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Glycine Betaine Catabolism Contributes to *Pseudomonas syringae* Tolerance to Hyperosmotic Stress by Relieving Betaine-Mediated Suppression of Compatible Solute Synthesis

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Many bacteria can accumulate glycine betaine for osmoprotection and catabolize it as a growth substrate, but how they regulate these opposing roles is poorly understood. In *Pseudomonas syringae* B728a, expression of the betaine catabolism genes was reduced by an osmotic upshift to an intermediate stress level, consistent with betaine accumulation, but was increased by an upshift to a high stress level, as confirmed by an accompanying increase in degradation of radiolabeled betaine. Deletion of the *gbcAB* betaine catabolism genes reduced osmotolerance at a high osmolarity, and this reduction was due to the relief of betaine-mediated suppression of compatible solute synthesis. This conclusion was supported by the findings that, at high osmolarity, the Δ*gbcAB* mutant accumulated high betaine levels and low endogenous solutes and exhibited reduced expression of the solute synthesis genes. Moreover, the Δ*gbcAB* mutant and a mutant deficient in the synthesis of the compatible solutes NAGGN and trehalose exhibited similar reductions in osmotolerance and also in fitness on bean leaves. Activation of betaine catabolism at high osmotic stress resulted, in part, from induction of *gbdR*, which encodes the transcriptional activator GbdR. Betaine catabolism was subject to partial repression by succinate under hyperosmotic stress conditions, in contrast to strong repression in the absence of stress, suggesting that betaine functions both in nutrition and as an intracellular signal modulating solute synthesis under hyperosmotic stress conditions. Collectively, these results begin to provide a detailed mechanistic understanding of how *P. syringae* transitions from reliance on exogenously derived betaine to the use of endogenous solutes during adaptation to hyperosmotic conditions.

Pseudomonas syringae pv. syringae is a foliar pathogen of common bean (*Phaseolus vulgaris* L.). It has been used as a model organism for understanding the cellular and molecular biology, ecology, and epidemiology of pathogen interactions with plants (1, 2). Due to the highly exposed nature of aerial plant leaves, *P. syringae* must confront rapid fluctuations in water availability during leaf colonization (3–5). One *P. syringae* strategy to tolerate limited water availability on leaves is to form aggregates; such aggregation has been associated with survival superior to that seen with solitary cells under dry conditions (5). Like other bacteria, *P. syringae* also accumulates soluble organic compounds, designated compatible solutes or osmolytes, to alleviate the deleterious effects of water limitation. *P. syringae* can synthesize several endogenous osmolytes, namely, the disaccharide trehalose, the dipeptide N-acetylglutaminylglutamine amide (NAGGN), and L-glutamate, in response to water limitation imposed by osmotic stress (6–8).

P. syringae can also benefit from the uptake of exogenous compounds for osmoprotection, with this uptake being energetically favored over *de novo* solute synthesis. The quaternary ammonium compound glycine betaine (here referred to as betaine) functions as a particularly effective protectant against osmotic stress in many organisms (9, 10). Although betaine is not synthesized *de novo* in *P. syringae* (7), it can accumulate following uptake of betaine or a betaine precursor (Fig. 1A). Betaine accumulation under conditions of high osmotic stress has been associated with a low accumulation of the endogenous osmolytes that are synthesized *de novo* (11–13). *P. syringae* has multiple transporters for the uptake of betaine and its precursors choline and phosphocholine (10, 14, 15), with a particularly high capacity for choline uptake (14). A primary source of these compounds is likely to be the major plant membrane lipid phosphatidylcholine, which may be degraded to choline and phosphocholine by plant and bacterial phospholipases (16, 17).

Whereas many microbes use betaine solely as a compatible solute, the pseudomonads *P. syringae* and *P. aeruginosa* can additionally use it for nutrition and energy under water-replete conditions (10, 18). *P. aeruginosa*, an animal pathogen, catabolizes it to glycine through the sequential action of enzymes encoded by *gbcAB*, *dgcAB*, and *soxBDAG*. The transcription of these genes requires the AraC family transcription activator GbdR with betaine or its downstream catabolite dimethylglycine as a coinducer (18, 19). Betaine catabolism appeared to be inhibited by hyperosmolarity in *P. aeruginosa* (12, 20), as well as in the plant symbiont *Sinorhizobium meliloti* (21), consistent with its accumulation as a compatible solute. This inhibition can be quite rapid, as illustrated by the suppression of betaine catabolism within only 10 min following an osmotic upshift of *S. meliloti* (21). Moreover, strong *P. aeruginosa* growth under conditions of high osmolarity required a large intracellular betaine pool, as indicated by the reduced growth rate when the pool was depleted by overexpressing *gbcAB* (20).

P. aeruginosa and *S. meliloti* have both been reported to catab-

Received 22 January 2013 Accepted 26 February 2013

Published ahead of print 22 March 2013

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00094-13.

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doi:10.1128/JB.00094-13

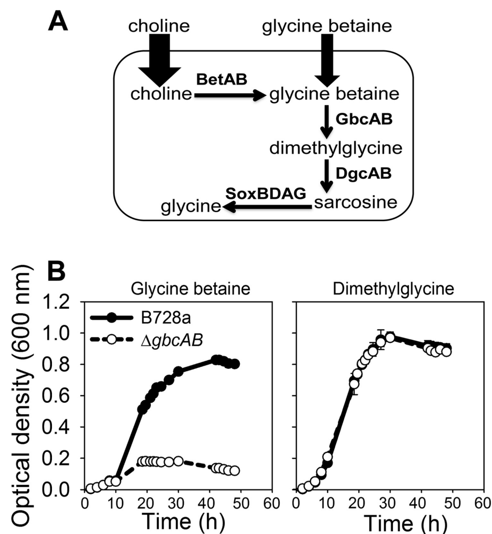

FIG 1 GbcAB is involved in the betaine catabolism pathway in *P. syringae* B728a. (A) Choline and betaine degradation pathway and the enzymes that are involved; B728a has a higher uptake capacity for choline than for betaine. (B) Growth of the Δ*gbcAB* mutant and B728a in MinA medium with 20 mM betaine or dimethylglycine based on optical density of cultures in test tubes. Values are means ± standard errors of the means (SEM) ($n = 3$).

olize betaine under hyperosmotic conditions, although the ramifications of this catabolism for betaine accumulation and osmotolerance were not explored. *P. aeruginosa* catabolism of betaine under hyperosmotic stress conditions is suppressed by succinate (11), suggesting that betaine catabolism is subject to catabolite repression and thus that betaine could be used as a nutrient source under these conditions. *S. meliloti* catabolism of betaine may be coordinated with endogenous osmolyte synthesis based on the preferential accumulation of betaine over endogenous osmolytes during early exponential growth but the transition to a predominance of endogenous osmolytes during late exponential growth and stationary phase (13).

