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Uptake of Amino Acids and Their Metabolic Conversion into the Compatible Solute Proline Confers Osmoprotection to *Bacillus subtilis*

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The data presented here reveal a new facet of the physiological adjustment processes through which *Bacillus subtilis* can derive osmostress protection. We found that the import of proteogenic (Glu, Gln, Asp, Asn, and Arg) and of nonproteogenic (Orn and Cit) amino acids and their metabolic conversion into proline enhances growth under otherwise osmotically unfavorable conditions. Osmoprotection by amino acids depends on the functioning of the ProJ-ProA-ProH enzymes, but different entry points into this biosynthetic route are used by different amino acids to finally yield the compatible solute proline. Glu, Gln, Asp, and Asn are used to replenish the cellular pool of glutamate, the precursor for proline production, whereas Arg, Orn, and Cit are converted into γ-glutamic semialdehyde/Δ^1-pyrroline-5-carboxylate, an intermediate in proline biosynthesis. The import of Glu, Gln, Asp, Asn, Arg, Orn, and Cit did not lead to a further increase in the size of the proline pool that is already present in osmotically stressed cells. Hence, our data suggest that osmoprotection of *B. subtilis* by this group of amino acids rests on the savings in biosynthetic building blocks and energy that would otherwise have to be devoted either to the synthesis of the proline precursor glutamate or of proline itself. Since glutamate is the direct biosynthetic precursor for proline, we studied its uptake and found that GltT, an Na^+-coupled symporter, is the main uptake system for both glutamate and aspartate in *B. subtilis*. Collectively, our data show how effectively *B. subtilis* can exploit environmental resources to derive osmotic-stress protection through physiological means.

Bacillus subtilis is a resident of the upper layers of the soil and of the rhizosphere, and it can also efficiently colonize root surfaces (1–3). The blueprint of its genome (4) bears the hallmarks of a bacterium that can exploit many plant-produced compounds for its growth. Accordingly, a considerable portion of the coding capacity of the *B. subtilis* chromosome (5) is devoted to high-affinity import systems (6) that allow the scavenging of a wide spectrum of nutrients. Reoccurring and persisting high osmolarity in the soil ecosystem (7) is a situation in which *B. subtilis* can take advantage of the import of plant-produced compounds (8–10) for its physiological adjustment to these unfavorable environmental conditions (7, 11).

As in many bacterial species (11–13), cellular adaptation of *B. subtilis* to both sudden and sustained increases in the external osmolarity involves a two-stage process (7, 14). It initially encompasses the uptake of large quantities of potassium as an emergency stress reaction to curb water efflux (14, 15) and, subsequently, the replacement of part of this ion by organic osmolytes, such as proline (Pro) and glycine betaine (GB), to decrease the ionic strength of the cytoplasm and to optimize its solvent properties (14, 16–19). These organic osmolytes, commonly referred to as compatible solutes, are highly compliant with cellular physiology and biochemistry (7, 12, 14). They are brought into the soil ecosystem through root exudates, decaying plant material, and osmotically downshocked or disintegrated microbial cells (8–10, 20). *B. subtilis* cells exposed to high salinity can capture them via osmotically inducible high-affinity transport systems to derive osmostress protection (7, 16).

Proline is the only compatible solute that *B. subtilis* can synthesize *de novo* (14), a process that is mediated through the ProJ-ProA-ProH biosynthetic route (18, 21) (Fig. 1). High-level production of proline is achieved through the osmotic induction of *proHJ* transcription (18) and probably through reduced feedback control (22, 23) of the activity of the ProJ enzyme by proline. Together, these two events lead to the buildup of cellular proline pools whose size is linearly related to the osmolarity prevalent in the environment at a given time (17, 18); they can reach values of about 500 mM in severely osmotically stressed cells (e.g., after growth in a minimal medium with 1.2 M NaCl) (17, 24). The genetic disruption of the osmostress-adaptive proline-biosynthetic route causes osmotic sensitivity of *B. subtilis* (18), highlighting the central role of compatible-solute accumulation in the cellular adjustment to high-osmolarity environments (7, 11). Relief from osmostress can also be accomplished through import of proline via the osmotically inducible OpuE transporter (25, 26), a system that is also involved in the recovery of newly synthesized proline that leaks, or is actively excreted, into the medium by *B. subtilis* cells continuously challenged by high osmolarity (27). Of all the compatible solutes imported by *B. subtilis* (7, 28, 29), proline is the only one that can also be catabolized (30) (Fig. 1), and

Received 26 August 2014 **Accepted** 15 October 2014

Accepted manuscript posted online 24 October 2014

Citation Zaprasis A, Bleisteiner M, Kerres A, Hoffmann T, Bremer E. 2015. Uptake of amino acids and their metabolic conversion into the compatible solute proline confers osmoprotection to *Bacillus subtilis*. Appl Environ Microbiol 81:250–259. doi:10.1128/AEM.02797-14.

Editor: A. M. Spormann

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02797-14.

