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Proline Utilization by *Bacillus subtilis*: Uptake and Catabolism

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L-Proline can be used by *Bacillus subtilis* as a sole source of carbon or nitrogen. We traced L-proline utilization genetically to the *putBCP* (*ycgMNO*) locus. The *putBCP* gene cluster encodes a high-affinity proline transporter (PutP) and two enzymes, the proline dehydrogenase PutB and the Δ^1-pyrroline-5-carboxylate dehydrogenase PutC, which jointly catabolize L-proline to L-glutamate. Northern blotting, primer extension, and *putB-treA* reporter gene fusion analysis showed that the *putBCP* locus is transcribed as an L-proline-inducible operon. Its expression was mediated by a SigA-type promoter and was dependent on the proline-responsive PutR activator protein. Induction of *putBCP* expression was triggered by the presence of submillimolar concentrations of L-proline in the growth medium. However, the very large quantities of L-proline (up to several hundred millimolar) synthesized by *B. subtilis* as a stress protectant against high osmolarity did not induce *putBCP* transcription. Induction of *putBCP* transcription by external L-proline was not dependent on L-proline uptake via the substrate-inducible PutP or the osmotically inducible OpuE transporter. It was also not dependent on the chemoreceptor protein McpC required for chemotaxis toward L-proline. Our findings imply that *B. subtilis* can distinguish externally supplied L-proline from internal L-proline pools generated through *de novo* synthesis. The molecular basis of this regulatory phenomenon is not understood. However, it provides the *B. subtilis* cell with a means to avoid a futile cycle of *de novo* L-proline synthesis and consumption by not triggering the expression of the *putBCP* L-proline catabolic genes in response to the osmoadaptive production of the compatible solute L-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 preformed 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 (64), as decomposed plant material (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). Here, we focus on the utilization by *B. subtilis* of L-proline as a sole carbon and energy source and as a sole nitrogen source.

Many bacteria can employ L-proline as a nutrient, and the catabolism of the amino acid typically involves its enzymatic oxidation to L-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 Δ^1-pyrroline-5-carboxylate (P5C). This intermediate spontaneously hydrolyzes to γ-glutamate-5-semialdehyde, which is then further oxidized by a NAD-dependent Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH) (EC 1.5.1.12) to L-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 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 L-proline-degradative enzyme and a cytoplasmic regulatory protein to repress the expression of the *putPA* proline utilization gene cluster when no proline is present in the growth medium (41, 46, 61, 71, 72). Transcription of the *putPA* genes is also regulated via the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex (13), thereby embedding L-proline utilization into a globally acting regulatory network that prioritizes the use of various carbon sources by microbial cells.

L-Proline utilization has also been investigated in microorganisms other than *E. coli* and *S. enterica* serovar Typhimurium. A common denominator of all these microbial proline utilization systems is the induction of *put* gene expression in response to an external supply of L-proline and the use of the concerted actions of PRODH- and P5CDH-type enzymes to catabolize L-proline to L-glutamate (61). However, the types of regulatory proteins and

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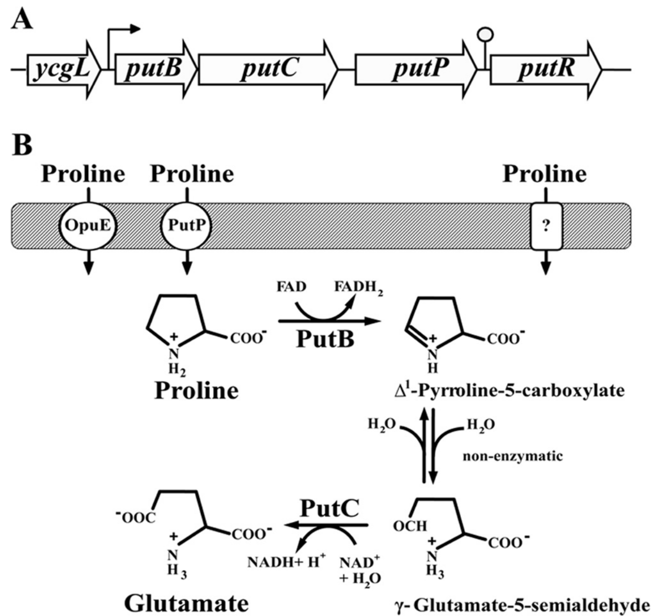

FIG 1 L-Proline utilization system of *B. subtilis*. (A) Genetic organization of the *putBCP* and *putR* region. The promoter and the predicted Rho-independent terminator for the *putBCP* gene cluster are indicated by an arrow and a lollipop, respectively. (B) Proposed route for L-proline uptake and catabolism in *B. subtilis*.

genetic circuits that control *put* expression in response to L-proline availability vary considerably in microorganisms (33, 35, 36, 47, 57, 64).

