Three Different Systems Participate in L-Cystine Uptake in *Bacillus subtilis*

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The symporter YhcL and two ATP binding cassette transporters, YtmJKLMN and YckKJI, were shown to mediate L-cystine uptake in *Bacillus subtilis*. A triple $\Delta yhcL \Delta ytmJKLMN \Delta yckK$ mutant was unable to grow in the presence of L-cystine and to take up L-cystine. We propose that *yhcL*, *ytmJKLMN*, and *yckKJI* should be renamed *tcyP*, *tcyJKLMN*, and *tcyABC*, respectively. The L-cystine uptake by YhcL ($K_m = 0.6 \mu\text{M}$) was strongly inhibited by seleno-DL-cystine, while the transport due to the YtmJKLMN system ($K_m = 2.5 \mu\text{M}$) also drastically decreased in the presence of DL-cystathionine, L-djenkolic acid, or S-methyl-L-cysteine. Accordingly, a $\Delta ytmJKLMN$ mutant did not grow in the presence of $100 \mu\text{M}$ DL-cystathionine, $100 \mu\text{M}$ L-djenkolic acid, or $100 \mu\text{M}$ S-methyl-L-cysteine. The expression of the *ytmI* operon and the *yhcL* gene was regulated in response to sulfur availability, while the level of expression of the *yckK* gene remained low under all the conditions tested.

Eukaryotic, bacterial, and archaeal cells contain a large number of integral membrane proteins and protein complexes involved in solute transport across the membrane. In *Escherichia coli* and *Bacillus subtilis* as many as 285 and 239 genes encode membrane transport proteins, respectively (25, 30), and 14% (*E. coli*) and 20% (*B. subtilis*) of these proteins are proposed to participate in amino acid transport (25). However, identification of permease function is often complex due to the existence of paralogues with similar or different substrate specificities and due to functional redundancy (14). Complete genome sequences and expression profiling experiments provide a powerful tool for analyzing global transcriptional patterns and for identifying gene function. DNA arrays have been used to investigate the changes in gene transcript levels during growth of the *B. subtilis* wild-type strain in the presence of sulfate or methionine as a sole sulfur source (2). Many sulfur-regulated genes encode transporters that are good candidates for the uptake of sulfur-containing compounds (2). *B. subtilis* can use methionine, homocysteine, cystathionine, cystine, sulfate, sulfite, thiosulfate, and sulfonates as sole sulfur sources, indicating that these sulfur compounds are efficiently taken up. A sulfate permease (CysP), a sulfonate ATP binding cassette (ABC) transporter (SsuABC), and a methionine ABC transporter (MetNPQ) have been experimentally characterized (15, 21, 43). In contrast, little is known about the transport of cysteine and its oxidized form cystine in this bacterium. Cysteine uptake has been investigated mainly in *Saccharomyces cerevisiae* and in enterobacteria (9, 17). In yeast, seven different permeases, which also transport other amino acids, participate in cysteine uptake. It therefore appears that cysteine is not taken up by one specific permease but rather is taken up by

multiple transporters with broad specificity (9). In *Salmonella enterica* serovar Typhimurium, L-cystine is taken up by three different systems, CTS-1 ($K_m = 2 \mu\text{M}$), CTS-2 ($K_m = 0.1 \mu\text{M}$), and CTS-3 (nonsaturable) (3). In *E. coli*, two kinetically identifiable cystine transport systems are present; one is shared with diaminopimelic acid and several cystine analogues, and the other is more specific (4). The first system, which is sensitive to osmotic shock, corresponds to an ABC transporter. The bacterial ABC importers include one or two ATP binding proteins localized to the inner side of the cytoplasmic membrane, one or two transmembrane proteins, and a high-affinity solute binding protein external to the cytoplasmic membrane (5, 13, 39). The periplasmic cystine binding protein from *E. coli* has been characterized and was identified as FliY (6, 24). Its synthesis was increased during sulfate starvation (27). The *fliY* gene forms an operon with *fliA* encoding σ^F , which participates in transcription of class III genes involved in flagellar synthesis (24). FliY is not required for motility, and its possible role in flagellar synthesis remains to be established. The YecS and YecC proteins probably correspond to the *E. coli* permease and ATP binding protein of a cystine ABC transporter, but this has never been substantiated by experimental data (8, 14). The *yecS* and *yecC* genes are separated from *fliY* only by the *yedO* gene encoding a D-cysteine desulfhydrase. It has been proposed that these genes could form an operon (36, 41). In gram-positive bacteria, the solute binding protein BspA has been shown to be required for L-cystine uptake in *Lactobacillus fermentum* (42). In this work, we tested the possible involvement in L-cystine transport of different permeases whose synthesis was increased in the presence of methionine (2). We characterized the three transporters involved in L-cystine uptake in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. subtilis* strains used in this work are listed in Table 1. *E. coli* cells were grown in Luria-Bertani broth (31). *B. subtilis* was grown in SP medium or in minimal medium (6 mM K_2HPO_4 , 4.4 mM KH_2PO_4 , 0.3 mM trisodium citrate, 4 mM MgCl_2 , 250 μM CaCl_2 , 10 μM

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TABLE 1. *B. subtilis* strains used