doi:10.1128/AEM.02797-14

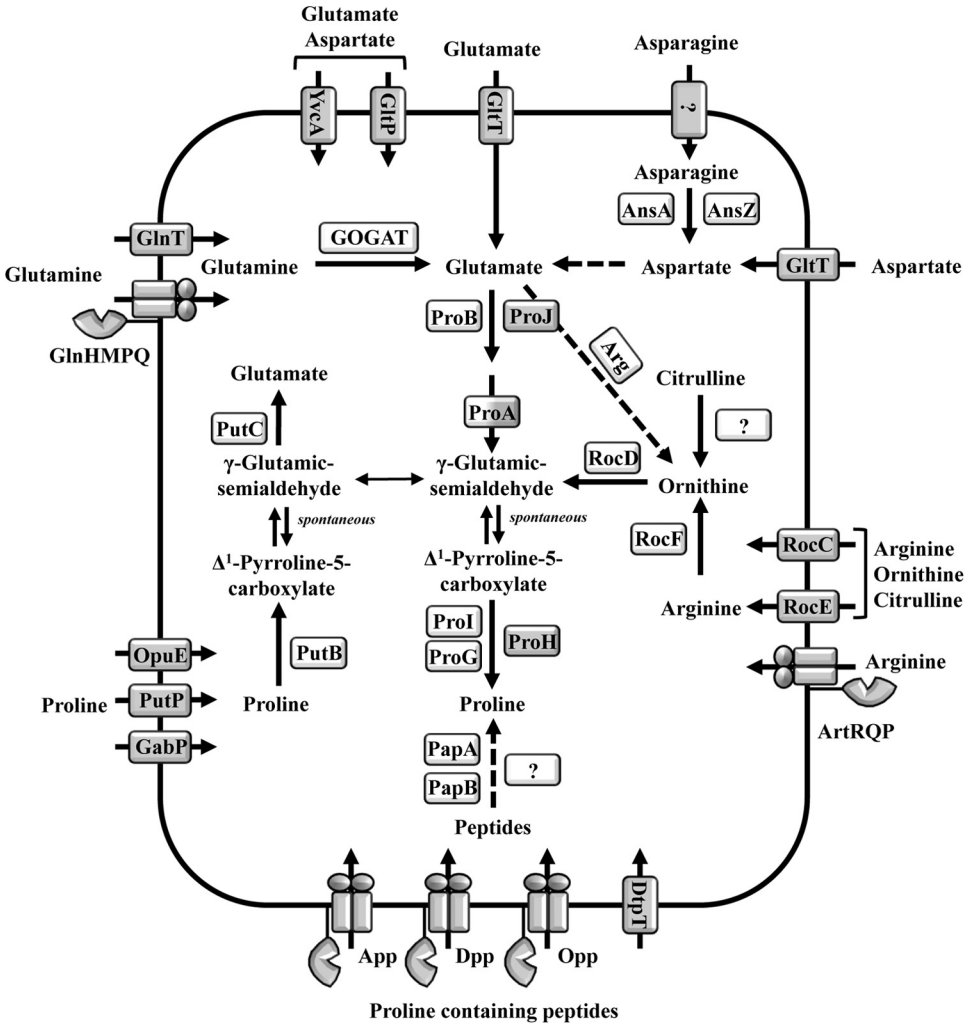

FIG 1 Overview of the uptake, synthesis, catabolism, and generation of L-proline through the metabolic conversion of several proteogenic and nonproteogenic amino acids by *B. subtilis*. The dashed arrows indicate that several enzymes are involved in the indicated steps.

this limits the effectiveness of an external supply of proline as an osmostress protectant in comparison to metabolically inert compatible solutes, such as glycine betaine or proline betaine (16, 24, 29). Curiously, induction of the proline-catabolic *putBCP* operon occurs only when the amino acid is present in the growth medium but not by the large amount of proline that is accumulated inside the cell via *de novo* synthesis under osmotic-stress conditions (24, 30). *B. subtilis* can also exploit proline-containing peptides of different lengths and compositions as osmoprotectants through uptake and their subsequent intracellular hydrolysis to yield free proline (24) (Fig. 1).

Here, we demonstrate that *B. subtilis* can achieve osmoprotection by proline in yet another way. We found that the cell imports a restricted set of proteinogenic and nonproteinogenic amino acids, all of which are present in the rhizosphere and in the soil (8–10), and subsequently converts them either into the proline-biosynthetic precursor glutamate (Glu) (31, 32) or into the intermediate in proline biosynthesis, γ-glutamic semialdehyde/Δ^1-pyrroline-5-carboxylate (33, 34) (Fig. 1). Both processes involve the previously identified osmostress-adaptive ProJ-ProA-ProH proline-biosynthetic enzymes (18), but in a way that allows the preservation of precious building blocks and biosynthetic resources.

MATERIALS AND METHODS

Chemicals. Amino acids, the ninhydrin reagent for the quantification of proline by a colorimetric assay (35), and the antibiotics chloramphenicol, kanamycin, tetracycline, and spectinomycin were all purchased from Sigma-Aldrich (Steinheim, Germany) or from Carl Roth GmbH (Karlsruhe, Germany). Radiolabeled L-[U-^{14}C]proline (269 mCi mmol^{-1}), L-[U-^{14}C]glutamate (253 mCi mmol^{-1}), and L-[U-^{14}C]aspartate (200 mCi mmol^{-1}) were purchased from GE Healthcare Lifesciences (Munich, Germany); L-[U-^{14}C]arginine (274.3 mCi mmol^{-1}) was purchased from PerkinElmer (Rodgau, Germany).

Bacterial strains, media, and growth conditions. The genetic properties of the *B. subtilis* strains used in this study are summarized in Table 1; they are all derived from the laboratory strain JH642 (36, 37). *B. subtilis* strains were routinely cultivated in Spizizen's minimal medium (SMM) (38) 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 requirements of strain JH642 (*trpC2 pheA1*) and its mutant derivatives (Table 1). A solution of trace elements was added to SMM to improve the growth of *B. subtilis* strains (38). The osmolarity of growth

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

Strain[a]	Relevant genotype	Source or reference
JH642	*trpC2 pheA1*	36
JSB8	Δ(*proHJ*::*tet*)*1*	21
GWB120[b]	Δ(*proBA*::*cat*)*2* SigA-P1; *rocDEF* promoter	33
ABB1	Δ(*putBCP*::*tet*)*2* Δ(*proHJ*::*neo*)*1*	24
ADB1	(*gltP*::*neo*)*1*	This study
ADB4	(*gltT* × pADK5)*1* (*spc*)[c]	This study
MDB43	Δ(*yveA*::*neo*)*1*	This study
MDB52	(*gltP*::*cat*)*2*	This study
MDB53	(*gltP*::*cat*)*2* Δ(*yveA*::*neo*)*1*	This study
MDB54	(*gltP*::*cat*)*2* Δ(*yveA*::*neo*)*1* (*gltT* × pUS19)*1* (*spc*)	This study

[a] All the strains are derivatives of the *B. subtilis* domesticated laboratory strain JH642 (37) and therefore carry the *trpC2 pheA1* mutations, in addition to the indicated genetic markers.

[b] The strain carries a mutation that activates a SigA-type cryptic promoter in front of the *rocDEF* operon, leading to enhanced transcription of the gene cluster in the absence of its natural RocR-dependent inducers (33).

[c] The pUS19-derived plasmid pADK5 is inserted into *gltT* via a single recombination event, thereby disrupting the integrity and function of that gene.

media was adjusted by adding NaCl from a 5 M stock solution. All *B. subtilis* cultures were inoculated from exponentially growing precultures in prewarmed (37°C) SMM to optical densities at 578 nm (OD_{578}) of about 0.1, and the cultures were then propagated at 37°C in a shaking water bath set to 220 rpm; 100-ml shake flasks filled with 20-ml medium were used for these experiments. When the use of glutamate and aspartate (Asp) by *B. subtilis* as sole nitrogen sources was assessed, the ammonium source [$(NH_4)_2SO_4$; 15 mM] was replaced by various concentrations of either L-Glu or L-Asp in preparing the SMM.