The use of L-proline as a nutrient by *B. subtilis* is well known (4, 6, 8), but the underlying systems for L-proline uptake and catabolism have so far not been studied in any detail. Recently, the understanding of the genetic control of the use of L-proline as a nutrient by *B. subtilis* was advanced by the identification of a proline-responsive activator protein (PutR) that controls the expression of the L-proline utilization *putBCP* (formerly *ycgMNO* [9]) gene cluster (7, 31). PutR activates *putBCP* transcription in response to proline availability but can be displaced by an active form of the negatively acting CodY regulatory protein (4, 44, 54) from the *putBCP* promoter region (7), thereby establishing repression of the gene cluster.

Here, we have focused on physiological aspects of L-proline utilization by *B. subtilis* and have studied the role of the *putBCP*-encoded proline transporter PutP and of the PutB (PRODH) and PutC (P5CDH) enzymes. Catabolism of L-proline by *B. subtilis* poses interesting questions, since L-proline also serves as an important osmostress protectant for the soil bacterium (9, 10). Both the osmotically inducible *de novo* synthesis of the compatible solute L-proline (11, 67) and its import via the osmotically inducible OpuE transporter (58, 65) confer stress resistance to high-osmolarity challenges.

We discovered that the expression of the catabolic *putBCP* operon of *B. subtilis* can be induced in a PutR-dependent fashion by very low concentrations (low μM range) of L-proline present in the growth medium but that the very large quantities of L-proline amassed via de *novo* synthesis under osmotic stress conditions (several hundred millimolar) do not trigger enhanced *putBCP* transcription. Physiologically, this allows the *B. subtilis* cell to 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-DL-proline (DHP), the chromogenic substrates for the TreA [phospho-α-(1,1)-glucosidase] enzyme *para*-nitrophenyl-α-D-glucopyranoside (α-PNPG) and for the ProB enzyme (*o*-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-[^{14}C(U)]proline (40 mCi mmol^{-1}) was purchased from DuPont de Nemour GmbH (Neu-Isenburg, Germany).

Bacterial strains. The *E. coli* strain DH5α (Invitrogen, Carlsbad, 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 minimal medium (SMM) (26) supplemented with 0.5% (wt/vol) glucose as the carbon source, a solution of trace elements (28), and the amino acids L-tryptophan (20 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 NH_4Cl 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 NH_4Cl 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, Manheim, 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 ϕ(*putB′-treA*)*1* transcriptional 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

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TABLE 1 *B. subtilis* strains used in this study

Strain[a]	Relevant genotype[b]	Source
JH642	*trpC2 pheA1*	J. Hoch; BGSC[c] 1A96
GNB37	Δ(*treA*::*erm*)*2*	G. Nau-Wagner
MBB1	Δ(*treA*::*neo*)*1*	M. Brosius
BLOB9	Δ(*opuE*::*tet*)*1*	65
SMB10	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*treA*::*neo*)*1*	This study
SMB11	Δ(*putP*::*spc*)*1*	This study
SMB12	Δ(*putP*::*spc*)*1* Δ(*opuE*::*tet*)*1*	This study
SMB14	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*putP*::*spc*)*1* Δ(*treA*::*neo*)*1*	This study
SMB27	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*putP*::*spc*)*1* Δ(*opuE*::*tet*)*1* Δ(*treA*::*neo*)*1*	This study
SMB28	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*opuE*::*tet*) Δ(*treA*::*neo*)*1*	This study
SMB32	Δ(*putC*::*neo*)*2*	This study
SMB34	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*putC*::*neo*)*2* Δ(*treA*::*erm*)*2*	This study
SMB42	Δ(*putB*::*spc*)*3*	This study
SMB45	Δ(*putBCP*::*tet*)*2*	This study
SMB46	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*putBCP*::*tet*)*2* Δ(*treA*::*erm*)*2*	This study
SMB49	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*putB*::*spc*)*3* Δ(*treA*::*erm*)*2*	This study
TSB2	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*putR*::*spc*) Δ(*treA*::*neo*)*1*	This study
TSB3	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*putC*::*neo*)*2* Δ(*putR*::*spc*) Δ(*treA*::*erm*)*2*	This study
ACB154	*amyE*::ϕ(*putB*′-*treA*)*1* Δ(*treA*::*kan*)*2* *mcpC*::*erm*	This study
BB3330	SMY Δ(*putR*::*cat*)	7
BB3530	SMY Δ(*putR*::*spc*)	This study
OI3280	*trpF7 hisH2 metC mcp*::*erm*	45

[a] All strains except BB3330, BB3530, and OI3280 are derivatives of *B. subtilis* strain JH642 and therefore carry, in addition to the genetic markers indicated, the *trpC2 pheA1* mutations.
[b] The designation *amyE*::ϕ(*putB*′-*treA*)*1* indicates that the *putB-treA* 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 ϕ(*putB*′-*treA*)*1* reporter fusion is linked to a chloramphenicol resistance gene (*cat*), thereby rendering all strains carrying the *amyE*::ϕ(*putB*′-*treA*)*1* construct resistant to the antibiotic chloramphenicol.
[c] BGSC, Bacillus Genetic Stock Center, Ohio State University, Columbus, OH.

