KtrAB and KtrCD: Two $K^+$ Uptake Systems in *Bacillus subtilis* and Their Role in Adaptation to Hypertonicity†

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Received 29 July 2002/Accepted 20 November 2002

Recently, a new type of $K^+$ transporter, Ktr, has been identified in the bacterium *Vibrio alginolyticus* (T. Nakamura, R. Yuda, T. Unemoto, and E. P. Bakker, J. Bacteriol. 180:3491-3494, 1998). The Ktr transport system consists of KtrB, an integral membrane subunit, and KtrA, a subunit peripherally bound to the cytoplasmic membrane. The genome sequence of *Bacillus subtilis* contains two genes for each of these subunits: yuaA (ktrA) and yqkB (ktrC) encode homologues to the *V. alginolyticus* KtrA protein, and yubG (ktrB) and ykrM (ktrD) encode homologues to the *V. alginolyticus* KtrB protein. We constructed gene disruption mutations in each of the four *B. subtilis* ktr genes and used this isogenic set of mutants for $K^+$ uptake experiments. Preliminary $K^+$ transport assays revealed that the KtrAB system has a moderate affinity with a $K_m$ value of approximately 1 mM for $K^+$, while KtrCD has a low affinity with a $K_m$ value of approximately 10 mM for this ion. A strain defective in both KtrAB and KtrCD exhibited only a residual $K^+$ uptake activity, demonstrating that KtrAB and KtrCD systems are the major $K^+$ transporters of *B. subtilis*. Northern blot analyses revealed that ktrA and ktrB are cotranscribed as an operon, whereas ktrC and ktrD, which occupy different locations on the *B. subtilis* chromosome, are expressed as single transcriptional units. The amount of $K^+$ in the environment or the salinity of the growth medium did not influence the amounts of the various ktr transcripts. A strain with a defect in KtrAB is unable to cope with a sudden osmotic upshock, and it exhibits a growth defect at elevated osmolalities which is particularly pronounced when KtrCD is also defective. In the ktrAB strain, the osmotically mediated growth defect was associated with a rapid loss of $K^+$ ions from the cells. Under these conditions, the cells stopped synthesizing proteins but the transcription of the osmotically induced *proHJ, opuA*, and *isiB* genes was not impaired, demonstrating that a high cytoplasmic $K^+$ concentration is not essential for the transcriptional activation of these genes at high osmolality. Taken together, our data suggest that $K^+$ uptake via KtrAB and KtrCD is an important facet in the cellular defense of *B. subtilis* against both suddenly imposed and prolonged osmotic stress.

All organisms lack the ability to actively transport water. Osmotic processes therefore determine cellular water content, and changes in external salinity or osmolality have a pronounced influence on the hydration status of the cytoplasm and the magnitude of turgor (58). These osmotically instigated water fluxes threaten the cell with dehydration and a collapse of turgor under hypersotmic conditions and with cell rupture under hyposotmic circumstances (10, 13, 40). To maintain turgor and cellular water content within physiologically acceptable limits, bacteria actively modulate their intracellular solute pools. They amass ions and organic osmolytes in growth conditions of high osmolality, and they expel these compounds through mechanosensitive channels when external osmolality drops (8, 33).

Many nonhalophilic bacteria respond to a sudden osmotic increase in their environment with a two-phase adaptation reaction (13). Initially, large amounts of $K^+$ are rapidly taken up by the cells (15) through specific transport systems (49) to compensate for the efflux of water that accompanies the increase in the external salinity or osmolality. Because high intracellular concentrations of intracellular $K^+$ have negative effects on protein function, DNA-protein interactions, and the synthesis of proteins (42, 43), this initial response is inadequate for coping with prolonged high osmolality. The second phase of osmoadaptation therefore frequently involves the synthesis or the uptake of compatible solutes (11, 59) and the efflux of $K^+$ (10, 13, 15).

Detailed studies by Whatmore and Reed (57) and Whatmore et al. (56) have provided evidence that the gram-positive soil bacterium *Bacillus subtilis* belongs to the group of microorganisms that uses the two-stage osmoadaptation strategy outlined above. In *B. subtilis*, turgor has been estimated at 1.9 MPa, and recovery of turgor subsequent to an osmotic upshock has been shown to be $K^+$ dependent (57). In exponentially growing cells subjected to a moderate osmotic increase that was elicited by the addition of 0.4 M NaCl, the cellular $K^+$ level rose from a basal value of 350 mM to 650 mM within 1 h (56). Since *B. subtilis* can sustain far higher salinities than 0.4 M NaCl (4), intracellular $K^+$ concentrations substantially higher than 650 mM can be expected for severely osmotically challenged cells. The cellular $K^+$ content not only responds to osmotic changes in the environment but is also modulated in response to the presence of compatible solutes in the cell.

† Dedicated to Karlheinz Altendorf on the occasion of his 60th birthday.