Construction of bacterial strains. The *gltP* gene was amplified by PCR from chromosomal DNA of strain JH642, and the resulting 1,079-bp DNA fragment was cloned into the EcoRI-ClaI sites of plasmid pBSK(−) (Stratagene), yielding pADK1. A kanamycin resistance cassette (*neo*), derived from plasmid pDG783 (39), was inserted into the HindIII site present in the coding region of *gltP*. DNA of the resulting plasmid, pADK2, was linearized by cleaving with SacII and ClaI and used to transform strain JH642 and to subsequently select for kanamycin-resistant colonies; the resulting strain was ADB1 (Table 1). A second mutant allele of *gltP* was constructed by removing the *neo* kanamycin resistance cassette from pADK2 via HindIII digestion and replacing it with a chloramphenicol resistance cassette (*cat*) derived via PCR from plasmid pJMB1 (M. Jebbar and E. Bremer, unpublished data); this yielded plasmid pMD27. DNA of plasmid pMD27 was linearized by cleaving with XhoI and ScaI and transformed into strain JH642, yielding strain MDB52 (Table 1). To construct a *gltT* mutant, the *gltT* gene was amplified from chromosomal DNA of strain JH642 by PCR, and the resulting DNA fragment was digested with PstI and XmnI. The 711-bp PstI-XmnI DNA fragment internal to the *gltT* coding region was then cloned into the PstI and SmaI sites of plasmid pUS19 (40) carrying a spectinomycin resistance determinant; this yielded plasmid pADK5. Plasmid pUS19 cannot replicate in *B. subtilis* (40); hence, transformation of pADK5 into strain JH642 results in its chromosomal integration via a single-crossover event that leads to the disruption of the *gltT* gene (strain ADB4) (Table 1). The *yveA*::*neo* mutation (41) was moved by transformation of chromosomal DNA obtained from the *B. subtilis* 168 genetic background into JH642, yielding strain MDB43 (Table 1). Combinations of the *gltP*, *gltT*, and *yveA* mutant alleles were obtained by transformation with chromosomal DNA prepared from appropriate *B. subtilis* donor strains (Table 1).

Northern blot analysis. Cells of the *B. subtilis* wild-type strain JH642 were gown in SMM in the absence or the presence of 1.2 M NaCl until the cultures reached an OD_{578} of 0.8 to 1.0. After harvesting of the cells by centrifugation, total RNA was prepared using the acidic phenol method (42). Total RNA (10 μg) was separated according to size on a 1.4% agarose gel, transferred to a Schleicher & Schuell NY13N membrane, and hybridized with a digoxigenin-labeled single-stranded antisense RNA probe specific for *gltT*. The *gltT* antisense RNA probe covered 575 bp of the *gltT* gene and was produced by *in vitro* transcription of a corresponding *gltT* PCR fragment that carried an artificial T7 promoter sequence on one of its ends and that was introduced by the reversed DNA primer used. The PCR product was amplified using the primers gltT5-ADK1 (5′-GCCATTATT CTCGGACTAGCCC-3′) and gltT3-ADK2 (5′-TCCATGATACGCGGA AGAACCG-3′). The procedures to synthesize *in vitro* the *gltT*-specific antisense RNA probe, its labeling with digoxigenin, the detection of the *gltT* mRNA with a Roche digoxigenin kit (Roche Diagnostics GmbH, Mannheim, Germany), and the fluorophore substrate ECF (GE Healthcare Lifesciences, Munich, Germany) have been described previously (18).

Measurements of intracellular proline pools. The intracellular proline content of *B. subtilis* cells was determined by a colorimetric assay that detects L-proline as a colored proline-ninhydrin complex that can be quantified by measuring the absorption of the solution at 480 nm (35). For these assays, the *B. subtilis* cells were grown in SMM with 1.2 M NaCl in the absence or presence of various amino acids until they reached an OD_{578} of about 1.8 to 2.0; the harvesting and processing of the cells, the details of the assay conditions, and the calculation of the intracellular volume of *B. subtilis* cells have been described previously (17, 24).

Tracing the metabolic conversion of glutamate, aspartate, and arginine (Arg) into proline in osmotically stressed *B. subtilis* cells. The *B. subtilis* strain JH642 was grown in minimal medium in the presence of 1.2 M NaCl. When the culture had reached the early exponential growth phase (OD_{578}, about 0.5), it was aliquoted (8-ml culture volume each) into four portions in prewarmed 100-ml Erlenmeyer flasks. The radiolabeled amino acids L-[U-^{14}C]glutamate, L-[U-^{14}C]aspartate, L-[U-^{14}C]arginine, and L-[U-^{14}C]proline were then added to the individual subcultures to a final concentration of 20 μM, corresponding to a specific radioactivity of 15 mCi per μmol. The cells were then incubated with shaking at 37°C, and 500-μl samples were withdrawn from each culture at 30-min intervals starting immediately after addition of the radiolabeled amino acid. The samples were harvested by centrifugation for 5 min at 13,000 rpm at room temperature in an Eppendorf tabletop centrifuge. The cell pellets were resuspended in 50 μl TE-lysozyme mixture (50 mM Tris-HCl, pH 8.0, 3 mg ml^{-1} lysozyme). After 15 min of incubation at 37°C, cell lysis was completed by the addition of 2 μl SDS (10%). Cell debris was removed by centrifugation for 10 min at 13,000 rpm and 4°C in an Eppendorf tabletop centrifuge. To separate the solutes of the cell extracts, 10-μl aliquots of the supernatants were spotted onto a 0.2-mm silica gel plate (Polygram Sil G; Macheray-Nagel, Düren, Germany) with a 20-cm path length. Separation of the compounds in the extracts was achieved overnight at room temperature with an acetone–*n*-butyl alcohol–glacial acetic acid–water (35:35:10:20) mixture as the mobile phase. Aliquots (1 μl) of L-[U-^{14}C]glutamate, L-[U-^{14}C]aspartate, L-[U-^{14}C]arginine, and L-[U-^{14}C]proline (50 nCi μl^{-1}) were run as standards in parallel with the samples derived from the cell extracts. After a 3- to 5-day exposure of the dried silica gel plates to a phosphor screen, radiolabeled compounds were detected with a Storm 860 phosphorimager (Amersham Biosciences, Freiburg, Germany).

Uptake of radiolabeled glutamate and aspartate by *B. subtilis*. Uptake rates for radiolabeled L-[U-^{14}C]glutamate and L-[U-^{14}C]aspartate were compared in cells of the *B. subtilis* wild-type strain JH642 and mutants derived from this strain that carried gene disruptions either in *gltT* (strain ADB4), in *gltP* (strain ADB1), or in *yveA* (strain MDB43) (Table 1). The cultures were grown in SMM with glucose as the carbon source to early exponential growth phase (OD_{578}, 0.5 to 0.7), and glutamate or aspartate spiked with radiolabeled L-[U-^{14}C]glutamate or L-[U-^{14}C]aspartate (specific activity for both solutes, 2.25 nCi $nmol^{-1}$) was added to 2-ml aliquots of the cultures. Uptake of these amino acids was followed at 30-s intervals by measuring the radioactivity accumulated by the cells as

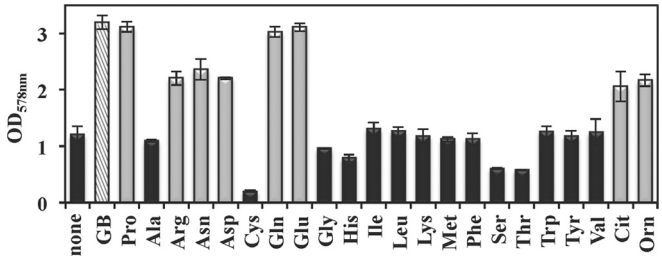

FIG 2 Effects of amino acids on the growth of *B. subtilis* at high osmolarity. Cultures of the *B. subtilis* wild-type strain JH642 were grown in SMM with 1.2 M NaCl in the absence or presence of individual proteogenic and nonproteogenic amino acids (final concentration, 1 mM). The growth yields of the cultures were determined after 16 h of incubation at 37°C by measuring the OD_{578}. The values shown represent the means of two independently grown cultures, and the error bars indicate standard deviations. The gray bars represent the amino acids that promote growth of *B. subtilis* at high salinity. The osmoprotectant GB (hatched bar) was used as a control for these experiments.