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 by the acidic phenol method (30). DIG-labeled single-stranded RNA probes specific for the *putB*, *putC*, and *putP* genes were prepared by *in vitro* transcription using the DIG RNA-Labeling Kit SP6/T3/T7 (Roche Diagnostics, Mannheim, Germany) according to the procedure described by the manufacturer. Derivatives of the cloning vector pBSK$^-$ containing the *putB* (pSM11), *putC* (pSM34), or *putP* (pSM35) gene were used as templates for the T3 RNA polymerase-mediated *in vitro* transcription reaction. RNA-RNA hybridization and detection of specific *put* transcripts were performed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Primer extension analysis of the proline-responsive *putBCP* promoter. The transcriptional initiation start site of the *putBCP* mRNA was determined by the primer extension method. Total RNA was isolated by the acidic-phenol method (30) from log-phase cells of the *B. subtilis* strain JH642 containing pSM13, a plasmid carrying the ′*ycgL-putB*′ 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 GCCATTTTATTGAGAAAGCCGC-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 Δ^1-pyrroline-5-carboxylate (P5C) by determining the formation of the P5C–*o*-aminobenzaldehyde from the chromogenic substrate *o*-aminobenzaldehyde in a spectrophotometer. The millimolar extinction coefficient of the P5C–*o*-aminobenzaldehyde 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 P5C formed per minute and mg protein (U mg $protein^{-1}$).

TreA enzyme activity assays. An aliquot (1.5 ml) from cultures of *putB-treA 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 TreA activity assays with *para*-nitrophenyl-α-D-glucopyranoside as the substrate (23). TreA 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 the addition of 0.6 M NaCl until they reached an optical density at 578 nm (OD_{578}) 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-mg 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-[^{14}C(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 (OD_{578}, 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-[^{14}C(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; Schleicher& Schuell, Dassel, Germany). The filters were washed two times with the proline-free cultivation medium of the cells and subsequently transferred to a scintillation analyzer (Coulter Liquid Scintilliation Analyser 1900CA) with 5 ml scintillation solution. The transport activity of cells is expressed as nmol substrate min^{-1} mg

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protein^{-1}. The kinetics of 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 μl 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 sequences were aligned and analyzed using ClustalW (63). *In silico* 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 Δ^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 1.5.99.8) (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 Δ^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 osmostress protectant (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, 57, 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 Δ^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 $\pm$ 0.05 U (mg protein^{-1}) to 2.69 $\pm$ 0.21 U (mg protein^{-1}). This increase in PRODH activity was abolished in a *putB* mutant that possessed an activity of 0.46 $\pm$ 0.02 U (mg protein^{-1}) in cells grown in the absence of L-proline and 0.45 $\pm$ 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

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TABLE 2 Use of L-proline as sole carbon and energy source and as sole nitrogen source by *B. subtilis*

Strain	Relevant genotype	Growth yield of cultures grown in the presence of[a]: Glucose and NH_4Cl	L-Proline and NH_4Cl	Glucose and L-proline
JH642	*putBCP*+	3.90	4.55	4.70
SMB45	Δ(*putBCP*::*tet*)*2*	3.90	0.20	0.90
SMB11	Δ(*putP*::*spc*)*1*	4.30	0.80	3.80

[a] Cells of the wild-type strain JH642 and its mutant 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 NH_4Cl (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 NH_4Cl (15 mM) as the nitrogen source.

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-[^{14}C]proline in strains BLOB9 (PutP+ OpuE−) and SMB11 (PutP− OpuE+). The PutP+ strain BLOB9 exhibits high-affinity L-proline transport activity (K_m, about 8 μM) with a rather modest capacity (V_{max}, about 29 nmol min^{-1} mg protein^{-1}) in cells that were grown in SMM. However, precultivation of this strain in SMM in the presence of 1 mM L-proline increased L-[^{14}C]proline uptake activity by about 6-fold (V_{max}, about 158 nmol min^{-1} mg protein^{-1}) without influencing the

