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# Osmotic Control of *opuA* Expression in *Bacillus subtilis* and Its Modulation in Response to Intracellular Glycine Betaine and Proline Pools

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**Glycine betaine is an effective osmoprotectant for *Bacillus subtilis*. Its import into osmotically stressed cells led to the buildup of large pools, whose size was sensitively determined by the degree of the osmotic stress imposed. The amassing of glycine betaine caused repression of the formation of an osmoadaptive pool of proline, the only osmoprotectant that *B. subtilis* can synthesize *de novo*. The ABC transporter OpuA is the main glycine betaine uptake system of *B. subtilis*. Expression of *opuA* was upregulated in response to both sudden and sustained increases in the external osmolarity. Nonionic osmolytes exerted a stronger inducing effect on transcription than ionic osmolytes, and this was reflected in the development of corresponding OpuA-mediated glycine betaine pools. Primer extension analysis and site-directed mutagenesis pinpointed the osmotically controlled *opuA* promoter. Deviations from the consensus sequence of SigA-type promoters serve to keep the transcriptional activity of the *opuA* promoter low in the absence of osmotic stress. *opuA* expression was downregulated in a finely tuned manner in response to increases in the intracellular glycine betaine pool, regardless of whether this osmoprotectant was imported or was newly synthesized from choline. Such an effect was also exerted by carnitine, an effective osmoprotectant for *B. subtilis* that is not a substrate for the OpuA transporter. *opuA* expression was upregulated in a *B. subtilis* mutant that was unable to synthesize proline in response to osmotic stress. Collectively, our data suggest that the intracellular solute pool is a key determinant for the osmotic control of *opuA* expression.**

In the microbial world, the amassing of compatible solutes is a widely used stress response to fend off the detrimental effects of high osmolarity on cellular water content, turgor, and physiology (1). The intracellular accumulation of compatible solutes strongly stimulates cell proliferation under otherwise growth-inhibiting high-osmolarity conditions. Compatible solutes can be scavenged by bacteria from environmental sources or synthesized. Genes encoding uptake systems for compatible solutes or enzymes for their synthesis are typically subjected to osmotic control at the transcriptional level (1–4). In addition, the activity of transporters for these compounds is often regulated in response to cytoplasmic or membrane-derived signals emanating from osmotic stress (5–8). Both regulatory mechanisms allow the microbial cells to sensitively adjust their compatible solute content in response to the osmotic conditions prevalent in their surroundings at a given time (5, 6, 8–11).

The soil bacterium *Bacillus subtilis* lives in a taxing habitat where desiccation processes frequently create high-salinity and high-osmolarity microniches, thereby exposing *B. subtilis* cells to sustained osmotic stress (12). *B. subtilis* makes extensive use of compatible solutes to cope with this challenge. Its initial cellular response to acute osmotic stress relies on the uptake of large amounts of potassium ions (13, 14). In the subsequent phase of acclimatization to sustained high-osmolarity surroundings, compatible solutes such as proline and glycine betaine are amassed via synthesis and uptake (9, 15–17). This leads, in turn, to a reduction in the cellular potassium content through the activities of K<sup>+</sup> efflux systems (13, 16, 18), which allows the cell to reduce the ionic strength of the cytoplasm without compromising its overall osmotic potential.

Proline is the only compatible solute that *B. subtilis* can synthesize

*de novo* in response to osmotic stress, and *B. subtilis* possesses a dedicated biosynthetic pathway for the production of proline as an osmoprotectant (Fig. 1) (9, 16, 19). Predictably, the genetic disruption of this osmoadaptive proline biosynthetic route causes osmotic sensitivity (9). *B. subtilis* is also equipped with a set of five osmotically inducible osmoprotectant uptake systems, the Opu family of transporters (Fig. 1), which allow *B. subtilis* to scavenge a broad spectrum of compatible solutes as a cellular defense against osmotic stress (12) and also against high- and low-temperature challenges (20, 21). With the exception of proline (22), none of the osmoprotectants used by *B. subtilis* can be exploited as nutrients; they are therefore bona fide stress protectants.

Among the compatible solutes employed, glycine betaine assumes an especially important role for *B. subtilis*, since it can be both synthesized and imported via three high-affinity and osmotically inducible transport systems. Synthesis of glycine betaine relies on the uptake of the precursor molecule choline (via OpuB and OpuC) (23), which then undergoes a two-step oxidation reaction catalyzed by the GbsB and GbsA enzymes to produce glycine betaine (Fig. 1) (24, 25). The ABC transporters OpuA and OpuC and the BCCT (betaine-choline-carnitine-transporter)-

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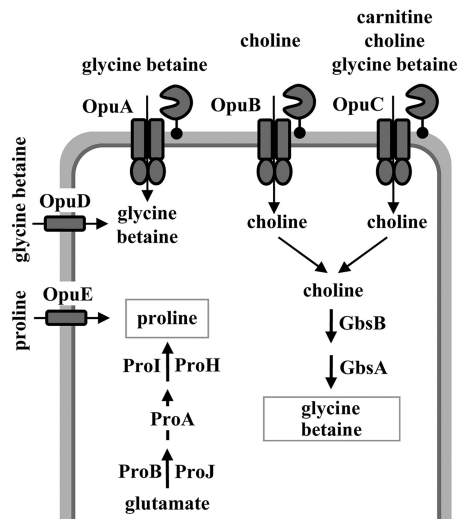
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**FIG 1** Compatible-solute uptake and synthesis in *B. subtilis*. Uptake systems and synthesis pathways for compatible solutes are summarized schematically. Detailed descriptions of the Opu transport systems and their substrate specificities have been provided elsewhere (12, 20). The synthesis of glycine betaine from the precursor choline (taken up via the OpuB and OpuC transporters) is catalyzed through a two-step oxidation process by the GbsB and GbsA enzymes, with glycine betaine aldehyde as the intermediate (24, 25). The anabolic and osmoadaptive proline biosynthetic routes and their genetic control have been described elsewhere (9, 19). In addition to the  $\Delta^1$ -pyrroline-5-carboxylase reductases ProI and ProH, shown here, a third protein (ProG) with  $\Delta^1$ -pyrroline-5-carboxylase reductase activity operates in *B. subtilis* (94); its physiological role is unclear.

type carrier OpuD mediate the import of glycine betaine (Fig. 1) (23, 26, 27). An assessment of the kinetic properties of the OpuA, OpuB, OpuC, and OpuD transporters revealed that the OpuA system is the dominant glycine betaine uptake system (27). OpuA consists of the ATPase OpuAA, the integral membrane protein OpuAB, and the solute receptor OpuAC, an extracellular high-affinity glycine betaine binding protein that is tethered to the membrane via a lipid anchor (Fig. 1) (26, 28–31).

The expression of the *B. subtilis* *opuA* gene cluster (*opuAA-opuAB-opuAC*) is inducible in response to an increase in external salinity (26). In addition, OpuA transport activity might also be osmotically controlled (32) in a fashion analogous to that discovered by B. Poolman and coworkers for the corresponding glycine betaine uptake system OpuA of *Lactococcus lactis*. In the latter system (33), two cystathionine-beta-synthase (CBS) domains present near the carboxy-terminal end of the OpuAA ATPase subunit serve as sensors for changes in the ion strength of the cytoplasm and set the overall transport activity of the OpuA transport system in response to external osmotic changes (34). A repressor protein (BusR) encoded by a gene positioned upstream of the *L. lactis* *opuA* locus participates in the osmotic induction of *opuA* transcription (35). Interestingly, the *in vitro* DNA-binding activity of BusR is responsive to ionic strength (36). No BusR-related regulatory protein is present in *B. subtilis*, and the mechanism(s) underlying the osmotic control of *opuA* gene expression (26) in this microorganism is unresolved.

Here we have addressed the osmotic control of *opuA* expression in response to both ionic and nonionic solutes under acute and sustained osmotic stress conditions and in response to intracellularly accumulated compatible solutes. We assessed the

buildup of glycine betaine pools in osmotically challenged *B. subtilis* cells and studied the influence of glycine betaine accumulation on the size of the osmostress-adaptive proline pool attained via *de novo* synthesis. Our data show that there is a linear relationship between the degree of the osmotic stress imposed on the *B. subtilis* cell and the size of the glycine betaine pool generated via import. As the glycine betaine content of the cell increases, a concomitant decrease in the size of the proline pool ensues. With respect to *opuA* expression, our data collectively indicate that the *B. subtilis* cell sensitively monitors its intracellular solute pool to precisely set the level of *opuA* transcription under high-osmolarity stress conditions.

## MATERIALS AND METHODS

**Chemicals.** Antibiotics, compatible solutes, the ninhydrin agent for the quantification of proline, and the chromogenic substrate for TreA enzyme assays (*para*-nitrophenyl- $\alpha$ -D-glucopyranoside [PNPG]) were obtained from Sigma-Aldrich (Steinheim, Germany). Radiolabeled [ $^{14}$ C]glycine betaine (55 mCi mmol<sup>-1</sup>) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO).