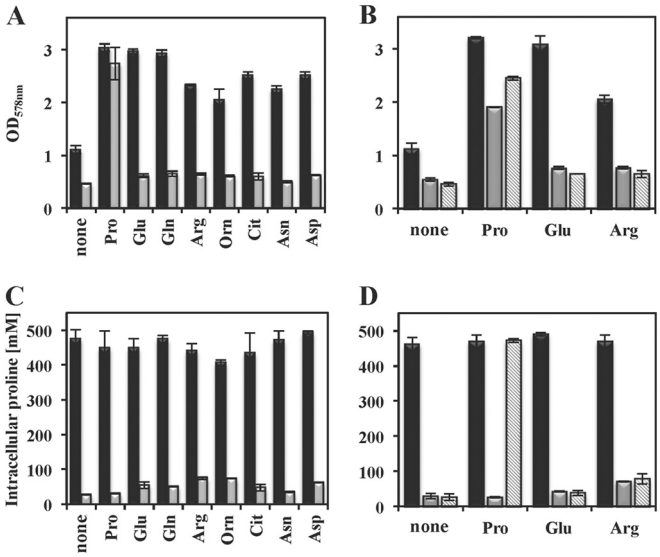

FIG 3 Osmoprotection by amino acids depends on the osmostress-adaptive proline-biosynthetic route. (A) Cultures of the *B. subtilis* wild-type strain JH642 (black bars) and of strain JSB8 [Δ(*proHJ*::*tet*)*1*] (gray bars) were grown in SMM with 1.2 M NaCl in the absence or presence of individual proteogenic and nonproteogenic amino acids (final concentration, 1 mM). The growth yields of the cultures were determined after 16 h of incubation at 37°C by measuring the OD_{578}. (B) Influence of the PutBCP catabolic system on the levels of osmostress protection by proline, glutamate, and arginine. The following strains were used: JH642 (black bars), JSB8 [Δ(*proHJ*::*tet*)*1*] (gray bars), and ABB1 [Δ(*proHJ*::*tet*)*1* Δ(*putBCP*::*tet*)*2*] (hatched bars). (C) Proline content of osmotically stressed cells. Cells grown in SMM containing 1.2 M NaCl were harvested once they reached an OD_{578} of about 1.8 and were then assayed for their intracellular L-proline pools. The following strains were used: JH642 (black bars) and JSB8 [Δ(*proHJ*::*tet*)*1*] (gray bars). (D) Influence of the PutBCP catabolic system on the buildup of intracellular proline pools. The following strains (grown as described above) were used: JH642 (black bars), JSB8 [Δ(*proHJ*::*tet*)*1*] (gray bars), and ABB1 [Δ(*proHJ*::*tet*)*1* Δ(*putBCP*::*tet*)*2*] (hatched bars). (C and D) The values shown represent the means of two independently grown *B. subtilis* cultures, and in each of these samples, the L-proline content was determined twice; the error bars indicate standard deviations.

described previously (43). To assess the kinetic parameters of the GltT transporter for glutamate and aspartate, the substrate concentrations were varied between 2 μM and 100 μM and the uptake rates were determined. The Michaelis-Menten kinetics was deduced by comparing the uptake velocities in relation to the substrate concentration in the uptake assays. Analysis and fitting of the transport data were performed using the GraphPad Prism 5 software (Graphpad Software, Inc., La Jolla, CA, USA). Three independent measurements were performed to determine the K_m and V_{max} values of the GltT carrier for its substrates, Glu and Asp.

Internet resources for *B. subtilis* and modeling of the GltT structure. The functional annotation of *B. subtilis* genes and their transcriptional profile measured across a comprehensive set of growth conditions (44) were assessed through *Subti*Wiki (http://subtiwiki.uni-goettingen.de/wiki/index.php/Main_Page) (45). The modeling of the *B. subtilis* GltT structure was carried out with resources provided by the I-TASSER protein structure prediction platform (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (46). Graphic representations of the retrieved *in silico* GltT model were prepared using the PyMOL software package (http://www.pymol.org).

RESULTS

Osmostress protection by amino acids. Both proteogenic and nonproteogenic amino acids can be found in the soil (8, 10), and they exhibit a high degree of turnover, a process to which microorganisms contribute substantially (47). We wondered whether the uptake of amino acids other than proline (25, 26) would aid *B. subtilis* in its process of adjustment to high-salinity surroundings. To test this idea, we grew the wild-type laboratory strain JH642 (Table 1) in a chemically defined minimal medium (SMM) containing 1.2 M NaCl in the absence or presence of individual proteogenic amino acids and the nonproteogenic amino acids ornithine (Orn) and citrulline (Cit). As expected, the osmostress protectants GB (16) and Pro (24, 25) enhance growth at high salinity (Fig. 2), but osmostress protection was also afforded by Glu, glutamine (Gln), Asp, asparagine (Asn), and Arg and by both Orn and Cit (Fig. 2).

Osmostress protection by amino acids is dependent on the ProJ-ProA-ProH proline-biosynthetic route. The proline and arginine synthesis and degradation routes of *B. subtilis* are highly interconnected (32, 34, 48, 49). By consulting descriptions of the appropriate synthesis and metabolic pathways and uptake systems in the literature (6, 34, 48, 49) (Fig. 1 shows a summary), it became apparent that each of the osmostress-relieving amino acids identified above could be converted into proline, whereas this is not the case for each of the amino acids that did not confer osmostress protection. Hence, it seemed possible that Glu, Gln, Asp, Asn, Arg, Orn, and Cit were not osmostress protectants *per se* but instead served as resources for proline synthesis (Fig. 1).

The key players in the osmostress-adaptive synthesis of proline from the precursor glutamate by *B. subtilis* are the ProJ-ProA-ProH enzymes (18, 21, 31) (Fig. 1). We therefore used a Δ*proHJ* mutant that is unable to synthesize osmostress-protective levels of proline to experimentally verify the anticipated roles of Glu, Gln, Asp, Asn, Arg, Orn, and Cit as precursors for osmoprotective proline biosynthesis. Except for Pro itself (25, 26), osmostress protection by each of these amino acids was lost when the ProJ-ProH enzymes were nonoperational (Fig. 3A). This was caused by the failure of the cells to build up the large proline pool required for adjustment to high external salinity (33) (Fig. 3C). Hence, the osmostress potentials of the seven tested amino acids were indeed dependent on their metabolic conversion into proline (Fig. 1).