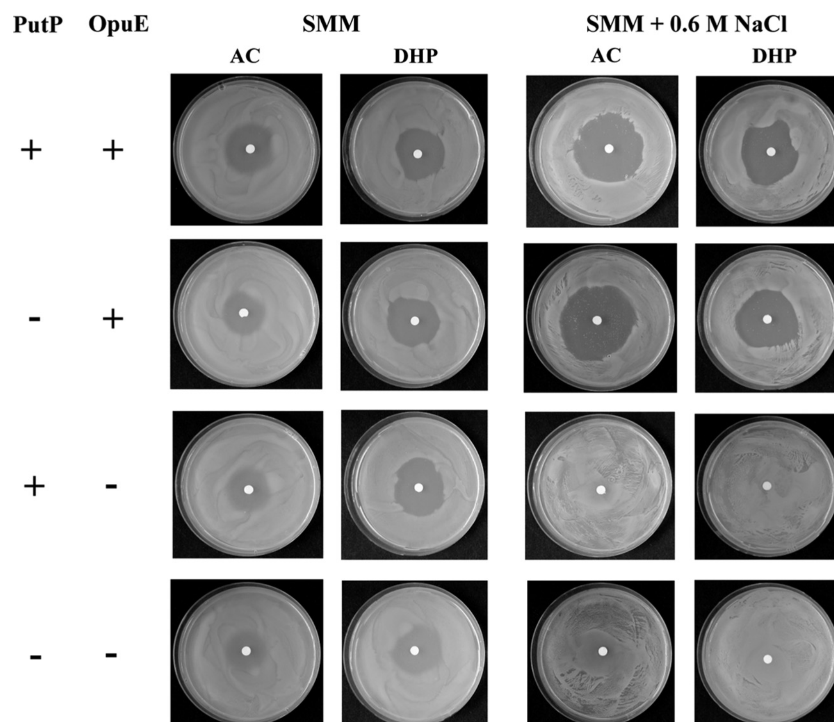

FIG 2 Sensitivity of *B. subtilis* and its *putP* and *opuE* mutant derivatives to toxic proline analogues. Cells of the wild-type strain JH642 and its mutant derivatives SMB11 (PutP− OpuE+), BLOB9 (PutP+ OpuE−), and SMB12 (PutP− OpuE−) were pregrown in SMM with and without the addition of 0.6 M NaCl. Aliquots (300 μl) of these cultures were then plated on SMM agar plates or SMM agar plates containing 0.6 M NaCl; a paper filter disk soaked with 10 μl of a 25-mg ml^{-1} solution of the toxic proline analogues AC and DHP was then placed in the middle of each of the agar plates. The agar plates were incubated at 37°C for 24 h (for cells grown on SMM plates) or for 48 h (for cells grown on SMM plates containing 0.6 M NaCl) before the formation of growth inhibition zones around the filter disk was recorded by photography.

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

Strain	Growth conditions[a]	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})
BLOB9 (PutP$^+$ OpuE$^-$)	SMM	8 ± 2	29 ± 2
	SMM + 1 mM Pro	8 ± 2	158 ± 5
	SMM + 0.4 M NaCl	6 ± 1	28 ± 2
	SMM + 0.4 M NaCl + 1 mM Pro	8 ± 2	68 ± 1
	SMM + 0.6 M NaCl + 1 mM Pro	11 ± 1	22 ± 2
SMB11 (PutP$^-$ OpuE$^+$)	SMM	12 ± 1	27 ± 3
	SMM + 1 mM Pro	12 ± 4	19 ± 3
	SMM + 0.4 M NaCl	12 ± 1	104 ± 14
	SMM + 0.6 M NaCl	23 ± 3	252 ± 10

[a] Cells were grown in SMM either in the absence or presence of the indicated concentrations of NaCl or L-proline to an OD_{578} 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 L-proline. For the uptake assays with L-[^{14}C]proline, the substrate concentration was systematically varied between 1 μM and 40 μM; the measured uptake rates were used for the calculation of K_m and V_{max} values according to Michaelis-Menten kinetics.

substrate affinity (K_m) of the PutP transport system (Table 3). Increases in the external salinity progressively decreased the L-proline transport capacity (V_{max}) of the PutP system to the non-induced level, despite the fact that the cells had been cultivated in the presence of L-proline (Table 3).

The OpuE-mediated L-[^{14}C]proline uptake activity was a mirror image of that of the PutP transporter. There was no induction of OpuE transport activity by L-proline, but growth of the cells in media with increased salinity resulted in progressively increased L-proline uptake activity (V_{max}) without strong effects on the affinity (K_m) of the OpuE import system (Table 3).

When interpreting the L-[^{14}C]proline transport data summarized in Table 3, one needs to keep in mind that L-proline can still enter a *putP opuE* double-mutant strain when 1 mM L-proline is provided in the growth medium (data not shown), and such a strain is also somewhat sensitive to AC and DHP (Fig. 2). However, the so-far-uncharacterized third proline importer operating in *B. subtilis* (Fig. 1A) must be a transport system with a rather modest capacity under the growth conditions we used. This is evident from our finding that there was no L-[^{14}C]proline uptake detectable in strain SMB12 [Δ(*putP*::*spc*)*1* Δ(*opuE*::*tet*)*1*] at the highest substrate concentration (40 μM) tested in the experiments assessing the kinetic parameters (Table 3) of the PutP and OpuE proline transporters (data not shown).

