Proline Utilization by Bacillus subtilis: Uptake and Catabolism

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1-Proline can be used by Bacillus subtilis as a sole source of carbon or nitrogen. We traced 1-proline utilization genetically to the putBCP (yegMNO) locus. The putBCP gene cluster encodes a high-affinity proline transporter (PutP) and two enzymes, the proline dehydrogenase PutB and the Δ1-pyrroline-5-carboxylate dehydrogenase PutC, which jointly catabolize 1-proline to 1-glutamate. Northern blotting, primer extension, and putB-treA reporter gene fusion analysis showed that the putBCP locus is transcribed as an 1-proline-inducible operon. Its expression was mediated by a SigA-type promoter and was dependent on the porters that harness electrochemical Na⁺ gradients to couple the flow of Na⁺ ions with the transport of solutes across biological membranes (48). The PutA protein is a trifunctional membrane-associated enzyme comprising both PRODH and P5CDH domains (Fig. 1B) (37, 52) and also contains an N-terminal ribbon-helix-helix DNA-binding domain that endows PutA with the ability to act as a transcriptional repressor (61, 71). Depending on the redox state (52, 70), PutA can switch between its functions as a membrane-associated 1-proline-degradative enzyme and a cytoplasmic regulatory protein to repress the expression of the putPA 1-proline catabolic gene locus in response to the osmoadaptive production of the compatible solute 1-proline.

The soil-dwelling Gram-positive bacterium Bacillus subtilis lives in a challenging habitat in which the supply of nutrients is often restricted (20, 27, 56). Amino acids are particularly valuable resources for bacteria because they not only can be used as pre-formed building blocks for protein synthesis but often can also be employed as sole carbon, energy, and nitrogen (or sulfur) sources (22). They enter the habitat of B. subtilis as root exudates (27), and as products of lysed or osmotically down-shocked microbial cells (66). B. subtilis can actively seek amino acids as nutrients through chemotaxis (49).

Many bacteria can employ 1-proline as a nutrient, and the catabolism of the amino acid typically involves its enzymatic oxidation to 1-glutamate (61, 72), a central metabolite positioned at the intersection of carbon and nitrogen metabolism in many microorganisms (14, 55). Oxidation of proline is catalyzed in a two-step reaction by a flavin-containing proline dehydrogenase (PRODH) (EC 1.5.99.8) to Δ1-pyrroline-5-carboxylate (PSC). This intermediate spontaneously hydrolizes to γ-glutamate-5-semialdehyde, which is then further oxidized by a NAD-dependent Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH) (EC 1.5.1.12) to 1-glutamate (Fig. 1B). These enzymatic steps can either be carried out by a single bifunctional protein comprising two domains (PRODH-P5CDH) (59) or by monofunctional proteins with separate PRODH and P5CDH activities (32, 68).

Particularly well-studied examples of proline utilization by microorganisms are the PutPA systems of the enterobacteria Escherichia coli and Salmonella enterica serovar Typhimurium (37, 52, 61, 70, 72). PutP is a high-affinity proline importer and a member of the sodium solute symporter (SSS) (TC2A.21) family, transporters that harness electrochemical Na⁺ gradients to couple the

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avoid a futile cycle of L-proline production and degradation when it faces high-osmolarity surroundings.

**MATERIALS AND METHODS**

**Chemicals.** L-Proline, trans-4-hydroxyproline, thioproline, L-azetidine-2-carboxylic acid (AC), 3,4-dehydro-L-proline (DHP), the chromogenic substrates for the TreA [phospho-α-(1,1)-glucosidase] enzyme para-nitrophenyl-α-D-glucopyranoside (α-PNG) and for the ProB enzyme (α-aminobenzaldehyde), and the ninhydrine reagent, as well as the antibiotics chloramphenicol, kanamycin, tetracycline, erythromycin, and spectinomycin, were purchased from Sigma-Aldrich (Steinheim, Germany). Dimethyl-proline (proline betaine) was purchased from Atkins Chemicals (Chengdu, China), and monomethyl-proline (1) was a kind gift from D. Le Rudulier (University of Nice, Nice, France). L-[14C(U)]proline (40 mCi mmol⁻¹) was purchased from DuPont de Nemour GmbH (Neu-Isenburg, Germany).

**Bacterial strains.** The E. coli strain DH5α (Invitrogen, Carlshard, CA) was used for routine cloning purposes and maintenance of cloning vectors and recombinant plasmids. These strains were grown and maintained on Luria-Bertani (LB) agar plates. Solid and liquid media contained, when necessary, antibiotics to select for the presence of plasmids. The B. subtilis wild-type strain JH642 (trpC2 pheA1) (a kind gift of J. Hoch, Scripps Research Institute) and its mutant derivatives were used throughout this study (Table 1).

**Media and growth conditions.** The B. subtilis strains were maintained on LB agar plates; liquid cultures were grown at 37°C in Spizizen’s mini medium (SMM) (26) supplemented with 0.5% (wt/vol) glucose as the carbon source, a solution of trace elements (28), and the amino acids threonine (50 mg liter⁻¹) and L-phenylalanine (20 mg liter⁻¹) to meet the auxotrophic needs of strain JH642 (trpC2 pheA1) and its derivatives. The medium contained 15 mM NH₄Cl as the nitrogen source. When L-proline was used as the sole carbon and energy source, glucose (28 mM) was replaced with 32 mM L-proline to provide the bacterial cells with the same molarity of carbon atoms available to the cells for catabolism. When L-proline was used as the sole nitrogen source, the NH₄Cl content (15 mM) of the SMM was replaced with 15 mM L-proline. The antibiotics chloramphenicol, kanamycin, tetracycline, erythromycin, and spectinomycin were used with B. subtilis strains at final concentrations of 5 μg ml⁻¹, 10 μg ml⁻¹, 15 μg ml⁻¹, 1 μg ml⁻¹, and 100 μg ml⁻¹, respectively. Ampicillin and chloramphenicol were used for E. coli cultures at final concentrations of 100 μg ml⁻¹ and 35 μg ml⁻¹, respectively.

**Recombinant DNA techniques.** The routine manipulations of plasmid DNA, the construction of recombinant DNA plasmids, the isolation of chromosomal DNA from B. subtilis, and transformation with plasmid or chromosomal DNA were carried out using standard procedures (28). For the detection of homologous sequences by Southern hybridization, we used digoxigenin (DIG)-labeled DNA probes. For the preparation of these hybridization probes and the detection of the hybridization signals with chromosomal DNAs of various B. subtilis strains, we used the DIG DNA Labeling and Detection kit (Roche Diagnostics, Mannheim, Germany). The DNA-DNA hybridization conditions used followed the experimental procedures suggested by the manufacturer of the labeling and detection kit. DNA restriction fragments were blotted on a Nytran 13N nylon membrane purchased from Schleicher and Schuell (Dassel, Germany).