In this work, we characterized the conditions under which *P. syringae* strain B728a catabolizes betaine in the presence of osmotic stress, the contribution of this catabolism to osmoadaptation, and the molecular mechanisms by which betaine catabolism influences B728a osmotolerance. Collectively, our data support a model in which maximal osmotolerance of *P. syringae* at high osmotic stress requires the upregulation of betaine catabolism to reduce the suppressive effect of betaine on endogenous compatible solute synthesis. Moreover, our data identify some of the molecular mechanisms underlying this upregulation, including the finding that suppression of compatible solute synthesis, activation of betaine catabolism, and production of a transcriptional activator of betaine catabolism are all regulated at the transcriptional level in response to hyperosmotic stress. Lastly, we show that betaine catabolism in B728a is subject to succinate-mediated repression at low osmotic stress, consistent with the use of betaine for nutrition, but is only partially repressed at high osmotic stress, consistent with a function for betaine as both an energy source and an intracellular signal modulating the accumulation of compatible solutes under hyperosmotic stress conditions.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. The *P. syringae* strains were grown on solid King's B medium (22) before inoculation into liquid MinA medium (23) which contained 10 mM pyruvate (MinA-pyruvate) or succinate (MinA-succinate) as a carbon source unless otherwise indicated. NaCl amendments were added to reach the stated final concentrations, and, unless otherwise indicated, betaine amendments were added to reach a final concentration of 1 mM. Cells were grown at 25°C with shaking. Antibiotics were added to the growth media as needed at the following concentrations ($\mu g\ ml^{-1}$): kanamycin (Km), 50; rifampin (Rif), 100; tetracycline (Tet), 20; spectinomycin (Spc), 60; and cycloheximide (Cm), 100. Cell growth was monitored either in test tubes based on the optical density at 600 nm (OD_{600}) or in microtiter plates based on measurements at both 630 nm and 450 nm to compensate for the optical interference of water condensation within the wells, with the OD_{630}/OD_{450} ratios subsequently converted to OD_{600} values using a standard curve.

Construction of mutant strains. Unmarked deletion mutants of B728a were generated as described previously (15). Briefly, two 1-kb fragments flanking the target locus were PCR amplified from B728a genomic DNA and a kanamycin (*kan*) cassette containing flanking FLP recombination target (FRT) sites was PCR amplified from pKD13 (24) using the primer pairs listed in Table S1 in the supplemental material. The three fragments were ligated together by splice-overlap-extension PCR using the original F1 and R2 primers to produce a single 3.5-kb fusion product consisting of the *kan* cassette and the flanking sequences. This product was cloned into SmaI-digested pTOK2T, which was then introduced into B728a cells by a triparental mating with pRK2013. The deletion mutants

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description or relevant genotype[a]	Reference or source
P. syringae strain		
B728a	Wild type; Rif[r]	36
B728a Δ*gbcAB*	B728a Δ*gbcAB*; Rif[r]	This work
B728a Δ*gbdR*	B728a Δ*gbdR*; Rif[r]	This work
B728a Δ*ggn*	B728a Δ*ggnAB*; Rif[r]	C. Chen and G. A. Beattie, unpublished data
B728a Δ*ggn* Δ*tre*	B728a Δ*ggnAB* ΔPsyr_2489-Psyr_2491 ΔPsyr_2992-Psyr_3001; Rif[r]	This work
Plasmid		
pTOK2T	pTOK2 with restored *lacZ* activity; Tet[r]	15
pKD13	Template for *kan* cassette flanked by FLP recombination target sites; Ap[r] Km[r]	24
pFlp2Ω	Encodes Flp recombinase, suicide vector in *P. syringae* derived from pFlp2 (37); Ap[r] Spc[r]	C. Chen and G. A. Beattie, unpublished data
pRK2013	RP4 transfer functions for mobilization; Km[r]	38

[a] Ap, ampicillin.

were identified as Rif^r Km^r Tet^s and confirmed by PCR. The *kan* cassette was excised by introducing pFlp2Ω, which was later cured using sucrose (20%) counterselection, and excision was confirmed by PCR and DNA sequencing. To construct the Δ*ggn* Δ*tre* mutant, the gene loci Psyr_2489 to Psyr_2491 and Psyr_2992 to Psyr_3001 were sequentially deleted in the Δ*ggn* mutant. The NAGGN deficiency of the Δ*ggn* mutant was confirmed by ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectroscopy (see Fig. S1A in the supplemental material). The trehalose deficiency of the Δ*ggn* Δ*tre* mutant was confirmed based on the lack of detectable trehalose using a trehalose assay kit (Megazyme, Ireland) (see Fig. S1B in the supplemental material).

Quantitative real-time PCR. Cells were grown in MinA medium containing pyruvate to an OD_{600} of 0.5, amended with betaine to 1 mM and NaCl to the indicated concentrations, and incubated with shaking in test tubes for the time indicated. Cells were diluted with RNAprotect Bacteria Reagent (Qiagen, Inc., Valencia, CA) before harvest, and RNA was purified using an RNeasy kit and on-column DNase I digestion to remove DNA (Qiagen, Inc., Valencia, CA). One-step reverse transcription (RT) conversion of RNA to cDNA and amplification was performed using a qScript 1-Step SYBR green quantitative RT-PCR (qRT-PCR) kit (Quanta BioSciences, Inc., Gaithersburg, MD). The PCR program was 50°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 30 s. The housekeeping gene *hemD* (Psyr_0061) was used as an internal control to normalize induction values for all genes. The fold change for each open reading frame (ORF) was calculated using $2^{(-\Delta\Delta CT)}$, where $\Delta\Delta CT = (CT$ [target] $- CT$ [*hemD*]) for the treatment − (CT [target] $- CT$ [*hemD*]) for the control, where *CT* is threshold cycle. The primers used are shown in Table S2 in the supplemental material. The specificity of the PCR amplification was examined based on the melting curve.