Northern blot analysis of the transcripts of the *putBCP* gene cluster. The *putB* and *putC* genes are separated by 16 bp, and the intergenic region between *putC* and *putP* is 69 bp, suggesting that the *putBCP* gene cluster is transcribed as an operon. We assessed the transcriptional profile of the *putBCP* locus by Northern blot analysis using total RNA extracted from cells that were grown in either SMM or SMM in the presence of 1 mM L-proline. Using a single-stranded antisense *putB*-specific RNA probe, we found that *putBCP* transcription was inducible by L-proline and that the transcripts detected in the wild-type strain JH642 were absent in strain SMB45 [Δ(*putBCP*::*tet*)*2*] carrying a deletion of the entire *putBCP* locus (Fig. 3A). The longest detected L-proline-inducible mRNA species, with a measured length of about 3,900 nucleotides, corresponds well to the size of a transcript comprising *putBCP*, which has a calculated length of 4,018 nucleotides. Hence, the *putBCP* gene cluster of *B. subtilis* is expressed as an L-proline-inducible operon. Consistent with the presence of a factor-independent transcriptional terminator in the *putP-putR* intragenic region (Fig. 1A), the *putBCP* transcript apparently does not extend into the flanking *putR* gene (Fig. 1A); in all likelihood, it ends in the vicinity of the predicted Rho-independent transcription terminator (Fig. 1A).

In addition to the full-length *putBCP* transcript, we observed two L-proline-inducible mRNA species with measured lengths of 2,500 and 800 nucleotides (Fig. 3A). Using *putB*- and *putC*-specific probes, we identified these mRNA species as transcripts that comprised either *putBC* or *putB* alone (Fig. 3B). The measured lengths of these transcripts correspond closely to the calculated lengths of the *putBC* (2,476 nucleotides) and *putB* (912 nu-

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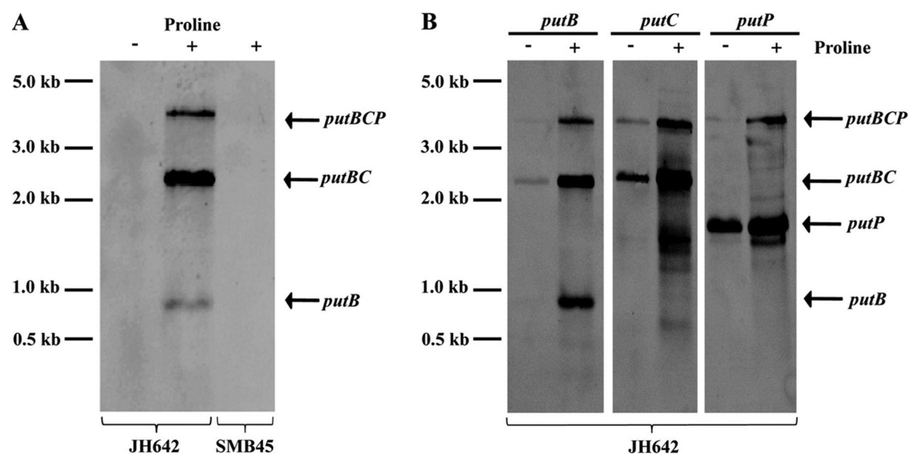

FIG 3 Northern blot analysis of the *putBCP* transcript(s). (A) Total RNA was isolated from log-phase cells of the wild-type strain JH642 (lanes 1 and 2) and its *putBCP* mutant derivative strain SMB45[Δ(*putBCP*::*tet*)*2*] (lane 3) after growth in SMM in the absence (−) or presence (+) of 1 mM L-proline and was then subjected to Northern blot analysis with a single-stranded antisense *putB*-specific DIG-labeled hybridization probe. (B) Total RNA isolated from the wild-type strain JH642 grown in SMM (−) or SMM with 1 mM L-proline (+) was reacted with single-stranded antisense *putB*-, *putC*-, and *putP*-specific DIG-labeled hybridization probes.