**Bacterial strains.** The *Escherichia coli* strain DH5 $\alpha$  (Invitrogen, Carlsbad, CA) was used for all routine cloning experiments. The *B. subtilis* strain JH642 (*trpC2 pheA1*) and its various mutant derivatives (Table 1)

**TABLE 1** Bacterial strains used in this study

Strain <sup>a</sup>	Relevant genotype	Construction or reference
JH642	<i>trpC2 pheA1</i>	J. Hoch
MBB9	<i>amyE::[Φ(opuAA'-treA)1 cat] Δ(treA::neo)</i>	This study
JSB8	$\Delta(\text{proHJ::tet})1$	19
MBB12	$\Delta(\text{opuA::tet})2 \Delta(\text{opuD::neo})2$ <i>opuC-20::Tn10(spc)</i>	This study
MBB13	$\Delta(\text{opuA::tet})2 \Delta(\text{opuD::neo})2$ <i>opuC-20::Tn10(spc) ΔtreA::ery</i>	This study
MBB14	<i>amyE::[Φ(opuAA'-treA)1 cat] Δ(opuA::tet)2</i> <i>opuC-20::Tn10(spc) Δ(opuD::neo)2</i> $\Delta\text{treA::ery}$	This study
MBB16	<i>amyE::[Φ(opuAA'-treA)1 cat]</i> $\Delta(\text{degS-degU::aphA3})1 \Delta\text{treA::ery}$	This study
MBB18	$\Delta(\text{opuA::tet})2 \Delta(\text{opuD::neo})2$ <i>opuB-20::Tn10(spc)</i>	This study
MBB19	<i>amyE::[Φ(opuAA'-treA)1 cat] Δ(sigB2::cat)</i> $\Delta\text{treA::ery}$	This study
MBB20	$\Delta\text{treA::ery} \Delta(\text{opuA::tet})2 \Delta(\text{opuD::neo})2$ <i>opuB-20::Tn10(spc)</i>	This study
MBB21	<i>amyE::[Φ(opuAA'-treA)1 cat] Δ(opuA::tet)2</i> $\Delta(\text{opuD::neo})2 \text{ opuB-20::Tn10(spc)}$ $\Delta(\text{treA::neo})$	This study
GNB37	$\Delta(\text{treA::ery})$	G. Nau-Wagner
FSB1	$\Delta(\text{treA::neo})$	54
BLOB22	<i>sigBΔ2::cat</i>	17
RMKB34	<i>opuBD::tet opuC-20::Tn10(spc)</i> $\Delta(\text{opuD::neo})2$	23
RMKB30	<i>opuC-20::Tn10(spc) Δ(opuD::neo)2</i>	R. Kappes
TMB117	<i>amyE::[Φ(opuAA'-treA)1 cat] Δ(treA::neo)</i> $\Delta(\text{proHJ::tet})1$	This study
TMB150	<i>amyE::[Φ(opuAA'-treA)1 cat] Δ(treA::neo)</i> $\Delta(\text{yceK::spc})$	This study
QB4238	<i>trpC2 Δ(degS-degU::aphA3)1</i>	95

<sup>a</sup> All strains, except for QB4238, are derivatives of the *B. subtilis* wild-type laboratory strain JH642 (37) and therefore carry the *trpC2* and *pheA1* mutations in addition to the genetic markers indicated. Strain QB4238 is a derivative of *B. subtilis* strain 168.

were used throughout this study. Strain JH642 is a member of the domesticated lineage of *B. subtilis* laboratory strains (37) and was kindly provided to us by James A. Hoch (Scripps Research Institute, La Jolla, CA).

**Media and growth conditions.** *B. subtilis* was cultivated in Spizizen's minimal medium (SMM) (38), with 0.5% (wt/vol) glucose as the carbon source and L-tryptophan (20 mg liter<sup>-1</sup>) and L-phenylalanine (18 mg liter<sup>-1</sup>) to satisfy the auxotrophic growth requirements of strain JH642 (*trpC2 pheA1*) (Table 1). A solution of trace elements was added to SMM (38). The osmolarity of the growth medium was increased by the addition of NaCl (5 M stock solution), KCl (1.6 M stock solution), glycerol (2.72 M stock solution), lactose (1.22 M stock solution), or sucrose (1.24 M stock solution). The osmolarities of the growth media were determined with a Wesco 5500 vapor pressure osmometer (Wesco Inc., Logan, UT). Glycine betaine, choline, and carnitine solutions were sterilized by filtration and added to the growth media from 100 mM stock solutions. All *B. subtilis* cultures were inoculated from precultures growing exponentially in prewarmed minimal medium to an optical density at 578 nm (OD<sub>578</sub>) of 0.1, and the cultures were propagated at 37°C in a shaking water bath set to 220 rpm. Typically, for the determination of TreA activity in *opuA-treA* fusion strains, RNA preparations, or growth curves, the *B. subtilis* cells were grown in 20-ml or 100-ml culture volumes in 100-ml or 500-ml Erlenmeyer flasks, respectively. The *B. subtilis* strains were routinely maintained on Luria-Bertani (LB) agar plates. The antibiotics chloramphenicol (5 µg ml<sup>-1</sup>), kanamycin (5 µg ml<sup>-1</sup>), erythromycin-lincomycin (0.4 µg ml<sup>-1</sup> and 15 µg ml<sup>-1</sup>), spectinomycin (100 µg ml<sup>-1</sup>), and tetracycline (10 µg ml<sup>-1</sup>) were used for the selection of gene disruption mutations in *B. subtilis* after the transformation of appropriate recipient strains with chromosomal DNA.

**Construction of *B. subtilis* mutant strains.** *B. subtilis* mutants with defects in the various *opu* loci were constructed by transformation with chromosomal DNA of previously described strains carrying antibiotic resistance cassettes in various *opu* genes (23, 26, 27). Transformants were selected on LB agar plates containing the appropriate antibiotics. The preparation of chromosomal DNA and the transformation of *B. subtilis* cells with chromosomal or plasmid DNA followed routine procedures (39); all the mutant strains constructed are listed in Table 1. A deletion of the presumptive *yceK* regulatory gene (40, 41) was constructed by amplifying the 5' and 3' regions bordering the *yceK* locus via PCR and connecting them by long-flanking region PCR (42) with a gene encoding a spectinomycin (*spc*) resistance cassette. The *spc* gene was derived from plasmid pDG1726 (43). The fused DNA fragment resulting from the long-flanking region PCR was used to transform competent cells of the *opuA-treA* reporter fusion strain MBB9 (Table 1), with subsequent selection for spectinomycin-resistant colonies. The proper insertion of the  $\Delta$ (*yceK::spc*) gene disruption into the chromosome of the resulting strain TMB150 (Table 1) was verified by PCR using DNA primers flanking the authentic *yceK* locus. Production of the extracellular  $\alpha$ -amylase AmyE by *B. subtilis* strains was tested by flooding the colonies grown on LB plates containing 1% starch with Gram's iodide stain (0.5% [wt/vol] iodide, 1% potassium iodide) for 1 min and scoring for zones of clearing around the colonies after the stain was decanted (39).

**Construction of plasmids and site-directed mutagenesis.** To clone the wild-type *opuA* promoter, we amplified a 976-bp *opuA* DNA fragment from the chromosome of strain JH642 by PCR using primers containing artificial EcoRV and BamHI restriction sites at their 5' ends (forward primer, EcoRV-*opuA* [5'-GCGCGATATCGAATTCAATTC AATAAAA G-3']; reverse primer, BamHI-*opuA* rev [5'-GCGCGGATCCCTTTGAA CGATGTTGCCG-3']). This DNA fragment was then inserted into a pBluescript SK(-) vector (Stratagene, Heidelberg, Germany) that had been cut with EcoRV and BamHI. This yielded plasmid pTMB1, and the nucleotide sequence of the inserted *opuA* fragment was verified by DNA sequence analysis. In parallel, we cloned the same PCR fragment carrying the *opuA* promoter and part of the *opuAA* coding region into the low-copy-number *treA* operon fusion vector pJMB1 (M. Jebbar and E. Bremer, unpublished results), thereby producing plasmid pMBB10

(*amyE::opuA-treA-cat::amyE*). This fusion construct was inserted into the chromosome of *B. subtilis* after linearization of the plasmid DNA by restriction digestion, transformation of an appropriate *B. subtilis* strain [e.g., strain FSB1 [ $\Delta$ (*treA::neo*)] (Table 1), and selection of chloramphenicol-resistant colonies on LB agar plates. This integrated the *amyE::opuA-treA-cat::amyE* construct, via a double homologous recombination event, stably into the nonessential chromosomal *amyE* locus without integrating the plasmid backbone of the pJMB1 reporter gene fusion vector as well. The integration of the *opuA-treA* reporter gene fusions into the *B. subtilis* chromosome at the *amyE* site was verified by scoring the AmyE-negative phenotype of such transformants. Mutations in the *B. subtilis* *opuA* promoter were isolated using the QuikChange site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) and a series of custom-synthesized mutagenic DNA primers. Plasmid pTMB1, carrying the wild-type *opuA* promoter, served as the template for the mutagenesis experiments. The mutant *opuA* promoter fragments were recovered from these pTMB1-derived plasmids by digestion with EcoRV and BamHI and were inserted into the *treA* operon fusion vector pJMB1. After linearization of the resulting plasmids by restriction digestion, the *B. subtilis* strain FSB1 [ $\Delta$ (*treA::neo*)] (Table 1) was transformed with the mutant *opuA-treA* fusion constructs, and their integration into the chromosomal *amyE* locus was selected for by chloramphenicol resistance and subsequent scoring of the AmyE phenotype. To construct a plasmid for primer extension analysis, we cloned the 976-bp *opuA* DNA fragment (derived from plasmid pMBB10) as an EcoRI-BamHI restriction fragment into the *E. coli-B. subtilis* shuttle vector pRB373 (44), thereby producing plasmid pMBB39.

