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GENETICS AND MOLECULAR BIOLOGY

OpuF, a New *Bacillus* Compatible Solute ABC Transporter with a Substrate-Binding Protein Fused to the Transmembrane Domain

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ABSTRACT The accumulation of compatible solutes is a common defense of bacteria against the detrimental effects of high osmolarity. Uptake systems for these compounds are cornerstones in cellular osmostress responses because they allow the energy-preserving scavenging of osmostress protectants from environmental sources. *Bacillus subtilis* is well studied with respect to the import of compatible solutes and its five transport systems (OpuA, OpuB, OpuC, OpuD, and OpuE), for these stress protectants have previously been comprehensively studied. Building on this knowledge and taking advantage of the unabated appearance of new genome sequences of members of the genus *Bacillus*, we report here the discovery, physiological characterization, and phylogenomics of a new member of the Opu family of transporters, OpuF (OpuFA-OpuFB). OpuF is not present in *B. subtilis* but it is widely distributed in members of the large genus *Bacillus*. OpuF is a representative of a subgroup of ATP-binding cassette (ABC) transporters in which the substrate-binding protein (SBP) is fused to the transmembrane domain (TMD). We studied the salient features of the OpuF transporters from *Bacillus infantis* and *Bacillus panaciterrae* by functional reconstitution in a *B. subtilis* chassis strain lacking known Opu transporters. A common property of the examined OpuF systems is their substrate profile; OpuF mediates the import of glycine betaine, proline betaine, homobetaine, and the marine osmolyte dimethylsulfoniopropionate (DMSP). An *in silico* model of the SBP domain of the TMD-SBP hybrid protein OpuFB was established. It revealed the presence of an aromatic cage, a structural feature commonly present in ligand-binding sites of compatible solute importers.

IMPORTANCE The high-affinity import of compatible solutes from environmental sources is an important aspect of the cellular defense of many bacteria and archaea against the harmful effects of high external osmolarity. The accumulation of these osmostress protectants counteracts high-osmolarity-instigated water efflux, a drop in turgor to nonphysiological values, and an undue increase in molecular crowding of the cytoplasm; they thereby foster microbial growth under osmotically unfavorable conditions. Importers for compatible solutes allow the energy-preserving scavenging of osmoprotective and physiologically compliant organic solutes from environmental sources. We report here the discovery, exemplary physiological characterization, and phylogenomics of a new compatible solute importer, OpuF, widely found in members of the *Bacillus* genus. The OpuF system is a representative of a growing subgroup of ABC transporters in which the substrate-scavenging function of the substrate-binding protein (SBP) and the membrane-embedded substrate translocating subunit (TMD) are fused into a single polypeptide chain.

KEYWORDS ABC transporter, *Bacillus*, osmoregulation, compatible solute, glycine betaine, substrate-binding protein

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In their natural habitats, most microorganisms are exposed to fluctuations in osmolarity (1–4). This frequently occurring environmental challenge requires well-coordinated genetic and physiological adjustment responses in order to prevent dehydration of the cytoplasm and collapse of turgor in cells confronted with high osmolarity (5, 6) and rupture of cells suddenly exposed to low-osmolarity milieus (7, 8). In those bacteria and archaea that follow the salt-out osmostress adjustment strategy (1, 9, 10), the accumulation of a particular set of organic osmolytes, the compatible solutes, is a central component of their overall cellular response to both suddenly imposed and sustained high-osmolarity/-salinity surroundings (1–5, 10). The physicochemical properties of compatible solutes make them compliant with cellular biochemistry and physiology (6, 11–13), attributes which permit their high-level accumulation (2, 3, 5). In this process, the severity of the osmotic stress imposed onto the microbial cell determines the cytoplasmic pool sizes of compatible solutes (14, 15). Hence, a precise level of cellular hydration, turgor, and molecular crowding conducive for growth can be attained under challenging osmotic circumstances (1, 5, 6, 10, 11, 13, 16, 17).

For energetic reasons (18), the import of preformed compatible solutes is preferred over their synthesis, and consequently, uptake systems for these stress-relieving compounds are frequently a cornerstone of the cellular osmostress response systems of bacteria (1, 4–6, 19, 20). A considerable number of compatible solute transporters have been identified in bacteria that belong to different transporter families, as follows: binding-protein-dependent ATP-binding cassette (ABC) systems (21) (e.g., the ProU, OpuA, OpuB, OpuC, BusA, OsmU, OusA, and Prb transporters [22–30]), members of the major facilitator superfamily (MFS) (31) (e.g., the ProP and OusA transporters [32–34]), members of the betaine-choline-carnitine transport (BCCT) systems (35) (e.g., the OpuD, BetP, BetS, EctT, EctP, EctM, and BetM transporters [33, 36–39]), members of the sodium-solute-symporter (SSS) family (40) (e.g., OpuE [41]), and members of the tripartite ATP-independent periplasmic transporters (TRAP-T) (42) (e.g., the TeaABC system [43]). In addition to the typical osmotic induction of the transcription of their structural genes (1, 3–5, 44–46), the activities of compatible solute transporters are also frequently enhanced by osmotic stress (6, 19, 20, 32, 35, 47, 48), thereby providing bacterial cells with increased uptake capacity for stress-relieving compounds both under acute and sustained high-osmolarity conditions.

Binding-protein-dependent ABC systems play important roles for the uptake of compatible solutes in many bacteria. These types of multicomponent importers (21, 49–51) rely on an extracellular substrate-binding protein (SBP) that scavenges the ligand(s) from environmental sources with high affinity and delivers it to the transmembrane components for its translocation into the cytoplasm, a process that depends on the hydrolysis of two ATP molecules per molecule of imported substrate (52).

Uptake and synthesis of compatible solutes have been particularly well studied in *Bacillus subtilis* (46, 53), the model organism for Gram-positive bacteria (54). Members of the ecophysiologically diverse *Bacillus* genus can be commonly found both in terrestrial and marine habitats (55–57). When confronted by high osmolarity, *B. subtilis* synthesizes large amounts of L-proline (14, 58, 59), a compatible solute widely used by both bacteria and plants (2, 60, 61), and an amino acid that serves also as a function-preserving chemical chaperone (62, 63). The massive osmostress-responsive synthesis of L-proline by *B. subtilis* (14, 58, 64) is an energy-demanding process because the production of just a single molecule of L-proline requires the expenditure of 20 ATP equivalents (65). To preserve precious energetic and biosynthetic resources (18), *B. subtilis* generally prefers the import of preformed osmostress protectants that can be found in its varied habitats (46, 53, 66–68). In turn, their accumulation suppresses the transcription of the osmostress-adaptive L-proline biosynthesis genes (14, 58, 64). *B. subtilis* can also synthesize the compatible solute glycine betaine (69, 70), but this process requires the prior import of the precursor choline for its subsequent two-step oxidation to glycine betaine (46, 53, 69, 70).

Five osmotically inducible osmostress protectant uptake systems (Opu) operate in *B. subtilis* (46, 53). Three of these (OpuA, OpuB, and OpuC) are multicomponent systems

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and members of the ABC transporter superfamily (21, 50, 51). In contrast, OpuD and OpuE are single-component transporters that belong to the BCCT (37) and SSS (41) families, respectively. In addition to the different ways in which the transporter activities of the Opu transporters are energized, each of them possesses a distinct substrate profile (46, 53). Together, the five *B. subtilis* Opu systems mediate the import of 17 osmostress protectants, thereby providing a considerable degree of flexibility to the cell to physiologically cope with high-osmolarity surroundings in its varied habitats (46, 53). With the notable exception of L-proline (71), none of the osmostress protectants scavenged by *B. subtilis* from environmental sources can be used by this bacterium as a nutrient (46, 53).

