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Two evolutionarily closely related ABC transporters mediate the uptake of choline for synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*

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Summary

Biosynthesis of the compatible solute glycine betaine in *Bacillus subtilis* confers a considerable degree of osmotic tolerance and proceeds via a two-step oxidation process of choline, with glycine betaine aldehyde as the intermediate. We have exploited the sensitivity of *B. subtilis* strains defective in glycine betaine production against glycine betaine aldehyde to select for mutants resistant to this toxic intermediate. These strains were also defective in choline uptake, and genetic analysis proved that two mutations affecting different genetic loci (*opuB* and *opuC*) were required for these phenotypes. Molecular analysis allowed us to demonstrate that the *opuB* and *opuC* operons each encode a binding protein-dependent ABC transport system that consists of four components. The presumed binding proteins of both ABC transporters were shown to be lipoproteins. Kinetic analysis of [^{14}C]-choline uptake via OpuB ($K_m = 1\ \mu M$; $V_{max} = 21$ nmol min^{-1} mg^{-1} protein) and OpuC ($K_m = 38\ \mu M$; $V_{max} = 75$ nmol min^{-1} mg^{-1} protein) revealed that each of these ABC transporters exhibits high affinity and substantial transport capacity. Western blotting experiments with a polyclonal antiserum cross-reacting with the presumed substrate-binding proteins from both the OpuB and OpuC transporter suggested that the expression of the *opuB* and *opuC* operons is regulated in response to increasing osmolality of the growth medium. Primer extension analysis confirmed the osmotic control of *opuB* and allowed the identification of the promoter of this operon. The *opuB* and *opuC* operons are located close to each other on the *B. subtilis* chromosome, and their high sequence identity strongly suggests that these systems have evolved from a duplication event of a primordial gene cluster. Despite the close relatedness of OpuB and OpuC, these systems exhibit a striking difference in substrate specificity for osmoprotectants that would not have been predicted readily for such closely related ABC transporters.

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Introduction

Many microbial, plant and mammalian cells accumulate compatible solutes as an adaptive strategy to high osmolality surroundings (Csonka and Hanson, 1991; Kempf and Bremer, 1998; Poolman and Glaasker, 1998). These substances are actively amassed by the cells, often up to molar concentrations, to curb the outflow of water under hyperosmotic conditions, thus preventing dehydration of the cytoplasm and loss of turgor. The types of compounds that serve as compatible solutes are the same across the kingdoms, reflecting fundamental constraints on the kind of solutes that are congruous with macromolecular and cellular functions (Yancey, 1994).

Glycine betaine is an excellent osmoprotectant and is widely used in both the prokaryotic and eukaryotic world. Many microorganisms can either synthesize it or acquire it from exogenous sources (Csonka and Hanson, 1991; Kempf and Bremer, 1998; Poolman and Glaasker, 1998). A number of bacteria produce glycine betaine by a stepwise methylation of glycine in which S-adenosyl-methionine serves as the methyl donor (Galinski and Trüper, 1994). However, most microorganisms are not capable of *de novo* glycine betaine synthesis. Instead, they use a two-step oxidation of choline via the intermediate glycine betaine aldehyde for its production, and thus depend on the efficient uptake of this precursor for the osmoregulatory glycine betaine production.

Although the physiological importance of the osmoregulatory choline to glycine betaine synthesis pathway has been documented in a wide variety of microorganisms (Pocard *et al*., 1989; Abee *et al*., 1990; Choquet *et al*., 1991; Graham and Wilkinson, 1992; Boch *et al*., 1994;

Cánovas *et al*., 1996; Kiene, 1998; Park and Gander, 1998), the molecular details of this pathway have been fully characterized only in *Escherichia coli*. Strøm and colleagues identified the *betTIBA* gene cluster (Lamark *et al*., 1991) and defined the physiological functions of its products (Landfald and Strøm, 1986; Stryvold *et al*., 1986; Røkenes *et al*., 1996). Import of the precursor choline is mediated at low external concentration by the high-affinity ($K_m = 8\ \mu M$) BetT transporter (Stryvold *et al*., 1986) and at a high substrate supply with low affinity ($K_m = 1.5$ mM) via the ABC uptake system ProU (Lamark *et al*., 1992). BetI functions as a regulatory protein and co-ordinates the expression of the divergently oriented *betT* and *betIBA* genes in response to the availability of choline in the growth medium, but it is not involved in their osmotic control (Lamark *et al*., 1996; Røkenes *et al*., 1996). The *betBA*-encoded proteins are responsible for the enzymatic conversion of choline and glycine betaine aldehyde to glycine betaine (Landfald and Strøm, 1986; Lamark *et al*., 1991). Genes closely related to *betIBA* have also been identified in the rhizosphere bacterium *Sinorhizobium meliloti* (Pocard *et al*., 1997; Østerås *et al*., 1998). Bacterial enzymes involved in glycine betaine synthesis have recently gained biotechnological attention in connection with attempts to engineer plants genetically with increased salt tolerance and resistance to drought (Holmstrøm *et al*., 1994; Nomura *et al*., 1995).

The natural habitats of the Gram-positive bacterium *Bacillus subtilis* are the soil and estuarine waters (Priest, 1993). To thrive in these environments with their fluctuations in osmolality, *B. subtilis* adjusts the intracellular level of compatible solutes either by synthesis (Whatmore *et al*., 1990; Boch *et al*., 1994) or by transport of preformed molecules (Kempf and Bremer, 1998). It possesses several dedicated transport systems for osmoprotectants, such as the osmoregulated proline transporter OpuE (osmoprotectant uptake) (von Blohn *et al*., 1997) and the glycine betaine uptake systems OpuA, OpuC and OpuD (Kempf and Bremer, 1995; Kappes *et al*., 1996). Physiological studies have shown that the OpuC system also serves as sole uptake route for the osmoprotectants ectoine, carnitine, γ-butyrobetaine, crotonobetaine and choline-*O*-sulphate (Jebbar *et al*., 1997; Kappes and Bremer, 1998; Nau-Wagner *et al*., 1999).

In addition to the scavenging of glycine betaine from environmental sources (Kempf and Bremer, 1998), its production is an important facet in the stress reaction of *B. subtilis* to high osmolality (Boch *et al*., 1994). This is accomplished by a two-step oxidation of the precursor choline via a type III alcohol dehydrogenase (GbsB) and a glycine betaine aldehyde dehydrogenase (GbsA) (Boch *et al*., 1996). In contrast to the genetic arrangement for the glycine betaine biosynthetic genes in *E. coli*, the *B. subtilis gbsAB* operon is not flanked by genes that could potentially encode a system mediating osmotically stimulated choline uptake (Boch *et al*., 1994). We report here that choline uptake in *B. subtilis* is mediated by two evolutionarily highly conserved multicomponent ABC transporters that probably evolved through the duplication of a primordial gene cluster. Despite their close relatedness, these transporters display striking differences in their substrate specificity and play different physiological roles in the osmostress response of *B. subtilis*.

Results

Selection of mutants defective in choline uptake

Glycine betaine aldehyde uptake competes with osmotically stimulated choline uptake in *B. subtilis*, suggesting that both compounds are transported via the same uptake system(s) (Boch *et al*., 1994). The chemically very reactive aldehyde is highly toxic for *B. subtilis* mutants impaired in glycine betaine production, because it cannot be converted to the metabolically well-tolerated end-product glycine betaine (Boch *et al*., 1996). Therefore, strains resistant to glycine betaine aldehyde should also have defects in choline uptake. To isolate such strains, we mutagenized strain JBB5 [Δ(*gbsAB*::*neo*)*2*)] with a mini-Tn*10*(*spc*) transposable element (Steinmetz and Richter, 1994), selected approximately 50 000 Spc^r colonies with chromosomal Tn*10* insertions on rich medium, pooled them and plated aliquots from this transposon pool on selective minimal plates containing 1 mM glycine betaine aldehyde and 0.4 M NaCl. Strains resistant to glycine betaine aldehyde were readily obtained, and two mutants (strains JBB33 and JBB38) were chosen for further characterization. The parental strain JBB5 showed the expected [^{14}C]-choline uptake activity (Boch *et al*., 1994), whereas both JBB33 and JBB38 were entirely defective in choline transport (Fig. 1). Thus, the selection procedure used yielded the desired types of *B. subtilis* mutants with defects in choline acquisition.