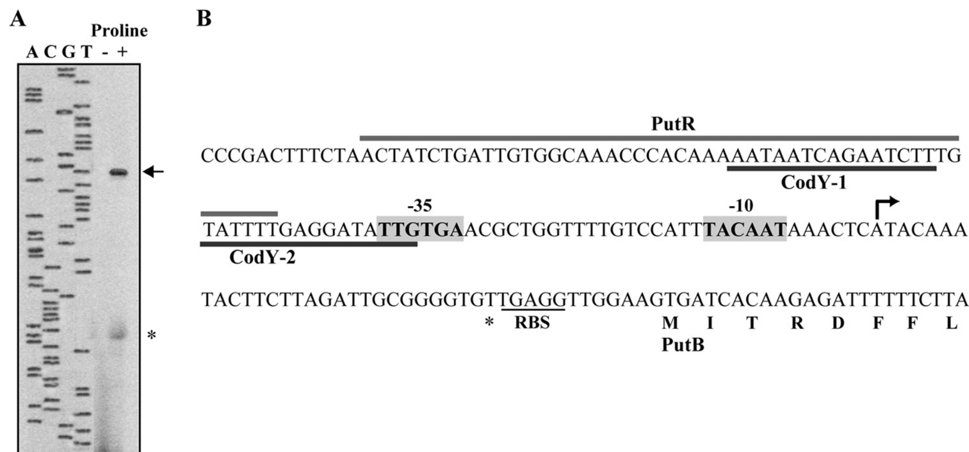

FIG 4 Mapping of the L-proline-responsive *putBCP* promoter by primer extension analysis. (A) Total RNA was isolated from log-phase cells of the *B. subtilis* strain JH642 [pSM13 (*putB′*)] cultivated in SMM with glucose as a carbon source or in SMM that contained 1 mM L-proline. A reverse transcription reaction was carried out with this RNA and a synthetic single-stranded DNA oligonucleotide marked with an IRD-800 fluorescent label; the primer used hybridizes to the 5′ region of the *putB* gene. The same oligonucleotide was used for DNA-sequencing reactions with DNA of plasmid pSM13 to size the 5′ end of the *putB* mRNA. The arrow marks the full-length reverse transcription product. The mRNA species marked by an asterisk is either a premature termination product of the reverse transcriptase reaction or a degradation product of the full-length reverse transcriptase reaction product. (B) DNA sequence of the *putBCP* regulatory region. The mapped 5′ end of the *putB* mRNA is indicated by an arrow; the −10 and −35 elements of the inferred SigA-type *putBCP* promoter are highlighted. The ribosome-binding site (RBS) and the reading frame of the *putB* gene are indicated. The marked binding regions for the CodY and PutR regulatory proteins have been mapped through DNA footprinting and mutant analysis (7).

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-N_{17}-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 (*′ycgL-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

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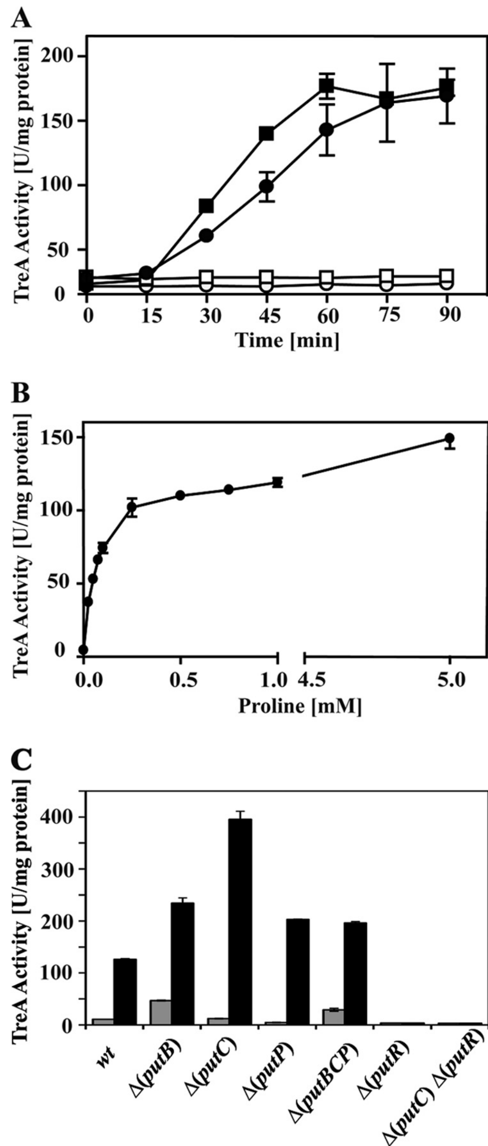

FIG 5 Induction of *put* expression in response to an external supply of L-proline. Expression of the *put* genes in response to L-proline in the growth medium was monitored with the aid of a chromosomal *putB-treA* operon fusion. (A) The reporter strain SMB10 [ϕ(*putB-treA*)*1*] was grown in SMM (squares) 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 [ϕ(*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) ϕ(*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 ϕ(*putB-treA*)*1* fusion strains were used: SMB10 (*putBCP*$^+$), SMB49 [Δ(*putB*::*spc*)*3*], SMB34 [*putB*$^+$ Δ(*putC*::*neo*)*2*], SMB14 [*putB*$^+$ *putC*$^+$ Δ(*putP*::*spc*)*1*], SMB46 [Δ(*putBCP*::*tet*)*2*], TSB2 [*putBCP*$^+$ Δ(*putR*::*spc*)], and TSB3 [*putB*$^+$ 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. wt, wild type. The error bars indicate standard deviations.