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Under high-salinity growth conditions, *B. subtilis* synthesizes large amounts of the osmoprotectant proline (36, 56) via a dedicated and osmotically controlled biosynthetic pathway (J. Brill and E. Bremer, submitted for publication). An essentially linear correlation exists between the salinity of the growth medium and the proline content of the cell (Brill and Bremer, submitted). As the intracellular proline level increases through de novo synthesis at high osmolality, the K⁺ pool level decreases (56). Moreover, *B. subtilis* is also capable of accumulating compatible solutes from the environment (10, 30). For example, the widely found compatible solute glycine betaine is taken up through the osmotically controlled transport systems OppA, OppC, and OppD (26, 27, 29, 34). When glycine betaine is added to *B. subtilis* cells grown at high osmolality, the intracellular K⁺ pool decreases to a level similar to that of non-osmotically-stressed cells (56). Thus, it is apparent that *B. subtilis* actively modulates its intracellular K⁺ level in response to changes in growth conditions (e.g., increases in osmolality) and to the presence of compatible solutes. It is therefore not surprising that *B. subtilis* exhibits an increased demand for K⁺ when grown in high-osmolality minimal medium lacking compatible solutes (48).

In *B. subtilis*, the systems responsible for the osmoregulated biosynthesis of the compatible solutes proline and glycine betaine (5; Brill and Bremer, submitted) and the systems for the uptake of preformed compatible solutes (10, 30) have already been studied in considerable detail. In comparison, only limited molecular and physiological information is available with respect to K⁺ acquisition in this microorganism. Sturr et al. (50) used complementation experiments with a K⁺ uptake-deficient *Escherichia coli* strain to isolate a *B. subtilis* gene that encodes an apparent homologue of TrkA, a subunit of the multicomponent Trk K⁺ transporter of *E. coli* (49). However, subsequent DNA sequence analysis revealed that the overall amino acid identity of the *B. subtilis* homologue to the *E. coli* TrkA protein is very low (50). There is also a chromosomally encoded multifunctional tetracycline-metal/H⁺ antipporter, TetA(L), in *B. subtilis* which exhibits a net K⁺ uptake activity. Mutants with defects in the TetA(L) system show reduced growth in medium containing a low concentration of K⁺ (55).

In addition, *B. subtilis* cells exhibit an inducible K⁺ uptake activity proposed to be associated with a K⁺-activated P-type ATPase (48). This K⁺ uptake activity is clearly not mediated by a KdpFABC-type K⁺ uptake system (1, 21, 44), since no kdp-related genes are found in the *B. subtilis* genome (31).

Recently, Ktr has been described as a new type of bacterial K⁺ uptake system (28, 38, 51). This system (17, 18) consists of a membrane-embedded subunit (KtrB, called Ntp in *Enterococcus hirae* [51]) that is evolutionarily related to the KcsA type of the K⁺ channel (16), which was first described for *Streptomyces lividans* (47), and a peripheral membrane protein (KtrA) that binds both NAD⁺ and NADH in different conformations. The interaction of these dinucleotides with KtrA has been proposed to regulate KtrB activity through a ligand-mediated conformational switch mechanism (45). The KtrA and KtrB proteins apparently cooperate in K⁺ translocation, because bacteria that have a ktrA gene always possess a ktrB gene and because KtrB alone is inactive (38). Two copies of ktrAB-like genes are present in the genome of *B. subtilis*, but as yet no functional studies have been performed to assess the role of ktr-encoded proteins in K⁺ acquisition. Here we show that *B. subtilis* possesses two Ktr-type K⁺ transport systems, KtrAB and KtrCD, which differ in their affinity for K⁺. A mutant with disruptions in both systems exhibits only residual K⁺ uptake activity; therefore, KtrAB and KtrCD must play a central role in K⁺ acquisition in *B. subtilis*. Consistent with the recognized role of K⁺ in osmoadaptation in microorganisms, we found that ktr mutants exhibit a major growth defect when they are challenged by high salinity. We also show that an osmotically induced increase in the cellular K⁺ concentration cannot be a signal for the expression of several osmotically regulated genes in *B. subtilis*.

### MATERIALS AND METHODS

#### Bacterial strains.

The *B. subtilis* strain JH642 (trpC2 pheA1 [BGSIC LA06]; a kind gift from J. Hoch) is a derivative of the *B. subtilis* wild-type strain 168. This strain and its isogenic-mutant derivatives (Table 1) were used throughout this study. Plasmids constructed by recombinant DNA procedures were introduced by electrotropistation into the *E. coli* strain DHZa (GBICO BRL, Egggenstein, Germany), and the resulting strains were propagated on Luria-Bertani medium containing an appropriate antibiotic.

#### Media, chemicals, and growth conditions.

Rich (Luria-Bertani) medium and minimal medium (SMM) for the growth of *B. subtilis* and *E. coli* strains were prepared as described previously (26, 29). The potassium-free medium for *B. subtilis* was a phosphate-buffered minimal medium containing 8.2 g of Na₂HPO₄, 3.2 g of NaCl, 1 g of sodium citrate, and 0.2 g of MgSO₄·7H₂O per liter. Glucose (0.5%) was provided as a carbon source, and the medium was supplemented with 2 mg of tryptophan and 1.8 mg of phenylalanine per liter and a solution of trace elements to satisfy the growth requirements of the *B. subtilis* strain JH642 and its derivatives (23). The K⁺ content of the minimal medium used was adjusted by the addition of KCl from a 5 M salt solution. When used for *E. coli*, the antibiotics chloramphenicol and ampicillin were employed at final concentrations of 30 µg ml⁻¹ and 100 µg ml⁻¹, respectively. Kanamycin, spectinomycin, and tetracycline were used for *B. subtilis* strains at final concentrations of 5 µg ml⁻¹, 100 µg ml⁻¹, and 12 µg ml⁻¹, respectively. [S]Methionine (1,000 µCi mmol⁻¹) was purchased from Amersham Biosciences (Freiburg, Germany).