**Northern blot analysis.** Total RNA was isolated from exponentially growing *B. subtilis* JH642 cultures (OD<sub>578</sub>, approximately 1.0) by the acid-phenol method (45). Two micrograms of total RNAs were denatured by heating (70°C, 5 min) in the presence of 50% formamide. The denatured RNA sample was then electrophoretically separated in a 1.4% agarose gel buffered with 20 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0). Northern blot analysis was performed as described previously (21). Antisense RNA probes for *opuAA* and *trnB-Ala* (a constitutively expressed gene for a *B. subtilis* tRNA) were prepared using an *in vitro* transcription system (Strip-EZ kit; Ambion, Austin, TX). DNA templates for *in vitro* transcription reactions were generated by PCR using the following primer pairs, with the reverse primer carrying an artificial T7 promoter sequence in addition to either the *opuAA*- or the *trnB-Ala*-specific sequence: *opuAA*-for195 (5'-GGTCTATCAGGGAGCGG-3') and *opuA*-rev969-T7 (5'-TAATACGACTCACTATAGGGAGGCTGTCGCCGATCGCAACGC-3') for the *opuAA* probe and primers *trnB-Ala*-for (5'-GGGCCCTTAGCTCAGCT-3') and *trnB-Ala*-rev-T7 (5'-TAATACGACTC ACTATAGGGAGGAAGTGGAGCCTAGCGGG-3'). By using the resulting PCR products as templates for *in vitro* transcription reactions, hybridization probes internal to the *opuAA* gene (519 nucleotides) or covering the entire *trnB-Ala* gene (76 nucleotides) were generated.

**Primer extension analysis.** Strain JH642 (pMBB39-*opuAA*') was grown either in SMM alone or in SMM with 0.4 M NaCl in the presence of 5 µg ml<sup>-1</sup> kanamycin to select for the presence of the plasmid. RNA was prepared from these cultures after they had reached the mid-exponential-growth phase (OD<sub>578</sub>, 0.8 to 1.0). Total RNA was isolated, and an *opuA*-specific antisense DNA primer (*opuA*-PE [5'-CCGCTCCCTGATAGAC CC-3']) labeled at its 5' end with the fluorescent dye IRD800 (purchased from MWG, Ebersberg, Germany) was hybridized to the *opuA* mRNA and was extended with avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI). The same primer was used for a DNA sequence reaction using plasmid pMBB39 as a template to allow the identification of the 5' end of the *opuA* mRNA. DNA sequencing was performed using the dideoxy chain termination method with the "Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-GTP" (Amersham Pharmacia Biotech, Freiburg, Germany). The products of the primer extension and DNA sequencing reactions were analyzed using a DNA sequencer (model 4000; Li-COR Biosciences, Bad Homburg, Germany).

**TreA reporter enzyme assays.** The expression of chromosomal *opuA-treA* reporter gene fusions was monitored by assaying TreA [phospho- $\alpha$ -(1,1)-glucosidase] enzyme activity (46) by using the chromogenic substrate *para*-nitrophenyl- $\alpha$ -D-glucopyranoside as detailed previously (19). One unit of TreA activity is defined as 1  $\mu$ mol of substrate converted per min per mg of protein. The protein concentrations of the samples were estimated from the optical density of the *B. subtilis* cell culture. In all of the *opuA-treA* gene fusion strains used, the natural *treA* gene in the chromosome of *B. subtilis* (47) was disrupted (Table 1), so that the measured TreA enzyme activity reflected only the activity of the TreA reporter enzyme produced by the cell in response to the transcriptional activity of the *opuA* promoter.

**Determination of intracellular glycine betaine pools.** To determine the sizes of intracellular glycine betaine pools, *B. subtilis* cultures were grown to the mid-exponential-growth phase ( $OD_{578}$ , about 1.5) in SMM with varying salinities in the presence of 1 mM glycine betaine; the glycine betaine sample used for this experiment was spiked with 0.64  $\mu$ M radiolabeled [ $1-^{14}C$ ]glycine betaine. Aliquots (300  $\mu$ l) of the cultures were withdrawn. The cells were collected by filtration onto filter discs (pore size, 0.45  $\mu$ M; Schleicher & Schuell, Dassel, Germany) and were then carefully washed with isoosmotic growth medium to remove extracellular [ $1-^{14}C$ ]glycine betaine. The radioactivity in the *B. subtilis* cells collected on the filters was then measured by scintillation counting, and the intracellular pool size of glycine betaine was calculated as described previously (21) using a *B. subtilis* cell volume of 0.67  $\mu$ l per 1  $OD_{578}$  unit of cell culture (S. Moses, E. P. Bakker, and E. Bremer, unpublished data). The *B. subtilis* cell volumes were calculated from the determination of the internal and total water spaces. To this end, aliquots of the cultures were incubated with  $^3H_2O$  and inulin- $^{14}C$ carboxylic acid (Amersham Life Science, Freiburg, Germany), and the distribution of these radiolabeled compounds was measured by scintillation counting after centrifugation through silicon oil (48).

**Measurements of cellular proline pools.** The intracellular proline contents of *B. subtilis* strains were determined by a colorimetric assay detecting proline as a colored proline-ninhydrin complex that can be quantified by measuring the absorption of the solution at 480 nm (49). Cells of the *B. subtilis* strain studied were grown in SMM in the absence or presence of glycine betaine, using NaCl and glycine betaine concentrations specified in the figures, until they reached an  $OD_{578}$  of about 1.5. The cells (8 ml) were then harvested by centrifugation and were frozen at  $-20^\circ C$  until further use. The soluble cellular content was extracted, and the amount of proline was assessed according to the procedure detailed by Bates et al. (49). Proline concentrations were determined by establishing a standard curve with L-proline for the colorimetric proline assay, and intracellular proline concentrations of *B. subtilis* were then calculated by using a *B. subtilis* cell volume of 0.67  $\mu$ l per 1  $OD_{578}$  unit of cell culture.

## RESULTS

**Glycine betaine and proline pools in osmotically stressed *B. subtilis* cells.** To monitor the buildup of an intracellular glycine betaine pool in osmotically stressed cells from an exogenous supply of glycine betaine, we grew the *B. subtilis* wild-type strain JH642 (*OpuA*<sup>+</sup> *OpuC*<sup>+</sup> *OpuD*<sup>+</sup>) (Fig. 1) in SMM containing various concentrations of NaCl in the presence of 1 mM glycine betaine. The glycine betaine solution used was spiked with 0.64  $\mu$ M radiolabeled [ $1-^{14}C$ ]glycine betaine, which allowed us to determine the glycine betaine content of the cells by scintillation counting after recovering the cells by filtration. We found that the concentration of the glycine betaine pool increased from about 160 mM in cells grown in SMM (about 350 mosmol kg<sup>-1</sup>) to about 570 mM in cells grown in SMM with 1 M NaCl (about 2,190 mosmol kg<sup>-1</sup>) (Fig. 2A). There was a perfect linear relationship between the glycine betaine content of the cells and the strength of the osmotic stress imposed (Fig. 2A). Notably, even in cells grown

