Osmotically induced synthesis of the dipeptide N-acetylglutaminylglutamine amide is mediated by a new pathway conserved among bacteria

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The dipeptide N-acetylglutaminylglutamine amide (NAGGN) was discovered in the bacterium *Sinorhizobium meliloti* grown at high osmolarity, and subsequently shown to be synthesized and accumulated by a few osmotically challenged bacteria. However, its biosynthetic pathway remained unknown. Recently, two genes, which putatively encode a glutamine amidotransferase and an acetyltransferase and are up-regulated by osmotic stress, were identified in *Pseudomonas aeruginosa*. In this work, a locus carrying the orthologous genes in *S. meliloti*, *asnO and ngg*, was identified, and the genetic and molecular characterization of the NAGGN biosynthetic pathway is reported. By using NMR experiments, it was found that strains inactivated in *asnO* and *ngg* were unable to produce the dipeptide. Such inability has a deleterious effect on *S. meliloti* growth at high osmolarity, demonstrating the key role of NAGGN biosynthesis in cell osmoprotection. β-Glucuronidase activity from transcriptional fusion revealed strong induction of *asnO* expression in cells grown in increased NaCl concentration, in good agreement with the NAGGN accumulation. The *asnO–ngg* cluster encodes a unique enzymatic machinery mediating nonribosomal peptide synthesis. This pathway first involves Ngg, a bifunctional enzyme that catalyzes the formation of the intermediate N-acetylglutaminylglutamine, and second AsnO, required for subsequent addition of an amide group and the conversion of N-acetylglutaminylglutamine into NAGGN. Interestingly, a strong conservation of the *asnO–ngg* cluster is observed in a large number of bacteria with different lifestyles, such as marine, symbiotic, and pathogenic bacteria, highlighting the ecological importance of NAGGN synthesis capability in osmoprotection and also potentially in bacteria host–cell interactions.

osmotic stress | rhizobium | peptide synthesis | GCN5-related N-acetyltransferase | glutamine amidotransferase

Bacteria that thrive in environments of elevated osmolarity possess specific mechanisms to adjust their internal osmotic status, and one of the widely used is the ability to accumulate low molecular weight organic osmolytes, the so-called compatible solutes (1), which do not interfere with cellular functions. The spectrum of compatible solutes identified is limited and comprises sugars, polyols, amino acids and derivatives, quaternary amines and their sulfonium analogues, sulfate esters, and the dipeptide N-acetylglutaminylglutamine amide (NAGGN). Usually, a given bacterium employs a selection of several osmolytes, accumulated from external source or from de novo synthesis, and the composition of intracellular compatible solutes largely depends on the growth conditions (2). The genetic processes involved in the accumulation of compatible solutes have been investigated in many bacteria, and various genes encoding uptake systems have been cloned and functionally characterized. In contrast, only few biosynthetic pathways of osmolytes have been completely elucidated at the molecular level, such as the most widely used ectoine, glycine betaine (GB), or trehalose. Genes involved in uptake or synthesis of compatible solutes are often regulated in an osmotically fashion at the transcriptional level, and this process contributes to the finely tuned osmolyte buildup (3).

Among endogenous synthesized osmolytes, the dipeptide NAGGN has been originally identified in osmotically stressed cultures of the alfalfa root-symbiont *Sinorhizobium meliloti* (4). Ever since, its presence has only been reported in a very limited number of other soil bacteria from the *Pseudomonas* genus, and in few marine bacteria (5, 6). Through physiological and biochemical approaches, some characteristics of NAGGN accumulation have been analyzed in *S. meliloti* (4) and in the human pathogen *Pseudomonas aeruginosa* (7). In both bacteria, the endogenous pool of NAGGN increases with osmolarity and, in the absence of exogenous osmoprotective compound, the dipeptide is the predominant solute at high salt concentration. Interestingly, when GB is added to the growth medium the amount of endogenously synthesized NAGGN is severely reduced (7, 8). In a pioneering study, Smith and collaborators have showed that the dipeptide synthesis does not occur ribosomally, and using an in vitro approach with crude extracts of *S. meliloti* cells, they have proposed that an intermediate, the N-acetylglutaminylglutamine (NAGG), can be produced from N-acetylglutamine and glutamine. In addition, they have suggested that the osmotically stimulated accumulation of the dipeptide requires a genetic induction. Although several mutants were isolated, none was completely devoid of NAGGN (9), and the molecular characterization of the dipeptide biosynthetic pathway has not been conducted so far. Recently, a microarray analysis of *P. aeruginosa* PAO1 transcriptional response to osmotic stress has revealed the up-regulation of two contiguous genes that putatively encode a glutamine amidotransferase and an acetyltransferase (10). Mutant strains in these two genes were impaired for growth in the presence of high NaCl concentration, and the authors made the presumption that these two genes might be implicated in the synthesis of NAGGN.

In this study, we report a genetic and molecular analysis of genes involved in the biosynthesis of NAGGN, the osmotically induced genes *asnO and ngg* of *S. meliloti*. We present evidence that Ngg triggers the formation of the dipeptide bond, producing the intermediate NAGG that is subsequently converted into NAGGN by AsnO. Remarkably, Ngg represents a unique enzymatic machinery mediating nonribosomal peptide synthesis. Furthermore, a comparison with all available bacteria sequences revealed that *ngg* and *asnO* are highly conserved in a large range of

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bacteria with different lifestyles, including halophiles, mesophiles, and both plant and human pathogens. Thus, contrary to the presumption that NAGGN biosynthesis is limited to a few bacterial species, this study provides a broader insight into the possible role of NAGGN in enhancing the ability of bacteria to adapt flexibly to varying conditions in their habitats and to colonize different ecological niches.

