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Genetic Control of Osmoadaptive Glycine Betaine Synthesis in *Bacillus subtilis* through the Choline-Sensing and Glycine Betaine-Responsive GbsR Repressor

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Synthesis of the compatible solute glycine betaine confers a considerable degree of osmotic stress tolerance to *Bacillus subtilis*. This osmoprotectant is produced through the uptake of the precursor choline via the osmotically inducible OpuB and OpuC ABC transporters and a subsequent two-step oxidation process by the GbsB and GbsA enzymes. We characterized a regulatory protein, GbsR, controlling the transcription of both the structural genes for the glycine betaine biosynthetic enzymes (*gbsAB*) and those for the choline-specific OpuB transporter (*opuB*) but not of that for the promiscuous OpuC transporter. GbsR acts genetically as a repressor and functions as an intracellular choline sensor. Spectroscopic analysis of the purified GbsR protein showed that it binds the inducer choline with an apparent K_D (equilibrium dissociation constant) of approximately 165 μM . Based on the X-ray structure of a protein (Mj223) from *Methanococcus jannaschii*, a homology model for GbsR was derived. Inspection of this GbsR *in silico* model revealed a possible ligand-binding pocket for choline resembling those of known choline-binding sites present in solute receptors of microbial ABC transporters, e.g., that of the OpuBC ligand-binding protein of the OpuB ABC transporter. GbsR was not only needed to control *gbsAB* and *opuB* expression in response to choline availability but also required to genetically tune down glycine betaine production once cellular adjustment to high osmolarity has been achieved. The GbsR regulatory protein from *B. subtilis* thus records and integrates cellular and environmental signals for both the onset and the repression of the synthesis of the osmoprotectant glycine betaine.

One of the challenges *Bacillus subtilis* faces in its soil habitat is frequent fluctuation in osmolarity (9). Desiccation of the soil creates a high-osmolarity surrounding that requires a highly integrated and timely cellular response to avoid cytoplasmic dehydration and a concomitant drop in turgor (62) to physiologically nonsustainable values (11, 63). The *B. subtilis* cell accomplishes this task by dynamically increasing the osmotic potential of its cytoplasm to promote water retention and reentry in tune with the prevailing osmolarity of the habitat. Initially, the cell takes up K^+ ions as an emergency reaction (31, 62), after which it accumulates compatible solutes such as proline (13, 61) and glycine betaine (4, 5, 35–37) for its long-term physiological adjustment to sustained high-osmolarity growth conditions (9, 11, 38).

Glycine betaine, a trimethylated derivative of the amino acid glycine, is without doubt the most widely used compatible solute in nature (64). *B. subtilis* makes extensive use of glycine betaine to promote growth when it is challenged by sustained high-osmolarity conditions. It possesses three high-affinity and osmotically inducible uptake systems for this compound: the two ABC-type transporters OpuA and OpuC and the BCCT-type transporter OpuD (Fig. 1) (35–37). In addition to taking up extracellular glycine betaine, the *B. subtilis* cell can also synthesize it (4–6), an osmoadaptive process that is the focus of this study.

Microorganisms can synthesize glycine betaine by one of two different routes: (i) through a stepwise methylation of the amino acid glycine (39, 46, 59) or (ii) through the import and subsequent oxidation of choline using various combinations of enzymes (5, 15, 42, 49, 51). *B. subtilis* uses this latter route to produce glycine betaine and imports for this purpose the precursor choline into the cell via two osmotically inducible ABC-type transport systems: the aforementioned OpuC transporter and the OpuB system (Fig. 1). The two systems exhibit comparable kinetic parameters for

choline uptake, with K_m values in the low- μM range and similar V_{max} values (36). However, OpuB is highly specific for the uptake of choline, whereas OpuC imports a broad range of osmoprotectants (Fig. 1) (9, 30). Once imported, choline is oxidized to glycine betaine in a two-step enzymatic reaction that involves the type III alcohol dehydrogenase GbsB and the glycine betaine aldehyde dehydrogenase GbsA, with glycine betaine aldehyde as the intermediate (Fig. 1) (5, 6).

It should be noted in this context that choline has no osmoprotective properties *per se* in *B. subtilis* and that neither choline nor glycine betaine can be used as a nutrient by this bacterium (4, 5). Hence, both the choline-dependent synthesis and the uptake of preformed glycine betaine serve exclusively for stress protection of the *B. subtilis* cell, either against high osmolarity (4, 5, 35–37) or against sustained high (52°C)- and low (13°C)-temperature challenges (12, 30, 31).

A previous study from our laboratory has shown that the transcription of the glycine betaine biosynthetic genes (*gbsAB*) from *B. subtilis* is upregulated in response to an exogenous supply of choline but is not stimulated *per se* in cells that are continuously cul-

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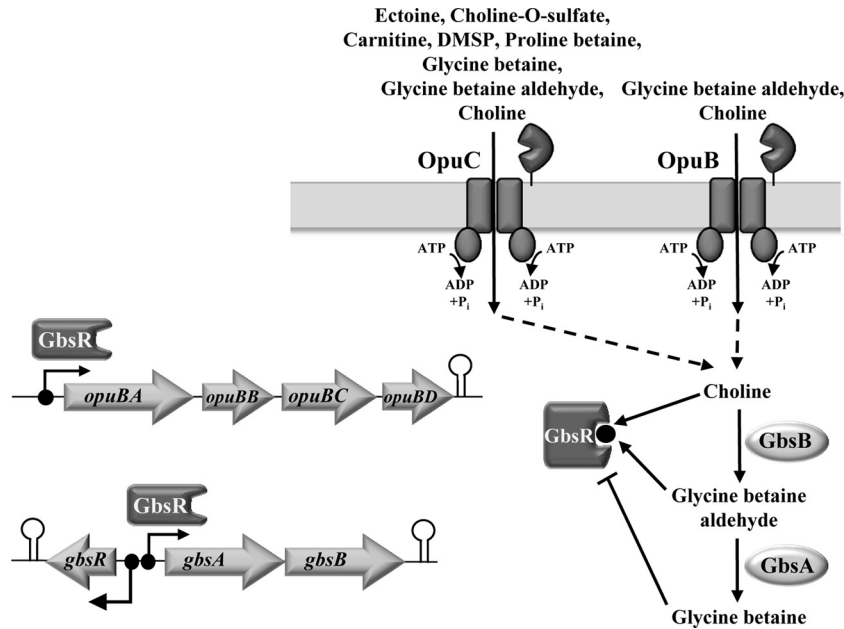


FIG 1 Systems for the uptake of compatible solutes by *B. subtilis* and the osmoadaptive choline-to-glycine betaine biosynthetic pathway. The substrate profiles of the OpuB and OpuC ABC transporters shown were compiled from the literature (9, 30), except those for proline betaine and DMSP, whose import systems are assigned based on unpublished data from our laboratory (T. Hoffmann, A. Bashir, C. Chen, and E. Bremer, unpublished data). The dots and arrows shown within the context of the *opuB* and *gbsAB* operons and the *gbsR* gene represent the experimentally mapped promoters (5, 36). The lollipops indicate predicted factor-independent transcriptional terminators.

tivated at high osmolarity (5). However, sustained high osmolarity enhances *gbsAB* induction by choline (5), indicating that an increase in the intracellular choline pool generated via the osmotically inducible OpuB and OpuC systems (36) is beneficial for the induction of *gbsAB* expression. These findings suggest (i) that choline, or a metabolic derivative of it, serves as an inducer for *gbsAB* transcription and (ii) that a regulatory protein that monitors the intracellular pool of the inducer and then sets the level of *gbsAB* expression is likely to exist.

Here we report the identification of the long-suspected regulatory protein (5) for the osmoadaptive glycine betaine synthesis pathway of *B. subtilis*, the repressor protein GbsR. We demonstrate that GbsR functions as an intracellular choline sensor controlling the expression of both the structural genes for the substrate-specific choline import system OpuB and those encoding the glycine betaine biosynthetic enzymes GbsAB (Fig. 1). In addition, we found that the GbsR protein not only functions as a regulatory switch for the onset of glycine betaine biosynthesis when choline is present in the environment but also that GbsR is also required to downregulate glycine betaine production once the *B. subtilis* cell has achieved adjustment to the osmotic conditions prevailing in its ecosystem.

