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Osmoprotection of *Bacillus subtilis* through Import and Proteolysis of Proline-Containing Peptides

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Bacillus subtilis **can attain cellular protection against the detrimental effects of high osmolarity through osmotically induced *de novo* synthesis and uptake of the compatible solute L-proline. We have now found that *B. subtilis* can also exploit exogenously provided proline-containing peptides of various lengths and compositions as osmoprotectants. Osmoprotection by these types of peptides is generally dependent on their import via the peptide transport systems (Dpp, Opp, App, and DtpT) operating in *B. subtilis* and relies on their hydrolysis to liberate proline. The effectiveness with which proline-containing peptides confer osmoprotection varies considerably, and this can be correlated with the amount of the liberated and subsequently accumulated free proline by the osmotically stressed cell. Through gene disruption experiments, growth studies, and the quantification of the intracellular proline pool, we have identified the PapA (YqhT) and PapB (YkvY) peptidases as responsible for the hydrolysis of various types of Xaa-Pro dipeptides and Xaa-Pro-Xaa tripeptides. The PapA and PapB peptidases possess overlapping substrate specificities. In contrast, osmoprotection by peptides of various lengths and compositions with a proline residue positioned at their N terminus was not affected by defects in the PapA and PapB peptidases. Taken together, our data provide new insight into the physiology of the osmotic stress response of *B. subtilis*. They illustrate the flexibility of this ubiquitously distributed microorganism to effectively exploit environmental resources in its acclimatization to sustained high-osmolarity surroundings through the accumulation of compatible solutes.**

When confronted with fluctuations in the external osmolarity, microorganisms need to balance the osmotic gradient across their cytoplasmic membrane in order to ensure a physiologically adequate level of cellular hydration and turgor, sustain growth, and safeguard cellular integrity (1, 2). Many microorganisms face up to this challenge through a dynamic increase in the osmotic potential of their cytoplasm via the accumulation of a selected class of organic compounds, the compatible solutes (3–5). These compounds are highly congruous with cellular physiology and biochemistry, and microorganisms can amass them through either synthesis or uptake (1, 3–5). The level to which compatible solutes are accumulated by the cell is finely tuned to the osmolarity prevailing in the environment (6, 7), and consequently, the outflow of water, drop in turgor, and the ensuing growth arrest are all counteracted. Hence, synthesis pathways and uptake systems for compatible solutes provide a flexible tool for microorganisms to acclimatize their physiology to sustained high-osmolarity surroundings (1, 3–5).

One of the compatible solutes most widely employed by members of the *Bacteria* is the amino acid L-proline (3–5). In addition to its function as a water-attracting osmolyte, proline also serves as a "chemical chaperone" (8) by aiding the proper folding of proteins and by preventing their aggregation in osmotically challenged cells (9). The soil-dwelling bacterium *Bacillus subtilis* makes use of proline as an osmoprotectant through both *de novo* synthesis and uptake (6, 10–12). It lives in a taxing habitat where desiccation processes create microniches with low water availability and high salinity, thereby necessitating appropriate osmostress-relieving cellular responses (13–16). *B. subtilis* extensively employs many preformed compatible solutes (e.g., glycine betaine) to fend off the detrimental effects of high osmolarity on cellular physiology and growth (17, 18) by importing them via several osmotically controlled uptake systems, the Opu family of transporters (14).

In the absence of an exogenous supply of osmoprotectants, *B. subtilis* has to rely on its own devices to cope with sustained high-osmolarity surroundings (13, 14). It does so by initially importing substantial amounts of potassium ions (19, 20) and by the subsequent synthesis of very large quantities of L-proline (6, 10, 12). Osmoadaptive proline synthesis in *B. subtilis* relies on the concerted activities of the ProJ-ProA-ProH enzymes (6), whereas anabolic proline production is catalyzed by the ProB-ProA-ProI enzymes (21) (Fig. 1). Predictably, the genetic disruption of the osmostress-responsive ProJ-ProA-ProH proline biosynthetic pathway causes osmotic sensitivity (6). *B. subtilis* can also take up free proline from the environment as an osmoprotectant, and proline is specifically imported for this physiological task via the osmotically inducible OpuE transporter (11, 22) (Fig. 1). This transporter also serves as a recycling system for newly synthesized proline that is released from high-salinity-grown *B. subtilis* cells, probably as a measure to fine-tune turgor (23).

Proline also serves as a nutrient for *B. subtilis*. The *B. subtilis* cell actively seeks proline for this purpose through chemotaxis (24) and then imports it through the OpuE-related PutP transporter for PutB-PutC-mediated catabolism to glutamate (25) (Fig. 1). Glutamate is a central metabolite positioned at the intersection of carbon and nitrogen metabolism (26, 27) and also serves as the

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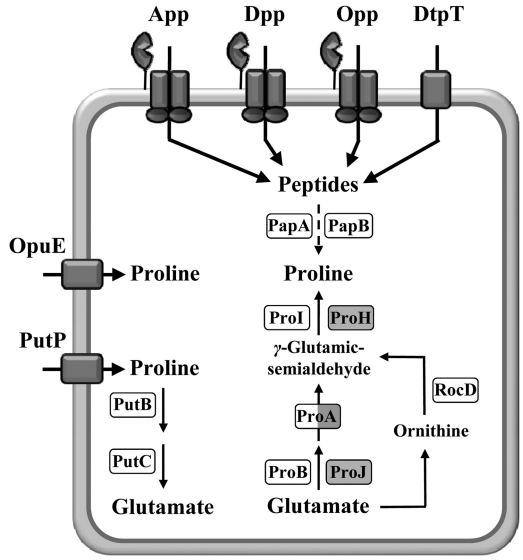

FIG 1 Import, synthesis, and catabolism of L-proline in *B. subtilis* and the generation of free proline through the uptake of proline-containing peptides and their proteolytic breakdown. The osmotically regulated proline transport system OpuE (11, 23), the PutBCP system for the uptake and catabolism of proline (25), and the ABC-type peptide transporters App, Dpp, and Opp (31–33, 39) have been described in detail. DtpT is a predicted peptide uptake system (34) and is a member of the POT transporter family (42). Proline biosynthesis in *B. subtilis* proceeds from the precursor glutamate and involves three enzymes: the γ-glutamate kinase (ProB, ProJ), the γ-glutamyl-phosphate reductase (ProA), and the Δ^1-pyrroline-5-carboxylase reductase (ProI, ProH) (28). Anabolic proline biosynthesis is catalyzed by the ProB-ProA-ProI route (marked by white boxes), and osmostress-adaptive proline biosynthesis is catalyzed by the ProJ-ProA-ProH route (marked by gray boxes). In addition to the shown Δ^1-pyrroline-5-carboxylase reductases ProI and ProH, an additional protein (ProG) with Δ^1-pyrroline-5-carboxylase reductase enzyme activity operates in *B. subtilis* (28); its physiological role is unclear. The *rocD*-encoded ornithine aminotransferase (RocD) catalyzes an enzymatic reaction that yields the same reaction product as the ProA enzyme and can therefore bypass the joint enzymatic activities of ProB and ProA in proline biosynthesis; a *proA rocD* double mutant is required to obtain a tight proline auxotrophic growth phenotype of *B. subtilis* (A. Zaprasis, G. Wünsche, and E. Bremer, unpublished data).

precursor for proline biosynthesis in *B. subtilis* (6, 21, 28). Expression of the catabolic *putBCP* operon is under the transcriptional control of the proline-responsive PutR activator protein (29). Strikingly, PutR-dependent induction of the *putBCP* gene cluster in response to proline availability occurs only when proline is present in the extracellular medium but not through the very large proline pools generated via *de novo* synthesis under high-osmolarity growth conditions (25).

