Genome-Driven Investigation of Compatible Solute Biosynthesis Pathways of *Pseudomonas syringae* pv. syringae and Their Contribution to Water Stress Tolerance[▽][†]

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The foliar pathogen *Pseudomonas syringae* pv. syringae exhibits an exceptional ability to survive on asymptomatic plants as an epiphyte. Intermittent wetting events on plants lead to osmotic and matric stresses which must be tolerated for survival as an epiphyte. In this study, we have applied bioinformatic, genetic, and biochemical approaches to address water stress tolerance in *P. syringae* pv. syringae strain B728a, for which a complete genome sequence is available. *P. syringae* pv. syringae B728a is able to produce the compatible solutes betaine, ectoine, *N*-acetylglutaminylglutamine amide (NAGGN), and trehalose. Analysis of osmolyte profiles of *P. syringae* pv. syringae B728a under a variety of *in vitro* and *in planta* conditions reveals that the osmolytes differentially contribute to water stress tolerance in this species and that they interact at the level of transcription to yield a hierarchy of expression. While the interruption of a putative gene cluster coding for NAGGN biosynthesis provided the first experimental evidence of the NAGGN biosynthetic pathway, application of this knockout strain and also a *gfp* reporter gene fusion strain demonstrated the small contribution of NAGGN to cell survival and desiccation tolerance of *P. syringae* pv. syringae B728a under *in planta* conditions. Additionally, detailed investigation of *ectC*, an orphan of the ectoine cluster (lacking the *ectA* and *ectB* homologs), revealed its functionality and that ectoine production could be detected in NaCl-amended cultures of *P. syringae* pv. syringae B728a to which sterilized leaves of *Syringa vulgaris* had been added.

Any bacterium growing in an environment with variable external wetness conditions will need a method of adjusting its own intracellular osmolarity. In order to counteract the deleterious effects of high osmolarity and a lack of water itself on cell physiology and the resulting loss of cytosolic water (dehydration), many bacteria rapidly synthesize or take up compatible solutes. These osmotically active small molecules are compatible with cellular function even at high concentrations and serve to maintain cell turgor by balancing the osmotic pressure across the cellular membrane without compromising protein folding or other processes which could be inhibited by high levels of cytosolic salts (26, 38, 42, 65, 66, 74).

Because they are necessarily inert, there are only a limited variety of compounds that can serve as compatible solutes, including certain polyols, amino acids, amino acid derivatives, and peptides. Typical prokaryotic osmolytes include glycine betaine, carnitine, proline, L-α-glutamate, mannitol, trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), *N*-acetylglutaminylglutamine amide (NAGGN), glucosylglycerol, ectoine, and hydroxyectoine (65). These osmolytes can be produced *de novo* but are often accumulated by uptake from the environment to reduce the energetic cost to the cell. It is unclear what proportion of its compatible solutes an epiphyte must produce *de novo*, although it has been hypothesized that choline is widely available in plants for uptake and conversion to betaine (also known as glycine betaine or *N,N,N*-trimethylglycine) (14). Regardless of the mode of accumulation, bacteria must be able to respond quickly, especially in harsh environments where water potentials can change dramatically in a short period of time.

Waxy leaf surfaces are a particularly hostile bacterial habitat due to their rapidly and frequently changing environment. In addition to coping with a limited heterogeneous nutrient supply and daily fluxes in solar UV irradiation and temperature, epiphytes must also respond to frequent fluctuations in moisture availability from factors such as dew, rainfall, and atmospheric water content (33, 43). The bacterial communities on the plant surface regularly experience water stresses in the forms of both osmotic and matric stresses. Osmotic stress can result from the concentration of leaf surface solutes as water films evaporate, while matric stress can occur even in the absence of solutes during periodic desiccation events (78). Therefore, water stress tolerance likely is very robust in epiphytes, although its comprehensive analysis in such bacteria has not been undertaken previously.

The Gram-negative gammaproteobacterium *Pseudomonas syringae* pv. syringae is a common epiphyte on a variety of plants and the causative agent of bacterial brown spot disease in *Phaseolus vulgaris* L. (snap bean). *P. syringae* pv. syringae establishes a large epiphytic population on healthy plants, serving as a source of inoculum for the organism to invade the plant tissue and cause infections under permissive conditions. Due to its importance as a plant pathogen and the fact that adequate chemical or biological control of such bacterial dis-

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics or purpose[a]	Reference(s) or source
Strains		
E. coli		
DH5α	Multipurpose cloning	69
S17-1	Conjugal transfer of mobilizable plasmids	73
P. syringae pv. syringae		
B728a	Rifampin-resistant mutant of a wild-type strain originally collected from bean leaves	45
B728a ΔPsyr_3747	Tet[r]	This study
B728a (pPnaggn:*gfp*)	Isolate B728a harboring NAGGN reporter plasmids containing transcriptional fusions of the promoter for Psyr_3747 with a GFP-encoding gene	This study
B728a (p519*gfp*)	Isolate B728a harboring constitutive p519*gfp*	52
P. syringae pv. tomato DC3000	Rifampin derivative of DC52 (wild-type *P. syringae* pv. tomato race 0 strain), pathogen of tomato and *Arabidopsis* spp.	20
P. stutzeri DSM 5190[T]	Wild type, contains the full functional *ectABC* gene cluster	67
H. elongata		
DSM 2581[T]	Wild type, contains the full functional *ectABC* gene cluster	79
WUB01	Isolate DSM2581 Δ*ectC* (in-frame deletion)	E. Witt and A. Ures, Galinski group, University of Bonn, Germany
WUB pPESyC	Isolate DSM2581 Δ*ectC* plus *ectC* gene of *P. syringae* pv. syringae B728a; Chl[r]	This study
WUB pPEStC	Isolate DSM2581 Δ*ectC* plus *ectC* gene of *P. stutzeri* DSM5190[T]; Chl[r]	This study
Plasmids		
pENTR/D-Topo	Entry vector	Invitrogen
pLVC-D	Destination vector	47
pPE	Cloning vector; pBBR1MCS derivative equipped with the salt-dependent *H. elongata* promoter region upstream of *H. elongata ectC*	12, 35, 36
pPESyC	*ectC* gene of *P. syringae* pv. syringae B728a subcloned in frame into pPE under the control of the *H. elongata* promoter	This study
pPEStC	*ectC* gene of *P. stutzeri* DSM5190[T] subcloned in frame into pPE under the control of the *H. elongata* promoter	This study
pTOPO-Pnaggn	Vector equipped with the upstream promoter region of the NAGGN biosynthetic gene cluster	This study
pPROBE-OT	Vector containing a promoterless *gfp* gene	54
pPnaggn:*gfp*	Cloning vector containing the upstream promoter region of the NAGGN cluster fused with the *gfp* gene	This study

[a] Chl[r], chloramphenicol resistant; Tet[r], tetracycline resistant.

eases is not available, the epidemiology, epiphytic lifestyle (33), and recently, also the genome of *P. syringae* pv. syringae strain B728a (27) have been intensively studied. *P. syringae* pv. syringae B728a is thus a good model bacterium for studying osmoprotection in an epiphyte.