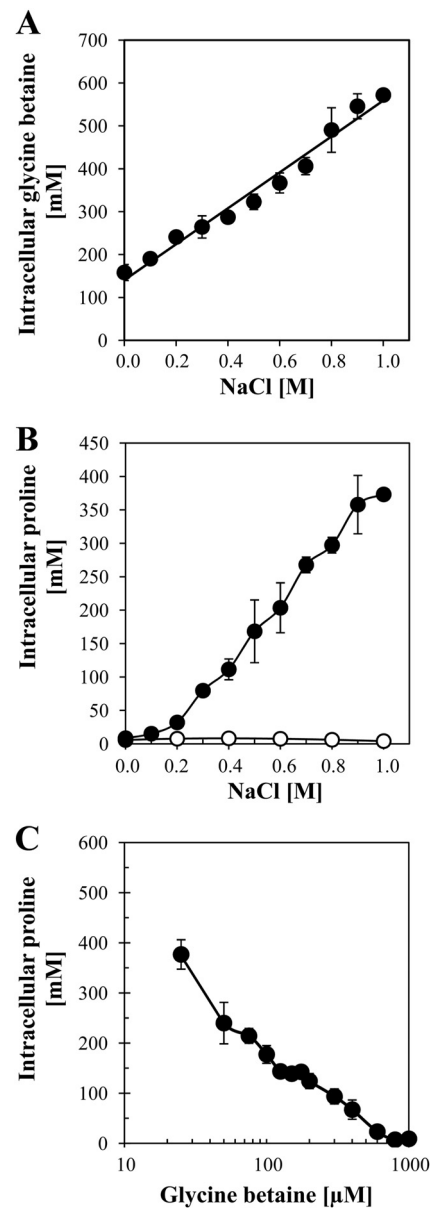


FIG 2 Adjustment of the intracellular compatible-solute pool of *B. subtilis* to external salinity and glycine betaine availability. (A) Cultures of the wild-type strain JH642 were grown in SMM to the mid-exponential-growth phase ( $OD_{578}$ , about 1.5). The medium contained the NaCl concentrations indicated and 1 mM glycine betaine spiked with 0.64  $\mu$ M radiolabeled [ $1-^{14}C$ ]glycine betaine. Intracellular glycine betaine (●) was quantified by scintillation counting. (B) For the analysis of cytoplasmic proline pools, cells of the *B. subtilis* wild-type strain JH642 were grown to the mid-exponential-growth phase ( $OD_{578}$ , about 1.5) in SMM with the NaCl concentrations indicated in the absence (●) or presence (○) of 1 mM glycine betaine. After extraction of the soluble solute pools from these cells, their proline content was determined by a colorimetric assay (49). (C) Cells of strain JH642 were grown to the mid-exponential-growth phase ( $OD_{578}$ , about 1.5) in SMM containing 1 M NaCl and the indicated concentrations of glycine betaine. Their proline content (●) was determined by a colorimetric assay (49). All data presented in this figure are the means for two independent cultures that were assayed for their internal solute concentrations in duplicate.

in SMM, a growth medium widely used for physiological studies of *B. subtilis* (38) and considered not to impose a significant degree of osmotic stress (15), a considerable glycine betaine pool could be detected (Fig. 2A) when this medium was supplemented with 1

mM glycine betaine. The buildup of the glycine betaine pool in cells grown in SMM in the absence of additional NaCl is probably largely dependent on the transport activity of the OpuA system, since its transport capacity ( $V_{\max}$ , about  $110 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ ) well exceeds those of the OpuC and OpuD glycine betaine importers, which exhibit  $V_{\max}$  values of about  $41 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$  and  $16 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ , respectively, under these growth conditions (27). All three glycine betaine transporters possess similar  $K_m$  values in the low micromolar range (27).

Brill et al. (9) have recently reported that the proline pool of osmotically stressed *B. subtilis* cells increases linearly in response to the degree of the osmotic stress imposed once the salinity of the growth medium, SMM, exceeds 0.2 M NaCl. We reconfirmed this pattern of proline accumulation and now provide quantitative measurements of the sizes of the cellular proline pools attained in osmotically stressed *B. subtilis* cells (Fig. 2B). The content of free proline rose from 8.5 mM in cells cultivated in SMM to about 373 mM in cells grown in SMM containing 1 M NaCl (Fig. 2B). Strikingly, when 1 mM glycine betaine was present in the growth medium, the buildup of an osmoadaptive proline pool was completely prevented. These cells contained a proline pool of just 8 mM, despite the fact that they were subjected to sustained osmotic stress by growth in SMM with 1 M NaCl (Fig. 2B). Consequently, it is clear that *B. subtilis* cells prefer to take up externally provided glycine betaine rather than engage in the *de novo* synthesis of the compatible solute proline to attain relief from osmotic stress.

In natural ecosystems, *B. subtilis* is unlikely to be exposed frequently to the glycine betaine concentration (1 mM) that we have used in our growth assays. Not only does the provision of this compatible solute through root exudates, decaying plant material, and osmotically downshocked microbial cells yield differing supplies of glycine betaine in natural settings, but also the concentrations typically should not exceed micromolar values (50–53). As a consequence of such low concentrations of glycine betaine in the environment, an osmoprotective pool made up of this compatible solute alone cannot be built up in fast-growing cells. We derive this conclusion from a calculation of the potential glycine betaine content of *B. subtilis* cells grown to an  $\text{OD}_{578}$  of 1.5 (corresponding to a cytoplasmic volume of  $0.975 \mu\text{l}$  per 1 ml culture) that suggests that in the presence of 25  $\mu\text{M}$ , 75  $\mu\text{M}$ , or 600  $\mu\text{M}$  glycine betaine, an intracellular pool size of 26 mM, 77 mM, or 615 mM, respectively, can be generated. It is assumed for this calculation that the externally provided glycine betaine is completely taken up by the cells. We realize that in natural settings (e.g., *B. subtilis* cells growing in the soil next to root hairs exuding glycine betaine or proline), the situation might be much more complex, since the cells do not grow fast and their population density is much lower than under laboratory conditions. Nevertheless, the estimate above presented suggests that the *B. subtilis* cell facing limiting concentrations of osmoprotectants in its environment builds up an intracellular compatible-solute pool consisting of a mixture of imported osmoprotectants and newly synthesized proline.

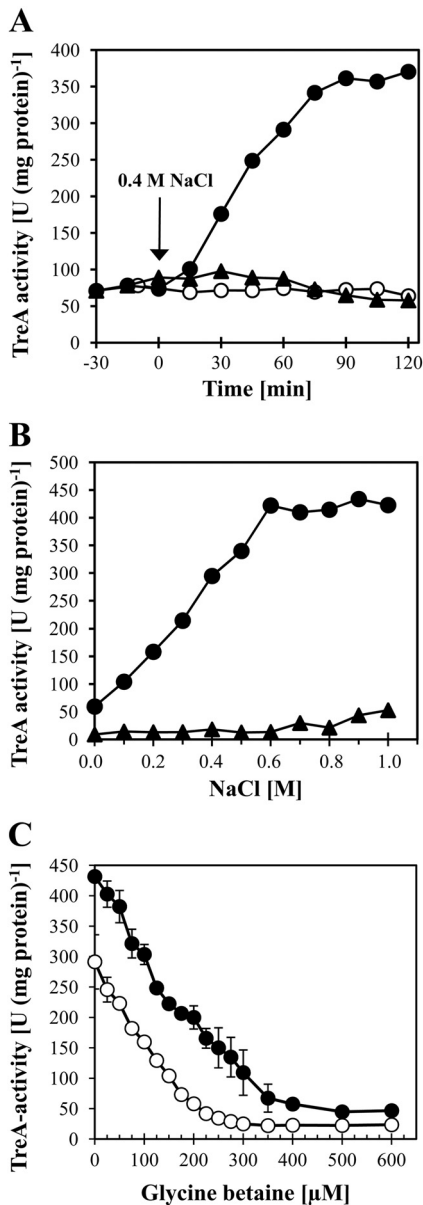
We therefore assessed the effects of various concentrations of glycine betaine on the size of the intracellular proline pool formed via *de novo* synthesis in osmotically stressed *B. subtilis* cells (grown in SMM with 1 M NaCl). For this experiment, we titrated the glycine betaine concentration present in the growth medium from 0  $\mu\text{M}$  to 1,000  $\mu\text{M}$ . Our results revealed a finely tuned influence of exogenously provided glycine betaine on the intracellular proline pool (Fig. 2C). Even very low (25  $\mu\text{M}$ ) glycine betaine concentra-

tions notably reduced the proline content of the cells, and the presence of this compatible solute at 75  $\mu\text{M}$  was sufficient to reduce the proline pool by about 50%. A supply of 600  $\mu\text{M}$  glycine betaine reduced the proline content to a basal level of 21 mM (Fig. 2C). Within the framework of the calculations presented above, an external supply of 600  $\mu\text{M}$  glycine betaine would theoretically be sufficient to attain a pool size of 615 mM. In agreement with these calculations, we actually measured a glycine betaine pool of about 570 mM in cells challenged with 1 M NaCl and grown in the presence of 1 mM glycine betaine (Fig. 2A).

**Induction of *opuA* expression in response to an osmotic upshock and sustained high salinity.** The OpuA uptake system is the dominating transporter for glycine betaine in *B. subtilis* (27). We therefore focus below on the regulation of the *opuA* operon in response to osmotic stress and the availability of compatible solutes. To provide a genetic tool for these studies, we constructed an *opuA-treA* operon fusion and ectopically inserted this reporter construct stably as a single copy into the *B. subtilis* chromosome at the nonessential *amyE* locus, yielding the *B. subtilis* strain MBB9. The *treA* gene encodes a highly salt tolerant phospho- $\alpha$ -(1,1)-glucosidase (46), and the TreA enzyme is therefore a reporter well suited for transcriptional studies involving high-salinity-stressed *B. subtilis* cells (9, 54, 55).