B. subtilis is well known for its highly active proteolytic system (30). It possesses several secreted or cell-wall-associated proteases, a plethora of peptidases, and several peptide uptake systems (Fig. 1). These peptide transport systems (31–34) allow the acquisition of peptides of various lengths and compositions for use as nutrients and as building blocks for protein synthesis after their proteolytic breakdown. They also enable the import of extracellular peptides as signaling molecules to fine-tune developmental programs leading to competence for DNA uptake and spore formation by *B. subtilis* (35–38). Three of these peptide import systems (Dpp, Opp, and App) (31–33, 39) belong to the ABC superfamily of binding-protein-dependent transporters that fuel substrate uptake through ATP hydrolysis. In the commonly used 168 lineage of *B. subtilis* laboratory strains, the App transporter is inoperative (40, 41), but revertants of the *appA168* mutation with restored App transporter function can readily be obtained (31). Inspection of the *B. subtilis* genome sequence (41) revealed the presence of a fourth peptide transporter, DtpT (YclF) (34), a member of the POT transporter family comprising proton motive force-dependent single-component peptide importers (42). DtpT is functionally not well characterized in *B. subtilis* (34), but based on studies with DtpT-type transporters from various microorganisms (43–46), it is predicted to catalyze the import of di- and tripeptides.

Given that *B. subtilis* can import a variety of di- and oligopeptides (31–33, 39), we wondered whether it might acquire the compatible solute proline as an osmostress protectant by first importing proline-containing peptides and then proteolytically breaking them down to liberate proline. Such a process has been shown to contribute to the development of osmostress resistance in *Listeria monocytogenes* (43, 44, 47), *Lactobacillus casei* (48), and *Oenococcus oeni* (49). The data reported here demonstrate that the *B. subtilis* cell can indeed generate sizeable intracellular pools of the compatible solute proline through the uptake and hydrolysis of selected proline-containing peptides, and we genetically identified two proline aminopeptidases that are involved in this process.

MATERIALS AND METHODS

Chemicals. The antibiotics ampicillin, chloramphenicol, erythromycin, lincomycin, tetracycline, kanamycin, and spectinomycin; the ninhydrin agent for the quantification of proline (50); and the chromogenic substrate *para*-nitrophenyl-α-D-glucopyranoside (PNPG) for the TreA enzyme (51) were purchased from Sigma-Aldrich (Steinheim, Germany). The antibiotic zeocin was obtained from Invitrogen (Carlsbad, CA). The various peptides used in this study were all purchased from Bachem (Bubendorf, Switzerland).

Bacterial strains, media, and growth conditions. The *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA) was used for the maintenance of recombinant plasmids. *E. coli* and *B. subtilis* strains were propagated at 37°C and maintained on Luria-Bertani (LB) agar plates. *B. subtilis* strains were cultivated in Spizizen's minimal medium (SMM) with 0.5% (wt/vol) glucose as the carbon source and L-tryptophan (20 mg liter^{-1}) and L-phenylalanine (18 mg liter^{-1}) to satisfy the auxotrophic growth requirements of the *B. subtilis* strain JH642 (*trpC2 pheA1*) and its derivatives (Table 1). A solution of trace elements was added to SMM (52). *B. subtilis* cultures were typically inoculated from exponentially growing precultures in prewarmed minimal medium to optical densities at 578 nm (OD_{578}) of about 0.1, and the cultures were then subsequently propagated at 37°C in a shaking water bath set to 220 rpm. The *B. subtilis* cells were grown in 20-ml culture volumes in 100-ml Erlenmeyer flasks. The osmolarity of SMM was increased by the addition of an appropriate amount of NaCl using a 5 M stock solution. Solutions of peptides, L-proline, and other additives were sterilized by filtration and added to the growth medium at the indicated concentrations; typically, the osmoprotectants proline, glycine betaine, and various peptides were added to the growth medium at a final concentration of 1 mM. The antibiotics chloramphenicol, kanamycin, tetracycline, erythromycin-lincomycin, spectinomycin, and zeocin were used with *B. subtilis* strains at final concentrations of 5, 5, 10, 0.4-15, 100, and 35 μg ml^{-1}, respectively. Ampicillin was used at a final concentration of 100 μg ml^{-1} for *E. coli* cultures. The *B. subtilis* wild-type strain JH642 (*trpC2 pheA1*) and its various mutant derivatives (Table 1) were used throughout this study. Strain JH642 is a member of the domesticated lineage of *B. subtilis* laboratory strains (40) and carries a frameshift mutation (*appA168*) inactivating the App oligopeptide transporter (31). Strain JH14115 is derived from JH642 and carries a functional App system caused by a reversion of the frameshift mutation in the *appA* gene (31).

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

Strain[a]	Relevant genotype	Source or reference
JH642	*trpC2 pheA1 appA168*	J. Hoch; BGSC 1A96[b]
JH14115[c]	JH642 *appA*$^+$-Neo^r	31
JSB8	JH642 Δ(*proHJ*::*tet*)1	6
SMB45	JH642 Δ(*putBCP*::*tet*)2	25
ABB1	JH642 Δ(*putBCP*::*tet*)2 Δ(*proHJ*::*neo*)1	A. Blumrich
ACB73	JH14115 Δ(*rocD*::*tet*)2 Δ(*proBA*::*cat*)2 *appA*$^+$-Neo^r	This study
ACB97[d]	JH14115 Δ(*rocD*::*tet*)2 Δ(*proBA*::*cat*)2 Δ(*papA*::*ery*)1 Δ(*papB*::*spc*)1 *appA*$^+$-Neo^r	This study
ACB108[d]	JH14115 Δ(*rocD*::*tet*)2 Δ(*proBA*::*cat*)2 Δ(*papA*::*ery*)1 Δ(*yclE*::*zeo*)1 *appA*$^+$-Neo^r	This study
ACB109	JH14115 Δ(*rocD*::*tet*)2 Δ(*proBA*::*cat*)2 Δ(*papB*::*spc*)1 Δ(*yclE*::*zeo*)1 *appA*$^+$-Neo^r	This study
ACB118	JH14115 Δ(*proHJ*::*tet*)1 Δ(*papB*::*spc*)1 Δ(*papA*::*ery*)1 *appA*$^+$-Neo^r	This study
ACB221	JH14115 Δ(*putBCP*::*tet*)2 *appA*$^+$-Neo^r	This study
ACB223	JH14115 Δ(*putBCP*::*tet*)2 Δ(*proHJ*::*ery*)2 *appA*$^+$-Neo^r	This study
ACB225	JH14115 *amyE*::[ϕ(*putB′-treA*)1 *cat*] Δ(*treA*::*ery*)2 *appA*$^+$-Neo^r	This study
GWB19[d]	JH642 Δ(*appDFABC*::*neo*)1 Δ(*dppABCDE*::*zeo*)1 Δ(*oppABCDF*::*spc*)1 Δ(*dtpT*::*ery*)1 Δ(*proHJ*::*tet*)1	This study
GWB100	JH14115 Δ(*proHJ*::*tet*)1 *appA*$^+$-Neo^r	This study

[a] All strains are derivatives of the *B. subtilis* wild-type strain JH642 (40) and therefore carry, in addition to the genetic markers indicated, also the *trpC2*, *pheA1*, and *appA168* mutations.
[b] BGSC, Bacillus Genetic Stock Center (Columbus, OH).
[c] The *appA*$^+$ allele is a reversion of a frameshift mutation in the *appA168* mutant allele and thus restores the functioning of the App oligopeptide transporter; it is genetically linked to a kanamycin (*neo*) resistance marker (31).
[d] The *papA*, *papB*, and *dtpT* genes are identical to the *yqhT*, *ykvY*, and *yclF* genes annotated in the updated genome sequence of the *B. subtilis* 168 strain (41). *yclE* codes for a putative peptidase (60); it has no role in Xaa-Pro peptide hydrolysis (A. Zaprasis and E. Bremer, unpublished data).

Strains JH642 and JH14115 were kindly provided to us by James A. Hoch (Scripps Research Institute, CA).