MATERIALS AND METHODS

Chemicals. Choline, glycine betaine aldehyde, glycine betaine, carnitine, proline, and the chromogenic substrates for the TreA enzyme, *para*-nitrophenyl- α -D-glucopyranoside (α -PNPG), and the antibiotics chloramphenicol, kanamycin, erythromycin and spectinomycin were purchased from Sigma-Aldrich (Steinheim, Germany). Proline betaine (dimethylproline) was purchased from Atkins Chemicals (Chengde, China); dimethylsulfoniopropionate (DMSP) and choline-O-sulfate were from laboratory stocks. Ectoine was a kind gift from G. Lentzen (Bitop AG, Witten, Germany) and [*methyl*-¹⁴C]choline (55 mCi mmol⁻¹) was purchased

from DuPont de Nemour GmbH (Neu-Isenburg, Germany). Avian myeloblastosis virus reverse transcriptase was obtained from Roche Diagnostics (Mannheim, Germany). The resin (*Strep*-Tactin Sepharose) for the affinity chromatography of the GbsR protein labeled with a Strep-Tag affinity peptide was purchased from IBA (Göttingen, Germany). The inducer for the *tet* promoter-mediated expression of the *gbsR* gene, anhydrotetracycline (AHT), was obtained from Acros Organics (Geel, Belgium).

Bacterial strains. The *Escherichia coli* K-12 strain DH5 α (Invitrogen, Carlsbad, CA) was used for routine cloning purposes and maintenance of cloning vectors and recombinant plasmids. The *E. coli* B strain BL21 (Stratagene, Heidelberg, Germany) was used for the overproduction of recombinant proteins. These *E. coli* strains were grown and maintained on Luria-Bertani (LB) agar plates, and solid and liquid media contained, when necessary, antibiotics to select for the presence of plasmids. The *B. subtilis* strain JH642 (*trpC2 pheA1*) and its mutant derivatives were used throughout this study (Table 1). JH642 is a member of the domesticated 168 lineage of *B. subtilis* laboratory strains (54) and was a kind gift of J. Hoch (Scripps Research Institute, CA).

Media and growth conditions. *B. subtilis* strains were maintained on LB agar plates; liquid cultures were grown at 37°C in Spizizen’s minimal medium (SMM) (25) supplemented with 0.5% (wt/vol) glucose as the carbon source, a solution of trace elements (26), and the amino acids L-tryptophan (20 mg liter⁻¹) and L-phenylalanine (20 mg liter⁻¹) to meet the auxotrophic needs of strain JH642 (*trpC2 pheA1*) and its derivatives (Table 1). Cultures of the *E. coli* strain BL21 used for the production of recombinant proteins were grown in minimal medium A (MMA) with glucose (0.5%) as the carbon source and supplemented with 0.5% Casamino Acids and thiamine (1 μ g ml⁻¹). The antibiotics chloramphenicol, kanamycin, erythromycin, and spectinomycin were used with *B. subtilis* strains at final concentrations of 5 μ g ml⁻¹, 10 μ g ml⁻¹, 1 μ g ml⁻¹, and 100 μ g ml⁻¹, respectively. Ampicillin and chloramphenicol were used for *E. coli* cultures at final concentrations of 100 μ g ml⁻¹ and 35 μ g ml⁻¹, respectively. Operon fusions of a promoterless *treA* reporter gene (22) to the regulatory regions of the *gbsAB*, *opuB*, and *opuC* gene clusters were

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

Strain	Relevant genotype ^a	Reference/source
JH642	<i>trpC2 pheA1</i>	J. Hoch; BGSC 1A96 ^b
JBB8	(<i>gbsR::neo</i>)1	5
RMKB25	<i>opuC-20::Tn10(spc)</i>	36
RMKB26	<i>opuB-20::Tn10(spc)</i>	36
BKB30	(<i>yvbF::tet</i>)1	This study
BKB31	(<i>yvaV::spc</i>)1	This study
GNB25	(<i>gbsR::neo</i>)1 <i>opuC-20::Tn10(spc)</i>	This study
GNB26	(<i>gbsR::neo</i>)1 <i>opuB-20::Tn10(spc)</i>	This study
GNB37	Δ (<i>treA::erm</i>)2	This study
GNB40	Δ (<i>treA::erm</i>)2 (<i>gbsR::neo</i>)1	This study
GNB45	Δ (<i>treA::erm</i>)2 [<i>amyE::</i> (Δ <i>gbsR</i>)2- Φ (<i>gbsA'</i> - <i>treA</i>)1]	This study
GNB46	Δ (<i>treA::erm</i>)2 Δ (<i>gbsAB::neo</i>)2 [<i>amyE::</i> (Δ <i>gbsR</i>)2- Φ (<i>gbsA'</i> - <i>treA</i>)1]	This study
GNB48	Δ (<i>treA::erm</i>)2 (<i>gbsR::neo</i>)1 [<i>amyE::</i> (Δ <i>gbsR</i>)2- Φ (<i>gbsA'</i> - <i>treA</i>)1]	This study
GNB55	Δ (<i>treA::erm</i>)2 (<i>yvbF::tet</i>)1 [<i>amyE::</i> (<i>gbsA'</i> - <i>treA</i> Δ (<i>gbsR</i>)2)1]	This study
GNB56	Δ (<i>treA::erm</i>)2 (<i>yvaV::spc</i>)1 [<i>amyE::</i> (<i>gbsA'</i> - <i>treA</i> Δ (<i>gbsR</i>)2)1]	This study
GNB67	Δ (<i>treA::erm</i>)2 [<i>amyE::yvbF</i> - Φ (<i>opuCA'</i> - <i>treA</i>)2]	This study
GNB69	Δ (<i>treA::erm</i>)2 (<i>gbsR::neo</i>)1 [<i>amyE::yvbF</i> - Φ (<i>opuCA'</i> - <i>treA</i>)2]	This study
GNB74	Δ (<i>treA::erm</i>)2 [<i>amyE::yvaV</i> - Φ (<i>opuBA'</i> - <i>treA</i> <i>yvaV</i> ⁺)2]	This study
GNB76	Δ (<i>treA::erm</i>)2 (<i>gbsR::neo</i>)1 [<i>amyE::yvaV</i> - Φ (<i>opuBA'</i> - <i>treA</i>)2]	This study

^a The designation *amyE::* Φ (*gbsA'*-*treA*) indicates that the *gbsA*-*treA* operon fusion is stably integrated via a double-recombination event into the chromosomal *amyE* gene of *B. subtilis* as a single copy, thereby rendering the fusion strains defective in the extracellular AmyE α -amylase. The Φ (*gbsA'*-*treA*)1 reporter fusion is linked to a chloramphenicol resistance gene (*cat*), thereby conferring resistance to the antibiotic chloramphenicol to all strains carrying the *amyE::*(*gbsA'*-*treA*) construct. This is true also for Φ (*opuCA'*-*treA*) and Φ (*opuBA'*-*treA*) reporter fusion strains. The designation (Δ *gbsR*)2- Φ (*gbsA'*-*treA*)1 indicates that the DNA fragment used for the construction of the Φ (*gbsA'*-*treA*) reporter fusion (Fig. 5) does not carry an intact *gbsR* gene.

^b Bacillus Genetic Stock Center, Columbus, Ohio.

constructed with the aid of plasmid pJMB1 (M. Jebbar and E. Bremer, unpublished data) (see the supplemental material). In this *treA* reporter fusion plasmid, a gene conferring resistance to chloramphenicol follows the operon fusion construct; the entire genetic construct is flanked by the 5' and 3' segments of the *B. subtilis* *amyE* gene. This allows the stable single-copy integration of the *treA* reporter gene fusion and of the *cat* gene via a double homologous recombination event into the chromosomal copy of the *B. subtilis* *amyE* locus by selecting for chloramphenicol-resistant colonies. This renders the resulting *treA* reporter fusion reporter strain defective in the production of the extracellular AmyE amylase.

Recombinant DNA techniques and construction of plasmids and *B. subtilis* strains. The routine manipulations of plasmid DNA, the construction of recombinant DNA plasmids, the isolation of chromosomal DNA from *B. subtilis*, and transformation with plasmid or chromosomal DNA were carried out using standard procedures (26). The construction of recombinant DNA plasmids and their use for the generation of *B. subtilis* mutants carrying chromosomal gene disruptions or *treA* reporter fusions integrated at the chromosomal *amyE* locus are detailed in the supplemental material. The genotypes of the *B. subtilis* strains used in this study are described in Table 1 (also see Table 4 and Fig. 8).

Choline transport assays. Uptake assays with [*methyl*-¹⁴C]choline as the substrate were conducted as described previously (35, 36). For these transport experiments, the *B. subtilis* cells were grown to early exponential growth phase (optical density at 578 nm [OD₅₇₈] of about 0.4) in either SMM or SMM containing 0.4 M NaCl. The final substrate concentration of choline used in these uptake assays was 10 μ M.

TreA enzyme activity assays. Aliquots (1.5 ml) from cultures of *B. subtilis* fusion strains carrying operon fusions to the promoterless *treA* reporter gene were withdrawn and assayed for TreA reporter enzyme activity as described previously, using *para*-nitrophenyl- α -D-glucopyranoside as the substrate (22). TreA [phospho- α -(1,1)-glucosidase]-specific activity is expressed in units per mg of protein; protein concentrations were estimated from the optical density of the cell culture (44).