Loss of choline transport activity requires two mutations

Southern hybridization experiments with a probe derived from the mini-Tn*10*(*spc*) element demonstrated that each of the mutant strains JBB33 and JBB38 contained only one chromosomal copy of the mini-Tn*10*(*spc*) inserted at different sites (data not shown). To test whether the resistance to glycine betaine aldehyde and loss of the choline transport activity was caused by the mini-Tn*10*(*spc*) insertion in these strains, we prepared chromosomal DNA from JBB33 and JBB38 and used it to transform the parent strain JBB5 [Δ(*gbsAB*::*neo*)*2*)] by selecting for Spc^r colonies. Such transformants were then assayed for [^{14}C]-choline transport activity and tested for resistance to glycine

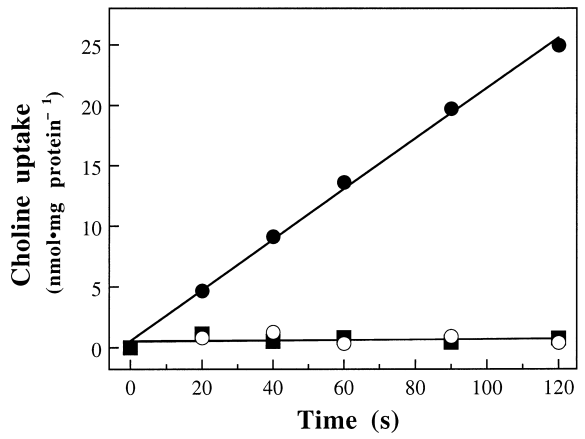

Fig. 1. Choline uptake in *B. subtilis*. Cultures of strains JBB5 [Δ(*gbsAB*::*neo*)*2*; ●] were grown in SMM with 0.4 M NaCl to mid-log phase and assayed for uptake of radiolabelled [^{14}C]-choline at a final substrate concentration of 10 μM. Choline transport was also measured for the glycine betaine aldehyde-resistant derivatives of JBB5, strain JBB33 [*opuB-21 opuC-20*::Tn*10*(*spc*); ■] and JBB38 [*opuB-20*::Tn*10*(*spc*) *opuC-21*; ○] grown in SMM with 0.4 M NaCl.

betaine aldehyde. Both original mutants yielded two types of Spc^r transformants: (i) strains that were resistant to the aldehyde and simultaneously defective in choline uptake; and (ii) strains that were sensitive to glycine betaine aldehyde and proficient in choline transport. Two explanations could account for these findings: (i) both JBB33 and JBB38 each contained a spontaneous mutation resulting in the loss of choline and glycine betaine aldehyde uptake that was genetically linked to, but independent of, the mini-Tn*10*(*spc*) insertions; or (ii) two closely spaced mutations (a spontaneous mutation and a mini-Tn*10*(*spc*) insertion) are required to confer full resistance to glycine betaine aldehyde and loss of choline transport activity.

To distinguish between these alternatives, we replaced the Spc^r marker in one of the mini-Tn*10*(*spc*) insertions with a chloramphenicol resistance gene (*cat*) by homologous recombination and used this mutant derivative for a series of genetic mapping experiments. These experiments showed that two genetically linked mutations were required to confer glycine betaine aldehyde resistance and loss of choline uptake activity. Consequently, the originally isolated mutant strains JBB33 and JBB38 were double mutants, and each contained a spontaneous mutation closely linked to a mini-Tn*10*(*spc*) insertion in a different locus. Subsequent DNA sequence analysis proved that the mini-Tn*10*(*spc*) elements were indeed inserted into different operons (Fig. 2). One of these loci encodes the previously physiologically characterized OpuC system serving for high-affinity glycine betaine uptake (Kappes *et al*., 1996) and transport of various structurally related trimethylammonium compounds (Kappes and Bremer, 1998; Nau-Wagner *et al*., 1999). We refer to the other locus marked by the mini-Tn*10*(*spc*) insertion as *opuB*.

To map genetically the distance between the *opuB* and *opuC* loci more precisely, we transformed the chromosomal *opuC*::Tn*10*(*spc*) insertion from the *opuB*$^+$ strain RMKB25 into the *opuC*$^+$ strain RMKB36 carrying the *opuB*::Tn*10*(*cat*) mutation. Among 574 tested Spc^r transformants, 251 strains (43.8%) were sensitive to chloramphenicol, and the remaining 323 colonies were resistant to both antibiotics. This two-factor cross thus establishes a tight genetic linkage between the *opuB*::Tn*10* and *opuC*::Tn*10* insertions with a calculated physical distance of approximately 4–6 kb (Hoch, 1991).

Molecular analysis of the opuB *and* opuC *loci*

The mini-Tn*10*(*spc*) element contains a plasmid origin of replication that is functional in *E. coli*, thus allowing the convenient rescue of genomic DNA flanking the mini-Tn*10*(*spc*) insertion (Steinmetz and Richter, 1994). We cloned the *opuB-20*::Tn*10*(*spc*) and *opuC-20*::Tn*10*(*spc*) insertions and their flanking genomic regions from the *B. subtilis* chromosome and determined the nucleotide sequence of the *opuB* and *opuC* loci. Each locus consists of four genes that are apparently organized in an operon (Fig. 2) and, in each case, the coding region is followed by DNA sequences with potential stem–loop structures typical of factor-independent transcription terminators in *B. subtilis*. The nucleotide sequence at both mini-Tn*10* insertion sites was confirmed by direct sequencing of

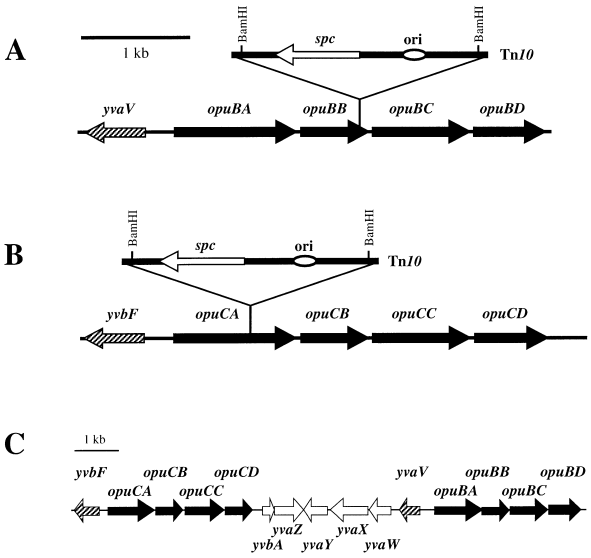

Fig. 2. Physical and genetic organization of the *opuB* and *opuC* regions.
A and B. Genetic organization of the *opuB* (A) and *opuC* (B) loci and their flanking regions (*yvaV* and *yvbF*). The position of the mini-Tn*10* element present in the *opuB-20*::Tn*10*(*spc*) and the *opuC-20*::Tn*10*(*spc*) mutations is indicated.
C. Representation of the genomic organization near the *opuB* and *opuC* operons. The genetic nomenclature of open reading frames with unknown function is according to Kunst *et al*. (1997).

appropriate polymerase chain reaction (PCR) products amplified from the genomic *opuB*$^+$ and *opuC*$^+$ loci. No rearrangements had occurred except for the typical duplication of 9 bp at the ends of the mini-Tn*10* transposon (Steinmetz and Richter, 1994). Using *opuB*- and *opuC*-specific hybridization probes, we also cloned the intact *opuB*$^+$ and *opuC*$^+$ loci from the wild-type strain JH642 into the low-copy-number plasmid pACYC184, but both operons were not functionally expressed in *E. coli* from their own transcription and translation initiation signals (data not shown).