#### Genetic procedures and construction of *B. subtilis* mutants.

Standard genetic techniques were used for the genetic manipulation and transformation of *B. subtilis* strains (14). Gene disruptions of the various *kat* genes of *B. subtilis* (Table 1) were constructed by transformation of linear plasmid DNA carrying the various *kat* mutations marked by antibiotic resistance genes into JH642 and selecting for the appropriate antibiotic resistance. Disruptions of the genes of interest were then confirmed by Southern hybridization with specific probes of the corresponding genes. Strain GHB14, which is deficient for KtrC ([ktrC::spec]) and KtrD ([ktrD::tet]), was constructed by transforming strain GHB6 ([ktrC::spec]) with chromosomal DNA of GHB12 ([ktrD::tet]) and selecting for tetracycline resistance and subsequently scoring of the spectinomycin resistance marker. The ktrAB ktrCD triple mutant GHB15 ([ktrAB::neo] [ktrC::spec] [ktrD::tet]) was obtained by transformation of GHB14 ([ktrC::spec] [ktrD::tet]) with chromosomal DNA from GHB1 [Δ(ktrB::neo)] and selection for kanamycin resistance and subsequent scoring of the spectinomycin and tetracycline resistance markers. Strain GHB16 ([ktrB::neo] [ktrC::spec])

### TABLE 1. *B. subtilis* strains used for this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin</th>
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<tr>
<td>JH642</td>
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<td>J. Hoch</td>
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<tr>
<td>GHB1</td>
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</tr>
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<td>(ktrB::neo)</td>
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<td>(ktrD::tet)</td>
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<tr>
<td>GHB15</td>
<td>(ktrD::tet) [ktrC::spec] Δ(ktrAB::neo)</td>
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<tr>
<td>GHB16</td>
<td>(ktrB::neo) [ktrC::spec]</td>
<td>This study</td>
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* All mutants are derivatives of strain JH642.
resulted from a transformation of chromosomal DNA of GHB6 ([ktrC::pect]::1) into GHB3 ([ktrB::neo]::1) and selection for spectinomycin resistance and subsequent scoring of the kanamycin resistance marker.

Methods used with nucleic acids. Routine manipulation of plasmid DNA, isolation of chromosomal DNA from B. subtilis, and detection of homologous sequences by Southern hybridization were carried out according to standard procedures (46). For Northern analyses using a High-Purity RNA isolation kit (Roche Diagnostics, Mannheim, Germany), we isolated total RNA from B. subtilis cultures grown to early log phase (optical density at 578 nm [OD578] 0.5 to 1). Digoxigenin (DIG)-labeled RNA probes were generated, using a DIG RNA-labeling kit (SP6/T7) (Roche Diagnostics). The DIG-labeled probes were hybridized with a DIG-labeled DNA probe specific for either ktrAB, ktrC, or ktrD. Hybridization was carried out at 68°C overnight in a hybridization solution containing 50% formamide, 5% SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2% blocking reagent (Roche Diagnostics), 0.1% N-lauroylsarcosine, and 7% sodium dodecyl sulfate. The membrane filters were washed according to standard procedures (46). The ktr transcripts were detected, using the chemiluminescent ECF-Vistra (Amersham Biosciences) and a Storm 860 phosphorimager (Amersham Biosciences).

Construction of plasmids. To construct plasmids for the disruption of the ktrB and ktrD genes, we first amplified the corresponding regions from the B. subtilis genome by PCR using custom-synthesized primers that hybridized to DNA and ktrD loci and their flanking open reading frames. The positions of the antibiotic resistance cassettes inserted into the ktrAB, ktrC, and ktrD genes for chromosomal gene disruption experiments are indicated.

FIG. 1. Genetic organization of the ktrAB, ktrC, and ktrD loci. The genetic organization of the ktrAB (A), ktrC (B), and ktrD (C) loci and their flanking open reading frames is shown. The positions of the antibiotic resistance cassettes inserted into the ktrAB, ktrC, and ktrD genes for chromosomal gene disruption experiments are indicated.

Ktr-DEPENDENT K TRANSPORT IN B. SUBTILIS 1291

Measurement of proline content of B. subtilis cells. The proline contents of growing cells were measured before and after osmotic upshift with 0.6 M NaCl were added to the cell suspension, and the cells were preincubated for 10 min by shaking at 150 rpm. Net K uptake was initiated by adding KCl at t = 0. At different time points, 1.0 ml sample from each flask was pipetted onto 0.2 ml of AR200 silicone oil (Serva, Heidelberg, Germany) in a 1.5-ml Eppendorf centrifuge tube, which was then centrifuged immediately for 2 to 3 min at 12,000 rpm in an Eppendorf tabletop centrifuge. The pellets of cells centrifuged through the silicone oil layer were cut out with a razor blade, and each pellet was added to a different tube containing 1.0 ml of 5% trichloroacetic acid. The tubes were closed and vortexed vigorously. Subsequently, their contents were frozen, thawed, heated for 5 min at 95°C, and diluted either with 3 ml of H2O (for K measurements by atomic adsorption) or with 3 ml of 6.7 mM CoCl2 (for K measurements by flame photometry). After the protein was removed by centrifugation, the concentration of K* was determined either by atomic adsorption or by flame photometry using KCl concentrations between 0 and 150 µM as a standard. In experiments in which the K* contents of growing cells were measured before and after osmotic upshift, cells were grown at 37°C in a minimal medium containing 4 mM KCl. After the culture reached an OD600 of about 0.7, an aliquot of preheated 5 M NaCl solution was added to give a final NaCl concentration of 0.65 M NaCl. The OD600 and K* values of the cells were determined as a function of time. Samples (1.0 ml each) were withdrawn from the suspension at various time points. The cell pellets were centrifuged through silicone oil, and the K* content of the cell pellet was determined as described above. Cell K* contents are expressed in nanomoles per milligram (dry weight) of cells. To calculate this parameter, it was essential to correlate the optical density of the B. subtilis cultures with the dry weight of cells per milliliter. We observed that cells with an OD600 value of 1.0 corresponded to 0.25 mg (dry weight) ml-1. This value was used for all calculations of cell K* contents. To calculate the cytoplasmic K* concentration, we used a value of 2.6 µmol of cytoplasmic water space mg-1 (dry weight) of cells, as determined for B. subtilis growing in the phosphate-buffered minimal medium (37).