In a previous study by Feil et al. in which they first analyzed the *P. syringae* pv. syringae B728a genome sequence, it was hypothesized that the strain is able to produce betaine and possibly ectoine as osmolytes (24). However, biochemical studies have not been undertaken to confirm these bioinformatic predictions, and other possible responses of *P. syringae* pv. syringae to water stress have not been addressed. In order to gain novel insight into the osmotic stress responses of B728a, this study investigated the strain's biosynthetic capability to produce osmolytes using a genome-driven strategy, complemented by genetic and biochemical experiments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. For identification of compatible solutes, *P. syringae* pv. syringae B728a and *Pseudomonas syringae* pv. tomato DC3000 were grown in Davis minimal broth without dextrose (41) but containing 20 mM glycerol (DMBgly), supplemented with 1% to 3% (wt/vol) NaCl for 72 h with shaking (160 rpm) at 25°C. For simulated *in planta* ectoine production, *P. syrin-

gae* pv. syringae B728a was cultivated for 8 days at 10°C in modified minimal medium MM63 (half of the glucose was replaced by fructose, and 1% NaCl was added) (40). In some experiments, fresh shredded surface-sterilized leaves of lilac (*Syringa vulgaris*) were added to the medium at the beginning of the experiment. Shredded leaves, incubated under identical conditions without added bacteria, and the extract of fresh shredded leaves served as negative controls. Restoration of the *Halomonas elongata* Δ*ectC* mutant WUB01 was tested in DMBgly with 5% added NaCl. Production of ectoine and betaine from precursors by *P. syringae* pv. syringae B728a and *P. syringae* pv. tomato DC3000 was tested in DMB containing 1% NaCl and amended with 2 mM *N*-γ-acetyldiaminobutyric acid or choline, respectively. Betaine uptake was tested in both Luria-Bertani (LB) medium (6) and antibiotic assay medium (assay broth) 3 (30) containing 1% NaCl. Production of solutes by *P. syringae* pv. syringae B728a and *P. syringae* pv. tomato DC3000 under pseudo *in planta* conditions was checked by cultivation in SRM medium (55) for 7 days at 25°C without shaking. *Escherichia coli* strain DH5α was maintained on LB agar and cultured at 37°C. The antibiotics kanamycin, rifampin, and tetracycline were used at final concentrations of 50, 100, and 15 μg/ml, respectively.

Construction of mutants. To generate the NAGGN synthesis-defective (ΔPsyr_3747) mutant, a DNA fragment of approximately 500 bases in length harboring an internal portion of Psyr_3747 was amplified from *P. syringae* pv. syringae B728a genomic DNA using the primers 3747-KO-F (5′-CACCTACG GCCCGCACGAAGACGAG-3′) and 3747-KO-R (5′-CCAGTGGTAACCGG CGAACAGTTCG-3′). This fragment was placed in the Gateway vector pENTR/D-Topo (Invitrogen, Carlsbad, CA), and then transferred to the destination vector pLVC-D, as described by Marco et al. (47). The resulting construct was transformed into *P. syringae* pv. syringae B728a, and tetracycline was used to select for single-crossover events, as in other studies (47). The resulting inter-

ruptions in Psyr_3747 were confirmed using PCR with primers specific to the pLVC-D vector and genomic DNA.

For the construction of a Pnaggn::*gfp* transcriptional fusion, the upstream promoter region of the *P. syringae* pv. syringae B728a Psyr_3747 NAGGN biosynthetic gene was amplified from genomic DNA by PCR with primers naggn-HindIII, 5′-TAAGCTTTGGAGACCGATCGGGGCTC-3′, and naggn-EcoRI, 5′-TGAATTCAAGTCCTTACCGGGTTTCGTTGGGG-3′, to generate a 400-bp promoter region (underlining shows corresponding restriction sites). PCR conditions used were as follows: 28 cycles of 95°C, 61°C, and 72°C for 1 min each, with a final extension time of 10 min at 72°C. The PCR product was first cloned into pTOPO Blunt (Invitrogen) to generate pTOPO-Pnaggn, and then transformed into *E. coli* DH5α. The insert was sequenced to verify its identity. pTOPO-Pnaggn was digested with HindIII and EcoRI, and the resulting fragment was cloned into pPROBE-OT (54), which contains a promoterless *gfp* gene, in order to generate pPnaggn:*gfp*. pPnaggn:*gfp* was electroporated into *P. syringae* pv. syringae B728a to yield the transformed reporter strain B728a (pPnaggn:*gfp*).

In order to confirm the activity of the ectoine synthase EctC of *P. syringae* pv. syringae B728a, the *ectC* gene from B728a was cloned into the vector pPE. Plasmids were then transformed into *E. coli* S17-1 for conjugal transfer into the *ectC*-deficient strain *H. elongata* WUB01, as described elsewhere (28). The *ectC* gene of ectoine-producing *Pseudomonas stutzeri* was included as a positive control.

Analysis of intracellular osmolyte contents. Extraction of freeze-dried cell material from an exponentially growing shaking culture followed a modified protocol of the technique of Bligh and Dyer (7) as described previously (27). Solute content was analyzed by isocratic high-performance liquid chromatography (HPLC) on a Macherey-Nagel Nucleosil NH_2 100-3 column (125 by 4 mm, 3 μ) and an 80:20 (vol/vol) acetonitrile/water ratio at a flow rate of 1 ml min^{-1} as a mobile phase, with simultaneous refractive index and UV detection (220 nm).

Bioinformatics and phylogenetic tree construction. In order to detect homologous protein sequences from each biosynthetic pathway, previously reported sequences, preferably from pseudomonads or bacteria related to *Pseudomonas*, were used. Searches for related proteins were performed with the BLASTP program at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/BLAST) (2), and sequences with minimum E values of 0.0001 without filtering were identified.

To estimate the phylogenetic relationships of EctC, a BLAST search for sequences with similarity either to that of EctC of *P. stutzeri* (YP_001170728), representing a full functional EctC derived from a classic three-component gene *ectABC* operon, or to that of EctC of *P. syringae* pv. syringae B728a (YP_233444), originating from a single and isolated *ectC* gene, was conducted. Sequence alignments were performed with ClustalW2 software using default values (39). The tree was visualized using the software PhyloDraw (16).