<i>B. subtilis</i> strain	Genotype ^a	Source or reference
168	<i>trpC2</i>	Laboratory stock
BSIP1214	<i>trpC2 ytlI::aphA3</i>	7
BSIP1256	<i>trpC2 amyE::(PytmI'-lacZ cat)</i>	pDIA5599 → 168
BSIP1321	<i>trpC2 amyE::(Pyhcl'-lacZ cat)</i>	pDIA5629 → 168
BSIP1389	<i>trpC2 ΔytmJKLMN::aphA3</i>	— ^b
BSIP1398	<i>trpC2 ΔytmJKLMN::aphA3 yhcL'::lacZ-erm ΔyhcL</i>	BSIP1389 → BFS1605
BSIP1534	<i>trpC2 ΔyhcL::spc</i>	pDIA5669 → 168
BSIP1570	<i>trpC2 ΔyxeMNO::cat</i>	—
BSIP1572	<i>trpC2 ΔytmJKLMN::aphA3 ΔyxeMNO::cat</i>	BSIP1389 → BSIP1570
BSIP1575	<i>trpC2 ΔyxeMNO::cat ΔyhcL::spc</i>	BSIP1570 → BSIP1534
BSIP1576	<i>trpC2 ΔytmJKLMN::aphA3 ΔyxeMNO::cat ΔyhcL::spc</i>	BSIP1534 → BSIP1572
BSIP1582	<i>trpC2 ΔytmJKLMN::aphA3 ΔyhcL::spc</i>	BSIP1389 → BSIP1534
BSIP1601	<i>trpC2 ΔytmJKLMN::aphA3 ΔyxeMNO::cat ΔyhcL::lacZ-erm ΔyhcL</i>	BSIP1570 → BSIP1398
BSIP1603	<i>trpC2 ΔytmJKLMN::aphA3 ΔyxeMNO::cat yhcL'::lacZ-erm ΔyhcL yckK::Tn10</i>	Transposon mutagenesis
BSIP1643	<i>trpC2 ΔytmJKLMN::aphA3 yckK'::lacZ-erm ΔyckK</i>	BSIP1389 → BFS4376
BSIP1644	<i>trpC2 ΔyxeMNO::cat yckK'::lacZ-erm ΔyckK</i>	BSIP1570 → BFS4376
BSIP1645	<i>trpC2 ΔyhcL::spc yckK'::lacZ-erm ΔyckK</i>	BSIP1534 → BFS4376
BSIP1646	<i>trpC2 ΔytmJKLMN::aphA3 ΔyxeMNO::cat yckK'::lacZ-erm ΔyckK</i>	BFS4376 → BSIP1572
BSIP1647	<i>trpC2 ΔyxeMNO::cat ΔyhcL::spc yckK'::lacZ-erm ΔyckK</i>	BFS4376 → BSIP1575
BSIP1648	<i>trpC2 ΔytmJKLMN::aphA3 ΔyxeMNO::cat ΔyhcL::spc yckK'::lacZ-erm ΔyckK</i>	BFS4376 → BSIP1576
BSIP1649	<i>trpC2 ΔytmJKLMN::aphA3 ΔyhcL::spc yckK'::lacZ-erm ΔyckK</i>	BFS4376 → BSIP1582
BSIP1689	<i>trpC2 amyE::(Pyhcl'-lacZ cat) ΔytmJKLMN::aphA3</i>	BSIP1389 → BSIP1321
BSIP1690	<i>trpC2 amyE::(PytmI'-lacZ cat) ΔytmJKLMN::aphA3</i>	BSIP1389 → BSIP1256
BSIP1691	<i>trpC2 amyE::(Pyhcl'-lacZ cat) ΔyhcL::spc</i>	BSIP1534 → BSIP1321
BSIP1692	<i>trpC2 amyE::(PytmI'-lacZ cat) ΔyhcL::spc</i>	BSIP1534 → BSIP1256
BSIP1693	<i>trpC2 amyE::(Pyhcl'-lacZ cat) ΔytmJKLMN::aphA3 ΔyhcL::spc</i>	BSIP1534 → BSIP1689
BSIP1694	<i>trpC2 amyE::(PytmI'-lacZ cat) ΔytmJKLMN::aphA3 ΔyhcL::spc</i>	BSIP1534 → BSIP1690
BSIP1701	<i>trpC2 amyE::(Pyhcl'-lacZ cat) ΔytlI::aphA3</i>	BSIP1214 → BSIP1321
BFS1605	<i>trpC2 yhcL'::lacZ-erm ΔyhcL</i>	S. Bron
BFS4376	<i>trpC2 yckK'::lacZ-erm ΔyckK</i>	K. Yamane

^a *cat*, pC194 chloramphenicol acetyltransferase gene; *aphA3*, *Enterococcus faecalis* kanamycin resistance gene; *spc*, *Staphylococcus aureus* spectinomycin resistance gene; *erm*, pMUTIN erythromycin resistance gene.

^b —, see Materials and Methods.

MnCl₂, 0.5% glucose, 50 mg of L-tryptophan liter⁻¹, 11 mg of ferric ammonium citrate liter⁻¹, 0.1% L-glutamine) supplemented with one of the following sulfur sources: 1 mM K₂SO₄, 1 mM L-methionine, 1 mM DL-homocysteine, 20 μM to 1 mM L-cystine, 0.1 to 1 mM DL-cystathionine, 0.1 to 1 mM L-djenkolic acid, or 0.1 to 1 mM S-methylcysteine. In this minimal medium, residual growth in the absence of any added sulfur source was observed. To avoid this problem, an exhausted minimal medium was obtained by growing *B. subtilis* 168 in a sulfur-free minimal medium and then centrifuging the culture and filtering the supernatant (15). Antibiotics were added at the following concentrations when required: ampicillin, 100 μg ml⁻¹; chloramphenicol, 5 μg ml⁻¹; spectinomycin, 100 μg ml⁻¹; kanamycin, 5 μg ml⁻¹; and erythromycin plus lincomycin, 1 and 25 μg ml⁻¹, respectively. Solid media were prepared by addition of 20 g of Noble agar (Difco) liter⁻¹. Standard procedures were used to transform *E. coli* and *B. subtilis* (19, 31).

The loss of amylase activity was detected as previously described (38). β-Galactosidase specific activity was measured as described by Miller (23) with cell extracts obtained by lysozyme treatment. Protein concentrations were determined by the method of Bradford. One unit of β-galactosidase activity was defined as the amount of enzyme that produced 1 nmol of *o*-nitrophenol min⁻¹ at 28°C. The mean values for at least three independent experiments are presented below. The standard deviations were less than 15%.

The resistance of *B. subtilis* strains to a toxic analogue of L-cystine, seleno-DL-cystine, was tested as follows. Overnight cultures were grown in minimal medium containing L-methionine. The cells were diluted to an optical density at 600 nm of 1 and spread on plates of the corresponding agar medium. A paper disk (diameter, 6 mm) was laid on the agar and soaked with 10 μl of a 50 mM seleno-DL-cystine solution. The area of growth inhibition was measured.

DNA manipulations. Plasmids from *E. coli* and chromosomal DNA from *B. subtilis* were prepared according to standard procedures. Restriction enzymes, *Taq* DNA polymerase, and phage T4 DNA ligase were used as recommended by the manufacturers. DNA fragments were purified from agarose gels with a Qiaquick kit (QIAGEN, Basel, Switzerland). DNA sequences were determined by using the dideoxy chain termination method with plasmid DNA as the template and a Thermo Sequenase kit (Amersham Pharmacia Biotech).

Plasmid and strain construction. Plasmid pAC6 (38) allowed construction of transcriptional fusions between the *yhcL* and *ytmI* promoter regions and the promoterless *lacZ* gene. The DNA fragments corresponding to a region upstream from *yhcL* (nucleotides -206 to +59 relative to the *yhcL* translational start site) or a region upstream from *ytmI* (nucleotides -131 to -9 relative to the *ytmI* translational start site) were amplified by PCR with the creation of EcoRI and BamHI sites. The PCR products were inserted into pAC6, yielding pDIA5629 (*Pyhcl*) and pDIA5599 (*PytmI*), respectively. These plasmids were linearized with ScaI, which allowed insertion of the transcriptional *lacZ* fusions as single copies at the *amyE* locus (Table 1).

A *yhcL* deletion mutant (BSIP1534) was constructed as follows. We cloned a 472-bp EcoRI/SmaI fragment, a SmaI/SmaI fragment carrying the *spc* spectinomycin cassette, and a 755-bp SmaI/BamHI DNA fragment between the EcoRI and BamHI sites of pJH101 (10) to obtain plasmid pDIA5669. The EcoRI/SmaI fragment and the SmaI/BamHI DNA fragment were generated by PCR by using oligonucleotides with the creation of restriction sites. These fragments corresponded to the 5' part of *yhcL* until codon 64 and the 3' part of *yhcL* with the 117 last codons. Plasmid pDIA5669 was linearized at the unique ScaI site and used to transform *B. subtilis* 168. Spectinomycin-resistant, chloramphenicol-sensitive integrants arose through a double-crossover event in which most of the *yhcL* coding sequence was deleted and replaced by the spectinomycin resistance gene (Table 1 and Fig. 1).

