inhibited by 50% (50% inhibitory concentration [IC_{50}]) when 2.9 mM Thr was added to the enzyme assay but was unaffected when 50 mM Lys was added individually (Table 2); in contrast, the addition of Lys stimulated the activity of Ask_LysC somewhat (Fig. 6A). However, a mixture of Thr and Lys already afforded 50% inhibition of enzyme activity at concentrations of 0.4 mM Lys and 0.4 mM Thr (Fig. 6A). Thus, although Lys has no inhibitory effect on Ask_LysC by itself, a combination of Lys and Thr inhibits the enzyme to the same extent at 10-fold lower concentrations than are observed for Thr alone (Fig. 6A). The Ask_Ect enzyme behaved similarly with respect to Thr (50% inhibition with 3.6 mM Thr

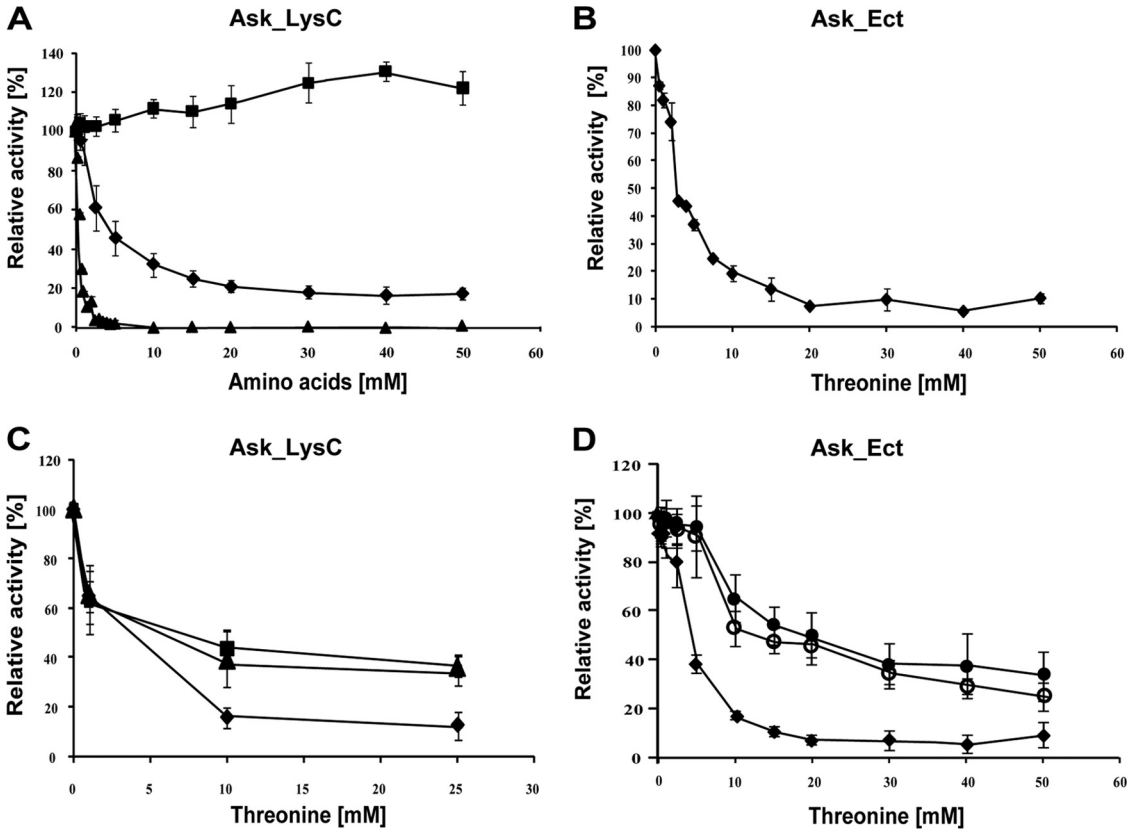

FIG. 6. Allosteric control of Ask_LysC and Ask_Ect in response to Thr and Lys. The purified Ask_LysC and Asl_Ect proteins were assayed for enzyme activity in the absence or presence of the amino acids Thr and Lys. (A) inhibition of Ask_LysC enzyme activity by the amino acid Thr (◆), Lys (■), and Thr and Lys combined (▲). (B) inhibition of Ask_Ect enzyme activity by the addition of Thr (◆). (C) inhibition of Ask_LysC enzyme activity by Thr without any additives (◆) or in the presence of 0.5 M ectoine (■) or 0.5 M hydroxyectoine (▲). (D) inhibition of Ask_Ect enzyme activity by Thr without (◆) or in the presence of 650 mM KCl (○) or 650 mM NaCl (●). Shown are the mean values and standard deviations based on three replicates.

added) but showed no additional response to Lys (Fig. 6B and Table 2).

Osmotically stressed microbial cells typically import very large amounts of potassium ions as an initial response to high osmolarity that are subsequently extruded again and replaced by compatible solutes in the second phase of osmotic adjustment (7, 31, 65, 66). Sodium ions also enter osmotically stressed cells either in cotransport with potassium (15) or via compatible solute transport systems that are driven by sodium gradients (69). We therefore asked if the allosteric feedback control of Ask_Ect in response to Thr is influenced by high concentrations of potassium