Recombinant DNA techniques and construction of *B. subtilis* mutants. The routine manipulations of plasmid DNA, PCR, the isolation of chromosomal DNA from *B. subtilis*, and the transformation of *B. subtilis* strains with chromosomal DNA or PCR products were all carried out using standard procedures (53, 54). To construct a deletion of a chosen gene in the *B. subtilis* genome, the flanking 5′ and 3′ regions were amplified by PCR from chromosomal DNA and connected by long-flanking-region PCR (55) with a gene encoding an antibiotic resistance cassette. The resulting PCR product was then used to transform *B. subtilis* strains, and the integration of the gene disruption construct into the chromosome was selected for by plating on LB agar plates containing the appropriate antibiotic. Individual colonies were then picked from the transformation plates and purified by restreaking. The presence of the desired gene disruption construct was verified by PCR using chromosomal DNA as the template and synthetic DNA primers flanking the deleted genomic segment. Different gene disruption mutations were genetically combined by transforming *B. subtilis* recipient strains with chromosomal DNA of *B. subtilis* donor strains carrying a gene disruption linked to an antibiotic resistance marker; transformants were selected on LB agar plates containing the appropriate antibiotic. The following primer pairs were used to perform the long-flanking-region PCR: for the deletion of the *rocD* gene, 5′-GAAATAACCCGTCTTCATAAAAATGTGTCA-3′/5′-ATTTCCTG GCCTTTATTATTGAAATAGCTC-3′; for the deletion of the *proBA* operon, 5′-CATTCGTGAAAAAAAATCATCACTTCAGGC-3′/5′-ATA TAACATACCCTCAATATTTGGCTTTGC-3′; for the deletion of the *papA* (*yqhT*) gene, 5′-CGGAACTTTATGTTTTGACATATTATCAAC-3′/5′-CAATTCGTATATTAAAAGATGCGATGTCAC; for the deletion of the *papB* (*ykvY*) gene, 5′-TGACCAAAAAGTGAATGAAATCAAACG GTT-3′/5′-TTCATGCCTTTTTTTCTTGCCAAACAGATG-3′. The various antibiotic resistance cassettes used in this study were derived from plasmids pDG1515 (*tet*), pDG642 (*ery*), pDG783 (*neo*), and pDG1727 (*spc*) (56); p7Z6 (*zeo*) (57); and pJMB1 (*cat*) (M. Jebbar and E. Bremer, unpublished data).

TreA reporter enzyme assays. The expression of chromosomal *putB-treA* reporter gene fusions (25) was monitored by assaying the TreA [phospho-α-(1,1)-glucosidase] enzyme (51) activity by using the chromogenic substrate *para*-nitrophenyl-α-D-glucopyranoside (PNPG) as detailed previously (21). One unit of TreA activity is defined as 1 μmol of substrate converted by the enzyme per min per mg protein. Protein concentrations of the samples were estimated from the optical density of the *B. subtilis* cell culture (58).

Quantification of proline. The intracellular proline content of *B. subtilis* strains was assessed by a colorimetric assay that detects proline as a colored proline-ninhydrin complex that can be quantified by measuring the absorption of the solution at 480 nm (50). For this assay, *B. subtilis* cells were grown in SMM under various osmotic conditions in the presence or absence of proline or proline-containing peptides until mid-exponential growth phase (OD_{578} of about 1.8 to 2.0). For the measurement of the intracellular proline contents of various *B. subtilis* strains, cells (8 ml) were harvested by centrifugation, extracted, and analyzed according to the procedure detailed by Bates et al. (50). Proline concentrations were determined by establishing a standard curve with L-proline, and intracellular proline concentrations were then 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). The cell volumes of *B. subtilis* cells were calculated from the determination of the internal and total water spaces. To this end, aliquots of the *B. subtilis* cells grown in SMM (or in SMM with various concentrations of NaCl) were simultaneously incubated with membrane-permeable ^3H_2O and membrane-impermeable [^{14}C]inulin carboxylic acid (Amersham Life Science, Freiburg, Germany). The distribution of these radiolabeled compounds in the internal and external water spaces was measured by scintillation counting after centrifugation of the cells through silicon oil (59).

Database searches, alignments of amino acid sequences of proteins, and *in silico* modeling of protein structures. Functional annotations of proteins and information on the expression profiles of *B. subtilis* genes were accessed via the SubtiWiki webserver (http://subtiwiki.uni-goettingen.de/wiki/index.php/Main_Page) (60). The alignment of amino acid sequences of proteins was performed with ClustalW (61). *In silico* models of the *B. subtilis* PapA (YqhT) and PapB (YkvY) proline aminopeptidases were obtained with the aid of the Swiss Model server (http://swissmodel.expasy.org) (62).

RESULTS

Proline-containing peptides can provide free proline for anabolic purposes. For our studies on the use of proline-containing

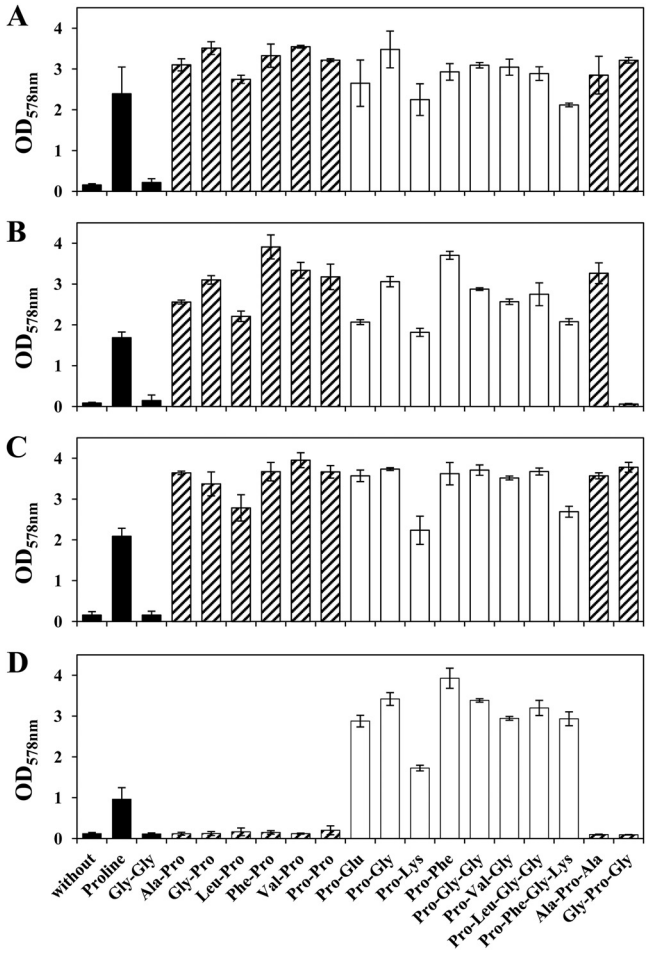

FIG 2 Proline-containing peptides allow growth of proline auxotrophic *B. subtilis* strains. Cultures of the proline auxotrophs ACB73 (*proBA rocD*) (A), ACB108 (*proBA rocD papA*) (B), ACB109 (*proBA rocD papB*) (C), and ACB97 (*proBA rocD papA papB*) (D) were grown in SMM in the absence or the presence of the indicated peptides (final concentration in the growth medium, 1 mM) or proline (1 mM). The cells were inoculated to an OD_{578} of 0.08 from precultures grown in SMM, and the growth yields of the cultures were determined by measuring the OD_{578} after 16 h of incubation at 37°C. The values given represent the means of two independently grown cultures, and the error bars indicate standard deviations. Striped bars indicate those proline-containing peptides whose hydrolysis is dependent on the PapA and PapB peptidases. The App, Dpp, Opp, and DtpT peptide transport systems are intact in all of the *B. subtilis* strains used in these experiments.