**Construction of plasmids, putB’-treA reporter strains, and chromosomal gene disruptions.** The construction of a plasmid that allows the expression of the putB’-treA reporter gene fusion on plasmids and their integration as a single copy into the B. subtilis chromosome at the amyE gene are detailed in the supplemental material. Strains with defects in individual genes of the chromosomal putBCP locus or the entire putBCP gene cluster were constructed by transforming strain JH642 with linearized plasmid DNA carrying the desired gene disruption mutation marked with an antibiotic resistance cassette into strain JH642 and by a subsequent selection for antibiotic-resistant colonies on LB agar plates. Details on the construction...
of the plasmids used for the generation of these mutant *B. subtilis* strains can be found in the supplemental material.

**Transcription analysis of the putBCP gene cluster by Northern blot analysis.** The transcriptional regulation of the putBCP gene cluster in response to the availability of l-proline in the growth medium and its genetic organization were analyzed by Northern blotting. Total RNA was isolated from *B. subtilis* strains with the acidic-phenol method (30) from log-phase cells of the *B. subtilis* strain JH64 containing pSM13, a plasmid carrying the *yclt-plutB* intragenic region that should contain the putBCP promoter (Fig. 1A). A reverse transcription reaction was carried out with 10 µg of total RNA isolated from these cells and 2 µmol of the synthetic oligonucleotide SM28 (5′-C GCCATTTTTATGGAGAAAGGGCGG-3′, bp 37 to 60 of the putB coding region) labeled at its 5′ end with the infrared dye IRD-800 (Eurofins MWG Operon, Ebersberg, Germany) as described previously (11). The reverse transcription reaction product was analyzed on a 6% DNA-sequencing gel run in a Li-Cor DNA sequencer (type 4000; Eurofins MWG Operon, Ebersberg, Germany). A sequencing ladder produced with the IRD-800-labeled SM28 primer and plasmid pSM13 as the DNA template was run in parallel with the primer extension reaction product on the same DNA-sequencing gel to determine the 5′ end of the putBCP mRNA.

**Determination of PutB enzyme activity.** PutB enzyme activity was measured in crude extracts of *B. subtilis* cells grown in SMM in the absence or presence of 1 mM proline. The assay used followed the method of Dendinger and Brill (17), which monitors the oxidation of proline to delta-1-pyrroline-5-carboxylate (PSC) by determining the formation of the PSC-o-aminoazulenoate from the chromogenic substrate o-aminoazulenoate in a spectrophotometer. The millimolar extinction coefficient of the PSC-o-aminoazulenoate complex is 2.71 mM⁻¹ cm⁻¹ (17). The specific activity of the PutB proline dehydrogenase (also sometimes referred to as proline oxidase) (4) in the crude cell extracts of *B. subtilis* strains is given as nmol PSC formed per minute and mg protein (U mg protein⁻¹).

**TrecA enzyme activity assays.** An aliquot (1.5 ml) from cultures of putB-trecA *B. subtilis* fusion strains was harvested by centrifugation for 2 min in an Eppendorf microcentrifuge (15,000 rpm) and resuspended in 0.5 ml Z buffer (42) adjusted to pH 7.0 and containing 1 mg ml⁻¹ lysozyme. After incubation for 10 min at 37°C in an Eppendorf thermomixer, cellular debris was removed by centrifugation (5 min at 12,000 rpm), and the supernatant was then used for TrecA activity assays with *para*-nitrophenyl-α-o-glucopyranoside as the substrate (23). TrecA specific activity is expressed in units per mg of protein; protein concentrations were estimated from the optical density of the cell culture (42).

**Sensitivity of B. subtilis strains to toxic proline analogues.** The proline analogues AC and DHP are toxic to microorganisms (39, 69). To test the sensitivity of *B. subtilis* strains to AC and DHP, cultures were grown in SMM with and without addition of 0.6 M NaCl until they reached an optical density at 578 nm (OD578) of 1.5. A 300-µl aliquot of each culture was then plated on SMM or SMM agar plates with 0.6 M NaCl before a 5-mm paper filter disk, soaked with 10 µl of a 25-µg ml⁻¹ solution of AC or DHP, was placed in the center of each agar plate. The formation of a growth inhibition zone around the filter disk was recorded after incubation of the agar plates at 37°C for 24 to 48 h.

**Transport assays with radiolabeled proline.** The kinetic parameters of proline transport via the PutP and OpuE transport systems of *B. subtilis* were determined in strain SMB11 (PutP⁺ OpuE⁺) and BLOB9 (PutP⁻ OpuE⁻) (Table 1) using L-[1-¹⁴C(U)]proline (40 mCi mmol⁻¹). The strains were cultivated in SMM or SMM containing either 0.4 M or 0.6 M NaCl under putBCP-inducing (growth of the cultures in the presence of 1 mM l-proline) or non-putBCP-induced (growth of the cultures in the absence of l-proline) conditions. Aliquots (2 ml) were removed from the culture when the cells reached the log phase (OD578, about 0.3 to 0.6). Those cultures that were grown in the presence of proline to induce putBCP expression were washed twice with proline-free cultivation medium that had been warmed to 37°C. Various concentrations (1 µM to 40 µM) of l-1-[¹⁴C(U)]proline were added to the cells, and aliquots (0.3 ml) were taken after 40, 80, and 120 s; the cells were then collected by filtration onto a cellulose filter (0.45 µm) and taken after 40, 80, and 120 s; the cells were then collected by filtration onto a cellulose filter (0.45 µm) and the sensitivity of

### Table 1. *B. subtilis* strains used in this study

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*All strains except BB330, BB350, and OI3280 are derivatives of *B. subtilis* strain JH642 and therefore carry, in addition to the genetic markers indicated, the trpC2 pheA1 mutations.

* The designation amyE::[putB⁻·treA]1 indicates that the putB-trecA operon gene fusion is stably integrated via a double-recombination event into the chromosomal amyE gene of *B. subtilis* as a single copy, thereby rendering the fusion strains defective in the extracellular amyE-α-amylase. The [putB⁻·treA]1 reporter fusion is linked to a chloramphenicol resistance gene (cat), thereby rendering all strains carrying the amyE::[putB⁻·treA]1 construct resistant to the antibiotic chloramphenicol.

* BGSC, Bacillus Genetic Stock Center, Ohio State University, Columbus, OH.

