

Synthesis of the Osmoprotectant Glycine Betaine in *Bacillus subtilis*: Characterization of the *gbsAB* Genes

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Synthesis of the osmoprotectant glycine betaine from the exogenously provided precursor choline or glycine betaine aldehyde confers considerable osmotic stress tolerance to *Bacillus subtilis* in high-osmolarity media. Using an *Escherichia coli* mutant (*betBA*) defective in the glycine betaine synthesis enzymes, we cloned by functional complementation the genes that are required for the synthesis of the osmoprotectant glycine betaine in *B. subtilis*. The DNA sequence of a 4.1-kb segment from the cloned chromosomal *B. subtilis* DNA was established, and two genes (*gbsA* and *gbsB*) whose products were essential for glycine betaine biosynthesis and osmoprotection were identified. The *gbsA* and *gbsB* genes are transcribed in the same direction, are separated by a short intergenic region, and are likely to form an operon. The deduced *gbsA* gene product exhibits strong sequence identity with members of a superfamily of specialized and nonspecialized aldehyde dehydrogenases. This superfamily comprises glycine betaine aldehyde dehydrogenases from bacteria and plants with known involvement in the cellular adaptation to high-osmolarity stress and drought. The deduced *gbsB* gene product shows significant similarity to the family of type III alcohol dehydrogenases. *B. subtilis* mutants with defects in the chromosomal *gbsAB* genes were constructed by marker replacement, and the growth properties of these mutant strains in high-osmolarity medium were analyzed. Deletion of the *gbsAB* genes destroyed the choline-glycine betaine synthesis pathway and abolished the ability of *B. subtilis* to deal effectively with high-osmolarity stress in choline- or glycine betaine aldehyde-containing medium. Uptake of radiolabelled choline was unaltered in the *gbsAB* mutant strain. The continued intracellular accumulation of choline or glycine betaine aldehyde in a strain lacking the glycine betaine-biosynthetic enzymes strongly interfered with the growth of *B. subtilis*, even in medium of moderate osmolarity. A single transcription initiation site for *gbsAB* was detected by high-resolution primer extension analysis. *gbsAB* transcription was initiated from a promoter with close homology to σ^A -dependent promoters and was stimulated by the presence of choline in the growth medium.

Soil microorganisms are subjected to frequent fluctuations in the osmotic conditions of their habitat due to drying and wetting of the soil. Since proper maintenance of turgor is essential for cell division and survival, bacteria must have active mechanisms to respond to changes in the environmental osmolarity in order to compete successfully for their ecological niche (8, 35). Exposure of *Bacillus subtilis* to a hypersaline environment incites an integrated physiological adaptation reaction that is aimed at restoring the disturbed cellular water balance, maintaining optimal turgor, and protecting cell components from the detrimental effects of high ionic strength. It consists of an initial rapid influx of K⁺ ions and the onset of increased proline biosynthesis (54, 55). In addition, the expression of a general stress regulon that is also responsive to other growth-limiting conditions and to stationary-phase signals is induced (20).

The induction of the general stress regulon, the uptake of potassium, and increased proline biosynthesis are, however, insufficient to ensure the growth of *B. subtilis* in harsh high-osmolarity environments (4). A more effective defense against these growth conditions is the accumulation of large amounts of osmoprotectants which can be amassed to high intracellular levels without disturbing essential functions of the cell (8, 35). One of the most important osmoprotectants is glycine betaine.

It is synthesized by plants and is thus brought into the habitat of *B. subtilis* through the degradation of plant tissues and root exudates (14, 44). Glycine betaine can be accumulated directly from the environment through three osmotically controlled uptake systems (25): OpuD, a single-component transporter, and OpuA and OpuC (ProU), multicomponent transport systems that are related to the binding protein-dependent transport system ProU from *Escherichia coli* (25, 26, 34).

In addition to directly acquiring glycine betaine from the environment, *B. subtilis* can also accumulate this osmoprotective compound through synthesis (4). Synthesis requires the presence of the precursor choline or glycine betaine aldehyde in the growth medium (4). Production of glycine betaine from choline is a two-step oxidation process with glycine betaine aldehyde as the intermediate. Uptake of the precursor molecules for glycine betaine synthesis is mediated by an efficient transport system that is osmotically regulated at the level of transport activity and the expression of its structural gene(s). In contrast, the systems mediating the enzymatic conversion of choline into glycine betaine are only marginally stimulated by an increase in medium osmolarity (4). *B. subtilis* shares the ability to oxidize choline to glycine betaine for osmoprotective purposes with a number of gram-negative and gram-positive bacteria (8, 15, 35). This process has been most intensively studied at both the molecular and biochemical levels for *E. coli* (30, 31). In *E. coli*, a membrane-bound and flavin adenine dinucleotide-dependent choline dehydrogenase (BetA) oxidizes choline to glycine betaine aldehyde, which is then further oxidized to glycine betaine at approximately the same rate. A second enzyme, a betaine aldehyde dehydrogenase (BetB), has

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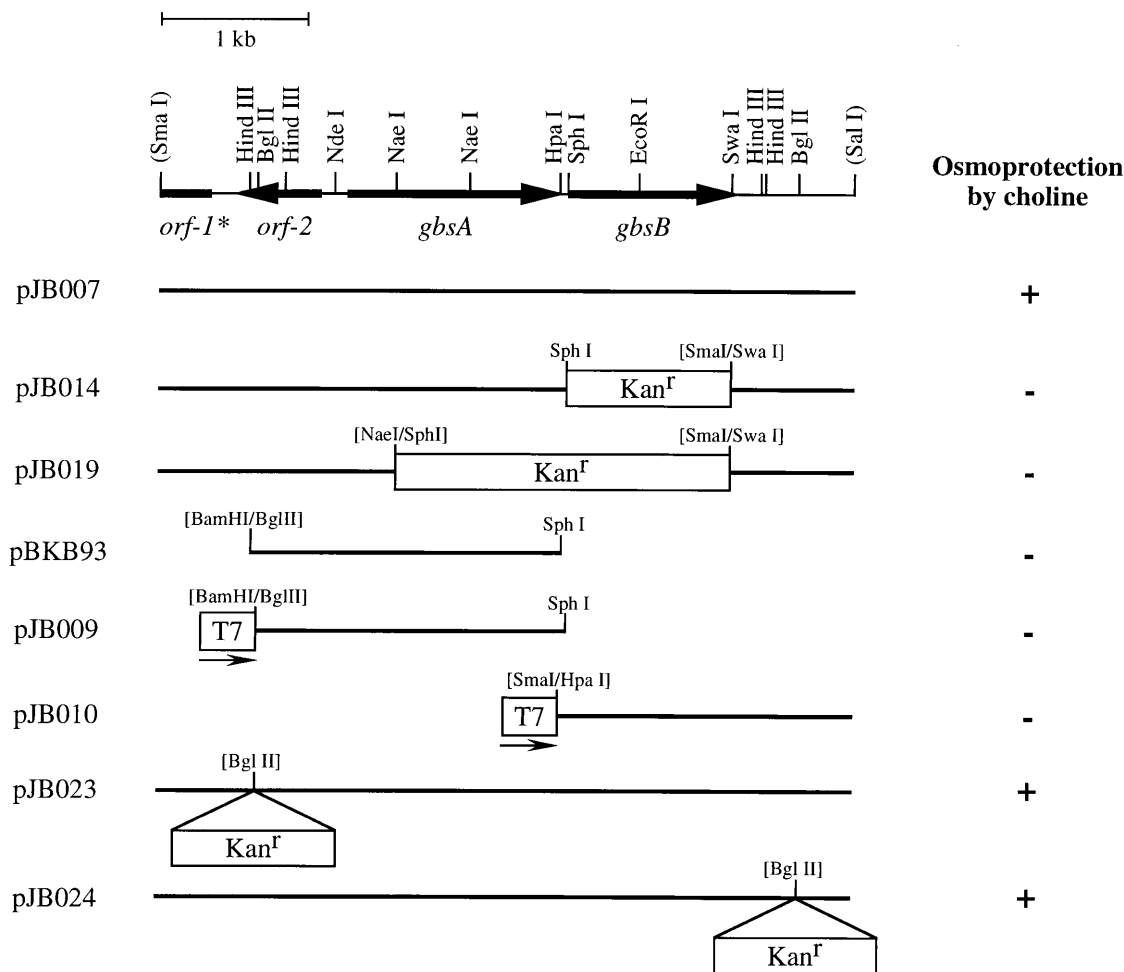