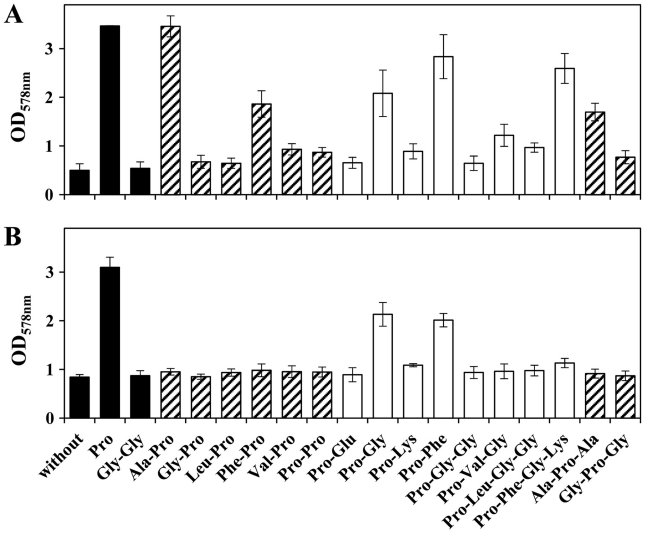

FIG 3 Osmoprotective effects of proline-containing peptides for *B. subtilis*. Cultures of strain GWB100 (*proHJ*) (A) and strain GWB19 (*proHJ app dpp opp dtpT*) (B) were grown in SMM with 1.2 M NaCl in the absence or the presence of the indicated peptides (final concentration, 1 mM, except for the Pro-Pro dipeptide, which was supplied at a concentration of 0.5 mM) and proline (1 mM). The cells were inoculated to an OD_{578} of 0.1 from precultures grown in SMM. The growth yields of the cultures were determined by measuring the OD_{578} after incubation for 16 h for strain GWB19 or 20 h for strain GWB100 at 37°C. The values given represent the means of two independently grown cultures, and the error bars indicate standard deviations.

peptides for either anabolic or osmoprotective purposes, we used the *B. subtilis* strain JH14115. All currently proven (App, Dpp, and Opp) and predicted (DtpT) peptide uptake systems (Fig. 1) are intact in this strain (31); JH14115 should therefore allow the uptake of a broad spectrum of peptides (31–34, 39).

For the studies assessing the use of proline-containing peptides for anabolic purposes, a process that obviously requires the proteolytic breakdown of these types of peptides, we introduced into the JH14115 genetic background simultaneous gene disruptions in the *proBA* and *rocD* loci, thereby creating a tight proline auxotrophic growth phenotype (Fig. 1). The data summarized in Fig. 2A demonstrate that all 16 peptides tested by us were able to complement the proline auxotrophic growth phenotype of the *B. subtilis* tester strain. The supply of the peptide Gly-Gly, studied as a control, was unable to do so, whereas an external supply of free proline showed the expected rescue of the growth defect of strain JH14115 (Fig. 2A). Hence, *B. subtilis* is able to proteolytically break down a considerable spectrum of proline-containing peptides with different compositions and lengths and then use the liberated proline to fuel protein biosynthesis.

A subset of proline-containing peptides provides osmoprotection. In a subsequent set of experiments, we used the same 16 proline-containing peptides in growth assays that monitor their osmoprotective potential. We used for this purpose a derivative of strain JH14115 carrying a *proHJ* deletion (strain GWB100) that abolishes osmostress-adaptive proline biosynthesis via the ProJ-ProA-ProH proline biosynthetic route (6) (Fig. 1) and therefore causes an osmotic-stress-sensitive growth phenotype that is much stronger that that exhibited already by the wild-type strain in a chemically defined medium (SMM) containing 1.2 M NaCl (6). Strain GWB100 can therefore be used to assess the osmoprotective potential of a given compound. We found that only a subset of the evaluated proline-containing peptides was able to confer osmoprotection, and the degree of osmoprotection varied considerably (Fig. 3A). Consistent with previous data (6, 11), growth inhibition of the *proHJ* mutant strain by high salinity (with 1.2 M NaCl) was alleviated by an exogenous supply of proline (Fig. 3A).

We also tested osmoprotection of proline-containing peptides in a *proHJ B. subtilis* mutant strain, GWB19, in which the App, Dpp, Opp, and DtpT peptide transporters were all nonfunctional due to the complete deletions of the corresponding structural genes (Table 1). With the exception of the Pro-Gly and Pro-Phe peptides, osmoprotection was abolished when these peptide transporters were not operational (Fig. 3B). Consequently, the osmoprotective effects of most tested peptides were dependent on

their import and not on extracellular hydrolysis. We currently cannot tell whether the osmoprotection that is conferred by the Pro-Gly and Pro-Phe peptides on the strain with the defects in the App, Dpp, Opp, and DtpT transporters is caused by an extracellular breakdown of these peptides and the subsequent import of the liberated proline, their passive diffusion through the cytoplasmic membrane, or their active uptake by a so-far-unrecognized peptide transport system and subsequent intracellular hydrolysis. It is also not clear if there is a preferential use of a particular peptide transport system under high-salinity growth conditions.

Osmoprotection by proline-containing peptides depends on their hydrolysis to release the compatible solute proline. The osmoprotective effects of proline-containing peptides could be caused by the accumulation of the peptides themselves, by their hydrolysis to release free proline (47–49), or by indirect effects on the nutritional status (63) of the osmotically stressed *B. subtilis* cells. To test for a potential beneficial nutritional influence of the proline-containing peptides on osmotically stressed *B. subtilis* cells, we compared the growth properties of strain JH14115, which possesses an intact PutBCP proline-degradative system, and its isogenic derivative strain ACB221, in which the proline-degradative PutBCP system (25) is disrupted. We monitored the growth of these strains in SMM or SMM containing 1.2 M NaCl in the presence or absence of proline or proline-containing peptides. None of the tested peptides (Ala-Pro, Pro-Gly, and Gly-Pro; the final concentration in the medium was 1 mM) provided a nutritional advantage to the two strains, regardless of whether they were grown in SMM or in SMM containing 1.2 M NaCl (Fig. 4). However, some beneficial effects of proline degradation via the PutBCP system on growth were noticeable (Fig. 4). The SMM used for these growth studies contained glucose and ammonium, two of the most preferred carbon and nitrogen sources of *B. subtilis* (26, 27, 64). The growth data summarized in Fig. 4 show that the presence of proline-containing peptides does not have a general stimulatory effect on cell growth, as they stimulate growth only under high-osmolarity cultivation conditions. The behavior of *B. subtilis* therefore differs from that of *E. coli*, where an improvement of the nutritional status of osmotically challenged cells by proline-containing peptides has been observed (63).

To test for a possible proteolytic breakdown of proline-containing peptides as the underlying explanation for their osmoprotective effects (Fig. 3A), we provided the osmoprotective peptides Ala-Pro, Pro-Gly, and Val-Pro and the peptide Gly-Pro, which does not confer osmoprotection (Fig. 3A), to high-salinity-challenged cultures (SMM with 1.2 M NaCl) of the *proHJ* mutant strain GWB100. We used for these experiments a *proHJ* mutant since it cannot produce osmoprotective levels of proline (6), and the buildup of proline pools resulting from the hydrolysis of proline-containing peptides should therefore be readily detectable. For these experiments, we grew the cultures to an OD_{578} of about 1.8 to 2.0 and then measured the free intracellular proline content of the cells. As expected, the *proHJ* mutant strain GWB100 did not accumulate osmoprotective levels of free proline (the intracellular proline content was about 24 mM), whereas in the cells that had been provided with the osmoprotective peptides Ala-Pro, Pro-Gly, and Val-Pro, large amounts of free proline were found (Fig. 5). In contrast, cells that were provided with the nonprotective peptide Gly-Pro did not accumulate proline (Fig. 5).