Isolation and purification of NAGGN. *P. syringae* pv. syringae B728a was grown for 72 h at 25°C with shaking (160 rpm). Two cultures in 1 liter of DMBgly supplemented with 1% (wt/vol) NaCl yielded 708.4 mg of crude extract. Chromatographic separation of the crude extract employing isocratic HPLC on a Macherey-Nagel Nucleosil NH_2 100-5 column (250 by 4.6 mm, 5 μ) and an 80:20 (vol/vol) acetonitrile/water ratio at a flow rate of 1 ml min^{-1} as a mobile phase yielded 4.7 mg of pure NAGGN.

NMR spectroscopy. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 DPX spectrometer. Spectra were calibrated to the solvent signal (^{13}C, d_6-dimethyl sulfoxide [d_6-DMSO], δ 39.5 ppm) and the signal contributed by the nondeuterated portion of the solvent (^1H, D_2O, δ 4.68 ppm; DSMO, δ 2.50 ppm).

Absolute configuration of the peptide moiety of NAGGN. NAGGN (0.9 mg) was dissolved in 600 μl 6 N HCl and hydrolyzed in a sealed vial at 105°C for 16 h. The solvent was removed by using a stream of dry N_2, and the sample was analyzed by chiral HPLC with a Phenomenex Chirex 3126 phase (4.6 by 250 mm, 2 mM $CuSO_4$-MeCN [95:5], 1.0 ml/min, UV detection at 254 nm). The retention times of the standards were 42.8 min for L-Glu and 45.6 min for D-Glu. The hydrolysate showed a peak coincident with that of the L-Glu standard (see the supplemental material).

Plant assays. Cells of *P. syringae* pv. syringae B728a strains were grown overnight on KB medium with appropriate antibiotic selection, harvested by scraping them from the plate surface, washed, and resuspended to a final cell concentration of 10^6 cells ml^{-1} in 1 mM potassium phosphate buffer. The cell suspension was then spray inoculated onto fully expanded trifoliate leaves of bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274; approximately 2 weeks old). Plants were placed in a humid chamber at room temperature (approximately 24°C) and periodically exposed to a fine mist of water to maintain high relative humidity

and to maintain thin films of water on the leaf surfaces. Artificial light was maintained for 16-h periods within the 24-h cycle. For epiphytic growth analysis, plants were taken out of the humid chamber after 48 h and placed on a greenhouse bench for an additional 24 h of drying. Cells were washed from individual trifoliate leaves with 5 min of sonication in 1 mM potassium phosphate buffer (pH 7.0), and epiphytic population sizes were determined by plating of appropriate dilutions, as previously described (63, 64). Alternatively, for the fluorescence analysis, after an initial 24 h of humidity the plants were exposed to the following conditions for 24 h: continued humidity for "wet plants," turning off the humidifier for "slow-dry plants" and moving the inoculated plants to a dry chamber with 40% humidity and at 28°C for "fast-dry plants."

Fluorescence assays. For analysis of expression of NAGGN *in vitro*, cells of the reporter *P. syringae* pv. syringae B728a (pPnaggn:*gfp*) and a constitutively fluorescent strain B728a (p519*gfp*) (52) were grown with shaking overnight in Davis minimal medium broth and then spiked with NaCl (final concentration, 1%); half of the NaCl cultures were also spiked with choline (2 mM), and all were incubated for an additional 4 h. Cells were then recovered by centrifugation, washed, and resuspended in phosphate buffer (10 mM, pH 7.5) to a concentration of approximately 3×10^8 cell ml^{-1} (optical density at 600 nm [OD_{600}] = 0.2). The green fluorescent protein (GFP) fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) with a 486-nm band-pass excitation filter and a 510- to 700-nm combination emission filter. A relative fluorescence unit (RFU) was defined as the fluorescence of the suspensions normalized for the suspension turbidity, measured as OD_{600}.

For *in vivo* applications, *P. syringae* pv. syringae B728a (pPnaggn:*gfp*) and B728a (p519*gfp*) were grown and inoculated as described above in plant assays. Cells were washed from individual leaves and recovered by successive rounds of centrifugation at 8,000 × g. Cells were mixed with a DAPI (4′,6-diamidino-2-phenylindol) solution (final concentration, 2 μg ml^{-1}) on a microscope slide and then flash frozen on dry ice to permeabilize the cells. Epifluorescent microscopy was then done using a Zeiss Axio Imager M1 fluorescence microscope using a 20× lens objective and a xenon light source. The GFP was excited with a band-pass filter at 470/40 nm. Emissions were collected using a band-pass filter at 525/50 nm. DAPI was excited with a band-pass filter at 350/50 nm, and the emissions were collected using a 400-nm long-pass filter for 0.4 s. Images were collected with a Hamamatsu Orca camera, using exposure times of 6 s for GFP and 0.4 s for DAPI. Images were captured, and relative GFP fluorescence was quantified using iVision software (BioVision Technologies).

RESULTS

Identification of compatible solute biosynthetic pathways in the *P. syringae* pv. syringae B728a genome. The complete genome sequence of B728a was investigated for the presence of open reading frames (ORFs) with similarity to genes known to code for compatible solutes typical for prokaryotes.

Trehalose. Using a BLAST search against proteins carried by the three typical eubacterial trehalose biosynthesis pathways TreXYZ, TS, and TPS/TPP (4, 17, 48–51), we identified a TreXYZ pathway (Psyr_2997, Psyr_2995, and Psyr_2993) and two potential TS pathways (Psyr_2736 and Psyr_2490) in *P. syringae* pv. syringae B728a (Fig. 1). The predicted Psyr_2997 protein shares 48% sequence identity with TreX of *Arthrobacter* sp. Q36, whereas the Psyr_2995 and Psyr_2993 proteins showed 65% and 66% sequence identity with *Pseudomonas fluorescens* Pf-5 maltooligosyl-trehalose synthase TreY and maltooligosyl-trehalose trehalohydrolase TreZ, respectively. Psyr_2736 and Psyr_2490 were significantly related (73% sequence identity each) to the two trehalose synthases of *Pseudomonas putida* W619 (PputW619_1821 and PputW619_1816) and are here referred to as TreS-1 and TreS-2, respectively. Genes encoding the TPS/TPP pathway (as found in *Pseudomonas savastanoi* and *P. stutzeri* A1501) were not found in the genome of *P. syringae* pv. syringae B728a.