and sodium. Whereas these ions in general had no influence on the overall enzyme activity of either Ask_LysC or Ask_Ect (data not shown), we found that the presence of either 650 mM NaCl or KCl reduced the inhibition by Thr of Ask_Ect. The IC_{50} was increased from 3.6 mM to 18.7 mM or 13.5 mM in the presence of 650 mM NaCl or KCl, respectively (Table 2) (Fig. 5D). Remarkably, the inhibition of Ask_LysC activity by either Thr or Thr plus Lys was not influenced by NaCl or KCl (tested concentration, 650 mM) (Table 2).

Osmotically challenged *P. stutzeri* A1501 cells amass large amounts of hydroxyectoine/ectoine (Fig. 3), and therefore, we also studied a possible influence of these compatible solutes on Ask_LysC and Ask_Ect enzyme activity; none was found up to a tested concentration of 650 mM either ectoine or hydroxyectoine (data not shown). However, the feedback control of Ask_LysC enzyme activity by Thr was somewhat reduced in the presence of these compatible solutes (Fig. 6C), whereas the allosteric control of Ask_Ect was not affected (data not shown).

Heterologous expression of the *ectABCD_ask_ect* gene cluster in *E. coli*. The cotranscription of the *ask_ect* gene with the *ectABCD* gene cluster present in *P. stutzeri* A1501 (Fig. 2) and its osmotic induction along with the *ectABCD* gene cluster (Fig. 4) suggest that the encoded Ask_Ect serves a specialized function during osmotic stress. In all likelihood, increased expression of *ask_ect* contributes to an adequate supply of the precursor, beta-aspartylphosphate, for ectoine/hydroxyectoine production in osmotically challenged *P. stutzeri* A1501 cells (Fig. 1). To test this, we constructed two recombinant plasmids that carry either the entire *ectABCD_ask_ect* gene cluster (pNST6) or a truncated *ectABCD* gene cluster lacking the *ask_ect* gene (pNST5). Both plasmids also carry 215 bp of DNA sequences located upstream of the *ectA* start codon, and hence, they should possess the osmotically controlled promoter for *ect* expression. We introduced pNST5 and pNST6 in the *E. coli* strain FF4169, which is defective in the osmoadap-