The proline content of the osmotically stressed cells that was generated through the hydrolysis of the Ala-Pro, Pro-Gly, and Val-Pro peptides steadily rose in proportion to the increase in the external salinity. The buildup of the proline pool followed the pattern exhibited by cells of strain JH14115 (Fig. 5), which, in contrast to strain GWB100, possesses an intact ProJ-ProA-ProH osmostress-adaptive proline biosynthetic route (Fig. 1). Its proline pool reached about 500 mM in cells cultivated in SMM that contained 1.2 M NaCl, and a similar proline pool (about 456 mM) was generated via the hydrolysis of the Ala-Pro dipeptide (Fig. 5). Overall, we noted a good correlation between the osmoprotective potential of the tested four peptides (Fig. 3A) and the size of the proline pool generated through proteolysis in osmotically challenged cells (Fig. 5).

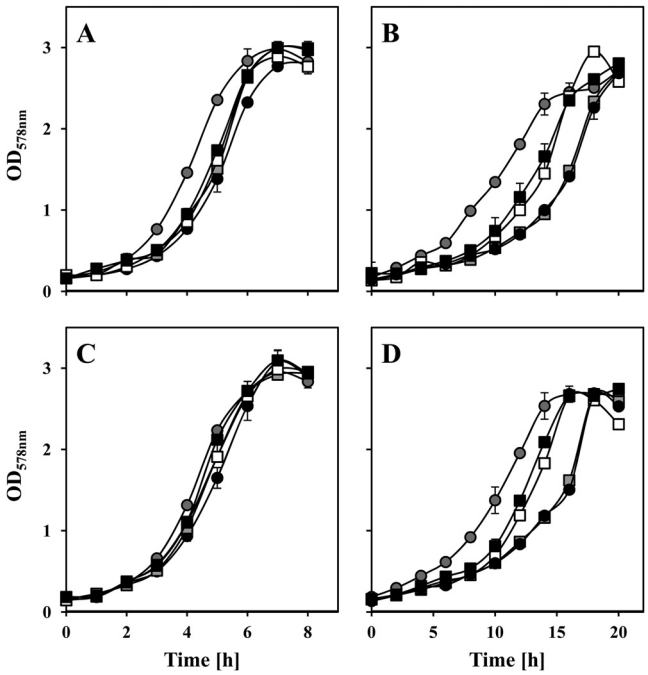

FIG 4 The effect of the *putBCP* deletion on the growth characteristics of *B. subtilis* in the presence of various osmoprotective peptides. Cultures were inoculated to optical densities (OD_{578}) of 0.14 from overnight cultures grown in SMM and were incubated at 37°C in SMM (A and C) or SMM with 1.2 M NaCl (B and D) either in the absence (black circles) or in the presence of L-proline (gray circles) or the peptides Ala-Pro (black squares), Pro-Gly (white squares), and Gly-Pro (gray squares) (final concentration of proline and of the various peptides was 1 mM). The *B. subtilis* wild-type strain JH14115 (A and B) and its *putBCP* mutant derivative, strain ACB221 (C and D), were used in this experiment. Growth of the cultures was monitored over time by measuring the OD_{578}. The values given represent the means of two independently grown cultures, and the error bars indicate standard deviations.

Role of the PapA and PapB proline aminopeptidases for the use of Xaa-Pro- and Xaa-Pro-Xaa-type peptides. Previously reported data on the hydrolysis of proline-containing osmoprotective peptides in *L. casei* and *O. oeni* (48, 49) and our consultation of the SubtiWiki database (60) alerted us to the *B. subtilis* YkvY and YqhT proteins. These paralogous proteins exhibit about 34% amino acid sequence identity to each other, and both belong to the M24B subfamily of peptidases according to the MEROPS database (65). The M24 family of peptidases contains two different types of peptidases: (i) Xaa-Pro peptidases (EC 3.4.13.9) (prolidase), which split dipeptides with a prolyl residue in the carboxy-terminal position but do not act on Pro-Pro peptides, and (ii) Xaa-Pro

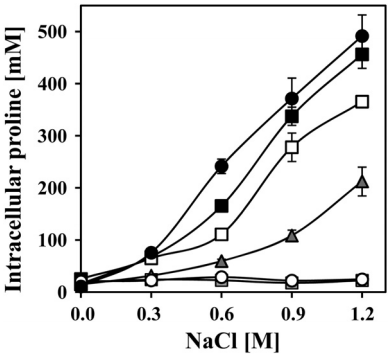

FIG 5 Intracellular proline content of osmotically stressed *B. subtilis* cells grown in the presence of osmoprotective peptides. Cultures of strain GWB100 (*proHJ*) were grown in SMM of various salinities either in the absence (white circles) or in the presence of the peptides Ala-Pro (black squares), Pro-Gly (white squares), Gly-Pro (gray squares), and Val-Pro (gray triangles) (final concentration of the various peptides was 1 mM) until they reached an OD_{578} of 1.8 to 2; the cells were then assayed for their proline content. The proline content of cultures of strain JH14115 (black circles) that was accumulated via *de novo* synthesis was measured as the control. For each sample analyzed, the intracellular proline content was determined twice. The values given represent the means of two independently grown cultures, and the error bars indicate standard deviations. The App, Dpp, Opp, and DtpT peptide transport systems are all intact in the *B. subtilis* strains GWB100 and JH14115 used for this experiment.

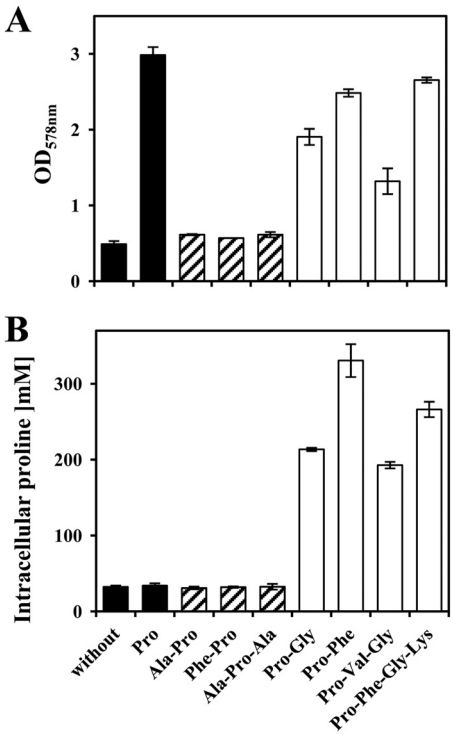

FIG 6 Influence of the PapA and PapB peptidases on osmoprotection by proline-containing peptides and on the intracellular proline pool. (A) Cells of strain ACB118 (*proHJ papA papB*) were grown in SMM with 1.2 M NaCl in the absence or the presence of the indicated peptides (final concentration, 1 mM) or in the presence of proline (1 mM). The cells were inoculated to an OD_{578} of 0.1 from a preculture grown in SMM at 37°C. The growth yield of the cultures was measured after 16 h of incubation. (B) The high-osmolarity-grown cells (SMM with 1.2 M NaCl) were harvested once they reached an OD_{578} of 1.8 to 2 and were then assayed for their proline content. For each sample analyzed, the intracellular proline content was determined twice. The values given represent the means of two independently grown cultures, and the error bars indicate standard deviations. Striped bars represent those osmoprotective peptides whose proteolytic breakdown is affected by the PapA and PapB peptidases.

aminopeptidases (EC 3.4.11.9) (66), which can release any N-terminal amino acid (including proline) adjacent to a proline residue from di- and oligopeptides. The active center of these types of metallopeptidases typically is comprised of two metal ions (67, 68). *In silico* model building using the Swiss Model web server (62) suggests that the *B. subtilis* YkvY and YqhT proteins are structurally related to the PepQ prolidase from the hyperthermophilic archaeon *Pyrococcus furiosus* (Protein Data Bank [PDB] accession code 1PV9) (69) and a structurally but not functionally characterized Xaa-Pro dipeptidase from *Bacillus anthracis* (PDB accession code 3Q6D). The residues known to be involved in metal binding of the *P. furiosus* PepQ prolidase (69) are conserved in the amino acid sequences of the *B. subtilis* YkvY and YqhT proteins (data not shown).