A previous study by Kempf and Bremer (26) established that enhanced expression of a plasmid-encoded *opuA-lacZ* translational fusion could be triggered in a high-salinity complex growth medium (LB medium with 0.5 M NaCl). To study the transcriptional response of *opuA* in response to increases in external salinity in greater detail and under better-controlled conditions, we grew the *opuA-treA* reporter strain MBB9 in a chemically defined medium (SMM) to early-log phase and then subjected it to a sudden osmotic upshift with 0.4 M NaCl. This increase in external salinity caused a rapid and strong transcriptional response of the *opuA-treA* fusion and increased the activity of the TreA reporter enzyme from a basal level of  $75 \text{ U (mg protein)}^{-1}$  to  $361 \text{ U (mg protein)}^{-1}$  within 90 min subsequent to the osmotic upshift (Fig. 3A). In addition to its ability to perceive sudden increases in external salinity, the *B. subtilis* cell was also able to react to incremental increases in external salinity when the *opuA-treA* reporter strain MBB9 was continuously grown in media containing different concentrations of NaCl. The strength of *opuA-treA* expression increased linearly as the external salinity increased up to 0.6 M NaCl and then leveled off at higher salinities (Fig. 3B). Osmotic induction of *opuA* expression was also evident when we assessed *opuA* transcription by Northern blot analysis (Fig. 4A). This experiment also proved that the *opuA* locus is transcribed as an operon, since the size of the *opuA* mRNA observed (about 3,000 nucleotides) (Fig. 4A) closely matches that calculated for the coding and intragenic regions (2,985 bp) of the *opuAA-opuAB-opuAC* gene cluster (26).

**Salt-induced *opuA* expression is not controlled by SigB, the DegS-DegU two-component regulatory system, or the ArsR-type regulator YceK.** Salt stress (56, 57) is one of the strongest inducers of the SigB-controlled general stress regulon of *B. subtilis* (58, 59); however, upregulation of the transcription of its members in response to salt stress is only transient (54, 57, 60). As expected from the data shown in Fig. 3B, which record high levels of *opuA-treA* expression under sustained salt stress, salt stress-mediated induction of *opuA-treA* expression was not prevented in a *sigB* mutant background (Table 2).



**FIG 3** Osmotic control of *opuA* expression and its response to glycine betaine availability. (A) A culture of the *opuA-treA* fusion strain MBB9 was grown in SMM to the early-exponential-growth phase ( $OD_{578}$ , approximately 0.5) and was divided into three portions. Two portions were subjected to an osmotic upshift with 0.4 M NaCl either in the absence (●) or in the presence (▲) of 1 mM glycine betaine; the third portion was not subjected to an osmotic upshift and did not receive glycine betaine (○). Aliquots of the cultures were withdrawn at the indicated times and were assayed for TreA reporter enzyme activity. This experiment was conducted in three biological replicates, and the data shown represent a typical data set. (B) The *opuA-treA* fusion strain MBB9 was grown in SMM with various salinities in the absence (●) or presence (▲) of 1 mM glycine betaine. Cells that had reached the same optical density ( $OD_{578}$ , 0.6 to 0.9) were assayed for TreA reporter enzyme activity. The data shown represent two independently grown cultures with two replicates of the TreA assays each. (C) The *opuA-treA* fusion strain MBB9 was grown in SMM with either 0.4 M NaCl (○) or 0.8 M NaCl (●) and the indicated concentrations of glycine betaine. Cells that had reached the same optical density ( $OD_{578}$ , about 1.5) were assayed for TreA reporter enzyme activity. The data shown represent two independently grown cultures with two replicates of the TreA assays each.

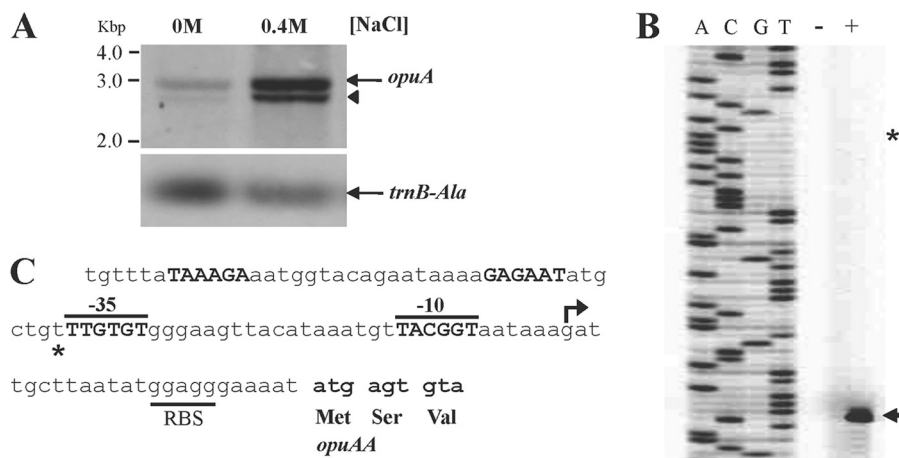
Several studies have implicated the two-component DegS-DegU regulatory system of *B. subtilis* in sensing salt stress (61, 62) and in developing osmotic stress resistance (63). There is also considerable overlap between high-salinity-induced *B. subtilis* genes and members of the DegS-DegU regulon (64). We therefore wondered whether the salt-stress-mediated expression of *opuA* was under the control of this two-component regulatory system and tested the influence of a *degSU* gene disruption on *opuA-treA* expression. None was observed (Table 2).

The *yceK* locus is transcribed divergently from the *opuA* operon and encodes a presumptive regulatory protein belonging to the ArsR family; it contains a helix-turn-helix DNA-binding motif (40, 41). Prompted by the vicinity of the *yceK* and *opuA* loci, we tested for a possible role of the predicted YceK transcriptional regulator in the osmotic control of *opuA* expression. None was observed (Table 2).

**The level of *opuA* induction differs in response to ionic and nonionic solutes.** To determine whether the observed increase in *opuA* expression (Fig. 3A and B) depends exclusively on high concentrations of NaCl or other salts, or whether it reflects a true osmotic response, we grew the *opuA-treA* reporter fusion strain MBB9 in a minimal medium (SMM) in the absence or presence of isoosmotic concentrations of both ionic (NaCl, KCl) and nonionic (lactose, sucrose) osmolytes. As documented in Table 3, an increased level of *opuA* transcription results from an increase in external osmolarity and therefore does not reflect a salt-specific response. The signal(s) that is perceived by the *B. subtilis* cell and induces *opuA* expression requires the establishment of an osmotically effective gradient across the cytoplasmic membrane. This conclusion can be inferred from an experiment where glycerol was used to increase the osmolarity of the growth medium. Glycerol, a compound that can permeate membranes at high concentrations and thus fails to impose osmotic stress on microbial cells, did not trigger a significantly enhanced level of *opuA* transcription (Table 3).

However, inspection of the data summarized in Table 3 revealed that the degree of *opuA* induction was much more pronounced when sugars were used to increase the osmolarity of the growth medium. The addition of lactose or sucrose increased the level of *opuA* expression about 17-fold, whereas NaCl and KCl enhanced *opuA* expression only about 5-fold. Dissimilar effects of isoosmotic ionic and nonionic osmolytes on potassium pools and osmotically regulated gene expression in *E. coli* (65, 66) and *Lactobacillus plantarum* have been reported before (67). In addition, studies on the osmotic induction of the *proHJ* promoter of *B. subtilis* revealed a considerably stronger effect of nonionic osmolytes than of ionic osmolytes on the transcription of this operon as well (9). Nevertheless, the differences in the degree of *opuA-treA* induction in response to isoosmotic concentrations of salts and sugars are unusual; we therefore sought independent confirmation for our observation. To this end, we measured the glycine betaine pools in strain RMKB20, a mutant with defective OpuC and OpuD glycine betaine transporters (Fig. 1) but with an intact OpuA system (27), in response to different types of osmolytes.