Results

***S. meliloti* Genome Contains a Locus Putatively Involved in NAGGN Synthesis.** A BLAST search for proteins homologous to the products of PA3459 and PA3460 from *P. aeruginosa* PAO1 (10) was performed against the *S. meliloti* genome database (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi). Two proteins encoded by the contiguous ORFs SMb20481 and SMb20482 of the pSymb replicon were identified. The SMb20481 product is homologous to the putative glutamine amidotranferase encoded by PA3459 (55% identical amino acids) and the SMb20482 product shares 53% identical amino acids with the putative acetyltransferase encoded by PA3460. These high identities extend over the entire length of the proteins, which have similar size (591 residues vs. 589 for the products of SMb20481 and PA3459, respectively, and 595 vs. 585 for the products of SMb20482 and PA3460, respectively). DNA sequence analysis suggested that the two coding sequences belong to the same transcriptional unit, as they are separated by only 1 bp. A cluster of five genes potentially encoding the four components of a dipeptide ABC transporter (SMb20476 to SMb20479) and a transcriptional regulator (SMb20480) is located 122 bp upstream of SMb20481. At the 3′ extremity of the locus, another putative transcriptional regulator is encoded by a gene (SMb20483) divergently transcribed compared with SMb20482 (Fig. 1*A*).

The product of SMb20481 has been previously named AsnO in view of its similarity with the AsnO protein of *Bacillus subtilis* (11). The sequence of both proteins shows residues conserved in members of the PurF family of glutamine-dependent amidotransferases, which use glutamine as nitrogen source for the biosynthesis of various compounds (12). Within this superfamily, proteins that belong to the classB asparagine synthetase family, like *Escherichia coli* AsnB or *B. subtilis* AsnO, catalyze specifically the transfer of the amide nitrogen of glutamine to aspartic acid, and produce asparagine (13, 14). Such specificity could not be ascribed to the *S. meliloti* AsnO, suggesting that another acceptor molecule is aminated by the enzyme (11). This work also reported that the product of SMb20482 encodes a protein with two distinct regions, an N-terminal domain conserved in acetyltransferases and a C-terminal domain conserved in cyanophycin synthetases. The refined sequence analysis conducted here reveals that this protein might be a bifunctional enzyme with N-acetylating and peptide bond–forming activities. For the reasons developed here and described later, the SMb20482 product was called Ngg for N-acetylglutaminylglutamine synthetase. The N-terminal domain is typical of N-acetyltransferases from the GCN5-related N-acetyltranferase (GNAT) superfamily. These enzymes are involved in the N-acylation of aminoglycosides, peptides, polyamines, proteins, and other molecules (15). They acylate their substrate by using an acylCoA and share a structurally conserved fold, the GNAT fold, involved in binding of the acyl donor (16). Alignment between a part of the Ngg N-terminal sequence and three GNAT members showed the presence of the motifs involved in this fold in the *S. meliloti* sequence (Fig. 1*B*), suggesting that Ngg catalyzes an N-acylation reaction. On the contrary to the N-terminal domain, the C-terminal domain of Ngg groups within the ATP-grasp family of ATP-dependent ligases. These enzymes, which catalyze the ATP-dependent ligation of a carboxylate containing molecule to an