NAGGN. An early study on the biosynthesis of the compatible solute NAGGN in *Rhizobium meliloti* reported that NAGGN is produced nonribosomally by an ATP-dependent
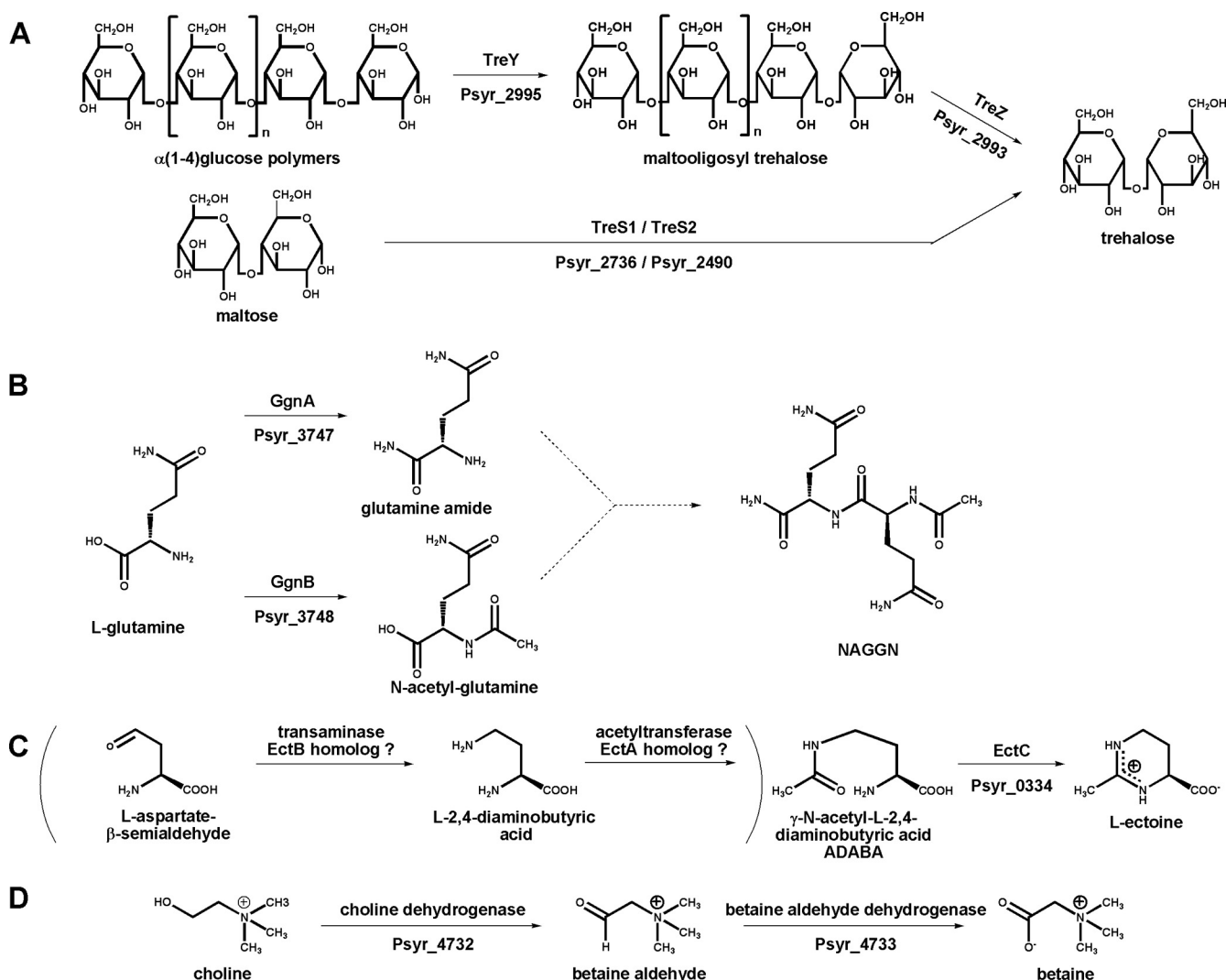
FIG. 1. Compatible solute biosynthesis pathways found in *P. syringae* pv. syringae B728a. (A) Three pathways leading from either maltose or glucose polymers to trehalose. (B) Proposed biosynthesis of *N*-acetylglutaminylglutamine amide (NAGGN). (C) Formation of the osmolyte ectoine from its precursor ADABA. The enzymatic steps in parentheses are speculative. (D) Conversion of choline into betaine by *P. syringae* pv. syringae B728a.

enzyme, although no candidate loci were identified in *R. meliloti* (76). This prediction aided our search for candidate genes in *P. syringae* pv. syringae B728a. As a working hypothesis, it was envisioned that two glutamines serve as precursors which are fused directly before N-acetylation and amidation occurs, or in turn, the free amino acids are first derivatized and subsequently connected to yield a dipeptide. Hence, a clustered set of at least three genes was hypothesized to be involved in the biosynthesis, i.e., an acetyltransferase, an amidotransferase, and a dipeptide synthase, possibly accompanied by regulatory genes. A BLAST search for annotated acetyltransferases in the genome of *P. syringae* pv. syringae B728a yielded numerous potential candidate genes. Exclusion of apparently specialized acetyltransferases halved the number of candidate genes. Closer inspection of flanking regions of the remaining candidate genes identified the acetyltransferase Psyr_3748 to be of particular interest due to the adjacent amidotransferase (Pysr_3747) and aminopeptidase (Psyr_3749). This hypothesis

was further corroborated by a microarray study by Aspedon and coworkers which demonstrated that the expression of orthologs of Psyr_3747 to Psyr_3749 in *Pseudomonas aeruginosa* (PA3459 to PA3461) was upregulated by 5- to 6-fold in osmotically shocked cells (3).

Ectoine. *P. syringae* pv. syringae B728a possesses a homolog of *ectC* (Psyr_0334), although ectoine biosynthesis is uncertain because there are no homologs of *ectA* and *ectB*, which synthesize the ectoine precursor *N*-γ-acetyldiaminobutyric acid (ADABA) (Fig. 1) and are usually spatially linked with *ectC* (24).

Betaine. Putative choline and betaine dehydrogenase enzymes (Psyr_4732 and Psyr_4733, respectively) were already annotated in the first analysis of the *P. syringae* pv. syringae B728a genome sequence (24). Like many other bacteria, this indicates that B728a can synthesize betaine only when supplied with its precursor choline (Fig. 1). Additionally, orthologs of osmoprotectant transporters BetT (Psyr_3758), OpuC
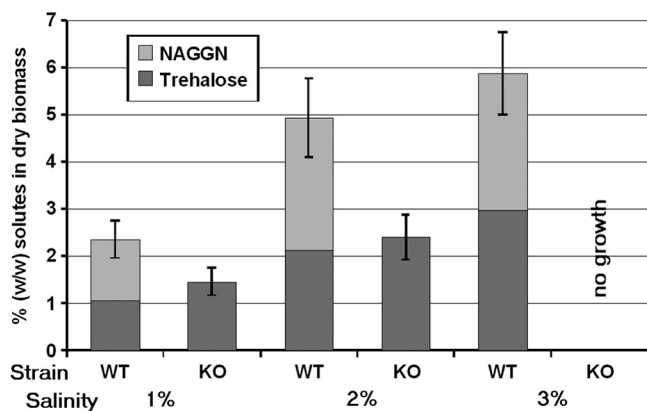
FIG. 2. Osmolytes accumulated by the *P. syringae* pv. syringae B728a wild type (WT) and NAGGN-deficient mutants (knockouts [KO]) grown at different salinities (wt/vol).