Determination of intracellular compatible solutes by ^{13}C-NMR spectroscopy. Cells were grown to the log phase in MinA medium containing pyruvate, and the reaction mixture was then amended with 0.6 M NaCl and 1 mM betaine and incubated for 5 and 30 h, and similar numbers of cells, as estimated based on optical density, were harvested from all cultures. The cells were washed twice in MinA medium containing 0.6 M NaCl and pyruvate and extracted in 80% ethanol. After centrifugation, the supernatant was evaporated to dryness in a Speed-Vac concentrator and dissolved in 0.8 ml D_2O. ^{13}C-NMR spectra were recorded with a Bruker Avance 700 MHz NMR spectrometer. DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) was added to each sample to adjust the chemical shift (indicated by peaks at 0 ppm).

Radiotracer assays. [*methyl*-^{14}C]glycine betaine was prepared enzymatically from [*methyl*-^{14}C] choline as previously described (10). Cells were grown in MinA medium with pyruvate or succinate to the log phase, amended with 0.6 M NaCl and 1 mM [*methyl*-^{14}C]glycine betaine, and grown in 250-ml sidearm flasks with shaking and with filter paper moistened with 5 M KOH in the side arm. Replicate flasks were prepared for sampling after 5 h and 30 h. At those times, the filter papers were removed and the $^{14}CO_2$ that was trapped was determined by measuring the radiolabel using a liquid scintillation counter as described in previous studies (11, 13). Measurements were expressed as counts per minute and were normalized to the OD_{600}.

Characterization of bacterial survival and growth on plants. Bacterial survival following inoculation onto plant surfaces was evaluated by growing bacteria on solid King's B medium, diluting a cell suspension to a density of 10^6 cells ml^{-1} in sterile H_2O containing 0.01% Silwet L-77 (Lehle Seeds, Round Rock, TX), and inoculating bean leaves (*Phaseolus vulgaris* cultivar Bush Blue Lake 274) by leaf immersion in the bacterial suspension. At various times after inoculation, the epiphytic bacterial populations were recovered from each of 4 to 8 leaves by gentle sonication for 7 min in phosphate buffer (10 mM, pH 7) containing 0.1% peptone and enumerated on King's B medium containing rifampin and cycloheximide.

Statistics. All *P* values reported are for comparisons between the B728a and a mutant using a two-tailed Student's *t* test or a Fisher's least significant difference test.

RESULTS

B728a exhibits differential responses to distinct levels of osmotic stress. The glycine betaine catabolism genes *gbcA* and *gbcB*, which encode products that catalyze the conversion of betaine to dimethylglycine in *P. aeruginosa* (18), were also required for the first step of betaine catabolism in *P. syringae* B728a, as evidenced by the poor growth of the Δ*gbcAB* mutant with betaine but not dimethylglycine (Fig. 1B). The expression levels of *gbcA* were distinct at different levels of NaCl in the presence of betaine (Fig. 2A): high expression at a low osmotic stress level (≤0.1 M NaCl), low expression at an intermediate stress level (0.2 to 0.4 M NaCl), and high expression at a high osmotic stress level (≥0.5 M NaCl). This osmoregulated expression was relevant to B728a growth at each osmotic stress level based on the greater differences in growth between the wild type and the Δ*gbcAB* mutant at the low and high osmotic stress levels than at the intermediate levels (Fig. 2B). Additional genes involved in the downstream steps of the betaine catabolic pathway, namely, *dgcA* and *soxA* (Fig. 1A), also showed higher expression at 0 and 0.6 M NaCl than at 0.3 M NaCl (Fig. 2C), implying that activation of betaine catabolism genes occurred primarily at low osmotic stress levels, potentially to promote cellular utilization of betaine for nutrition, and at high osmotic stress levels. The expression of these catabolic genes was low in the first 30 min of exposure to 0.6 M NaCl but was greatly increased by 30 h (Fig. 2D), suggesting that betaine catabolism is initially low after an upshift to a high osmotic stress level, which would allow betaine to accumulate, but that its catabolism eventually increases.

To directly evaluate if betaine is catabolized at high osmotic stress levels, we provided cells with 1 mM [*methyl*-^{14}C]glycine betaine and 0.6 M NaCl and measured the resulting $^{14}CO_2$ after 5 and 30 h. Whereas B728a produced a significant amount of $^{14}CO_2$ by 5 h and dramatically more after 30 h (Fig. 2E), consistent with the kinetics of the transcription of the betaine catabolic genes (Fig. 2D), the Δ*gbcAB* mutant released little $^{14}CO_2$, even after 30 h of incubation, as expected. This reduction in $^{14}CO_2$ was not due to a lower rate of betaine uptake (data not shown). Collectively, these data indicate that B728a catabolizes betaine following an upshift to high osmotic stress.

Betaine catabolism contributes to tolerance of high osmotic stress by relieving the betaine-mediated suppression of trehalose and NAGGN synthesis. When grown with betaine as an osmoprotectant, the Δ*gbcAB* mutant showed reduced growth in 0.6 M NaCl, as detected in the early log phase (Fig. 3). In contrast, the Δ*gbcAB* mutant grew like the wild type in 0.3 M NaCl but exhibited slightly reduced population densities in the stationary phase (Fig. 3). Thus, betaine catabolism contributed to osmotolerance, and this was most dramatic at high stress levels. Based on a previous report that decreased betaine accumulation in *S. meliloti* was associated with increased endogenous compatible solutes (13), we hypothesized that betaine catabolism is required to enable the accumulation of trehalose, NAGGN, and/or glutamate in *P. syringae.* To test this, we examined the composition of the organic osmolytes accumulated in osmotically stressed cells using natural-abundance ^{13}C-NMR spectroscopy following exposure for 5 and 30 h to 0.6 M NaCl in the presence of betaine. During the early