Overproduction and purification of recombinant GbsR protein in *E. coli*. To obtain purified GbsR protein, we used the *gbsR* overexpression plasmid pDH1. Plasmid pDH1 carries the *B. subtilis* *gbsR* gene under the

control the anhydrotetracycline (AHT)-inducible *tet* promoter, and its AHT-dependent expression leads to the production of a hybrid GbsR protein with an N-terminal Strep-Tag II affinity peptide. Derivatives of *E. coli* strain BL21 carrying pDH1 were grown at 37°C in a chemically defined medium (MMA) (44) to avoid binding of possible ligands (e.g., choline or glycine betaine) to the GbsR protein that might be provided through components (e.g., yeast extract) of rich growth media during protein overproduction. Expression of the pDH1-carried *gbsR* gene was induced by adding AHT (final concentration, 0.2 μ g ml⁻¹) to the cells, when the cultures reached an OD₅₇₈ of about 0.5. The cultures were then grown for an additional 2 h, and the cells were then harvested by centrifugation, resuspended in a lysis buffer (100 mM Tris-HCl [pH 7.5], 2.5% glycerol, 2 mM dithiothreitol, 0.4 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM benzamidine), and disrupted by passage several times through a French pressure cell. A cleared cell lysate was prepared from these disrupted cells by ultracentrifugation (1 h at 4°C at 140,000 \times g), and the soluble proteins were loaded onto a Strep-Tactin column for affinity purification according to procedures described by the manufacturer of the affinity resin (IBA, Göttingen, Germany). Elution fractions containing the purified GbsR protein were pooled, and the proteins were concentrated using VivaSpin columns (VivaSciences Ltd., Stonehouse, United Kingdom) with a simultaneous exchange of the elution buffer to a buffer containing 10 mM Tris-HCl (pH 7). The purity of the isolated GbsR protein was assessed by SDS-polyacrylamide gel electrophoresis.

Determination of the dissociation constants of the GbsR/choline complex. The dissociation constant of GbsR for choline was determined by intrinsic tyrosine and tryptophan fluorescence spectroscopy as described by Pittelkow et al. (48) for the OpuBC/choline complex using a Carry Eclipse fluorescence spectrometer (Varian, Surry, United Kingdom). Purified GbsR protein (5 μ M) (in 10 mM Tris-HCl [pH 7]) was equilibrated for 5 min in the cuvette, and various concentrations (from 25 μ M to 1,000 μ M) of choline were then titrated to the GbsR protein solution. The excitation wavelength of the fluorescence spectrometer was set to 280 nm, the slit width was 5 nm, and an emission spectrum was recorded (from 290 nm to 400 nm). For these experiments, the photomul-

tiplier tube voltage (PMT) of the fluorescence detector was set to 800 V. Upon choline binding by the purified GbsR protein (see Fig. 6A), the intrinsic fluorescence intensity decreases in response to the choline concentration in the assay (see Fig. 6B). Differences in the fluorescence intensities of the GbsR protein solution observed at 335 nm for the GbsR protein solutions incubated with choline (see Fig. 6B) were used to calculate the apparent K_D (equilibrium dissociation constant) value (see Fig. 6C) as previously reported (48).

Database searches, alignments of amino acid sequences, and modeling of the GbsR structure. Proteins that are homologous to the *B. subtilis* GbsR protein were searched for via the Web server of the Department of Energy (DOE) Joint Genome Institute (JGI) (<http://www.jgi.doe.gov/>) or that of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) using the BLAST algorithm (2). Protein sequences related to the *B. subtilis* GbsR and the *Methanococcus jannaschii* Mj223 (50) proteins were aligned and analyzed using ClustalW (57). The genome context of finished and unfinished microbial genomes in the vicinity of *gbsR*-related and Mj223-type genes was assessed using the gene neighborhood tool provided by the JGI Web server. An *in silico* model of the *B. subtilis* GbsR protein was generated with the aid of the SWISS Model server (<http://swissmodel.expasy.org>) (3) using crystallographic data deposited in the Protein Data Bank (PDB) file 1ku9 for the *M. jannaschii* Mj223 protein (50) as the template. Figures of the crystal structure of the *M. jannaschii* Mj223 protein, of the *in silico*-modeled *B. subtilis* GbsR protein, and of the OpuBC protein in complex with its ligand choline (PDB code 3r6u) were prepared with PyMol (<http://www.pymol.org/>).

RESULTS

Induction of *gbsAB* transcription in response to choline and glycine betaine aldehyde. To provide quantitative data on the regulation of the *gbsAB* operon in response to external stimuli, we constructed a *gbsA-treA* transcriptional fusion and integrated it stably as a single copy into the *amyE* gene in the *B. subtilis* chromosome through a homologous double-recombination event. Expression of the *gbsA-treA* fusion was very low when the resulting reporter strain GNB45 was grown in minimal medium (SMM) with glucose as the carbon source. However, the addition of 1 mM choline or 1 mM glycine betaine aldehyde to the growth medium led to a strong induction of *gbsA-treA* transcription: 29-fold in response to choline and 22-fold in response to glycine betaine aldehyde (Fig. 2A). In contrast to the inducing effects of the precursor and intermediate for glycine betaine synthesis (Fig. 1), the end product of the glycine betaine biosynthetic route did not trigger enhanced *gbsA-treA* expression (Fig. 2A). The addition of 25 μ M choline to the growth medium of strain GNB45 already resulted in a significant increase in the level of *gbsA-treA* expression, and the addition of about 100 to 125 μ M choline was sufficient to fully induce the expression of the reporter fusion (data not shown).

Sustained growth of the cells of the *gbsA-treA* reporter strain GNB45 at high osmolarity (SMM with 0.4 M NaCl) did not trigger enhanced *gbsA-treA* expression. However, the addition of 1 mM choline to the osmotically stressed cells resulted in a strong induction of the reporter gene fusion, and the degree of *gbsA-treA* expression exceeded that observed in cells grown in the presence of choline alone (Fig. 2A). The enhancing effect of high salinity on the induction of *gbsA-treA* expression by an exogenous supply of choline can be rationally explained by the fact that the expression of the operons encoding the choline importers OpuB and OpuC is stimulated by high-osmolarity growth conditions (24, 36, 55).

GbsR is a negative regulator of *gbsAB* expression. The *yuaC*

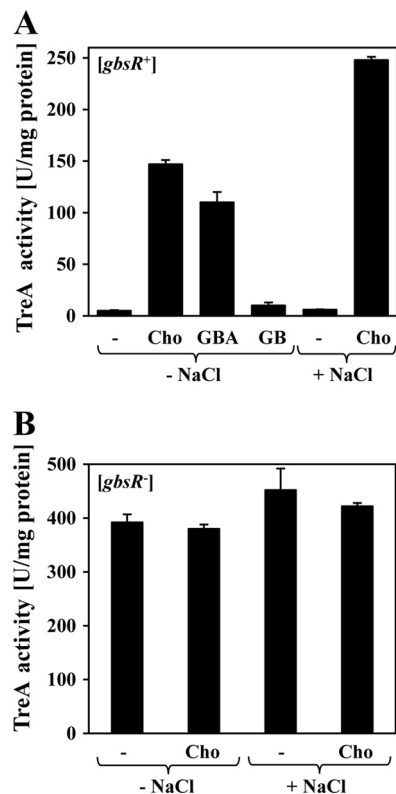


FIG 2 Genetic control of *gbsAB* expression in response to the presence of external inducers, high salinity, and the GbsR regulator. Cells of the *gbsA-treA* reporter strains GNB45 (*gbsR⁺*) (A) or GNB48 (*gbsR::neo*) (B) were grown at 37°C either in SMM or in SMM with 0.4 M NaCl to an OD₅₇₈ of 0.25; 1 mM choline (Cho), glycine betaine aldehyde (GBA), or glycine betaine (GB), respectively, was then added to some of the cultures, and the activity of the TreA reporter enzyme was determined after 90 min of further cultivation.

gene (locus tag BSU31070) is oriented divergently from the *gbsAB* operon (Fig. 1) and encodes a 180-amino-acid-comprising protein (21 kDa). Our database searches and computer predictions suggest that it is a regulatory protein carrying a winged-helix DNA-binding motif (20, 33) between amino acids 49 and 73 (see below). YuaC-related proteins are encoded by genes found in close vicinity of functionally characterized glycine betaine biosynthetic genes from *Staphylococcus xylosum* (51), *Halobacillus dabanensis* (23), and *Halobacillus halophilus* (15). We therefore considered the possibility that the *B. subtilis* YuaC protein might function as a transcriptional regulator for the *gbsAB* operon. To test this idea, we constructed a gene disruption in the chromosomal *yuaC* gene and introduced this mutation (*yuaC::neo*) into the above-described *gbsA-treA* reporter strain. In the resulting strain, GNB48, transcription of *gbsA-treA* was strongly derepressed when the cells were grown in the absence of choline, and the availability of choline in the growth medium of cells cultivated in either SMM or SMM with 0.4 M NaCl no longer enhanced *gbsA-treA* transcription (Fig. 2B). These data therefore show that the *yuaC*-encoded protein serves as a regulator for *gbsAB* expression and genetically functions as a repressor. We refer in the following to this protein as the GbsR repressor (glycine betaine synthesis regulator) and redesignate the *yuaC* locus as the *gbsR* structural gene (Fig. 1).