The SBPs (OpuAC, OpuBC, and OpuCC) operating in conjunction with the *B. subtilis* OpuA, OpuB, and OpuC ABC transporters are tethered to the outer face of the cytoplasmic membrane through lipid modifications of a Cys residue at their mature N termini (24, 26, 72). Crystal structures of lipidless versions of these proteins in complex with various osmostress protectants have been reported (73–76), thereby providing insights into the molecular determinants that allow high-affinity binding of types of organic solutes that are otherwise preferentially excluded from protein surfaces (12, 77–79). A key feature of the architecture of the ligand-binding sites present in these SBPs is a cage-like structure formed by the side chains of either three (OpuAC) (73, 74) or four (OpuBC and OpuCC) (75, 76) aromatic amino acids. This aromatic cage permits the flexible binding of natural and synthetic compatible solutes with fully methylated and positively charged headgroups via cation-π interactions (46, 66, 67, 73–76).

Members of the Opu transporter family were genetically identified and functionally characterized (24, 26, 37, 41) before the genome sequence of *B. subtilis* was reported (54, 80). Now, thousands of representatives of these types of osmostress protectant uptake systems can be found by searching microbial genome databases. Their annotation in genome projects has profited from the comprehensive functional characterization of the *B. subtilis* Opu family of compatible solute transporters (46, 53). By building on this knowledge and by taking advantage of the unabated appearance of new genome sequences of members of the genus *Bacillus*, we report here the discovery, phylogenomics, and physiological characterization of a new member of the Opu transporters, OpuF. This compatible solute importer is not present in *B. subtilis* but is found in many members of this large genus. OpuF is a representative of a subgroup of ABC transporters in which the SBP is fused to the transmembrane domain (81).

RESULTS

Phylogenomics of the OpuF ABC transporter. Among the five Opu transporters operating in *B. subtilis* (46), the OpuB and OpuC systems stand out because these ABC transporters have likely evolved through a gene duplication event (26). As a consequence, the amino acid sequences of their components are closely related, yet their substrate profiles are strikingly different. OpuB is a rather specific transporter (mainly for choline), while OpuC is highly promiscuous (46, 53, 82). We used the amino acid sequence of OpuCC, the SBP of the OpuC system (26, 46, 76, 82), as a query to analyze the phylogenomic distribution of ABC transport systems in members of the genus *Bacillus*. For this database search, we relied on the Integrated Microbial Genomes & Microbiomes (IMG/M) comparative data analysis system of the U.S. Department of Energy (83). At the time of the search (8 May 2018), the IMG/M database contained 1,362 deposited genome sequences of members of the genus *Bacillus*. For a phylogenetic analysis, we focused on one strain per species for which 16S rRNA gene sequence information was also available through the SILVA database (84). This yielded a final data set of 175 genome sequences and 86 representatives of OpuC-type ABC transporters, and 29 representatives of OpuB-type ABC transporters were identified (Fig. 1). Their SBPs (OpuBC and OpuCC, respectively) are lipoproteins (85) tethered to the outer face of the cytoplasmic membrane (26) and possess an amino acid sequence identity of 71%. We distinguished between putative OpuB- and OpuC-type ABC transporters recovered in our BLAST search (86) by individually comparing the amino acid sequence

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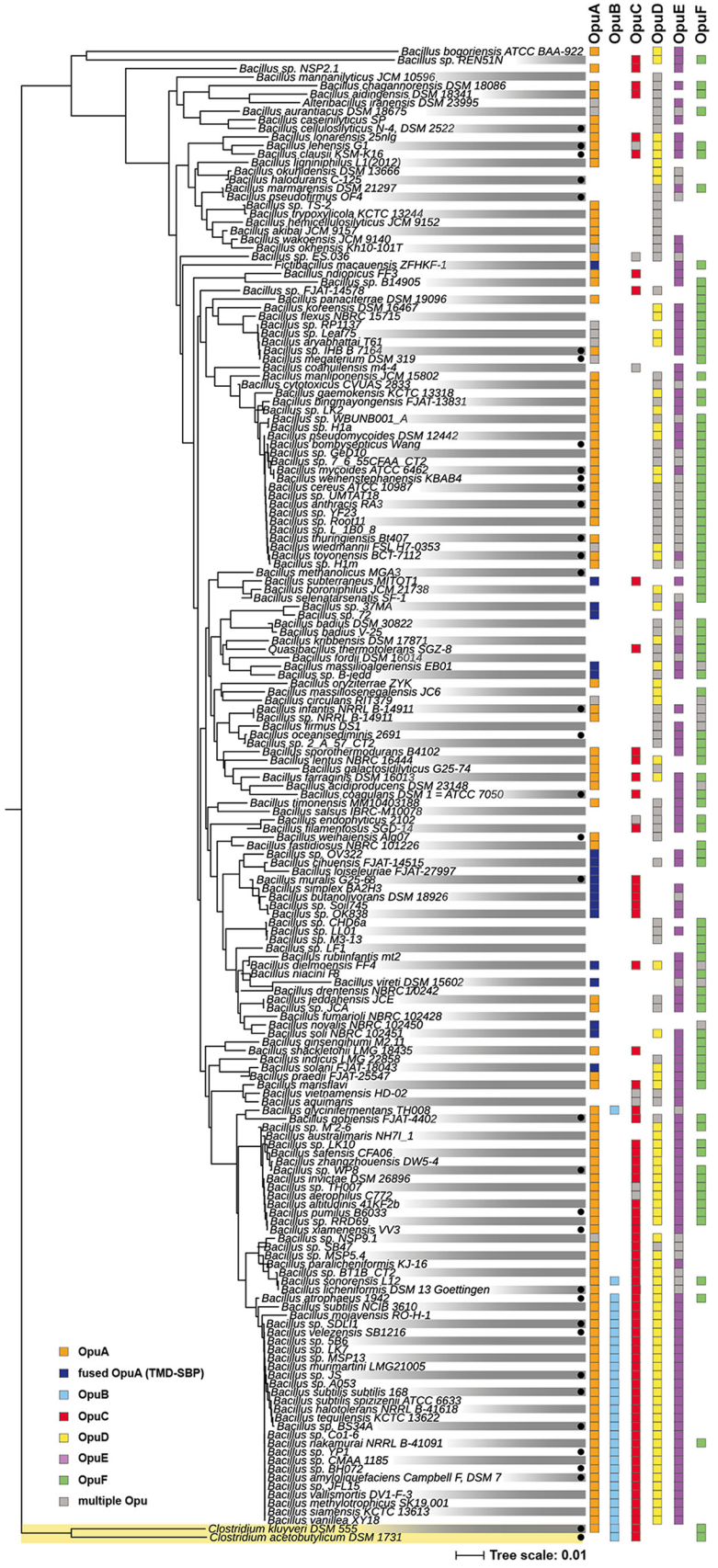

FIG 1 Phylogenomics of the Opu osmostress protectant uptake systems in members of the genus *Bacillus*. The filled-in circles indicate those genomes whose sequences are complete.