The YkvY and YqhT proteins are predicted, according to the MEROPS database (65), to serve as Xaa-Pro aminopeptidases (EC 3.4.11.9). Since a number of peptides tested by us as sources of proline for anabolic (Fig. 2A) and osmoprotective (Fig. 3A) purposes in *B. subtilis* contain Pro residues at the second position, we investigated a possible role of the potential aminopeptidases YkvY and YqhT in these processes. The individual disruptions of the *ykvY* and *yqhT* genes had no noticeable effects on the use of six Xaa-Pro-type dipeptides as proline sources, and this also did not affect the use of the Ala-Pro-Ala peptide (Fig. 2B and C). However, the use of the Gly-Pro-Gly peptide as a proline source was affected in the *papA* but not in the *papB* mutant strain (Fig. 2B and C). When a *papA papB* double mutant was tested, all di- and tripeptides with a Pro residue at the second position were no longer able to function as proline sources; this included the tested Pro-Pro peptide (Fig. 2D). Since the YqhT and YkvY peptidases can hydrolyze Pro-Pro-type peptides and since they can also act on tripeptides (Fig. 2D), they seem to function as proline aminopeptidases. We therefore refer below to YqhT as the PapA protein and to YkvY as the PapB protein (Pap = proline aminopeptidase) (Fig. 1).

Fully consistent with the assignment of the PapA and PapB proteins as proline aminopeptidases was our finding that the use of di-, tri-, and pentapeptides in which the Pro residue was positioned at the N terminus did not affect their function as proline sources in a *papA papB* double mutant strain (Fig. 2D).

The PapA and PapB proteins possess overlapping substrate specificities (Fig. 2), but their substrate spectra are not identical. The latter feature came to light when the Ala-Pro-Ala and Gly-Pro-Gly tripeptides were tested as possible proline sources for anabolic purposes. Use of the Gly-Pro-Gly tripeptide was abolished in the *papA* mutant strain ACB108, but Ala-Pro-Ala peptide use was unaffected (Fig. 2B); loss of the PapB protein did not affect the use of either of these two peptides (Fig. 2C). In a *papA papB* double mutant (strain ACB97), however, the function of both the Ala-Pro-Ala and Gly-Pro-Gly tripeptides as a proline source for protein biosynthesis was abolished (Fig. 2D).

Building on these data, we then assessed the role of the PapA and PapB peptidases in the use of a selected set of proline-containing peptides as osmoprotectants by *B. subtilis* (Fig. 6). The osmoprotective potential of the Ala-Pro, Phe-Pro, and Ala-Pro-Ala peptides (Fig. 6A) was abolished in strain ACB118 lacking the

PapA and PapB peptidases. This was caused by the inability of this strain to build up a proline pool large enough for osmoprotection through the hydrolysis of the provided peptides (Fig. 6B). Hence, the PapA and PapB proteins not only are involved in releasing proline from peptides for use as a building block for protein synthesis (Fig. 2D) but also are critical for the release of proline from Xaa-Pro- and Xaa-Pro-Xaa-type peptides for their use as osmoprotectants (Fig. 6). Fully consistent with the data shown in Fig. 2D, the use of peptides as osmoprotectants with an N-terminally positioned proline residue and the concomitant buildup of a large intracellular proline pool were not affected by the simultaneous loss of the PapA and PapB aminopeptidases (Fig. 6).

Osmoprotection of a ProHJ-proficient *B. subtilis* strain by an external supply of the Ala-Pro dipeptide. The experiments reported so far on the osmoprotective potential of proline-containing peptides (Fig. 3A) employed *B. subtilis* strains that are defective in the osmoadaptive ProJ-ProA-ProH proline biosynthetic route (Fig. 1) and are therefore intrinsically sensitive to high salinity (6). These types of strains were used to unambiguously assess the hydrolysis of proline-containing peptides and the concomitant buildup of an osmoprotective intracellular proline pool, two tasks that would be impossible to perform with *B. subtilis* strains proficient in the endogenous buildup of the very large proline pool derived from osmostress-dependent *de novo* synthesis (6, 10, 23).

To test whether osmoprotection by proline-containing peptides can also be accomplished in a *B. subtilis* strain that can synthesize proline via the ProJ-ProA-ProH route under osmotic-stress conditions (Fig. 1), we subjected the wild-type strain JH14115 to osmotic stress (in SMM containing 1.2 M NaCl) in the absence or the presence of either of the Ala-Pro and Pro-Gly dipeptides; these two peptides confer good osmoprotection on a *proHJ* mutant strain (Fig. 3A), and their hydrolysis yields large intracellular proline pools (Fig. 5). Both peptides provided osmoprotection to the strain proficient in osmostress-adaptive proline biosynthesis (Fig. 4B). We note, however, that they are somewhat less effective osmoprotectants than is an exogenous supply of proline. As expected, the Gly-Pro dipeptide that does not allow the buildup of large proline pools in a *proHJ* mutant strain (Fig. 5) did not stimulate growth of the wild-type strain under high-salinity conditions (Fig. 4B and D).

We then investigated whether the feeding of the osmoprotective Ala-Pro and Pro-Gly dipeptides to osmotically stressed cells of strain JH14115 would influence the size of the free proline pool. As shown in Fig. 7 and as expected from data previously reported by Brill et al. (6) and Hoffmann and Bremer (17), an increase in the external salinity strongly stimulated the proline content of the JH14115 cells that were grown in the absence of any externally provided osmoprotective compounds. However, the addition of the Ala-Pro and Pro-Gly peptides, or of free proline, to the growth medium did not result in a further increase in the cellular proline pool (Fig. 7). These observations suggest that at a given osmolarity of the environment, the proline pool is sensitively set by the *B. subtilis* cell to a particular osmostress-relieving level (6, 23) and that the provision of either additional external proline or proline intracellularly liberated by proteolysis from peptides does not greatly boost the total size of the proline pool.

Interdependence between proline degradation and osmoprotection by externally provided proline. Inspection of the data shown in Fig. 6 and 7 yielded a very surprising observation: the addition of proline to osmotically stressed cells of *proHJ* mutant strains provided osmoprotection (Fig. 6A) but, contrary to expectation (11), did not lead to the buildup of a large proline pool (Fig. 6B and 7). We suspected that this phenomenon was connected with the ability of *B. subtilis* to use exogenously provided proline as a nutrient, a process that relies on the import of proline via the OpuE-related high-affinity PutP transporter and its catabolism to glutamate by the PutB and PutC enzymes (Fig. 1) (25).

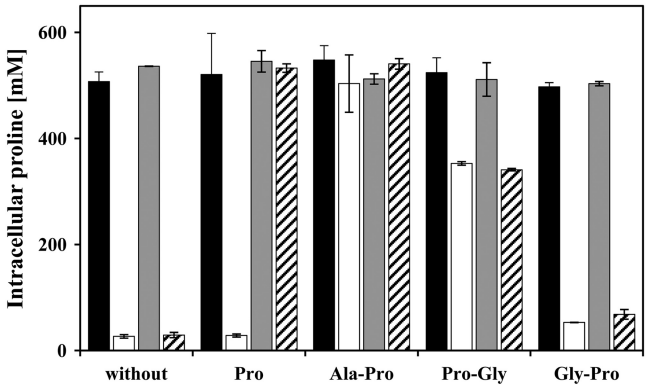

FIG 7 Influence of the catabolic PutBCP system on proline pools formed in response to an external supply of either proline or proline-containing peptides. Cultures of the strain JH14115 (black bars) and its mutant derivatives strain GWB100 (*proHJ*) (white bars), strain ACB221 (*putBCP*) (gray bars), and strain ACB223 (*proHJ putBCP*) (striped bars) were pregrown in SMM and used to inoculate SMM containing 1.2 M NaCl in the absence or presence of L-proline (1 mM) or the peptides Ala-Pro, Pro-Gly, and Gly-Pro (final concentration, 1 mM) to an OD_{578} of 0.1. The cultures were grown until they reached an OD_{578} of 1.8 to 2 and were subsequently assayed for their proline content. The values given represent the means of two independently grown cultures, and the error bars indicate standard deviations. For each sample analyzed, the intracellular proline content was determined twice.