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protein $^{-1}$. The kinetics of $\text{L-}^{14}$C(U)]proline uptake was analyzed according to the method of Michaelis-Menten.

**Measurements of cellular proline pools.** Cells of the *B. subtilis* strain SMB10 (Table 1) were cultivated in 20 ml SMM or SMM with 1 M NaCl until they reached an OD$_{578}$ of 1. One set of the cultures received 5 mM L-proline, and both sets of cultures were then incubated further until they reached an OD$_{578}$ of 2. Aliquots of the cells were harvested prior to (at an OD$_{578}$ of 1) and after (at an OD$_{578}$ of 2) the addition of L-proline, washed with their growth medium (without L-proline), and then assayed for TreA activity to monitor putB-treA expression and proline content. The intracellular content of proline was determined by the method described by Bates et al. (5), which monitors the proline content of samples as a dark-red proline-ninhydrin complex that is measured photometrically at a wavelength of 480 nm. To correlate the colored proline-ninhydrin complex with the proline concentration, a calibration curve was established by treating standard solutions with a known L-proline concentration (0 mM to 10 mM) in the same way as the whole-cell extracts. Intracellular proline concentrations were calculated using a volume for a *B. subtilis* cell of 0.67 $\mu$l per 1 OD$_{578}$ unit of cell culture (S. Moses, E. P. Bakker, and E. Bremer, unpublished data).

Database searches and alignments of amino acid sequences of proteins related to the PutB, PutC, and PutP proteins. Proteins that are homologous to the proline catabolic PutB and PutC enzymes, to the proline transporter PutP, and to the proline-responsive PutR activator protein from *B. subtilis* were searched for via the Web server of the Department of Energy (DOE) Joint Genome Institute (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 (2). Protein models of the *B. subtilis* PutB and PutC proteins were generated with the aid of the SWISS Model server (http://swissmodel.expasy.org) (3).

**RESULTS**

Predicted functions of the putBCP-encoded proteins for proline utilization. The ycgMNO gene cluster from *B. subtilis* encodes two enzymes (*YcgM* and *YcgN*) predicted to be involved in L-proline catabolism and a transport protein (*YcgO*) predicted to mediate L-proline uptake. Since we show below that the ycgMNO-encoded proteins are required for the utilization of L-proline as a nutrient by *B. subtilis*, we refer here to this gene cluster as putBCP (proline utilization) (Fig. 1A). We avoided the use of *putA* as a gene designation because the PutA protein in enterobacteria comprises both proline dehydrogenase (PRODH) and $\Delta^1$-pyrroline-5-carboxylate dehydrogenase (P5CDH) enzyme activities (37, 52, 59), whereas the PutB and PutC proteins from *B. subtilis* represent monofunctional PRODH and P5CDH enzymes (see below) (32, 68). The putBCP gene cluster is followed in the same transcriptional orientation by the *putR* (*ycgP*) gene, which encodes the PutR protein, the proline-responsive activator of putBCP expression (7, 31). A 155-bp spacer region separates the putBCP gene cluster and the *putR* gene, and this intergenic region contains a predicted Rho-independent transcriptional terminator sequence (Fig. 1A) and the promoter for the *putR* gene (7, 31).

Database searches suggest that the *B. subtilis* PutB protein (303 amino acids) is a monofunctional PRODH (EC 1.5.9.8) (68). PutB exhibits 25% amino acid sequence identity to the PRODH domain (from amino acid 261 to amino acid 612) of the PutA enzyme from *E. coli* (61). The PutC protein (515 amino acids) from *B. subtilis* exhibits 37% amino acid sequence identity to the P5CDH domain (from amino acid 650 to amino acid 1130) of the PutA enzyme from *E. coli* and is predicted to function as a monofunctional NAD-dependent $\Delta^1$-pyrroline-5-carboxylate dehydrogenase (P5CDH) (EC 1.5.1.12). It contains the catalytically active residues (Glu-286 and Cys-320) typically found in P5CDH enzymes (32, 61). The crystal structures of the monofunctional PRODH and P5CDH enzymes from *Thermus thermophilus* were recently reported (32, 68). The amino acid sequence of the PutB protein of *B. subtilis* is 41% identical to that of the PRODH from *T. thermophilus*, and the PutC protein sequence is 50% identical to that of the P5CDH from the microorganism, suggesting that the *B. subtilis* enzymes possess folds similar to those of the PRODH and P5CDH enzymes from *T. thermophilus*.

The PutP protein (491 amino acids) is predicted to function as a high-affinity proline importer that belongs to the sodium solute symporter family, whose members couple the import of the substrate with the inflow of Na$^+$ ions (48). The *B. subtilis* PutP transporter exhibits 54% amino acid sequence identity to the well-studied proline importer PutP from *E. coli* (48). The *E. coli* PutP protein is predicted to comprise 13 membrane-spanning segments, and those residues known to be involved in L-proline and Na$^+$ binding are conserved in the *B. subtilis* PutP protein (proline binding, W-85, Y-166, W-270, and Y-274; Na$^+$ binding, Y-15, A-79, M83, A-359, S-362, and T-363) (40, 48). The *B. subtilis* PutP protein exhibits 61% amino acid sequence identity to the osmotically inducible OpuE transporter that is used by *B. subtilis* under high-salinity stress conditions to import L-proline as an osmoprotectant (58, 65).

**Biochemical and genetic assessment of the putBC-encoded proteins in L-proline catabolism.** The bioinformatic analysis of the predicted enzymatic functions of the PutB and PutC proteins and of the PutP transporter suggests that *B. subtilis* can import L-proline and oxidize it to L-glutamate via the pathway depicted in Fig. 1B, an L-proline degradation route found in many microorganisms (18, 33, 35, 36, 47, 59, 61, 64, 72). It is known from previous studies that growth of *B. subtilis* in the presence of L-proline triggers the induction of a proline-catabolizing enzyme (referred to by Atkinson et al. as proline oxidase) that converts L-proline to $\Delta^1$-pyrroline-5-carboxylate (4).

To provide biochemical evidence for the suggested L-proline catabolic pathway (Fig. 1B), we assayed proline dehydrogenase (PutB) activity in cleared cell extracts of cultures grown in SMM with glucose or in SMM-grown cultures that received 1 mM L-proline for 80 min prior to cell harvest. The addition of L-proline to *B. subtilis* cultures grown in SMM increased PRODH activity 5-fold from 0.48 ± 0.05 U (mg protein$^{-1}$) to 2.69 ± 0.21 U (mg protein$^{-1}$). This increase in PRODH activity was abolished in a *putB* mutant that possessed an activity of 0.46 ± 0.02 U (mg protein$^{-1}$) in cells grown in the absence of L-proline and 0.45 ± 0.02 U (mg protein$^{-1}$) in cells cultivated in the presence of L-proline.