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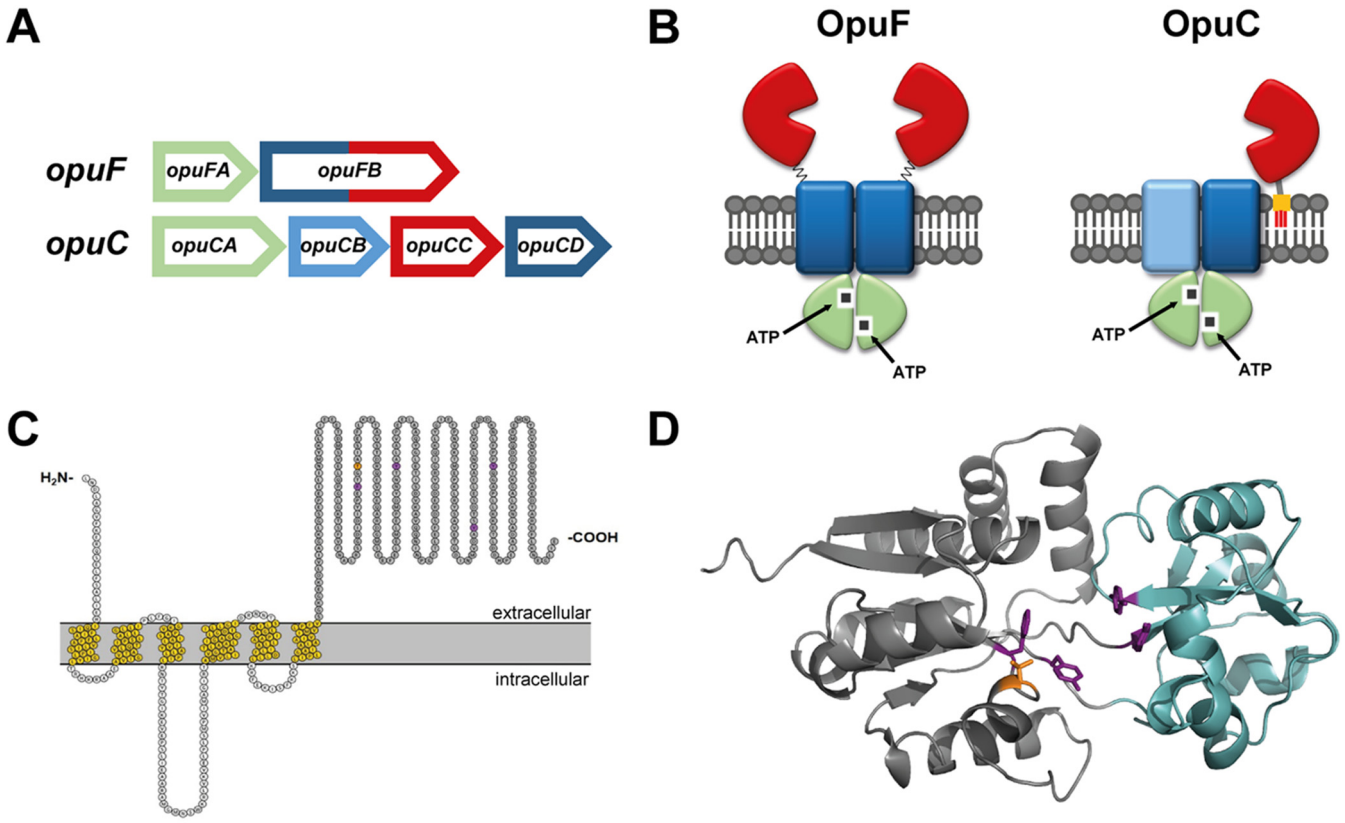

FIG 2 *In silico* analysis of the OpuF transporter and its coding genes. (A) Organization of genes (*opuCA-opuCB-opuCC-opuCD*) encoding the *B. subtilis* OpuC ABC transporter and those (*opuFA-opuFB*) of the OpuF ABC transporter from *B. infantis*. (B) Schematic representation of the predicted subunit composition of the OpuC system from *B. subtilis* (26) and of the OpuF ABC transport systems from *B. infantis*. (C) Predicted topology of the hybrid OpuFB protein from *B. infantis* carrying an N-terminal transmembrane domain (TMD) and a C-terminal domain representing the substrate-binding protein (SBP). (D) *In silico* model of the *B. infantis* SBP domain of OpuFB. The two lobes of the SBP are marked in different colors, and the four amino acids forming the predicted aromatic cage for ligand binding are indicated as violet sticks. The Thr residue discussed in the text is represented as an orange stick.

identities of each of the recovered 115 SBPs with those of the *B. subtilis* OpuBC and OpuCC proteins. In addition, by using the gene neighborhood analysis tool provided through the IMG/M website (83), we ascertained that the genes of the other components of the OpuB (OpuBA-OpuBB-OpuBC-OpuBD) and OpuC (OpuCA-OpuCB-OpuCC-OpuCD) transporters (26) were present in the queried genome sequences as well. This was the case for each of the inspected *opuB* and *opuC* gene clusters. When the occurrence of the genes for the OpuB and OpuC systems was projected onto a 16S rRNA gene-derived phylogenetic tree, it became apparent that representatives of the OpuC ABC transporter were widely distributed in members of the genus *Bacillus*, whereas OpuB was only present in a rather restricted and phylogenetically very closely related number of species and strains (Fig. 1).

In addition to the identification of OpuB- and OpuC-type systems, the BLAST search of the IMG/M database recovered a different type of ABC transporter (112 representatives). In contrast to OpuB and OpuC, these ABC systems consisted of only two, instead of the four, components typical of the OpuB and OpuC transporters (26). The corresponding two genes were arranged in an operon-like configuration and encoded an ATPase and a hybrid protein that consisted of a transmembrane domain (TMD) fused to an SBP (Fig. 2A and B). We refer in the following to this type of ABC transport system as OpuF; it consists of the ATPase OpuFA and the TMD-SBP dual-functional protein OpuFB (Fig. 2A and B). When the occurrence of the OpuF system was projected onto the 16S rRNA gene-derived phylogenetic tree (Fig. 1), we found that OpuF is one of the most predominant predicted transporters for compatible solutes in the genus *Bacillus* (46, 53). Notably, OpuF is absent in *B. subtilis* (Fig. 1).

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***In silico* assessment of the OpuF components.** In comparison with most ATPases from binding-protein-dependent ABC import systems (21, 49, 50), the ATPases (OpuBA and OpuCA) of the OpuB and OpuC transporters possess a long carboxy-terminal extension (Fig. 2A). This segment of the OpuBA and OpuCA proteins contains two cystathionine β-synthase (CBS) structural domains. As shown through studies with the ATPases of the OpuC systems from *Listeria monocytogenes*, *Staphylococcus aureus*, and *Streptococcus agalactiae*, the CBS domains bind the secondary messenger c-di-AMP (87–89) and thereby contribute to the activity regulation of the OpuC-type compatible solute transporter operating in these Gram-positive bacteria (88). No information is currently available in this respect for the OpuB and OpuC systems from *B. subtilis*. While a few OpuFA-type ATPases possess both CBS domains (19 representatives), the vast majority (93 representatives) only possess one of these domains.