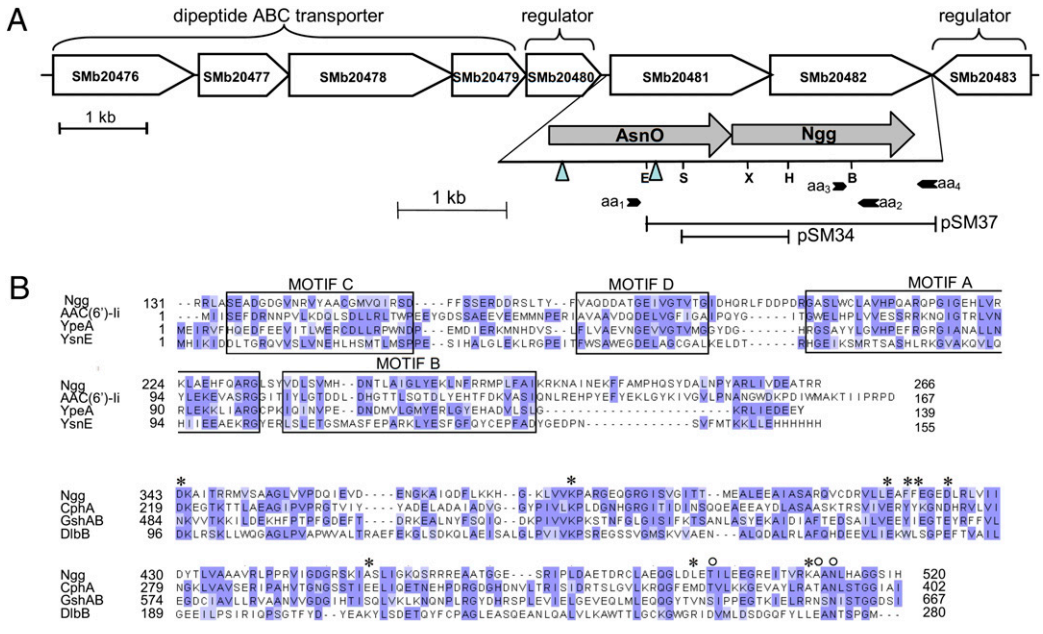

Fig. 1. The NAGGN encoding region of *S. meliloti*. (*A*) Genetic organization of the *asnO–ngg* region on the pSymb replicon. The location of the Tn5 insertions (arrowheads) and the position of the restriction sites and primers used for *ngg* cloning in pSM37 and for *ngg* inactivation by pSM34 integration are indicated. (B, BamHI, E, EcoRI, H, HindIII, S, SalI, X, XhoI.) (*B*) Homology of Ngg with members of the GNAT and ATP-grasp superfamilies. (*Upper*) Alignment of the N-terminal region of Ngg with three GNAT proteins. [AAC(6′)-Ii, aminoglycoside 6′-N-acetyltransferase type Ii from *Enterococcus faecium* (accession no. 1N71D); YpeA, N-acetyltransferase from *E. coli* (accession no. A1ADU8); YsnE, acetyltransferase from *B. subtilis* (accession no. 1YX0A).] The boxes correspond to the regions designated motifs C, D, A, and B by Neuwald and Landsman (16). (*Lower*) Alignment of the C-terminal region of Ngg with the conserved regions in three ATP-grasp proteins. [CphA, cyanophycin synthetase from *Synechocystis* sp. strain PCC6308 (accession no. AAF43647); GshAB, glutathione biosynthesis bifunctional protein from *Streptococcus agalactiae* (accession no. Q8DXM9); DlbB, D-alanine:D-alanine ligase from *E. coli* (accession no. BAB96660).] *DlbB residues interacting with ATP; °DlbB residues interacting with Mg^{2+} (17).

amino or thiol group–containing molecule, present an ATP-grasp fold with conserved amino acid residues in the regions of the phosphate-binding loop and the Mg^{2+} binding site (Fig. 1*B*) (17). They participate to the synthesis of molecules containing peptide bonds such as cyanophycin, peptidoglycan, or glutathione (18, 19).

In view of the fact that NAGGN is a modified dipeptide formed by an N-acetylated glutamine and a glutamine carrying an extra amide group, the potential catalytic properties of AsnO and Ngg are consistent with their involvement in NAGGN biosynthesis, as we propose in Fig. 2. In a first step, Ngg catalyzes both the N-acetylation of one glutamine and the formation of a peptide bond with a second glutamine, producing the intermediate NAGG. In a second step, the protein AsnO transfers the amide nitrogen of another free glutamine to the second glutamine of NAGG, giving rise to NAGGN.

***asnO* or *ngg* Inactivation Stops NAGGN Synthesis.** To assess the role of the *asnO–ngg* cluster in NAGGN synthesis by *S. meliloti*, we compared the capacity of the WT strain Rm2011, an *asnO* mutant, isolated after transposon mutagenesis (20) and called here AsnO°, and an *ngg* mutant (strain UNA442) to accumulate NAGGN. Cultures were grown to late exponential phase in MCAA growth medium containing 0.3 M NaCl, and the cellular content was analyzed by ^{13}C NMR. NAGGN and glutamate were accumulated in the WT strain as previously observed (4), whereas only glutamate could be detected in both mutant strains (Fig. 3*A*), demonstrating that the integrity of *asnO–ngg* cluster is required for NAGGN synthesis.

Ngg Is Involved in the Synthesis of NAGG. According the pathway proposed earlier (Fig. 2), a strain deficient in AsnO should accumulate a NAGGN intermediate generated by Ngg. To explain why this intermediate was not characterized from the AsnO° extracts, we postulated that the Tn5 insertion in *asnO* gene had a polar effect on the expression of the downstream *ngg* gene because the *asnO–ngg* cluster is an operon. Consequently, an *asnO* mutant created by a potentially leakier insertion and called here $AsnO^{1/2}$ was analyzed as described before. In this strain, the transposon was inserted 1,103 bp downstream the beginning of *asnO* coding sequence instead of 164 bp in AsnO° (Fig. 1 and Table S1; also see http://www.cebitec.uni-bielefeld.de/CeBiTec/rhizogate). A ^{13}C NMR spectrum of an extract from salt-stressed cells of $AsnO^{1/2}$ showed the presence of fair unidentified signals in addition to the already observed glutamate signals (Fig. 3*B*). To confirm the correlation between these signals and a functional *ngg*, this gene was expressed from a stable plasmid (pSM37) in the

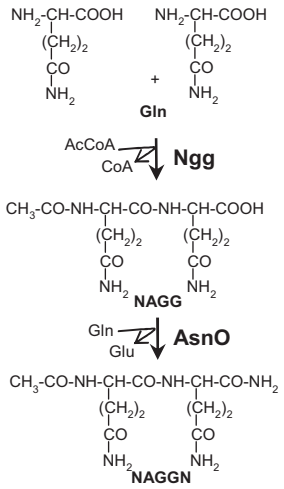

Fig. 2. Pathway for the biosynthesis of NAGGN in *S. meliloti*.