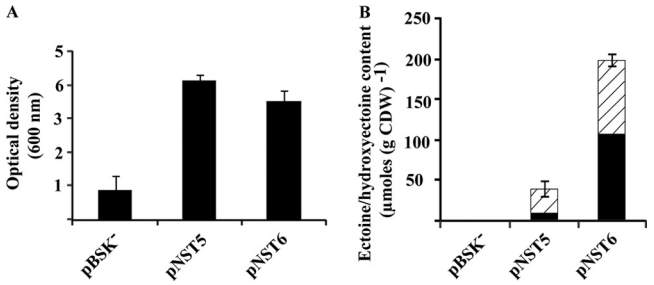

FIG. 7. Osmoprotection and heterologous production of ectoine and hydroxyectoine in *E. coli*. The *E. coli* strain FF4169 (*otsA1*::Tn*10*), defective in the synthesis of the compatible solute trehalose, was transformed either with the vector pBSK(−), pNST5 (*ectABCD*$^+$), or pNST6 (*ectABCD_ask_ect*$^+$) and cultivated in MM63 containing 0.3 M NaCl. (A) growth of the recombinant strains was determined by measuring the absorbance at 600 nm. (B) the intracellular content of ectoine (black bars) or hydroxyectoine (hatched bars) was determined by HPLC analysis. Shown are mean values and standard deviations based on three independent measurements.

tive synthesis of the compatible solute trehalose and is therefore rather osmotically sensitive (18). It should be noted that *E. coli* does not naturally synthesize ectoine (28). Strains FF4169(pNST5) and FF4169(pNST6) were grown in MM63 containing either 50 mM or 300 mM NaCl to raise the osmolarity. Both cultures grew like a control culture of the same strain carrying the empty cloning vector pBluescript SK(−) [pBSK(−)] in low-salt MM63 (50 mM NaCl), but the presence of either pNST5 or pNST6 effectively complemented the osmotically sensitive growth phenotype of the host strain FF4169 (Fig. 7A) in high-salt MM63 (300 mM NaCl), whereas a control culture of FF4169 carrying the pBSK(−) cloning vector remained osmotically sensitive. This growth pattern was indicative of the production of the osmoprotectants ectoine/hydroxyectoine in the recombinant strains, and we therefore analyzed the intracellular ectoine and hydroxyectoine contents of the cells by HPLC. The cells of FF4169(pNST5) and FF4169(pNST6) grown at low osmolarity contained only very low levels of about 0.825 μmol g CDW ectoine^{-1} and 1.89 μmol g CDW hydroxyectoine^{-1} in strain FF4169(pNST5) and 2.0 μmol g CDW ectoine^{-1} and 2.0 μmol g CDW hydroxyectoine^{-1} in strain FF4169(pNST6). As expected (28), no ectoine was found in strain FF4169[pBSK(−)]. However, growth of strains FF4169(pNST5) and FF4169(pNST6) at high osmolarity (MM63 with 300 mM NaCl) led to a very strong increase in the ectoine and hydroxyectoine contents: 10.32 μmol g CDW ectoine^{-1} and 29.95 μmol g CDW hydroxyectoine^{-1} were found in the strain carrying the *ectABCD* plasmid pNST5 and 107 μmol g CDW ectoine^{-1} and 91 μmol g CDW^{-1} were found in the strain carrying the *ectABCD_ask_ect* plasmid pNST6 (Fig. 7B). Hence, ectoine and hydroxyectoine formation was strongly stimulated in both recombinant strains by high-salinity growth conditions, indicating that the *ect* genes present on both recombinant plasmids pNST5 and pNST6 were expressed from their authentic and osmotically regulated promoter(s). The strain carrying the *ectABCD_ask_ect* plasmid pNST6 produced about 5-fold more ectoine and hydroxyectoine (both compounds combined) than its counterpart strain FF4169(pNST5) expressing only the *ectABCD* genes (Fig. 7B). Since plasmids pNST5 and pNST6 differ only in the presence of the *ask_ect* gene, it is apparent that the added Ask_Ect enzyme significantly enhances the recombinant production of the compatible solutes ectoine and hydroxyectoine in an *E. coli* host strain (Fig. 7B).