Since OpuA is the only functional glycine betaine transporter operating in strain RMKB20, the glycine betaine pools attained reflect only the steady-state transport activity of the OpuA system. In agreement with the transcriptional data on *opuA-treA* expression reported above (Table 3), the sugars lactose and sucrose triggered the buildup of glycine betaine pools substantially larger than



**FIG 4** Osmoregulated transcription of the *opuA* operon occurs from a SigA-type promoter. (A) Northern blot analysis of the *opuAA-opuAB-opuAC* (26) in cells grown either in SMM alone or in SMM containing 0.4 M NaCl. *opuA* mRNA was detected by hybridization using a digoxigenin-labeled *opuAA* antisense RNA probe. The full-length *opuA* mRNA is indicated by an arrow; the shorter *opuA*-specific hybridization signal (indicated by an arrowhead) is believed to represent either a degradation or a premature termination product of the *opuAA-opuAB-opuAC* transcript. As a control for the total RNA quantities blotted onto the membrane in this experiment, an antisense RNA probe against the transcript of a constitutively expressed tRNA gene (*trnB-Ala*) was used. (B) Primer extension analysis of the *opuA* transcript in cells of strain JH642 (pMBB39 *opuAA'*) grown either in SMM alone (–) or in SMM with 0.4 M NaCl (+) to the mid-exponential-growth phase (OD<sub>578</sub>, approximately 1). The arrow indicates the position of the 5' end of the *opuA* mRNA; the position where the second *opuA* mRNA species had been identified previously (26) is marked by a star. (C) DNA sequence of the *opuA* promoter region. The mapped transcriptional start site for the osmoregulated *opuA* mRNA is marked by an arrow, and the corresponding –35 and –10 regions of the osmotically controlled *opuA* promoter are indicated (marked in boldface letters and overlined). The position of the promoter that was previously thought to direct a constitutively produced second *opuA* mRNA species (26) is shown in boldface letters, and the corresponding previously suggested transcriptional start site is indicated by a star. The ribosome binding site (RBS) and the beginning of the *opuAA* coding region are indicated.

those elicited by the addition of the salts NaCl and KCl to the growth medium (Table 3). Consequently, there can be no doubt that isosmotic concentrations of ionic and nonionic compounds exert different effects on OpuA-mediated glycine betaine import by eliciting transcriptional responses of different magnitudes from the *opuA* operon (Table 3). The distinct effects of ionic and nonionic osmolytes on the OpuA-mediated buildup of glycine betaine pools are smaller than those found for the corresponding transcriptional activity of the *opuA-treA* reporter fusion (Table 3). Our data do not address the possibility that sugars and salts might also have disparate effects on the transport activity of the OpuA system in cells challenged by high osmolarity on a long-term basis.

**The compatible solute glycine betaine exerts a major influence on *opuA* expression.** Previous studies with different microorganisms have shown that in addition to functioning as an osmoprotectant, glycine betaine can also influence gene expression. Genes that are involved in the synthesis or uptake of various types of compatible solutes are particularly strongly affected, and their

transcriptional activity is downregulated under osmotic stress conditions (for examples, see references 9, 35, 54, and 68 to 72). To test whether glycine betaine would also influence *opuA* expression, we included 1 mM glycine betaine in the growth medium of the *opuA-treA* reporter strain MBB9, in which all three glycine betaine transport systems (OpuA, OpuC, and OpuD) operating in *B. subtilis* are intact (Fig. 1). The presence of this concentration of glycine betaine strongly downregulated the osmotic induction of the reporter *opuA-treA* fusion in response to either ionic or nonionic osmolytes (Table 3). Likewise, the presence of 1 mM glycine betaine fully prevented the induction of *opuA-treA* expression subsequent to a sudden osmotic upshift with 0.4 M NaCl (Fig. 3A) and under sustained high-salinity growth conditions (Fig. 3B). Hence, the presence of glycine betaine dramatically influences the pattern of *opuA* expression under osmotic stress conditions.

To further evaluate the influence of glycine betaine on *opuA* expression, we titrated its concentration in media containing either 0.4 M or 0.8 M NaCl. Even the addition of very small concentrations of glycine betaine (e.g., 25  $\mu$ M) to the growth medium had measurable effects on the transcriptional activity of the *opuA-treA* reporter fusion (Fig. 3C). Incremental increases in the glycine betaine content of the growth medium successively decreased *opuA-treA* expression down to a basal level (Fig. 3C). The amount of glycine betaine required to fully shut down *opuA* transcription was dependent on the salinity of the growth medium. Approximately 300  $\mu$ M glycine betaine was required to prevent osmotic induction of *opuA-treA* expression in cells grown in the presence of 0.4 M NaCl, whereas about 500  $\mu$ M glycine betaine was required to accomplish this in cells grown in the presence of 0.8 M NaCl (Fig. 3C).

**TABLE 2** Salt induction of *opuA* transcription is independent of the alternative transcription factor SigB, the two-component DegSU system, and the ArsR-type YceK regulator

Strain <sup>a</sup>	TreA activity (U [mg protein] <sup>-1</sup> )	
	Without NaCl	With NaCl
MBB9 (wild type)	75 $\pm$ 4	431 $\pm$ 37
MBB16 $\{\Delta([\textit{degS-degU}::\textit{aphA3}]1)\}$	81 $\pm$ 1	373 $\pm$ 3
MBB19 $[\Delta(\textit{sigB2}::\textit{cat})]$	66 $\pm$ 2	338 $\pm$ 2
TMB150 $[\Delta(\textit{yceK}::\textit{spc})1]$	69 $\pm$ 5	460 $\pm$ 39

<sup>a</sup> Cells of the various *opuA-treA* fusion strains were grown to the mid-exponential-growth phase (OD<sub>578</sub>, approximately 1) in SMM in the absence or presence of 0.4 M NaCl and were assayed for TreA reporter enzyme activity.



TABLE 3 Osmotic induction of *opuA* transcription

Medium	Osmolarity (mosmol kg <sup>-1</sup> )	TreA activity (U [mg protein] <sup>-1</sup> ) <sup>a</sup>		
		Without glycine betaine	With glycine betaine (1 mM)	Intracellular glycine betaine pool (mM) <sup>b</sup>
SMM	356	55 ± 6	12 ± 1	126 ± 1
SMM with 0.68 M glycerol	1,100	62 ± 10	20 ± 2	162 ± 33
SMM with 0.4 M NaCl	1,188	251 ± 17	25 ± 2	304 ± 78
SMM with 0.4 M KCl	1,178	246 ± 33	25 ± 3	299 ± 5
SMM with 0.61 M lactose	1,143	987 ± 73	68 ± 11	776 ± 67
SMM with 0.62 M sucrose	1,118	947 ± 87	63 ± 13	785 ± 89

<sup>a</sup> Cells of the *opuA-treA* fusion strain MBB9 were grown to the mid-exponential-growth phase (OD<sub>578</sub>, approximately 1) in SMM alone or SMM with increased osmolarities that were generated by the addition of the indicated ionic or nonionic osmolyte.

<sup>b</sup> Cultures of strain RMKB30 (OpuA<sup>+</sup> OpuC<sup>-</sup> OpuD<sup>-</sup>) were grown to the mid-exponential-growth phase (OD<sub>578</sub>, approximately 1) in SMM alone or SMM with increased osmolarities in the presence of 1 mM glycine betaine spiked with 0.64 μM radiolabeled [1-<sup>14</sup>C]glycine betaine.

### Role of the uptake and intracellular accumulation of glycine betaine in controlling *opuA* expression.

Since glycine betaine apparently exerts a strong influence on *opuA* expression, we investigated this regulatory phenomenon in greater detail. We asked the following four questions. (i) Does the regulatory influence of glycine betaine on *opuA* expression require its uptake? (ii) Is uptake of glycine betaine via the OpuA system a prerequisite for the reduction of *opuA* expression, or can this effect also be exerted when glycine betaine is imported by a different Opu transport system? (iii) Can glycine betaine synthesis from the precursor choline exert regulatory effects on *opuA* gene expression as well? (iv) Is the repressing effect on *opuA* gene expression specifically caused by glycine betaine, or can it also be conferred by other compatible solutes that function as effective osmoprotectants for *B. subtilis*?

To test whether the uptake of glycine betaine by osmotically stressed cells is required for the reduction in *opuA* expression, we used strain MBB14, which is defective in the OpuA, OpuC, and OpuD glycine betaine transporters (Fig. 1). In this strain, glycine betaine is no longer able to repress *opuA-treA* expression (Table 4), demonstrating that glycine betaine needs to be taken up by the cell in order to exert its repressive effect on *opuA* transcription.

Strain MBB21 is defective in the OpuA and OpuD glycine betaine transport systems but allows glycine betaine uptake via the OpuC transporter (Fig. 1). The addition of glycine betaine to high-salinity-grown cells of MBB21 reduced *opuA-treA* expres-

sion to a degree similar to that observed for strain MBB9, which can acquire glycine betaine through the OpuA, OpuC, and OpuD transporters (Table 4). Consequently, the repressing effect of glycine betaine on *opuA* expression is independent of its uptake via the OpuA transporter.

Choline, the biosynthetic precursor for glycine betaine synthesis by *B. subtilis* (Fig. 1) (24, 25), does not function as a compatible solute by itself; its osmoprotective effects rely entirely on its enzymatic conversion to glycine betaine (24). The addition of choline to the growth medium repressed *opuA-treA* expression to an extent similar to that observed for the addition of preformed glycine betaine (Table 4). Hence, glycine betaine does not need to be imported in order to influence *opuA* transcription; it can also exert its effect on *opuA* transcription when it is newly produced.