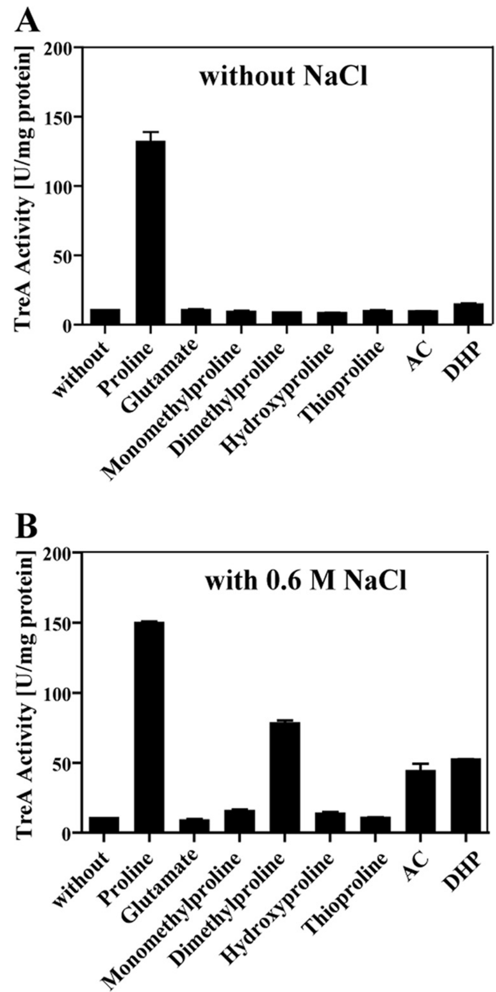

FIG 6 Induction of *put* expression in response to an external supply of L-proline, proline-derived compounds, and proline analogues. The reporter strain SMB10 [ϕ(*putB-treA*)*1*] was pregrown in either SMM (A) or SMM with 0.6 M NaCl (B) overnight, and the cultures were used to inoculate fresh cultures 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 dimethyl-proline (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

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analogues 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 OD_{578} of 1; the two sets of cultures then received 5 mM L-proline and were further grown until they reached an OD_{578} of 2. Samples were withdrawn prior to and after the addition of L-proline and assayed for their L-proline contents and TreA reporter enzyme activities. As documented in Fig. 7A, the L-proline pool of the cells grown in SMM with 1 M NaCl rose 34-fold in comparison to that of cells cultivated in SMM; it increased from about 10 mM to about 340 mM. However, the expression of the *putB-treA* reporter fusion was not triggered by this strong increase in the cellular L-proline content (Fig. 7B). In contrast, the addition of 5 mM L-proline to the growth medium of the cells, regardless of whether they were cultivated in SMM or in SMM with 1 M NaCl, triggered strong expression of the *putB-treA* reporter fusion (Fig. 7B) but did not lead to a greatly increased cellular proline pool (Fig. 7A). Hence, the *B. subtilis* cell is "blind" to internally produced L-proline with respect to the induction of *putB-treA* transcription.

L-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

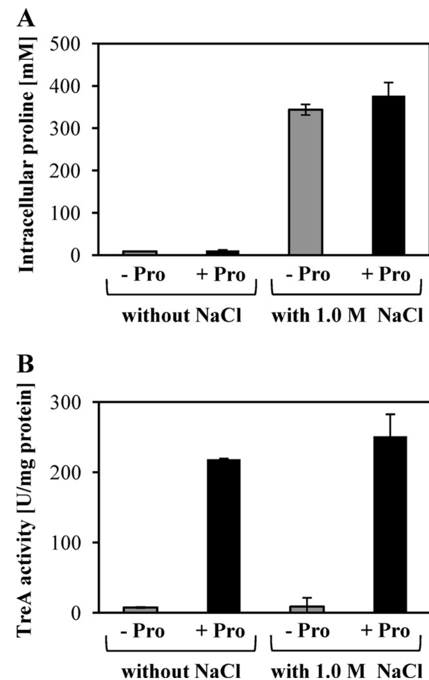

FIG 7 Synthesis of L-proline by high-salinity-stressed cells does not trigger *ϕ(putB-treA)1* expression. Cells of the *putB-treA* reporter strain SMB10 were grown either in SMM or in SMM with 1 M NaCl to an OD_{578} of 1, and one part of each culture then received 5 mM L-proline (black bars) while the other part remained untreated (gray bars). The cells were cultivated until they had reached an OD_{578} 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.