We therefore constructed an isogenic set of *B. subtilis* strains in which the *proHJ* and *putBCP* operons were either disrupted or intact and then asked if an external supply of proline to osmotically stressed cells would lead to the buildup of a large intracellular proline pool. Loss of the degradative PutBCP system did not influence the buildup of osmoprotective levels of proline by *de novo* synthesis via the ProJ-ProA-ProH route, and it also did not influence the size of the proline pool when 1 mM proline was present in the growth medium (Fig. 7, gray bars). However, in a *putBCP* deletion strain with a defect in the ProJ-ProA-ProH route, a striking difference from its isogenic *putBCP*$^+$ counterpart was observed: a large proline pool was now built up from an exogenous supply of proline (Fig. 7, striped bars). Consequently, *B. subtilis* degrades proline not only when it is supplied to the cells in a standard laboratory medium (SMM) with a moderate degree of osmolarity (25) but also when the cells are cultured under high-salinity stress conditions.

Using a *putB-treA* transcriptional reporter fusion, we found (building on previous data [25]) that the expression of the proline-degradative *putBCP* gene cluster can be induced by low concentrations of proline (1 mM) in both low- and high-salinity media (Fig. 8). In contrast, the same concentration of the proline-containing dipeptides Ala-Pro, Pro-Gly, and Gly-Pro did not trigger significantly enhanced levels of *putB-treA* expression, despite the fact that the hydrolysis of Ala-Pro and Pro-Gly peptides allows the buildup of very substantial intra-

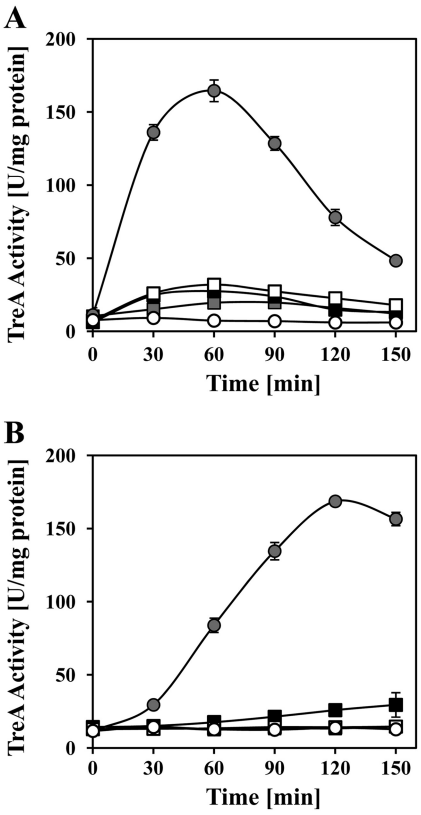

FIG 8 The effect of externally provided L-proline and osmoprotective peptides on the induction of *put* expression. The reporter strain ACB225 [ϕ(*putB-treA*)1] was grown in SMM (A) or in SMM with 1.2 M NaCl (B) to early exponential phase. At time zero, the cells were provided either with L-proline (gray circles) or with the peptide Ala-Pro (black squares), Pro-Gly (white squares), or Gly-Pro (gray squares) (final concentration of proline and of the various peptides was 1 mM). One culture did not receive proline or a peptide (white circles). At the indicated time points, the cells were 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.

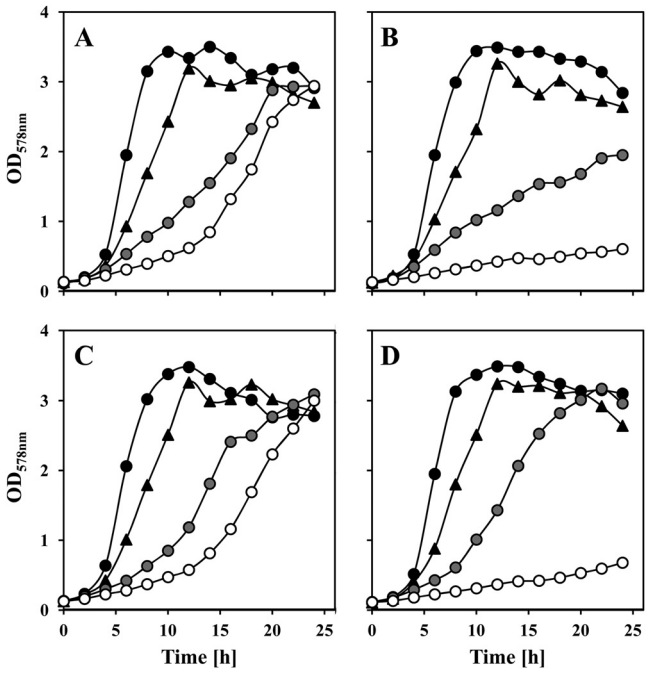

FIG 9 The effect of externally provided proline on growth of different *B. subtilis* mutant strains. Cultures of the *B. subtilis* wild type-strain JH642 (A) and its mutant derivatives JSB8 (*proHJ*) (B), SMB45 (*putBCP*) (C), and ABB1 (*proHJ putBCP*) (D) were inoculated to optical densities (OD_{578}) of 0.1 from overnight cultures pregrown in SMM and were incubated at 37°C in SMM (black circles) or in SMM with 1.2 M NaCl either in the absence (white circles) or in the presence of glycine betaine (black triangles), or L-proline (gray circles) (final concentrations of glycine betaine and proline were 1 mM). Growth of the cultures was monitored over time by measuring the OD_{578}.

cellular proline pools (Fig. 5 and 7). These different effects of free external proline and of the internally hydrolyzed proline-containing peptides on the expression of the *putB-treA* reporter fusion are in full agreement with our previous notion that *B. subtilis* can somehow distinguish external from internal proline as far as the proline-responsive induction of *putBCP* transcription is concerned (25).

We found in growth assays that the osmoprotective properties of externally provided proline for *B. subtilis* (11) are rather modest in a direct comparison to the potent osmoprotectant glycine betaine (18) (Fig. 9A). We observed that the loss of the PutBCP catabolic system improves the osmoprotective potential of externally provided proline (compare Fig. 9A with 9C). However, proline was a good osmoprotectant only in a *B. subtilis* strain that cannot synthesize proline by itself and that simultaneously lacks the PutBCP system (Fig. 9D). Consequently, the osmoprotective potential of proline for *B. subtilis* is limited by the induction of the *putBCP* operon by externally provided proline and the ensuing use of this amino acid as a nutrient (25).

DISCUSSION

The data reported here add an additional dimension to the use of L-proline as an osmoprotectant by *B. subtilis* (6, 10–12). We show that a noticeable level of osmotic stress resistance and a considerable intracellular pool of free proline can be attained through the import of proline-containing peptides and their proteolytic breakdown. The degree of osmoprotection that can be achieved in this way is correlated with the size of the intracellular proline pool, thereby suggesting that free proline, and not the peptide from which it is proteolytically derived, is responsible for the osmoprotective effect. We have proven this notion directly for two Xaa-Pro-type dipeptides and one Xaa-Pro-Xaa-type tripeptide that completely lose their osmoprotective potential in a *B. subtilis* mutant with simultaneous defects in the PapA (YqhT) and PapB (YkvY) proline aminopeptidases. The behavior of *B. subtilis* thus resembles that of *L. monocytogenes* (43, 44, 47), *L. casei* (48), and *O. oeni* (49), all of which can achieve osmoprotection through the hydrolysis of proline-containing peptides, but it differs from that observed in *E. coli*, where the osmoprotective effects of peptone have been traced to the import of free proline and the nutritional effects of peptides (63). By comparing the growth properties of cells with an intact or defective proline catabolic system (PutBCP) in the presence or absence of proline-containing peptides in media of low or high salinity, we clearly can rule out a nutritional contribution of peptide hydrolysis to the observed osmoprotection by these types of peptides (Fig. 4) under the tested growth conditions (high levels of glucose and ammonium as carbon and

nitrogen sources in a minimal medium, respectively). However, nutritional effects by externally provided proline on the growth of osmotically stressed *B. subtilis* cells were noticeable (Fig. 4).