FIG. 1. Genetic organization of the *gbsAB* region. A restriction map of the chromosomal fragment present in plasmid pJB007 is shown. This DNA segment was originally cloned as a fragment from partial *Sau*3A digestion of chromosomal DNA of *B. subtilis* JH642 and inserted into the *Bam*HI site of the polylinker present in plasmid pHSG575 (51). The *Sma*I and *Sal*I restriction sites flanking the original *Bam*HI site are derived from the cloning vector and are therefore shown in parentheses. The ability of choline to protect *E. coli* MKH13(pJB004 [*betT*⁺]) carrying the indicated plasmids from the detrimental effects of high osmolarity is shown (+, growth; -, no growth). Growth of the bacterial strains was tested on high-osmolarity minimal agar plates (MMA with 0.7 M NaCl) in the presence of 1 mM choline; strain MKH13(pJB004 [*betT*⁺]) by itself cannot grow on these plates.

a high substrate specificity and converts glycine betaine aldehyde to the osmoprotectant glycine betaine. Uptake of the precursor molecule choline is mediated with high affinity by the BetT transporter and with low affinity via the glycine betaine transport system ProU (32).

The choline-glycine betaine synthesis pathway is an important facet of the process of cellular adaptation of *B. subtilis* to high-osmolarity stress (4). However, the genetic and biochemical details governing choline uptake and glycine betaine synthesis have remained largely unknown. This prompted us to study the choline-glycine betaine synthesis pathway more closely at the molecular level.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. *B. subtilis* JBB4 [Δ (*gbsB::neo*)1] and JBB5 [Δ (*gbsAB::neo*)2] are derivatives of JH642 (*trpC2 pheA1*) (BGSC 1A96) and were constructed in this study by transforming JH642 with *Sma*I-*Sal*I restriction fragments isolated from plasmids pJB014 [Δ (*gbsB::neo*)1] (Fig. 1) and pJB019 [Δ (*gbsAB::neo*)2] (Fig. 1), respectively. The *E. coli* K-12 strain MKH13 [Δ (*betTIBA*)U169 Δ (*putPA*)101 Δ (*proP*)2 Δ (*proU*)608], the *E. coli* B strain BL21 (λ DE3), and the plasmids pBR322, pRB373, pPD100, and pHSG575 have been described previously (5, 7, 11, 17, 49, 51). The kanamycin resistance cassette used for gene disruption experiments was isolated from plasmid pAT21 (52), and the

recombinant *betTIBA* λ phage [135]6E6(+) was from the Kohara bacteriophage λ clone miniset collection (27).

Growth conditions, media, and chemicals. Bacteria were maintained on Luria-Bertani medium (37). When minimal medium was used, *B. subtilis* strains were grown in Spizizen's minimal medium (SMM) with 0.5% glucose as the carbon source and supplemented with L-tryptophan (20 mg/liter), L-phenylalanine (18 mg/liter), and a solution of trace elements (19). *E. coli* strains were grown in minimal medium A (MMA) with 0.2% glucose as the carbon source (37). Expression of the *gbsA* or *gbsB* gene under T7 ϕ 10 control was carried out in cultures of strain BL21(λ DE3) grown in M9 minimal medium (37) containing 0.2% Casamino Acids and 0.2% glucose. The antibiotics ampicillin, chloramphenicol, and kanamycin were used with *E. coli* strains at final concentrations of 100, 30, and 30 μ g/ml, respectively. For the selection of kanamycin-resistant *B. subtilis* strains, a final concentration of 5 μ g of the antibiotic per ml was used. Radiolabelled [*methyl*-¹⁴C]glycine betaine was synthesized as described previously (4).

Methods used with nucleic acids and construction of plasmids. Routine manipulations of plasmid DNA, the isolation of chromosomal DNA from *B. subtilis*, and the detection of homologous sequences by Southern hybridization were all carried out by standard techniques (9, 48). Sequencing of double-stranded plasmid DNA and of single-stranded DNA segments cloned in phage M13BM20 or M13BM21 (Boehringer, Mannheim, Germany) was determined with the Sequenase 2.0 kit and 7-deaza-dGTP (U.S. Biochemical Corp., Braunschweig, Germany). The transcription initiation site of the *gbsA* gene was determined by primer extension analysis with a synthetic primer (5'-TGCAATTTCTTCTTGATTAA ATGGG-3') complementary to the *gbsA* mRNA (bp 1358 to 1334 [see Fig. 3]) and avian myeloblastosis virus reverse transcriptase as described previously (26).

A library of chromosomal DNA segments from the *B. subtilis* wild-type strain JH642 was prepared by partially cleaving chromosomal DNA with *Sau3A* and ligating the resulting restriction fragments into the *Bam*HI site of the polylinker of the *lacZ* α -complementing plasmid pHSG575. To clone the *betTBA* gene cluster from *E. coli* (30), DNA from the λ phage [135]6E6(+) (27) was cleaved with *Bam*HI, and a 9.0-kb restriction fragment was inserted into the *Bam*HI site of pBR322, yielding pJB003. A subclone carrying the *betT*⁺ genes was prepared by isolating a 4.4-kb *Bam*HI-*Sca*I fragment from plasmid pJB003 and ligating it into pBR322 DNA that had been digested with *Bam*HI and *Ssp*I, resulting in plasmid pJB004. The physical structures of the plasmids carrying either all or part of the *gbsAB* gene cluster are shown in Fig. 1. Plasmids pJB007, pJB014, pJB019, pJB023, and pJB024 are derived from pHSG575. Plasmids pJB009 and pJB010 are derivatives of the T7 ϕ 10 expression vector pPD100, and plasmid pKB93 is an *E. coli*-*B. subtilis* shuttle plasmid derived from pRB373.