It is apparent from the interconnected proline and arginine synthesis and degradation routes in *B. subtilis* (34, 48) that stress relief by Glu, Gln, Asn, and Asp should be dependent on a functional γ-glutamyl-phosphate reductase (ProA) (Fig. 1). In con-

trast, osmostress protection afforded by Arg, Orn, and Cit should be independent of the functioning of the ProA enzyme, since these amino acids can be metabolized to γ-glutamic semialdehyde/Δ^1-pyrroline-5-carboxylate, a process that requires the activity of the ornithine aminotransferase (RocD) (33, 34). This intermediate can be fed into the proline synthesis pathway at an intersection subsequent to the ProA-catalyzed step (Fig. 1).

To substantiate this hypothesis, we used growth experiments with the *B. subtilis* strain GWB120, a mutant that is defective in the *proBA* genes and simultaneously carries a promoter mutation that allows enhanced *rocDEF* transcription in the absence of its natural RocR-dependent inducers (33). We note in this context that in this strain the function of ProB can be substituted for by its paralogue ProJ (Fig. 1); however, only a single ProA-type enzyme is present in *B. subtilis* (18, 21, 31). Fully consistent with the working hypothesis outlined above, osmostress protection by Glu, Gln, Asn, and Asp was dependent on a functional ProA protein, whereas that afforded by Arg, Orn, and Cit was not (see Fig. S1 in the supplemental material). Consequently, osmostress protection by Glu, Gln, Asp, and Asn on one hand and Arg, Orn, and Cit on the other hand is dependent on different entry points into the osmostress-adaptive ProJ-ProA-ProH proline biosynthesis route (Fig. 1).

As noted previously (24), externally provided proline confers osmostress protection (Fig. 3A), but surprisingly, the cells do not build up a sizable proline pool in a Δ*proHJ* mutant (18) (Fig. 3C). This phenomenon is not fully understood but is related to proline catabolism via the PutBCP system, a proline import (PutP) and degradation (PutBC) route that can be transcriptionally induced by externally provided proline but not by proline synthesized by the cell (24, 30, 50). Accordingly, the disruption of the PutBCP system in a Δ*proHJ* genetic mutant background led to the buildup of a very large proline pool in osmotically stressed cells that were grown in the presence of 1 mM proline in the growth medium. The size of this proline pool matched that of the pool formed through *de novo* synthesis in the wild-type strain in the absence of an external proline supply (Fig. 3D).

Influence of the uptake of osmostress-protective amino acids on the size of the intracellular proline pool. We wondered whether growth of the *B. subtilis* cells under high-salinity conditions in the presence of the osmostress protectants Glu, Gln, Asp, Asn, Arg, Orn, and Cit (Fig. 3A) would increase the cellular proline pool size and would thereby enhance growth under osmotically unfavorable conditions. However, this was not the case. When presented with an osmostress-protective amino acid, the cells accumulated the same amount of proline as cells provided with no amino acid (Fig. 3C). Consequently, the question arose as to how osmostress protection by Glu, Gln, Asp, Asn, Arg, Orn, and Cit is achieved by *B. subtilis*.

Tracing the metabolic conversion of osmostress-protective amino acids into proline. Synthesis of the direct proline precursor glutamate (Fig. 1) is energetically demanding (32, 34), and osmotically stressed *B. subtilis* cells successively drain the substantial glutamate pool (14), concomitant with the onset of enhanced proline production (18). It thus seems possible that the cells would save energy and biosynthetic building blocks for the *de novo* production of glutamate (32, 51) either by importing it directly or by metabolically converting exogenously provided Gln, Asp, and Asn into glutamate (Fig. 1). Conversely, Arg, Orn, and Cit could exert their osmostress-protective effects through their conversion into γ-glutamic semialdehyde/Δ^1-pyrroline-5-carboxylate through the metabolic activities of the RocDEF enzymes (Fig. 1) (34, 48, 52).

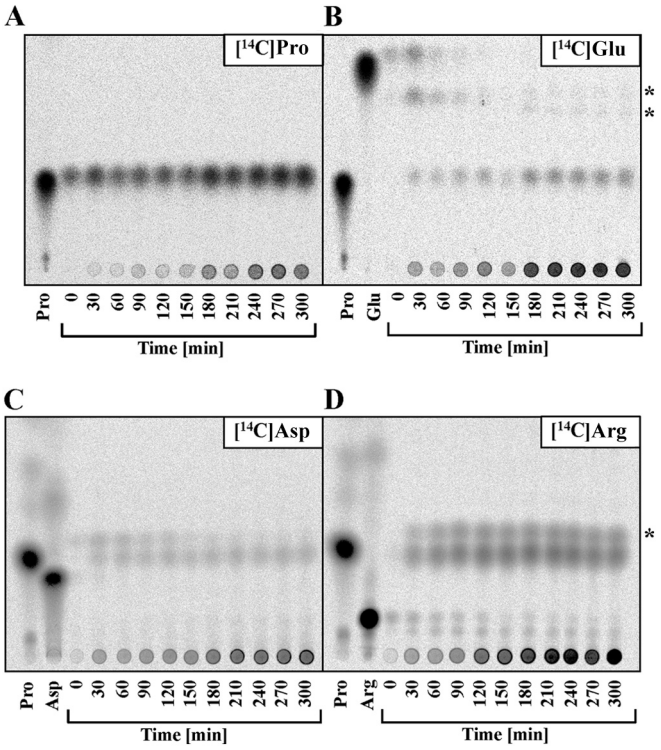

FIG 4 Conversion of Glu, Asp, and Arg into Pro in *B. subtilis* wild-type cells growing at high salinity. Cultures of the *B. subtilis* strain JH642 were grown in SMM with 1.2 M NaCl. When the cultures reached an OD_{578} of 0.5, L-[U-^{14}C]proline (A), L-[U-^{14}C]glutamate (B), L-[U-^{14}C]aspartate (C), and L-[U-^{14}C]arginine (D) were separately added to the cells (final concentration, 20 μM). Cell samples were withdrawn at the indicated time points, and the cells were harvested by centrifugation. Soluble extracts of the cell pellets were prepared and separated by thin-layer chromatography; spots corresponding to Pro, Glu, Asn, and Arg were identified through comigrating radiolabeled reference standards. Unidentified radiolabeled compounds, in all likelihood metabolic intermediates of the imported amino acids, are indicated by asterisks.

To follow the anticipated metabolic conversion of these amino acids into proline, we separately fed radiolabeled Pro, Glu, Asp, and Arg (final concentration of each amino acid, 20 μM) to cells that were grown in SMM with 1.2 M NaCl. We harvested the cells at 30-min intervals over a time span of 5 h and then separated the soluble fraction of cell extracts by thin-layer chromatography, along with appropriate radiolabeled reference standards. L-[U-^{14}C]Pro was accumulated in unmodified form (Fig. 4A), whereas L-[U-^{14}C]Glu, L-[U-^{14}C]Asp, and L-[U-^{14}C]Arg were taken up by the cells and were then metabolically converted into proline (Fig. 4B, C, and D). These metabolic-labeling experiments are thus consistent with the working hypothesis outlined above, which invokes the metabolic conversion of osmostress-protective amino acids into the compatible solute proline. We note that in each of the experiments, part of the imported radiolabeld amino acid was incorporated into cellular material that did not migrate in the thin-layer chromatography, and the amount of this material increased over time (Fig. 5). At least part of the material might be newly synthesized proteins.