To construct strain BSIP1389, in which the *ytmJKLMN* genes were replaced by a kanamycin resistance cassette, a four-primer PCR procedure was used (44). The regions upstream from *ytmJ* (nucleotides -873 to +224 relative to the translational start site of *ytmJ*) and downstream from *ytmN* (nucleotides -614 to +420 relative to the translational stop site of *ytmN*) were amplified by PCR so that 21-bp fragments corresponding to the *aphA3* gene were introduced at one of the ends. The *ytmJ* upstream region and the *ytmN* downstream region overlapping the *aphA3* gene at one of the ends then served as long primers for PCR with *aphA3* as the template. In this second PCR, two external primers were added. The final product, corresponding to the two regions flanking the *ytmJ* and *ytmN* genes with the inserted *aphA3* cassette between them, was used to transform *B. subtilis* 168, giving strain BSIP1389 (Table 1 and Fig. 1). To limit the polar effect

FIG. 1. Genetic organization and disruption of the different transporters. The putative promoters of the *ymtI*, *yxeK*, *yckK*, and *yhcL* genes or operons and the transcriptional terminators are indicated. The checked boxes correspond to the ATP binding cassettes of the ABC transporters, the cross-hatched boxes correspond to the membrane permeases, and the striped boxes correspond to the solute binding proteins.

on the genes located downstream from *ytmN*, the terminator of the *aphA3* gene was absent and the cassette was transcribed in the same orientation as the *ymtI* operon. To confirm transcription of the downstream genes, we showed that a *ymtJ'-lacZ* fusion (BFS70) was expressed in the $\Delta ytmJKLMN$ mutant.

To construct strain BSIP1570, in which the *yxeMNO* genes were replaced by a chloramphenicol resistance cassette (*cat* gene), a four-primer PCR procedure was used as described above for deletion of the *ytmJKLMN* genes. The regions upstream from *yxeM* (nucleotides -983 to +42 relative to the translational start site of *yxeM*) and downstream from *yxeO* (nucleotides +317 to +1191 relative to the translational start site) were amplified by PCR. To limit the polar effect on the genes located downstream from *yxeO*, the terminator of the *cat* gene was absent and the cassette was transcribed in the same orientation as the *yxeK* operon (Fig. 1).

We used some strains disrupted by fusion with the *lacZ* reporter gene within the framework of European Union and Japanese projects for functional analysis of the *B. subtilis* genome (<http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.oper1> and <http://bacillus.genome.ad.jp>) (16).

Transposon mutagenesis. A transposon bank was constructed by introduction of the mini-Tn10 delivery vector pLC333 (37) into the *B. subtilis* 168 strain. Several thousand independent clones were pooled, and nine samples of chromosomal DNA were prepared for further use (33). To obtain selenocystine-resistant clones, *B. subtilis* BSIP1601 ($\Delta ytmJKLMN::aphA3 \Delta yxeMNO::cat yhcL::lacZerm \Delta yhcL$) was transformed with chromosomal DNA corresponding to the prepared transposon bank (33). Clones were selected on SP plates containing spectinomycin (60 $\mu\text{g ml}^{-1}$). By using velvet replicas, clones were transferred onto minimal medium plates containing methionine (250 μM), selenocystine (10 μM), and spectinomycin (60 $\mu\text{g ml}^{-1}$). After 24 h of growth, selenocystine-

resistant clones were isolated. The single-transposon insertion event was confirmed by backcrossing into strain BSIP1601, and the strain was checked for selenocystine resistance. To determine the location of the transposon insertion, chromosomal DNA was prepared, digested with *EcoRI*, and self-ligated. After transformation in *E. coli*, spectinomycin-resistant clones were selected, and the corresponding plasmids were sequenced. The following primers were used for sequencing of transposon insertions: Tn10 left (5'-GGCCGATTCATTAATGCAAGG3') and Tn10 right (5'-CGATATTCACGGTTTACCCAC3').

L-Cystine uptake and substrate specificity of transporters. Cells were grown in minimal medium in the presence of L-methionine as the sole sulfur source to the middle of the exponential growth phase. They were harvested by centrifugation for 10 min and washed twice with medium A (6 mM K_2HPO_4 , 4.4 mM KH_2PO_4 , 0.3 mM trisodium citrate, 200 mM xylose, 0.5% glucose, 0.2 mM K_2SO_4 , 60 μg of chloramphenicol ml^{-1}). The substrate L-[^{14}C]cystine was added at a concentration of 20 μM , and the reaction mixture was incubated at 37°C. Samples (200 μl) were withdrawn at intervals and filtered through 0.45- μm -pore-size Durapore membranes (HVLP02500; Millipore). The filters were washed with 5 ml of medium A, dried, and transferred to vials containing 15 ml of a counting scintillant (NBCS 104; Amersham Biosciences) for determination of the radioactivity.

To determine the apparent K_m values of YhcL and YtmJKLMN, we used strains BSIP1646 ($\Delta ytmJKLMN::aphA3 \Delta yxeMNO::cat yckK::lacZ erm \Delta yckK$) and BSIP1647 ($\Delta yxeMNO::cat \Delta yhcL::spc yckK::lacZ erm \Delta yckK$) lacking all the other possible L-cystine transport systems, respectively. To estimate the K_m of YhcL, the rates of uptake were determined at 20°C to slow down the uptake and thus facilitate measurement of the initial rates. The experiments were performed at 37°C for YtmJKLMN.

The effects of addition of unlabeled amino acids or sulfur compounds at a

FIG. 2. Growth of the wild-type strain and of different mutants with several transporters inactivated in the presence of L-cystine or sulfate. Growth curves for the following strains are shown: 168 (■), BSIP1582 ($\Delta ytmJKLMN::aphA3 \Delta yhcL::spc$) (○), BSIP1576 ($\Delta yxeMNO::cat \Delta ytmJKLMN::aphA3 \Delta yhcL::spc$) (▲), BSIP1649 ($\Delta ytmJKLMN::aphA3 \Delta yhcL::spc yckK'::lacZ-erm \Delta yckK$) (●), and BSIP1648 ($\Delta yxeMNO::cat \Delta ytmJKLMN::aphA3 \Delta yhcL::spc yckK'::lacZ-erm \Delta yckK$) (△). Cells were grown in exhausted minimal medium containing either 20 μ M cystine (A), 1 mM cystine (B), or 40 μ M sulfate (C). OD 600 nm, optical density at 600 nm.

concentration of 50 μ M to the reaction mixture containing 5 μ M L-[14 C]cystine were determined. A 3-min transport kinetics analysis was performed to ensure linearity of the curves. The inhibition of L-cystine uptake at 2 min was determined.

RESULTS

***B. subtilis* transport systems whose synthesis is derepressed in the presence of methionine.** Transcriptome experiments revealed that several genes encoding transporters (*yhcL*, *ytmJ*, *ytmK*, *ytmL*, *ytmM*, *ytmN*, *yxeM*, and *yxeO*) were expressed more in the presence of methionine than in the presence of sulfate (2). The *yhcL* gene encodes a membrane protein belonging to the dicarboxylate amino acid:cation (Na^+ and/or H^+) symporter family (TC 2.A.23) (30). This type of transporter, which is found in eukaryotes, archaeobacteria, and bacteria, is involved in the transport of dicarboxylic acids, of semi-polar and neutral amino acids (Ala, Ser, Cys, and Thr), of both neutral and acidic amino acids, or of dibasic amino acids (29). The bacterial dicarboxylate amino acid:cation symporters are about 450 amino acids long (range, 420 to 491 amino acids) and have 10 to 12 putative transmembrane segments (35). In *B. subtilis*, four members of this family are present; they are two glutamate transporters (GltP and GltT), a C_4 -dicarboxylic acid transporter (DctP), and YhcL (1, 18, 40).