In addition to these enzymatic studies, proline utilization was also assessed genetically through targeted deletion analysis of the *putBCP* catabolic genes. The *B. subtilis* wild-type strain JH642 was able to use L-proline effectively both as a sole carbon and energy source and as a sole nitrogen source (Table 2). Deletion of the entire *putBCP* locus from the *B. subtilis* chromosome abolished L-proline utilization (Table 2). Noticeably, a strain with intact *putB* and *putC* genes that carries a defect in the L-proline transporter PutP could not use L-proline as a sole carbon and energy source but was proficient in the use of the amino acid as a sole nitrogen source (Table 2). We attribute this finding to reduced, but still significant, import of L-proline in a *putP* mutant; such a strain is apparently able to import enough L-proline through other
transport systems (see Fig. 8) to provide the cells with an adequate level of L-proline for use as a sole nitrogen source.

PutP- and OpuE-dependent uptake of L-proline. The PutP and OpuE transporters both belong to the sodium solute symporter family (48). The role of OpuE as an L-proline transporter has already been established; OpuE catalyzes the import of L-proline as an osmostress protectant (65), and the transcription of its structural gene (opuE) is induced under high-osmolarity growth conditions (58). We first analyzed the sensitivity of B. subtilis against the toxic proline analogues AC and DHP, which are typically imported into microbial cells through proline transport systems (39, 69). The growth-inhibiting effects of AC and DHP result from the incorporation of these compounds into proteins that then are prone to misfolding. The B. subtilis wild-type strain JH642 is sensitive to both AC and DHP, and the sensitivity to these proline analogues increases under high-salinity growth conditions (Fig. 2). A comparison of the sensitivities to AC and DHP in an isogenic pair of strains expressing either the PutP or OpuE transporter alone showed that AC and DHP sensitivity at high salinity is exclusively dependent on the activity of OpuE (Fig. 2). Thus, either PutP transport activity is inhibited by high salinity or the PutP protein is not present in cells exposed to high-saline growth conditions (Fig. 2). We observed residual AC and DHP sensitivity in a putP opuE double-mutant strain on SMM agar plates (Fig. 2), suggesting that a still uncharacterized L-proline transporter(s) is present in B. subtilis (Fig. 1B). This residual AC and DHP sensitivity is abrogated at high salinity (SMM agar plates containing 0.6 M NaCl) (Fig. 2).

L-Proline transport in B. subtilis was then analyzed directly by measuring the kinetic parameters of the PutP and OpuE systems with radiolabeled L-[14C]proline in strains BLOB9 (PutP+/OpuE+) and SMB11 (PutP−/OpuE+). The PutP+ strain BLOB9 exhibits high-affinity L-proline transport activity (Km, about 8 μM) with a rather modest capacity (Vmax, about 29 nmol min−1 mg protein−1) in cells that were grown in SMM. However, precultivation of this strain in SMM with 1 mM L-proline increased L-[14C]proline uptake activity by about 6-fold (Vmax, about 158 nmol min−1 mg protein−1) without influencing the

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<th>Relevant genotype</th>
<th>Growth yield of cultures grown in the presence of:</th>
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<td>JH642</td>
<td>putBCP+</td>
<td>Glucose and NH4Cl: 3.90, L-Proline and NH4Cl: 4.55, L-Proline: 4.70</td>
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<tr>
<td>SMB45</td>
<td>Δ[putBCP::tet]2</td>
<td>Glucose and NH4Cl: 3.90, L-Proline and NH4Cl: 0.20, L-Proline: 0.90</td>
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<tr>
<td>SMB11</td>
<td>Δ[putP::spc]1</td>
<td>Glucose and NH4Cl: 4.30, L-Proline and NH4Cl: 0.80, L-Proline: 3.80</td>
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a Cells of the wild-type strain JH642 and its mutants Δ[putBCP::tet]2 and Δ[putP::spc]1 derivatives were cultivated in shake flasks containing (i) SMM with glucose (28 mM) as the carbon and energy source and NH4Cl (15 mM) as the nitrogen source, (ii) glucose (28 mM) as the carbon and energy source and L-proline (15 mM) as the nitrogen source, and (iii) L-proline (32 mM) as the sole carbon and energy source and NH4Cl (15 mM) as the nitrogen source.
substrate affinity ($K_w$) of the PutP transport system (Table 3). Increases in the external salinity progressively decreased the $\text{L-}\text{proline}$ transport capacity ($V_{\max}$) of the PutP system to the noninduced level, despite the fact that the cells had been cultivated in the presence of $\text{L-}\text{proline}$ (Table 3).

The OpuE-mediated $\text{L-[14C]}\text{proline}$ uptake activity was a mirror image of that of the PutP transporter. There was no induction of OpuE transport activity by $\text{L-}\text{proline}$, but growth of the cells in SMM or SMM with the indicated salt concentrations but in the absence of $\text{L-}\text{proline}$. The cells were then harvested by centrifugation and washed twice in prewarmed (37°C) growth medium (SMM or SMM with the indicated salt concentrations) but in the absence of $\text{L-}\text{proline}$. For the uptake assays with $\text{L-[14C]}\text{proline}$, the substrate concentration was systematically varied between 1 mM and 40 mM; the measured uptake rates were used for the calculation of $K_w$ and $V_{\max}$ values according to Michaelis-Menten kinetics.