(Psyr_4249), and CbcXWV (Psyr_4709 to Psyr_4711), responsible for the transport of the essential precursor choline, have already been identified by Beattie and coworkers (13–15).

Other compatible solutes. Searches of the *P. syringae* pv. syringae B728a genome for homologs of GgpPS from *Pseudomonas mendocina* YMP (32) (accession no. YP_001188605) that encode glucosylglycerol biosynthesis did not yield any significant matches, suggesting that glucosylglycerol is not produced by B728a. Due to their dual role in primary metabolism, it was not possible to attribute genes for amino acid-based osmolytes (e.g., proline or glutamate) or mannitol exclusively to water stress tolerance.

Identification of compatible solutes of salt-stressed *P. syringae* pv. syringae B728a. In order to test the bioinformatic hypotheses, B728a was subjected to salt stress (1% NaCl) and analyzed for compatible solute production. First, natural-abundance ^{1}H and ^{13}C NMR spectroscopy of the crude polar extract was employed to identify the osmolyte composition of *P. syringae* pv. syringae B728a. Inspection of the ^{1}H NMR spectrum and particularly the ^{13}C NMR spectrum (see the supplemental material) indicated the presence of NAGGN (δ_H D$_2$O: 1.85 m + 1.98 m, 1.89 s, 2.23 t, J = 7.3 Hz, 4.14 m; δ_C D$_2$O: 179.3, 179.2, 177.2, 175.9, 175.1, 54.7, 54.3, 2 × 32.4, 28.1, 28.0, and 23.0) and trehalose (δ_H D$_2$O: 3.21 to 3.74 m, 5.03 d 3.7; δ_C D$_2$O: 94.6, 73.8, 73.5, 72.4, 71.0 and 61.9) as the two principal osmolytes. The identity of these compounds was readily established by direct comparison with literature data (22, 75) and the spectra of reference substances. Additionally, by two-dimensional NMR (2D-NMR), high-resolution mass spectrometry (HR-MS), and chiral amino acid analysis, NAGGN was semipreparatively purified, and its identity was further confirmed (see the supplemental material).

Inactivation of the *P. syringae* pv. syringae B728a NAGGN biosynthesis pathway. In order to confirm that the pathway proposed from the genome data analysis (Psyr_3747 to Psyr_3749) was involved in the production of NAGGN, a mutant was constructed by chromosomal inactivation of the first gene (Psyr_3747) of the pathway, encoding a putative amidotransferase. While the mutant still produced trehalose under conditions of 1% and 2% salinity, it was unable to accumulate NAGGN (Fig. 2; see also Fig. S10 in the supplemental material).

Osmotic regulation of NAGGN and trehalose in salt-stressed cells. The dependence of osmolyte accumulation on medium osmolality was examined further by determining the intracellular levels of NAGGN and trehalose as a function of salinity. The levels of both of the osmolytes increased at a 1:1 dilution ratio as the concentration of NaCl was increased (Fig. 2). In the absence of NaCl, NAGGN and trehalose were undetectable, indicating that both compounds are osmoregulated. The mutant of *P. syringae* pv. syringae B728a deficient in NAGGN production responded to 1% and 2% salinity exclusively with the synthesis of trehalose, and although the amount of trehalose produced was always slightly higher than that produced by the wild type at any given level of NaCl, this increase did not fully compensate for the loss of NAGGN. The limited compensation for increased trehalose production is also evident in the lack of growth of the NAGGN mutant in 3% NaCl. While no difference in growth was observed between the wild type and the NAGGN mutant without salt stress, the mutant strain was severely impaired in growth compared to that of the wild-type strain when the osmolarity of the medium was raised to 3% NaCl (see Fig. S13 in the supplemental material). As further proof of osmotic regulation, the transcription of the NAGGN biosynthetic locus was estimated by measuring the GFP fluorescence of cells harboring a fusion of the promoter for Psyr_3747 to a promoterless *gfp* reporter gene. As with accumulation of NAGGN, the expression of Psyr_3747 increased directly with the increasing NaCl concentration of the culture media (see Fig. S14 in the supplemental material).

Betaine as a compatible solute in *P. syringae* pv. syringae B728a. *P. syringae* pv. syringae B728a is not expected to be able to perform *de novo* biosynthesis of betaine due to its predicted inability to produce the precursor choline, which was further confirmed when no betaine was detected in osmotically stressed cells. Therefore, choline was supplied in order to probe the functionality of the predicted betaine biosynthetic pathway. HPLC analysis of cellular extracts revealed that betaine was produced when choline was added to the growth medium (Fig. 3). Moreover, the accumulation of betaine was accompanied by a decrease of the other endogenously synthesized osmoprotectants. The production of NAGGN was nearly abolished upon addition of choline to culture media, while the accumulation of trehalose appeared to be reduced to only 20% of that seen in media without choline (Fig. 3). A direct uptake of betaine was also demonstrated by growing *P. syringae* pv. syringae B728a in a betaine-containing media; after 48 h of growth in the presence of betaine and 1% NaCl, HPLC analysis of cellular extracts revealed a prominent betaine peak (Fig. 3), thus confirming that B728a possesses efficient transport mechanisms for this compatible solute, as noted by Beattie et al. (13–15). Similar to the addition of choline, there was a near-complete elimination of NAGGN biosynthesis but only a slight decrease in trehalose production upon addition of betaine to culture media in the presence of NaCl (Fig. 3). Furthermore, transcription of Psyr_3747 involved in NAGGN production was strongly downregulated in the presence of choline and NaCl compared to that in the presence of NaCl alone (see Fig. S14 in the supplemental material).