Mapping of the *gbsR* promoter. The transcriptional start site

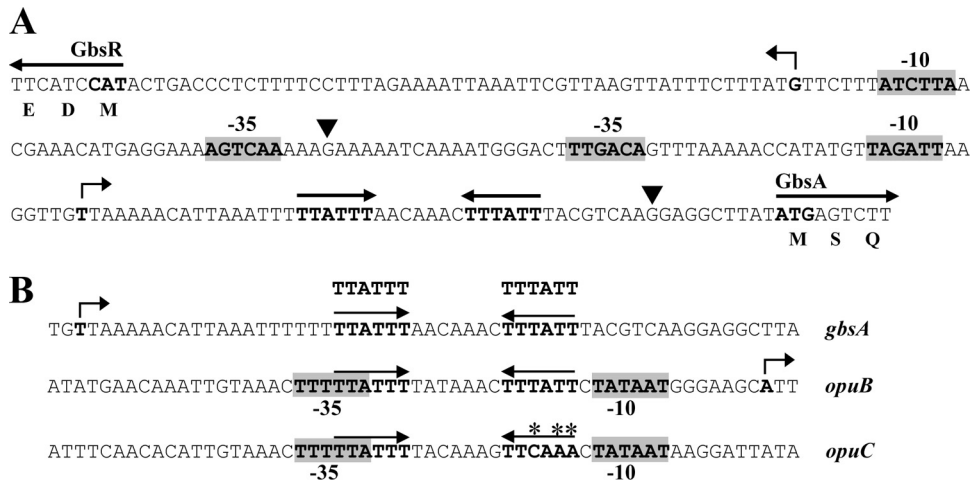


FIG 3 DNA sequence of the intergenic *gbsR-gbsAB* regulatory region. (A) The -10 and -35 sequences of the SigA-type promoters for the *gbsR* gene and the *gbsAB* operon are indicated, and the mapped transcriptional start sites for these genes are shown by bent arrows. The transcriptional start site for the *gbsAB* operon was mapped by Boch et al. (5) and that of the *gbsR* gene was determined in this study (see Fig. S1 in the supplemental material). The putative GbsR binding site positioned downstream of the *gbsAB* transcription initiation site is indicated by a pair of inverted arrows. The beginning of the coding regions for the GbsR and GbsA proteins is marked. The two black triangles indicate the extent of the inferred minimal DNA fragment (Fig. 5) that permits GbsR-mediated induction of *gbsAB* transcription in response to choline. (B) Putative GbsR binding sites in the *gbsAB* and *opuC* regulatory regions. The -10 and -35 sequences of the SigA-type promoters for the *opuB* and *opuC* operons are indicated; the transcriptional start site for the *opuB* mRNA has been experimentally determined (36) (indicated by a bent arrow), and the promoter for the *opuC* operon was predicted by DNA sequence gazing. The putative GbsR binding sites are indicated by pairs of inverted arrows; the asterisks (*) mark differences in the DNA sequences of the putative GbsR binding site in the *opuB* and *opuC* regulatory regions.

for the *gbsAB* operon has previously been mapped by primer extension analysis by Boch et al. (5). This allowed the identification of the positions of -10 and -35 regions characteristic for SigA-type promoters (28); the *gbsAB* promoter possesses a canonical spacing of 17 bp between the -10 and -35 sequences (Fig. 3A). We now have mapped the transcriptional start site for the *gbsR* mRNA by primer extension analysis, and a single transcript was found (see Fig. S1 in the supplemental material). Upstream of this mRNA start site, DNA sequences that resemble -35 and -10 regions of SigA-type promoters are present. The inferred *gbsR* promoter possesses a spacing of 16 bp (Fig. 3A), a suboptimal spacing for -10 and -35 regions of typical SigA-dependent promoters, suggesting that the *gbsR* promoter is probably not a particularly strong promoter (28). The promoter regions of the divergently transcribed *gbsR* and *gbsAB* genes are compactly organized, but the two promoters do not overlap; their -35 regions are separated by a 22-bp A-T-rich DNA segment (Fig. 3A).

GbsR controls the expression of the *opuB* operon but not that of the *opuC* operon. Synthesis of glycine betaine by *B. subtilis* depends on the GbsAB enzymes but requires the prior import of the precursor choline through the OpuB or OpuC ABC transport systems (4, 5, 36) (Fig. 1), since *B. subtilis* cannot synthesize choline. To test whether the *gbsR::neo* gene disruption mutation would influence choline uptake, we introduced this allele into strain JH642 (*opuB*⁺ *opuC*⁺ *gbsR*⁺) and compared the transport activity of the parent strain JH642 with the resulting *gbsR::neo* mutant derivative, strain JBB8. As reported previously (36), uptake of [¹⁴C]choline was stimulated in cells that were grown at high salinity (SMM with 0.4 M NaCl) in comparison to those cultivated in SMM without additional salt (Fig. 4A). However, the levels of choline uptake by the *gbsR::neo* mutant strain JBB8 with or without osmotic induction well exceeded the choline import activity exhibited by the *gbsR*⁺ strain JH642 (Fig. 4A). This

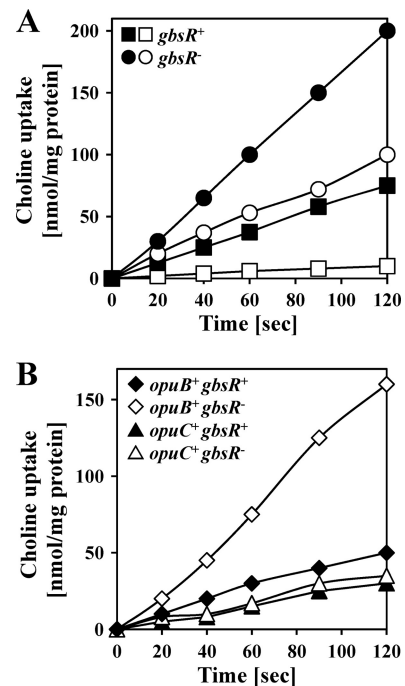


FIG 4 Influence of GbsR on choline import via the OpuB and OpuC ABC transporters. Cells were cultivated at 37°C in SMM or in SMM containing 0.4 M NaCl until they had reached an OD₅₇₈ of about 0.4. Subsequently, uptake of [¹⁴C]choline was assayed at a final substrate concentration of 10 μM. (A) Uptake of [¹⁴C]choline in the wild-type strain JH642 (*opuB*⁺ *opuC*⁺ *gbsR*⁺) (□, ■) and strain JBB8 (*opuB*⁺ *opuC*⁺ *gbsR::neo*) (○, ●) grown in SMM (□, ○) or SMM with 0.4 M NaCl (■, ●). (B) Uptake of [¹⁴C]choline in strain RMKB25 [*opuB*⁺ *opuC*⁺:Tn10 (*spc*) *gbsR*⁺] (◆), GNB25 [*opuB*⁺ *opuC*⁺:Tn10 (*spc*) *gbsR::neo*] (◇), strain RMKB26 [*opuB*⁺:Tn10 (*spc*) *opuC*⁺ *gbsR*⁺] (▲) and GNB26 [*opuB*⁺:Tn10 (*spc*) *opuC*⁺ *gbsR::neo*] (△); cells were grown at 37°C in SMM containing 0.4 M NaCl.

TABLE 2 Influence of GbsR on the transcriptional activity of the *opuB* and the *opuC* genes^a

Strain	<i>tre</i> fusion	<i>gbsR</i> ^b	TreA activity (U/mg protein)	
			Without NaCl	1.2 M NaCl
GNB74	<i>opuB-treA</i>	+	38 ± 5	104 ± 2
GNB76	<i>opuB-treA</i>	–	308 ± 5	724 ± 16
GNB67	<i>opuC-treA</i>	+	34 ± 3	ND ^c
GNB69	<i>opuC-treA</i>	–	36 ± 5	ND

^a Cells carrying chromosomal copies of the indicated *opuB-treA* and *opuC-treA* operon fusions were cultivated in SMM or SMM with 1.2 M NaCl to mid-log phase and were then harvested for TreA reporter enzyme activity.

^b Strains carrying a *gbsR* mutation (denoted with –) harbored the *gbsR::neo* allele.

^c ND, not determined.

finding suggested that GbsR is involved in controlling the expression of either the *opuB* or *opuC* operons or that of both loci.