Repression of *opuA-treA* expression was also caused by the addition to the growth medium of carnitine (Table 4), a highly effective osmoprotectant for *B. subtilis* that is taken up exclusively via the OpuC transport system (Fig. 1) (73). It should be noted in this context that carnitine is not metabolized by *B. subtilis* to glycine betaine (73). As observed for glycine betaine, carnitine needs to enter the *B. subtilis* cell in order to repress *opuA-treA* expression, as evidenced by the fact that in the *opuC* mutant strain MBB14, carnitine was no longer able to reduce *opuA* transcription.

Taken together, these data show that the repressing effect of glycine betaine on *opuA-treA* expression was independent of the way (transport or synthesis) it was amassed by the osmotically stressed *B. subtilis* cell, that this regulatory effect was independent of the functioning of the OpuA uptake system, and that a reduction in *opuA* expression can also be accomplished by other compatible solutes used by *B. subtilis* as osmoprotectants.

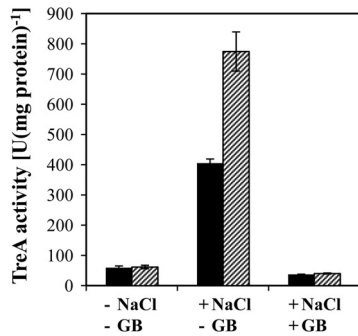
**The strength of *opuA* expression is modulated by the proline pool of osmotically stressed cells.** Since the intracellular accumulation of the compatible solutes glycine betaine and carnitine downregulated *opuA* expression at high salinity (Table 4), we wondered if the production of proline, the only compatible solute that *B. subtilis* can synthesize *de novo* under osmotic stress conditions (9, 16), would affect the level of *opuA* transcription as well. To test this notion, we introduced the *opuA-treA* fusion into a *B. subtilis* strain that is defective in the ProJ-ProA-ProH-mediated osmoadaptive proline biosynthesis route (Fig. 1) due to the presence of the  $\Delta(\text{proHJ}::\text{tet})1$  mutation (9). We then compared the expression level of the reporter gene fusion present in the  $\Delta(\text{proHJ}::\text{tet})1$  genetic background with that present in an iso-

TABLE 4 Repression of *opuA* transcription by compatible solutes

Compatible solute in growth medium <sup>a</sup>	TreA activity (U [mg protein] <sup>-1</sup> ) <sup>b</sup>					
	Without NaCl			With 0.4 M NaCl		
	MBB9 (wild type)	MBB14 ( <i>opuB</i> <sup>+</sup> )	MBB21 ( <i>opuC</i> <sup>+</sup> )	MBB9 (wild type)	MBB14 ( <i>opuB</i> <sup>+</sup> )	MBB21 ( <i>opuC</i> <sup>+</sup> )
None	49 ± 9	48 ± 10	49 ± 6	218 ± 25	205 ± 9	204 ± 24
Glycine betaine	12 ± 5	50 ± 12	16 ± 2	19 ± 3	210 ± 6	59 ± 4
Choline	23 ± 12	18 ± 3	27 ± 1	17 ± 5	38 ± 7	57 ± 8
Carnitine	15 ± 8	39 ± 4	11 ± 3	32 ± 4	203 ± 17	28 ± 2

<sup>a</sup> Cells of the various *opuA-treA* fusion strains were grown to the mid-exponential-growth phase (OD<sub>578</sub>, approximately 1) in SMM alone or in SMM containing 0.4 M NaCl. The presence in the growth medium of the compatible solutes glycine betaine and carnitine and the precursor for glycine betaine synthesis (choline) is indicated. The final concentration of the various osmoprotectants was 1 mM.

<sup>b</sup> The presence of osmoprotectant uptake systems operating in the wild-type strain MBB9 (OpuA<sup>+</sup> OpuB<sup>+</sup> OpuC<sup>+</sup> OpuD<sup>+</sup>) and its mutant derivatives strain MBB14 (OpuA<sup>-</sup> OpuB<sup>+</sup> OpuC<sup>-</sup> OpuD<sup>-</sup>) and strain MBB21 (OpuA<sup>-</sup> OpuB<sup>-</sup> OpuC<sup>+</sup> OpuD<sup>-</sup>) is indicated.



**FIG 5** Osmoregulated expression of *opuA-treA* expression is responsive to the cellular proline pool. Cells of the *opuA-treA* reporter strains MBB9, carrying a genetically intact *proHJ* operon (filled bars), and TMB117, carrying a disruption [ $\Delta(\textit{proHJ}::\textit{tet})1$ ] of the *proHJ* locus (hatched bars), were grown in SMM alone or in SMM with 0.6 mM NaCl in the absence (–) or presence (+) (1 mM) of glycine betaine (GB). The TreA activities given are means from two independently grown cultures whose TreA activities were determined in duplicate.

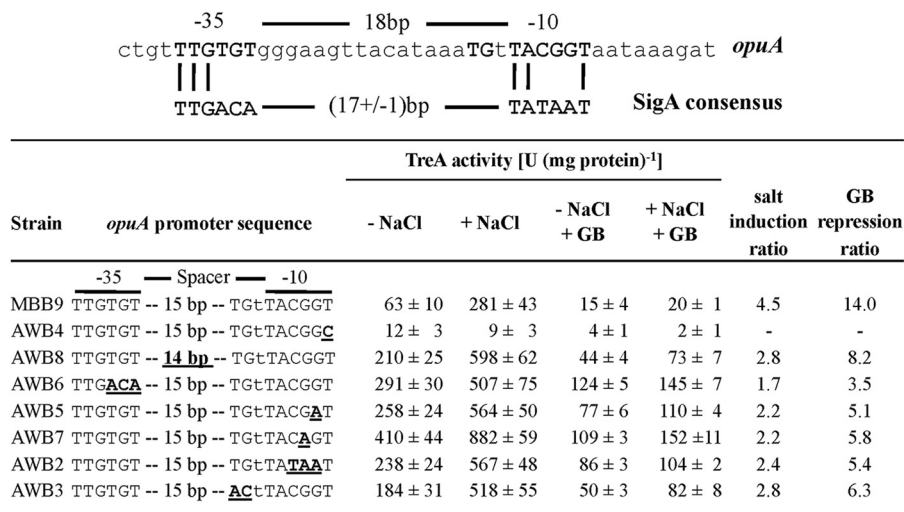
genic strain that is proficient in osmoadaptive proline production. The expression of the *opuA-treA* fusion was osmotically inducible in both strains, but the level of *opuA-treA* expression was considerably higher in the strain carrying the  $\Delta(\textit{proHJ}::\textit{tet})1$  mutation (Fig. 5). Thus, the activity of the *opuA* promoter is sensitive to the compatible-solute pool present in osmotically stressed *B. subtilis* cells. Osmotic induction of *opuA-treA* expression was fully repressible by the addition of 1 mM glycine betaine to the growth medium, regardless of whether the reporter strain was able to synthesize osmoadaptive levels of proline or not (Fig. 5).

***opuA* transcription depends on a single, osmotically inducible promoter.** The transcriptional landscape of the *opuA* regulatory region has been analyzed previously by Kempf and Bremer (26) via primer extension analysis, and two *opuA* mRNA species with 5' ends spaced 38 bp apart were found (Fig. 4C). The longer

transcript was constitutively produced at high levels in cells cultivated in LB medium alone and in LB medium containing 0.5 M NaCl, whereas the amount of the shorter transcript was strongly inducible when increased amounts of NaCl were present in the growth medium. We reevaluated the transcriptional start site(s) of the *opuA* operon by primer extension analysis with total RNA isolated from cells of strain JH642(pMBB39) grown in a chemically defined medium (either in SMM alone or in SMM with 0.4 M NaCl) to early-log phase. In contrast to the previously reported primer extension analysis data (26), our new primer extension experiment shows that there is only a single *opuA* promoter and that the activity of this promoter is responsive to increases in the salinity of the growth medium (Fig. 4B). This conclusion is fully consistent with data from the recently reported genomewide transcriptional profiling study of Nicolas et al. (74) on the condition-dependent transcriptome of *B. subtilis*, where only a single *opuA* mRNA start site was detected under a large number of growth conditions (74).

We assessed the functionality and validity of the proposed osmostress-responsive *opuA* promoter (26) by site-directed mutagenesis. The first T · A base pair in the –10 regions of SigA-type promoters (Fig. 6) is highly conserved and is typically critical for promoter activity (75). We changed this A · T base pair in the *opuA* promoter into a C · G base pair via site-directed mutagenesis and found that this point mutation abolished osmotic induction of *opuA* expression and allowed only a very basal level of promoter activity (Fig. 6). The properties of this mutant *opuA* promoter are therefore fully consistent with our finding in the new primer extension experiment that there is only a single promoter driving *opuA* transcription (Fig. 4B and C). We do not have any satisfying explanation for the differences between the previous primer extension data (26) and our current primer extension data (Fig. 4B).