Verification of the functionality of *P. syringae* pv. syringae B728a EctC. To test if *P. syringae* pv. syringae *ectC*
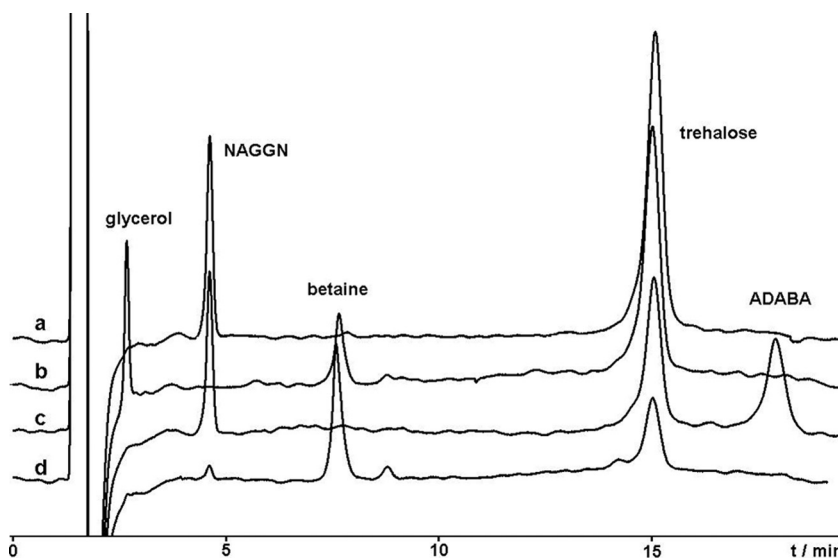
FIG. 3. Compatible solute profile of the *P. syringae* pv. syringae B728a wild type grown under different conditions at 1% salinity (amounts of solutes are given in mg [dry biomass] per g). (a) DMBgly (trehalose, 10.7; NAGGN, 2.3); (b) betaine-containing AB1 medium (trehalose, 7.7; NAGGN, 0.0; betaine, 0.9); (c) DMBgly supplemented with the ectoine precursor ADABA (trehalose, 4.9; NAGGN, 1.9; ADABA, 2.3); (d) DMBgly supplemented with choline (trehalose, 2.3; NAGGN, 0.1; betaine, 2.0).

encodes a functional cyclase, the gene was transferred into the *ectC*-deficient strain *Halomonas elongata* WUB01, which does not produce ectoine, and ectoine production was quantified. This strain retains functional *ectA* and *ectB* genes, so it can produce the ectoine precursor ADABA. Ectoine production was restored in the *ectC*-deficient *H. elongata* strain complemented with pPESyC to produce half the level of ectoine compared with that produced by the wild-type strain harboring this plasmid, indicating that *P. syringae* pv. syringae *ectC* encodes a fully functional but less efficient ectoine synthase. Although no ectoine was detected in cells exposed to NaCl, it was hypothesized that B728a might be able to produce ectoine *de novo* but that appropriate conditions for its synthesis were not provided under these culture conditions. Since the production of secondary metabolites depends on culture conditions (9) and since many genes in *P. syringae* pv. syringae are modulated by plant signal molecules (47, 55), simulated *in planta* conditions were explored to determine if the *ectC* gene and, hence, ectoine production might be conditional in its expression. Surprisingly, of the many conditions tested, only the addition of surface-sterilized leaves of lilac (the organism from which *P. syringae* pv. syringae was first isolated), which were devoid of other microbes, was shown to induce ectoine production in wild-type B728a (Fig. 4). While such leaves would be expected to contain arbutin, a compound previously shown to induce toxin production in *P. syringae* pv. syringae (55), arbutin-containing medium had no influence on the osmolyte production profile, suggesting that arbutin itself is not a signal for ectoine production (data not shown). Additionally, an osmolyte profile of *P. syringae* pv. syringae B728a grown in media containing the ectoine precursor ADABA revealed that ADABA was readily taken up (Fig. 3), demonstrating the presence of the respective transport capacity in B728a. ADABA uptake was accompanied by a decrease in the production of NAGGN and trehalose, since it may also be used as a compatible solute (58). However,

feeding of ADABA alone was not sufficient to yield ectoine production in *P. syringae* pv. syringae B728a.

Role of NAGGN in colonization of plant surfaces by *P. syringae* pv. syringae B728a. In order to assess the role of NAGGN in the osmoadaption of *P. syringae* pv. syringae B728a *in planta*, wild-type and NAGGN-knockout strains of B728a were inoculated onto the surface of bean plants (*Phaseolus vulgaris* L.) that were subsequently exposed to various environmental conditions. The NAGGN-deficient mutant grew to population sizes equal to those of the wild-type strain under humid conditions, exhibiting about 100-fold epiphytic growth after 2 days. Given that moist leaves probably confer little water stress to epiphytic bacteria, the lack of difference in growth between the NAGGN mutant and wild-type strains under humid conditions was not surprising. However, little difference in survival of the NAGGN mutant compared to that of the wild-type strain was also seen on dry leaves (see Fig. S15 in the supplemental material). Water stress conditions were imposed by a slight increase in temperature and a great reduction in leaf surface moisture after the initial moist growth conditions. Both strains exhibited about a 10-fold decrease in population size on the leaf surface in response to the drying of the leaf environment, and the presence or absence of NAGGN biosynthesis had no effect on this response.

In order to clarify if NAGGN is actually produced by *P. syringae* pv. syringae B728a on leaves, an expression analysis of the NAGGN biosynthetic genes using *gfp* reporter gene fusion strains of wild-type B728a (pPnaggn:*gfp*) and its corresponding NAGGN mutant (p519*gfp*) was performed. In agreement with the epiphytic growth experiments, only small differences in the levels of GFP fluorescence under all conditions tested on leaves were observed, indicating that there is no evidence of NAGGN biosynthesis induction on leaves, even under desiccation conditions.
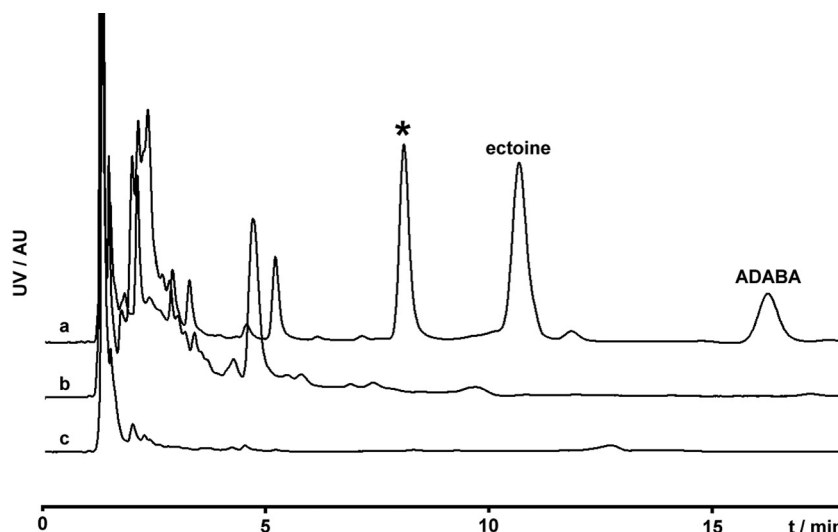
FIG. 4. (a) UV-HPLC osmolyte profile of *P. syringae* pv. syringae B728a grown in the presence of shredded lilac leaves. The asterisk indicates an unknown component of the extract. In the negative controls, which comprised shredded leaves incubated under identical conditions but without bacteria added (b) and fresh shredded leaves (c), peaks for the ectoine precursor ADABA or for ectoine itself were not observed. AU, arbitrary units.