The YtmJKLMN and YxeMNO proteins belong to the polar amino acid uptake transporter family of the ABC transporters (TC 3.A.1.3) (14, 28). The YtmJ, YtmK, and YxeM polypeptides exhibit similarities to the L-cystine binding protein of *E. coli*, FliY (28, 28, and 35% identity, respectively) (6). The YtmL, YtmM, and YxeN proteins are similar to *E. coli* YecS (35, 32, and 48% identity, respectively), while YtmN and YxeO exhibit 50 and 52% identity to YecC. The *ytmJKLMN* genes belong to the large *ytmIJKLMNO-ytmIJ-ribR-ytmLM* operon (7, 34), while the *yxeMNO* genes are part of the *yxeKLMNOPQR* operon (Fig. 1). Remarkably, six of the eight products of the latter operon exhibit sequence similarities with the proteins encoded by the *ytmI* operon. These two operons could have evolved from the same ancestral operon by sequence duplications and rearrangements.

Due to their similarities with the L-cystine uptake system from *E. coli*, the YxeMNO and YtmJKLMN proteins are good candidates for L-cystine transporters in *B. subtilis*. The *yhcL* gene, which is expressed in the same conditions as the *ytmI* and *yxeK* operons, could also be involved in the uptake of this compound. To test the role of YxeMNO, YtmJKLMN, and YhcL in L-cystine transport, single and multiple mutants were constructed. We disrupted the coding regions of the *yxeMNO*, *ytmJKLMN*, and *yhcL* genes by double-crossover events that resulted in marker replacement (Fig. 1) (see Materials and Methods). In order to avoid major polar effects on downstream genes, the *ytmJKLMN* and *yxeMNO* genes were replaced by antibiotic resistance genes inserted in the same orientation with their transcription terminators deleted.

Strain BSIP1576 ($\Delta yxeMNO::cat \Delta ytmJKLMN::aphA3 \Delta yhcL::spc$), in which the three systems were inactivated, could still grow in the presence of 1 mM L-cystine (Fig. 2B). This indicates that at least one more L-cystine transporter exists in *B. subtilis*.

Screening of a transposon library for selenocystine-resistant mutants in a $\Delta yhcL \Delta ytmJKLMN \Delta yxeMNO$ strain. Selenocystine is a toxic analogue of L-cystine. We therefore used this molecule to identify *B. subtilis* mutants with inactivated L-cystine uptake systems. The wild-type strain was sensitive to this toxic compound, and the growth inhibition area on plates was 3.8 cm wide in the presence of 10 μ l of a 50 mM selenocystine solution. The growth inhibition of strain BSIP1576 was quite similar to that of the wild-type strain, but spontaneous selenocystine-resistant mutants appeared close to the paper disk containing selenocystine (data not shown). These spontaneous mutants showed no growth inhibition in the same conditions. This strongly suggested that a unique active cystine transporter was present in the triple mutant BSIP1576 ($\Delta yxeMNO::cat \Delta ytmJKLMN::aphA3 \Delta yhcL::spc$).

To identify this additional transporter, random transposition mutagenesis was performed. Both strain BSIP1576 and the mini-Tn10 transposon (37) carried a spectinomycin resistance marker. Next, we constructed strain BSIP1601 ($\Delta yxeMNO::cat \Delta ytmJKLMN::aphA3 yhcL'::lacZerm$). In this strain the spectinomycin cassette disrupting the *yhcL* gene in strain BSIP1576

was replaced by a *lacZ-erm* gene obtained from strain BFS1605 (*yhcL::lacZ-erm ΔyhcL*). Strain BSIP1601 was transformed with a random transposon library (see Materials and Methods). Mutants were selected for growth in the presence of 10 μM seleno-DL-cystine on plates containing L-methionine as the sole sulfur source. In order to ascertain that the selenocystine-resistant phenotype did not come from secondary mutations but was directly related to the transposon insertion, chromosomal DNA was extracted from each putative mutant and back-transformed into strain BSIP1601, with selection for the transposon antibiotic marker. The selenocystine resistance phenotype was subsequently checked, and a mutant that passed the test was retained. The insertion site of the mini-Tn10 transposon was then determined. Sequence analysis revealed that the transposon had been inserted into codon 182 of the *yckK* gene, which encodes the solute binding protein of a polar amino acid ABC transporter (<http://genolist.pasteur.fr/SubtiList>). Interestingly, the *yckK* gene seems to form an operon with the *yckJ* and the *yckI* downstream genes. YckJ corresponds to the permease, and YckI corresponds to the ATP binding protein of an ABC transporter.

Phenotypes of mutants with the four different uptake systems inactivated. To investigate the relative roles of the *yxeMNO*, *ytmJKLMN*, *yhcL*, and *yckKJI* genes in L-cystine transport, several mutants carrying one to four mutations were constructed. To do this, a *yckK* mutant, BFS4376 (*yckK'::lacZ-erm ΔyckK*) obtained during the *Bacillus* Functional Analysis Program, was used (Table 1). We examined the effects of *yxeMNO*, *ytmJKLMN*, *yhcL*, and *yckK* gene disruptions on *B. subtilis* growth in sulfur-exhausted minimal medium containing either 20 μM L-cystine, 1 mM L-cystine, or 40 μM sulfate (Fig. 2). Single *yxeMNO*, *ytmJKLMN*, *yhcL*, and *yckK* mutants grew similar to the wild-type strain in the presence of sulfate or L-cystine at both concentrations (data not shown). Strains with two different transporters inactivated were therefore grown with 20 μM or 1 mM L-cystine. Strains BSIP1572, BSIP1575, BSIP1643, BSIP1644, and BSIP1645 grew similar to the wild-type strain at both concentrations (data not shown). In contrast, strain BSIP1582 (*ΔytmJKLMN::aphA3 ΔyhcL::spc*) displayed significant growth retardation with both L-cystine concentrations (Fig. 2A and B), while it grew as well as the wild-type strain with sulfate (Fig. 2C). The doubling times of this mutant were 220 and 60 min in the presence of 20 μM and 1 mM L-cystine, respectively, instead of the 40 min observed for the wild-type strain (Fig. 2A and B). To test the participation of each component of the ABC transporter encoded by the *ytmI* operon, we used the *ytmJ*, *ytmK*, *ytmL*, and *ytmM* mutants constructed during the *Bacillus* Functional Analysis Program (16). In these mutants, the downstream genes are expressed under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *Pspac* promoter to avoid major polar effects (Fig. 1). Like strain BSIP1582 (*ΔytmJKLMN::aphA3 ΔyhcL::spc*), all the double mutants with mutations in *yhcL* and *ytmJ*, *ytmK*, *ytmL*, or *ytmM* poorly grew in the presence of 20 μM cystine and IPTG (data not shown). The disruption of the downstream genes *ytmO* and *ytmN* (Fig. 1) in a *ΔyhcL* background led to normal growth in the same conditions. The results strongly suggest that the YtmJKLMN and YhcL systems are involved in L-cystine transport. In contrast, participation of YxeMNO seems unlikely

since strains BSIP1582 (*ΔytmJKLMN::aphA3 ΔyhcL::spc*) and BSIP1576 (*ΔyxeMNO::cat ΔytmJKLMN::aphA3 ΔyhcL::spc*) grew similarly in the presence of 20 μM or 1 mM L-cystine (Fig. 2A and B).