### TABLE 3 PutP- and OpuE-mediated uptake of $\text{L-[14C]}\text{proline}$ by *B. subtilis*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth conditionsa</th>
<th>$K_w$ (µM)</th>
<th>$V_{\max}$ (nmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOB9 (PutP⁺)</td>
<td>SMM</td>
<td>8 ± 2</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>OpuE⁻)</td>
<td>SMM + 1 mM Pro</td>
<td>8 ± 2</td>
<td>158 ± 5</td>
</tr>
<tr>
<td></td>
<td>SMM + 0.4 M NaCl</td>
<td>6 ± 1</td>
<td>28 ± 2</td>
</tr>
<tr>
<td></td>
<td>SMM + 0.4 M NaCl +</td>
<td>8 ± 2</td>
<td>68 ± 1</td>
</tr>
<tr>
<td></td>
<td>1 mM Pro</td>
<td>11 ± 1</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>SMB11 (PutP⁻)</td>
<td>SMM</td>
<td>12 ± 1</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>OpuE⁺)</td>
<td>SMM + 1 mM Pro</td>
<td>12 ± 4</td>
<td>19 ± 3</td>
</tr>
<tr>
<td></td>
<td>SMM + 0.4 M NaCl</td>
<td>12 ± 1</td>
<td>104 ± 14</td>
</tr>
<tr>
<td></td>
<td>SMM + 0.6 M NaCl</td>
<td>23 ± 3</td>
<td>252 ± 10</td>
</tr>
</tbody>
</table>
| a Cells were grown in SMM either in the absence or presence of the indicated concentrations of NaCl or $\text{L-}\text{proline}$ to an OD₅₇₈ of about 0.3 to 0.6. The cells were then harvested by centrifugation and washed twice in prewarmed (37°C) growth medium (SMM or SMM with the indicated salt concentrations) but in the absence of $\text{L-}\text{proline}$. For the uptake assays with $\text{L-[14C]}\text{proline}$, the substrate concentration was systematically varied between 1 mM and 40 mM; the measured uptake rates were used for the calculation of $K_w$ and $V_{\max}$ values according to Michaelis-Menten kinetics.

![FIG 3](http://jb.asm.org/Downloaded from http://jb.asm.org)
cleotides) mRNA species. We cannot distinguish between the possibilities that the putB or putBC mRNA species represents either premature transcription termination products or stable degradation products of the full-length putBCP mRNA. Interestingly, when we used a putP hybridization probe, we also detected an mRNA comprising putP that was present not only in cells cultivated in SMM with l-proline, but also in cells grown in SMM in the absence of l-proline (Fig. 3B). Judging from the length of this constitutively produced mRNA species (estimated length, 1,700 nucleotides), and assuming that it has the same 3' end as the full-length putBCP transcript, it must be produced from a promoter that is located within the 3' region of the putC gene, since the length of the calculated putP mRNA segment is only about 1,420 nucleotides. Manual inspection of the corresponding region within the 3' segment of the putC gene revealed a putative promoter with −35 and −10 regions (TTCAAC-N17-TATCGT) corresponding reasonably well to SigA-type promoter consensus sequences, and this promoter also possessed the TG motif that is frequently found at position −16 in B. subtilis promoters (29). This putative promoter is present 140 bp upstream of the putC stop codon and might therefore direct the constitutive synthesis of the observed 1,700 nucleotide ‘putC-putP’ mRNA species (Fig. 3B).

Mapping of the proline-responsive putBCP promoter by primer extension analysis. To map the 5' end of the putBCP mRNA, we carried out a primer extension analysis with total RNA isolated from cells of the B. subtilis wild-type strain harboring plasmid pSM13 (‘ygl-putB’) (Fig. 1A) that were grown in either SMM or SMM with 1 mM l-proline. A major l-proline-inducible transcript was detected (Fig. 4A) whose 5' end corresponds to an A·T base pair located 40 bp upstream of the predicted GTG start codon for the putB reading frame (Fig. 4B), in agreement with other recently reported determinations of the putBCP transcription initiation site (7, 31). Upstream of the transcriptional start site, −10 and −35 elements are present, with features typical of SigA-dependent promoters of B. subtilis (29). The spacing between the −10 and −35 regions of the put promoter is 18 bp, a suboptimal spacing for SigA-type promoters (29).

A minor l-proline-inducible mRNA species was also detected in our primer extension experiments (Fig. 4A). However, since its 5' end is positioned close to the predicted ribosome-binding site of the putB gene (Fig. 4B) and since we did not detect any typical promoter elements in the vicinity of its 5' end, we interpreted this short mRNA species either as a degradation product of the full-length primer extension reaction product or as a product of a stalling event of the reverse transcriptase.

Use of putB-treA reporter fusions to study induction of gene expression in response to exogenously provided l-proline and proline-related compounds. To monitor the expression of the putBCP operon in greater detail, we constructed a putB-treA transcriptional reporter gene fusion and integrated it as a single copy into the amyE locus of the B. subtilis genome. We studied the expression of this fusion in a putBCP+ wild-type genetic background (strain SMB10). First, we analyzed the influence of an exogenous supply of l-proline (1 mM) on the time course of the induction of the putB-treA gene fusion. Expression reached its maximal level about 60 min after the addition of the inducer l-proline to the growth medium; during this time span, we did not observe any enhanced expression of the putB-treA fusion in the control culture that had received no l-proline (Fig. 5A). We then analyzed the dependence of the strength of putB-treA expression on the amount of l-proline that was added to the growth medium. An inducing effect of l-proline was already noticeable when it was present at a concentration of 25 μM in the growth medium (Fig. 5B). It should be noted in this context that the cellular proline pool of B. subtilis cells grown in a minimal medium with glucose as the carbon source has been measured to lie in a range between 16 mM (67) and 10 mM (see Fig. 7). Apparently, this substantial internal
L-proline pool does not cause high-level expression of the put gene cluster (Fig. 3A and 5). The very low level of putB-treA expression observed in SMM-grown cells and the enhanced expression of the reporter gene fusion in the presence of an exogenous supply of L-proline were both dependent on the L-proline-responsive PutR regulator (7) (Fig. 5C).

We also tested the proline-related compounds thioproline, trans-4-hydroxyproline, monomethyl-proline, and dimethylproline (proline betaine) for the ability to induce the expression of the putB-treA reporter fusion; none of these compounds functioned as an inducer for put expression in SMM-grown cells (Fig. 6A). This was also the case for the toxic proline analogues AC and DHP (Fig. 6A). This picture changed, however, when we tested the inducing effects of the above-mentioned proline derivatives and}

**FIG 5** Induction of put expression in response to an external supply of L-proline. Expression of the put genes in response to L-proline in the growth medium was monitored with the aid of a chromosomal putB-treA operon fusion. (A) The reporter strain SMB10 [Δ(putB-treA)]1 was grown in SMM (squares) or SMM with 0.6 M NaCl (circles) to early exponential phase; two cultures (filled symbols) received 1 mM L-proline at time zero, and the induced and noninduced cells were assayed for TreA reporter enzyme activity at the indicated time intervals. (B) The reporter strain SMB10 [Δ(putB-treA)]1 was grown in SMM to early exponential phase, and the cells were assayed for TreA reporter enzyme activity 60 min after they received the indicated amounts of L-proline. (C) Δ(putB-treA)1 reporter gene fusion activity was measured in cells of various B. subtilis strains that were grown in either the absence (gray bars) or presence (black bars) of 1 mM L-proline; TreA reporter enzyme activity was recorded 60 min after the addition of L-proline to the cultures. The following Δ(putB-treA)1 fusion strains were used: SMB10 [putBCP::spc]3, SMB34 [Δ(putB::spc)3], SMB49 [Δ(putB::spc)3], SMB84 [Δ(putB::spc)3], SMB14 [Δ(putB::spc)3], SMB46 [Δ(putBCP-tet)2], TSB2 [putBCP::spc], and TSB3 [Δ(putC::neo)2] [Δ(putR::spc)]. The values for the TreA activity given represent two independently grown cultures, and for each sample analyzed, the TreA activity was determined twice. The error bars indicate standard deviations.