The fused TMD-SBP hybrid protein OpuFB is the more interesting component of the OpuF (OpuFA-OpuFB) transporter. SBPs of ABC transporters are typically either freely diffusible in the periplasmic space of Gram-negative bacteria or are tethered to the outer face of the cytoplasmic membrane of Gram-positive bacteria through a lipid-modification of a Cys residue present at the N terminus of the mature secreted protein (21, 85). However, a subgroup of microbial binding-protein-dependent ABC import systems exists in which one, or even multiple, SBPs are fused to the TMD (81, 90, 91). Prominent examples are the osmotically regulated glycine betaine uptake system OpuA (BusAB) systems from *Lactococcus lactis* (25, 29) and *S. agalactiae* (89), and the L-asparagine, L-glutamine, and L-glutamate GlnPQ importer from *L. lactis* (90).

Taking the *Bacillus infantis* OpuFB protein (511 amino acids) as an example, OpuFB consists of an N-terminal domain (amino acids 1 to 204) that functionally represents the TMD of the OpuF transporter; it contains six predicted transmembrane segments (Fig. 2C), a number typically found in TMDs of microbial binding-protein-dependent ABC transporters (21, 49–51). The remaining carboxy-terminal part of the OpuFB protein (amino acids 205 to 511) has a predicted extracellular location and functionally represents the substrate-binding portion of the hybrid OpuFB protein. Both the N and C termini of the OpuFB protein face the extracellular milieu (Fig. 2C). The subunit composition of typical binding-protein-dependent ABC transporters is as follows: two ATPases, two TMDs (either homo- or heterodimers), and the SBP (21, 81). Following this canonical arrangement, the components of the OpuF system are schematically depicted in Fig. 2B and suggest that a homodimer of the ATPase (OpuFA) and two copies of the TMD-SBP hybrid protein (OpuFB) are assembled in a functional OpuF ABC transporter.

The crystal structures of the *B. subtilis* OpuBC and OpuCC SBPs are known (75, 76), and the SBP portion of the *B. infantis* OpuFB protein exhibits amino acid sequence identities of 33% and 34%, respectively, to the mature forms of these proteins. SBPs of type I ABC transporters possess an evolutionarily highly conserved structural fold (92, 93). We used the SWISS-MODEL server (94) to derive an *in silico* model of the SBP domain of the OpuFB protein. The relevant data for the top 10 hits onto which the SWISS-MODEL server build the *in silico* model of the OpuFB SBP domain are summarized in Table S1 in the supplemental material; they primarily contained SBPs from ABC transporter with substrate specificity for compatible solutes.

The *B. subtilis* OpuCC (76) (TM score: 0.823) and OpuBC (75) (TM score: 0.756) crystal structures were found as the six and seventh hits in the data set generated by the SWISS-MODEL server (94) (Table S1). The TM score describes the overall quality of a structural alignment between a crystalized protein and the *in silico*-predicted structure of the query amino acid sequence (94). The second hit on this list was the glycine betaine/proline betaine-binding protein ProX (TM score: 0.93) from the hyperthermophilic archaeon *Archaeoglobus fulgidus* (95, 96). Somewhat surprising was the top hit (TM score: 0.973) on the list, the BilE protein from *Listeria monocytogenes* (97). This is the substrate-binding domain of the BilEB TMD-SBP hybrid protein of the BilEA/BilEB ABC transporter, a resistance determinant against bile and a system that contributes to the colonization of the gastro-

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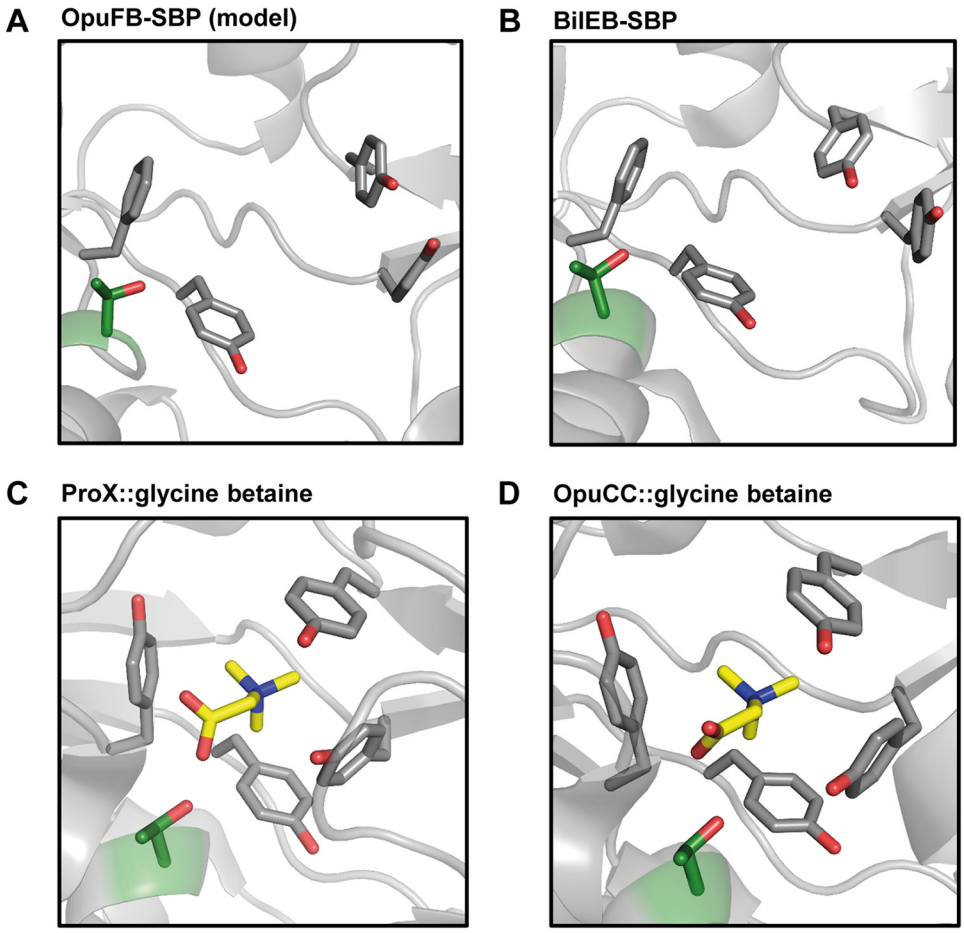

FIG 3 Architecture of the substrate-binding site of various ligand-binding proteins. (A) *In silico* model of the SBP domain of the *B. infantis* OpuFB TMD-SBP hybrid protein. It was automatically generated by the SWISS-MODEL server (94) based upon the crystal structure of the SBP domain of the BilEB protein from *L. monocytogenes* (PDB ID 4Z7E) (97). (B) Crystal structure of the SBP domain of the BilEB protein from *L. monocytogenes* (97). (C) Crystal structure of the ProX SBP from the archaeon *A. fulgidus* in complex with glycine betaine (PDB ID 1SW2) (95). (D) Crystal structure of the OpuCC SBP from *B. subtilis* in complex with glycine betaine (PDB ID 3PPP) (76). The representation of the predicted (A) and experimentally determined (B to D) structures were generated with PyMOL. Residues predicted or experimentally proven in substrate binding and (when known) ligands are highlighted.