Since both the OpuB and OpuC systems can mediate high-affinity choline transport, we genetically constructed an isogenic set of strains in which either only the OpuB transporter or only the OpuC transporter was functional and which differed in the genetic status of their *gbsR* loci. Transport assays with radiolabeled [*methyl*-¹⁴C]choline revealed that the uptake activity via the choline-specific OpuB transporter (36, 48) was strongly enhanced in a *gbsR::neo* mutant strain (Fig. 4B), whereas choline import via the OpuC transporter, an uptake system with a broad substrate specificity (19, 30, 36) (Fig. 1), was not affected by the *gbsR::neo* gene disruption (Fig. 4B).

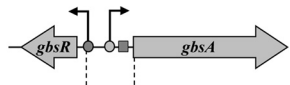
The different effects of a *gbsR::neo* mutation on the choline import activity of the OpuB and OpuC transporters was further corroborated by testing the effect of this mutation on the expression level of *opuB-treA* and *opuC-treA* reporter gene fusions. As documented in Table 2, and fully consistent with the [*methyl*-¹⁴C]choline transport assays shown in Fig. 4A, loss of GbsR affected only *opuB* expression. Hence, the entire choline-to-glycine betaine biosynthetic route of *B. subtilis*, i.e., the *gbsAB* operon encoding the glycine betaine biosynthetic enzymes (5) and the *opuB* operon encoding the choline-specific import system OpuB (36) (Fig. 1), is under the control of the GbsR repressor.

High salinity increases the expression of the *opuB* operon (24, 36, 55). We therefore wondered whether a *gbsR* gene disruption would impinge on the osmotic induction of *opuB* transcription. The data summarized in Table 2 show that this is not the case. Under the conditions tested, a *gbsR* mutant increased *opuB-treA* expression about 8-fold in cells grown in SMM, and a similar level of *opuB-treA* derepression (about 7-fold) was observed in cells grown in SMM that contained 1.2 M NaCl. Likewise, osmotic induction of *opuB-treA* expression was not significantly affected by the presence of a *gbsR* mutation: transcription of the reporter fusion was increased about 2.6-fold in the *gbsR*⁺ strain GNB74 and about 2.3-fold in the *gbsR::neo* strain GNB76 (Table 2). In contrast to the *opuB* operon (24, 36, 55), expression of the *gbsAB* gene cluster is not enhanced in cells cultivated under sustained high-salinity conditions (Fig. 2A) (5), but it responds to an osmotic stimulus in cells subjected to a severe and growth-restricting sudden osmotic upshift with 1.7 M NaCl (24).

Delineation of the GbsR-binding site within the *gbsAB* regulatory region by deletion analysis. To narrow down the DNA

segment required for the GbsR-mediated and choline-responsive control of *gbsAB* expression, we performed a deletion analysis of the *gbsAB-treA* reporter gene fusions carrying the *gbsAB* promoter and flanking DNA sequences of various lengths. These transcriptional fusions were integrated in a single copy into the genome of a strain with an intact *gbsR* gene and also into that of a *gbsR::neo* mutant. We then determined the expression level of the various *gbsA-treA* reporter fusions in response to the presence of choline in the growth medium and the functionality of the GbsR regulator. Each of the reporter fusions tested responded to choline availability and was controlled by GbsR (Fig. 5). By viewing these data together, we infer that a DNA fragment with a length of just 100 bp is sufficient to confer GbsR-mediated and choline-responsive control of *gbsAB* expression (Fig. 5). This regulatory fragment comprises the *gbsAB* promoter, a 51-bp-long region downstream of the –10 region and a 19-bp-long region upstream of the –35 region. This DNA fragment, whose sequence is highlighted in Fig. 3A, does not carry any part of the *gbsR* promoter. Hence, GbsR binding is expected to occur at or very close to the *gbsAB* promoter sequence.

Since GbsR also controls the expression of *opuB*, but not that of *opuC* (Table 2), we inspected the regulatory regions of these gene



Fusion strain	Promoter fragment size	Promoter region	<i>gbsR</i>	TreA activity [U/mg protein]	
				non-induced	induced
GNB45	914 bp	— (Δ)	+	3 ± 1	247 ± 24
GNB48	914 bp	— (Δ)	–	454 ± 24	420 ± 21
GNB52	576 bp	— (Δ)	+	4 ± 1	175 ± 3
GNB63	576 bp	— (Δ)	–	399 ± 25	280 ± 15
GNB53	232 bp	—	+	5 ± 1	186 ± 8
GNB64	232 bp	—	–	312 ± 10	304 ± 5
GNB54	464 bp	—	+	6 ± 1	722 ± 39
GNB65	464 bp	—	–	1295 ± 85	1129 ± 120
DHB4	118 bp	—	+	5 ± 1	174 ± 22
DHB12	118 bp	—	–	362 ± 16	331 ± 23
DHB5	124 bp	—	+	5 ± 1	150 ± 9
DHB13	124 bp	—	–	216 ± 12	216 ± 12
	100 bp	—		predicted minimal fragment	

FIG 5 Choline-responsive and GbsR-dependent regulation of *gbsA-treA* expression. Strains with either an intact *gbsR* gene (+) or a *gbsR::neo* mutation (–) carrying chromosomal *gbsA-treA* fusions of various lengths were pre-grown in SMM to early log phase (OD₅₇₈ of 0.25), and aliquots were assayed for TreA activity (noninduced condition). At this time point, 1 mM choline (final concentration) and 0.4 M NaCl (final concentration) were added to the cultures; after further growth for 90 min, the cells were harvested for assays of the TreA reporter enzyme (induced condition). NaCl was added to these cultures to elicit enhanced uptake of the inducer choline via the osmotically stimulated OpuB and OpuC transport systems (36). The symbol (Δ) indicates that the corresponding segment of the *gbsR* gene was deleted from the DNA fragment fused to the *treA* reporter gene. All fusion strains are derivatives of the *B. subtilis* strain JH642 (*trp phe*).

TABLE 3 Influence of choline and glycine betaine on the activity of the *gbsA* promoter^a

Strain	<i>gbsAB</i> ^b	<i>gbsR</i>	Compound present	TreA activity (U/mg protein)
GNB45	+	+	–	6 ± 1
	+	+	Choline	106 ± 3
	+	+	Choline + glycine betaine	5 ± 1
GNB46	–	+	–	6 ± 1
	–	+	Choline	534 ± 7
	–	+	Choline + glycine betaine	5 ± 1
GNB48	+	–	–	478 ± 16
	+	–	Choline	569 ± 19
	+	–	Choline + glycine betaine	515 ± 27

^a Cells of the various *gbsA-treA* reporter fusion strains were cultivated in SMM without (–) or with 1 mM choline or in the presence of 1 mM choline and 1 mM glycine betaine until the cultures had reached an OD₅₇₈ of 1.5; samples were then assayed for TreA reporter enzyme activity.

^b The strains carrying mutations in the *gbsAB* operon [$\Delta(gbsAB::neo)$] or in the *gbsR* gene [*gbsR::neo*] are indicated by –.

clusters for sets of common DNA sequences that might serve as operator sequences for the GbsR repressor. We found an identical A·T-rich inverted repeat (5'-TTATTT-N₇-TTTATT-3') that is present downstream of the transcription initiation site of the *gbsAB* operon (Fig. 3B) and which overlaps the –35 and spacer region of the previously mapped (36) *opuB* promoter (Fig. 3B). This DNA element is not intact in the presumed promoter region of the *opuB* operon (Fig. 3B). While this type of DNA sequence gazing is certainly suggestive, DNA-binding assays and footprinting studies with the purified GbsR protein have to be conducted in the future to assess the functionality of the indicated inverted repeat sequence for GbsR binding. If the DNA sequences highlighted in Fig. 3B are indeed the operator sites for GbsR, GbsR would likely repress the transcription of *gbsAB* through a road-block mechanism impeding progress of the RNA polymerase and that of *opuB* through a mechanism relying on competition with RNA polymerase for access to the promoter region.

GbsR is a choline-sensing protein and binds choline directly. Since the addition of choline to the growth medium induces *gbsAB* expression (Fig. 2A), it seemed to us that the GbsR repressor might serve as an intracellular choline-sensing protein. To investigate this further genetically, we constructed a *gbsA-treA* reporter strain (GNB46) that is defective in the *gbsAB* operon. In such a strain, choline accumulated via the OpuB and OpuC transporters cannot be removed by the cell via oxidation to glycine betaine (Fig. 1) and is thus predicted to accumulate to high intracellular levels (5). The addition of choline to the growth medium triggered *gbsA-treA* expression in both the *gbsAB* wild-type strain and the *gbsAB* mutant strain, but the level of *gbsA-treA* expression was considerably higher (about 5-fold) in strain GNB46 (*gbsAB::neo*) lacking the glycine betaine biosynthetic enzymes (Table 3). As a matter of fact, the level of *gbsA-treA* expression in strain GNB46 was, within experimental error, practically identical with that measured in *gbsR::neo* mutant strain GNB48, in which the *gbsA-treA* reporter fusion is completely derepressed and no longer answers to choline availability in the growth medium (Table 3). This experiment therefore suggests that choline serves as the inducer of GbsR-controlled gene expression and that the *B. subtilis* cell continuously removes

this inducer from the cytoplasm by oxidizing choline to glycine betaine via the action of the GbsAB enzymes (Table 3). This conclusion can be inferred by comparing the level of *gbsA-treA* promoter activity in strain GNB45, which is proficient in the GbsAB enzymes, with that of strain GNB46, which lacks these enzymes (Table 3).