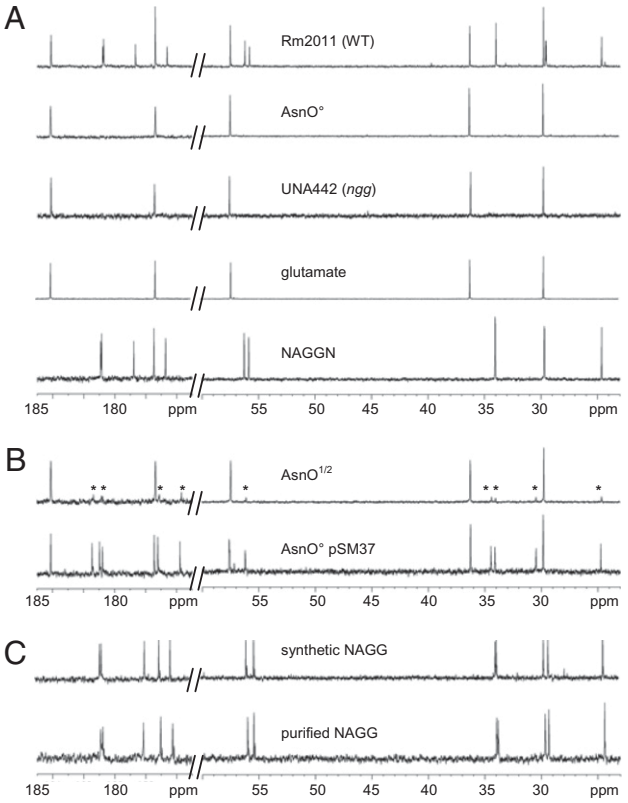

Fig. 3. The *asnO–ngg* cluster is involved in NAGGN synthesis pathway in *S. meliloti*. The solutes accumulated by strains (*A*) Rm2011 (WT), AsnO°, UNA442; and (*B*) $Asno^{1/2}$, AsnO° pSM37, grown in MCAA medium containing 0.3 M NaCl were extracted and analyzed by ^{13}C NMR. Spectra of chemically synthesized glutamate and NAGGN are also shown. Asterisks indicate the fair signals detected in addition to glutamate signals in $AsnO^{1/2}$ spectrum. (*C*) Carbon 13 NMR spectra of synthetic NAGG and of purified NAGG from AsnO° pSM37. Only regions that contain osmolyte signals (ranges of 185–175 and 60–24 ppm) are shown.

AsnO° mutant. Carbon 13 NMR analysis of osmolytes accumulated in such salt-stressed cells revealed the signals detected in the cell extracts of $AsnO^{1/2}$, and thus corroborated their Ngg origin (Fig. 3*B*). To obtain structural information on the product synthesized by Ngg, a combination of one-dimensional and 2D NMR analyses was performed on the extracts from the strain AsnO° pSM37. These analyses offer evidence for the formation of a peptide compound structurally similar to NAGG (*SI Materials and Methods*, Table S2, and Fig. S1). Additional support for this structural assignment was obtained by comparison of NMR data for the purified compound with those for a synthetically prepared NAGG (Fig. 3*C* and *SI Materials and Methods*). The slight variations in chemical shift positions between the NAGG in cellular extracts and after purification (compare Fig. 3*B* with Fig. 3*C*) are attributed to differences in the osmolyte environment. Furthermore, the purified NAGG exhibited the expected molecular mass (*SI Materials and Methods* and Fig. S2). Altogether, these results demonstrated that the *ngg* gene mediated the NAGG synthesis and confirmed the proposed pathway.

Incapacity to Synthesize NAGGN Is Deleterious for Growth of *S. meliloti* at High Osmolarity and Fairly Compensated by Exogenous GB. NAGGN is accumulated only at high osmolarity and thus, has been considered as a compatible solute. To get direct evidence, various growth experiments using, in parallel, the WT strain Rm2011 and the AsnO° mutant strain were performed. Clearly, growth of the *asnO* mutant was more affected than growth of the WT strain in

MCAA medium containing 0.4 or 0.6 M NaCl, whereas the two strains had a similar growth rate in MCAA medium without NaCl (Fig. 4*A*). Inhibition was drastic upon the addition of 0.6 M NaCl, a growth condition in which NAGGN has been shown to be the major osmolyte in WT strain (8). Growth of the two strains was also compared in the presence of GB, a very efficient osmoprotectant in *S. meliloti* (6). The addition of 1 mM GB in MCAA medium containing 0.4 or 0.6 M NaCl improved the growth rate of the *asnO* mutant (Fig. 4*B*), which suggested that the accumulated GB counteracted the defect in NAGGN. Taken together, these results demonstrated that NAGGN synthesis and accumulation are important for bacterial growth and division at high NaCl concentrations in the absence of exogenous osmoprotective compound.

Osmotic Induction of *asnO* and Reverse Effect of GB. The accumulation of NAGGN has been shown to increase as a function of salt concentration in the growth medium, and more largely to depend on medium osmolarity; such accumulation is limited by the addition of GB (8, 21). To examine if these factors control the expression of NAGGN biosynthesis genes, β-glucuronidase activity was monitored in the AsnO° strain, which contains an *asnO–gus* transcriptional fusion. First, gene expression was analyzed in cultures grown for 24 h in media of various osmolarities (Fig. 4*C*). The level of *asnO–gus* expression increased with NaCl concentration and reached a maximum value at 0.5 M NaCl, showing that the induction level of NAGGN synthesis genes largely reflected the amount of NAGGN previously quantified. Similarly, high osmolarity resulting from the addition of KCl or sucrose induced *asnO–gus* expression (Fig. 4*C*). Second, the effect of exogenous GB on *asnO* expression was investigated by comparing β-glucuronidase activity in AsnO° cultures grown in MCAA medium containing 0.4 M or 0.6 M NaCl, with or without 1 mM GB. After 24 h growth in the presence of GB, the NaCl stimulation of the β-glucuronidase activity was reduced by approximately twofold, suggesting that the accumulated GB modulated the osmotic induction of *asnO* expression (Fig. 4*D*). In cells grown in the absence of NaCl, exogenous GB had no effect on *asnO* expression. Thus, the accumulation of NAGGN results from a tight control of the expression of genes involved in the dipeptide synthesis relative to external osmolarity and GB availability.