Genomic context of the *ectABCD_ask_ect* gene cluster in *P. stutzeri* A1501. There are currently 19 complete genomes of *Pseudomonas* species publicly available in the NCBI and JGI databases. With the exception of *P. stutzeri* 1501, none carries a complete ectoine biosynthetic gene cluster. This suggests that the *ectABCD_ask_ect* operon might have been acquired by *P. stutzeri* A1501 via lateral gene transfer. We therefore analyzed the genetic context of the *ectABCD_ask_ect* gene cluster in *P. stutzeri* A1501 and the corresponding regions in other *Pseudomonas* genomes. The *ectABCD_ask_ect* gene cluster has an average G+C content of 61.4%, a value that is marginally lower than the average G+C content of the entire *P. stutzeri* A1501 genome, 63.8% (67). The *ectABCD_ask_ect* operon is located between genes coding for a putative peptidase (Pep) and a putative glucose-6-phosphate dehydrogenase (G6P-DH) (Fig. 8). Both genes could also be identified in an identical arrangement in genome sequences of various *P. aeruginosa* strains (e.g., *P. aeruginosa* PA7) (51) (Fig. 8), pointing to the insertion of the *ectABCD_ask_ect* locus as an entire gene block by lateral gene transfer into the intergenic region of the abovementioned peptidase and glucose-6-phosphate dehydrogenase genes of *P. stutzeri* A1501 (Fig. 8).

We used BLAST searches to look for a possible origin of the *ectABCD_ask_ect* genes of *P. stutzeri* A1501 by employing the ectoine (EctABC), hydroxyectoine (EctD), and Ask_Ect proteins as search queries. This approach identified the marine bacterium *Marinomonas* sp. strain MWYL1 as a potential source for the *ectABCD_ask_ect* genes of *P. stutzeri* A1501. The degree of amino acid sequence identity between the EctABCD and the Ask_Ect proteins of *P. stutzeri* A1501 and the *Marinomonas* strain MWYL1 orthologues ranges between 63% for the EctA protein and 48% for the EctD protein; the two Ask_Ect proteins are 56% identical. *Marinomonas* MWYL1 is a member of the *Gammaproteobacteria*, to which *P. stutzeri* A1501 also belongs (36). Interestingly, of all members of the *Gammaproteobacteria* that are putative ectoine producers, *Marinomonas* MWYL1 is the only representative that also possesses an *ectD* gene integrated into the *ectABC* cluster, as well as an *ask_ect* gene (see Fig. S5 in the supplemental material). Although *P. stutzeri* A1501 was originally isolated from soil of a rice paddy field and not from a marine environment (67), it is well known that many strains of the widely distributed *P. stutzeri* species live in marine habitats (36).

Genetic coorganization of the ectoine biosynthetic genes with *ask_ect*. Colocalization of the ectoine/hydroxyectoine biosynthetic genes and of *ask_ect* is not uncommon in microorganisms (39, 45, 57, 62). We extend previous analysis by inspecting the presently available bacterial (1,426) and archaeal (80) genome sequences for *ask_ect* genes associated with ectoine/hydroxyectoine biosynthetic gene clusters. By using the EctC protein as a search query, we identified 220 microorganisms as putative ectoine producers; this number probably slightly overestimates the occurrence of complete ectoine biosynthetic gene clusters, since some microorganisms posses only an *ectC* gene but lack readily identifiable *ectAB*