The opuB *and* opuC *operons encode two closely related ABC transport systems*

A comparison of the protein sequences deduced from the *opuB*- and *opuC*-encoded genes with the databases revealed that both operons encode a multicomponent binding protein-dependent transport system that is a member of the ATP-binding cassette (ABC) superfamily of transporters (Higgins, 1992; Boos and Lucht, 1996). The first gene of both operons encodes an evolutionarily highly conserved ATPase (OpuBA; OpuCA) (Fig. 3) that is responsible for the energetization of the transport and, by analogy with other well-characterized binding protein-dependent ABC transporters, is likely to function as a dimer. The proteins (OpuBB and OpuBD; OpuCB and OpuCD) encoded by the second and fourth genes of the *opuB* and *opuC* operons are related to each other (38% and 41% identity) and exhibit alternating hydrophobic and hydrophilic segments typical of integral membrane proteins. These four components of the OpuB and OpuC transporters display limited homology to membrane proteins that mediate substrate translocation across the cytoplasmic membrane in several other binding protein-dependent ABC transporters (Boos and Lucht, 1996). In both the *opuB* and the *opuC* operons, the third gene encodes a hydrophilic polypeptide (OpuBC; OpuCC) with an N-terminal signal sequence exhibiting the characteristic features of lipoproteins (Braun and Wu, 1994). The OpuBC and OpuCC proteins are thus likely to form extracellular substrate-binding proteins tethered to the cytoplasmic membrane by an N-terminal lipid modification (Tam and Saier, 1993; Sutcliffe and Russel, 1995).

A pairwise comparison of the components of the OpuB and OpuC transporters revealed striking sequence identities of 83% for the OpuBA/OpuCA, 85% for the OpuBB/OpuCB, 69% for the OpuBC/OpuCC and 85% for the OpuBD/OpuCD proteins (Fig. 3). This unusual degree of sequence conservation between two bacterial binding

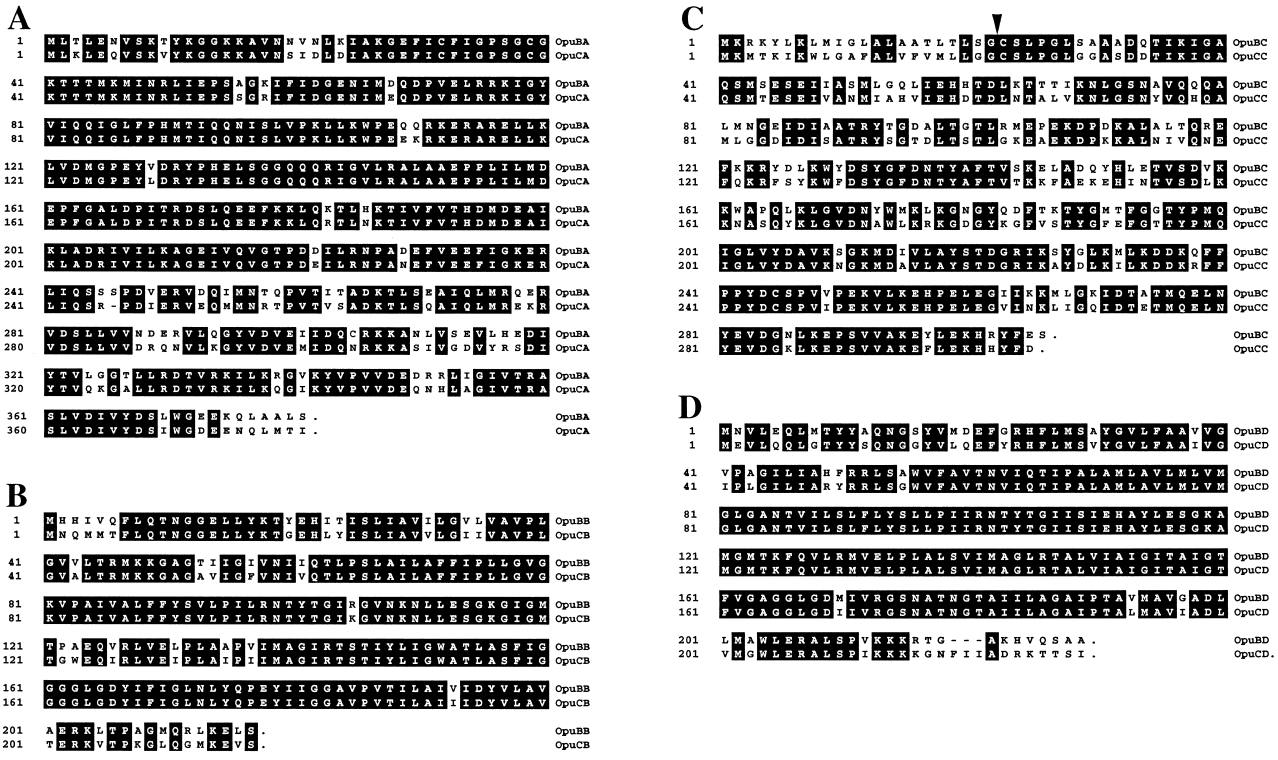

Fig. 3. Comparison of the components of the OpuB and OpuC transport systems. The ATPases (OpuBA and OpuCA) (A), the integral membrane components (OpuBB and OpuCB) (B) and (OpuBD and OpuCD) (D), and the substrate-binding proteins (OpuBC and OpuCC) (C) of both ABC transport systems are aligned. The putative processing sites for the pro-OpuBC and pro-OpuCC lipoproteins are indicated by an arrowhead (▼).

protein-dependent ABC transporters (Boos and Lucht, 1996) strongly suggests that their structural genes have evolved through a gene duplication event of a primordial operon. This suggestion is supported by the high level of sequence identity that exists between the coding regions (≈70%) of the two operons. This high degree of sequence identity also extends to the open reading frames (*yvaV* and *yvbF*) located upstream of the *opuB* and *opuC* operons (Fig. 2), whereas essentially no sequence homology exists outside the *yvaV–opuB* and *yvbF–opuC* genomic segments.

OpuBC and OpuCC are lipoproteins

A characteristic feature of lipoproteins in both Gram-negative and Gram-positive bacteria is that proteolytic processing of their signal sequence via signal peptidase II during translocation through the cytoplasmic membrane is inhibited by the cyclic peptide antibiotic globomycin (Inukai *et al*., 1978). In contrast, proteolytic processing of non-lipoproteins, which is mediated by signal peptidase I, is not affected by globomycin (Braun and Wu, 1994; Sutcliffe and Russel, 1995). To test the influence of globomycin on pro-OpuBC processing, we expressed the *opuBC*$^+$ gene under the control of a T7Φ10 promoter on a low-copy-number plasmid in the *E. coli* host strain BL21(λDE3) and labelled the plasmid-encoded proteins selectively with [^{35}S]-methionine. Overproduction of secreted proteins with a T7Φ10 expression system frequently results in the appearance of unprocessed precursors (Dersch *et al*., 1994), and such incomplete processing was also observed for the pro-OpuBC protein (Fig. 4A). The addition of 120 μg ml^{-1} globomycin to the cells completely prevented the proteolytic processing of the OpuBC precursor (Fig. 4A). A lipid modification of OpuBC was then directly proven by labelling this protein with [^{3}H]-palmitic acid (Fig. 4B). The same set of experiments carried out with the OpuCC protein produced the same results (data not shown), demonstrating that this presumed substrate-binding protein is also lipid modified.

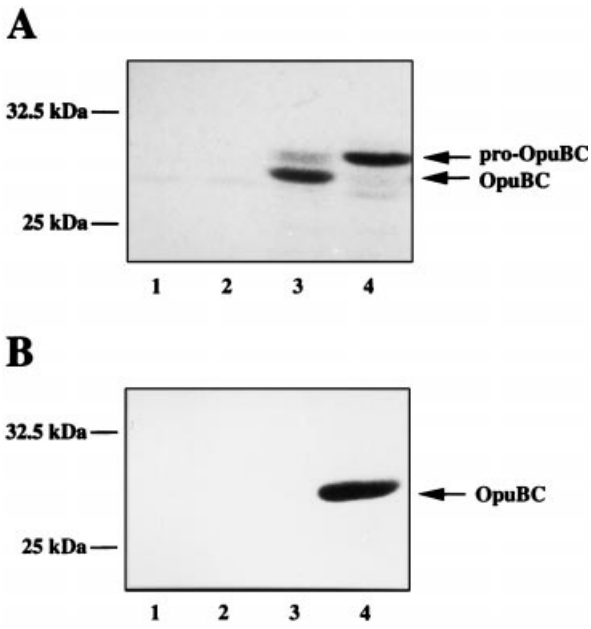

Fig. 4. OpuBC is a lipoprotein.
A. Effect of globomycin on the processing of the pro-OpuBC protein. IPTG-induced cultures of strains BL21(λDE3)(pPD101) (lanes 1 and 2) and BL21(λDE3)(pBKB89) (lanes 3 and 4) were grown in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 120 μg ml^{-1} globomycin and were labelled with [^{35}S]-methionine.
B. Labelling of OpuBC with [^{3}H]-palmitic acid. Cultures of BL21(λDE3)(pPD101) (lanes 1 and 2) and BL21(λDE3) (pBKB89) (lanes 3 and 4) were grown to mid-exponential phase in the presence of [^{3}H]-palmitic acid. A portion of ≈800 kcpm of the delipidated cell extracts was loaded per lane. Lanes 1 and 3, uninduced cultures; lanes 2 and 4, induced cultures. A longer exposure (10 days) of the gel revealed the presence of several other host-encoded lipoproteins.