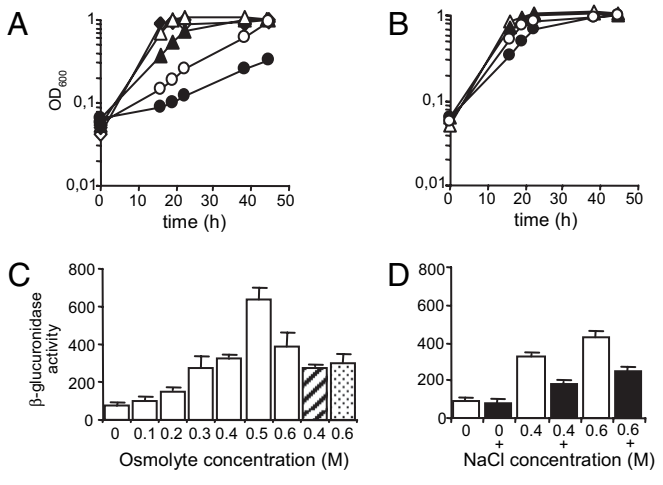

Fig. 4. Role of NAGGN in *S. meliloti* and regulation of NAGGN synthesis. (*Upper*) Growth curves of strains Rm2011 (white symbols) and AsnO° (black symbols) in MCAA medium containing (*A*) 0 NaCl (◇, ◆), 0.4 M NaCl (△, ▲), or 0.6 M NaCl (○, ●). (*B*) GB (1 mM) and 0.4 M NaCl (△, ▲) or 0.6 M NaCl (○, ●). (Lower) Effects of osmolytes on *asnO* expression. β-Glucuronidase activity of AsnO° (*asnO–gus* fusion) cells grown in MCAA medium containing (*C*) increasing NaCl concentrations (white bars), KCl (dashed bars), or sucrose (dotted bars), and (*D*) 0, 0.4, or 0.6 M NaCl with (+) or without 1 mM GB. Results are means of three independent cultures, and SDs are shown.

NAGGN Biosynthesis Pathway Is Conserved. Until now, accumulation of NAGGN has been demonstrated only in few bacteria such as various *Pseudomonas* species (22). To investigate the possible presence of NAGGN synthesis pathway in other organisms, a search for proteins homologous to AsnO and Ngg has been conducted. Such proteins have been detected only in the bacterial databases; 60 species have a gene encoding a protein closely related to AsnO, and among them 39 have an *ngg* homologue. The *asnO* and *ngg* genes are always contiguous and form a potential operon in which *asnO* is the first gene transcribed. The identities between AsnO and Ngg from *S. meliloti* and their respective homologues are particularly high, varying in the case of AsnO from a maximum of 94% identical residues (*Sinorhizobium medicae*) to a minimum of 51% (*Mycobacterium* sp. MCS), and in the case of Ngg from 91% (*S. medicae*) to 49% (*Hahella chejuensis*; Table S3).

The *asnO–ngg* arrangement is present in the genome of bacteria from Proteobacteria (α, β, γ classes), Actinobacteria (actinobacteria class), and chlorobi (chlorobia class) phyla. It is scattered within a few families and can occur in certain species of a given genus and not in others in which the complete sequence is available, e.g., an *ansO–ngg* type cluster is present in *Methylobacterium nodulans* and sp. 4-46 but not in *Methylobacterium radiotolerans*, *Methylobacterium populi*, or *Methylobacterium extorquens*, or in several *Mycobacterium* species, but not in *Mycobacterium leprae*, *Mycobacterium tuberculosis, Mycobacterium marinum, or Mycobacterium ulcerans*. This type of sporadic distribution in taxonomically distant bacteria is often associated to operon genes encoding secondary metabolites and inherited by horizontal gene transfer (23). Phylogenetic relationships among homologues of AsnO and Ngg are consistent with an acquisition via horizontal gene transfer, as the corresponding phylogenetic trees revealed clustering patterns that are not congruent with the taxonomic status of the strains based on the topology of 16S rRNA gene tree (Fig. S3).

Several marine bacteria possess an *asnO–ngg* cluster, i.e., the three chlorobia, several proteobacteria (*Aurantimonas* sp. SI85-9A1, *Fulvimarina pelagi*, α-*proteobacterium*, *H. chejuensis*, *Stappia aggregata*, *Labrenzia alexandrii*, *Brevundimonas* sp. BAL3), and actinobacteria (*Salinospora arenicola* and *Salinospora tropica*). Among them, the chlorobia *Prosthecochloris aestuarii* and *Chlorobaculum parvum* and the two members of the *Salinospora* genus are seawater-requiring bacteria undoubtedly adapted to permanent high salinity. The cluster also occurs in strains coping with frequent salinity fluctuations such as *Magnetococcus* sp. MC-1 strain isolated in Pettaquamscutt Estuary (Rhode Island, United States) and *Mycobacterium vanbaaleni* strain isolated in the watershed of Redfish Bay (Texas, United States). Soil bacteria, also exposed to large modulation of osmolarity, living in a free-living form (*Azotobacter vinelandii*, *M*. sp. 4-46, *Mycobacterium gilvum*, *Mycobacterium smegmatis*, *Kineococcus ratiotolerans*) or frequently associated to plant or human (*Aromatoleum aromaticum* sp. EbN1, *M. nodulans*, *Sinorhizobium* and *Pseudomonas* species, *Mycobacterium avium*, *Ochrobactrum anthropi*) also contain an *asnO–ngg*. Taken together, these observations suggested that this region has been widespread between bacteria living in different ecosystems and, although detailed studies are required to verify and characterize the effective production of NAGGN in such bacteria, underscore the physiological relevance of the dipeptide in organism adaptation to fluctuations imposed by the environment.