Metabolic labeling with [35S]methionine. To measure incorporation of [35S]methionine into newly synthesized proteins, B. subtilis cells were grown in minimal medium. Aliquots of 2 ml of the cell suspension were transferred to Eppendorf tubes containing 5S-labeled methionine at a final concentration of 2 µCi ml-1 and 2 µM unlabeled l-methionine; the Eppendorf tubes were preheated to 37°C. The cell suspensions were incubated with shaking at 37°C, and at various time points, 0.5-ml samples were withdrawn, immediately mixed with ice-cold 10% trichloroacetic acid (TCA), and then incubated on ice for 30 min. Subsequently, the samples were vortexed, filtered through nitrocellulose filters (Schleicher & Schuell), and washed three times with 1 ml of 5% TCA. The radioactivity of the samples was then measured in a liquid scintillation counter.

Measurement of proline content of B. subtilis cells. The proline contents of B. subtilis cells before and after an osmotic upshift with 0.6 M NaCl were measured by using a colorimetric assay for proline (3).
RESULTS

The ktr genes of B. subtilis. In connection with the characterization of the KtrAB K⁺ transport system of *V. alginolyticus*, Nakamura et al. (38) noted the presence of two pairs of *ktrAB*-related genes in the genome of *B. subtilis* (31). These were *yuaA* and *yubG*, located adjacent to each other at 272.3° of the chromosome, *ykqB* at 128.9°, and *ykrM* at 120.9° (Fig. 1). The *yubG* and *ykrM* genes encode proteins that are homologues to the integral membrane K⁺-translocating subunit KtrB (called NtpJ in *E. hirae* [51]), whereas the *yuaA* and *ykqB* genes are homologues to the membrane surface-associated component KtrA of this K⁺ uptake system. Our database searches revealed that YubG and YkrM exhibit 44 and 34% amino acid sequence identity, respectively, to KtrA from that organism. Since we show below that these proteins indeed constitute two K⁺ transport systems in *B. subtilis*, we refer in the following observations to *yuaA* and *yubG* as the *ktrAB* genes and to *ykqB* and *ykrM* as *ktrC* and *ktrD*, respectively. We call the corresponding K⁺ transporters KtrAB and KtrCD.

Transcriptional organization of the *ktrAB*, *ktrC*, and *ktrD* loci. We first investigated the transcriptional organization of the four *ktr* genes by Northern blot analysis. A single-stranded antisense RNA probe comprising parts of both *ktrA* and *ktrB* hybridized with a 2-kb mRNA species (Fig. 2A), in close agreement with the size (2,025 bp) deduced from the DNA sequence of the coding regions of the *ktrAB* genes and their intergenic space. Our data therefore demonstrate that the *ktrAB* genes are cotranscribed as an operon. A probe directed against the *ktrC* reading frame (calculated length, 663 bp) hybridized with a 700-nucleotide mRNA species (Fig. 2), showing that this gene is transcribed alone, although it is flanked on both sides by reading frames oriented in the same direction as *ktrC* (Fig. 1). The transcription of *ktrD* (calculated length, 1,347 bp) was so weak that its transcript was detected only when additional copies of *ktrD* were present in the wild-type strain JH642 on the *E. coli*-B. *subtilis* shuttle vector pGH16. The *ktrD* transcript detected then was 1.3 kb in length (Fig. 2A), demonstrating that this gene is expressed as a single transcriptional unit as well (Fig. 1).

In the same set of experiments, we determined whether the expression of the various *B. subtilis* *ktr* genes was influenced by the K⁺ concentration of the growth medium. For this purpose, total RNA (from cells of JH642 grown in minimal medium with either 50 μM or 200 mM KCl) was extracted and analyzed by Northern blotting using *ktrAB*, *ktrC*, and *ktrD* probes. The amount of each *ktr* transcript was clearly independent of the externally provided K⁺ concentration (Fig. 2A). Increased K⁺ uptake is among the first cellular responses of *B. subtilis* to elevated salinity of the growth medium (56). We therefore tested whether *ktrAB*, *ktrC*, and *ktrD* transcription changed when a *B. subtilis* culture of strain JH642 was grown in minimal medium with elevated osmolality (SMM with 0.6 M NaCl). Northern blot analyses demonstrated that this was not the case (data not shown).