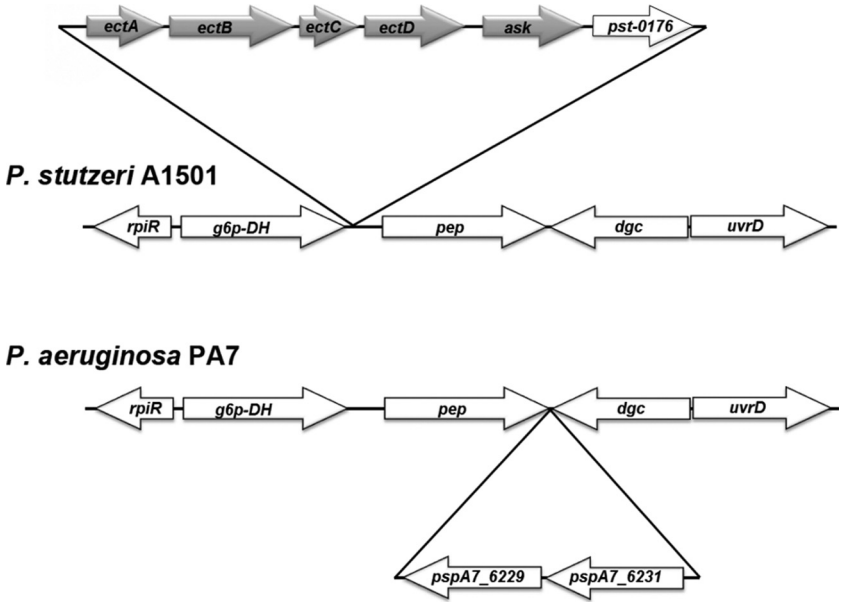

FIG. 8. A possible scenario for the acquisition of the *ectABC_ask_ect* gene cluster of *P. stutzeri* A1501 by lateral gene transfer. A DNA segment of the genomic organization of the *P. aeruginosa* PA7 strain (51), which is a close phylogenetic relative of *P. stutzeri* A1501 (67), is shown. The *ectABCD_ask_ect* gene cluster and the gene (*Pst_0176*) for a protein of unknown function were derived from an unknown donor organism and inserted as a block into the intergenic region between the *P. stutzeri* A1501 genes encoding a putative glucose-6-phosphate dehydrogenase (*g6p-DH*) and a putative peptidase (*pep*).

genes. Of these 220 putative ectoine/hydroxyectoine producers, 84 also possess an *ask* gene coorganized within the same gene cluster with either the *ectABC* or the *ectABCD* operon or, very rarely, located adjacent to an orphan *ectC* gene (see Fig. S5 in the supplemental material). As already recognized by Lo et al. (39) in their analysis of microbial genomes available in the year 2009, *ask_ect*-containing genomes were exclusively from members of the *Proteobacteria*. Most of the *ectABC*(*D*)-*ask_ect*-containing microorganisms were from members of the *Alphaproteobacteria* (19 genomes) and *Gammaproteobacteria* (59 genomes that included 46 *Vibrio* species), two *ectABC_ask_ect*-containing genomes originate from the *Deltaproteobacteria*, one from the *Epsilonproteobacteria*, and one from the *Betaproteobacteria* (see Fig. S5 in the supplemental material). Since ectoine and hydroxyectoine biosynthetic genes can readily be found outside the phylum *Proteobacteria* (9, 45, 50), the strong association of the ectoine biosynthetic genes with *ask_ect*-type genes exclusively in members of the *Proteobacteria* is a surprising observation. With two exceptions, all *ask_ect*-containing bacteria are associated with marine habitats. The majority of this group contain an *ectABC_ask_ect* cluster; only eight microorganisms are currently known that possess an *ectABCD_ask_ect* cluster with a genetic organization identical to the one we have identified in *P. stutzeri* A1501 (see Fig. S5 in the supplemental material). We aligned the amino acid sequences of the 84 Ask_Ect proteins and found that the proteins have amino acid sequence identities that range between 56% and 39% (see Fig. S3 in the supplemental material). With two exceptions, all of them possess the conserved K-I-G-G-T motif that has been proposed by Lo et al. (39) as a hallmark of the Ask_Ect subgroup of aspartokinases.

DISCUSSION

To fine tune specific biosynthesis routes that branch from the aspartate-beta-semialdehyde hub (Fig. 1), many microorganisms have evolved multiple forms of aspartokinase (14, 39, 46). The compatible solutes and osmoprotectants ectoine and hydroxyectoine are derived from aspartate-beta-semialdehyde (40, 44) (Fig. 1), and the data presented here provide evidence for the existence of a specialized Ask enzyme in *P. stutzeri* A1501 that is associated with the production of these compounds. Our database searches hint at the widespread occurrence of such specialized aspartokinases (Ask_Ect) in many ectoine/hydroxyectoine-synthesizing microorganisms (see Fig. S3 and S5 in the supplemental material).