Discussion

In this study, we presented a functional characterization of genes involved in NAGGN synthesis, a dipeptide previously identified as an osmolyte in only few bacteria, and we showed that these genes and their organization are conserved among many divergent bacterial species. We demonstrated that NAGGN synthesis in *S. meliloti* required the integrity of *asnO–ngg* cluster,

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Ngg triggering first the synthesis of the intermediate NAGG and AsnO its subsequent conversion into NAGGN. These two proteins represent a unique enzymatic machinery mediating nonribosomal peptide synthesis in bacteria, which differs from the nonribosomal peptide synthetases, the poly-γ-glutamate, cyanophycin, and glutathione synthetases, the D-alanine:D-alanine ligase, the L-amino acid α-ligase, and others (24).

The dipeptide formation is mediated by Ngg, which appears to be a bifunctional protein also catalyzing an acetylation reaction. Peptide synthetases with modular organization have been described previously. However, conversely to Ngg and excepting some nonribosomal peptides (25), they catalyze only peptide-forming reactions. For example, CyaH incorporates an Asp or Arg residue into a cyanophycin primer using two distinct ATP-dependent ligase activities (19), and the synthesis of the tripeptide glutathione (γ-glutamyl-cysteinylglycine) in *S. agalactiae* is mediated by a bifunctional enzyme, GshAB, that contains the γ-glutamylcysteine and glutathione synthetase activities usually found on two distinct proteins (26). By its N-terminal domain, the Ngg protein belongs to the enormous family of GCN5-related N-acetyltransferases. In bacteria, these enzymes participate in important metabolic pathways such as the synthesis of spermidine and mycothiol, or in the extension mechanism of pepdidoglycan in Gram-positive bacteria (15, 27). An example of a bifunctional enzyme containing a GNAT domain is GlmU, which catalyzes the acetylation of glucosamine-1-phophate and its subsequent uridylylation to give UDP-N-acetylglucosamine (28). Multiple representatives of this family are present on any sequenced genome, but most of them have not been functionally characterized and their substrates are still unknown. Within the *S. meliloti* genome, 22 of the 38 ORFs annotated as encoding acetyltransferases belong to the GNAT family, two have been ascribed as acetylating ribosomal proteins, and one as a GlmU homologue. The demonstration that Ngg is involved in the biosynthesis of NAGG is a unique functional characterization of a GNAT protein in this symbiotic bacterium. Our results give a molecular support to the previous in vitro experiments that have proposed the formation of NAGGN with NAGG as a potential intermediate (9). However, conversely to these preliminary results, the bioinformatic analysis of the Ngg sequence indicated that NAGG formation can occur directly from two glutamines and not from one N-acetylglutamine and one glutamine. A possible explanation for this discrepancy is the absence of AcCoA in the in vitro reaction mixture, and thus only the Ngg activity making a peptide bond between N-acetylglutamine and glutamine could be seen. Nevertheless, additional biochemical work is still needed to establish (*i*) the sequence of the two reactions catalyzed in vivo by Ngg and (*ii*) if N-acetylglutamine can directly be used in vivo as a substrate.

The *asnO* gene is required for the final step of NAGGN synthesis, i.e., the addition of a NH_2 group to the NAGG moiety thus creating a C-terminal amide group. As a member of the PurF family, AsnO most likely catalyzes the removal of the NH_2 group from glutamine via its N-terminal domain, then transfers it to NAGG within its C-terminal domain. This is an example of an enzyme similar to the *E. coli* AsnB that is not involved in asparagine synthesis (29, 30). Sequence comparisons indicate that the proteins closely related to the *S. meliloti* AsnO group into the amidotransferase AsnB family, but that they are distinct from previously identified members of this family (Fig. S4). Such distribution supports the idea that AsnO-like proteins have their own catalytic properties. Consequently, the presence of AsnO homologues in bacteria that do not encode a Ngg homologue, such as in *Frankia alni* or in *Methylobacterium extorquens*, remains intriguing, and its biological significance is still far from clear.

The high degree of sequence identity among the AsnO and Ngg proteins and the preservation of the *asnO–ngg* organization in various bacteria showed that the NAGGN biosynthetic pathway is evolutionally well conserved in the bacterial kingdom. It is also obvious that NAGGN is produced within a taxonomically and physiologically diverse set of bacterial species. In particular, the presence of *asnO–ngg* cluster on the genome of several marine bacteria and in bacteria subjected to osmolarity fluctuations in their environment indicate that NAGGN could participate in the responsiveness to seawater salinity and osmolarity variation. Our results (Fig. 4*A*) show that the growth of an *asnO* mutant that does not produce any NAGGN is much more affected than the growth of the WT strain in a medium of elevated osmolarity, and thus offer definitive evidence of the efficiency of the dipeptide for osmoprotection of the cells. Interestingly, the *asnO–ngg* cluster of *S. meliloti* is located within a region of the pSymb replicon that has been shown recently to be important for osmoprotection, and hence for the survival of the free-living form in the rhizosphere (31). Our analysis with an *asnO* transcriptional fusion revealed that, in the absence of exogenous osmoprotective compound, an increasing NaCl concentration triggers an increase in the expression of the *asnO–ngg* operon (Fig. 4*C*). It is also noteworthy that the presence of exogenous compatible solute, such as GB, has a reverse effect on this induction (Fig. 4*D*). Thus, it is obvious that the cells sensitively adjust the expression of the *asnO and ngg* genes with a direct consequence on the NAGGN content. It has already been assumed that the biosynthesis of organic compatible solute is energetically more costly than the uptake of osmolytes from the environment when available (32).