intestinal tract by the pathogen *L. monocytogenes* (98). Although it was initially suggested that BilE was involved in the uptake of osmostress protectants (glycine betaine, carnitine, and choline), subsequent studies showed that it does not serve this physiological function and instead is somehow involved in the exclusion of bile from the *L. monocytogenes* cell (97, 98). We detected in our *in silico*-generated model of the SBP domain of the *B. infantis* OpuFB hybrid protein a putative ligand-binding site carrying an aromatic cage that is formed by the side chains of three tyrosine residues and of a phenylalanine (Fig. 2D). The architecture of this putative substrate-binding site closely resembles those present in the crystal structures of the glycine betaine- and proline betaine-binding protein ProX from *A. fulgidus* (95, 96), the choline-binding protein OpuBC from *B. subtilis* (75), and the promiscuous compatible solute OpuCC-binding protein from *B. subtilis* (76) (Fig. 3). Although the BilEB SBP from *L. monocytogenes* apparently does not bind compatible solutes (97), the architecture of the ligand-binding site revealed through structural analysis nevertheless resembles that of the ProX, OpuBC, and OpuCC SBPs (Fig. 3). The overall fold of the *in silico*-generated SBP domain of the *B. infantis* OpuFB protein suggests that it belongs to the subcluster F-III in the structure-based classification scheme of SBPs (92, 93), a cluster of SBPs of ABC transporters mainly involved in the import of osmostress protectants. Notably, in an alignment of the SBP domains of 112 OpuFB-type proteins (Fig. S1), aromatic amino acids

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

Strain[a]	Relevant genotype/description[c]	Origin and/or reference
JH642	*trpC2 pheA1*	J. Hoch (110)
TMB118	Δ(*opuA*::*tet*)3 Δ(*opuB*::*ery*)1 Δ(*opuC*::*spc*)3 Δ(*opuD*::*kan*)2	82
CAB5	*B. subtilis* strain 168 Δ(*gbsR*::*zeo*)	C. Arnold
HKB9[b]	Δ(*opuA*::*tet*)3 Δ(*opuB*::*ery*)3 Δ(*opuC*::*spc*)3 Δ(*opuD*::*kan*)2 Δ(*gbsR*::*zeo*) *amyE*::*opuFA-opuFB*$_{B.i.}$ (Cml^r)	This study
HKB12[b]	Δ(*opuA*::*tet*)3 Δ(*opuB*::*ery*)3 Δ(*opuC*::*spc*)3 Δ(*opuD*::*kan*)2 Δ(*gbsR*::*zeo*) *amyE*::*opuC*$^+$ (Cml^r)	This study
HKB13[b]	Δ(*opuA*::*tet*)3 Δ(*opuB*::*ery*)3 Δ(*opuC*::*spc*)3 Δ(*opuD*::*kan*)2 Δ(*gbsR*::*zeo*) *amyE*::pX (Cml^r)	This study
HKB15[b]	Δ(*opuA*::*tet*)3 Δ(*opuB*::*ery*)3 Δ(*opuC*::*spc*)3 Δ(*opuD*::*kan*)2 Δ(*gbsR*::*zeo*) *amyE*::*opuFA-opuFB*$_{B.p.}$ (Cml^r)	This study
LTB52	Δ(*opuA*::*tet*)3 Δ(*opuC*::*spc*)3 Δ(*opuD*::*kan*)2 Δ(*opuB*::*ery*) Δ(*gbsR*::*zeo*)	This study

[a]All strains used in this study are derivatives of the domesticated *B. subtilis* strain JH642 (110), except for strain CAB5, which is a derivative of *B. subtilis* strain 168 (80).

[b]These strains all carry either the integration vector pX (99), or recombinant derivatives of it, integrated via a double-homologous recombination event into the chromosomal *amyE* gene; the backbone of the pX vector carries a chloramphenicol resistance (Cml^r) gene allowing the selection of the chromosomal integrants. The designation *opuFA-opuFB*$_{B.i.}$ refers to the *opuF* operon from *B. infantis*, and the corresponding designation *opuFA-opuFB*$_{B.p.}$ refers to the *opuF* gene cluster from *B. panaciterrae*.

[c]Numbers outside the parentheses [e.g., the "3" in Δ(*opuA*::*tet*)3] are allele numbers.

were always present at the positions corresponding to the residues forming the predicted aromatic cage in the *B. infantis* OpuFB protein (Fig. 3A).

Functional characterization and substrate profile of selected OpuF transport systems. The *in silico* analysis of the OpuF system suggests that its members function in the uptake of osmostress protectants. To experimentally assess this hypothesis, we focused on two OpuF systems (from *B. infantis* and from *Bacillus panaciterrae*) for functional analysis. For these studies, we cloned the *opuFA-opuFB* gene cluster along with their 5′-regulatory regions into a plasmid (pX), which allows the stable, single-copy integration of the cloned regions into the chromosomal *amyE* locus of *B. subtilis* (99). We chose for these experiments a *B. subtilis* chassis strain, LTB52 (Table 1), lacking all Opu transporters except for the L-proline transporter OpuE (41).

We performed osmostress protection assays with the two heterologous OpuF systems in a chemically defined medium using a set of nine compatible solutes that are known to serve as potent osmostress protectants for *B. subtilis* (46, 53). The compatible solutes assessed in this growth assay were glycine betaine, proline betaine, homobetaine, choline (the precursor for glycine betaine), choline-*O*-sulfate, DMSP, carnitine, crotonobetaine, and γ-butyrobetaine. L-Proline was used as an internal control since the used *B. subtilis* strains all possess an intact OpuE system. Each of the tested compatible solutes proved to be osmostress protective for a *B. subtilis* strain (HKB12) that possessed (in addition to OpuE) the promiscuous OpuC transporter (82) expressed from a copy of the *opuC* operon inserted into the chromosomal *amyE* gene (Fig. 4). As expected, the control strain HKB13 lacking the OpuA, OpuB, OpuC, and OpuD systems (but possessing a functional OpuE transporter) (Table 1) was only protected by the addition of L-proline to the growth medium (Fig. 4). From the set of the nine tested compatible solutes, the OpuF systems from *B. infantis* and *B. panaciterrae* served for the uptake of glycine betaine, proline betaine, homobetaine, and DMSP, but none of the studied OpuF systems used carnitine, crotonobetaine, choline, or choline-*O*-sulfate as their substrate (Fig. 4). In the osmostress protection growth assays, the two recombinant *B. subtilis* strains carrying the *opuFA-opuFB* gene clusters from *B. infantis* and *B. panaciterrae* yielded optical density at 578 nm (OD_{578}) values similar to those of the *B. subtilis* control strain HKB12 (Fig. 4).

To assess whether the substrate profile of the recombinant OpuF systems transplanted into the LTB52 *B. subtilis* chassis strain (Fig. 4) reflected the osmostress protection profile of their authentic parent strains, we used *B. infantis* as an example.