Physiological roles of OpuB and OpuC in the choline and glycine betaine uptake

We studied the physiological roles of the OpuB and OpuC systems for the acquisition of choline and glycine betaine aldehyde. The addition of 1 mM glycine betaine, choline or glycine betaine aldehyde to high-osmolality medium protected the *opuB*$^+$ *opuC*$^+$ wild-type strain JH642 from the detrimental effects of high osmolality. Both precursors for glycine betaine conferred a considerable degree of osmoprotection but were somewhat less efficient than glycine betaine (Fig. 5A); this might be explained by the fact that the cell must first convert choline and glycine betaine aldehyde enzymatically into glycine betaine (Boch *et al*., 1994). Osmoprotection by choline and glycine betaine aldehyde was completely abolished in the *opuB opuC* double mutant strain RMKB47 (Fig. 5B). Glycine betaine was still fully osmoprotective in this strain, as it can be accumulated not only via OpuC but also through the OpuA and OpuD transporters (Kempf and Bremer, 1995; Kappes *et al*., 1996). We also tested osmoprotection by choline in strains RMKB20 [*opuB*$^+$ *opuC-20*::Tn*10*(*spc*)] (Fig. 5C) and RMKB22 [*opuB-20*::Tn*10*(*spc*) *opuC*$^+$] (Fig. 5D) and, in both single mutant strains, choline was still osmoprotective but was less effective than in strain JH642 (*opuB*$^+$ *opuC*$^+$). Hence, under the growth conditions tested, both the ABC transporters OpuB and OpuC are able to provide the cell with enough choline to sustain growth under unfavourable circumstances, although a more efficient osmoadaptation is achieved when both transport systems are functional. To test a possible involvement of the OpuB system in glycine betaine uptake, we performed osmoprotection assays with an isogenic set of strains that lacked the OpuA and OpuD glycine betaine

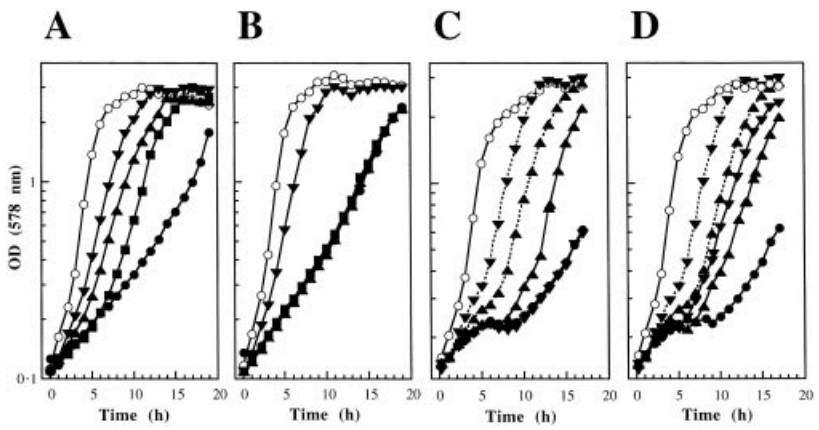

Fig. 5. Choline and glycine betaine aldehyde uptake via the OpuB and OpuC systems. Cultures were inoculated to an optical density (OD_{578}) of ≈0.12 from overnight cultures grown in SMM. The various *B. subtilis* strains were grown in 75 ml of minimal media of low (SMM) or high (SMM with 1.2 M NaCl) osmolality in 500 ml Erlenmeyer flasks at 37°C in a shaking water bath set at 220 r.p.m. Growth curves of strain JH642 (wild type; $OpuA^+$ $OpuB^+$ $OpuC^+$ $OpuD^+$) (A), RMKB47 ($OpuA^+$ $OpuB^-$ $OpuC^-$ $OpuD^+$) (B), RMKB20 ($OpuA^-$ $OpuB^+$ $OpuC^-$ $OpuD^-$) (C) and RMKB22 ($OpuA^-$ $OpuB^-$ $OpuC^+$ $OpuD^-$) (D) in SMM (○), SMM with 1.2 M NaCl (●), SMM with 1.2 M NaCl and 1 mM glycine betaine (▼), SMM with 1.2 M NaCl and 1 mM choline (▲) and SMM with 1.2 M NaCl and 1 mM glycine betaine aldehyde (■). C and D. Growth of the wild-type strain JH642 in SMM with 1.2 M NaCl and 1 mM choline (▲) or 1 mM glycine betaine (▼) is shown as a control (dashed lines).

transporters and harboured either intact OpuB (strain RMKB20) or OpuC (strain RMKB22) systems. Glycine betaine was osmoprotective in strain RMKB22 (Fig. 5D) but not in strain RMKB20 (Fig. 5C), demonstrating unambiguously that OpuB is not involved in glycine betaine transport, despite its close sequence relatedness to OpuC (Fig. 3).

Kinetics of choline transport via OpuB and OpuC

The [^{14}C]-choline transport in *B. subtilis* is moderately stimulated by increases in medium osmolality (Boch *et al*., 1994). To determine the kinetic parameters for choline transport via OpuB and OpuC, we measured the initial uptake of [^{14}C]-choline in strain RMKB20 ($OpuB^+$ $OpuC^-$) and strain RMKB22 ($OpuB^-$ $OpuC^+$) over a range of substrate concentrations (1–40 μM) in cultures grown in SMM or SMM with 0.4 M NaCl. The OpuB system exhibits a high affinity for choline, with a K_m value of 1 μM and a V_{max} of 5 nmol min^{-1} mg^{-1} protein. Increases in medium osmolality had no effect on the affinity of choline transport ($K_m = 1$ μM) but stimulated the maximal uptake rate (V_{max}) approximately fourfold to 21 nmol min^{-1} mg^{-1} protein. Choline transport via OpuC was not detectable in cells grown in SMM, but uptake activity was readily measurable in cultures propagated in SMM with 0.4 M NaCl. The OpuC transporter has a lower affinity ($K_m = 38$ μM) but a higher transport capacity ($V_{max} = 75$ nmol min^{-1} mg^{-1} protein) for choline than the OpuB system under identical growth conditions.

Osmoregulation of opuB *and* opuC *expression*

We raised a polyclonal antiserum against the purified lipidless OpuBC protein in a rabbit. In Western blot experiments with *B. subtilis* whole-cell extracts, this antiserum recognized the OpuBC protein and also the sequence-related OpuCC polypeptide, allowing the convenient simultaneous detection of both proteins (Fig. 6). Although both OpuBC ($M_r = 32$) and OpuCC ($M_r = 31.7$) have a very similar molecular mass, they do not migrate to the same positions on 13.5% SDS–polyacrylamide gels. The amount of both OpuBC and OpuCC increased in cells of the wild-type strain JH642 grown at elevated osmolality (SMM with 0.4 M NaCl) (Fig. 6, lanes 1 and 2), suggesting that the expression of the *opuB* and *opuC* operons is under osmotic control. The antiserum also cross-reacted with a polypeptide unrelated to OpuBC and OpuCC that was already recognized by the preimmune serum (data not shown), and this polypeptide thus serves as an internal control for the osmotically stimulated increase in OpuBC and OpuCC synthesis (Fig. 6, lanes 1 and 2). The degree of osmotic induction of both the OpuBC and the OpuCC proteins is reduced in strains synthesizing only one of the OpuB or OpuC systems (Fig. 6, lanes 3–6). This observation might indicate that uninduced cultures of the *opuB* and *opuC* mutant strains compensate for the loss of one of

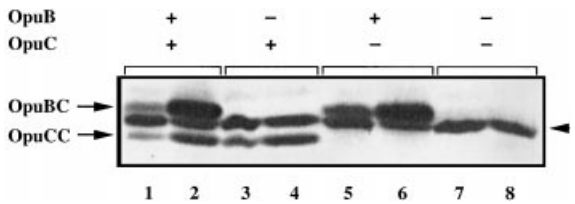

Fig. 6. Immunological detection of the OpuBC and OpuCC proteins. Total cellular proteins from various *B. subtilis* strains grown in SMM (lanes 1, 3, 5 and 7) or SMM with 0.4 M NaCl (lanes 2, 4, 6 and 8) were probed with a polyclonal antiserum raised against OpuBC. The presence of the OpuB and OpuC systems in the various strains is indicated. Strain JH642 (lanes 1 and 2), strain RMKB22 (lanes 3 and 4), strain RMKB20 (lanes 5 and 6) and strain RMKB47 (lanes 7 and 8). The polypeptide indicated by an arrowhead was already recognized by the preimmune serum.