All the genomes carrying the *asnO–ngg* cluster encode a conserved putative peptidase, with the exception of *Mesorhizobium* sp., *M. avium*, *M. vanbaalenii*, *M. gilvum*, *M. smegmatis*, and *M.* sp. MCS. Two different organizations are observed: (*i*) the gene encoding the peptidase is located immediately downstream *ngg* for 26 of the 40 *asnO–ngg* clusters, such as in *P. aeruginosa* (ORF PA3461), or (*ii*) this gene is found elsewhere on the genome in eight cases, like in *S. meliloti* (ORF SMb20466). This peptidase belongs to the M42 peptidase family that contains aminopeptidases hydrolyzing acylated N-terminal residues (33). Despites the fact that its role still remains to be established, one can suggest that such peptidase could play a role in balancing NAGGN pool during adaptation to osmotic fluctuations. In addition, particularly during an osmotic down-shock, a direct release of the dipeptide cannot be excluded. The presence of genes encoding a putative dipeptide ABC transporter located directly upstream of *asnO* might suggest such possibility or an efflux of hydrolysates.

Another attractive and intriguing feature about the *asnO–ngg* cluster comes from the work of Berges and collaborators (11). The authors have shown that a mutation in the *asnO* gene impaired the activity of FixT, an antikinase that inhibits the FixL–FixJ two-component system that controls the expression of nitrogen fixation genes in bacteroid, the symbiotic form of *S. meliloti*. It is thus tempting to argue that the NAGGN biosynthetic pathway could play an important role inside the nitrogen-fixing nodules. These data, together with the presence of an *asnO–ngg* cluster on the genome of pathogenic or symbiotic bacteria, offer insights into the relationships between osmoadaptation and host interactions. In view of the variety of stresses encountered by pathogenic bacteria during the course of infection, a number of osmoprotective compounds have already been linked to the virulence potential of certain pathogens (34). Therefore, our study raises questions about the effectiveness of NAGGN as a latent virulence factor in pathogenic bacteria. AsnO and Ngg proteins, which are conserved in animal and human pathogens (*M. avium*, *P. aeruginosa*, *Pseudomonas mendocina*, and *O. anthropi*), but absent in eukaryotes, could be consequently attractive targets for antibacterial drugs.

Finally, the characterization of the *asnO–ngg* cluster offers a set of biosynthetic genes for metabolic engineering technology, to install the production of NAGGN in bacteria or agriculturally important crop plants to improve their tolerance to osmotic stress.

The introduction of novel pathways in various organisms increases the demand for precursors, and impacts metabolic fluxes, pool sizes, and gene expression (35). Because glutamate and glutamine are abundant compounds in most organisms, engineering NAGGN production might overcome the problem of precursor limitation. Furthermore, in vitro biotechnological applications would benefit of the *asnO–ngg* cluster for an efficient manufacturing production of the dipeptide.

Materials and Methods

Bacterial Strains and Growth Conditions. Strains and plasmids used in this study are listed in Table S1. *S. meliloti* strains were maintained at 30 °C in LB medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$, then grown in MCAA semisynthetic medium (4). The medium osmolarity was increased by the addition of NaCl as indicated, or by the addition of 0.4 KCl or 0.6 M mannitol, which are osmotically equivalent to 0.4 M NaCl.

Enzyme Assay. β-Glucuronidase was measured from 50 to 200 μL culture with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 1 mM BSA) containing 0.01% SDS, 0.8 mg/mL *p*-nitrophenyl β-D-glucuronide, and 40 mM β-mercaptoethanol. Reaction mixtures (1 mL) were incubated at 30 °C and the reactions were stopped with 250 μL 2M Na_2CO_3. After 1 min centrifugation at room temperature, the absorbance of the supernatant was read at 405 nm. β−Glucuronidase specific activity was calculated as (1,000 × OD_{405}) / (time × mg protein).

NMR Spectral Determination of Intracellular Osmolytes. Ethanolic extraction of cellular osmolytes and sample preparation were performed as previously (21). Details on NAGG purification and NMR spectral analyses are given in *SI Materials and Methods*.

MS Analyses. Purified and synthetic NAGG were analyzed by electrospray ionization (ESI) MS. Low-resolution MS (i.e., ESIMS) were obtained with a Bruker Esquire 3000 Plus spectrometer in the positive and negative mode. High-resolution ESIMS (HRESIMS) were conducted on an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). Details are given in *SI Materials and Methods*.