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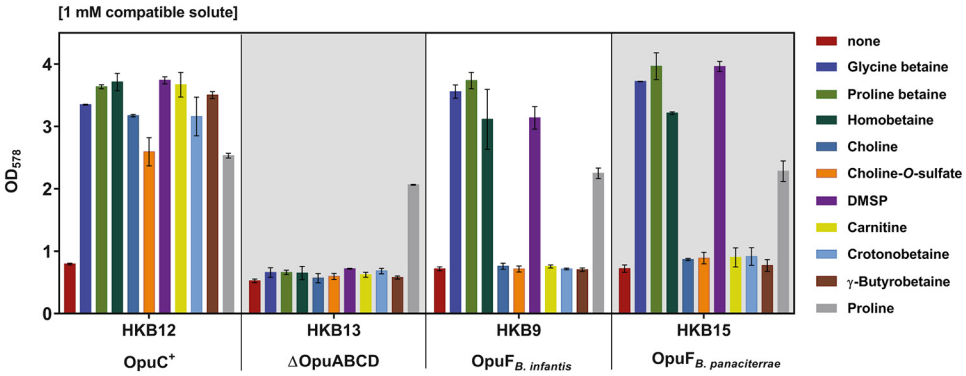

FIG 4 Osmostress protection growth assays assessing the substrate profile of the OpuF transport systems from *B. infantis* and from *B. panaciterrae*. The various *B. subtilis* strains were grown in SMM with 1.2 M NaCl in the absence or presence of a 1 mM concentration of the indicated osmostress protectant for 17 h at 37°C in a shaking water bath (20-ml cultures in 100-ml Erlenmeyer flasks). The *B. subtilis* strain HKB12 expresses a copy of the *opuC* operon inserted into the chromosomal *amyE* gene. Strain HKB13 is a *B. subtilis* control strain lacking the OpuA, OpuB, OpuC, and OpuD compatible solute transport systems and carries the empty pX integration vector in the *amyE* gene. Strains HKB8, HKB9, and HKB15 expressing the *opuFA-opuFB* genes from the indicated *Bacillus* species are derivatives of the *B. subtilis* chassis strain LTB52 (inactive OpuA, OpuB, OpuC, and OpuD transporter systems). These strains carry the *opuFA-opuFB* gene cluster as single-copy constructs inserted into the *amyE* gene. As a control for the functioning of the osmostress protectant growth assays, all strains harbor an intact osmostress-responsive OpuE L-proline transporter (41). All cultures were grown as two replicates, and the mean and standard deviation of the growth yield (measured at OD_{578}) are indicated.

As shown in Fig. S2, *B. infantis* used glycine betaine, proline betaine, homobetaine, and DMSP as osmostress protectants. It should be noted, however, as predicted from the genome sequence (100), that this *Bacillus* species possesses OpuA-, OpuD-, OpuE-, and OpuF-type osmolyte transporters (Fig. 1), a genetic configuration that precludes the assignment of substrate profiles to a particular Opu transporter.

Transport characteristics of selected OpuF systems for glycine betaine. Having assessed the substrate profile of the two inspected OpuF systems through physiological studies (Fig. 4), we focused our analysis in somewhat greater detail on the OpuF-dependent import of glycine betaine, which is probably the most widely used compatible solute on Earth (101). We monitored the osmostress protective effects of glycine betaine under hyperosmotic growth conditions in a minimal medium containing 1.2 M NaCl and that was supplemented with various concentrations of this compatible solute (0.025 mM, 0.05 mM, 0.1 mM, and 1 mM). Like the authentic OpuC transporter from *B. subtilis*, the heterologous OpuF systems from *B. infantis* and *B. panaciterrae* already provided a growth advantage to the cells when the medium contained as little as 25 μM glycine betaine (Fig. S3).

Collectively, these growth data suggest that the *B. subtilis* strains carrying the *B. infantis* and *B. panaciterrae opuFA-opuFB* genes should import glycine betaine with high affinity. We tested this directly by determining the kinetic parameters (K_m and V_{max}) of the two OpuF transporters under conditions of moderate salt stress (0.4 M NaCl was present in the growth medium and the assay solution). Representative data for these transport assays with the recombinant *B. subtilis* strains are documented in Fig. S4, and the derived kinetic data are summarized in Table 2. The kinetic parameters obtained for the authentic *B. subtilis* OpuC transporter determined as a control (K_m of about 7 μM; V_{max} of about 108 nmol · min^{-1} · mg $protein^{-1}$) closely match previously reported data (K_m of about 5 μM; V_{max} of about 100 nmol · min^{-1} · mg $protein^{-1}$) (82). The OpuF transporters from *B. infantis* and *B. panaciterrae* exhibited the same high-affinity K_m values as the *B. subtilis* OpuC transporter, but notably, their V_{max} exceeded that of OpuC by approximately 2-fold (Table 2).

DISCUSSION

Based on an extensive bioinformatics analysis of *Bacillus* genome sequences (Fig. 1), we report here the identification and functional characterization of a new type of

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TABLE 2 Kinetic parameters for the uptake of glycine betaine by *B. subtilis* and its recombinant derivatives possessing heterologous OpuF ABC transport systems[a]

Transporter	V_{max} (nmol · min^{-1} · mg^{-1})	K_m (μM)
$OpuC_{B.s.}$[b]	108 ± 4	7 ± 1
$OpuF_{B.i.}$	185 ± 6	4 ± 1
$OpuF_{B.p.}$	208 ± 5	5 ± 1

[a]The strains were grown in SMM with 0.4 M NaCl to an OD_{578} of about 0.3. Two-milliliter samples were withdrawn and used to measure at different time intervals the import of radiolabeled [1-^{14}C]glycine betaine at different substrate concentrations. The amount of the imported [1-^{14}C]glycine betaine was determined by scintillation counting (14). The glycine betaine concentration used in the experiments varied between 3 μM and 120 μM and was spiked with a 1 μM concentration of the radiolabeled substrate.

[b]The strain (HKB12) possessing the intact OpuC transporter harbors a copy of the *opuC* operon inserted into the chromosomal *amyE* locus. Recombinant *B. subtilis* strains possessing the *B. infantis* (strain HKB9) or *B. panaciterrae* (strain HKB15) OpuF transporters carry a single copy of the respective *opuFA-opuFB* gene cluster inserted into the *amyE* gene. In the *B. subtilis* chassis strain (LTB52) used for the construction of each of the above-described recombinant strains, all native osmostress protectant import systems (OpuA, OpuB, OpuC, and OpuD) are nonfunctional, except for the proline-specific OpuE transporter (46).

compatible solute importer, OpuF (Fig. 2B). The wide phylogenomic distribution of the structural genes (*opuFA-opuFB*) (Fig. 1) encoding this ABC transport system indicates that it is an integral part of the cellular osmostress defense (46, 53) of many members of the genus *Bacillus*, ecophysiologically important inhabitants of both terrestrial and marine ecosystems (54–57, 102). Notably, OpuF is found neither in the legacy *B. subtilis* laboratory strain 168 (80, 102) nor in the undomesticated and biofilm-forming *B. subtilis* strain NCIB 3610 (103, 104) (Fig. 1).

OpuF is a member of a growing subgroup of ABC importers (81) that possess a separate ATPase (e.g., OpuFA) but in which the substrate-scavenging functions of the SBP and the membrane-embedded substrate translocating subunit (TMD) are fused into a single polypeptide chain (e.g., OpuFB; Fig. 2C) (25, 29, 81, 89–91). Building on the established subunit composition of microbial ABC-type importers, the OpuF transporter is thus predicted to possess two fixed SBPs, whereas the traditional SBP-dependent systems from Gram-positive bacteria (e.g., OpuC from *B. subtilis* [26]) rely on lipid-modified SBPs anchored in the outer leaflet of the cytoplasmic membrane (21, 85) (Fig. 2B). The stoichiometry of lipid-modified SBPs with reference to the canonical homo- or heterodimeric TMD components of ABC importers (21, 49–51) is unknown.