GltT-mediated import of glutamate and aspartate. Since glu-

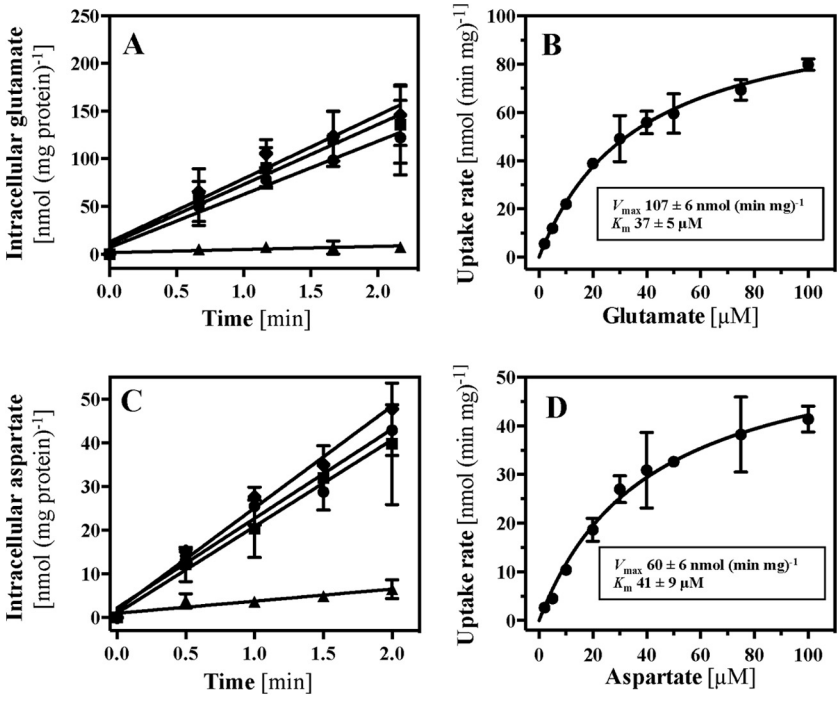

FIG 5 Uptake of radiolabeled glutamate and aspartate by *B. subtilis* and its *gltP*, *gltT*, and *yveA* mutant derivatives. (A and C) Strains JH642 (wild type) (●), ADB1 (*gltP*) (■), ADB4 (*gltT*) (▲), and MDB43 (*yveA*) (◆) were grown in SMM, and the initial uptake of L-[U-^{14}C]glutamate (A) and L-[U-^{14}C]aspartate (C) was measured at a final substrate concentration of 20 μM. (B and D) Michaelis-Menten kinetics were deduced from uptake rates for the GltT substrates L-[U-^{14}C]glutamate (B) and L-[U-^{14}C]aspartate (D) in strain MDB53, which possesses an intact GltT system but is defective in the GltP and YveA transporters.

tamate is the direct precursor for the synthesis of proline (32, 34, 48), we wondered how the amino acid is imported by *B. subtilis*. The GltP and YveA transporters have been implicated in glutamate uptake by *B. subtilis*, but the process is not satisfactorily understood. GltP, a glutamate/aspartate H^+ symporter, has been cloned by functional complementation in *Escherichia coli*, and the purified protein has also been reconstituted in membrane vesicles (53); however, its physiological and transport properties have not been directly assessed in *B. subtilis* through mutant analysis. YveA, a member of the amino acid/polyamine/organocation (APC) superfamily, has been implicated in glutamate uptake, but the YveA transporter is not substrate specific, since it mediates the import of a broad range of amino acids, including that of aspartate (41). A third glutamate/aspartate transporter, GltT, is predicted from the *B. subtilis* genome sequence (5, 6), but its physiological contribution to glutamate import has not been studied. The amino acid sequence of this putative glutamate/aspartate Na^+ symporter (6) is related to biochemically studied GltT homologs from *Bacillus caldotenax* and *Bacillus stearothermophilus* (54, 55), and it possesses overall degrees of amino acid sequence identity of 65% and 66%, respectively, to these proteins.

To characterize the contribution of the GltP, GltT, and YveA transporters to glutamate uptake, we constructed an isogenic set of *B. subtilis* mutant strains in which the corresponding genes were separately disrupted. We then measured the initial uptake of radiolabeled glutamate at a low substrate concentration (20 μM) in cells that were grown at 37°C in SMM. Loss of the GltP and YveA systems did not affect the uptake of glutamate, whereas the inactivation of GltT practically abrogated its import (Fig. 5A). Since the GltP, YveA, and GltT transporters are also potential uptake systems for aspartate (41, 53), we tested the uptake of radiolabeled aspartate in the same set of strains and found that only the disruption of *gltT* affected its uptake (Fig. 5C). Hence, GltT is an important uptake system for Glu and Asp when they are present at low (μM) external substrate concentrations and presented to *B. subtilis* cells cultured to early exponential phase in a minimal medium with glucose as the carbon and $(NH_4)_2SO_4$ as the nitrogen source.

We determined the kinetic parameters of GltT for glutamate and aspartate in a strain (MDB53) that was simultaneously defective in the GltP and YveA transporters by varying the substrate concentration between 2 μM and 100 μM. GltT-mediated import of glutamate and aspartate exhibited Michaelis-Menten kinetics, and the affinities of the transporter for the two substrates were very similar, with K_m values of 37 ± 5 μM for glutamate and 41 ± 9 μM for aspartate (Fig. 5B and D). GltT is not only a high-affinity transporter, it also possesses a substantial transport capacity, with V_{max} values of 107 ± 6 nmol (min mg protein)$^{-1}$ for glutamate and 60 ± 6 nmol (min mg protein)$^{-1}$ for aspartate (Fig. 5B and D).