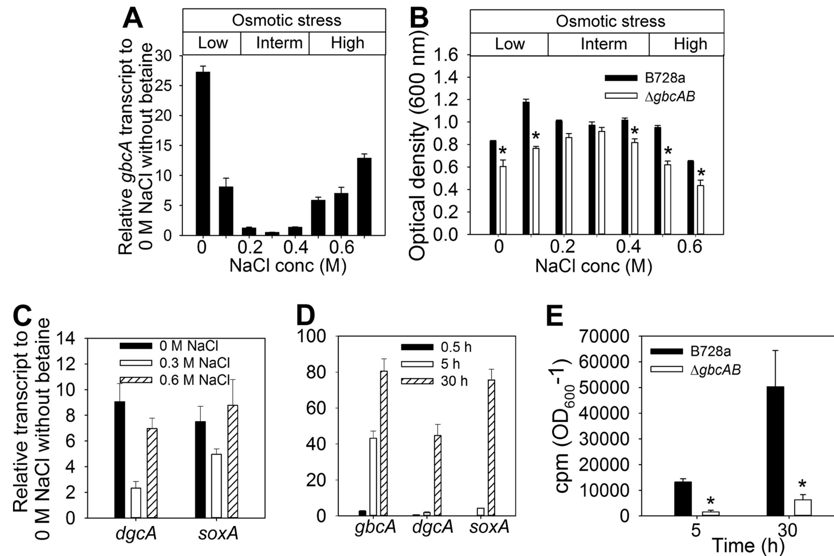

FIG 2 Effect of osmolarity on betaine catabolism. (A) qRT-PCR analysis of *gbcA* in B728a at 0.5 h after amending cells with 0 to 0.7 M NaCl and betaine. Values are expressed relative to the *gbcA* transcript level at 0 M NaCl without betaine. Interm, intermediate; conc, concentration. (B) Optical density of cultures of B728a and the Δ*gbcAB* mutant grown in microtiter plates in MinA-pyruvate containing 0 to 0.6 M NaCl and betaine for 45 h. (C) qRT-PCR analysis of *dgcA* and *soxA* in B728a at 30 min after amending cells with 0, 0.3, or 0.6 M NaCl and betaine. Values are expressed relative to transcript levels at 0 M NaCl without betaine. (D) qRT-PCR analysis of *gbcA*, *dgcA*, and *soxA* in B728a at 0.5, 5, and 30 h after amending cells with 0.6 M NaCl and betaine. (E) $^{14}CO_2$ released by B728a and the Δ*gbcAB* mutant during their growth in MinA-pyruvate with 0.6 M NaCl and [*methyl*-^{14}C]glycine betaine for 5 and 30 h. cpm, counts per minute. Asterisks indicate differences between B728a and the Δ*gbcAB* mutant at the NaCl concentration or time points indicated ($P < 0.05$). Values are means ± SEM ($n = 3$ for panels A to D; $n = 2$ for panel E).

growth stage (5 h), the B728a and Δ*gbcAB* strains showed similar compositions of osmolytes, with betaine dominating over the other solutes (Fig. 4A and B). After 30 h, when B728a had reached a higher cell density than the Δ*gbcAB* mutant, as demonstrated by an optical density at 600 nm of 2.3 for B728a and 1.5 for the Δ*gbcAB* mutant, the betaine levels in B728a had decreased whereas the trehalose, NAGGN, and glutamate levels had increased (Fig. 4C). These data suggest that B728a catabolized betaine and accumulated endogenous osmolytes over time after the osmotic upshift. In contrast, the Δ*gbcAB* mutant showed increased betaine levels at 30 h, with no detectable accumulation of the endogenous osmolytes (Fig. 4D). These data support a model in which the high level of betaine accumulated in the Δ*gbcAB* mutant inhibits the accumulation of the endogenous osmolytes.

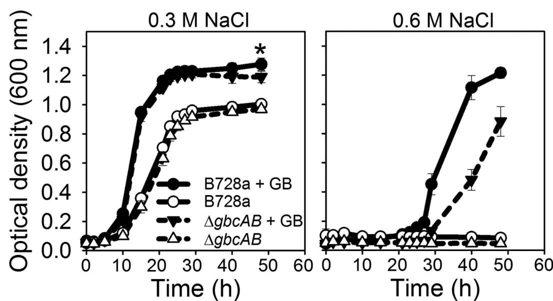

FIG 3 Contribution of betaine catabolism to osmotolerance. B728a and the Δ*gbcAB* mutant were grown in MinA-succinate with 0.3 M or 0.6 M NaCl and betaine, and growth in microtiter plates was monitored based on optical density. The asterisk indicates differences between B728a and the Δ*gbcAB* mutant at 0.3 M NaCl ($P < 0.05$). Values are means ± SEM ($n = 4$). GB, glycine betaine.

To further explore a possible inhibitory effect of betaine on *de novo* trehalose and NAGGN synthesis, we examined the transcription of *ggnA* and *treX*, which encode enzymes involved in NAGGN and trehalose synthesis, respectively (7). Their levels of transcription were similar in B728a and the Δ*gbcAB* mutant when cells were grown for up to 5 h at 0.6 M NaCl with or without betaine, but the level was much lower in the Δ*gbcAB* mutant than in B728a at 30 h in the presence of betaine (Fig. 5A and B). These data support an inhibitory role for accumulated, intracellular betaine in endogenous solute synthesis and indicate that this inhibition occurs, at least in part, at the transcriptional level.

Last, we compared the growth of the wild-type B728a strain to that of the Δ*ggn* Δ*tre* mutant, which is deficient in NAGGN and trehalose production (see Fig. S1 in the supplemental material), in 0.6 M NaCl amended with betaine. The Δ*ggn* Δ*tre* mutant showed a reduction in growth that was similar to that of the Δ*gbcAB* mutant (Fig. 5C), suggesting that although betaine can protect B728a from the deleterious effects of osmotic stress, it cannot fully compensate for the loss of endogenous osmolyte synthesis during adaptation to high osmolarity.

Betaine catabolism contributes to the fitness of B728a on bean leaves. In a previous study examining the transcriptome of B728a cells grown on bean leaves for 3 days under controlled conditions (25), the betaine catabolic genes were significantly induced in cells recovered from epiphytic and apoplastic, or intercellular, leaf sites (Table 2). The induction of *gbcA* was particularly strong. These results suggest that betaine catabolism might contribute to the survival of B728a under conditions of limited water availability in the phyllosphere, particularly on leaf surfaces (Table 2).