To determine the involvement of the YckKJI ABC transporter in the residual growth observed for a *ΔyhcL ΔytmJKLMN* double mutant, we tested the effect of inactivation of *yckK* in a BSIP1582 mutant. In the presence of a low or high L-cystine concentration, the growth of strain BSIP1582 (*ΔytmJKLMN::aphA3 ΔyhcL::spc*) was completely abolished by introduction of a *yckK* gene disruption (Fig. 2A and B). As a control, we verified that all the strains grew similar to the wild-type strain with sulfate (Fig. 2C). These results strongly suggest that YckK participates in L-cystine transport. Inactivation of the transporters encoded by the *ytmI* operon, the *yhcL* gene, and the possible *yckKJI* operon led to a complete absence of growth of *B. subtilis* with L-cystine, indicating that three L-cystine uptake systems are present in this bacterium. Moreover, it seems that YtmJKLMN and YhcL are higher-affinity transporters than YckKJI. Indeed, the double mutant BSIP1582 (*ΔytmJKLMN::aphA3 ΔyhcL::spc*) displayed growth retardation with 20 μM cystine, while strains BSIP1643 (*ΔytmJKLMN::aphA3 yckK'::lacZ-erm*) and BSIP1645 (*ΔyhcL::spc yckK'::lacZ-erm*) did not (Fig. 2A and data not shown).

L-Cystine uptake by the YhcL, YtmJKLMN, and YckKJI transporters. To confirm the role of the symporter and the two ABC transporters in L-cystine uptake, the abilities of *B. subtilis* strains 168, BSIP1534 (*ΔyhcL::spc*), BSIP1389 (*ΔytmJKLMN::aphA3*), BSIP1582 (*ΔytmJKLMN::aphA3 ΔyhcL::spc*), and BSIP1649 (*ΔytmJKLMN::aphA3 ΔyhcL::spc yckK'::lacZ-erm*) to take up this compound were tested. The data for uptake of L-[¹⁴C]cystine at a concentration of 20 μM were compared for the different backgrounds after growth of the strains with L-methionine, which allowed a high level of expression of the *ytmI* operon and the *yhcL* gene (2). A decrease in L-[¹⁴C]cystine uptake was observed in the *ΔytmJKLMN* or *ΔyhcL* mutant compared to the wild-type strain (Fig. 3A). The initial rates of L-cystine uptake were 1.9 nmol/min/mg of protein for the wild-type strain, 1.4 nmol/min/mg of protein for the *ΔytmJKLMN* mutant, and 0.85 nmol/min/mg of protein for the *ΔyhcL* mutant. The L-cystine uptake detected in the double mutant (*ΔyhcL::spc ΔytmJKLMN::aphA3*) was strongly reduced compared to the transport in single mutants (Fig. 3A). However, a low uptake rate (initial rate, 0.120 nmol/min/mg of protein) was obtained with strain BSIP1582, and uptake was completely abolished in strain BSIP1649 (*ΔytmJKLMN::aphA3 ΔyhcL::spc yckK'::lacZ-erm*) (Fig. 3B). This indicates that YckK was involved in the residual L-cystine transport observed in a *ΔyhcL ΔytmJKLMN* mutant. Three systems participated in L-cystine uptake in *B. subtilis*: the YhcL symporter and the two ABC transporters encoded by the *ytmI* and *yckKJI* operons. We then used strains BSIP1646 (*ΔytmJKLMN::aphA3 ΔyxeMNO::cat yckK'::lacZ-erm*) and BSIP1647 (*ΔyxeMNO::cat ΔyhcL::spc yckK'::lacZ-erm*) to estimate the K_m values of the YhcL and YtmJKLMN transporters, respectively. The apparent K_m values for L-cystine were 0.6 μM for the YhcL symporter and 2.5 μM for the YtmJKLMN ABC transporter.

Substrate specificity of the YtmJKLMN and YhcL systems. To estimate the substrate specificity of YhcL and YtmJKLMN, different sulfur-containing compounds and several amino acids

FIG. 3. Time course for L-[14 C]cystine uptake into *B. subtilis* cells. (A) Comparison of L-cystine uptake in strains 168 (■), BSIP1389 ($\Delta ytmJKLMN::aphA3$) (●), BSIP1534 ($\Delta yhcL::spc$) (▲), and BSIP1582 ($\Delta ytmJKLMN::aphA3 \Delta yhcL::spc$) (◆). (B) Comparison of L-cystine uptake in strains BSIP1582 ($\Delta ytmJKLMN::aphA3 \Delta yhcL::spc$) (◆) and BSIP1649 ($\Delta ytmJKLMN::aphA3 \Delta yhcL::spc yckK':lacZ-erm \Delta yckK$) (◇). Cells were grown at 37°C in minimal medium containing 250 μ M L-methionine. The cystine uptake analysis was performed in minimal medium in the presence of 20 μ M L-[14 C]cystine as described in Materials and Methods.

were tested. To do this, we measured the inhibition of labeled L-cystine uptake in the presence of a 10-fold excess of nonradioactive compounds in strains BSIP1647 and BSIP1646 containing the YtmJKLMN and YhcL transporters, respectively. In the presence of 50 μ M L-cystine, 50 μ M L-djenkolic acid, 50 μ M DL-cystathionine, 50 μ M seleno-DL-cystine, or 50 μ M S-methyl-L-cysteine, the YtmJKLMN-dependent L-[14 C]cystine uptake was reduced by 88, 89, 80, 80, and 73%, respectively (Table 2). Additionally, the levels of inhibition for diaminoimelic acid, DL-lanthionine, L-methionine sulfoxide, L-methionine, L-arginine, L-glutamine, L-histidine, and DL-homocysteine were 24 to 45%, while there was no significant inhibition by L-cystine or oxidized glutathione (Table 2). These results suggest that the ABC transporter encoded by the *ytmI* operon participates in the uptake of L-cystine, djenkolic acid, cystathi-

onine, selenocystine, and S-methylcysteine and that it can also recognize a much broader range of substrates.

For YhcL, a decrease in L-[14 C]cystine uptake was observed when a 10-fold excess of nonradioactive L-cystine (92%) or seleno-DL-cystine (67%) was added (Table 3). The levels of inhibition for L-leucine, L-alanine, and L-valine were 48, 43, and 42%, respectively. Decreases in L-cystine uptake (18 to 40%) were also detected in the presence of S-methylcysteine, L-cysteine, L-djenkolic acid, DL-cystathionine, L-methionine, and DL-homocysteine (Table 3). The YhcL symporter seems to be more specific for L-cystine, but it could also transport a much broader range of amino acids and sulfur compounds.

We then tested the effect of an *ytmJKLMN* gene disruption on utilization of djenkolic acid, cystathionine, and S-methylcysteine, a set of substrates more specific for this ABC transporter (Table 2). Growth of strain BSIP1389 ($\Delta ytmJKLMN::$

TABLE 2. Inhibition of YtmJKLMN-dependent L-[14 C]cystine uptake by different compounds

Competitor ^a	% Inhibition
L-Cystine.....	88 $\pm$ 4
L-Djenkolic acid	89 $\pm$ 2
DL-Cystathionine	80 $\pm$ 6
Seleno-DL-cystine	80 $\pm$ 3
S-Methyl-L-cysteine.....	73 $\pm$ 8
L-Histidine.....	45 $\pm$ 2
L-Methionine	40 $\pm$ 1
DL-Diaminopimelic acid	39 $\pm$ 1
DL-Lanthionine	32 $\pm$ 3
L-Glutamine.....	32 $\pm$ 4
L-Arginine	28 $\pm$ 2
DL-Homocysteine	24 $\pm$ 2
L-Cysteine.....	<10
Oxidized glutathione.....	<10

^a L-[14 C]cystine was used at a concentration of 5 μ M, and the unlabeled amino acids or sulfur compounds were present at a concentration of 50 μ M. The initial L-[14 C]-cystine uptake rate was measured.