The *B. subtilis* GltT protein is related to the structurally characterized Glt_{Ph} and Glt_{Tk} proteins from the archaea *Pyrococcus horikoshii* (56) and *Thermococcus kodakarensis* (57), with an overall degree of amino acid sequence identity of approximately 32% (see Fig. S2 in the supplemental material). The last two proteins are aspartate transporters, and the residues that have been implicated in substrate binding are for the most part functionally conserved in the *B. subtilis* GltT protein (see Fig. S2 in the supplemental material). The Glt_{Ph} and Glt_{Tk} proteins are trimers, with each monomer possessing structurally separable transport and trimerization domains (56–59). A modeling study of the *B. subtilis* GltT protein using the I-TASSER Web server resources (46) auto-

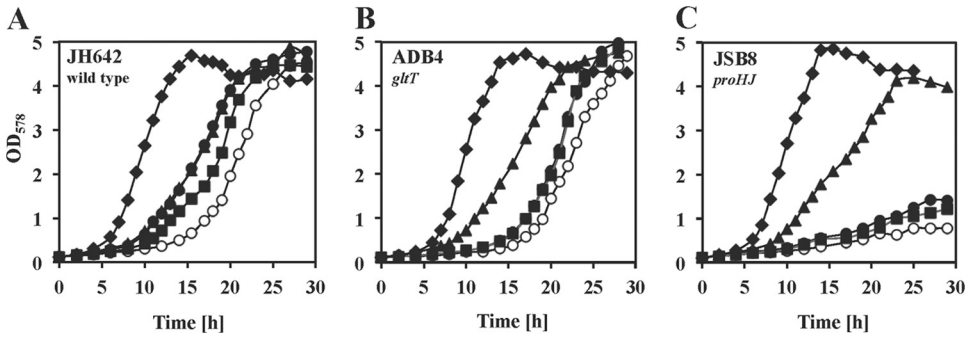

FIG 6 Osmostress protection of *B. subtilis* by glutamate and aspartate is dependent on the GltT transporter and the functioning of the ProHJ proline-biosynthetic system. Cells of the wild-type strain JH642 (A), strain ADB4 (*gltT* × pUS19) (B), and strain JSB8 [Δ(*proHJ*::*tet*)*1*] (C) were grown at high salinity (SMM with 1.2 M NaCl) in the absence (○) or the presence of glutamate (●), aspartate (■), proline (▲), and glycine betaine (◆); the final concentration of these compounds in the growth medium was 1 mM.

matically chose the *P. horikoshii* Glt_{Ph} crystal structure containing the aspartate ligand (Protein Data Bank [PDB; http://www.rcsb.org/pdb/home/home.do] accession code 2NWX) (56) as an appropriate template. In Fig. S3 in the supplemental material, we show an overlay of a GltT monomer with one protomer of the Glt_{Ph} trimeric crystal structure. This *in silico* model suggests that the overall fold of the *B. subtilis* GltT protein resembles that of Glt_{Ph} and Glt_{Tk} (56–59), with structurally distinct transport and trimerization domains.

GltT is involved in osmostress protection afforded by glutamate and aspartate. Having identified GltT as a system for glutamate and aspartate in exponentially growing *B. subtilis* cells in a minimal medium with glucose as the carbon source, we studied the role of GltT in glutamate- and aspartate-mediated osmostress protection. For these experiments, we conducted growth studies in SMM containing 1.2 M NaCl and compared the performance of these osmoprotectants with that of the compatible solutes glycine betaine (16) and proline (26, 33). Glutamate and aspartate exhibited an osmostress-protective potential similar to that afforded by proline in the wild-type strain JH642, but glycine betaine was a considerably better osmoprotectant than these compounds (Fig. 6A). In a *gltT* mutant strain (ADB4), osmoprotection by glutamate and aspartate was significantly reduced, and the growth of these cultures resembled that of a culture that had not received any osmostress protectant. In contrast, the osmoprotective effects of proline and glycine betaine were not affected by a *gltT* mutation (Fig. 6B). As expected from the data presented above, osmoprotection by glutamate and aspartate was abolished in the *proHJ* mutant strain JSB8 (Fig. 6C), whereas that afforded by proline and glycine betaine was unaffected (Fig. 6B and C). Taken together, our data show that the GltT transporter is an important contributor for the use of glutamate and aspartate as osmostress protectants by *B. subtilis*.

Transcription of *gltT* is not induced in response to osmotic stress. The expression of *opuE*, the dominant importer for the use of exogenously provided proline as an osmostress protectant (25, 26), is upregulated in response to increases in the environmental osmolarity (25, 60). Given the involvement of GltT in the use of glutamate and aspartate as osmostress protectants (Fig. 6), we wondered whether *gltT* transcription would be induced in response to sustained high salinity, as well. However, Northern blot analysis with a *gltT*-specific antisense mRNA probe showed that this is not the case (see Fig. S4 in the supplemental material).

GltT contributes to glutamate or aspartate import when they are used as the sole nitrogen source. Amino acids can be used by *B. subtilis* as sole nitrogen sources (49), and we verified that this was also the case for glutamate and aspartate (Fig. 7). SMM contains 15 mM $(NH_4)_2SO_4$ as the ammonium source. When the ammonium source was reduced to 2.5 mM, growth of the cells was limited (Fig. 7); the same growth yield was afforded through the supply of either glutamate or aspartate when an equivalent nitrogen concentration of these amino acids (5 mM was added to the medium) was used. Under these nitrogen-limiting conditions, loss of the GltT system had a strong effect on the growth yields of the cultures, whereas the simultaneous disruption of the glutamate/aspartate transporters GltP and YveA (41, 53) in a strain with an intact GltT transporter had no influence on glutamate and aspartate utilization as a nitrogen source (Fig. 7). Of note is our observation that glutamate and aspartate could both still be used as sole nitrogen sources in a triple-mutant strain (MDB54) simultaneously lacking the GltT, GltP, and YveA systems (Fig. 7), and this effect became more pronounced when the concentrations of these amino acids were raised to either 15 or 30 mM (Fig. 7). Hence, our growth assays not only revealed an important role of GltT in the use of glutamate and aspartate as nitrogen sources, but also uncovered the existence of a yet undisclosed glutamate and aspartate transport system in *B. subtilis*.

DISCUSSION

B. subtilis is resourceful in the ways in which it can derive osmostress protection by the compatible solute proline. Four routes are now known through which this can occur: (i) through osmotically stimulated *de novo* synthesis (14, 18), (ii) through osmostress-induced import of free proline via the OpuE transporter (25, 27), (iii) through the import of proline-containing peptides and their subsequent intracellular hydrolysis (24), and, as shown here for the first time, (iv) through the uptake of proteogenic and nonproteogenic amino acids that can be metabolically converted into proline (Fig. 2 and 3). For the last process, the osmotically controlled ProJ-ProA-ProH biosynthetic route (18) (Fig. 3) is required, but different entry points into this pathway are used by different osmostress-protective amino acids to finally yield the compatible solute proline (Fig. 1).