We evaluated the impact of the *gbcAB* deletion on B728a fitness on bean leaves based on enumeration of cells recovered from leaf

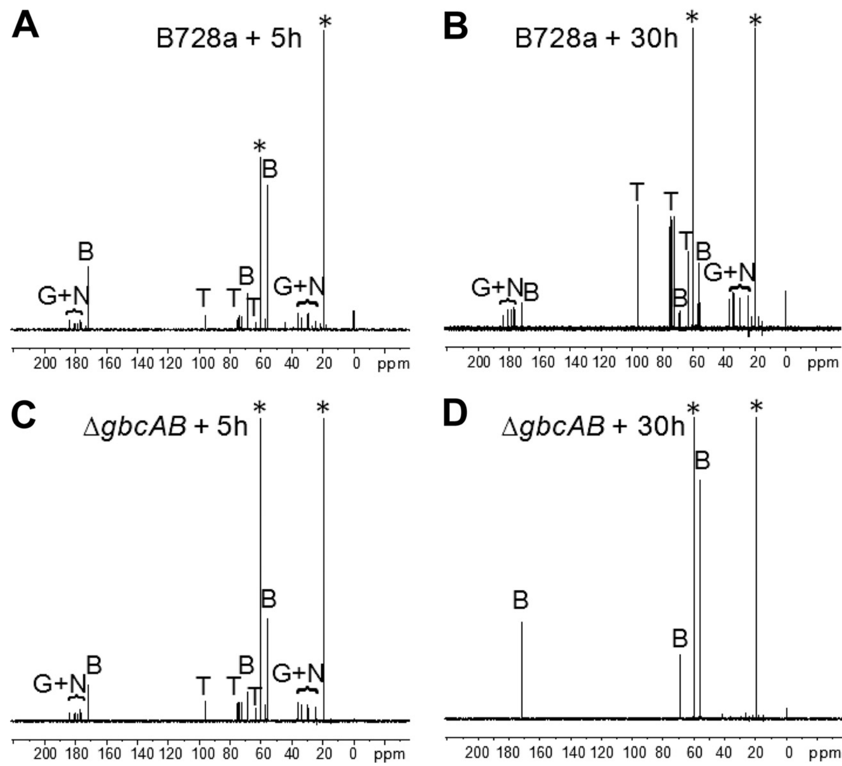

FIG 4 Effect of betaine catabolism on the composition of the accumulated compatible solutes of B728a (A and C) and the Δ*gbcAB* mutant (B and D) after 5 h (A and B) and 30 h (C and D) of growth. Cells were collected for ^{13}C-NMR at 5 and 30 h after cultures were amended with 0.6 M NaCl and betaine. Resonances (peaks) corresponding to betaine (B), L-glutamate (G), the dipeptide NAGGN (N), and trehalose (T) are indicated. Asterisks indicate peaks for residual ethanol from sample preparation.

surfaces by viable plate counts. The populations of the Δ*gbcAB* mutant were significantly smaller than those of B728a at 4 and 8 days postinoculation (Fig. 6A), demonstrating that betaine catabolism contributed to epiphytic fitness under these conditions. This contribution could have been due to nutrition and/or tolerance of water limitation. We therefore evaluated the fitness of the Δ*ggn* Δ*tre* mutant under the same conditions. Populations of this mutant were also significantly smaller than those of the B728a at 4 days postinoculation (Fig. 6B). Thus, B728a likely experienced water limitation on leaves under these conditions, and the contribution of betaine catabolism to epiphytic fitness was probably due, at least in part, to its contribution to tolerating water limitation.

The transcriptional regulator GbdR is responsible for the differential expression of betaine catabolic genes at distinct osmotic stress levels. To begin to understand how betaine catabolism is differentially regulated at low versus high osmotic stress levels, we investigated its regulation by GbdR, a transcriptional regulator that induces the betaine catabolic genes in *P. aeruginosa* (18). Deletion of the *gbdR* homolog in B728a greatly reduced its growth on betaine and dimethylglycine, consistent with the role of these compounds as coinducers for GbdR activation of *gbcA* and

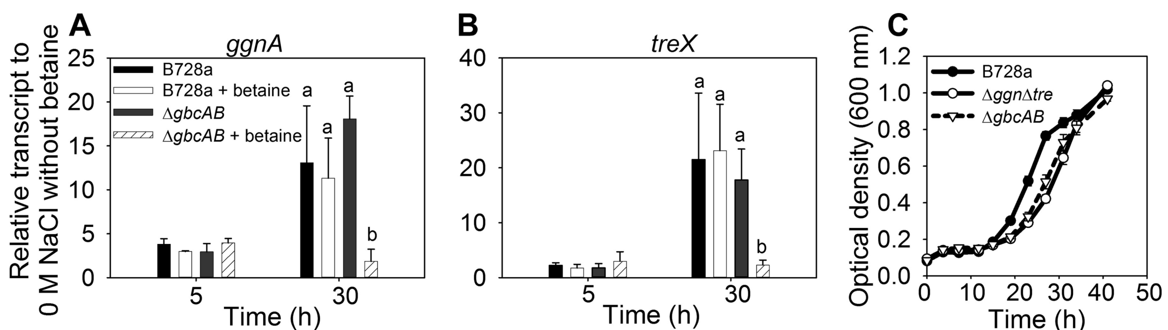

FIG 5 Role of betaine catabolism in relieving betaine-mediated inhibition of endogenous solute synthesis. (A and B) qRT-PCR analysis of *ggnA* (A) and *treX* (B) in B728a and the Δ*gbcAB* mutant at 5 and 30 h after amending cells with 0.6 M NaCl and betaine. Values are expressed relative to transcript levels at 0 M NaCl without betaine. Values indicated with the same letter do not differ significantly ($P < 0.05$). (C) B728a and the Δ*ggn* Δ*tre* and Δ*gbcAB* mutants were grown in MinA-succinate with 0.6 M NaCl and betaine, and growth in microtiter plates was monitored based on optical density. Values are means ± SEM ($n = 3$ for panels A and B; $n = 4$ for panel C).

TABLE 2 The change in the *in planta* transcript levels of the betaine catabolic genes of *P. syringae* B728a[a]

Locus	Gene	Fold change[b]	
		Epiphytic	Apoplastic
Psyr_4776	*gbcA*	12.6	5.2
Psyr_4775	*gbcB*	2.4	1.6
Psyr_4782	*dgcA*	5.7	4.7
Psyr_4781	*dgcB*	1.7	2.7
Psyr_4708	*gbdR*	6.7	2.3
Psyr_4715	*soxA*	4.5	2.8
Psyr_4713	*soxB*	7.4	4.3
Psyr_4714	*soxD*	6.3	4.9
Psyr_4716	*soxG*	4.1	3.6

[a] Data are derived from Gene Expression Omnibus accession no. GSE42544.
[b] The fold change values represent the change in transcript abundance in leaf surface (epiphytic) sites and in intercellular (apoplastic) sites relative to the transcript abundance in a defined medium in culture. Data are adapted from reference 25.