DISCUSSION

Genomic mining for compatible solutes in *P. syringae* pv. syringae B728a revealed that this plant pathogen has the capacity to produce a wide repertoire of cell-protecting osmolytes to counteract water stress. The predicted compatible solutes of *P. syringae* pv. syringae B728a appear to be typical of the osmolytes produced by other pseudomonads (Table 2). While we demonstrated that all four predicted osmoprotectants could actually be produced by *P. syringae* pv. syringae, their production in many cases was very context dependent; while trehalose and NAGGN were readily biosynthesized in NaCl-containing growth medium, betaine and ectoine were produced only when provided with medium conditions mimicking *in planta* conditions, and the supplementation of exogenous osmolytes such as betaine suppressed the production of many of these compounds, suggesting that osmolyte production itself is generally inhibited when osmolyte uptake is possible.

Compatible solutes in *P. syringae* pv. syringae B728a. Under the moderate-salt stress conditions of 1% NaCl, *P. syringae* pv. syringae B728a produces trehalose and NAGGN in equivalent amounts. With increasing salinity, the strain increases the biosynthesis of both compatible solutes. Although we have determined only one biosynthetic cluster for NAGGN and demonstrated its sufficiency for NAGGN biosynthesis, the production of trehalose may be conferred by several biosynthetic pathways. In fact, not only are there multiple biosynthetic pathways for trehalose production in B728a, but there are even multiple copies of some of these pathways. Such redundancy is a commonly observed phenomenon in halotolerant and halophilic bacteria (4), which may be due to a need for rapid accumulation of trehalose under changeable environmental conditions and substrate availability. Trehalose appears to be a particularly effective osmolyte; production of trehalose enables *P. syringae* pv. syringae B728a to tolerate up to 2% NaCl, while

trehalose in combination with NAGGN raises the halotolerance to 3% NaCl (Fig. 2). At salinities of 3% and higher, *P. syringae* pv. syringae B728a was hardly cultivable. Overall, the *in vitro* osmolyte profile and the mechanisms of osmotic stress adaption of *P. syringae* pv. syringae B728a were largely similar to those of other pseudomonads (22).

Choline participates in the osmotolerance of *P. syringae* pv. syringae B728a in complex ways. A large number of bacteria are not able to synthesize betaine *de novo* (57) but require the presence of the precursor choline or glycine betaine aldehyde in the growth medium for betaine formation (8). This study showed that *P. syringae* pv. syringae B728a actually produces betaine as bioinformatically predicted but only if its precursor choline is added to the growth medium. Under these conditions, betaine becomes the dominant osmoprotectant, replacing the endogenously synthesized osmolytes trehalose and NAGGN. Such shifts in osmolyte preference by exogenously added compatible solutes or their precursors are well known (18, 19, 22) and can be explained by the fact that the production of the small 5-carbon-containing betaine is energetically less costly than the larger 12-carbon-containing trehalose or NAGGN molecules (60). *P. syringae* pv. syringae B728a is somewhat more flexible in osmolyte accumulation than some other bacteria, because even in the presence of exogenous betaine, it still produces trehalose (Fig. 3). With its ability to derive better osmoprotection from choline than from betaine, *P. syringae* pv. syringae B728a is distinct from most other characterized bacterial strains. However, this behavior may be explained by the higher capacity for uptake of choline than that for uptake of betaine by the concerned transporter systems OpuC, BetT, and CbcXWV (13–15). Since choline is ubiquitous in plants, whereas betaine is produced by only some plants (77), it has been speculated that *P. syringae*, as a plant-associated organism living in the phyllosphere, particularly evolved the preference of choline rather than betaine in order to en-

TABLE 2. Compatible solutes of pseudomonads

Pseudomonas strain	*De novo* biosynthesized compatible solute results								Uptake/conversion of betaine[c]	Reference(s)
	Alanine	Glutamate[b]	NAGGN	Trehalose	Mannitol	Glucosyl-glycerol	Ectoine	OH-ectoine		
P. aeruginosa ATCC 17933		+							ND/ND	34
P. aeruginosa PAO1		+	+	+					U/C	22
P. chlororaphis L10-2-1[a]			+	+					ND/ND	53
P. fluorescens		+	+	+					ND/ND	75
P. fluorescens ATCC 11250		+							ND/ND	34
P. fluorescens DSM 8569[a]			+	+					ND/ND	53
P. fluorescens DSM 50090[T]			+	+					−/C	This study
P. fluorescens EPS62e			+	+		+			U/ND	10
P. fluorescens Pf-5			+	+					U/C	This study
P. fluorescens ST		+							ND/ND	34
P. halophila DSM 3050							+	+	ND/C	72
P. halosaccharolytica CCM 2851	+						+	+	U/C	72
P. mendocina ATCC 25411		+	+			+			ND/ND	62
P. mendocina SKB70		+	+	+					U/ND	62
P. pseudoalcaligenes ATCC 14440		+	+			+			ND/ND	62
P. psychrotolerans BO001	+	+	+	+					−/−	31; this study
P. putida Idaho NRRL B-18435		+	+		+				ND/ND	34
P. putida L30-3-8[a]			+	+					ND/ND	53
P. putida PpG1 ATCC 17453		+	+		+				ND/ND	34
P. putida S12			+		+				U/ND	34
P. stutzeri DSM 5190[T]			+	+			+	+	U/C	37, 71; this study
P. syringae L20-3-1[a]			+	+					ND/ND	53
P. syringae pv. syringae B728a			+	+			(+)[d]		U/C	This study
P. syringae pv. tomato DC3000		+	+	+					U/C	25; this study

[a] Amino acids and other charged osmolytes were not determined in this study.
[b] The role of glutamate as a compatible solute is under debate (70).
[c] U, uptake occurred; C, conversion occurred; ND, not determined in the corresponding study.
[d] (+), production occurs only when medium conditions mimic *in planta* conditions.

hance its survival rate in or on plants. Based on these facts, we hypothesize that the low efficacy of the bacterial transporter systems for betaine uptake may restrict the ability of *P. syringae* pv. syringae B728a to accumulate enough betaine for optimal osmotolerance, and it thus retains the capacity for *de novo* synthesis of trehalose for maximal osmoprotection.