TABLE 3. Inhibition of YhcL-dependent L-[14 C]cystine uptake in the presence of different compounds

Competitor ^a	% Inhibition
L-Cystine.....	92 $\pm$ 3
Seleno-DL-cystine	67 $\pm$ 4
L-Leucine.....	48 $\pm$ 6
L-Valine	43 $\pm$ 2
L-Alanine.....	42 $\pm$ 7
S-Methyl-L-cysteine.....	40 $\pm$ 1
L-Cysteine.....	38 $\pm$ 1
L-Djenkolic acid	20 $\pm$ 1
DL-Cystathionine	19 $\pm$ 1
L-Methionine	19 $\pm$ 1
DL-Homocysteine	18 $\pm$ 2
DL-Homocysteine	<10
Oxidized glutathione.....	<10
Reduced glutathione.....	<10

^a L-[14 C]cystine was used at a concentration of 5 μ M, and the unlabeled amino acids or sulfur compounds were present at a concentration of 50 μ M. The initial L-[14 C]cystine uptake rate was measured.

FIG. 4. Growth of the wild-type strain and of different mutants in the presence of DL-cystathionine, L-djenkolic acid, or S-methyl-L-cysteine. (A) Growth curves for strain 168 (solid symbols) and BSIP1389 ($\Delta ytmJKLMN::aphA3$) (open symbols) in the presence of 100 μ M DL-cystathionine (circles), 100 μ M L-djenkolic acid (triangles), or 100 μ M S-methyl-L-cysteine (squares). (B) Growth curves for strain 168 (■), strain BSIP1389 ($\Delta ytmJKLMN::aphA3$) (□), strain BSIP1649 ($\Delta ytmJKLMN::aphA3 \Delta yhcL::spc yckK::lacZ-erm \Delta yckK$) (▲), and strain BSIP1648 ($\Delta yxeMNO::cat \Delta ytmJKLMN::aphA3 \Delta yhcL::spc yckK::lacZ-erm \Delta yckK$) (◆) in the presence of 1 mM S-methyl-L-cysteine. OD 600 nm, optical density at 600 nm.

aphA3) in the presence of 100 μ M djenkolic acid, 100 μ M cystathionine, or 100 μ M S-methylcysteine was strongly reduced compared to growth of the wild-type strain (Fig. 4A). The ABC transporter encoded by the *ytmI* operon was therefore required for growth with these products at a concentration of 100 μ M. We also tested the ability of this mutant to utilize these sulfur compounds at a higher concentration. The growth of the $\Delta ytmJKLMN$ mutant with 1 mM cystathionine or 1 mM djenkolic acid remained reduced compared to the growth of the wild-type strain (data not shown). However, the residual growth observed was abolished in a $\Delta ytmJKLMN \Delta yhcL \Delta yckK$ mutant (data not shown). In contrast, the growth of strain BSIP1389 was restored in the presence of 1 mM S-methylcysteine (Fig. 4B). Significant growth retardation was observed only in a $\Delta ytmJKLMN \Delta yhcL \Delta yckK$ mutant. Introduction of a deletion of the *yxeMNO* genes in this triple mutant led to a further decrease in growth (Fig. 4B). This indicates that YhcL, YckK, and YxeMNO are probably involved to the import of S-methylcysteine together with YtmJKLMN. The residual growth of the quadruple mutant could have been due either to the existence of an additional, uncharacterized transporter or to the presence of traces of another sulfur compound in S-methylcysteine.

Regulation of expression of the genes encoding L-cystine transporters. The level of transcription of the *ytmI* gene is high in the presence of methionine, taurine, or glutathione and very low in the presence of sulfate, thiosulfate, and cysteine (7). To determine whether the *yhcL*, *ytmI*, and *yckK* genes are coregulated, expression of a *yhcL'-lacZ*, *ytmI'-lacZ*, or *yckK'-lacZ* transcriptional fusion was measured after growth with 1 mM L-methionine, 1 mM sulfate, or 20 μ M or 1 mM L-cystine (Table 4). The expression of the *yckK'-lacZ* fusion was low in all the conditions tested. The β -galactosidase activities of the *yhcL'-lacZ* and *ytmI'-lacZ* fusions were 30- and 1,000-fold higher in the presence of methionine than in the presence of

sulfate, respectively (Table 4) (2). The expression of these genes was also reduced in the presence of 1 mM cystine (Table 4). In the presence of 20 μ M L-cystine, the expression of the *yhcL'-lacZ* fusion was quite similar during exponential growth and at the beginning of stationary phase, while *ytmI* expression increased when cells reached the stationary phase (Table 4). This increase in expression of the *ytmI-lacZ* fusion during the stationary phase was not observed in the presence of 1 mM L-cystine. Since L-cystine at a concentration of 20 μ M is most probably the limiting growth factor, L-cystine depletion could lead to an increase in *ytmI* transcription.

We then tested the effect of inactivation of the two major L-cystine transport systems, YhcL and YtmJKLMN, on their own expression (Table 4). In the presence of 20 μ M or 1 mM L-cystine, significant increases in expression of the *ytmI'-lacZ* and *yhcL'-lacZ* fusions were observed in a $\Delta yhcL$ mutant but not in a $\Delta ytmJKLMN$ background. The upregulation of the *ytmI'-lacZ* fusion was more important in a $\Delta yhcL \Delta ytmJKLMN$ background. Deletion of the genes encoding the transporters had no effect on their expression in the presence of sulfate (Table 4). The increase in expression of the *ytmI* and *yhcL* genes in a *yhcL* mutant was specific for L-cystine.

The *ytlI* gene is located upstream of the *ytmI* operon and is transcribed divergently. YtlI is a LysR-type regulator that positively controls the expression of the *ytmI* operon (7). The role of YtlI in the regulation of expression of *yhcL* was investigated. Inactivation of the *ytlI* gene did not modify the expression pattern of the *yhcL'-lacZ* fusion (Table 4). YtlI is therefore not involved in the regulation of *yhcL* gene expression in response to sulfur availability.

DISCUSSION

Twenty-one families of secondary carriers, 11 families of ABC transporters, and three families of channel proteins me-

20 μ M is also very low in a mutant containing only TcyABC, and the initial rate observed corresponds to less than 10% of the rate of the wild-type strain (Fig. 3).