Assay for glycine betaine synthesis activity. To determine the abilities of *E. coli* strains to synthesize glycine betaine from exogenously provided choline, the fate of radiolabelled [*methyl*-¹⁴C]choline was monitored by thin-layer chromatography. One milliliter of MMA minimal medium containing glucose as the carbon source, 150 mM NaCl to increase the osmolarity of the growth medium, and 38 μ M [*methyl*-¹⁴C]choline (5.3 mCi/mmol) was inoculated with 0.1 ml of an overnight culture. The cells were then grown with aeration at 37°C, and samples (0.5 ml) were taken after 1 and 16 h. These cells were pelleted by centrifugation. The cell pellet and 0.2 ml of the supernatant were collected and frozen at -20°C until further analysis. The cells were broken by treatment with a solution (50 μ l) containing NaOH (0.2 M) and 1% sodium dodecyl sulfate (SDS). Cellular debris was removed by centrifugation, and 5 μ l of the soluble fraction and 5 μ l of the frozen supernatant of the cells were spotted onto thin-layer chromatography plates (Silica Gel G plates; Macherey-Nagel, Düren, Germany). The thin-layer chromatography plates were developed with a methanol-0.88 M ammonia (75:25) solution as the running solvent and subsequently autoradiographed (4). Glycine betaine synthesis in *B. subtilis* was monitored in a similar fashion except that the growth medium was SMM containing 400 mM NaCl and the cells were lysed by treatment with lysozyme and SDS exactly as described by Boch et al. (4). Assays of radiolabelled [*methyl*-¹⁴C]choline (5.3 mCi/mmol) uptake in *E. coli* and *B. subtilis* strains (at a final substrate concentration of 10 μ M) were performed as described previously (4).

Expression of the *gbsA* and *gbsB* genes under T7 ϕ 10 control and N-terminal sequencing of the GbsA and GbsB proteins. The *E. coli* B strain BL21(λ DE3) carrying the gene for the T7 RNA polymerase in the chromosome under *lacPO* control (49) was used as the host strain for the expression of the *gbsA* and *gbsB* genes under T7 ϕ 10 control under the conditions described by Dersch et al. (11). Two-dimensional polyacrylamide gel electrophoresis of the whole-cell extracts, elution of the GbsA and GbsB proteins from the gel, and microsequencing of their amino-terminal ends were performed as described by Graumann et al. (16).

Computer analysis. Searches for homologies were performed at the National Center for Biotechnology Information with the BLAST programs (1) and the current version of the databases (January 1996).

Nucleotide sequence accession number. The nucleotide sequence data presented here were submitted to the GenBank database and assigned accession number U47861.

RESULTS

Cloning of the genes required for glycine betaine synthesis.

The *E. coli* genes encoding the uptake system for choline (*betT*) and its enzymatic conversion into glycine betaine (*betBA*) are adjacent on the chromosome together with the regulatory gene *betI* (30). Assuming that the corresponding genes from *B. subtilis* (4) are also tightly genetically linked, we initially tried to clone them by functional complementation of *E. coli* MKH13 [Δ (*betTBA*)] (26). Despite repeated attempts and the use of different gene libraries from *B. subtilis*, no MKH13 derivatives that could grow on high-osmolarity minimal plates (MMA with 0.7 M NaCl) with 1 mM choline were found.

Our failure to recover the *B. subtilis* genes encoding the choline uptake system and the glycine betaine-biosynthetic enzymes could result from the fact that they are not clustered on the chromosome. We therefore modified our selection strategy and focused on the cloning of the *B. subtilis* genes coding for the glycine betaine-biosynthetic enzymes. A derivative of strain MKH13 that harbored the *E. coli* choline transport gene *betT* on a pBR322-derived plasmid (pJB004; Amp^r) was constructed. Strain MKH13(pJB004) can thus accumulate choline, but it cannot oxidize it to the osmoprotectant glycine betaine. Since choline has no osmoprotective properties per se (50), strain MKH13(pJB004) cannot grow on high-osmolarity minimal

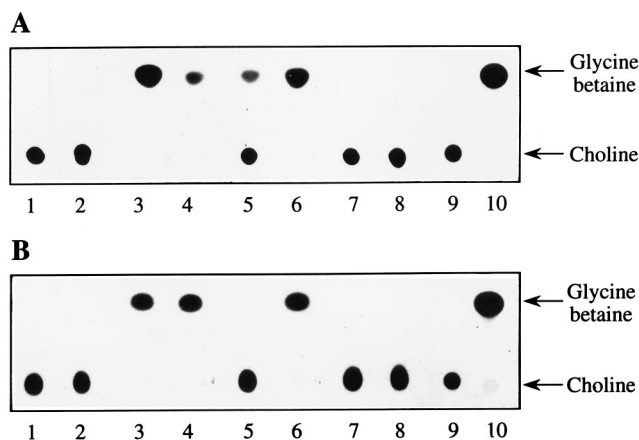


FIG. 2. The cloned *gbsAB* region mediates glycine betaine synthesis. Derivatives of *E. coli* MKH13 carrying various plasmids were grown with aeration in minimal medium (MMA with 0.15 M NaCl) and incubated with [*methyl*-¹⁴C]choline (final concentration, 38 μ M). Samples (0.5 ml) were taken at 1 and 16 h, the cells were pelleted by centrifugation, and the soluble components of the disrupted cells (A) and the supernatants of the cultures (B) were separated by thin-layer chromatography on Silica Gel G plates. The radiolabelled compounds were visualized by autoradiography. Lanes: 1 and 2, strain MKH13(pJB004 [*betT*⁺]); 3 and 4, strain MKH13(pJB003 [*betTBA*⁺]); 5 and 6, strain MKH13(pJB004 [*betT*⁺], pJB007 [*gbsAB*⁺]); 7 and 8, strain MKH13(pJB004 [*betT*⁺], pHSG575 [vector]). The samples shown in lanes 1, 3, 5, and 7 were taken after 1 h of growth of the cultures; those shown in lanes 2, 4, 6, and 8 were taken after 16 h of growth of the cultures. Lanes 9 and 10, radiolabelled choline and glycine betaine, respectively. Choline does not migrate on the Silica Gel G plates with the solvent system used (methanol-0.88 M ammonia [75:25]).

plates containing choline. We then transformed the *B. subtilis* gene library prepared in pHSG575 (Cm^r) into strain MKH13 (pJB004) and selected for chloramphenicol-resistant colonies that could grow under high-osmolarity conditions (MMA with 0.7 M NaCl) in the presence of 1 mM choline. Osmotolerant transformants were readily recovered. Five of these strains were chosen for further analysis, and each of them contained in addition to pJB004 (*betT*⁺) a pHSG575-derived plasmid with a cloned DNA segment. Restriction analysis of these plasmids revealed that the cloned regions differed slightly in size but were all related to each other. Plasmid pJB007 was chosen for further characterization, and a restriction map of the cloned DNA segment is presented in Fig. 1. There was no growth of MKH13(pJB004, pJB007) when choline was omitted from the high-osmolarity plates, and there was no growth when plasmid pJB007 was transformed into strain MKH13 lacking the *betT*⁺ plasmid pJB004. Furthermore, uptake studies with radiolabelled choline revealed no choline transport activity in strain MKH13(pJB007). Thus, the cloned segment from the *B. subtilis* genome carried genes required to convert choline into an osmoprotective compound, but it did not encode a functional choline transport system.