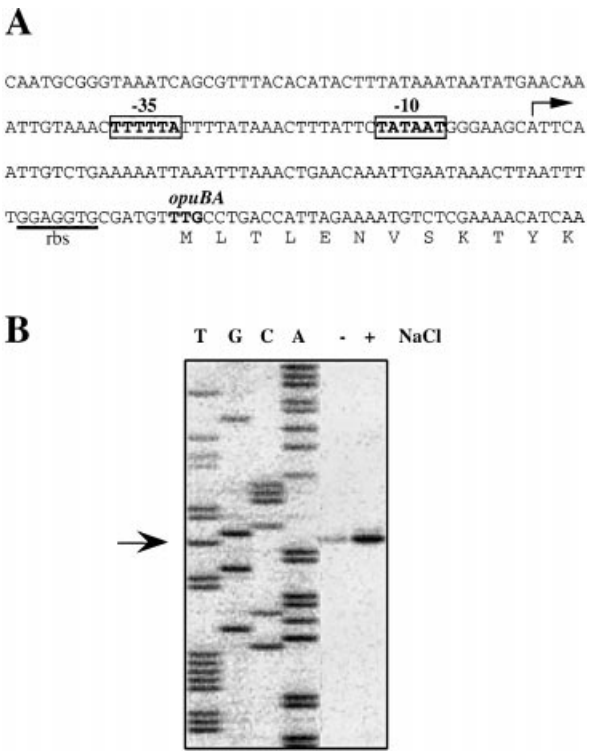

Fig. 7. Mapping of the *opuB* transcription start site.
A. Nucleotide sequence of the *opuB* promoter region.
B. Total RNA was prepared from cells of the *B. subtilis* strain JH642 (pBKB105; *opuB'*) grown in SMM (−) and SMM with 0.4 M NaCl (+), and the transcription initiation site of the *opuB* locus was identified by primer extension analysis using an *opuBA*-specific primer labelled with the infrared dye IRD-41. The reaction products were analysed on an automatic sequencer, and the same primer was used for sequencing reactions to size the *opuB* transcript.

these transport systems by overproducing the remaining transporter to a certain extent.

To identify the *opuB* promoter(s), we mapped the transcription initiation site(s) by primer extension analysis. We detected a single, *opuB*-specific RNA species whose production was under osmotic control (Fig. 7). Inspection of the DNA sequence upstream of the mRNA initiation site revealed the presence of putative −10 and −35 sequences that resemble the consensus sequence of promoters recognized by the main vegetative sigma factor (σ^A) of *B. subtilis* (Helman, 1995). The *opuB* promoter does not show the unusually high G + C content characteristic of the Pribnow boxes of the osmoregulated σ^A-dependent promoters of the *opuA* and *opuE* loci from *B. subtilis* and the *proP* and *proU* loci from *E. coli* (Kempf and Bremer, 1995; von Blohn *et al*., 1997; Spiegelhalter and Bremer, 1998).

We also attempted to map the *opuC* transcription initiation site by primer extension analysis. Despite repeated attempts, we were not able to identify primer extension products that were reproducible between different RNA preparations, and the various detected 5′ ends of the mRNA species were not preceded by recognizable promoter elements. The reasons for the difficulties in mapping the *opuC* transcription initiation site(s) remain unresolved.

Orthologues and paralogues of the OpuB and OpuC transporters

We compared the products of the *opuB* and *opuC* operons sequenced by us with those determined independently in the course of the *B. subtilis* sequencing project (Kunst *et al*., 1997) and found that the components of the OpuB and OpuC transporters determined in these two studies were completely identical, with the exception of two adjacent amino acid substitutions in the OpuCC protein. Database searches revealed that sequence-related ABC transport systems with a subunit composition identical to OpuB and OpuC are present in *E. coli* (YehZYXW), in the Gram-positive bacterium *Mycobacterium tuberculosis* (ProVWXZ) and in the archaeon *Archaeoglobus fulgidus* (ProV, ProW-1, ProW-2, ProX), but their physiological function is unknown. The components of both the OpuB and OpuC systems are also related to the binding protein-dependent glycine betaine transport systems OpuA from *B. subtilis* (Kempf and Bremer, 1995) and ProU from *E. coli* (Gowrishankar, 1989). Related ABC transporters are also present in the Lyme disease spirochaete *Borrelia burgdorferi* and the gastric pathogen *Helicobacter pylori*, but their substrate specificities have not yet been determined. The most extensive sequence identity was detected between the components of the OpuB and OpuC transporters and those of an ABC transporter (ProU) encoded by an artificially constructed chimeric *proU* operon of a subtilin-producing *B. subtilis* strain LH45 (Lin and Hansen, 1995). OpuB from the wild-type strain JH642 exhibits the highest sequence identity (between 89% and 94%) to the ProU components from LH45, but important strain differences must exist between this mutant and JH642. Disruption of the hybrid *proU* locus in strain LH45 significantly reduces osmoprotection by glycine betaine (Lin and Hansen, 1995), whereas OpuB is not involved in glycine betaine uptake (Fig. 5C), and mutations in *opuC* have only a minor influence on the overall glycine betaine uptake resulting from the presence of the effective transport systems OpuA and OpuD (Kempf and Bremer, 1995; Kappes *et al*., 1996).

Discussion

Increases in the external osmolality impose considerable strain on the water balance of the cell and necessitate active countermeasures to prevent dehydration and cessation of growth. One of these measures is the biosynthesis of glycine betaine from choline. *B. subtilis* cannot

synthesize choline *de novo* and, as its natural habitats (e.g. soil and estuarine waters) generally contain only very low concentrations of choline (Kortstee, 1970; Kiene, 1998), it requires effective transport systems to take up this trimethylammonium compound from exogenous sources. The data presented in this communication characterize two evolutionarily closely related ABC transport systems (OpuB and OpuC) for high-affinity, osmotically stimulated uptake of choline in *B. subtilis*. Together with the previously identified genes for the glycine betaine biosynthetic enzymes (GbsA and GbsB) (Boch *et al*., 1996), these transport systems complete the first osmoregulatory choline to glycine betaine synthesis pathway characterized at the molecular level in a Gram-positive bacterium (Fig. 8).

A comparison with the analogous pathway operating in *E. coli* (Lamark *et al*., 1991) reveals that the overall scheme of glycine betaine synthesis is conserved in the model organisms for the Gram-positive and Gram-negative branches of bacteria. However, considerable differences exist with respect to the uptake of choline and its enzymatic conversion into glycine betaine. A single-component choline transporter (BetT) driven by the proton motive force is found in *E. coli*, whereas two multicomponent, binding protein-dependent ABC transporters (OpuB and OpuC) are present in *B. subtilis*. Each of these transporters is well suited for its physiological task, as BetT, OpuB and OpuC possess a high degree of uptake velocity and recognize choline effectively, with K_m values in the low micromolar range. Common to the biosynthetic pathway in both organisms is the presence of an evolutionarily well-conserved and highly salt-tolerant glycine betaine aldehyde dehydrogenase (GbsA, BetB) (Falkenberg and Strøm, 1990; Boch *et al*., 1997). This enzyme not only functions in the second step of glycine betaine production (Fig. 8), but also serves to keep the level of the highly toxic intermediate, glycine betaine aldehyde, very low. We exploited the toxic effects caused by an increase in the intracellular glycine betaine aldehyde concentration (Boch *et al*., 1996) to select *B. subtilis* mutants resistant to this aldehyde and found that these strains were simultaneously deficient in choline transport as well. Double mutations in the *opuB* and *opuC* loci were required for these phenotypes.