If choline is the true inducer for the GbsR repressor, then one might be able to biochemically demonstrate the binding of this ligand to the GbsR protein. To test this, we produced the *B. subtilis* GbsR repressor in *E. coli* as a fusion protein with an NH₂-terminal Strep-Tag and purified the recombinant protein by affinity chromatography to apparent homogeneity (Fig. 6A). GbsR carries two Trp and six Tyr residues, and we reasoned that one could exploit changes in intrinsic fluorescence of this protein to monitor conformational changes that might occur upon ligand binding. We have previously successfully used this technique to determine the binding constant for choline and glycine betaine of ligand-binding proteins associated with bacterial ABC transport systems (32, 47, 48, 53, 58). The addition of 1 mM choline to the purified GbsR protein resulted in a strong quenching of the intrinsic Trp/Tyr fluorescence of GbsR with a simultaneous shift in the wavelength of the emission maximum (Fig. 6B). Titration experiments with choline were then used to assess the binding constant for choline (Fig. 6C), and a K_D value of $165 \pm 15 \mu\text{M}$ was calculated. This value is 1 to 2 orders of magnitude higher than that measured for choline binding by ligand-binding proteins associated with high-affinity ABC transport systems: ChoX (K_D , 2 μM) from *Sinorhizobium meliloti* (47), OpuBC (K_D , 30 μM) from *B. subtilis* (48), and ChoX (K_D , 2 μM) from *Agrobacterium tumefaciens* (1).

Glycine betaine tunes down choline-mediated and GbsR-dependent induction of *gbsAB* expression. The level to which glycine betaine is amassed by the cell from exogenous sources (30, 31) is linearly dependent on the prevalent osmolarity of the growth medium (T. Hoffmann and E. Bremer, unpublished data). This observation suggests the existence of a not-yet-understood physiological and/or genetic regulatory circuit that links the intracellular glycine betaine pool with the osmolarity of the particular ecological niche that *B. subtilis* happens to colonize. It therefore follows that the osmoadaptive glycine betaine synthesis pathway needs to be shut down once the cell has balanced the osmotic gradient across its cytoplasmic membrane through synthesis of the compatible solute glycine betaine.

We therefore asked if an exogenous supply of glycine betaine would repress *gbsA-treA* expression and found that this was indeed the case (Table 3). The presence of 1 mM glycine betaine in the growth medium repressed even the very high level of transcription of this reporter fusion in a *gbsAB* mutant strain that cannot remove the inducer choline from the cytoplasm by oxidizing it to glycine betaine (Table 3). Strikingly, repression of *gbsA-treA* transcription in response to the presence of glycine betaine was abolished in a *gbsR* mutant strain (Table 3), showing that the GbsR repressor protein is required for the inhibitory effect of glycine betaine on *gbsAB* expression.

We then employed the intrinsic fluorescence-based ligand-binding assay used to assess the binding of choline to GbsR (Fig. 6B and C) to test whether glycine betaine would bind to the *B. subtilis* GbsR protein as well. However, we did not detect any effects of glycine betaine (1 mM) on the intrinsic fluorescence of the purified GbsR protein (Fig. 6D) that could be interpreted as an indication for binding of glycine betaine to GbsR. We conducted a

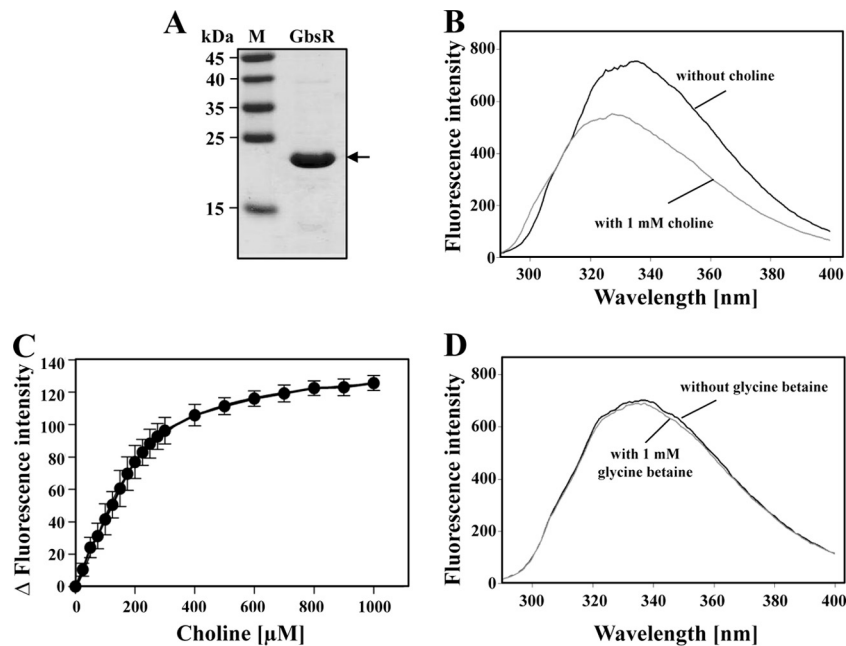


FIG 6 Binding of choline by the GbsR regulatory protein. (A) SDS-polyacrylamide gel electrophoresis of the purified GbsR protein. (B) Fluorescence spectrum of the purified GbsR protein (5 μ M) in the absence or the presence of 1 mM choline. (C) Binding kinetics of choline to the purified GbsR protein (5 μ M) as assessed by intrinsic fluorescence spectroscopy. (D) Intrinsic fluorescence spectrum of the purified GbsR protein (5 μ M) in the absence or the presence of 1 mM glycine betaine.

further series of competition experiments where we preincubated the GbsR protein in the presence of 1 mM, 10 mM, and 100 mM glycine betaine, subsequently added 1 mM the inducer choline to the reaction mixture, and then recorded the intrinsic fluorescence of GbsR. No noteworthy influence of glycine betaine on the choline-induced emission spectrum of the GbsR protein was observed (see Fig. S2 in the supplemental material). This observation indicates that there is no significant degree of competition between the inducer choline and the “counterinducer” glycine betaine for occupancy of the choline-binding site in GbsR (see Fig. S2) in a ligand-binding assay that relies on changes in the intrinsic fluorescence of the purified GbsR protein (Fig. 6B). However, since this assay provides only an indirect readout of ligand binding, some caution in the interpretation of the data is warranted. Mechanisms other than competition of choline and glycine betaine for the same ligand-binding site come to mind: (i) binding of glycine betaine to an allosteric effector site in GbsR influencing the occupancy of the choline-binding site and (ii) opposing effects of choline and glycine betaine on dimer formation of GbsR. Alternatively, glycine betaine might not bind directly to GbsR at all and might instead exert its regulatory effects indirectly through its “chemical chaperone” function (16, 17) that can influence protein folding and conformation (7, 8, 56).

Osmoprotectants other than glycine betaine can tune down choline-mediated GbsR-dependent induction of *gbsAB* expression. Since glycine betaine is the end product of the choline-dependent biosynthetic route for this compatible solute (Fig. 1), we wondered whether osmoprotectants other than glycine betaine would exert a repressing effect on *gbsA-treA* transcription as well. From the six additional compounds tested that function as osmoprotectants for *B. subtilis* (9, 30, 31), proline betaine and dimethylsulfoniopropionate (DMSP) repressed *gbsA-treA* transcription

to an extent similar to that of glycine betaine (Fig. 7A). On the other hand, carnitine repressed the expression of the reporter gene fusion only modestly (Fig. 7A), whereas choline-*O*-sulfate, proline, and ectoine did not significantly influence *gbsA-treA* expression (Fig. 7A). As observed for glycine betaine (Table 3), repression of *gbsA-treA* expression by proline betaine, by DMSP, and to a minor degree by carnitine was dependent on an intact GbsR protein (Fig. 7B). Hence, compatible solutes used by *B. subtilis* that are not associated with glycine betaine production (Fig. 1) can reduce the level of expression of the glycine betaine *gbsAB* biosynthetic genes either very effectively (e.g., proline betaine), modestly (e.g., carnitine), or not at all (e.g., ectoine) (Fig. 7A). However, the interpretation of the data shown in Fig. 7A is not straightforward, since the effects of these compounds on gene expression cannot be directly correlated with the osmoprotective potential that these compatible solutes possess for *B. subtilis*. For instance, glycine betaine, carnitine, proline betaine, and choline-*O*-sulfate confer a similar degree of osmoprotection to high-salinity-challenged *B. subtilis* cells (34, 35, 45, 53), yet they have quite disparate effects on *gbsA-treA* transcription (Fig. 7A).