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1. Csonka LN, Hanson AD (1991) Prokaryotic osmoregulation: Genetics and physiology. *Annu Rev Microbiol* 45:569–606.
2. Ventosa A, Nieto JJ, Oren A (1998) Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* 62:504–544.
3. Bremer E, Krämer R (2000) *Bacterial Stress Responses*, eds Stortz GT, Hengge Aronis R (ASM, Washington, DC), pp 79–97.
4. Smith LT, Smith GM (1989) An osmoregulated dipeptide in stressed *Rhizobium meliloti*. *J Bacteriol* 171:4714–4717.
5. Imhoff JF (2001) True marine and halophilic anoxygenic phototrophic bacteria. *Arch Microbiol* 176:243–254.
6. Le Rudulier D, Mandon K, Dupont L, Trinchant JC (2002) *Encyclopedia of Environmental Microbiology* (Wiley, Hoboken, NJ), pp 2774–2789.
7. D'Souza-Ault MR, Smith LT, Smith GM (1993) Roles of N-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress. *Appl Environ Microbiol* 59:473–478.
8. Smith LT, et al. (1994) Osmoregulation in *Rhizobium meliloti*: Mechanism and control by other environmental signals. *J Exp Zool* 268:162–165.
9. Smith LT, Allaith AM, Smith GM (1994) Mechanism of osmotically regulated N-acetylglutaminylglutamine amide production in *Rhizobium meliloti*. *Plant Soil* 161: 103–108.
10. Aspedon A, Palmer K, Whiteley M (2006) Microarray analysis of the osmotic stress response in *Pseudomonas aeruginosa*. *J Bacteriol* 188:2721–2725.
11. Bergès H, et al. (2001) A glutamine-amidotransferase-like protein modulates FixT anti-kinase activity in *Sinorhizobium meliloti*. *BMC Microbiol* 1:6.
12. Boehlein SK, Richards NG, Schuster SM (1994) Glutamine-dependent nitrogen transfer in *Escherichia coli* asparagine synthetase B. Searching for the catalytic triad. *J Biol Chem* 269:7450–7457.
13. Larsen TM, et al. (1999) Three-dimensional structure of *Escherichia coli* asparagine synthetase B: A short journey from substrate to product. *Biochemistry* 38:16146–16157.
14. Yoshida K, Fujita Y, Ehrlich SD (1999) Three asparagine synthetase genes of *Bacillus subtilis*. *J Bacteriol* 181:6081–6091.
15. Vetting MW, et al. (2005) Structure and functions of the GNAT superfamily of acetyltransferases. *Arch Biochem Biophys* 433:212–226.
16. Neuwald AF, Landsman D (1997) GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. *Trends Biochem Sci* 22: 154–155.
17. Galperin MY, Koonin EV (1997) A diverse superfamily of enzymes with ATP-dependent carboxylate-amine/thiol ligase activity. *Protein Sci* 6:2639–2643.
18. Fan C, Moews PC, Shi Y, Walsh CT, Knox JR (1995) A common fold for peptide synthetases cleaving ATP to ADP: Glutathione synthetase and D-alanine:d-alanine ligase of *Escherichia coli*. *Proc Natl Acad Sci USA* 92:1172–1176.
19. Berg H, et al. (2000) Biosynthesis of the cyanobacterial reserve polymer multi-L-arginyl-poly-L-aspartic acid (cyanophycin): Mechanism of the cyanophycin synthetase reaction studied with synthetic primers. *Eur J Biochem* 267:5561–5570.
20. Pobigaylo N, Szymczak S, Nattkemper TW, Becker A (2008) Identification of genes relevant to symbiosis and competitiveness in *Sinorhizobium meliloti* using signature-tagged mutants. *Mol Plant Microbe Interact* 21:219–231.
21. Talibart R, et al. (1997) Transient accumulation of glycine betaine and dynamics of endogenous osmolytes in salt-stressed cultures of *Sinorhizobium meliloti*. *Appl Environ Microbiol* 63:4657–4663.
22. Pocard JA, Smith LT, Smith GM, Le Rudulier D (1994) A prominent role for glucosylglycerol in the adaptation of *Pseudomonas mendocina* SKB70 to osmotic stress. *J Bacteriol* 176:6877–6884.
23. Fischbach MA, Walsh CT, Clardy J (2008) The evolution of gene collectives: How natural selection drives chemical innovation. *Proc Natl Acad Sci USA* 105:4601–4608.
24. Yagasaki M, Hashimoto S (2008) Synthesis and application of dipeptides; Current status and perspectives. *Appl Microbiol Biotechnol* 81:13–22.
25. Sieber SA, Marahiel MA (2005) Molecular mechanisms underlying nonribosomal peptide synthesis: Approaches to new antibiotics. *Chem Rev* 105:715–738.
26. Janowiak BE, Griffith OW (2005) Glutathione synthesis in *Streptococcus agalactiae*. One protein accounts for gamma-glutamylcysteine synthetase and glutathione synthetase activities. *J Biol Chem* 280:11829–11839.
27. Rajan LA, et al. (2008) Characterization and phylogenetic analysis of ectoine biosynthesis genes from *Bacillus halodurans*. *Arch Microbiol* 190:481–487.
28. Olsen LR, Roderick SL (2001) Structure of the *Escherichia coli* GlmU pyrophosphorylase and acetyltransferase active sites. *Biochemistry* 40:1913–1921.
29. Ren H, Liu J (2006) AsnB is involved in natural resistance of *Mycobacterium smegmatis* to multiple drugs. *Antimicrob Agents Chemother* 50:250–255.
30. Miller MT, Bachmann BO, Townsend CA, Rosenzweig AC (2001) Structure of beta-lactam synthetase reveals how to synthesize antibiotics instead of asparagine. *Nat Struct Biol* 8:684–689.
31. Domínguez-Ferreras A, et al. (2006) Transcriptome profiling reveals the importance of plasmid pSymB for osmoadaptation of *Sinorhizobium meliloti*. *J Bacteriol* 188: 7617–7625.
32. Oren A (1999) Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev* 63:334–348.
33. Ando S, et al. (1999) Thermostable aminopeptidase from *Pyrococcus horikoshii*. *FEBS Lett* 447:25–28.
34. Sleator RD, Hill C (2002) Bacterial osmoadaptation: The role of osmolytes in bacterial stress and virulence. *FEMS Microbiol Rev* 26:49–71.
35. Rontein D, Basset G, Hanson AD (2002) Metabolic engineering of osmoprotectant accumulation in plants. *Metab Eng* 4:49–56.