The first step in glycine betaine synthesis in *B. subtilis* and *E. coli* is performed by two different types of enzymes. A soluble, metal-containing, type III alcohol dehydrogenase (GbsB) functions in *B. subtilis* to convert choline into glycine betaine aldehyde (Fig. 8) (Boch *et al*., 1996), whereas this reaction is catalysed in *E. coli* by a FAD-containing, membrane-bound choline dehydrogenase, which can also oxidize glycine betaine aldehyde to glycine betaine at the same rate (Landfald and Strøm, 1986). Both *B. subtilis* and *E. coli* synthesize glycine betaine as a metabolically inert stress compound (Landfald and Strøm, 1986;

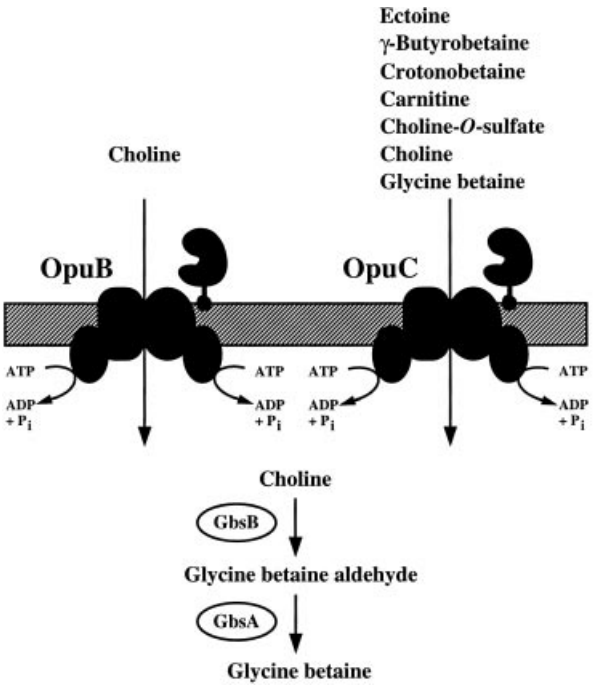

Fig. 8. The choline to glycine betaine pathway of *B. subtilis*.

Boch *et al*., 1994), but glycine betaine and choline have both osmoregulatory and nutritional roles in *S. meliloti* (Bernard *et al*., 1986).

Our previous physiological and mutant studies have established a pivotal role for OpuC in supplying *B. subtilis* with a variety of osmoprotectants (Kappes *et al*., 1996; Jebbar *et al*., 1997; Kappes and Bremer, 1998; Nau-Wagner *et al*., 1999). The experiments described here provide the molecular details of this transport system. Its closely related cousin OpuB is an entirely new addition to the arsenal of transporters used by *B. subtilis* 168 for the acquisition of osmoprotectants or their biosynthetic precursors (Kempf and Bremer, 1998). OpuC plays a unique role among the osmoprotectant uptake systems operating in *B. subtilis*. It functions as the sole and high-affinity uptake route for the potent osmoprotectants carnitine, crotonobetaine, γ-butyrobetaine and choline-*O*-sulphate and as a low-affinity transporter for ectoine (Jebbar *et al*., 1997; Kappes and Bremer, 1998; Nau-Wagner *et al*., 1999). It also serves in various combinations with OpuA and OpuD not only in glycine betaine transport, but also in the effective acquisition of proline betaine, β-alanine betaine, dimethylsulphonioacetate (DMSA) and dimethylsulphoniopropionate (DMSP) from environmental sources (unpublished results). OpuB does not participate in the transport of any of these compounds and shares with OpuC only choline and glycine betaine aldehyde as its substrates (Fig. 8). Such a striking difference in substrate specificity and physiological function would not have been

readily predicted for two such closely related ABC transporters (Fig. 3).

The most surprising finding in our study was the identification of two choline uptake systems that were evolutionarily so closely related. Inspection of the nucleotide sequence of the entire *B. subtilis* genome revealed that more than a quarter of the genes show molecular traces of duplication events (Doolittle, 1997; Kunst *et al*., 1997). It is generally thought that gene duplications allow the modification of gene function during evolution via the accumulation of mutations in one of the duplicated DNA segments, thus providing new metabolic and physiological diversity to an organism (Henikoff *et al*., 1997; Tatusov *et al*., 1997). The strikingly different substrate specificity of the OpuB and OpuC transporters (Fig. 8) provides evidence for this concept. Inspection of the nucleotide sequence of the *B. subtilis* genome (Kunst *et al*., 1997) confirmed our genetic mapping data and revealed that the *opuB* and *opuC* operons are both located at 296° on the genetic map, are transcribed in the same direction and are separated by only 4.3 kb (Fig. 2). The amplified unit comprises approximately 4.2 kb and contains, in addition to the four-gene *opuB* and *opuC* operons, two divergently transcribed open reading frames (*yvaV* and *yvbF*) of unknown function (Fig. 2); the YvaV and YvbF proteins display a high degree of sequence identity (80%). Outside this region, there is essentially no sequence identity at either the DNA or the protein level.

Microbial binding protein-dependent ABC transport systems show a common global organization with respect to the number and function of their individual components (Higgins, 1992; Doige and Ferro-Luzzi Ames, 1993; Boos and Lucht, 1996). Repeated duplication and gene fusion events have played important roles in the spread of this type of transporter and their eukaryotic counterparts through the kingdoms in the course of evolution and during their specialization towards different substrates (Higgins, 1992; Doige and Ferro-Luzzi Ames, 1993; Tam and Saier, 1993; Saurin and Dassa, 1994). The close relatedness of the integral inner membrane proteins OpuBB/OpuBD and OpuCB/OpuCD (Fig. 3) is thus likely to reflect such an incident. Sequence similarities between all components of transport systems involved in the uptake of structurally related substrates (e.g. histidine and octopine; di- and oligopeptides; Mathiopoulos *et al*., 1991; Valdivia *et al*., 1991; Koide and Hoch, 1994) are likely to reflect gene duplication events of an entire primordial gene cluster. The OpuB and OpuC systems for the uptake of the trimethylammonium compounds choline and glycine betaine are striking examples of such gene duplication and paralogue evolution. Despite the close sequence relatedness of the OpuB and OpuC systems, these transporters exhibit widely different substrate specificities (Fig. 8). This finding raises intriguing questions with respect to the specificity of the interactions between the substrate-binding proteins and their corresponding inner membrane components. Consequently, the OpuB and OpuC uptake systems might provide interesting models for studying the evolution of bacterial ABC transporters to understand further the molecular determinants governing substrate recognition and translocation across the cytoplasmic membrane.

Experimental procedures

Growth conditions, media and chemicals

Rich and minimal media for the growth of *B. subtilis* and *E. coli* strains were prepared as described previously (Kempf and Bremer, 1995; Kappes *et al*., 1996). The osmotic strength of growth media was increased by adding sodium chloride from a 5 M stock solution, and their osmolality was then determined with a vapour pressure osmometer (model 5500; Wescor). The osmolarity of Spizizen's minimal medium (SMM) was 340 mosmol kg^{-1}, of SMM with 0.4 M NaCl was 1100 mosmol kg^{-1} and of SMM with 1.2 M NaCl was 2700 mosmol kg^{-1}. The antibiotics ampicillin, chloramphenicol and kanamycin were used with *E. coli* strains at final concentrations of 100, 30 and 50 $\mu g\,ml^{-1}$ respectively. Kanamycin, spectinomycin, tetracycline, erythromycin and chloramphenicol were used with *B. subtilis* strains at final concentrations of 5, 100, 15, 1 and 5 $\mu g\,ml^{-1}$ respectively. [*methyl*-^{14}C]-Choline chloride (54 mCi $mmol^{-1}$) was purchased from NEN, and [1-^{14}C]-glycine betaine (55 mCi $mmol^{-1}$) was custom synthesized by ARC (American Radiolabeled Chemicals). [9,10(*n*)-^{3}H]-Palmitic acid (toluene solution; 52.0 mCi $mmol^{-1}$) and [^{35}S]-methionine (523 mCi $mmol^{-1}$) were obtained from Amersham Buchler. Choline, glycine betaine and glycine betaine aldehyde were purchased from Sigma.