Compared with the very broad substrate profile of the *B. subtilis* OpuC transporter (46, 53), the substrate specificity of OpuF is more restricted and comprises in the two studied examples (from *B. infantis* and *B. panaciterrae*) the nitrogen-containing compatible solutes glycine betaine, proline betaine, and homobetaine and the sulfur-containing compatible solute DMSP (Fig. 4), an osmostress protectant abundant in marine ecosystems (66). A common denominator of the architecture of the ligand-binding site present in SBPs operating in conjunction with ABC transporters for osmostress protectants is an aromatic cage that allows the high-affinity binding of various compatible solutes with fully methylated head groups (46, 53). The aromatic cage, along with residues coordinating the tail(s) of the various substrate within the ligand-binding site, can be structurally configured in a way that yields either a rather specific (25, 73–75, 105) or a very broad (76) substrate profile of the entire transporter. This type of compatible solute-binding site is found both in archaeal (95, 96) and bacterial (25, 73–76, 95, 105–107) SBPs, and it is also present in the membrane-spanning portion of the single-component BCCT-type (35) glycine betaine importer BetP from *Corynebacterium glutamicum* (108). These findings indicate that nature has found a common physicochemical solution to facilitate the high-affinity binding of organic osmolytes that are otherwise preferentially excluded from protein surfaces (11, 12, 77, 79). Our assessment of the *in silico*-derived structure of the SBP domain of the OpuFB TMD-SBP transporter component (Fig. 2D) suggests the presence of an aromatic cage as well (Fig. 3A). The four amino acid residues predicted to form it are completely conserved in the data set of the 112 OpuFB-type proteins inspected by us (Fig. S1).

The amino acid at position 74 of the mature OpuBC and OpuCC SBPs of *B. subtilis* plays

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TABLE 3 DNA primers used in this study

Primer name	Primer sequence (5′–3′)
BI-fusitransp-for	AAAGGATCCGGGTAGGTAATAGAGTTAATGGAGTGAGGTGG
BI-fusitransp-rev	AAAGGATCCGGCATTTTAAACGCTTTTAAATGGAACCCGCG
BP-fusitransp-for	AAACAGCTGGTGGAGAGGATAAAAAGTAAGGAGGACAGC
BP-fusitransp-rev	AAACAGCTGCTGGACAACTAATTCATCTGCTCTACAGGG
BI-seq1	CGATCGTGTTTGTGACGCATGATATGCAGG
BI-seq2	GCACTTCTGCCGATTTTGAGGAATACGTAC
BP-seq1	GTTTCTCTTGATGGACGAGCCGTTTAGTGC
BP-seq2	CGATATTTGGAATAGGAACCGTACCTGCTG

a decisive role in trapping the substrate stably during closure of the two lobes of the SBP (92, 93) and in setting the substrate specificity of the entire OpuB and OpuC systems (75, 76). In the *B. subtilis* OpuBC protein and its orthologs, this residue is always an Asp residue, while a Thr residue is almost invariably present in OpuCC-type SBPs. When one views the corresponding position in the *in silico* model of the OpuFB SBP domain, a Thr residue is present (Fig. 2D and 3A), and it is strictly conserved in 112 OpuFB proteins compiled in this study (Fig. S1). It thus appears from the analysis of the substrate profile of the OpuB, OpuC, and OpuF transporters and the architecture of their corresponding SBPs that an aromatic cage combined with a Thr residue at a location critical for domain closure of the SBP hints at a broader substrate specificity of the corresponding compatible solute uptake system. We stress here, however, that such a structural configuration of the ligand-binding site in the SBP is insufficient to predict the substrate profile of the entire ABC transporter with confidence, as exemplified by the substantial differences in the substrate profiles of the *B. subtilis* OpuC on one hand and the *B. infantis* and *B. panaciterrae* OpuF systems on the other hand (Fig. 4).

The description and functional characterization of the OpuF transporter reported in this study have ramifications for an understanding of the osmostress adjustment systems not only for members of the genus *Bacillus*. Our extended database searches of genome sequences of *Bacillales* revealed that OpuF-type transporters are widely found in two important genera of the *Bacillales*, *Listeria* (84 out of 84 fully sequenced genomes) and *Staphylococcus* (180 out of 180 fully sequenced genomes) (Table S2), genera that comprise important human pathogens.

MATERIALS AND METHODS

Chemicals and reagents. The antibiotics ampicillin, chloramphenicol, erythromycin, kanamycin, spectinomycin, and tetracycline were all obtained from Sigma-Aldrich (Steinheim, Germany). The antibiotic zeocin was purchased from InvivoGen (San Diego, CA, USA). All compatible solutes used in this study were from laboratory stocks (46). Radiolabeled [1-^{14}C]glycine betaine (55 mCi · mmol^{-1}) was purchased from American Radiolabeled Chemicals (St. Louis, MO).

Bacterial strains, media, and growth conditions. *Escherichia coli* and *B. subtilis* strains were routinely maintained on Luria-Bertani (LB) agar plates or cultured in LB liquid medium at 37°C. Long-term storage of bacilli occurred at −80°C as frozen glycerol stocks. All *B. subtilis* strains used in this study are listed in Table 1. The concentrations of the antibiotics used for *B. subtilis* strains were as follows: chloramphenicol, 10 μg · ml^{-1}; erythromycin, 1 μg · ml^{-1}; kanamycin, 5 μg · ml^{-1}; spectinomycin, 100 μg · ml^{-1}; tetracycline, 10 μg · ml^{-1}; and zeocin, 50 μg · ml^{-1}. When ampicillin was used with *E. coli* strains carrying plasmids, the antibiotic was added to the growth medium to a final concentration of 100 μg · ml^{-1}. For osmostress protection assays and transport studies, the used *B. subtilis* strains were grown in a chemically defined medium (Spizizen minimal medium [SMM]) with 0.5% glucose (wt/vol) as a carbon source, a solution of trace elements (109), and L-tryptophan (20 mg · liter^{-1}) and L-phenylalanine (18 mg · liter^{-1}) to satisfy the auxotrophic requirements of the *B. subtilis* laboratory wild-type strain JH642 (110) and its mutant derivatives (Table 3). The osmolarity of SMM was increased by the addition of NaCl from a 5 M stock solution, and compatible solutes were typically added to the growth medium to a final concentration of 1 mM, unless specified otherwise. Cultures of *B. subtilis* cells were inoculated from exponentially grown precultures in SMM to an optical density (OD_{578}) of 0.1 in 20-ml culture volumes. Cultivation was carried out in 100-ml Erlenmeyer flasks in a shaking water bath set to 220 rpm.

B. infantis (BGSC 29A3) and *B. panaciterrae* (DSM 19096) strains were obtained either from the Bacillus Genetic Stock Center (BGSC; Columbus, OH, USA) or purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). *B. infantis* cultures were maintained on LB-agar plates and incubated at 37°C; *B. panaciterrae* cultures were maintained on R2A medium (111) and incubated at 30°C. To study the osmostress protection of *B. infantis* by various compatible solutes, an overnight culture of this strain was prepared in morpholinepropanesulfonic acid (MOPS)-buffered basal medium (112) that contained

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TABLE 4 Plasmids used in this study[a]

Plasmid	Genotype/description	Reference or source
pX	Vector for the integration of genes into the *amyE* gene	99
pChen3	*opuC* operon with native promoter cloned into pX-*amyE*	82
pHK2	$opuF_{B.i.}$ operon with native promoter cloned into pX-*amyE*	This study
pHK3	$opuF_{B.p.}$ operon with native promoter cloned into pX-*amyE*	This study

[a]All plasmids carried the genes *bla*, which confers resistance against the antibiotic ampicillin in *E. coli* but not in *B. subtilis*, and *cat*, which confers resistance against the antibiotic chloramphenicol in *B. subtilis*.