The *ytmI* operon and the *tcyP* gene are expressed differently in response to sulfur availability, while the level of expression of the *tcyABC* operon remains low in all the conditions tested. The expression of genes encoding amino acid permeases is often increased when the imported amino acid is depleted (3, 12, 15, 22, 26, 32). In particular, the synthesis of the high-affinity cystine ABC transporter from *S. enterica* serovar Typhimurium is decreased in the presence of cysteine as part of the CysB regulon (3). The expression of the genes encoding the two high-affinity transporters of *B. subtilis* is increased in conditions under which there is probable cysteine limitation; these conditions include the beginning of the stationary phase for the *ytmI* operon, the absence of the TcyP transporter, and maybe also the presence of methionine as the sole sulfur source. However, there are several differences in the regulation of expression of *tcyP* and *ytmI*: (i) the *ytmI* operon seems to be more sensitive to L-cystine starvation; (ii) the expression of the *ytmI* operon is repressed more than 200-fold in the presence of sulfate or L-cystine instead of the 30-fold observed for the *tcyP* gene (Table 4); (iii) the expression of some genes of the *ytmI* operon is induced by disulfide stress (20); (iv) a common target in the promoter region of *tcyP* and *ytmI* was not identified; and (v) the YtlI activator does not play a role in the regulation of *tcyP* expression. This indicates that YtlI is specifically involved in the control of the *ytmI* operon and that there is an uncharacterized regulator for the *tcyP* gene (2, 7; this study). It is also noteworthy that the high level of regulation of the *ytmI* operon could be due to the existence of a cascade of regulation and that the expression of *ytlI* is itself regulated (2).

In *E. coli*, the FliY/YecS/YecC ABC transporter displays a broader specificity for a variety of cystine analogues, while the second uncharacterized system is much more specific for L-cystine (4). This is reminiscent of the properties of the *B. subtilis* TcyJKLMN and TcyP transporters, respectively. Indeed, we found that the TcyJKLMN system is the major transporter of djerkolic acid, cystathionine, and, to a lesser extent, S-methylcysteine (Fig. 5). Two solute binding proteins exhibiting 57% identity, TcyJ and TcyK, are encoded by the *ytmI* operon. Characterization of the substrate specificities of TcyJ and TcyK, which might be different, requires further investigation. The FliY/YecS/YecC ABC transporter can also transport diaminopimelic acid (4). The L-cystine uptake by the TcyJKLMN transporter seems to be decreased in the presence of diaminopimelic acid (39% inhibition). A transporter specific for diaminopimelic acid, which is not involved in L-cystine uptake, is present in *Bacillus megaterium* (11). In contrast, it has been shown that *B. subtilis* is not able to take up diaminopimelic acid after growth in the presence of sulfate (11). This could be due to repression of the synthesis of the TcyJKLMN system in these conditions. Further work is needed to determine whether the TcyJKLMN transporter is able to efficiently take up diaminopimelic acid.

Probable L-cystine symporters, which are highly similar to TcyP, are present in *Bacillus anthracis*, *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Oceanobacillus ihayensis*, and *Enterococcus faecalis*. A TcyP-like protein is also

found in several γ -proteobacteria, including *E. coli*, *Salmonella enterica* serovar Typhi, *S. enterica* serovar Typhimurium, *Haemophilus influenzae*, *Photobacterium luminescens*, *Yersinia pestis*, *Vibrio cholerae*, and *Pseudomonas putida*. Interestingly, the YdjN polypeptide from *E. coli*, which exhibits 45% identity to TcyP, could correspond to the second uncharacterized L-cystine transporter (4). The ABC transporter encoded by the *ytmI* operon is present only in *Listeria* species (<http://genolist.pasteur.fr>). The TcyKLMN proteins exhibit 65 to 75% identity with the corresponding *Listeria* polypeptides. However, a unique solute binding protein is present in *Listeria* (Lmo2349 or Lin2443) instead of the two proteins present in *B. subtilis*, TcyJ and TcyK. A TcyABC-like ABC transporter is found in *B. anthracis*, *B. cereus*, *Bacillus halodurans*, *S. aureus*, *S. epidermidis*, *Clostridium acetobutylicum*, *Neisseria meningitidis*, *H. influenzae*, and probably several lactobacilli. The L-cystine binding protein BspA from *L. fermentum* (42) exhibits 36% identity to TcyA and less than 27% identity to TcyJ and TcyK. For the polar amino acid ABC transporter, identification of specific ligands is complex, and apparent extensive duplication and divergence of the sequence have occurred through evolution. The conservation of L-cystine binding proteins appears to be low (6, 42; this study). Further investigations are needed to analyze the sequence diversity of L-cystine binding proteins and to determine the substrate specificities of these different proteins.

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REFERENCES

- Asai, K., S. H. Baik, Y. Kasahara, S. Moriya, and N. Ogasawara. 2000. Regulation of the transport system for C₄ dicarboxylic acids in *Bacillus subtilis*. *Microbiology* **146**:263–271.
- Auger, S., A. Danchin, and I. Martin-Verstraete. 2002. Global expression profile of *Bacillus subtilis* grown in the presence of sulfate or methionine. *J. Bacteriol.* **184**:5179–5186.
- Baptist, E. W., and N. M. Kredich. 1977. Regulation of L-cystine transport in *Salmonella typhimurium*. *J. Bacteriol.* **131**:111–118.
- Berger, E. A., and L. A. Heppel. 1972. A binding protein involved in the transport of cystine and diaminopimelic acid in *Escherichia coli*. *J. Biol. Chem.* **247**:7684–7694.
- Boos, W., and J. M. Lucht. 1996. Periplasmic binding protein-dependent ABC transporters, p. 1175–1209. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli and Salmonella: cellular and molecular biology*, 2nd ed. ASM Press, Washington, D.C.
- Butler, J. D., S. W. Levin, A. Facchiano, L. Miele, and A. B. Mukherjee. 1993. Amino acid composition and N-terminal sequence of purified cystine binding protein of *Escherichia coli*. *Life Sci.* **52**:1209–1215.
- Coppee, J. Y., S. Auger, E. Turlin, A. Sekowska, J. P. Le Caer, V. Labas, V. Vagner, A. Danchin, and I. Martin-Verstraete. 2001. Sulfur-limitation-regulated proteins in *Bacillus subtilis*: a two-dimensional gel electrophoresis study. *Microbiology* **147**:1631–1640.
- Dassa, E., M. Hofnung, I. T. Paulsen, and M. H. Saier. 1999. The *Escherichia coli* ABC transporters: an update. *Mol. Microbiol.* **32**:887–889.
- During-Olsen, L., B. Regenberg, C. Gjermansen, M. C. Kiehlbrandt, and J. Hansen. 1999. Cysteine uptake by *Saccharomyces cerevisiae* is accomplished by multiple permeases. *Curr. Genet.* **35**:609–617.
- Ferrari, F. A., A. Nguyen, D. Lang, and J. A. Hoch. 1983. Construction and properties of an integrable plasmid for *Bacillus subtilis*. *J. Bacteriol.* **154**:1513–1515.
- Gally, D., C. R. Harwood, and A. R. Archibald. 1991. Diaminopimelic acid uptake by *Bacillus megaterium*: influence of growth conditions and other amino acids. *Lett. Appl. Microbiol.* **12**:54–58.