Disruption of the KtrAB and KtrCD systems causes a defect in K⁺ uptake. To examine whether the *ktr* gene products are involved in the uptake of K⁺ in *B. subtilis*, we constructed chromosomal disruptions of the corresponding genes. In each of these gene disruption constructs, antibiotic resistance cassettes were cloned into the various *ktr* genes (Fig. 1), and the antibiotic resistance cassettes chosen for these experiments were selected in such a way that it was possible to subsequently construct appropriate double and triple *ktr* mutants. We performed growth experiments with this isogenic set of strains (Table 1) to test the K⁺ demand of the KtrAB-, KtrC-, and KtrD-deficient strains. For these experiments, the wild-type strain JH642 and the various *ktr* mutants were grown in a minimal medium with various concentrations of K⁺ and the growth yields of these cultures were then determined after 16 h (Fig. 3). Strain JH642 already exhibited a significant growth yield at 0.1 mM K⁺, and it reached its maximal growth yield at 0.5 mM K⁺ (Fig. 3A). The growth yield of strains GHB6 (*ktrC::spc*), GHB12 (*ktrD::tet*), and GHB14 (*ktrC::spc ktrD::tet*)
was indistinguishable from that of the wild type (data not shown). By contrast, the growth characteristics of the (ktrAB::neo) mutant strain GHBI1 were different. It needed at least 1 mM K⁺ for any significant growth (Fig. 3A). The same phenotype was observed for strain GHBI3, which carries an intact ktrA gene and a disruption in ktrB (ktrB::neo) (data not shown). These results revealed that KtrAB is involved in K⁺ uptake by *B. subtilis*. A function of KtrC and KtrD in K⁺ acquisition became apparent when the (ktrAB::neo) mutation was combined with the ktrC::spc and ktrD::tet alleles. The resulting triple mutant strain, GHBI5 (Table 1), exhibited a severe defect in growth yield in that more than 20 mM K⁺ was required for cell growth (Fig. 3B). Such a severe growth defect was also manifested when the ktrAB::neo mutation was combined with either the ktrC::spc or the ktrD::tet allele (data not shown).

Taken together, these growth experiments suggest that at least two different K⁺ uptake systems operate in *B. subtilis*. As judged by the growth behavior of the various ktr mutants, these two K⁺ transporters appear to differ in their affinities for K⁺.

**Lack of cross talk between subunits of the KtrAB and KtrCD systems.** The KtrA and KtrB proteins are thought to function together in K⁺ uptake, and consequently their structural genes are always found together in microorganisms that appear to possess Ktr-type K⁺ uptake systems. *B. subtilis* organisms are among the few bacteria that possess two sets of ktrA-type and ktrB-type genes (38). We therefore determined whether a KtrA/KtrD hybrid system is active in vivo in *B. subtilis* and constructed for this purpose a strain (GHBI16) which carried intact ktrA and ktrD genes but which was defective in the ktrB and ktrC loci (Table 1). When the growth of strain GHBI6 was tested as a function of the external K⁺ concentration, we found that this strain behaved exactly like a strain that is defective in both KtrAB and KtrCD (e.g., strain GHBI5; Fig. 3B). This finding demonstrates that a hybrid KtrA/KtrD system is not functional in *B. subtilis*.

**Upregulation of the ktrAB, ktrC, and ktrD gene expression in response to a disruption of the other Ktr system.** Expression of the ktr genes was independent of the K⁺ content of the growth medium (Fig. 2A). To investigate whether ktr transcription is affected when the other Ktr system is inactive, we analyzed the level of ktr transcription in different genetic backgrounds. The level of the ktrAB transcripts was determined in strain GHB14, which lacks KtrCD, whereas the expression of ktrC and ktrD was analyzed in strain GHBI1, in which KtrAB is absent. In comparison to its level in the wild-type strain, ktrAB transcription was upregulated in the ktrCD strain; the same phenomenon was observed for the expression of the ktrC and ktrD genes when KtrAB was missing (Fig. 2B). In addition, a chromosomally encoded ktrD transcript was now detected, whereas this transcript was not detected in the wild-type strain (Fig. 2B).

**Net K⁺ uptake in *B. subtilis*: major contributions of the KtrAB and KtrCD transport systems.** The growth experiments documented in Fig. 3 suggested that KtrAB and KtrCD have different affinities with respect to K⁺. To investigate this further, we determined the kinetic parameters of K⁺ uptake of the two Ktr systems. Such experiments are generally done with K⁺-depleted cells (20). *B. subtilis* cells were depleted of about 90% of their K⁺ contents by washing them once with a high Na⁺ buffer free of KCl (K0 buffer). Even this very simple procedure led to some inactivation of the cells with respect to K⁺ uptake, since these cells only reaccumulated about 0.5 to 0.7 μmol of K⁺ mg⁻¹ (dry weight) of cells from the 1.5 μmol of K⁺ mg⁻¹ (dry weight) present in the cells before K⁺ depletion. These values of reaccumulated K⁺ correspond to cytoplasmic K⁺ concentrations of 0.18 to 0.26 M. In comparison, Whatmore et al. (56) reported a value of 0.3 M for *B. subtilis* cells growing at low osmolality.