The cellular levels of ectoine and hydroxyectoine are typically linked to the prevailing osmolarity of the environment (8, 9, 32, 33, 43, 45) and can reach molar concentrations in severely osmotically stressed cells (4). Hence, the demand for the substrate (aspartate-beta-semialdehyde) of the first ectoine biosynthetic enzyme EctB, L-2,4-diaminobutyric acid transaminase (40, 44), will vary greatly with growth conditions. Consequently, osmotic control of the *ectABCD-ask_ect* gene cluster, as observed in *P. stutzeri* A1501 (Fig. 4), leads to simultaneous increases in both the ectoine/hydroxyectoine biosynthetic enzymes EctABCD and Ask_Ect under growth conditions that trigger enhanced ectoine/hydroxyectoine production (Fig. 3). An aspartokinase that is specially adapted to the ectoine/hydroxyectoine biosynthetic route is thus likely to benefit osmotically challenged cells by preventing biosynthetic constraints for these compatible solutes caused by an insufficient supply of the precursor aspartate-beta-semialdehyde (Fig. 1) (5, 32). In support of this notion, we found that the

expression of the *ectABCD_ask_ect* gene cluster from *P. stutzeri* A1501 under its natural osmotically controlled promoter(s) in an *E. coli* host strain leads to a significantly (about 5-fold) larger ectoine/hydroxyectoine pool in direct comparison to a strain that expressed only the *ectABCD* genes (Fig. 7B).

We found that *P. stutzeri* A1501 is currently the only one of 19 *Pseudomonas* species with a finished genome sequence that possesses a complete cluster of ectoine/hydroxyectoine biosynthetic genes. This suggests that the *ectABCD_ask_ect* gene cluster present in *P. stutzeri* A1501 has been acquired via lateral gene transfer (Fig. 8). Lateral gene transfer is a major source of genetic and physiological innovation in the microbial world (59). The acquisition of the capacity to produce ectoine/hydroxyectoine, in addition to the synthesis of the compatible solutes trehalose and the dipeptide NAGGN that are commonly found in pseudomonads (35, 58), should enhance the ability of *P. stutzeri* A1501 to cope flexibly with osmotic stress in its varied habitats. In the *P. stutzeri* type strain DSM5190^T, whose genome has not yet been sequenced, an *ectABCD_ask_ect* gene cluster is also present (58), and Seip et al. observed that hydroxyectoine dominates the compatible solute pool of stationary-phase cells with minor amounts of ectoine and with more limited contributions of the disaccharide trehalose and the dipeptide NAGGN (58).

The Ask_Ect enzyme belongs to the cohesion group 02 defined recently by Lo et al. (39). These authors concluded from amino acid sequence comparisons that members of this subgroup possess either no allosteric control or allosteric control of an unknown type. The Ask_Ect enzyme from *P. stutzeri* A1501 is the first representative of cohesion group 02 that has been studied biochemically (Tables 1 and 2). The data presented here show that the Ask_Ect enzyme is indeed subjected to allosteric control and that feedback inhibition is exclusively mediated by Thr (Fig. 6B and D; see Fig. S4 in the supplemental material). High ionic strength modulates the degree of Thr-mediated feedback control of Ask_Ect (Table 2) (Fig. 6D), but high concentrations of ectoine or hydroxyectoine do not influence its enzyme activity. Hence, the Ask_Ect enzyme is well adapted to operate in an at least temporarily high-ionic-strength cytoplasmic environment (7, 31, 65, 66) and under conditions where hydroxyectoine and ectoine are amassed to exceedingly high cellular levels (4).