dgcA (see Fig. S2 in the supplemental material). The Δ*gbdR* mutant was also reduced in growth on choline (see Fig. S2 in the supplemental material), since growth on choline requires the betaine catabolic pathway. We also evaluated the influence of osmotic stress on GbdR regulation. Differences in *gbcA* transcript levels between B728a and the Δ*gbdR* mutant at various NaCl concentrations showed that GbdR positively regulated *gbcA* transcription at both low and intermediate osmotic stress levels (0 to 0.3 M NaCl) but not at high stress levels (0.6 to 0.7 M NaCl) following a 40-min exposure period (Fig. 7A). After 24 h of exposure to a high stress level, however, GbdR functioned as a positive regulator (Fig. 7B). Positive regulation at high osmotic stress was confirmed based on a significantly reduced liberation of $^{14}CO_2$ from [*methyl*-^{14}C]glycine betaine in the Δ*gbdR* mutant compared to B728a (Fig. 7C). The importance of this catabolism for osmoadaptation was again illustrated by the reduced growth of the Δ*gbdR* mutant under conditions of high osmotic stress, similar to the reduced growth initially observed for the Δ*gbcAB* mutant (Fig. 3 and 7D).

The expression of *gbdR* under various osmotic stress conditions in the presence of betaine showed that *gbdR* was subject to an expression pattern similar to that of *gbcA*, although the absolute expression levels were much lower for *gbdR* than *gbcA* (Fig. 2A and 8). This result indicates that the osmotic stress-dependent changes in *gbcA* transcription are probably due, at least in part, to osmotic stress-dependent changes in the transcription of the activator gene *gbdR*. Thus, activation of betaine catabolism at high osmotic stress likely resulted, in part, from transcriptional activation of *gbdR*. We used qRT-PCR to evaluate if GbdR directly regulates the endogenous solute synthesis genes in addition to betaine catabolism and found that the Δ*gbdR* mutant exhibited a betaine-mediated reduction of the expression of the trehalose and NAGGN synthesis genes (see Fig. S3 in the supplemental material), indicating that GbdR is not required for this regulation.

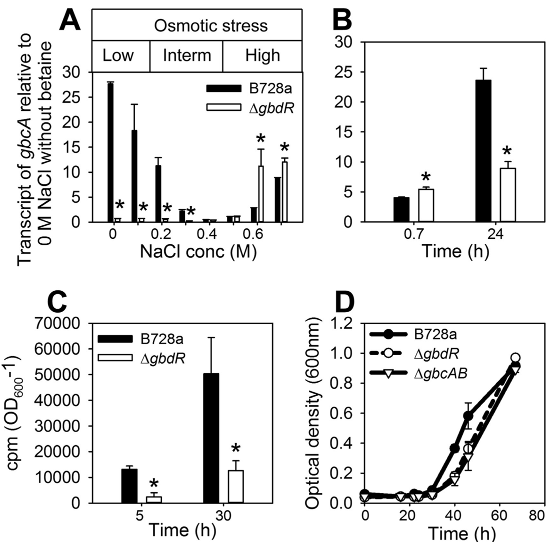

FIG 7 GbdR regulates betaine catabolism under conditions of low and high osmotic stress. (A) qRT-PCR analysis of *gbcA* in B728a and the Δ*gbdR* mutant at 40 min (0.7 h) after amending cells with 0 to 0.7 M NaCl and betaine. Values are expressed relative to *gbcA* transcript levels in B728a at 0 M NaCl without betaine. (B) qRT-PCR analysis of *gbcA* in B728a and the Δ*gbdR* mutant at 0.7 and 24 h after amending cells with 0.6 M NaCl and betaine. (C) $^{14}CO_2$ released by B728a and the Δ*gbdR* mutant during their growth in MinA-pyruvate with 0.6 M NaCl and [*methyl*-^{14}C]glycine betaine for 5 and 30 h. (D) B728a and the Δ*gbcAB* and Δ*gbdR* mutants were grown in MinA-succinate with 0.6 M NaCl and betaine, and growth in microtiter plates was monitored based on optical density. Asterisks indicate differences between B728a and the Δ*gbdR* mutant at the indicated concentrations or times ($P < 0.05$). Values are means ± SEM ($n = 3$ for panels A to C; $n = 4$ for panel D).

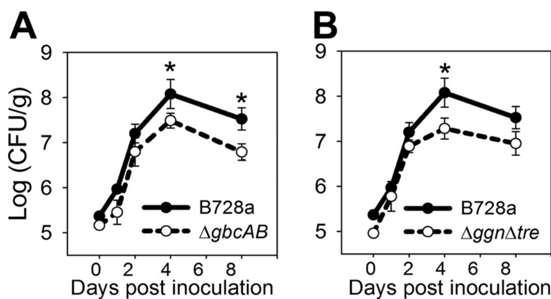

FIG 6 Contribution of betaine catabolism to the epiphytic fitness of B728a. Bean leaves were inoculated with B728a and the Δ*gbcAB* (A) and Δ*ggn* Δ*tre* (B) mutants, and the population sizes were estimated at 0, 1, 2, 4, and 8 days postinoculation based on viable plate counts. Values are means ± SEM ($n = 4$ for days 0 and 1 and $n = 8$ for days 2, 4, and 8). Asterisks indicate differences between B728a and the mutant at the indicated times ($P < 0.05$).

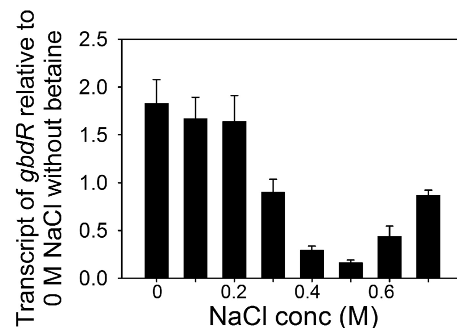

FIG 8 Effect of osmolarity on *gbdR* expression. qRT-PCR analysis of *gbdR* in B728a at 40 min after amending cells with 0 to 0.7 M NaCl and betaine. Values are expressed relative to transcript levels in B728a at 0 M NaCl without betaine. Values are means ± SEM ($n = 3$).