The effectiveness with which proline-containing peptides confer osmoprotection on *B. subtilis* varies considerably (Fig. 3A). Furthermore, not all of the tested peptides that complement a proline auxotrophic growth requirement (Fig. 2A) also serve as osmoprotectants for *B. subtilis* (Fig. 3A). The most striking examples in this respect are the dipeptides Pro-Gly and Gly-Pro. Pro-Gly is osmoprotective, and its intracellular hydrolysis yields large pools of free proline, whereas Gly-Pro has no osmoprotective properties and there is no buildup of a sizeable proline pool (Fig. 4 and 5). The differences between the efficiency in the use of proline-containing peptides for the purpose of protein biosynthesis and that in their role as osmoprotectants can probably be attributed to different rates of import of these peptides and the efficiency with which the internalized peptides are then hydrolyzed to yield a proline pool large enough for osmostress protection. Indeed, considerable differences in the catalytic efficiency of the *E. coli* PepQ prolidase, an enzyme that hydrolyzes dipeptide substrates with proline residues at the C terminus, have been measured *in vitro* with Xaa-Pro-type peptides as the substrates (70). In addition, the affinity of the *B. subtilis* OppA peptide-binding protein for its ligands also varies notably (71).

We found that peptides of various lengths and compositions can provide osmoprotection to *B. subtilis* (Fig. 3A). The longest peptides that we have tested for their osmoprotective potential are peptides with a length of four amino acids. The upper size limits of the peptides that can be imported via the Opp and App oligopeptide ABC transporters of *B. subtilis* for nutritional purposes are unexplored, but the import of peptides with up to five residues via these systems has been reported (31), and the ligand-binding protein (AppA) of the App transporter can bind nonapeptides (71, 72). The Opp and App uptake systems also reimport different Phr-type penta- and hexapeptides excreted by *B. subtilis* into the growth medium to promote cell-to-cell communication (73) and contribute to the genetic regulation of competence development, sporulation, and extracellular functions in *B. subtilis* (35–38, 74). Our data now ascribe an additional physiological role to the App, Dpp, Opp, and DtpT peptide transporters by implicating them in the indirect acquisition of the osmoprotectant proline (Fig. 1), but since we have not studied the individual involvement of these transporters in peptide import under osmotic-stress conditions, we cannot tell whether there is a preferential use by *B. subtilis* of a particular peptide uptake system under high-osmolarity growth conditions.

In view of the involvement of the peptide transporters and of the PapA and PapB peptidases in the physiology of the acclimatization process of the *B. subtilis* cell to high-salinity surroundings, it is pertinent to ask if their structural genes are upregulated in response to osmotic stress. Judging from the genome-wide transcriptional data set of salt-stressed *B. subtilis* cells recently reported by Nicolas et al. (75), the expression of the *papA* (*yqhT*) and *papB* (*ykvY*) genes is not induced in response to salt or osmotic stress and neither is the expression of those encoding the Dpp, Opp, App, and DtpT (YclF) peptide transport systems. This contrasts with the transcriptional profile of the *proHJ* operon and of the *opuE* gene in the study by Nicolas et al. (75), fully consistent with our previous reports on the strong upregulation of these genes in response to high osmolarity (6, 11, 22). We note in this context that the transcription of the structural genes for the various peptide transporters operating in *B. subtilis* is not upregulated in response to the presence of peptides in the growth medium (31–34, 39). The basal level of expression in low- and high-salinity media is apparently high enough to allow a sufficient level of peptide import.

The hydrolysis of all Xaa-Pro-type dipeptides that we examined was dependent on the activities of the PapA (YqhT) and PapB (YkvY) peptidases, whereas all other peptides that we have tested for their osmoprotective effects possess a Pro residue at their N terminus and are not attacked by the PapA and PapB proline aminopeptidases (Fig. 2 and 6). Hence, the existence of peptidases in *B. subtilis* able to specifically liberate N-terminally positioned Pro residues becomes apparent from our data.

Osmoprotection by proline-containing peptides was recorded not only for strains with defects in the osmoadaptive ProJ-ProA-ProH proline biosynthetic route but also for strains that were proficient in osmoadaptive proline biosynthesis (Fig. 4). In the latter type of strain, the proline pool generated in the presence of hydrolyzable proline-containing peptides was not higher than that attained in their absence (Fig. 7). Since strains with an intact *proHJ* operon have the potential to produce very large proline pools, we cannot tell if the proline pool present in the wild-type strain is made up of a mixture of newly synthesized proline and proline proteolytically released from the imported peptides or whether the proline liberated from proline-containing peptides preferentially represses (perhaps for energetic reasons [76]) the osmotically induced *de novo* production of proline by *B. subtilis*.

The proline content of osmotically stressed cells increases linearly over a wide range of increases in the external osmolarity (6, 23). This behavior implies that a mechanism must exist in *B. subtilis* that prevents the overaccumulation of the compatible solute proline at a given degree of environmentally imposed osmotic stress (23). Indeed, the feeding of proline to osmotically stressed cells does not significantly increase the proline content of the cells (Fig. 7). The physiological or genetic mechanism through which the proline pool is rather precisely tied to the external osmolarity (6, 23), regardless of whether additional free proline is present in the medium or whether additional proline can be liberated from imported peptides (Fig. 7), remains to be discovered.

We made the unusual observation that an external supply of proline provided osmoprotection to a *B. subtilis* strain with a defect in the ProJ-ProA-ProH osmostress-adaptive proline biosynthetic route without causing the expected (11) buildup of a sizeable proline pool. We traced the missing proline pool to the induction (Fig. 8) of the catabolic PutBCP system that degrades proline to glutamate (25, 29) (Fig. 1). Consistent with this finding, the disruption of the PutBCP system improves the osmostress-protective potential of externally provided proline (Fig. 9). Glutamate is synthesized as the sole compatible solute by a number of *Bacillus* species with a moderate capacity to withstand salt stress (7, 77). Possibly, the moderate osmoprotective effects of externally provided proline for a strain possessing an intact ProJ-ProA-ProH system might be exerted by indirectly contributing to the glutamate pool of the *B. subtilis* cells, a molecule that is energetically rather costly to synthesize (26, 27).

Our finding of osmoprotection of *B. subtilis* by an external supply of proline without the concomitant buildup of a proline pool is not without precedent. The compatible solute ectoine serves as an osmoprotectant for *Sinorhizobium meliloti*, but in

contrast to glycine betaine, it is not accumulated by the cells and instead triggers the synthesis of endogenous osmolytes such as glutamate and trehalose (78). As is the case for proline in *B. subtilis* (25), ectoine can be catabolized by *Sinorhizobium meliloti* (79). Apparently, in those cases where a given compatible solute can be used both as an osmoprotectant and as a nutrient, sophisticated physiological and genetic regulatory circuits have to be put in place to avoid cellular confusion about the physiological tasks at hand. We note in this context that, of all known osmoprotectants for *B. subtilis* (14, 17), proline is the only one that also can be used as a nutrient.

The data reported here provide a fresh view on the physiology of *B. subtilis* cells confronted with sustained salt stress (1, 13, 14). Peptides are expected to be present in practically all ecosystems (e.g., the upper layers of the soil, on the surface of plant roots, inside plants, marine environments, the intestines of humans and animals, and the guts of insects) that are inhabited by members of the ubiquitously distributed genus *Bacillus* (80, 81). Hence, the fact that *B. subtilis* can derive osmoprotection through the import and proteolysis of proline-containing peptides is probably of substantial ecological relevance in the varied natural habitats of *B. subtilis*. This process is also likely to help the proliferation of food-spoiling and food-poisoning members of the genus *Bacillus* (e.g., *Bacillus cereus*) (82) to overcome commonly employed preservation measures such as the salting of food ingredients.

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