**FIG 6** Induction of put expression in response to an external supply of L-proline, proline-derived compounds, and proline analogues. The reporter strain SMB10 [Δ(putB-treA)]1 was pregrown in either SMM (A) or SMM with 0.6 M NaCl (B) overnight, and the cultures were used to inoculate fresh cultures that were then allowed to grow to early exponential phase. To part of the cultures, we added 1 mM L-proline, proline-derived compounds, toxic proline analogues, and glutamate; the cells were then propagated for a further 60 min and were subsequently harvested for TreA reporter enzyme assays. The values for the TreA activity given represent two independently grown cultures, and for each sample analyzed, the TreA activity was determined twice. The error bars indicate standard deviations.

L-proline pool does not cause high-level expression of the put gene cluster (Fig. 3A and 5). The very low level of putB-treA expression observed in SMM-grown cells and the enhanced expression of the reporter gene fusion in the presence of an exogenous supply of L-proline were both dependent on the L-proline-responsive PutR regulator (7) (Fig. 5C).

We also tested the proline-related compounds thioproline, trans-4-hydroxyproline, monomethyl-proline, and dimethylproline (proline betaine) for the ability to induce the expression of the putB-treA reporter fusion; none of these compounds functioned as an inducer for put expression in SMM-grown cells (Fig. 6A). This was also the case for the toxic proline analogues AC and DHP (Fig. 6A). This picture changed, however, when we tested the inducing effects of the above-mentioned proline derivatives and
analognes on putB-treA expression in cells cultivated in high-osmolarity medium (SMM containing 0.6 M NaCl). L-Proline still functioned as an inducer under high-salinity growth conditions, but in contrast to SMM-grown cells, both AC and DHP and dimethyl-proline (proline betaine) now functioned as inducers, whereas monomethyl-proline and thioproline still did not function as inducers for putB-treA expression (Fig. 6B). The toxic proline analogues AC and DHP enter the B. subtilis cell under high-salinity growth conditions via the osmotically inducible OpuE (65) transporter (Fig. 2), and their enhancing effects on putBCP expression in osmotically stressed cells (Fig. 6B) can therefore be rationally understood. The fact that they do not induce the expression of the putB-treA reporter fusion in cells cultivated in the absence of salt hint either that these compounds are not substrates for PutP or that the affinity of the PutP transporter for AC and DHP is so low so that they cannot trigger enhanced putB-treA expression or cause increased sensitivity to these toxic compounds (Fig. 2). Notably, the end product of the L-proline degradation pathway, L-glutamate (Fig. 1B), did not trigger significantly enhanced transcription of the putB-treA reporter fusion at either low or high salinity (Fig. 6A and B).

**Induction of put expression is not triggered by the large amounts of L-proline synthesized under osmotic stress conditions.** High salinity per se did not trigger putB-treA expression (Fig. 5A and 6B), a rather surprising finding, since it is well known that B. subtilis synthesizes considerable amounts (several hundred millimolar) of the compatible solute L-proline as a cellular defense against high-osmolarity surroundings (11, 67). Induction of the putB-treA reporter fusion by an exogenous supply of L-proline in high-salinity (0.6 M NaCl)–grown cells was still possible, although the kinetics of putB-treA induction was somewhat delayed in comparison to SMM-grown cells (Fig. 5A).

The data presented in Fig. 5A and 6B hint that the osmotically induced L-proline biosynthesis does not trigger putB-treA expression. To investigate this in greater detail, we grew the putB-treA fusion strain SMB10 in either SMM or SMM with 1 M NaCl to an OD₅₇₈ of 1; the two sets of cultures then received 5 mM L-proline (black bars) while the other part remained untreated (gray bars). The cells were cultivated until they had reached an OD₅₇₈ of 2, harvested by centrifugation, and assayed for their L-proline contents (A) and TreA reporter enzyme activities (B). The values for the L-proline contents and the TreA activities given represent two independently grown cultures, and for each sample analyzed, the L-proline pool and TreA activity were determined twice. The error bars indicate standard deviations.

![FIG 7](http://jb.asm.org/)

**FIG 7** Synthesis of L-proline by high-salinity-stressed cells does not trigger putBCP expression. Cells of the putB-treA reporter strain SMB10 were grown either in SMM or in SMM with 1 M NaCl to an OD₅₇₈ of 1, and one part of each culture then received 5 mM L-proline (black bars) while the other part remained untreated (gray bars). The cells were cultivated until they had reached an OD₅₇₈ of 2, harvested by centrifugation, and assayed for their L-proline contents (A) and TreA reporter enzyme activities (B). The values for the L-proline contents and the TreA activities given represent two independently grown cultures, and for each sample analyzed, the L-proline pool and TreA activity were determined twice. The error bars indicate standard deviations.

**1-Proline-mediated induction of putB-treA expression is independent of the putBCP gene products but is dependent on PutR.** The PutA protein from E. coli and S. enterica serovar Typhimurium not only functions as an L-proline-catabolizing bifunctional enzyme (61), it also controls the transcription of the divergently oriented putA and putP genes in response to an external supply of L-proline (72). It possesses an N-terminal ribbon-helix-helix DNA-binding motif (71). This DNA-binding domain is not present in the crystallographically characterized monofunctional PRODH or P5CDH enzyme from T. thermophilus (32, 68), and the PutB and PutC proteins from B. subtilis also lack recognizable DNA-binding motifs.

To test whether any of the putBCP-encoded proteins from B. subtilis would influence the expression of this operon, we introduced the putBCP-treA reporter gene fusion into an isogenic set of strains that carried various lesions in the putBCP locus. Expression of the reporter gene fusion remained inducible by L-proline in a strain with a deletion of the entire putBCP operon (Fig. 5C). This finding excludes any direct influence of either the proline-catabolizing PutB and PutC enzymes or the proline transporter PutP on putBCP expression. The expression of the putB-treA fusion in a putBCP⁻ wild-type background was entirely dependent on PutR (Fig. 5C), fully consistent with data recently reported by Belitsky (7) and by Huang et al. (31).