Plasmid pJB007 mediates glycine betaine synthesis from choline. We then tested whether plasmid pJB007 could confer the ability to synthesize glycine betaine from exogenously provided choline to the *betBA* deletion strain MKH13(pJB004). Small cultures (1 ml of MMA with 150 mM NaCl) of various MKH13 derivatives were inoculated with 0.1 ml from overnight cultures, supplemented with radiolabelled [*methyl*-¹⁴C]choline, and incubated with aeration at 37°C. Samples were taken after 1 and 16 h, and the nature of the radiolabelled compounds that had accumulated in the cells was determined by thin-layer chromatography and subsequent autoradiography (Fig. 2A). Derivatives of strain MKH13 carrying the plasmid-encoded choline *E. coli* uptake system (*betT*) or the entire *betTBA* gene

cluster were used as controls. As expected, strain MKH13 (pJB004 [*betT*⁺]) accumulated choline but was unable to oxidize it to glycine betaine, whereas choline was quantitatively converted into glycine betaine in strain MKH13(pJB003 [*bet-TIBA*⁺]) (Fig. 2A, lanes 1 to 4). The *B. subtilis* DNA fragment present in pJB007 conferred the ability to synthesize glycine betaine on the *E. coli* host strain (Fig. 2A, lanes 5 and 6), whereas the presence of the vector pHSG575 did not result in the production of glycine betaine (Fig. 2A, lanes 7 and 8). Thus, these data clearly show that the cloned *B. subtilis* region encodes enzymes capable of converting exogenously providing choline into the osmoprotectant glycine betaine. We refer to this *B. subtilis* locus as *gbs* (for glycine betaine synthesis) below.

Since substantial radioactivity remained in the supernatants of the cultures even after 16 h of growth in the presence of [*methyl*-¹⁴C]choline, we analyzed the nature of the radiolabelled compounds by thin-layer chromatography (Fig. 2B). Only [*methyl*-¹⁴C]choline could be found in the supernatants of the cultures of strains deficient in glycine betaine synthesis (Fig. 2B, lanes 1, 2, 7, and 8), indicating that this compound either was not taken up completely or leaked from the bacterial cells once it had been accumulated. Surprisingly, glycine betaine was detected in the culture medium of the two strains proficient in glycine betaine synthesis (Fig. 2, lanes 3, 4, and 6), suggesting that these MKH13-derived strains, which are defective in the glycine betaine transport systems ProP and ProU (35), are incapable of retaining all of the newly synthesized glycine betaine inside the cell. This loss of the newly synthesized glycine betaine probably reflects the presence of specific export systems for this osmoprotectant (28, 32).

DNA sequence analysis of the *gbs* locus. Analysis of the *gbs*⁺ plasmid pJB007 indicated that a 3.7-kb *Bgl*II restriction fragment was sufficient to confer osmoprotection to MKH13 (pJB004) in the presence of choline (Fig. 1). To gain insight into the genetic organization of the cloned *gbs* locus from *B. subtilis*, we determined the nucleotide sequence of a 4,106-bp DNA segment from plasmid pJB007 (Fig. 3). Inspection of the sequenced region revealed the presence of three complete open reading frames (ORFs) and one incomplete ORF. Two of these ORFs constitute the *gbs* locus (see below), and we refer to them as the *gbsA* and *gbsB* genes (Fig. 1). The *gbsA* gene starts with an ATG codon at bp 1263 and ends with a TAA stop codon at bp 2735. It encodes a 490-residue protein (GbsA) with a calculated molecular mass of 53.67 kDa. Inspection of the DNA sequence suggested two alternative start sites for the *gbsB* gene. It could start either with the ATG codon at bp 2752 or with the ATG codon at bp 2772; the *gbsB* frame ends with a TAA stop codon at bp 3960 (Fig. 3). Both ATG codons are preceded at an appropriate distance by a ribosome-binding site, and hence the *gbsB* reading frame could code for either a 402-residue (43.425-kDa) protein or a 393-residue (42.391-kDa) protein. Downstream of the *gbsB* gene is an inverted repeat that potentially could form a stem-loop structure that might function as a factor-independent transcription termination signal. No such inverted repeat is present in the short intergenic region between the *gbsA* and *gbsB* genes, indicating that they might form an operon (Fig. 3).

Divergently oriented with respect to the *gbsAB* genes is an ORF (*orf-2*) of 540 bp that potentially can encode a protein of 180 amino acids with a calculated molecular mass of 21.07 kDa. The *orf-2* coding region is preceded by a potential ribosome-binding site and is followed by a possible stem-loop structure formed by inverted repeats (Fig. 3). Comparison of the deduced Orf-2 protein sequence with proteins in the data banks revealed no significant homology to any other protein. The intergenic region (198 bp) of the divergently oriented

gbsAB genes and *orf-2* is highly A · T rich (Fig. 3), a hallmark of *B. subtilis* regulatory and promoter regions (21). Downstream of *orf-2* we found the 5' end of an incomplete ORF (*orf-1*^{*}) (Fig. 3). A data bank search revealed that the Orf-1^{*} protein shows significant homology (44 identical residues in a 108-amino-acid segment) to a 16.7-kDa protein (the *ipa-74d* gene product) from *B. subtilis* with unknown function. Disruption of *orf-2* by the insertion of a kanamycin resistance cassette (pJB023 [Fig. 1]) or by the insertion of the same drug resistance cassette a few hundred base pairs downstream of the *gbsAB* genes (pJB024 [Fig. 1]) did not abolish osmoprotection of *E. coli* MKH13(pJB004) in the presence of choline. These findings indicate that in addition to *gbsA* and *gbsB*, no other genes are required for glycine betaine synthesis.

The *gbsA* gene encodes a glycine betaine aldehyde dehydrogenase. The amino acid sequence of GbsA was compared with the protein sequences deposited in the databases. Striking sequence identities between GbsA and members of a large superfamily of specialized and nonspecialized aldehyde dehydrogenases of prokaryotes and eukaryotes were detected (18). Thus, the GbsA protein is likely to function as an aldehyde dehydrogenase. The aldehyde dehydrogenase superfamily comprises seven enzymes of bacterial and plant origin with known involvement in the synthesis of the osmoprotectant glycine betaine from its precursor glycine betaine aldehyde. These are the BetB proteins from *E. coli* (13) and *Rhizobium meliloti* (41) and betaine aldehyde dehydrogenases from plants (*Spinacia oleracea*, *Beta vulgaris*, *Atriplex hortensis*, *Sorghum bicolor*, and *Hordeum vulgare*) involved in the cellular adaptation to salinity and drought (24, 36, 53, 56, 57). The *B. subtilis* enzyme exhibits 39% sequence identity to the BetB proteins from *E. coli* and *R. meliloti*. We found identities between GbsA and the corresponding plant enzymes of 45% (*B. vulgaris*), 43% (*S. oleracea* and *H. vulgare*), 42% (*A. hortensis*), and 37% (*S. bicolor*).