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 *putB-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

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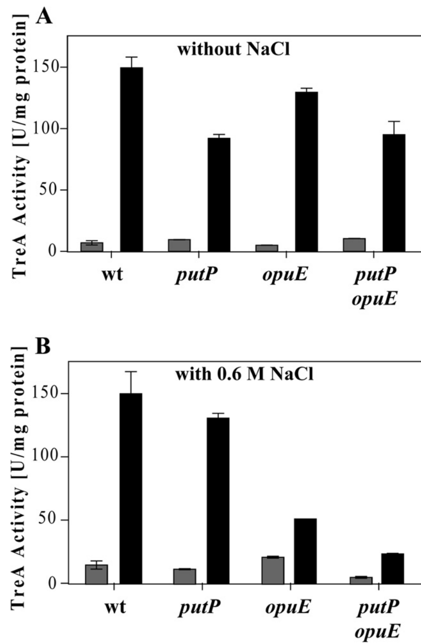

FIG 8 Induction of ϕ(*putB-treA*)*1* reporter gene activity does not depend on intact PutP and OpuE proline transport systems. Cells of the ϕ(*putB-treA*)*1* reporter strains were grown in SMM (A) or SMM with 0.6 M NaCl (B) in the absence (gray bars) or presence (black bars) of 1 mM L-proline. The cells were harvested for TreA reporter enzyme assays 60 min after the cultures received the inducer L-proline. The following ϕ(*putB-treA*)*1* reporter fusion strains were used: SMB10 (*putBCP*$^+$), SMB14 [Δ(*putP*::*spc*)*1*], SMB28 [Δ(*opuE*::*tet*)], and SMB27 [Δ(*putP*::*spc*)*1* Δ(*opuE*::*tet*)]. 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.

its spontaneously formed derivative γ-glutamate-5-semialdehyde (Fig. 1B), compounds that might function as effector molecules for PutR. Alternatively, P5C might cause feedback inhibition of the PutB enzyme activity and thus lead to the accumulation of L-proline, the inducer of PutR (7). We note in this context that the Δ(*putB*::*spc*)*3* and Δ(*putC*::*neo*)*2* mutants used for this experiment might exert polar effects on downstream-positioned genes in the *putBCP* 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. 5C).

The two *putBC*-encoded catabolic enzymes, the monofunctional PutB PRODH and the monofunctional PutC Δ^1-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

	Relevant genotype[b]		TreA activity (U mg protein^{-1})	
Strain[a]	*putR*	*mcpC*	With L-proline	Without L-proline
SMB10	+	+	7 ± 2	166 ± 6
TSB2	−	+	14 ± 2	14 ± 4
ACB154	+	−	6 ± 1	156 ± 9

[a] Each of the *B. subtilis* strains is derived from the wild-type strain JH642 and carries the same ϕ(*putB′-treA*)*1* 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] +, present; −, absent.

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(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 158 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 (Fig. 6B); these compounds enter the cell under high-salinity growth conditions via the OpuE (65) transporter (Fig. 2). Quite interesting is the inducing effect of dimethyl-proline (proline betaine) on *put* expression in high-salinity-grown cells (Fig. 6B). This plant-derived compound functions as a compatible solute (25) and is imported by salt-stressed *B. subtilis* cells through the osmoprotectant uptake systems OpuA, OpuC, and OpuD (9, 53) and not through the proline importer OpuE or PutP (A. Bashir, B. Kempf, and E. Bremer, unpublished results). Since proline betaine, in contrast to L-proline, is metabolically inert in *B. subtilis* (B. Kempf and E. Bremer, unpublished results), it should function as a gratuitous inducer for the PutR regulatory protein in high-salinity-grown cells.

The most intriguing finding of our study is certainly the observation that enhanced *putBCP* expression is not triggered by the very large quantities of L-proline (about 340 mM in cells cultivated in the presence of 1 M NaCl) produced via *de novo* synthesis (11, 67) by osmotically stressed *B. subtilis* cells (Fig. 7). It thus emerges from our data that the *B. subtilis* cell can somehow distinguish exogenously provided L-proline from an intracellular L-proline pool built up via *de novo* synthesis for either anabolic stress (pool size, about 10 to 16 mM) (12, 67) or osmostress (11, 67) protective purposes.

The molecular mechanism(s) responsible for these different effects of external and internal L-proline on the induction of the catabolic *putBCP* operon is unexplored. One way by which the *B. subtilis* cell could accomplish this would be to monitor the influx of L-proline through the PutP transporter and then to communicate this information to the PutR activator. Such a regulatory circuit is found, for instance, in *E. coli*, where the membrane-integrated transcriptional activator CadC, a member of the ToxR family of sensors/regulators, senses lysine availability in the environment indirectly via interactions with the lysine permease LysP (62).

Our data strongly argue against a scenario in which the *B. subtilis* cell senses the availability of L-proline in its environment by monitoring PutP-mediated L-proline import to upregulate 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-unidentified 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-[^{14}C]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

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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. Zaprasis, 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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