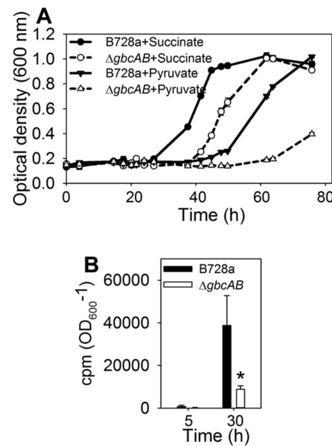

FIG 9 Evaluation of succinate repression of betaine catabolism at high osmolarity. (A) B728a and the Δ*gbcAB* mutant were grown in MinA-succinate or MinA-pyruvate with 0.6 M NaCl and betaine, and growth in microtiter plates was monitored based on optical density. (B) $^{14}CO_2$ released by B728a and the Δ*gbcAB* mutant during their growth in MinA with 20 mM succinate and 0.6 M NaCl and [*methyl*-^{14}C]glycine betaine for 5 and 30 h. Asterisks indicate differences between B728a and the Δ*gbcAB* mutant at the indicated times ($P < 0.05$). Values are means ± SEM ($n = 4$ for panel A; $n = 2$ for panel B).

Succinate mediates partial repression of betaine catabolism at a high osmotic stress level. *P. syringae* B728a grew better than the Δ*gbcAB* mutant in a succinate-containing high osmotic stress medium (Fig. 3), suggesting that succinate does not fully repress betaine catabolism at a high osmotic stress level, as it does in *P. aeruginosa* (11, 26). *P. syringae*, however, was similar to *P. aeruginosa* in exhibiting succinate-mediated repression of betaine catabolism in the absence of NaCl amendment, as illustrated by the reduced transcription of the betaine-dependent GbdR-regulated genes *gbcA*, *plcA*, and *pchP* in succinate- versus pyruvate-containing media (see Fig. S4 in the supplemental material). In a direct comparison of the impact of succinate to that of pyruvate on growth at high osmolarity, B728a and the Δ*gbcAB* mutant each grew better with succinate than with pyruvate (Fig. 9A), probably due to a higher energy yield or better uptake of succinate than pyruvate, but the difference in growth between B728a and the Δ*gbcAB* mutant was much larger when cells were provided with pyruvate than when they were provided with succinate (Fig. 9A), suggesting that succinate partially represses betaine catabolism. The fact that B728a grew better than the Δ*gbcAB* mutant with succinate indicated that succinate did not fully repress betaine catabolism, and this catabolic activity was confirmed by the liberation of significantly more $^{14}CO_2$ from [*methyl*-^{14}C]glycine betaine by B728a than by the Δ*gbcAB* mutant in a succinate-containing high-osmotic-stress medium (Fig. 9B). The contrast between the strong succinate-mediated repression of betaine catabolism in the absence of osmotic stress and the partial repression in its presence suggests that, under high-osmotic-stress conditions, betaine serves not only as a source of energy or nutrition but also as an intracellular signal to regulate endogenous solute synthesis.

DISCUSSION

The mechanisms by which bacteria change their intracellular osmolyte pools during osmoadaptation are not well understood. Following an osmotic upshift in the presence of betaine, many proteobacteria and other prokaryotes rapidly import betaine and restore cellular homeostasis. Our data are consistent with a model in which, following an osmotic upshift large enough to induce betaine uptake, cells eventually transit from a dependence on betaine to a dependence on endogenous compatible solutes. Most importantly, our data identify some of the molecular mechanisms underlying this transition in *P. syringae.* These mechanisms include upregulation of the transcriptional activator gene *gbdR*, resulting in a GbdR-mediated increase in the expression of the betaine catabolism genes and therefore in activation of betaine catabolism. They also include the relief of betaine-mediated suppression of genes required for the synthesis of the endogenous solutes trehalose and NAGGN. This betaine catabolism-driven transition was required for maximal osmotolerance in cells exposed to a high osmotic stress level, as detected by the reduced growth of early log-phase cells of the betaine catabolic Δ*gbcAB* mutant, but also contributed to osmotolerance at an intermediate osmotic stress level, as detected by slightly reduced growth at a later growth phase (Fig. 3). This observation suggests an expansion of the model of cellular adaption following an osmotic upshift in the presence of betaine to include the provision that the larger the osmotic upshift, the earlier the transition from a dependence on betaine to a dependence on endogenous osmolytes.

For bacteria that import betaine under hyperosmotic conditions, the size of the intracellular betaine pool is regulated by balancing this import with catabolism or export. Our work provides evidence that *P. syringae* regulates its betaine pools, at least in part, via catabolism based on the higher betaine levels in the *gbcAB* catabolic mutant. Previous studies with *P. aeruginosa* and *S. meliloti* have shown that these organisms also catabolize betaine under conditions of high osmotic stress (11, 13), with the *S. meliloti* studies documenting a steady depletion of betaine due to catabolism during log-phase growth. Interestingly, upon reaching stationary phase, the intracellular betaine pools in *S. meliloti* rapidly decreased, likely due to export, consistent with the need for a lower level of osmolytes to maintain turgor in stationary-phase cells (13). Our data showing a difference between the Δ*gbcAB* mutant and B728a during the stationary phase under conditions of an intermediate osmotic stress (Fig. 3) suggest that catabolism continued to be important into the stationary phase for *P. syringae*, but we cannot exclude the possibility of additional loss through export. Organisms that cannot catabolize betaine, including *Escherichia coli*, *Bacillus subtilis*, and *Corynebacterium glutamicum*, regulate intracellular betaine pools by attenuating import, such as via the BetP transporter of *C. glutamicum* (27), or efflux, as shown for the mechanosensitive channels MscCG (28) and the transporter protein EmrE (29) in *E. coli*. The versatility of these balancing mechanisms is further illustrated by the recent finding that *B. subtilis* regulates intracellular pools of the compatible solute proline and betaine using a mechanism that involves both release and recapture (30).

Few studies have addressed how organisms that can catabolize betaine at high osmotic stress levels actually regulate this catabolism. Smith and colleagues (1988) found that the activity of the betaine-catabolic enzymes in *S. meliloti* decreased following a 2-h exposure to osmotic stress and concluded that this decreased activity contributes to the accumulation of betaine for osmoprotection. Moreover, using *in vitro* enzyme assays, they showed that this inhibition was not due to a direct effect of salt on the enzymes (21). Such inhibition has not been studied in *P. aeruginosa* or *P. syringae*, and the enzyme catalyzing the putative rate-limiting step

in betaine catabolism is different in *Pseudomonas* spp. from that in *S. meliloti* (20, 21). Our work is the first to provide evidence for osmotic regulation of betaine catabolism at the transcriptional level. We demonstrated not only osmotic regulation of the *gbcA*, *dgcA*, and *soxA* genes, which encode enzymes involved in all three steps converting betaine to glycine, but also osmotic regulation of the *gbdR* gene, which encodes the GbdR transcriptional activator. The dependence of *gbcA*, *dgcA*, and *soxA* on GbdR for expression (18) suggests that their osmoregulation is probably due to the osmoregulated availability of GbdR. The osmotic regulation of *gbdR* expression itself could result, in part, from autoregulation by GbdR under hyperosmotic conditions, although such autoregulation was not observed under nonstressful conditions (18). Cellular factors that influence the osmoregulation of *gbdR* expression have not yet been identified.