The *gbsB* gene encodes an alcohol dehydrogenase. Computer searches revealed a significant similarity between GbsB and a family of alcohol dehydrogenases (type III) (43). This family of proteins comprises NAD/NADP-dependent alcohol dehydrogenases from several microorganisms, *Saccharomyces cerevisiae*, and *Entamoeba* species. The sequence identity between the GbsB protein and members of the type III alcohol dehydrogenase family varied from 21% for the 4-hydroxybutyrate dehydrogenase (4Hbd) from *Clostridium kluyveri* to 38% for the 1,3-propanediol dehydrogenase (DhaT) from *Citrobacter freundii*. The enzymes belonging to the type III alcohol dehydrogenase family usually have a subunit size of approximately 371 to 424 amino acid residues, except for the multifunctional AdhE proteins from *E. coli* (891 residues) and *Clostridium acetobutylicum* (862 residues) and the EhADH2 protein from *Entamoeba histolytica* (872 residues). These large proteins represent fusions of an aldehyde dehydrogenase (at the N-terminal end) with a type III alcohol dehydrogenase located at the carboxy terminus of the polypeptide chain (43). An iron-binding motif has been postulated for the type III alcohol dehydrogenases (2), and this motif is completely conserved in those enzymes with an experimentally demonstrated Fe²⁺ requirement for their catalytic activity (e.g., Adh2 from *Zymomonas mobilis*, FucO and AdhE from *E. coli*, and DhaT from *Citrobacter freundii*) (for references, see reference 10). The GbsB protein of *B. subtilis* lacks one of the three histidine residues implicated in metal binding, thus leaving the nature of the metal cofactor for this enzyme unclear.

High-level expression of the *gbsAB* genes and identification of their gene products. To visualize the GbsA and GbsB proteins, we expressed their structural genes separately under the control of the T7 ϕ 10 promoter in *E. coli*. Induction resulted in

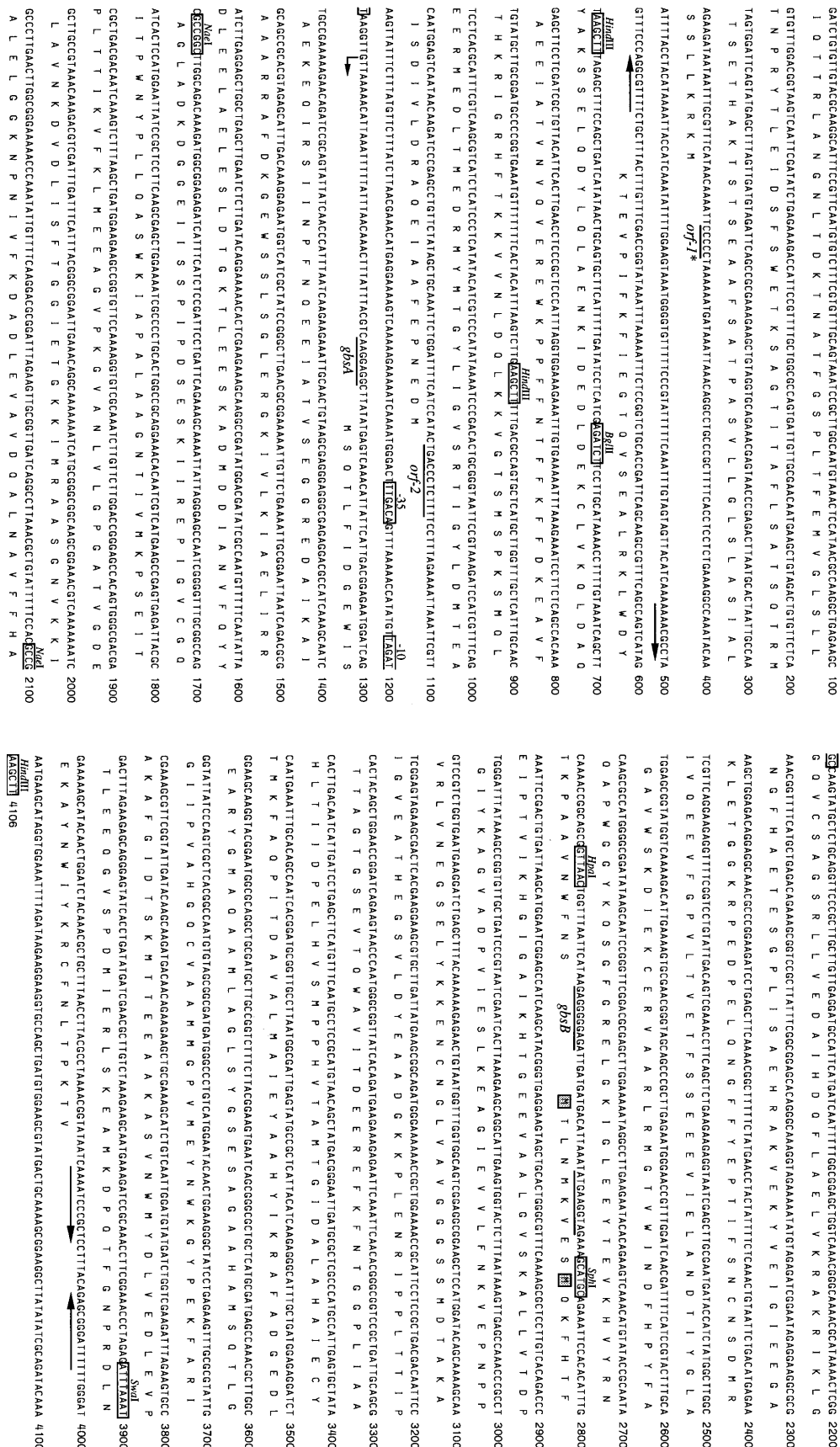


FIG. 3. Nucleotide sequence of the *gbsAB* region. The DNA sequence originally used to clone the chromosomal DNA segment to the first *HindIII* site downstream of the *gbsAB* region (Fig. 1) is shown. The deduced amino acid sequence of the *gbsA* and *gbsB* genes, of *orf2*, and of the incomplete ORF *orf1** is shown. The putative ribosome-binding sites are underlined, and the positions of inverted repeats that possibly could function in transcription termination are shown by pairs of inverted arrows. Recognition sites for several restriction enzymes are indicated. The -35 and -10 promoter regions are boxed, and the start site of the *gbs* mRNA is marked by a bent arrow. The alternative GhsB amino termini are marked by shaded squares.