Due to the problem encountered in our experiments with respect to refilling the K⁺-depleted *B. subtilis* cells with K⁺, we consider the data determined for the kinetic parameters of net K⁺ uptake by the cells to be preliminary. These data are as follows. (i) The *Kₘ* values for K⁺ transport were about 1 mM, 10 mM, and at least 110 mM for cells that contained the KtrAB system (strain JH642, GHBI12, or GHBI14), cells that contained the KtrCD system (strain GHBI1), and cells that contained neither of these systems (strain GHBI5), respectively. (ii) The *Vₘₐₓ* values were similar for these three types of cells, with values observed of 40 to 100 nmol of K⁺ taken up min⁻¹ mg⁻¹ (dry weight) of cells. These results demonstrate that the dif-
Role of the KtrAB system in the initial adaptation to a sudden osmotic upshift. Accumulation of K$^+$ is the initial adaptation response of many bacterial cells when they are suddenly exposed to elevated osmolality (10, 13, 15, 42, 49, 56). We investigated the involvement of the KtrAB K$^+$ uptake system in the initial stress response of *B. subtilis* to a sudden osmotic challenge. Cells of the wild-type strain JH642 and the KtrAB-deficient strain GHB1 were inoculated from a preculture containing 2 mM K$^+$ to an OD$_{578}$ of 0.1 in minimal medium containing the same concentration of K$^+$. The cells then were further grown for about three generations. Under these conditions both the KtrAB$^-$ and KtrABC$^-$ strains exhibited the same growth rate (Fig. 4A). We then suddenly increased the salinity of the growth medium in both cultures through the addition of 0.6 M NaCl. After a short lag phase, the wild-type strain resumed growth with a lower growth rate, whereas growth stopped altogether in the KtrAB-deficient strain (Fig. 4A). Apparently, under conditions of a sudden osmotic upshift the ktrA$k^+$ mutant GHB1 cannot accumulate and maintain an intracellular K$^+$ level sufficient to sustain growth. The central role of K$^+$ in the resumption of growth in the KtrAB mutant after an osmotic upshift is supported by the observation that the addition of a substantial concentration of K$^+$ (50 mM) to the culture of GHB1 (ktrA$^+$ ktrB$^+$ neo $^{::}$) eventually allowed cell growth in the mutant to resume (Fig. 4A).

More information on the growth defect of the KtrAB-deficient strain was obtained by measuring the K$^+$ content of the cells under osmotic upshift conditions. Before the osmotic upshift, the K$^+$ content of growing cells of all strains tested was approximately 1.4 to 1.6 $\mu$mol mg$^{-1}$ (dry weight). Subsequent to an osmotic upshift with 0.65 M NaCl in the presence of 4 mM KCl, both the KtrAB$^+$ KtrCD$^+$ wild-type strain JH642 and the KtrAB$^+$ KtrD$^+$ strain GHB12 maintained this cellular level of K$^+$ (Fig. 4C). In striking contrast, the KtrAB$^+$ KtrCD$^+$ strain GHB1 was unable to maintain its cellular K$^+$ content under these conditions: it lost approximately 85% of its K$^+$ content within 20 min after the osmotic upshift (Fig. 4C).

Role of the KtrAB and KtrCD systems in the long-term adaptation of *B. subtilis* to high osmolality. The severe growth defect of the KtrAB-deficient strain subsequent to a sudden osmotic upshift prompted us to test also the long-term effects of high osmolality. Exponentially growing cells in a minimal medium containing 4 mM K$^+$ were subjected to a sudden osmotic upshift with 0.65 M NaCl at $t = 0$. The cellular K$^+$ content was then determined as a function of time. $\bigcirc$, strain JH642 (KtrAB$^+$ KtrC$^+$ KtrD$^+$); $\blacktriangle$, strain GHB12 (KtrAB$^+$ KtrC$^+$ KtrD$^+$); $\blacktriangleleft$, strain GHB1 (KtrAB$^+$ KtrC$^+$ KtrD$^+$); $\blacktriangledown$, strain GHB1 (KtrAB$^+$ KtrCD$^+$); $\blackdiamondsuit$, strain GHB1 (KtrAB$^+$ KtrC$^+$ KtrD$^+$).

FIG. 4. (A) Effect of a deletion of the KtrAB system on the ability of *B. subtilis* to cope with a sudden osmotic upshift. Cells of the wild-type strain JH642 (●) and the KtrAB-deficient strain GHB1 (○), $\bullet$ were grown in minimal medium containing 2 mM K$^+$ at the indicated time (downward-pointing arrow), the salinity of the growth medium was suddenly increased by the addition of NaCl from a 5 M stock solution to a final concentration of 0.6 M. For one of the cultures of GHB1 (○), we added 50 mM K$^+$ at the indicated time (arrowhead). (B) Growth of KtrAB- and KtrABCD-deficient strains at elevated salinity levels. The wild-type strain JH642 (●) and KtrAB-derivative strain GHB1 (○) were grown in minimal medium containing 2 mM K$^+$, whereas the KtrAB KtrCD double mutant strain GHB15 (■) was grown in a minimal medium that contained 25 mM K$^+$. Each of the cultures was supplemented with the indicated NaCl concentration. The growth yield of the cultures was determined after 16 h by measuring the OD$_{578}$. The various cultures were inoculated to an OD$_{578}$ of 0.05 from a preculture that contained the appropriate amount of K$^+$ (2 mM K$^+$ for strains JH642 and GHB1 and 25 mM K$^+$ for strain GHB15).
of the loss of the KtrAB and KtrCD systems in osmotically challenged cells. We therefore compared the growth yields of the wild-type strain JH642, its KtrAB derivative GHB1, and the KtrAB and KtrCD mutant GHB15 in media of different osmolalities. Precultures of JH642 and GHB1 were grown in minimal medium containing 2 mM K\(^+\), whereas GHB15 was grown in a minimal medium with 25 mM K\(^+\). These precultures were then used to inoculate cultures containing the same K\(^+\) concentrations but different concentrations of NaCl; the growth yield of the cultures was then monitored after 16 h. On exposure to high salinity, cultures of the wild-type strain showed the same pattern of growth yield (Fig. 4B) as that previously observed by Boch et al. (4). In contrast, both the KtrAB mutant GHB1 and the KtrAB and KtrCD double mutant GHB15 were unable to cope effectively with the high-salinity conditions (Fig. 4B), demonstrating that effective potassium uptake is required for the long-term adaptation of B. subtilis to high-osmolality growth conditions. This effect was particular pronounced in strain GHB15, in which both the KtrAB and KtrCD systems are defective.