The feeding of osmoprotective amino acids does not enhance the proline pool beyond the size that is already found in osmotically stressed cells (Fig. 3C). This finding rules out a model that

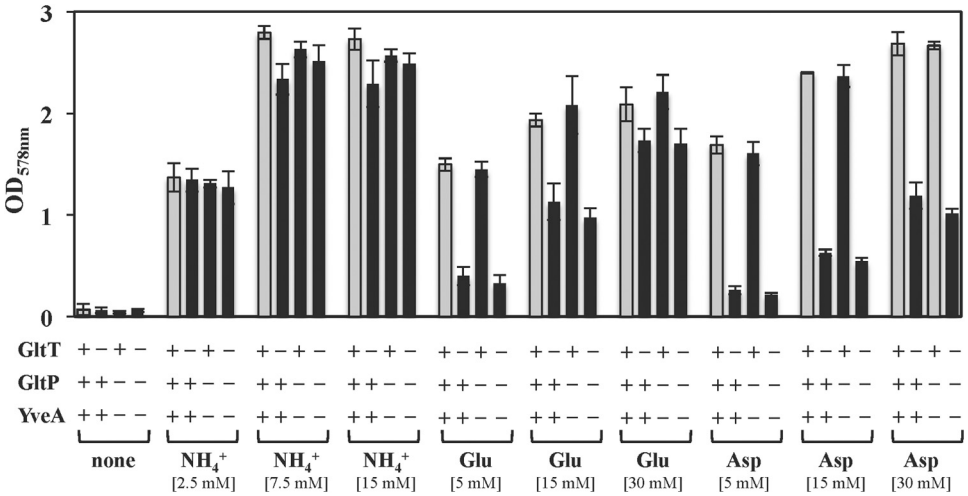

FIG 7 The *B. subtilis* wild-type strain JH642 (gray bars) and its mutant derivatives that were defective in the indicated glutamate/aspartate transporter GltT, GltP, or YveA (black bars) were tested for the ability to use glutamate or aspartate as the sole nitrogen source. We grew these strains in minimal medium without a nitrogen source in the presence of either 2.5 mM, 7.5 mM, or 15 mM $(NH_4)_2SO_4$ or with 5 mM, 15 mM, or 30 mM glutamate or aspartate, respectively, and then measured the growth yields of the cultures after 15 h of incubation at 37°C. For each growth condition tested, four independent cultures were used, and the means and standard deviations are shown.

would rely on a larger than normal cytoplasmic pool of proline to explain the osmostress-protective effects of an external supply of certain amino acids (Fig. 2 and 3). We are thus led to the conclusion that the higher growth yield of osmotically stressed cells cultured in the presence of Glu, Gln, Asp, Asn, Arg, Orn, and Cit than of those that did not receive an osmoprotective amino acid (Fig. 2) rests on the saving of precious biosynthetic building blocks and energy sources that would otherwise have to be devoted under high-osmolarity growth conditions (61) to the synthesis of the proline precursor glutamate (31, 32) or of proline itself (31, 51). The highly interconnected proline and arginine synthesis and degradation pathways of *B. subtilis* (34, 48, 49) (Fig. 1) thus provide the physiological foundation for the osmoprotective effects of a selected set of proteogenic and nonproteogenic amino acids.

We note that each of the amino acids that we have identified as osmoprotectants has been detected in the soil (8–10, 47), one of the prime habitats of *B. subtilis* (1). High-affinity transport systems should allow their scavenging from scarce environmental resources. Transporters for Pro, Gln, and Arg have been identified in *B. subtilis* (25, 26, 30, 52, 62–65), whereas the identities of the import systems for Asn, Orn, and Cit are either less certain or unknown (52, 62) (Fig. 1 shows an overview). A full understanding of the import routes for Glu and Asp in *B. subtilis* is also lacking. We found that the activity of the Na^+-coupled symporter GltT, a transporter that has not been previously functionally studied in *B. subtilis*, is an important contributor to the uptake of Glu and Asp by exponentially growing cells when their external concentrations are either low (20 μM) (Fig. 4), moderate (1 mM) (Fig. 6), or high (5 mM to 15 mM) (Fig. 6 and 7).

Transcription of the gene (*opuE*) for the main uptake system (OpuE) of the osmoprotectant proline in *B. subtilis* is induced by high salinity (25, 60), but this was not the case for the *gltT* gene (see Fig. S3 in the supplemental material). Inspection of the transcriptional profile of *gltT* obtained through a genome-wide tiling array study (44) revealed that it is expressed under a large set of growth conditions at similar levels, except in stationary or sporulating cells, where *gltT* expression is downregulated (44). Notably, expression of *gltT* is under the control of CodY, a globally acting transcription factor regulating the expression of a large set of metabolic genes in *B. subtilis*, in particular, those involved in amino acid metabolism (66). This fits nicely with our finding that the GltT transporter plays an important role in the import of Glu and Asp when these amino acids are used as sole nitrogen sources under conditions where they are supplied in growth-limiting amounts (Fig. 7).

The GltP and YveA transporters have also been implicated in the uptake of these amino acids by *B. subtilis* (41, 53). However, transport assays conducted by us in exponentially growing cells (Fig. 5) and osmostress protection assays (Fig. 6) did not uncover a substantial contribution of these carriers to the import of Glu and Asp. Furthermore, when these amino acids were used as sole nitrogen sources, effects of the GltP and YveA transporters were also not noticeable (Fig. 7). The transport activities of GltP and YveA might thus be physiologically relevant under growth conditions different from those tested by us. Indeed, the level of the *gltP* transcript varies in response to the type of carbon source available (67). Furthermore, transcriptional data obtained in a tiling array study examining more than 100 growth conditions (44) demonstrate that *gltP* expression is upregulated in cells that swarm, grow on solid media, enter stationary phase, or proceed to form spores. Under these conditions, very strong upregulation in the transcription of *yveA* is also observed, and the sporulation-specific transcription factor SigG has been implicated in its genetic control (68). Moreover, upregulation of *yveA* is observed under anaerobic growth conditions when the *B. subtilis* cells respire nitrate or undergo fermentation (44).

The cellular adjustment of *B. subtilis* to high-osmolarity environments is a well-staged and complex process (61, 69–71). However, the effective management of water fluxes in or out of the cell (12, 72) and the fine-tuning of the solvent properties of the cytoplasm (19, 73) are key events that allow cell proliferation under otherwise growth-inhibiting conditions (7, 11, 13). The amassing of the compatible solute proline plays an important part in the acclimatization process of the *B. subtilis* cell to unfavorably os-

motic conditions. We surmise that the import of proteogenic and nonproteogenic amino acids that can be metabolically converted into the osmoprotectant proline should enhance the competitiveness of the bacterium in its varied natural habitats (1–3).

ACKNOWLEDGMENTS

We greatly value the expert technical assistance of Jutta Gade and thank Milton Saier (UC San Diego, La Jolla, CA, USA) for providing us with the *B. subtilis yveA* mutant strain. We are indebted to Vickie Koogle for her kind help in the language editing of our manuscript. E.B. greatly appreciated the hospitality of Tom Silhavy during a sabbatical at the Department of Molecular Biology of Princeton University (Princeton, NJ, USA).

Funding for this study was provided by a grant from the BMBF via the Bacell-SysMo2 consortium, by contributions from the LOEWE program of the state of Hessen (via the Center for Synthetic Microbiology, Marburg, Germany), and by the Fonds der Chemischen Industrie. A.Z. was an associate member of the International Max Planck Graduate School for Environmental, Cellular and Molecular Microbiology (IMPRS-Mic; Marburg, Germany) and gratefully acknowledges its support.

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