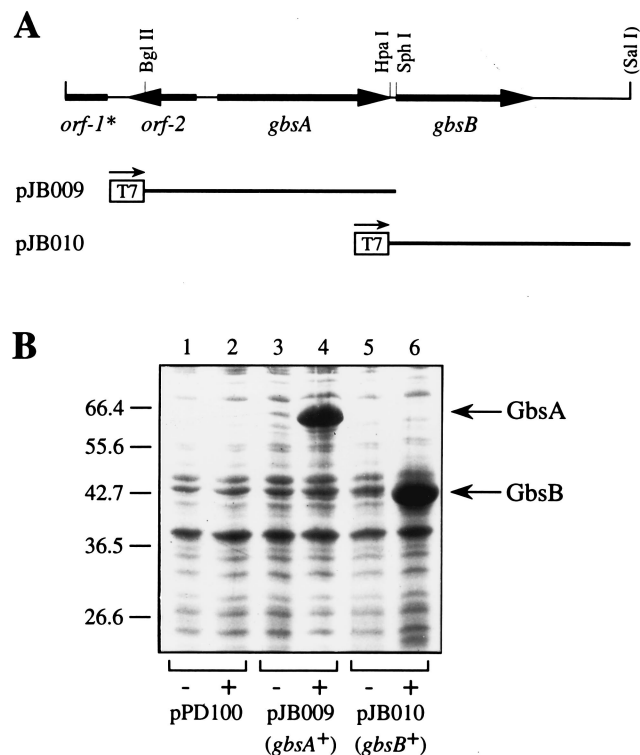


FIG. 4. Expression of the *gbsA*- and *gbsB*-encoded proteins under the control of the T7 ϕ 10 promoter. (A) Physical and genetic structures of the *gbsA*⁺ and *gbsB*⁺ plasmids pJB009 and pJB010, respectively. (B) T7 ϕ 10-mediated gene expression was performed with derivatives of *E. coli* BL21(λ DE3) carrying the vector pPD100 (uninduced [lane 1] or induced with 1 mM IPTG [isopropyl- β -D-thiogalactopyranoside] [lane 2]), plasmid pJB009 (*gbsA*⁺) (uninduced [lane 3] or induced [lane 4]), or plasmid pJB010 (*gbsB*⁺) (uninduced [lane 5] or induced [lane 6]). The proteins were electrophoretically separated on an SDS-12% polyacrylamide gel and visualized by staining with Coomassie brilliant blue. The positions of the GbsA and GbsB proteins are indicated by arrows, and the molecular masses of marker proteins (in kilodaltons) are indicated on the left.

the strong production of a protein with an apparent molecular mass of 62 kDa in the presence of the *gbsA*⁺ plasmid pJB009 and of a protein with an apparent molecular mass of 43 kDa in the presence of the *gbsB*⁺ plasmid pJB010 (Fig. 4). The apparent molecular mass of the GbsB protein is in excellent agreement with the molecular mass (43.425 or 42.391 kDa) of this protein deduced from the *gbsB* DNA sequence. However, the apparent molecular mass of the glycine betaine aldehyde dehydrogenase GbsA (62 kDa) inferred from its mobility on an SDS-polyacrylamide gel (Fig. 4) deviates considerably from the molecular mass (53.671 kDa) deduced from the *gbsA* gene. Interestingly, the same phenomenon was observed by Weretilnyk and Hanson (53) for the betaine aldehyde dehydrogenase from spinach.

The proteins from the cell extracts prepared from the GbsA- and GbsB-overproducing strains were separated by two-dimensional gel electrophoresis, and the GbsA and GbsB proteins were eluted from the gel and used for microsequencing of their amino termini; for each protein, 25 amino acid residues were determined. The amino terminus of the GbsA protein was in perfect agreement with the protein sequence deduced from the *gbsA* reading frame (Fig. 3). Two amino termini were found for the GbsB protein: approximately two-thirds of the protein preparation started with a methionine residue corresponding to the ATG start codon at bp 2752, and the rest of the protein sample had an amino terminus that corresponded to

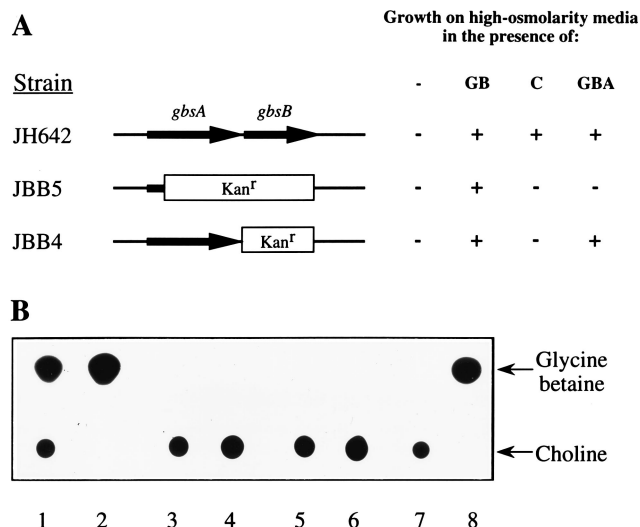


FIG. 5. Influence of mutations in the *gbsAB* locus on the use of osmoprotectants and synthesis of glycine betaine by *B. subtilis*. (A) Genetic organizations of the *gbsAB* regions of the *B. subtilis* wild-type strain JH642 and its chromosomal deletion derivative strains JBB4 [$\Delta(gbsB::neo)1$] and JBB5 [$\Delta(gbsAB::neo)2$]. Growth of the strains on high-osmolarity minimal agar plates (SMM with 1.2 M NaCl) in the absence of an osmoprotectant (-) or in the presence of 1 mM glycine betaine (GB), choline (C), or glycine betaine aldehyde (GBA) was determined. Growth of the strains was scored after 3 days of incubation at 37°C (+, growth; -, no growth). (B) Cultures of *B. subtilis* JH642 (lanes 1 and 2), JBB4 (lanes 3 and 4), and JBB5 (lanes 5 and 6) were grown in minimal medium (SMM with 0.4 M NaCl) in the presence of 38 μ M [*methyl*-¹⁴C]choline. Samples (0.5 ml) were taken at 1 and 16 h, the cells were pelleted by centrifugation and disrupted, and the soluble components were separated by thin-layer chromatography on Silica Gel G plates. The radiolabelled compounds were visualized by autoradiography. The samples shown in lanes 1, 3, and 5 were taken after 1 h of growth of the cultures; the samples shown in lanes 2, 4, and 6 were taken after 16 h of growth. Lanes 7 and 8 display radiolabelled choline and glycine betaine, respectively, used as standards.

the ATG start codon at bp 2779 (Fig. 3). Thus, at least when overexpressed in the heterologous host *E. coli*, the *B. subtilis* GbsB protein appears to be translated from two alternative initiation sites.