**Insufficient K\(^+\) accumulation at high salinity causes a block in protein biosynthesis.** Whatmore et al. (56) observed that in resting, osmotically challenged B. subtilis cell suspensions which were starved for K\(^+\), there was no longer an accumulation of the compatible solute proline via de novo synthesis. In contrast, cells provided with K\(^+\) accumulated large amounts of proline under such conditions. These observations led Whatmore et al. to speculate that the intracellular K\(^+\) concentration was involved in regulating the proline pool of osmotically stressed B. subtilis cells (56). We investigated this issue further by first testing whether under K\(^+\)-limiting conditions there still was transcription of the proHJ operon, which encodes the enzymes required for increased proline biosynthesis under osmotic stress conditions (Brill and Bremer, submitted). Cells of strain JH642 and the KtrAB-deficient strain GHB1 were grown to early log phase in minimal medium with 2 mM K\(^+\) and were then subjected to an osmotic upshift with 0.6 M NaCl. Prior and subsequent to the increase in medium salinity, we removed cell samples, extracted their total RNA, and probed the level of proHJ transcription in a dot blot experiment. As expected from the known osmotic control of proHJ transcription (Brill and Bremer, submitted), we found increased levels of proHJ mRNA in osmotically stressed cultures. This was true for both the wild-type strain JH642 and the KtrAB-deficient strain GHB1 (Fig. 5). Although the ktrAB mutant was unable to resume growth subsequent to an osmotic upshift (Fig. 4A), the osmotic control of proHJ transcription was still operational (Fig. 5). This was also true for the osmotically controlled opuA operon that encodes a high-affinity glycine betaine transport system (29) and for the gsiB gene (36), which showed that there is no increased proline synthesis under K\(^+\)-limiting conditions in osmotically challenged B. subtilis cells. Taken together, these findings demonstrated that although osmotically controlled proHJ transcription still occurs in the K\(^+\)-limited strain GHB1, there is no production of proline. We therefore speculated that protein synthesis is affected by severe K\(^+\) limitation, since the ribosome contains a K\(^+\) ion in its active center (39). We tested this hypothesis by pulse-labeling cultures of strains JH642 and GHB1 prior and subsequent to an osmotic challenge with 0.6 M NaCl, using \[^{35}\text{S}\]methionine. Both cultures incorporated the same level of radioactivity into newly made proteins prior to the osmotic upshift (Fig. 6A), but the levels of \[^{35}\text{S}\]methionine incorporation were drastically different between strains JH642 and GHB1 subsequent to the osmotic challenge: cultures of JH642 continued to incorporate \[^{35}\text{S}\]methionine, whereas there was essentially no incorporation of this radiolabeled amino acid into proteins by the KtrAB-deficient strain GHB1 (Fig. 6B).

**DISCUSSION**

K\(^+\) movement across the cytoplasmic membrane is involved in the regulation of both cytoplasmic pH (6) and cell turgor in response to changes in environmental osmolality (13). For these physiological processes, bacteria have evolved several types of K\(^+\) uptake systems (49), one of which is the Ktr transporter system (38, 51). The data presented in this communication show that the gram-positive soil bacterium B. subtilis possesses two active Ktr-type transporter systems, KtrAB and KtrCD, which differ in their affinity for K\(^+\) by a factor of approximately 10 and which constitute the major K\(^+\) uptake activities in this bacterium. Disruption of the KtrAB and KtrCD transporters leaves only a residual K\(^+\) uptake activity for which no molecular basis has been established so far. At present we do not know whether this residual K\(^+\) uptake activity is mediated by one of the K\(^+\) transport systems described previously by others for B. subtilis (48, 50, 55).
Ktr-type K\(^+\) transporters consist of two subunits: the membrane-embedded KtrB protein and the membrane-associated KtrA protein (38). The KtrB protein has an obvious function in K\(^+\) translocation across the cytoplasmic membrane, and it is evolutionarily related (17, 18) to the KcsA K\(^+\) channel from S. lividans (16). The KtrA subunit exhibits homology to the dinucleotide-binding site [NAD(H)] sequences of many other proteins (18, 38), and recent experiments suggest that the binding of these dinucleotides to the KtrA protein regulates KtrB K\(^+\) transport activity through a ligand-mediated conformational switch mechanism (45). It is thought that KtrA combines with the transmembrane KtrB protein to form a functional K\(^+\)-translocating protein complex. Our own experiments show that both components of an individual Ktr system in B. subtilis are required for K\(^+\) uptake activity. Disruption of the ktrB gene destroys the function of the KtrAB system, and the inactivation of either ktrC or ktrD abolishes KtrCD activity. These data are in full agreement with the findings of Nakamura et al. (38) for the V. alginolyticus Ktr system, which show that both components of this system are required for its functioning as well. Our mutant studies and the preliminary K\(^+\) uptake experiments clearly show that two Ktr-type transporters are operational in B. subtilis. Both the membrane-embedded (KtrB, KtrD) and the membrane-associated (KtrA, KtrC) subunits of these transporters are pairwise similar. This finding raised the question of whether B. subtilis can take up K\(^+\) via hybrids of the two systems. We investigated this issue by constructing a mutant that expressed only the ktrA and the ktrD genes and found that this strain behaves like a mutant in which both the KtrAB and the KtrCD are simultaneously defective, demonstrating that no hybrid Ktr systems operate in B. subtilis.