A somewhat higher level of putB-treA induction was noticed in a mutant lacking an intact putB gene (Fig. 5C). Since the PutR activator protein responds directly to L-proline in an *in vitro* transcription assay system (7), enhanced putB-treA expression can probably be rationalized by the inability of a putB mutant strain to degrade the inducer L-proline. An approximately 4-fold-higher level of putB-treA induction was observed in a strain that has an intact PutB protein but is defective in PutC (Fig. 5C). Such a strain is predicted to accumulate the PRODH reaction product P5C or
with respect to monitoring of L-proline import via the PutP transporter and the elegant way by which this could be accomplished would be the intact PutP and OpuE proline transport systems. Cells of the subsequent communication of this event to the PutR activator of the transporter (61% amino acid sequence identity). The expression defects in the proline transporter PutP or the PutP-related OpuE reporter gene fusion in a set of isogenic mutant strains with treA of the transporter (Fig. 5C) therefore needs to be viewed with some caution. The two putBCP reporter strains were grown in SMM (A) or SMM with 0.6 M NaCl (B) in the absence (gray bars) or presence (black bars) of 1 mM L-proline. The cells were harvested for TreA reporter enzyme assays 60 min after the cultures received the inducer L-proline. The following Δ(putB::spc)3 and Δ(putC::neo)2 mutants used for this experiment might exert polar effects on downstream-positioned genes in the putB-treA operon, and the interpretation of the data obtained with respect to putB-treA induction in the putB and putC mutant strains (Fig. 5C) therefore needs to be viewed with some caution.

Proline-mediated induction of put expression does not depend on PutP- or OpuE-catalyzed proline uptake. The data documented in Fig. 7 illustrate that B. subtilis can somehow physiologically distinguish between an external supply of L-proline and the L-proline pool amassed through de novo synthesis under osmotic stress conditions (11, 67) to induce put expression. One elegant way by which this could be accomplished would be the monitoring of L-proline import via the PutP transporter and the subsequent communication of this event to the PutR activator protein.

To test this hypothesis, we analyzed the expression of the putB-treA reporter gene fusion in a set of isogenic mutant strains with defects in the proline transporter PutP or the PutP-related OpuE transporter (61% amino acid sequence identity). The expression of the putB-treA reporter gene fusion remained L-proline inducible in both mutant strains, and this was the case even when we tested a putP opuE double mutant (1 mM L-proline was present in the growth medium) (Fig. 8A). Taken together, these data strongly suggest that the above-outlined hypothesis cannot adequately explain the different effects of an external L-proline supply and of an internal L-proline pool on the induction of the putB-treA reporter gene fusion as far as the PutP and OpuE L-proline importers are concerned.

Consistent with the data on cell growth (Fig. 2) and the influence of high salinity on PutP- and OpuE-mediated L-proline uptake activity (Table 3) obtained with the toxic proline analogues, we observed a significant decrease in the degree of L-proline-mediated induction of the putB-treA reporter gene fusion in strains lacking the osmotically inducible OpuE transporter (58, 65) under high-salinity growth conditions (Fig. 8B).

Proline-mediated induction of put expression does not depend on the McpC chemoreceptor. Ordal and coworkers have shown that B. subtilis actively seeks L-proline via chemotaxis (49) and that this behavioral response is independent of proline import (51) but depends on the functioning of the membrane-bound methyl-accepting chemotaxis protein McpC (45). We therefore considered the possibility that the induction of putB-treA expression by external L-proline was dependent on the chemoreceptor McpC. To test this hypothesis, we introduced an mcpC::erm gene disruption mutation into the putB-treA reporter strain SMB10 and monitored the L-proline-dependent induction of putB transcription in the resulting strain, ACB154. The data documented in Table 4 conclusively show that the functioning of the McpC chemoreceptor is not required for the induction of put expression in response to an external supply of L-proline.

DISCUSSION

The fact that B. subtilis can actively seek L-proline via chemotaxis (45, 49) underscores the function of the amino acid as a nutrient for the soil bacterium (4, 6, 8, 22). We found that the use of L-proline by B. subtilis as a sole carbon and energy or a sole nitrogen source can be traced genetically to the putBCP operon (Table 2). This catabolic gene cluster (Fig. 1A) is linked to the structural gene for the PutR activator protein controlling proline-responsive putBCP expression (7, 31) (Fig. 3C).

The two putBC-encoded catabolic enzymes, the monofunctional PutB PRODH and the monofunctional PutC Δ1-pyrroline-5-carboxylate dehydrogenase (PD5CD), carry out enzymatic reactions that lead to the degradation of L-proline to L-glutamate.

| Table 4: Influence of the McpC chemoreceptor on putBCP expression |
|---------------------------------|---------|--------|--------|--------|
| Strain | putR | mcpC | With L-proline | Without L-proline |
| SMB10 | + | + | 7 ± 2 | 166 ± 6 |
| TSB2 | − | + | 14 ± 2 | 14 ± 4 |
| ACB154 | + | − | 6 ± 1 | 156 ± 9 |

* Each of the B. subtilis strains is derived from the wild-type strain JH662 and carries the same Δ(putB::spc)3 reporter gene fusion integrated as a single copy into the chromosomal amyE gene. The strains were pregrown in SMM overnight, and the cultures were used to inoculate fresh cultures that were then allowed to grow to early exponential phase. To part of the cultures, 1 mM L-proline was added, and the cells were then propagated for another 60 min and subsequently harvested for TreA reporter enzyme assays. The values for the TreA activity represent three independently grown cultures, and for each sample analyzed, the TreA activity was determined twice.

b 1, present; −, absent.
(Fig. 1B), a proline utilization pathway present in many microorganisms (61, 72). In contrast to the situation found in E. coli and S. enterica serovar Typhimurium (41, 46, 61, 71, 72), our genetic analysis shows that the proline catabolic enzymes PutB and PutC of B. subtilis do not directly participate in the l-proline-mediated regulation of the putBCP gene cluster (Fig. 5C).

The putP-encoded l-proline transporter is a member of the SSS family (48). Its high affinity (K_m, about 8 μM) for its substrate and its substantial transport capacity (V_max about 138 nmol min^{-1} mg protein^{-1} in cells grown in the presence of l-proline) (Table 3) make the PutP uptake system well suited to scavenge this amino acid from scarce environmental sources (27, 66). In addition to the l-proline-inducible full-length putBCP mRNA (Fig. 3A), we detected a putP mRNA species in cells grown in the absence of l-proline (Fig. 3B). This mRNA species is probably produced from a promoter residing in the 3' region of the putC gene. As a consequence of the translation of the PutP protein from this constitutively synthesized 'putC-putP' mRNA species, the B. subtilis cell is predisposed for PutP-dependent scavenging of l-proline from the environment and the ensuing induction of putBCP transcription by the proline-responsive PutR activator protein (7, 31).