The *gbsAB* genes are essential for the utilization of choline as a precursor for glycine betaine synthesis. To further analyze the physiological function of the *gbsAB*-encoded proteins in the adaptation reaction of *B. subtilis* to high-osmolarity stress, we constructed chromosomal deletion mutations and determined the growth properties of these strains in high-osmolarity medium. For these studies, two constructs were made. In plasmid pJB019, both the *gbsA* and *gbsB* genes were destroyed and replaced by a kanamycin resistance cassette (Fig. 1). In plasmid pJB014, we retained an intact *gbsA* gene but replaced the *gbsB* gene with a kanamycin resistance marker (Fig. 1). Both constructs were then inserted by marker replacement into the chromosome of the *gbsAB*⁺ strain JH642, yielding strains JBB5 [$\Delta(gbsAB::neo)2$] and JBB4 [$\Delta(gbsB::neo)1$], respectively (Fig. 5A). The presence and the expected structures of these *gbs* mutations in the chromosome of strains JBB4 and JBB5 were verified by Southern hybridization (data not shown).

We found that both *gbs* mutant strains were unable to grow on high-osmolarity minimal plates (SMM with 1.2 M NaCl) in the presence of 1 mM choline, whereas the *gbsAB*⁺ parent strain JH642 readily grew under these conditions. Strains JBB5 and JBB4 were, however, protected from the detrimental effects of high osmolarity by the presence of 1 mM glycine betaine in the growth medium (Fig. 5A), demonstrating that the $\Delta(gbsAB::neo)2$ and $\Delta(gbsB::neo)1$ mutations do not confer

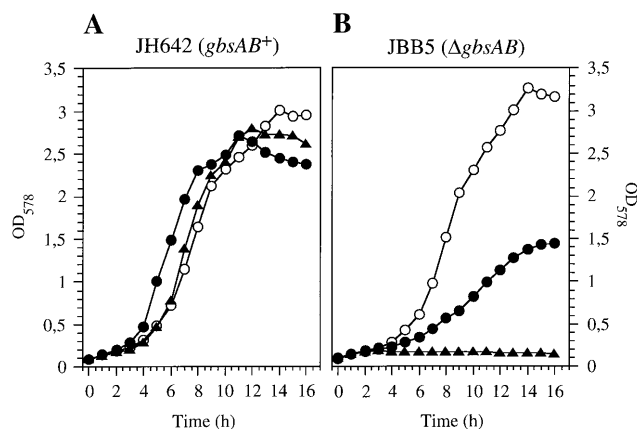


FIG. 6. Choline and glycine betaine aldehyde are detrimental to the growth of the *gbsAB* mutant strain JBB5. Cultures (75 ml in 500-ml Erlenmeyer flasks) were inoculated from overnight cultures of the *gbsAB*⁺ strain JH642 (A) and the Δ (*gbsAB::neo*)2 strain JBB5 (B) and were grown in a shaking water bath (200 to 220 rpm) at 37°C in SMM with 0.4 M NaCl. The cultures were grown either in the absence of any osmoprotectant (○) or in the presence of 1 mM choline (●) or 1 mM glycine betaine aldehyde (▲). OD₅₇₈, optical density at 578 nm.

a general sensitivity to high-osmolarity growth conditions. Thus, the *gbsAB* genes are absolutely required for the utilization of choline as an osmoprotectant by *B. subtilis*. Mutations in the *gbsAB* and *gbsB* genes confer different growth phenotypes in high-osmolarity medium in the presence of glycine betaine aldehyde. The Δ (*gbsAB::neo*)2 mutant strain JBB5 could not use this compound for osmoprotection, whereas the *gbsA*⁺ *gbsB* strain JBB4 was able to grow in high-osmolarity medium containing 1 mM glycine betaine aldehyde (Fig. 5A). The inability of strains JBB5 and JBB4 to use choline as an osmoprotectant is a reflection of a defect in the glycine betaine synthesis pathway in these strains (Fig. 5B). The *gbsAB*⁺ strain JH642 accumulated choline and converted it quantitatively into glycine betaine (Fig. 5B, lanes 1 and 2), whereas choline was taken up by strains JBB5 and JBB4 but no glycine betaine was synthesized (Fig. 5B, lanes 3 to 6). Thus, the *gbsAB* gene cluster is essential for the synthesis of glycine betaine in *B. subtilis*.

Glycine betaine aldehyde and choline are detrimental to cell growth in the *gbsAB* deletion mutant. We observed that the precursors for glycine betaine synthesis, choline and glycine betaine aldehyde, are highly detrimental to cell growth when these compound cannot be enzymatically oxidized to glycine betaine. We grew the *gbsAB*⁺ strain JH642 and its Δ (*gbsAB::neo*)2 mutant derivative, strain JBB5, in a medium of moderate osmolarity (SMM with 0.4 M NaCl). These osmotic conditions impair cell growth of *B. subtilis* only marginally but allow maximal accumulation of exogenously provided choline and glycine betaine aldehyde (4). Both compounds had little effect on the growth of the wild-type strain JH642 (Fig. 6A), but the Δ (*gbsAB::neo*)2 mutant strain JBB5 showed considerably reduced growth in the presence of choline and was totally inhibited in growth by glycine betaine aldehyde (Fig. 6B). Thus, a block in the choline-glycine betaine synthesis pathway strongly interferes with cell growth when the precursors for glycine betaine synthesis are present in the medium. In contrast, exogenously provided glycine betaine did not impair the growth of the wild-type or the mutant strains (data not shown).

Identification of the *gbsA* transcription initiation site. To identify the promoter for the *gbsAB* gene cluster, we mapped the transcription initiation site by primer extension analysis. Total

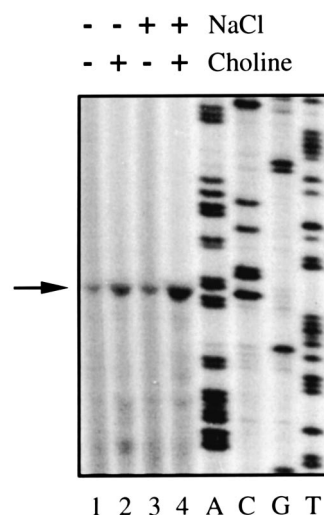


FIG. 7. Mapping of the *gbsA* transcription initiation site. Total RNA was prepared from cells of strain JH642(pBKB93) grown in either low (SMM)- or high (SMM plus 0.4 M NaCl)-osmolarity minimal medium with or without the addition of 1 mM choline as indicated. The RNA (18 μ g) was hybridized to a primer complementary to the *gbsA* mRNA in the presence of radiolabelled ³⁵S-dATP and extended with avian myeloblastosis virus reverse transcriptase. DNA sequencing reactions primed with the same oligonucleotide were employed as a standard to size the *gbsA* mRNA.

RNA was prepared from log-phase cultures of strain JH642 (pBKB93) (Fig. 1) grown either in low-osmolarity (SMM) or high-osmolarity (SMM plus 0.4 M NaCl) minimal medium in the presence or absence of 1 mM choline. A *gbsA*-specific primer was annealed to the RNA and extended with reverse transcriptase in the presence of ³⁵S-dATP, and the reaction products were then separated on a DNA sequencing gel and visualized by autoradiography (Fig. 7). A single mRNA species, whose 5' end corresponds to the G residue at bp 1208, 55 bp upstream of the start codon for the *gbsA* gene (Fig. 3), was detected. Inspection of the DNA sequence upstream of the *gbsA* transcription initiation site revealed the presence of putative -10 and -35 sequences (Fig. 3) that closely resemble the consensus sequence of promoters recognized by the main vegetative sigma factor (σ^A) of *B. subtilis* (21). The -10 and -35 regions are separated by 17 bp, a typical spacing for σ^A -dependent promoters. High osmolarity had no appreciable influence on the amount of the *gbsA* mRNA (Fig. 7, lanes 1 and 3), whereas the presence of choline in the growth medium induced *gbsA* expression in both low- and high-osmolarity-grown cells (Fig. 7, lanes 2 and 4). The most pronounced stimulation of *gbsA* expression occurred in high-osmolarity medium in the presence of choline (Fig. 7, lane 4).