12. Glansdorff, N. 1996. Biosynthesis of arginine and polyamines, p. 409–433. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
13. Higgins, C. F. 1992. ABC transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* **8**:67–113.
14. Hsieh, A. H. F., and P. S. Poole. 2001. Bacterial ABC transporters of amino acids. *Res. Microbiol.* **152**:259–270.
15. Hullo, M. F., S. Auger, E. Dassa, A. Danchin, and I. Martin-Verstraete. 2004. The *metNPQ* operon of *Bacillus subtilis* encodes an ABC permease transporting methionine sulfoxide, D- and L-methionine. *Res. Microbiol.* **155**:80–86.
16. Kobayashi, K., S. D. Ehrlich, A. Albertini, G. Amati, K. K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, P. Bessieres, F. Boland, S. C. Brignell, S. Bron, K. Bunai, J. Chapuis, L. C. Christiansen, A. Danchin, M. Debarbouille, E. Dervyn, E. Deuerling, K. Devine, S. K. Devine, O. Dresen, J. Errington, S. Fillinger, S. J. Foster, Y. Fujita, A. Galizzi, R. Gardan, C. Eschevins, T. Fukushima, K. Haga, C. R. Harwood, M. Hecker, D. Hosoya, M. F. Hullo, H. Kakeshita, D. Karamata, Y. Kasahara, F. Kawamura, K. Koga, P. Koski, R. Kuwana, D. Imamura, M. Ishimaru, S. Ishikawa, I. Ishio, D. Le Coq, A. Masson, C. Mauel, R. Meima, R. P. Mellado, A. Moir, S. Moriya, E. Nagakawa, H. Nanamiya, S. Nakai, P. Nygaard, M. Ogura, T. Ohanan, M. O'Reilly, M. O'Rourke, Z. Pragai, H. M. Pooley, G. Rapoport, J. P. Rawlins, L. A. Rivas, C. Rivolta, A. Sadaie, Y. Sadaie, M. Sarvas, T. Sato, H. H. Saxild, E. Scanlan, W. Schumann, J. Seegers, J. Sekiguchi, A. Sekowska, S. J. Seror, M. Simon, P. Stragier, R. Studer, H. Takamatsu, T. Tanaka, M. Takeuchi, H. B. Thomaides, V. Vagner, J. M. van Dijk, K. Watabe, A. Wipat, H. Yamamoto, M. Yamamoto, Y. Yamamoto, K. Yamane, K. Yata, K. Yoshida, H. Yoshikawa, U. Zuber, and N. Ogasawara. 2003. Essential *Bacillus subtilis* genes. *Proc. Natl. Acad. Sci. USA* **100**:4678–4683.
17. Kredich, N. M. 1996. Biosynthesis of cysteine, p. 514–527. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
18. Krom, B. P., J. B. Warner, W. N. Konings, and J. S. Lolkema. 2003. Transporters involved in uptake of di- and tricarboxylates in *Bacillus subtilis*. *Antonie Leeuwenhoek* **84**:69–80.
19. Kunst, F., and G. Rapoport. 1995. Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J. Bacteriol.* **177**:2403–2407.
20. Leichert, L. I. O., C. Scharf, and M. Hecker. 2003. Global characterization of disulfide stress in *Bacillus subtilis*. *J. Bacteriol.* **185**:1967–1975.
21. Mansilla, M. C., and D. de Mendoza. 2000. The *Bacillus subtilis* *cysP* gene encodes a novel sulphate permease related to the inorganic phosphate transporter (Pit) family. *Microbiology* **146**:815–821.
22. Merlin, C., G. Gardiner, S. Durand, and M. Masters. 2002. The *Escherichia coli* *metD* locus encodes an ABC transporter which includes Abc (MetN), YaeE (MetI), and YaeC (MetQ). *J. Bacteriol.* **184**:5513–5517.
23. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. Mytelka, D. S., and M. J. Chamberlin. 1996. *Escherichia coli* *flaAZY* operon. *J. Bacteriol.* **178**:24–34.
25. Paulsen, I. T., M. K. Sliwinski, and M. H. Saier. 1998. Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. *J. Mol. Biol.* **277**:573–592.
26. Pittard, A. J. 1996. Biosynthesis of aromatic amino acids, p. 458–484. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
27. Quadroni, M., W. Staudenmann, M. Kertesz, and P. James. 1996. Analysis of global responses by protein and peptide fingerprinting of proteins isolated by two-dimensional gel electrophoresis. Application to the sulfate-starvation response of *Escherichia coli*. *Eur. J. Biochem.* **239**:773–781.
28. Quentin, Y., G. Fichant, and F. Denizot. 1999. Inventory, assembly and analysis of *Bacillus subtilis* ABC transport systems. *J. Mol. Biol.* **287**:467–484.
29. Saier, M. H. 2000. Families of transmembrane transporters selective for amino acids and their derivatives. *Microbiology* **146**:1775–1795.
30. Saier, M. H., S. R. Goldman, R. R. Maile, M. S. Moreno, W. Weyler, N. Yang, and I. T. Paulsen. 2002. Transport capabilities encoded within the *Bacillus subtilis* genome. *J. Mol. Microbiol. Biotechnol.* **4**:37–67.
31. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. Sarsero, J. P., E. Merino, and C. Yanofsky. 2000. A *Bacillus subtilis* gene of previously unknown function, *yhaG*, is translationally regulated by tryptophan-activated TRAP and appears to be involved in tryptophan transport. *J. Bacteriol.* **182**:2329–2331.
33. Sekowska, A., and A. Danchin. 2002. The methionine salvage pathway in *Bacillus subtilis*. *BMC Microbiol.* **2**:8.
34. Sekowska, A., S. Robin, J. J. Daudin, A. Henaut, and A. Danchin. 2001. Extracting biological information from DNA arrays: an unexpected link between arginine and methionine metabolism in *Bacillus subtilis*. *Genome Biol.* **2**:0019.
35. Slotboom, D. J., W. N. Konings, and J. S. Lolkema. 1999. Structural features of the glutamate transporter family. *Microbiol. Mol. Biol. Rev.* **63**:293–307.
36. Soutourina, J., S. Blanquet, and P. Plateau. 2001. Role of D-cysteine desulfhydrase in the adaptation of *Escherichia coli* to D-cysteine. *J. Biol. Chem.* **276**:40864–40872.
37. Steinmetz, M., and R. Richter. 1994. Easy cloning of mini-Tn10 insertions from the *Bacillus subtilis* chromosome. *J. Bacteriol.* **176**:1761–1763.
38. Stülke, J., I. Martin-Verstraete, M. Zagorec, M. Rose, A. Klier, and G. Rapoport. 1997. Induction of the *Bacillus subtilis* *ptsGHI* operon by glucose is controlled by a novel antiterminator, GlcT. *Mol. Microbiol.* **25**:65–78.
39. Tam, R., and M. H. Saier. 1993. Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. *Microbiol. Rev.* **57**:320–346.
40. Tolner, B., T. Ubbink-Kok, B. Poolman, and W. N. Konings. 1995. Characterization of the proton/glutamate symport protein of *Bacillus subtilis* and its functional expression in *Escherichia coli*. *J. Bacteriol.* **177**:2863–2869.
41. Tomii, K., and M. Kanehisa. 1998. A comparative analysis of ABC transporters in complete microbial genomes. *Genome Res.* **8**:1048–1059.
42. Turner, M. S., T. Woodberry, L. M. Hafner, and P. M. Giffard. 1999. The *bspA* locus of *Lactobacillus fermentum* BR11 encodes an L-cysteine uptake system. *J. Bacteriol.* **181**:2192–2198.
43. van der Ploeg, J. R., N. J. Cummings, T. Leisinger, and I. F. Connerton. 1998. *Bacillus subtilis* genes for the utilization of sulfur from aliphatic sulfonates. *Microbiology* **144**:2555–2561.
44. Wach, A. 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* **12**:259–265.