Experiments in which we addressed the transcriptional control of the putBCP gene cluster by either Northern blotting (Fig. 3A), primer extension (Fig. 4A), or putB-treA reporter gene studies (Fig. 5 and 6) consistently showed induction of transcription by an exogenous supply of l-proline. In full agreement with recently reported data on put transcription (7, 31), l-proline-mediated induction of the putB-treA reporter fusion used throughout this study was strictly dependent on the PutR activator protein (Fig. 5C). A noticeable effect of the inducer l-proline on putB-treA expression was already recorded in our experiments when 25 μM l-proline was present in the growth medium (Fig. 5B). This low threshold level of the inducer required to trigger enhanced expression of the putBCP operon should permit B. subtilis to use l-proline effectively as a nutrient in natural settings with a predicted low and variable supply of l-proline, e.g., the soil. Indeed, corn root exudates have been shown to contain enough l-proline to induce the expression of the catabolic putAP gene cluster of Pseudomonas putida (64).

In vitro transcription assays with the putBCP regulatory region as the DNA template have shown that PutR responds directly to l-proline to activate putBCP transcription (7). Our in vivo experiments with the putB-treA reporter gene fusion strain suggest possible additional effector molecules for PutR. We found that the toxic proline analogues AC and DHP are also moderately effective in stimulating PutR-dependent putBCP expression. Addition of proline (1 mM) to the growth medium triggered putB-treA expression regardless of whether the PutP transporter was intact, and this was the case even when the PutP-related l-proline transporter OpuE was simultaneously missing, as well (Fig. 8A). It is therefore obvious that there is no direct channeling of externally provided proline via the PutP transporter to the PutR regulator, and a direct role of PutP in sensing the presence of the inducer of PutR in the growth medium is clearly ruled out. Our data (Fig. 2 and 8A) show that, in addition to the high-affinity l-proline transporters PutP and OpuE (Table 3), a yet-identified third proline transporter is present in B. subtilis. However, this proline import system exhibits rather moderate transport activity under the growth conditions tested, since we did not detect l-[14C]proline uptake in a putP opuE double mutant at a substrate supply of 40 μM (Table 3), but at high proline concentrations (1 mM) it allows enough l-proline import to induce putBCP expression (Fig. 8B). It seems highly unlikely to us that this yet-uncharacterized transport system would be specifically used to monitor the influx of l-proline into the B. subtilis cell in order to regulate the activity of PutR (Fig. 5B).

Another way by which B. subtilis could possibly monitor the presence of l-proline in its environment to induce putBCP expression is the use of the membrane-embedded chemoreceptor protein McpC. L-Proline taxis by B. subtilis (50) is independent of l-proline uptake (51) and strictly requires the functioning of McpC (45). Hence, it seemed possible that B. subtilis would exploit the McpC chemoreceptor to monitor the presence of external l-proline and then, in a deviation from its well-established role in chemotaxis signaling, would communicate this information to the cell to turn on putBCP transcription via the PutR regulator. Our data clearly rule out any role of McpC for the induction of putBCP expression (Table 4).

An alternative scenario worth considering with respect to the noninducing effects of the large amounts of l-proline synthesized by osmotically challenged B. subtilis cells on put expression (Fig. 7) is that the cell actually does not distinguish between externally provided and internally synthesized l-proline. Rather, it seems...
possible that the cell is somehow blinded to the intracellular proline signal that results from the osmotically instigated high-level proline synthesis (11, 67) either because the PutR activator cannot interact efficiently with the inducer proline or is compartmentalized in such a way that it is not accessible to L-proline or because the PutR protein complexed with proline cannot interact properly with the put regulatory region to induce transcription. However, our finding that an external supply of proline to severely salt-stressed cells still affords put induction (Fig. 5A, 7, and 8) argues that such a scenario is unlikely.

A phenomenon related to but different from the issue we focus on here with respect to the osmoadaptive L-proline biosynthesis and the catabolic use of the amino acid by B. subtilis has been addressed in E. coli and S. enterica serovar Typhimurium (15, 19, 21, 43). These two Gram-negative bacteria can achieve osmoprotection via uptake of the compatible solute L-proline through the osmotically inducible ProP and ProU transporters, but in contrast to B. subtilis (11, 67), they do not synthesize L-proline as an osmoprotectant (16, 38). However, the overproduction of L-proline as a consequence of feedback-resistant ProB variants leads to enhanced osmotic tolerance of S. enterica serovar Typhimurium cells (15). Interestingly, in these strain backgrounds, mutants that are defective in putA possess higher levels of proline than their putA counterparts, indicating that part of the newly produced L-proline is catabolized in these artificial proline overproducers (15). On the other hand, Ekena and Maloy (21) reported that the degradation of proline pools accumulated under high salinity is limited due to direct inhibition of the PutA proline degradative enzyme, but this might also be a nonspecific consequence of a more general inhibition of enzyme activity observed in severely osmotically stressed cells (19). In B. subtilis, exogenously provided L-proline is a moderately effective osmoprotectant in direct comparison with the metabolically inert compatible solute glycine betaine (34, 65). This is partially due to putBCP-dependent L-proline degradation (A. Zapris, H. Barzantny, T. Hoffmann, and E. Bremer, unpublished data). However, we found that exogenously provided L-proline is, in contrast to the amino acid arginine and the sugar glucose, a carbon source used very inefficiently by osmotically stressed B. subtilis cells (see Fig. S1 in the supplemental material).

It is evident that we do not yet understand an important aspect of the genetic control of the PutBCP-dependent L-proline utilization pathway of B. subtilis with respect to the overall process of cellular adjustment to high-osmolarity environments (24, 60). Whatever the underlying molecular control mechanism might be that prevents strong induction of the catabolic putBCP genes under osmotic stress conditions through newly synthesized L-proline (Fig. 5A and 7), our data highlight the fact that the B. subtilis cell actively prevents the onset of a wasteful and futile cycle of L-proline synthesis and degradation of the newly produced L-proline when it faces high-osmolarity surroundings. Failure to do so would certainly make osmoadaptive L-proline synthesis (11, 67) less effective and thereby in all likelihood render the overall cellular response of B. subtilis to osmotic stress less robust (9, 10).

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REFERENCES


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56. White TA, Krishnan N, Becker DF, Tanner JJ. 2007. Structure and