DISCUSSION

We have identified two new *B. subtilis* genes, *gbsA* and *gbsB*, whose gene products serve to convert choline into the osmoprotectant glycine betaine. Deletion of the *gbsAB* genes from the *B. subtilis* chromosome completely abolishes the oxidation of choline to glycine betaine and prevents the use of glycine betaine aldehyde as a precursor for glycine betaine production. Thus, the function of these genes in the osmoregulatory choline glycine betaine pathway (4) cannot be substituted for by other chromosomal genes of *B. subtilis*. In the course of the genome sequencing project, an aldehyde dehydrogenase that shows extensive similarities to the betaine aldehyde dehydrogenases from *E. coli*, beet, and spinach was discovered (39).

This protein also shows a high level of sequence identity (34.6%) to GbsA, but it apparently cannot oxidize glycine betaine aldehyde into glycine betaine at a level sufficient for osmoprotection. In the *gbsAB* mutant strain, choline can still be accumulated, but it has no osmoprotective properties per se. Such different effects of choline and glycine betaine for osmoadaptation have also been described for the gram-negative bacterium *E. coli* (50).

The ability to synthesize glycine betaine from choline is found in many cells of microbial, plant, and animal origin, and three different enzymatic systems can effect this synthesis. In *Arthrobacter pascens*, *Arthrobacter globiformis*, and an *Alcaligenes* sp., a bifunctional soluble choline oxidase that catalyzes the oxidation of choline and glycine betaine aldehyde to glycine betaine with the concomitant consumption of oxygen and the production of hydrogen peroxide has been found (23, 40, 47). In plants, glycine betaine synthesis occurs primarily in the chloroplasts and involves a soluble choline monooxygenase in combination with a soluble betaine aldehyde dehydrogenase (6, 36, 53). In a number of microorganisms and mammals, a membrane-bound choline dehydrogenase (which also can oxidize glycine betaine aldehyde to glycine betaine) and a soluble glycine betaine aldehyde dehydrogenase with a high substrate specificity are used for the synthesis of glycine betaine (33, 38, 46). Our analysis of the *B. subtilis* choline-glycine betaine synthesis pathway has revealed that a novel combination of enzymes is used in this soil bacterium. The GbsA protein exhibits strong sequence identities to both bacterial and plant glycine betaine aldehyde dehydrogenases. However, the GbsB protein does not exhibit any homology to the membrane-bound flavin adenine dinucleotide-containing choline dehydrogenase BetA from *E. coli* (30) and instead belongs to a family of soluble alcohol dehydrogenases (type III). Our mutant analysis of the *gbsAB* genes shows that the GbsB protein is absolutely required for the use of choline as a precursor for glycine betaine synthesis, but it is unclear whether GbsB can also oxidize glycine betaine aldehyde to glycine betaine.

The tight spacing of the *gbsA* and *gbsB* genes (Fig. 3) suggests that these genes are likely to form an operon. In *E. coli*, the genes (*betTIBA*) encoding the systems for choline uptake and glycine betaine synthesis are adjacent on the chromosome (30). The data presented here show that this is not the case in *B. subtilis*, since the genes encoding the glycine betaine-biosynthetic enzymes are separated from the locus encoding the choline transport system. We also did not detect, either upstream (Fig. 3) or downstream (3) of the *gbsAB* gene cluster, an ORF that encodes a polypeptide homologous to the BetI regulatory protein. Genetic studies have revealed the influence of a number of environmental factors and compounds (e.g., osmolarity, temperature, oxygen, choline, and glycine betaine) on the expression of the *E. coli bet* genes (12, 31, 33). Induction by choline is mediated by BetI, a member of the helix-turn-helix class of DNA-binding proteins (45). The presence of choline in the growth medium stimulates the expression of the *gbsAB* genes (Fig. 7), strongly suggesting the existence of a regulatory protein for the choline glycine betaine pathway of *B. subtilis*. High osmolarity alone did not stimulate *gbsAB* expression, in complete agreement with our previous report that glycine betaine synthesis is only marginally influenced by medium osmolarity (4). However, the combination of both high medium osmolarity and the presence of choline appeared to maximally stimulate *gbsAB* expression (Fig. 7), suggesting that these two environmental factors act in concert to control the level of *gbsAB* transcription. The DNA sequence of the *gbsAB* region reported here and the identification of the *gbsAB* promoter

should facilitate further studies on the genetic control of *gbsAB* expression in response to a changing environment.

Significant quantities of glycine betaine are intracellularly accumulated by *B. subtilis* from exogenous sources or through synthesis from choline even in media of low or moderate osmolarity (4, 54). This phenomenon is probably connected with the maintenance of the very high turgor found in *B. subtilis* (55). The maintenance of high turgor would most likely favor the accumulation of osmotically active solutes compatible with the normal functioning of the cell instead of ionic osmolytes which are deleterious at high concentrations (4, 15, 29). The presence of 1 mM choline or glycine betaine aldehyde in a minimal medium with 0.4 M NaCl has little effect on the growth of the *gbsAB*⁺ wild-type strain JH642 but strongly impairs the growth of the *gbsAB* mutant JBB5 (Fig. 6). Thus, when choline and glycine betaine aldehyde can be accumulated but not enzymatically oxidized to glycine betaine, the precursors for glycine betaine synthesis are highly toxic to the *B. subtilis* cells. Such a toxic effect of glycine betaine aldehyde has also been reported for the growth of plant cells (*Nicotiana tabacum*) incapable of glycine betaine synthesis (22, 42). Glycine betaine aldehyde is a highly reactive compound that can readily form Schiff bases with free amino groups. The noxious effect of glycine betaine aldehyde on the growth of *B. subtilis* in the absence of the detoxifying glycine betaine aldehyde dehydrogenase can thus be easily understood. The alcohol choline is far less chemically reactive, and its negative effect on the growth of JBB5 ($\Delta gbsAB$) might be connected with the accumulation of a large number of positively charged molecules inside the cell. Choline and glycine betaine aldehyde are accumulated in *B. subtilis* through the same osmotically controlled transport system (4). The strong inhibitory effect of glycine betaine aldehyde on the growth of a strain lacking the enzymes for glycine betaine synthesis should allow the isolation of glycine betaine aldehyde-resistant mutants with defects in the uptake of choline and glycine betaine aldehyde. Such mutants will pave the way for a detailed characterization of the choline and glycine betaine aldehyde transport system of *B. subtilis*.

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