Gene *ectC* of *P. syringae* pv. syringae B728a. Ectoine biosynthetic enzymes are usually encoded by the gene cluster *ectABC* (46, 59) (Fig. 1C). Intriguingly, under simulated *in planta* conditions, the wild-type strain of *P. syringae* pv. syringae B728a is able to produce the osmolyte ectoine despite the absence of the acetyltransferase EctA and the transaminase EctB. Two possible explanations for this observation can be envisioned, as follows: (i) the precursor ADABA is supplied by the host plant (11, 23, 44), or (ii) ADABA may be produced by a nonorthologous set of enzymes which do not reside in an operon with *ectC* (Psyr_0334). Several genes coding for *N*-acetyltransferases, as well as Psyr_1925 encoding a diaminobutyrate aminotransferase, were suggested to fulfill the functions of *ectA* and *ectB*, respectively (24). From some ectoine-producing microorganisms, it was already known that EctB is not essential because its respective ΔectB mutants were still capable of biosynthesizing ectoine (29; D. Stumpfe and M. Kurz, unpublished data). However, there is no precedence in the literature showing that EctA can also be replaced. This observation raises the question of the origin and the development of the *ectC* fragment in the genome of *P. syringae* pv. syringae B728a. A phylogenetic analysis (see Fig. S12 in the supplemental material) which compared EctC of *P. syringae* pv. syringae B728a with all other currently known bacterial ectoine synthases revealed (i) that several other bacteria, particularly plant-associated bacteria, possess a solitary *ectC* fragment that might be functional and (ii) that the products of solitary *ectC* genes differ significantly from those of clustered *ectC* genes, hence forming a distinctive subgroup. It can therefore be envisioned that the solitary *ectC* gene fragments represent a remnant of originally complete *ectABC* clusters, which evolved in their ecological niche toward a minimalistic cluster by evolution. It is possible that the corresponding regulatory mechanisms also coevolved and depend on a signal factor from the host plant, since simple addition of ADABA did not yield ectoine production.

NAGGN-biosynthetic pathway of *P. syringae* pv. syringae B728a. The analysis of the available *P. syringae* pv. syringae B728a genome sequence data led to the proposal of a 4.7-kb biosynthesis gene cluster for the osmolyte NAGGN. The pathway consists of the three contiguous genes, Psyr_3747 to Psyr_3749 (see Fig. S11 in the supplemental material), for which the designation *ggnABC* is proposed. The product of

ggnA (Psyr_3747) is predicted to code for an amidotransferase and is hypothesized to perform an amidation at the C-terminal end of its substrate, glutamine. Chromosomal disruption of *ggnA* abolished NAGGN production and proved *ggnA* to be involved and essential for NAGGN biosynthesis. The adjacent *ggnB* gene (Psyr_3748) encodes an acetyltransferase acetylating the N terminus of another glutamine. The reaction by which the two amino acids are fused is unknown at this time. Since no dipeptide synthase was detected in addition to their regular predicted function, we assume that the corresponding catalytic activity is embedded in either GgnA or GgnB, or elsewhere, e.g., by an unspecific peptide synthase. GgnC is predicted to be an aminopeptidase, with similarity to deblocking aminopeptidases. Aminopeptidases, particularly deblocking aminopeptidases, act at the amino terminals of peptides to release amino acids from regular peptides or peptides modified with amino-terminal acyl-type blocking groups, such as N-terminal acylated amino acids. Hence, GgnC might be necessary to cleave the dipeptide product from either GgnA or GgnB upon assembly. Alternatively, it could be hypothesized that GgnC cleaves NAGGN into glutamine amide and *N*-acetylglutamine and thereby possesses negative regulatory properties. A more detailed understanding of the roles of GgnA, GgnB, and GgnC requires more in-depth biochemical studies and is currently being investigated in our laboratories. A survey of all available *Pseudomonas* sp. genomes revealed that the NAGGN biosynthetic gene cluster is both widespread and highly conserved across the genus (see Fig. S11 in the supplemental material).

Contribution of the compatible solutes of *P. syringae* pv. syringae B728a to its epiphytic fitness. Depending on the bioavailability of precursors and on the given water availability conditions, *P. syringae* pv. syringae B728a can choose from a variety of osmolytes to assemble an optimal balance of compatible solutes. This flexibility in its stress response allows B728a to accomplish osmoregulation under a wide variety of conditions and reflects an evolutionary necessity for this bacterium to counteract desiccation stress in the harsh leaf environment. It can therefore be hypothesized that compatible solute production in *P. syringae* pv. syringae B728a contributes to the epiphytic fitness of the strain. The osmolyte NAGGN plays, in this context, a minor role, as demonstrated by reporter gene expression assays. *In planta*, the NAGGN biosynthetic genes were found to be weakly expressed in *P. syringae* pv. syringae B728a, and a deficient mutant of B728a exhibited the same survival on dry leaves as the wild-type strain. A possible explanation for this observation could be that the surviving bacteria are protected from desiccation by other osmoprotectants (i.e., betaine, trehalose, or ectoine), other mechanisms (i.e., aggregation [56], polysaccharides, or biofilms), or a combination of both, which renders NAGGN biosynthesis superfluous. In particular, the first hypothesis is in agreement with the finding that exogenous betaine or choline acts to suppress production of NAGGN. Although betaine is most likely not present in the phyllosphere, the betaine precursor choline is hypothesized to be ubiquitous and relatively abundant (77, 80) because it represents a component of the major membrane lipid phosphatidylcholine. Choline bound to phospholipids could be made disposable for epiphytic microorganisms through the action of phospholipases (1, 5), which in turn are activated in response to wounding (68) and microbial infection (21, 61). Rapid uptake of choline has been demonstrated in *Pseudomonas syringae* pv. tomato DC3000 (14), and B728a is predicted to have a homologous choline transport system. This transport is so efficient that DC3000 derives more osmoprotection from choline (which it must convert to betaine) than betaine itself, unlike bacteria from other environments where betaine is more abundant. Indeed, our studies found that addition of choline to the medium resulted in more intracellular betaine accumulation than addition of betaine itself (Fig. 3). Aside the choline/betaine system, trehalose can be further envisioned as a major compatible solute of B728a, particularly since its role in survival and population maintenance in the phyllosphere was very recently demonstrated for *P. syringae* pv. tomato DC3000 (25). However, the osmoadaption profile of *P. syringae* pv. syringae B728a in the presence of lilac strongly suggests that ectoine is the major compatible solute utilized by the bacterium *in planta*, particularly since no production of betaine or trehalose could be observed under these culture conditions (Fig. 4). This fact would also explain the better epiphytic performance exhibited by *P. syringae* pv. syringae B728a compared with that exhibited by *P. syringae* pv. tomato DC3000, because both strains exhibit comparable osmolyte profiles (Fig. 3; see also Fig. S9 in the supplemental material) and the only biosynthetic difference identified between the strains was the capacity to produce the compatible solute ectoine.

In summary, with this study we obtained a comprehensive and detailed overview on genetic and metabolic levels of the strategies used by *P. syringae* pv. syringae B728a to confer with osmotic stress. The results presented here thus serve as an important step toward a better understanding of the ecological roles of osmolytes in *P. syringae* biology. In addition to these findings, we identified during our explorative study the biosynthetic gene cluster coding for NAGGN and gained insights into a new minimalistic biosynthetic locus for ectoine production.

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