The structural genes for the KtrAB and KtrCD systems appear to be constitutively expressed, since their level of transcription is not modulated in response to the K\(^+\) concentration of the growth medium or the osmolarity of the environment (Fig. 2A). They differ in this respect from the high-affinity K\(^+\) uptake system KdpFABC of E. coli, whose transcription is responsive to both increases in osmolarity (32) and K\(^+\) limitation (25). We observed compensatory changes in the level of ktrAB, ktrC, and ktrD transcription when either the KtrAB or the KtrCD system was defective (Fig. 2B). These compensatory changes are likely made by the cell for the purpose of sufficient K\(^+\) acquisition, but it is unclear how the cell can detect a defect in one of its major K\(^+\) transporters and then transmit the signal to the transcription apparatus.

Findings regarding the temporal sequence of events during the initial phase in osmoadaptation in bacteria (e.g., the rapid uptake of K\(^+\) and the subsequent genetic induction of systems for the synthesis and uptake of compatible solutes) (15) have led to the proposal that the accumulation of K\(^+\) and its counterion glutamate act as secondary messengers for osmoregulatory processes (7, 13, 19). In apparent support of this hypothesis, Whatmore et al. (56) observed that in resting, osmotically challenged B. subtilis cell suspensions which were starved for K\(^+\), de novo biosynthesis of the compatible solute proline ceased. But the addition of K\(^+\) to these cells then reestablished the capacity for proline biosynthesis. We investigated the osmoregulated transcription of the proHJ operon, a locus that encodes the central enzymes required for proline buildup in osmotically stressed cells (Brill and Bremer, submitted), in the KtrAB-deficient strain GHB1 under conditions of an osmotic upshock. In this situation, GHB1 cannot acquire and maintain a K\(^+\) pool sufficiently large to support growth (Fig. 4A and C), but osmotic induction of the proHJ operon and the locus encoding the high-affinity glycine betaine uptake system OpuA (29) still occurred (Fig. 5). In contrast to the wild-type strain, the KtrAB-deficient mutant GHB1 experienced a major defect in its protein synthesis capacity under osmotic upshock conditions, as shown by pulse-labeling with \(^{35}\)S)methionine. The inability of the mutant GHB1 to produce large amounts of proline is thus readily understandable. These data led us to conclude that the growth defect of the KtrAB mutant GHB1 after the osmotic upshift is primarily due to the inability of the cells to synthesize proteins. We therefore consider it likely that the data of Whatmore et al. (56) reflect the inability of the cell to synthesize proteins under K\(^+\) limitation rather than a disturbance in the signaling cascade that leads to osmoregulated proHJ transcription.

**FIG. 6.** Incorporation of \(^{35}\)S)methionine into osmotically challenged cells. Cells of the wild-type strain JH642 and the ktrAB mutant GHB1 were grown in minimal medium with glucose as the carbon source until an OD\(_{578}\) of 0.5 was achieved and were then pulse-labeled with radiolabeled \(^{35}\)S)methionine. At various time points, aliquots of the cell suspension were withdrawn, immediately mixed with ice-cold 10% TCA, and then incubated on ice for 30 min. Subsequently, the samples were vortexed and collected by filtration, the filters were washed with 1 ml of 5% TCA, and the radioactivity was then measured in a liquid scintillation counter. (A) Incorporation of \(^{35}\)S)methionine into proteins of strains JH642 (○) and GHB1 (○) prior to osmotic upshift. (B) Cultures of strain JH642 (○) and GHB1 (○) were osmotically challenged by the addition of 0.6 M NaCl, and 30 min after the osmotic upshift, the incorporation of \(^{35}\)S)methionine was measured.
Collectively, the data presented in this communication identify the two major K⁺ uptake activities (KtrAB and KtrCD) of *B. subtilis* at a molecular level. These K⁺ transport systems differ in their affinities for K⁺ by a factor of approximately 10. The simultaneous disruption of the KtrAB and KtrCD systems caused a severe (greater than 20 mM) K⁺ demand for the cell. Not only did the KtrAB and KtrCD systems make major contributions to net K⁺ uptake in *B. subtilis* but they were also centrally involved in acquiring cell K⁺ under high osmolality conditions and are thus an integral part of the osmoprotection response network (9) operating in this soil bacterium.

ACKNOWLEDGMENTS

We appreciate the help of V. Koogle in editing the manuscript.

REFERENCES