A structure-guided *in silico* model for the GbsR repressor and inference of a putative binding site for the inducer choline. Database searches with the amino acid sequence of the *B. subtilis* GbsR protein as the search template alerted us to the crystal structure of the Mj223 protein (50) from the extreme thermophilic methanogenic archaeon *Methanococcus jannaschii* (14). Using the crystallographic data set deposited for the Mj223 protein (PDB code 1ku9) (50) (Fig. 8A) showing a graphic representation of the Mj223 protein) and by relying on the Web-based SWISS Model server (3), we were able to derive an *in silico* model of the GbsR protein from *B. subtilis* (Fig. 8B).

The *M. jannaschii* Mj223 protein is a DNA-binding protein

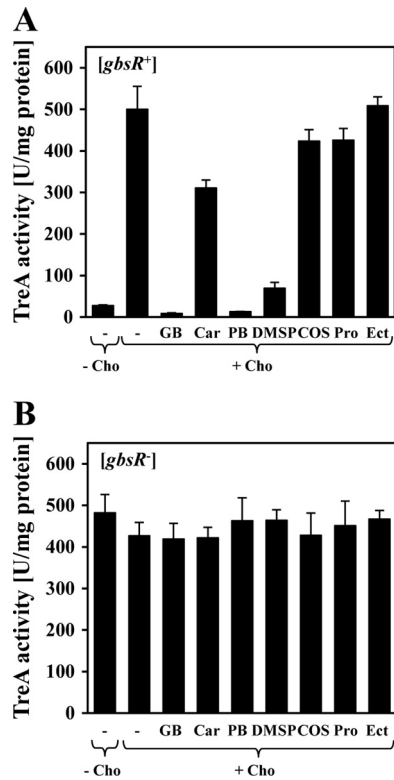


FIG 7 Cells of the *gbsA-treA* fusion strain GNB46 (*gbsAB::neo gbsR⁺*) (A) and of strain GNB48 (*gbsAB⁺ gbsR::neo*) (B) were grown either in SMM without a compatible solute (–) or in SMM containing 1 mM choline (Cho) or 1 mM choline in combination with various compatible solutes: glycine betaine (GB), carnitine (Car), proline betaine (PB), dimethylsulfoniopropionate (DMSP), choline-*O*-sulfate (COS), L-proline (Pro), or ectoine (Ect). Cells were harvested for TreA reporter assays when they reached an OD₅₇₈ of 1 to 1.5.

that possesses a winged-helix DNA binding motif (20, 33), and it is a dimer both in solution and in the crystal structure (50) (Fig. 8A). No ligand that could give a firm clue with respect to the physiological process in which Mj223 might be involved is present in the crystal structure of the Mj223 protein. However, based on the structural similarity of the *M. jannaschii* Mj223 protein to the *B. subtilis* BmrR and *E. coli* MarR transcriptional regulators, Ray et al. (50) speculated that it could be involved in controlling the expression of genes involved in multidrug resistance, but no concrete target gene of the Mj223 DNA-binding protein in the *M. jannaschii* genome is known. We inspected the genetic context of the structural gene for the Mj223 protein in the genome of *M. jannaschii* (14), but no genes whose products could be associated with glycine betaine synthesis or the uptake of choline or with any other type of osmoprotectant were identifiable.

Mj223 is a two-domain protein, with the N-terminal module containing the DNA reading head with a winged-helix DNA-binding motif (20, 33) and an extended C-terminal α -helical module that supports the dimerization of the protein (50) (Fig. 8A). In their analysis of the X-ray structure of the *M. jannaschii* Mj223 protein, Ray et al. (50) noticed that the determined crystal structure (Fig. 8A) would not be conducive for DNA binding of the Mj223 protein to a standard duplex B-form of DNA. Major spatial rearrangements of both the dimerization domain and the DNA-reading head relative to each other have to occur in order to

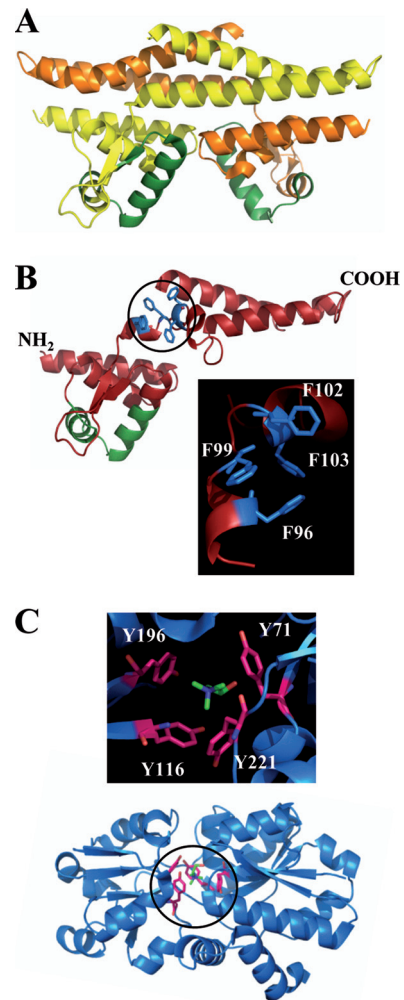


FIG 8 *In silico* model of the *B. subtilis* GbsR protein. (A) X-ray structure of the dimer of the *M. jannaschii* Mj223 protein (PDB code 1ku9) (50). The monomers are shown in either yellow or orange; the winged-helix DNA binding region is shown in green. (B) Monomer of the *in silico*-derived GbsR structure (red color). The predicted winged-helix DNA binding region is shown in green, and the four Phe residues predicted to form part of the choline-binding box are shown in blue. The insert enlarges a section of the GbsR *in silico* model and focuses on the spatial arrangement of the aromatic cage predicted to accommodate the trimethylammonium head group of choline. (C) Overall crystal structure (PDB code 3r6u) (48) of the *B. subtilis* OpuBC solute binding protein in complex with its ligand choline. The insert enlarges a section of the OpuBC crystal structure and focuses on the spatial arrangement of the aromatic cage entrapping the choline ligand.

properly fit the two winged helices of the Mj223 dimer into the major groove of the DNA. These movements pivot around the flexible linker region of the Mj223 protein connecting the DNA-binding and dimerization domains (50).

According to our GbsR *in silico* model, the N-terminal DNA reading head extends from amino acids 1 to 73 and contains the predicted winged-helix DNA-binding motif (20, 33) between amino acids 49 and 73; the predicted C-terminal dimerization domain of GbsR comprises amino acids 95 to 161. The predicted flexible linker region connecting these two domains extends from amino acids 74 to 94 (Fig. 8B).

Since GbsR can directly bind the inducer choline (Fig. 6B and

C), we manually inspected our GbsR *in silico*-derived model for a possible ligand-binding site. This search was based on three high-resolution crystal structures of solute-receptor proteins associated with microbial ABC transporters in complex with their choline ligand: the choline-binding protein ChoX protein from *S. meliloti* (47) and the OpuBC (48) and OpuCC (19) proteins from *B. subtilis*. The precise geometries of the choline-binding site vary somewhat among these ligand-binding proteins, but four aromatic residues that are spatially arranged in the form of an aromatic cage (10) are always part of the substrate-binding sites. The bulky head group of choline is wedged into this aromatic cage and is stabilized by cation- π interactions (18) between the positively charged trimethylammonium group of choline and the electronegative surface potential of side chains of the aromatic residues. The alcohol function of the choline molecule protrudes out of this aromatic cage and is stabilized by additional contacts (19, 47, 48).

We detected a region in our GbsR *in silico* model that could possibly correspond to an aromatic cage for the coordination of the trimethylammonium head group of the choline ligand. It is formed by four Phe residues that occur in close proximity in the amino acid sequence of the GbsR protein: Phe-96, Phe-99, Phe-102, and Phe-103 (Fig. 8B). The spatial arrangement of an aromatic cage used for the entrapment of choline by the ligand-binding protein, OpuBC, of the *B. subtilis* OpuB ABC transporter (48) is shown in Fig. 8C for comparison to the putative choline-binding site suggested here for the GbsR protein (Fig. 8B).

Genome context of *gbsR*-related genes. We used the amino acid sequence of the *B. subtilis* GbsR protein as the search template in a BLAST analysis (2) of unfinished and finished microbial genomes and inspected the genome context of structural genes for the recovered GbsR-related proteins. GbsR-related proteins are found ubiquitously in members of the *Bacillus* and *Staphylococcus* genera, where they are typically found in the vicinity of either glycine betaine biosynthetic gene clusters or potential uptake systems for compatible solutes.