0.4% (wt/vol) Casamino Acids. Using this preculture, a main culture (5 ml) was inoculated to an OD_{578} of about 0.1 in basal medium containing 0.9 M NaCl but lacking Casamino Acids. Compatible solutes were added to this medium to a final concentration of 1 mM. The *B. infantis* cultures were grown at 37°C for 29 h, and their growth yield was then determined by measuring their OD_{578} values.

Recombinant DNA procedures. DNA sequences relevant for the cloning of the various *opuFA-opuFB* gene clusters were retrieved from the deposited genome sequences of *B. infantis* (BGSC 29A3) and *B. panaciterrae* (DSM 19096), which are available under accession numbers 1367477 and 1121093, respectively. Chromosomal DNA of these bacilli was prepared and used as a PCR template to amplify the *opuFA-opuFB* gene clusters harboring their native promoter; the primer pairs BI-fusitransp-for and BI-fusitransp-rev and BP-fusitransp-for and BP-fusitransp-rev were used for these PCR amplification reactions (Table 3). The obtained PCR products and the *B. subtilis amyE*-integration vector pX (99) were cleaved either with BamHI (for *opuFA-opuFB*$_{B.i.}$) or with PvuII (for *opuFA-opuFB*$_{B.p.}$). DNA purification and ligation yielded plasmids pHK2-(*opuFA-opuFB*$_{B.i.}$) and pHK3-(*opuFA-opuFB*$_{B.p.}$), respectively (Table 4). The DNA sequences of the cloned *opuFA-opuFB* regions were verified by DNA sequence analysis (Eurofins MWG Operon, Ebersberg, Germany) using custom-synthesized primers (Microsynth AG, Balgach, Switzerland).

To physiologically characterize the OpuF ABC transport systems from *B. infantis* and *B. panaciterrae*, we integrated each of the cloned *opuFA-opuFB* operons under the transcriptional control of their native promoters as a single copy into the chromosome (at the *amyE* gene) of the *B. subtilis* chassis strain LTB52 (Table 1). This chassis strain was constructed by transformation of strain TMB118 (strain JH642 with Δ*opuA*::*tet*, Δ*opuC*::*spc*, Δ*opuD*::*kan*, and Δ*opuB*::*ery* mutations) (82) with chromosomal DNA of the *B. subtilis* strain CAB5 (strain 168 with a Δ*gbsR*::*zeo* mutation), thereby yielding strain LTB52, a derivative of TMB118 carrying the Δ(*gbsR*::*zeo*) gene disruption mutation (Table 1). To obtain appropriate control strains for our experiments, we inserted the empty pX vector (99) and plasmid pChen3 carrying the *B. subtilis opuC* wild-type operon (82) into the genome of the LTB52 chassis strain, yielding strains HKB12 (*amyE*::pChen3) and HKB13 (*amyE*::pX), respectively (Table 1).

Transport assays. To determine the kinetic parameters of the OpuF ABC transport systems, *B. subtilis* strains possessing the *opuFA-opuFB* gene clusters from *B. infantis* or from *B. panaciterrae* (strains HKB9, and HKB15; Table 1) were grown in SMM containing 0.4 M NaCl to an OD_{578} of about 0.3 at 37°C. The uptake of [1-^{14}C]glycine betaine at various substrate concentrations was followed over time, and the amount of the imported [1-^{14}C]glycine betaine was determined by scintillation counting. The glycine betaine concentration used in the experiments varied between 3 μM and 120 μM and was spiked with a 1 μM concentration of the radiolabeled substrate. The details of this type of transport assay haven been reported (24, 37, 82).

Database searches and *in silico* analysis of proteins. Genome sequences of members of the genus *Bacillus* were retrieved from the IMG/M database (https://img.jgi.doe.gov/cgi-bin/m/main.cgi) (113) (date of the database search, 8 May 2018) for which 16S rRNA gene data from the SILVA database (84) also were available. This set of genome sequences was manually curated so that only one representative of individual species (or not-yet taxonomically completely defined strains) was represented (final data set, 175 entries). The phylogenetic tree was built based on a 16S rRNA gene alignment of the chosen *Bacillus* species/strain provided by the Joint Genome Institute (JGI) Web server (113). This Web server builds a phylogenetic tree using the Distance Tree Tool (https://img.jgi.doe.gov/cgi-bin/m/main.cgi?section=DistanceTree&page=tree), which relies on an alignment of 16S rRNA genes present in the SILVA database (84) using the dnadist and neighbor tools from the PHYLIP package (http://evolution.genetics.washington.edu/phylip/doc/). As an outgroup for our phylogenetic analysis of members of the *Bacillus* genus, two genomes of the genus *Clostridium*, a superfamily within the *Firmicutes*, were used. The amino acid sequence of the SBP (OpuCC) of the OpuC ABC transporters was retrieved from the genome sequence of the domesticated *B. subtilis* strain JH642 (26) and used as a query for a BLASTP database search at the IMG/M database (https://img.jgi.doe.gov/cgi-bin/w/main.cgi) maintained by the Department of Energy (DOE) Joint Genome Institute (113). By inspecting the corresponding gene neighborhood of the retrieved OpuBC/OpuCC protein sequences using a tool provided by the IMG/M website, we ascertained that the other components of the OpuB and OpuC ABC transport systems were also present. Additionally, we used the *B. subtilis* OpuAC SBP of the OpuA ABC transport system (24, 73) and the OpuD (37) and OpuE (41) osmolyte transporter proteins for a BLASTP database search to find related transporters within the genus *Bacillus*. The OpuCC and OpuBC amino acid sequences retrieved through the BLASTP search were aligned using MAFFT (114) for the inspection of conserved residues, in particular for those that form the aromatic cages in the ligand-binding sites of the OpuBC and OpuCC SBPs (75, 76).

The topological arrangement of the OpuFB TMD-SBP hybrid protein from *B. infantis* with respect to the plane of the cytoplasmic membrane was predicted using the TOPCONS server (http://topcons.cbr.su.se/) (115). For the *in silico* modeling of the SBP domain of the *B. infantis* OpuFB protein, we used the

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SWISS-MODEL server (https://swissmodel.expasy.org/) (94). The *in silico*-derived structural model for the OpuFB SBP domain was visualized with PyMOL (https://pymol.org/2/) (116). The phylogenetic relationship of the *B. infantis* OpuFB SBP domain with compatible-solute-binding proteins whose crystal structure has been determined was analyzed by using an amino acid sequence alignment using the MAFFT server (https://mafft.cbrc.jp/alignment/server/) (114) with the bootstrapping setting (100 bootstraps) provided by the website. The resulting data set was visualized in a neighbor-joining phylogenetic tree using the Interactive Tree of Life (iTOL) Web resources (https://itol.embl.de/) (117). The structural data of the crystalized proteins were obtained from the RCSB Protein Data Bank (PDB) (https://www.rcsb.org/) (118) and visualized with PyMOL.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01728-18.

SUPPLEMENTAL FILE 1, PDF file, 4.9 MB.

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