Judging from the inspection of completed genome sequences, about a third of putative ectoine/hydroxyectoine-producing microorganisms possess genetically linked *ask_ect*-type genes (84 out of 220). This genetic configuration, however, is restricted to members of the *Proteobacteria*, and most of the bacteria that possess *ectABC*(*D*)-*ask_ect* gene clusters live in marine habitats (see Fig. S5 in the supplemental material). We have observed in a recombinant *E. coli* strain expressing the *ectABCD_ask_ect* operon substantially increased production of ectoine/hydroxyectoine in comparison to a recombinant *E. coli* strain expressing just the *ectABCD* gene cluster (Fig. 7B). Ectoine/hydroxyectoine producers can readily be detected outside the *Proteobacteria* (45, 50). One therefore wonders why not all microorganisms that synthesize these compatible solutes possess a specialized *ask_ect* gene cotranscribed with the osmotically controlled *ect* biosynthetic genes to foster ectoine/hydroxyectoine production.

Hydroxyectoine has been shown in a number of instances to possess cytoprotective and protein-stabilizing properties that are superior to those of ectoine (38, 45, 50). Although we did not make any attempts to maximize hydroxyectoine production, *P. stutzeri* A1501 synthesized hydroxyectoine almost exclusively in exponential-phase cells (Fig. 3). Most other characterized ectoine/hydroxyectoine producers contain mixtures of these solutes with a considerable portion of ectoine (8, 9, 58), and this requires cost-intensive separation processes for the retrieval of pure hydroxyectoine for commercial purposes (38). Since members of the *P. stutzeri* species are typically nonpathogenic (36), *P. stutzeri* A1501, studied by us, and *P. stutzeri* DSM5190^T (58), studied by Seip et al. (58), could be interesting candidates for the development of an efficient biotechnological production process for hydroxyectoine.

Attempts are being made to establish production processes for ectoine and hydroxyectoine that are based on recombinant systems hyperexpressing the *ect* biosynthetic genes in easy-to-manipulate host strains, e.g., *E. coli* (5, 40, 49, 56, 58). Since *E. coli* does not naturally synthesize ectoine (28), there is no adequate metabolic and genetic adaptation of the aspartokinases and their structural genes in this host (14) to effectively supply the ectoine biosynthetic precursor aspartate-beta-semialdehyde under osmotic stress conditions. Hence, for an efficient recombinant ectoine/hydroxyectoine production system, this bottleneck needs to be opened in *E. coli* (5) or other potential production strains, such as *Bacillus subtilis* (32). We have not yet made any attempts to optimize yields or the relative proportions of ectoine and hydroxyectoine in the recombinant *E. coli*(pNST6 [*ectABCD_ask_ect*]) system (Fig. 7), but we consider *ectABCD_ask_ect*-type gene clusters a promising starting point for the genetic engineering of improved ectoine/hydroxyectoine biosynthetic routes in heterologous host strains.

Collectively, our data provide novel insights into the biochemical properties of the specialized Ask_Ect enzyme and clues to improved recombinant production of two interesting compatible solutes that have already found wide commercial applications. With respect to the allosteric regulatory properties of the Ask_Ect enzyme, the characterization of mutants resistant to feedback inhibition by Thr might be rewarding in terms of a greater biochemical understanding of this enzyme. Furthermore, such types of mutants might also be helpful to boost the yield of a recombinant production system that employs *ectABCD_ask_ect* gene clusters.

ACKNOWLEDGMENTS

Financial support for this study was provided through the LOEWE program of the State of Hessen via the Centre for Synthetic Microbiology (SynMicro, Marburg, Germany) (to E.B. and J.H.) and the Fonds der Chemischen Industrie (to E.B).

We are very grateful to Claudine Elmerich for providing us with *P. stutzeri* A1501, to Arne Strom for sending the trehalose-deficient *E. coli* strain, and to Gregor Lentzen (Bitop AG) for a generous gift of ectoines. We thank Maritha Lippman for dedicated and expert technical assistance and greatly appreciate the help of Vickie Koogle with the editing of the manuscript.

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