B. subtilis *strains and genetic procedures*

The *B. subtilis* strains used in this study are listed in Table 1 and are all derivatives of the wild-type strain JH642 (*trpC2 pheA1*) (BGSC 1A96; J. Hoch). Mutations in the various uptake systems for osmoprotectants were constructed by transforming (Cutting and Vander Horn, 1990) appropriate recipient strains with chromosomal DNA prepared from *B. subtilis* mutants with previously described lesions in the *opuA* or *opuD* locus (Kempf and Bremer, 1995; Kappes *et al*., 1996). The Δ(*opuBD*::*tet*)*23* mutation was constructed in the course of this study and was isolated by transforming linear DNA of plasmid pRMK49 (cleaved with *Xmn*I) into strain JH642. Tetracycline-resistant transformants were selected and tested for the absence of the vector-encoded spectinomycin resistance. The physical structure of the chromosomal Δ(*opuBD*::*tet*)*23* in one of these Tet^r Spc^s colonies, strain RMKB13, was verified by Southern hybridization using an *opuBD*-specific probe prepared by PCR. To convert the mini-Tn*10*-encoded *spc* resistance marker (Steinmetz and Richter, 1994) in the *opuC-20*::Tn*10*(*spc*) (strain RMKB25) and *opuB-20*::Tn*10*(*spc*) (strain RMKB26) to a chloramphenicol resistance gene (*cat*), we transformed strains RMKB25 and RMKB26 with plasmid pRMK66 and selected for Cm^r colonies. Plasmid pRMK66 cannot replicate in *B. subtilis* and

Table 1. *B. subtilis* strains used.

Strain[a]	Genotype	Origin
JH642	*trpC2 pheA1*	J. Hoch
GNB8	Δ(*opuA*::*erm*)*4*	G. Nau-Wagner
JBB5	Δ(*gbsAB*::*neo*)*2*	Boch *et al*., 1996
JBB33	Δ(*gbsAB*::*neo*)*2 opuB-21 opuC-20*::Tn*10*(*spc*)	This study
JBB38	Δ(*gbsAB*::*neo*)*2 opuB-20*::Tn*10*(*spc*) *opuC-21*	This study
RMKB13	Δ(*opuB*::*tet*)*23*	This study
RMKB20	Δ(*opuA*::*erm*)*4 opuB*$^+$ *opuC-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	This study
RMKB22	Δ(*opuA*::*erm*)*4 opuB-20*::Tn*10*(*spc*) *opuC*$^+$ Δ(*opuD*::*neo*)*2*	This study
RMKB24	Δ(*opuA*::*erm*)*4* Δ(*opuB*::*tet*)*23 opuC-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	This study
RMKB25	*opuC-20*::Tn*10*(*spc*)	This study
RMKB26	*opuB-20*::Tn*10*(*spc*)	This study
RMKB33	Δ(*opuA*::*erm*)*4* Δ(*opuB*::*tet*)*23 opuC-20*::Tn*10*(*spc*) *opuD*$^+$	This study
RMKB34	*opuA*$^+$ Δ(*opuB*::*tet*)*23 opuC-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	This study
RMKB36[b]	*opuB-22*::Tn*10*(*cat*)	This study
RMKB37[b]	*opuC-22*::Tn*10*(*cat*)	This study
RMKB39	Δ(*gbsAB*::*neo*)*2 opuB-20*::Tn*10*(*spc*)	This study
RMKB40	Δ(*gbsAB*::*neo*)*2 opuC-20*::Tn*10*(*spc*)	This study
RMKB47	*opuB-20*::Tn*10*(*spc*) *opuC-22*::Tn*10*(*cat*)	This study
RMKB50	Δ(*gbsAB*::*neo*)*2 opuB-20*::Tn*10*(*spc*) *opuC-22*::Tn*10*(*cat*)	This study

a. The strains are all derivatives of JH642.
b. The Tn*10*-encoded *spc* gene was converted through homologous recombination events with pRMK66 to a *cat* gene.

integrates into chromosomal copies of the mini-Tn*10* element by homologous recombination involving DNA segments flanking the drug resistance gene. A double recombination event between the mini-Tn*10* element and pRMK66 can thus replace the originally present *spc* gene with the *cat* gene without altering the original mini-Tn*10* insertion site. These genetic manipulations yielded strains RMKB36 [*opuB-22*::Tn*10*(*cat*)] and RMKB37 [*opuC-22*::Tn*10*(*cat*)] (Table 1).

To isolate mutants resistant to glycine betaine aldehyde, we used the temperature-sensitive mini-Tn*10*(*spc*) mutagenesis system (Steinmetz and Richter, 1994). We isolated a pool of over 50 000 derivatives of strain JBB5 [Δ(*gbsAB*::*neo*)*2*] with chromosomal Tn*10*(*spc*) insertions on LB agar plates containing spectinomycin. Appropriate dilutions of this pool were then spread onto SMM agar plates with spectinomycin containing 0.4 M NaCl and 1 mM glycine betaine aldehyde. Growth of the parent strain JBB5, which is defective in glycine betaine synthesis, on these plates is prevented because the chemically highly reactive glycine betaine aldehyde is accumulated to toxic levels (Boch *et al*., 1994). A number of colonies resistant to glycine betaine aldehyde were picked and purified by restreaking on the same medium. Examples of such mutants are strains JBB33 and JBB38 (Table 1).

Methods used with nucleic acids and construction of plasmids

Routine manipulations of plasmid DNA, the isolation of chromosomal DNA and the detection of homologous sequences by Southern hybridization were carried out according to standard procedures (Sambrook *et al*., 1989). The mini-Tn*10*(*spc*) insertion present in the *opuB* [*opuB-20*::Tn*10* (*spc*)] and *opuC* [*opuC-20*::Tn*10* (*spc*)] operons and their flanking DNA segments from the *B. subtilis* chromosome were recovered by plasmid rescue as Spc^r colonies in the *E. coli* host strain DH5α (Steinmetz and Richter, 1994). Plasmids pRMKB38, pRMKB44, pRMKB50 and pRMKB51 were obtained after digestion of chromosomal DNA from JBB38 [*opuB-20*::Tn*10* (*spc*)] with *Eco*RI, *Pvu*II, *Eco*RV and *Hpa*I respectively. In an analogous way, we rescued the *opuC-20*::Tn*10* (*spc*) insertion from strain JBB33 after digestion of chromosomal DNA with *Eco*RI (pRMK48), *Ssp*I (pRMK72) and *Pvu*I (pRMK73).

To clone the intact *opuB* and *opuC* operons, chromosomal DNA from the wild-type strain JH642 was digested with *Cla*I or *Hpa*I, and restriction fragments carrying the *opuB* or *opuC* loci were identified by Southern hybridization using *opuB* or *opuC* probes derived from a PCR product using *opuBC*-specific primers and from plasmid pRMK48 (*opuC*) respectively. The *opuB* operon was present on a 5.0 kb *Cla*I fragment, and *opuC* was carried by an 8.1 kb *Hpa*I restriction fragment. Genomic restriction fragments of the appropriate size were obtained from preparative agarose gels and ligated into plasmid pACYC184 (Cm^r, Tet^r) (Chang and Cohen, 1978) cut with *Cla*I or *Xmn*I respectively. These limited gene libraries were transformed into strain DH5α and grouped into 10 pools of 50 colonies each. Plasmid DNA isolated from these pools was subjected to Southern hybridization using *opuB* or *opuC* probes. Plasmid DNA was then prepared from individual colonies of positive pools and subjected to Southern hybridization. This resulted in the isolation of plasmids pBKB119 (*opuB*$^+$) (Cm^r, Tet^r) and pBKB144 (*opuC*$^+$) (Cm^r, Tet^r).