Analyses of the Δ*gbcAB* mutant and the wild-type strain indicated an inverse association between durable accumulation of cytosolic betaine and endogenous osmolyte accumulation, consistent with observations in *P. aeruginosa* (11, 12), *S. meliloti* (13), and *Salmonella enterica* serovar Typhimurium (31). These observations have repeatedly led to the prediction that betaine inhibits endogenous solute synthesis. We provided experimental evidence supporting this as-yet-untested prediction. Using the Δ*gbcAB* mutant to achieve elevated cytosolic betaine levels, we demonstrated that betaine accumulation inhibited the transcription of the biosynthetic genes *ggnA* and *treX* required for NAGGN and trehalose synthesis, respectively, under hyperosmotic conditions (Fig. 5A and B). Furthermore, the loss of NAGGN and trehalose synthesis recapitulated the loss of betaine catabolism in reduced osmotolerance in culture and reduced fitness on leaves (Fig. 5C and 6), supporting our model positing that a major consequence of betaine catabolism is relieving the repression of endogenous compatible solute synthesis. We focused on only two of the three known endogenous compatible solutes in *P. syringae*, NAGGN and trehalose, because their biosynthetic loci are known. Nevertheless, our ^{13}C-NMR analysis indicated that durable accumulation of betaine also inhibited the third solute, L-glutamate, but the mechanisms underlying osmoinduced L-glutamate accumulation are not known. The similarity of the Δ*ggn* Δ*tre* mutant to the Δ*gbcAB* mutant in osmotolerance and fitness on leaves, however, suggests that NAGGN and trehalose were the primary contributors to osmotolerance under these conditions.

GbdR was previously identified as a transcriptional activator that enables *P. aeruginosa* to grow on betaine and its derivatives as sole carbon or nitrogen sources in the absence of osmotic stress (18). Here, we expanded the role for GbdR to include transcriptional activation of the betaine catabolic genes under hyperosmotic conditions. Unlike the rapid activation of catabolic genes such as *gbcA* at low or intermediate osmolarity, however, GbdR functioned as a repressor of *gbcA* at 40 min (0.7 h) after an upshift to high osmolarity but, by 24 h, had resumed activity as an activator (Fig. 7A and B). When the cells were grown in the presence of betaine before the upshift, GbdR did not exhibit repressor activity (data not shown). This finding suggests that intracellular betaine is required for GbdR activator activity, consistent with the known requirement for betaine and its degradation products as coinducers of GbdR (18), and that 40 min was insufficient for establishing an intracellular betaine pool. The repressor activity of GbdR in the absence of its coinducer may be similar to that of AraC in the absence of arabinose (32), which was suggested to result from the formation of a repressive DNA loop structure upstream of the regulated genes. The increased *gbcA* expression under high-salt conditions in the absence of GbdR (Fig. 7A) may result from a salt-mediated change in supercoiling (33).

Whereas betaine catabolism in *P. aeruginosa* was subject to complete repression by succinate in the absence (26) and presence (11) of osmotic stress, betaine catabolism in *P. syringae* was subject to only partial repression under hyperosmotic conditions (Fig. 9). The difference between these findings could reflect differences in the levels of stress used in the assays. Specifically, if succinate-mediated repression is relieved under highly stressful conditions, consistent with the need for betaine to serve a dual role in energy generation and as an intracellular signal, then the 0.5 M NaCl amendment used when evaluating catabolite repression in *P. aeruginosa* may not have conferred a sufficiently high level of stress to attenuate the succinate-mediated repression (11); in contrast, the 0.6 M NaCl amendment used for *P. syringae* may have been sufficiently stressful. These findings support the notion of the presence of an osmotically responsive regulator of succinate-mediated catabolite repression in *P. syringae.*

This report provides evidence that betaine catabolism is relevant to the biology of *P. syringae* during its interactions with plants. Previously, we obtained transcriptome data (25) showing the expression of genes for betaine catabolism *in planta* (Table 2). Here, we showed that betaine catabolism contributes to the ability of B728a to establish large populations on leaves of its host plant, bean (Fig. 6A). The reduced epiphytic fitness of the Δ*ggn* Δ*tre* mutant on leaves under the same conditions provides strong evidence that B728a is water limited during its growth on leaf surfaces, as we recently concluded based on our transcriptome data (25). Moreover, the similarity of the reduced fitness of the Δ*ggn* Δ*tre* mutant to that of the Δ*gbcAB* mutant strongly supports the hypothesis that a major role of the betaine catabolism on leaves is to relieve the repression of the synthesis of trehalose and NAGGN, although we cannot exclude the possibility that betaine also provides a nutritional and energy benefit to these bacteria *in planta*. The results strongly support the prediction that B728a can access available, exogenous pools of betaine or a betaine precursor on leaves. We predict that these pools consist primarily of choline and its precursors based on previous studies on B728a transporters that suggested its adaptation to life in choline-rich environments (10, 14), the presence of phosphatidylcholine as a major membrane lipid in plants, and the demonstrated abundance of choline in plants (34).

An issue that remains unresolved by this work is why cells transition from reliance on a continuing supply of exogenous betaine to reliance on endogenous osmolytes. From an ecological perspective, perhaps this transition is a strategy to ensure self-reliance. From a cellular perspective, at least in the case of high osmotic stress levels, perhaps the levels of betaine required to restore cellular homeostasis are toxic to the cells, similar to the reported toxicity of betaine for *Salmonella enterica* serovar Typhimurium (35) and toxicity of glycine for *S. meliloti* (21). And last, from a molecular perspective, perhaps the osmoprotection conferred by the endogenous solutes, either singly or as a set, is superior to the osmoprotection conferred by betaine. That last theory is supported by the finding that, in many bacteria, trehalose becomes an increasingly abundant osmolyte over time (11–13), consistent with the particularly potent protection it provides to proteins and membranes under environmentally stressful conditions.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation (no. MCB-0920156).

We thank Chiliang Chen for comments on the manuscript.

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