Rosenstein et al. (51) previously characterized a glycine betaine synthesis gene cluster (*cudTCAB*) in *Staphylococcus xylosum* comprising the glycine betaine biosynthetic CudAB enzymes and the choline transporter CudT, a member of the BCCT family of transporters (65). CudC is a regulatory protein exhibiting 54% identity to the GbsR protein from *B. subtilis* (51). Our database searches showed that the *cudTCAB* gene cluster is present in every sequenced *Staphylococcus* genome, suggesting that CudC/GbsR-type proteins function widely as the regulatory protein for glycine betaine synthesis in staphylococci. In the functionally characterized glycine betaine biosynthetic gene clusters of *Halobacillus dabanensis* (23) and *Halobacillus halophilus* (15), *gbsR*-type genes are present as well and are divergently orientated from the *gbsAB* genes and cotranscribed with a gene (referred to as *gbsT* and *gbsU*, respectively) encoding an orphan ligand-binding protein that bears the hallmarks of a substrate-binding protein associated with ABC transport system possessing a specificity for glycine betaine or other osmoprotectants with a trimethylammonium or a dimethylsulfonium head group (47, 52, 53, 58). Burkhardt et al. (15) demonstrated the induction of the transcription of the *H. halophilus gbsAB* and *gbsRU* gene clusters in response to choline availability and their repression by glycine betaine but did not experimentally correlate these transcriptional responses with the *gbsR*-encoded protein.

We detected two types of genome contexts of *gbsR*-related

TABLE 4 Role of the GbsR-related YvaV and YvbF *B. subtilis* proteins on *gbsA* promoter activity^a

Strain	<i>gbsR</i> ^b	<i>yvaV</i>	<i>yvbF</i>	TreA activity (U/mg protein)
GNB45	+	+	+	10 ± 3
GNB48	–	+	+	568 ± 13
GNB55	+	–	+	16 ± 2
GNB56	+	+	–	14 ± 2
GNB57	–	–	+	574 ± 24
GNB58	–	+	–	564 ± 33
GNB59	–	–	–	565 ± 5
GNB60	+	–	–	15 ± 4

^a Cells of the various *gbsA-treA* reporter fusion strains were cultivated in SMM until the cultures had reached on OD₅₇₈ of 1.5; samples were then assayed for TreA reporter enzyme activity. The strains carry the same *gbsA-treA* reporter gene fusion and are present in a *B. subtilis* JH642 (*trp phe*) strain background.

^b The strains carrying mutations in the *gbsR* gene (*gbsR::neo*), the *yvaV* gene (*yvaV::spc*) or the *yvbF* gene (*yvbF::tet*) are indicated by –; the presence of wild-type alleles is indicated by +.

genes in members of the *Bacillus* genus. One group of these genes is associated with *gbsAB*-type glycine betaine biosynthetic genes in a genetic configuration identical with that found in *B. subtilis* (Fig. 1). The second group of *gbsR*-related genes is associated with gene clusters annotated as ABC-type uptake systems for compatible solutes. Some bacilli possess more than one *gbsR*-type gene, and these are found in both mentioned genomic contexts. One example is *B. subtilis* itself. In addition to the *gbsR* gene found next to the *gbsAB* gene cluster (Fig. 1), *gbsR*-related genes (*yvbF* and *yvaV*) are also present upstream of the *opuB* and *opuC* operons, and the encoded proteins possess amino acid sequence identities to GbsR of 34% (similarity of 58%) and 35% (similarity of 57%), respectively. Nothing is currently known about a possible function of these putative regulatory proteins, but we noted that both the YvbF and YvaV proteins each possess three out of the four aromatic residues forming the predicted choline-binding site in GbsR (Fig. 8B). We therefore tested a possible involvement of YvbF and YvaV on *gbsAB* expression, but none was found regardless of whether the *yvbF* and *yvaV* genes were disrupted individually, tested as part of a double mutant, or tested in various combinations with the *gbsR::neo* mutation (Table 4).

DISCUSSION

The data presented here identify a regulatory protein (GbsR) controlling the osmoadaptive choline-to-glycine betaine synthesis pathway of *B. subtilis* (Fig. 1). Coregulation of the *opuB* and *gbsAB* operons by GbsR in response to choline availability is physiologically fitting, since glycine betaine production by *B. subtilis* relies on the import of the precursor molecule choline (4, 5). It is equally physiologically fitting that GbsR does not control the expression of the *opuC* operon (Table 2), since the OpuC ABC transporter does not only function for the import of choline (36) but mediates the uptake of a wide spectrum of other osmoprotectants (9, 30) (Fig. 1). Regulation of *opuC* expression in response to choline availability would therefore be counterproductive for osmotically stressed *B. subtilis* cells.

The precursor and inducer for glycine betaine production is present in the soil habitat of *Bacillus subtilis* in physiologically relevant concentrations (41), primarily as a result of the degradation of phosphatidylcholine, a major component of membranes of

eukaryotic cells. Glycine betaine aldehyde, the intermediate in glycine betaine synthesis (Fig. 1), is also an inducer of *gbsAB* expression (Fig. 2A) but is unlikely to be present in significant amounts in natural ecosystems due to its high chemical reactivity. Its function as an inducer might therefore serve as a fail-safe mechanism to ensure the synthesis of the glycine betaine aldehyde dehydrogenase GbsA, an enzyme that efficiently converts the noxious glycine betaine aldehyde (5, 36) into the physiologically well-tolerated glycine betaine molecule (6).

Our genetic and biochemical data show that GbsR acts as a repressor monitoring the presence of choline inside the cell (Table 3) and that the GbsR protein binds this ligand directly (Fig. 6B and C). We took advantage of an *in silico* model for GbsR to search for a possible choline-binding site (Fig. 8B) that resembles those found in several choline solute receptors of known crystal structure (19, 47, 48), e.g., the ligand-binding protein (OpuBC) of the OpuB transporter (Fig. 8C). These types of ligand-binding proteins accommodate the choline ligand via cation- π interactions (18) between the bulky and positively charged head group of choline with the electronegative surface potential of the side chains of aromatic amino acids spatially arranged in the form of an open cage (10). The predicted aromatic choline-binding cage in GbsR is strategically positioned close to the flexible linker region connecting the N-terminal DNA-reading head with its winged-helix DNA-binding motif and the extended C-terminal dimerization domain (Fig. 8B). Ligand-induced conformational changes, as we have detected by fluorescence spectroscopy of the purified GbsR protein in response to choline (Fig. 6B), can therefore readily be envisioned as altering the spatial orientation of the N-terminal DNA-binding domain relative to the C-terminal dimerization domain and thereby relieve the repressing effects of GbsR on *gbsAB* and *opuB* expression. Indeed, the modeling studies conducted by Ray et al. (50) for the *M. jannaschii* Mj223 protein strongly suggest that the conformations of the DNA-bound and the DNA-free forms (Fig. 8A) of the Mj223 protein differ greatly. These conformational changes in Mj223 pivot around the flexible linker region (50) (Fig. 8A), in whose vicinity we envision the ligand-binding site for the inducer choline in our GbsR *in silico* model (Fig. 8B).

One issue that needs to be reckoned with in the physiological context of the osmoadaptive glycine betaine synthesis route of *B. subtilis* (Fig. 1) is the cellular and genetic events that lead to a repression of glycine betaine production once the osmotically challenged cell has accomplished a physiologically sufficient degree of cellular hydration and raised turgor again. The presence of choline in the soil habitat of *B. subtilis* (41) will inevitably lead to choline uptake caused by the basal activity of the OpuB and OpuC transporters (36) (Fig. 4A and Table 2). Import of the inducer choline will then relieve GbsR-mediated repression of the *opuB* and *gbsAB* operons and will thereby trigger enhanced choline uptake and continued synthesis of glycine betaine, regardless of whether the *B. subtilis* cell is osmotically stressed or not. If glycine betaine synthesis would remain unchecked, an undue rise in turgor would ensue, and this would force the *B. subtilis* cell eventually to jettison part of the newly synthesized glycine betaine either through the transient opening of mechanosensitive channels (27, 29, 60) or through specialized excretion systems (21, 40, 43). The genetic data obtained by us suggest that *B. subtilis* has found an elegant way to avoid such a wasteful behavior, since the properties of the GbsR repressor are responsive to the presence of glycine

betaine, which can counteract the inducing effects of choline (Table 3 and Fig. 7).

Whatever might be the precise molecular mechanism that allows glycine betaine to promote the repressor function of the GbsR protein in the presence of the inducer choline, the data presented here show that the GbsR regulator integrates signals required both for the onset of glycine betaine synthesis from the precursor choline and for tuning down glycine betaine production when the *B. subtilis* cell has relieved itself from osmotic stress through the accumulation of this compatible solute.

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