The *opuBC* gene was positioned under the control of the T7Φ10 promoter by cloning a 1.8 kb *Bam*HI–*Eco*RI restriction

fragment from plasmid pRMK38 into the *Bam*HI and *Eco*RI sites in the polylinker of the low-copy-number expression vector pPD101 (Dersch *et al*., 1994), resulting in plasmid pBKB89. A 1.8 kb *Pst*I–*Bam*HI restriction fragment carrying the *opuB* regulatory region was isolated from plasmid pRMK38 and inserted into the *Pst*I and *Bam*HI sites of the *E. coli–B. subtilis* shuttle vector pRB373 (Brückner, 1992), resulting in the isolation of plasmid pBKB105. We used the following steps to construct a plasmid (pRMK66) that allows the switching of the spectinomycin resistance determinant carried by strains with chromosomal insertions of the mini-Tn*10* element (Steinmetz and Richter, 1994) to a chloramphenicol resistance marker by homologous recombination. We first isolated a 1.7 kb *Sau*3A–*Nde*I fragment carrying a chloramphenicol resistance gene from plasmid pJH101 (Ferrari *et al*., 1983), then filled in the 5′ overhanging ends with the DNA polymerase Klenow fragment and, finally, cloned this DNA fragment into the *Hin*cII site of plasmid pUC19, yielding plasmid pRMK59. Plasmid pRMK38 was cut with *Bam*HI and religated. The resulting plasmid pRMK65 was cleaved with *Pst*I and *Xba*I, and the plasmid backbone was ligated with a *Pst*I–*Xba*I fragment derived from pRMK59. This construction replaced most of the mini-Tn*10*-encoded *spc* gene with the chloramphenicol resistance determinant and positioned this antibiotic resistance marker between DNA segments derived from the left and right ends of the mini-Tn*10* element. These flanking DNA sequences thus provide homology for recombination events between a chromosomal mini-Tn*10*(*spc*) insertion and plasmid pRMK66.

Sequencing reactions of double-stranded plasmid DNA were performed using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP, as recommended by the supplier (Amersham Pharmacia Biotech). The nucleotide sequence of the *opuB* and *opuC* operons was established for both DNA strands and was performed by primer walking using custom-synthesized oligonucleotides with an infrared label (IRD-41; MWG Biotech). Sequencing products were analysed on a LI-COR DNA sequencer (model 4000; MWG Biotech). DNA primers [5′-GCGCGTTGGCCGATTC-3′, 113 bp to 98 bp, and 5′-GATATTCACGGTTTAC-3′, 2234 bp to 2249 bp] hybridizing to the ends of the mini-Tn*10* (Steinmetz and Richter, 1994) were used to establish the nucleotide sequence flanking the *opuB-20*::Tn*10* (*spc*) and *opuC-20*::Tn*10* (*spc*) insertion (Fig. 2). Total RNA was prepared from exponentially growing cells of the *B. subtilis* strain JH642 (pBKB105) using the Total RNA Midi kit (Qiagen). The amount of RNA isolated was determined spectrophotometrically (an A_{260} of 1 corresponds to $\approx$40 μg ml^{-1} RNA; Sambrook *et al*., 1989). To determine the *opuB* transcription initiation site, we used primer extension analysis. Approximately 5 μg of total RNA was hybridized with 2 pmol of a synthetic oligonucleotide complementary to the *opuB* mRNA (5′-GCCGC-CCTTGTATGTTTTCGAGAC-3′; position 899 bp to 922 bp). This oligonucleotide was labelled at its 5′ end with the infrared dye IRD-41. The primer was extended with avian myoblastosis virus reverse transcriptase (Promega) in the presence of 0.32 mM each dNTP at 42°C for 1 h. The reaction products were purified by phenol extraction, and the nucleic acids were precipitated with ethanol and resuspended in 6 μl of sequencing stop solution. One microlitre of this solution was applied to a 6% DNA sequencing gel, and the reaction products were analysed on the LI-COR DNA sequencer. A sequencing ladder produced with the same primer was run in parallel to determine the position of the 5′ end of the *opuB* mRNA.

*Metabolic labelling of the OpuBC protein with [9,10(*n*)-^{3}H]-palmitic acid*

The *opuBC* gene was expressed selectively under the control of the T7Φ10 promoter using the low-copy-number expression vector pPD101 (Dersch *et al*., 1994) and an *E. coli* strain [BL21(λDE3)] carrying a chromosomal copy of the gene for T7 RNA polymerase under *lacPO* control as the host. For the labelling of OpuBC with [9,10(*n*)-^{3}H]-palmitic acid, we used published procedures (Ehlert *et al*., 1995; Kempf *et al*., 1997). To study the influence of the peptide antibiotic globomycin (Inukai *et al*., 1978) on the proteolytic processing of the pro-OpuBC protein, we radiolabelled the OpuBC precursor with [^{35}S]-methionine in the absence or presence of globomycin (final concentration 120 μg ml^{-1}) in cells of strain BL21(λDE3) (pBKB89) expressing the *opuBC* gene under T7Φ10 control, as detailed previously (Kempf and Bremer, 1995). An analogous set of experiments was carried out using plasmid pBMW1, a derivative of pPD101 carrying the *opuCC*$^+$ gene under T7Φ10 control.

Preparation of an OpuBC antiserum and immunological detection of the OpuBC protein

We used the maltose-binding protein (MBP) fusion technique to purify a lipidless OpuBC protein. The *opuBC* coding region was amplified by PCR using the following primers: 5′-GCG-CGAGTACTTCGCTTCCGGGTCTCAGCGCCGC-3′; 5′-GA-CATGAGAAAGTGGCGG-3′). The resulting DNA fragment was cut with *Sca*I and *Eco*RI and ligated into plasmid pMAL-c2 (New England Biolabs), such that the *malE′* and *′opuBC* reading frames were properly aligned. The resulting MBP–OpuBC hybrid protein lacked the OpuBC signal sequence and the cysteine residue required for lipid modification. The fusion of both genes was engineered so that the cleavage site for the protease Xa was present at the MBP–OpuBC junction. The *malE–opuBC* fusion plasmid pRMK39 was introduced into *E. coli* strain MKH13 [Δ(*putPA*)*101* Δ(*proP*)*2* Δ(*proU*::*spc*)*608* Δ(*betTIBA*)] (Kempf and Bremer, 1995), and the expression of the hybrid gene was induced with 0.3 mM IPTG in LB medium with 0.2% glucose, resulting in the production of large amounts of soluble MBP–OpuBC fusion protein. The hybrid protein was purified by affinity chromatography on an amylose resin and cleaved with Factor Xa. The OpuBC moiety was isolated after rechromatography on the amylose column. To raise a polyclonal antiserum against the recombinant OpuBC protein, 100 μg of OpuAC was mixed with 500 μg of AdjuPrime Immune Modulator (Pierce) and injected into a rabbit; there were nine subsequent booster injections. The quality of the OpuBC antiserum was tested by Western blot analysis (Sambrook *et al*., 1989) using OpuB$^+$ and OpuB$^-$ total cell extracts of *B. subtilis* strains separated on SDS–13.5% polyacrylamide gels. The OpuBC antibody complexes were visualized with a second goat anti-rabbit immunoglobulin G–alkaline phosphatase-conjugated antibody

(Sigma) using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride (Boehringer Mannheim) as substrates. In an analogous series of experiments, we constructed plasmid pBMW2 carrying a *malE–opuCC* hybrid gene and used overproduced OpuCC protein to raise a polyclonal antiserum.

Transport assays

Uptake of radiolabelled [*methyl*-^{14}C]-choline chloride in exponentially growing cultures was measured as described previously (Kempf and Bremer, 1995; Kappes *et al*., 1996). For kinetic studies, the substrate concentration in the uptake assay was varied between 1 μM and 40 μM.

Computer analysis

DNA and protein sequences were assembled and analysed with the LASERGENE program (DNAstar) on an Apple Macintosh computer. Searches for homologies were performed at the National Centre for Biotechnology Information (NCBI) using the BLAST programs (Altschul *et al*., 1990). Protein sequences were aligned using the CLUSTAL algorithm provided with the LASERGENE program.

Nucleotide sequence accession numbers

The nucleotide sequences of the *opuB* and *opuC* operons and their flanking regions have been deposited in GenBank and have been assigned the accession numbers AF008930 and AF009352 respectively.

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