**Determinants of the osmotically controlled *opuA* promoter.** The *opuA* promoter deviates in both its –35 and –10 sequences from the consensus sequence of SigA-type promoters, and it also



**FIG 6** Mutational analysis of the osmotically controlled *opuA* promoter. (Top) Comparison of the DNA sequence of the *opuA* promoter to the consensus sequence of the –10 and –35 regions of SigA-type promoters (75, 83). (Bottom) Transcriptional data of *opuA-treA* fusion strains with the indicated promoter mutations. Cells were grown either in SMM alone (– NaCl) or in SMM with 0.4 M NaCl (+ NaCl) and in the presence (1 mM) or absence of glycine betaine (GB). The salt induction ratio compares the values of TreA activities in cells grown in the presence versus the absence of NaCl. The GB repression ratio compares TreA activities in cells grown at high salinity in the presence versus the absence of GB. The TreA activities given are means from two independently grown cultures whose TreA activities were determined in duplicate.

possesses a spacer length of 18 bp (Fig. 4C and 6), which is suboptimal spacing for this group of promoters (75). Since only a few osmotically controlled SigA-type promoters from *B. subtilis* have been analyzed in some detail (9, 54, 55), and since osmoregulation of gene expression might proceed without a classical regulatory protein and might instead depend on solute-mediated interactions between the RNA polymerase and a given promoter (76–79), we studied the contributions of key elements of the *opuA* promoter to osmotic control and functionality via site-directed mutagenesis.

First, we shortened the length of the spacer segment separating the –10 and –35 regions from 18 to 17 nucleotides, a configuration most frequently found in SigA-type promoters (75). In this promoter mutant, both the basal and osmotically induced levels of *opuA* expression increased considerably, but with a concomitant reduction in the degree of osmotic induction of transcription (Fig. 6). The –10 regions of SigA-type promoters from *B. subtilis* are typically very AT rich (75), but the corresponding region of the *opuA* promoter possesses an unusual enrichment of G · C base pairs (Fig. 6). The *B. subtilis opuA* promoter shares this feature with other osmotically controlled promoters from *B. subtilis*, *E. coli*, and *Salmonella enterica* serovar Typhimurium (54, 55, 78, 80–82). Mutations that improved the match of the *opuA* –10 region, or of the –35 region, with the consensus sequence of SigA-type promoters substantially increased both the basal and osmotically induced levels of *opuA* expression. Each of these *opuA* promoter variants still permitted osmotic control of gene expression to some extent (Fig. 6).

A TG motif at position –16 is frequently found in *B. subtilis* SigA-type promoters (75), and mutations affecting this motif typically strongly decrease promoter activity (83). In contrast to expectations, the change of the TG motif present in the wild-type *opuA* promoter (Fig. 6) to an AC variant did not inactivate the promoter. Instead, this promoter mutant displayed increased promoter activity both at low salinity and at high salinity, with some remaining degree of osmotic induction (Fig. 6).

Since the accumulation of glycine betaine strongly affects the strength of *opuA* expression (Fig. 2C and 3C), we also tested the susceptibility of all newly constructed *opuA* promoter variants to this repressing effect. Each *opuA* promoter variant retained control by glycine betaine to some extent (Fig. 6).

## DISCUSSION

The nearly linear relationship between the salinity of the environment and the pools of proline (formed by *de novo* synthesis) and glycine betaine (formed by import) implies that the *B. subtilis* cell can perceive incremental increases in external salinity. This allows it to modulate the intracellular levels of these two compatible solutes over a broad range of external salinities and to keep them within rather confined boundaries at a given salinity. *B. subtilis* achieves this through coordinated adjustments in the transcriptional activity of the *proHJ* proline biosynthetic genes (9) and of the loci (*opuA*, *opuC*, and *opuD*) encoding glycine betaine uptake systems (23, 26, 27), and probably also through posttranscriptional control mechanisms (32, 34) regulating the activity of these transporters. In natural ecosystems, *B. subtilis* is exposed to different types and mixtures of compatible solutes (50). The *B. subtilis* cell can take advantage of a wide spectrum of osmoprotectants via their import through the Opu transport systems (12, 20). The data reported here for glycine betaine show that the *B. subtilis* cell is

able to physiologically integrate compatible-solute import with its osmotic-stress-adaptive proline biosynthetic activities (9, 16).

The molecular mechanism(s) that allows *B. subtilis* to detect osmotic changes in its environment and then set the level of gene expression on a genomewide scale (56, 64) is rather poorly understood. Also unclear are the genetic processes that control *opuA* transcription in response to increases in external osmolarity elicited by either ionic or nonionic compounds. Our data reveal a major impact on *opuA* expression of the intracellular solute pools formed through the synthesis of proline and glycine betaine (formed by the oxidation of choline) (Fig. 1) and through the uptake of preformed osmoprotectants. By linking *opuA* transcription to the intracellular pool sizes of glycine betaine and proline, *B. subtilis* prevents uncontrolled and wasteful overaccumulation of glycine betaine, and probably also of several other compatible solutes (31), via the OpuA transport system.

A process of release and recapture of compatible solutes by continuously osmotically stressed *B. subtilis* cells, which we have recently discovered for newly produced proline (84), also exists for glycine betaine synthesized from the precursor choline (S. Moses and E. Bremer, unpublished data). This activity will aid the cell in finely tuning the glycine betaine pools at a given salinity. Nothing is currently known about the nature of the observed release of glycine betaine from osmotically stressed *B. subtilis* cells and its influence on the steady-state pool of this compatible solute, but the existence of specific betaine efflux systems has been suggested from experiments conducted with *S. enterica* serovar Typhimurium (85) and *Lactobacillus plantarum* (86). Release of glycine betaine by a MscS-type channel has been observed in *Corynebacterium glutamicum* (87), and such channels also exist in *B. subtilis* (88, 89). However, any role of MscL- or MscS-type channels in the release of proline from continuously osmotically challenged *B. subtilis* cells has been ruled out (84).

The size of the intracellular proline pool of *B. subtilis* is dependent not only on external salinity but also on the presence of the osmoprotectant glycine betaine in the growth medium. Optimal use of energetic resources available to salt-stressed cells might underlie this phenomenon (90), but the preference of glycine betaine accumulation via import over *de novo* synthesis of proline might also be rooted in the cells' aim to adjust and optimize cytoplasmic solvent properties by accumulating different kinds of compatible solutes (91). Hints that such processes might operate in microbial cells come from studies where salinity-dependent switching of the type of compatible solute synthesized is observed (11, 92, 93).

The amassing of compatible solutes impinges on the steady-state intracellular levels of potassium in osmotically stressed *B. subtilis* cells (13, 16). Given that potassium, and its counterion glutamate, is involved in the osmotic stress response of many bacterial species (1–4, 8, 76, 91), we consider it likely that these solute species also participate in setting the level of *opuA* transcription. Whether this is accomplished through yet unrecognized regulatory proteins (e.g., proteins such as the ion-responsive BusR repressor of *L. lactis* [35, 36]) or through solute-modulated interactions of the RNA polymerase (76, 77, 79) at the *B. subtilis opuA* promoter region remains to be determined. Mutational analysis of the *opuA* promoter revealed that its deviations from the consensus sequence of SigA-type *B. subtilis* promoters (75, 83) tune and optimize it both with respect to the osmotic inducibility of its transcriptional activity and with respect to its repressibility in response to intracellular compatible-solute pools.

The reduction in the level of osmotically induced transcription of genes involved in the synthesis or uptake of compatible solutes in response to the presence of these compounds in the growth medium is a well-known phenomenon (for examples, see references 9, 35, 54, and 68 to 72). In contrast to most of the previously reported studies, we not only evaluated the effects of substantial concentrations of glycine betaine (typically 1 mM) on gene expression but also used glycine betaine at concentrations (micromolar) that in all likelihood are more representative of the amounts of compatible solutes found in natural ecosystems (50–53). This experiment revealed a very finely tuned response of the level of *opuA* transcription to intracellular glycine betaine pools, which, to the best of our knowledge, has not been recorded before in any study addressing the influence of compatible solutes on gene expression.

We find it remarkable that the expression of *opuA* can be completely shut down by intracellular glycine betaine pools despite the fact that the cells remained exposed to high-salinity surroundings. This finding implies that the *B. subtilis* cell does not sense environmental osmolarity *per se* when it sets the level of *opuA* expression but reacts to the degree of osmotic stress it perceives. The accumulation of glycine betaine alleviates osmotic stress (15), and this either blinds the *B. subtilis* cell to the environmentally imposed osmotic signal or renders the cell unable to process this information into an output response leading to enhanced *opuA* expression. Mutants that will allow *opuA* expression in the presence of glycine betaine might thus pry open the door to a clearer picture of the inner workings of the osmotic sensory and response system(s) of *B. subtilis*.

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