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Identification and Characterization of a NaCl-Responsive Genetic Locus Involved in Survival during Desiccation in *Sinorhizobium meliloti*

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The *Rhizobiaceae* are a bacterial family of enormous agricultural importance due to the ability of its members to fix atmospheric nitrogen in an intimate relationship with plants. Their survival as naturally occurring soil bacteria in agricultural soils as well as popular seed inocula is affected directly by drought and salinity. Survival after desiccation in the presence of NaCl is enabled by underlying genetic mechanisms in the model organism *Sinorhizobium meliloti* 1021. Since salt stress parallels a loss in water activity, the identification of NaCl-responsive loci may identify loci involved in survival during desiccation. This approach enabled identification of the loci *asnO* and *ngg* by their reduced ability to grow on increased NaCl concentrations, likely due to their inability to produce the osmoprotectant N-acetylglutaminylglutamine (NAGGN). In addition, the mutant harboring *ngg*::Tn*5luxAB* was affected in its ability to survive desiccation and responded to osmotic stress. The desiccation sensitivity may have been due to secondary functions of Ngg (N-acetylglutaminylglutamine synthetase)-like cell wall metabolism as suggested by the presence of a D-alanine-D-alanine ligase (dAla-dAla) domain and by sensitivity of the mutant to β-lactam antibiotics. *asnO*::Tn*5luxAB* is expressed during the stationary phase under normal growth conditions. Amino acid sequence similarity to enzymes producing β-lactam inhibitors and increased resistance to β-lactam antibiotics may indicate that *asnO* is involved in the production of a β-lactam inhibitor.

The *Rhizobiaceae* are a bacterial family of enormous agricultural importance due to their ability to fix atmospheric nitrogen in an intimate relationship with plants (1). They occur naturally in most agricultural soils, and their survival is affected directly by both drought and salinity (2). Unfortunately, changes in climate patterns are occurring and, as a direct consequence, salinification and desertification are some of the major threats to agricultural land use. It is estimated that over 40% of arable land will be affected by desiccation and salinity by 2025 (2). Furthermore, production of seed inocula often includes a drying phase negatively affecting CFU of added rhizobia (3, 4), potentially resulting in desiccation-induced viable but nonculturable cells (5).

A multitude of conditions affecting survival during desiccation of agriculturally important *Rhizobiaceae* have been studied (6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20). These conditions include the intrageneric differences to cope with desiccation stress which affect survival (5, 6, 9, 10, 13, 18, 21, 22). These data suggest that no single gene affects the ability of rhizobia to survive desiccation but that several mechanisms are likely responsible.

One of the conditions affecting the ability of *Sinorhizobium meliloti* to survive desiccation is NaCl availability (10, 13); hence, we studied the response of *S. meliloti* 1021 to drought in conjunction with different salt stresses (13). We showed that survival of the strain in the presence of desiccation when exponentially growing cells were resuspended in water was worse than under conditions of resuspension in water containing 400 mM NaCl. In contrast, increasing amounts (200 and 400 mM) of sodium chloride added to standard media such as yeast mannitol broth (YMB) and phosphate mannitol medium (PMM) were found to enhance survival. This effect was mainly caused by the presence of anions rather than cations in the growth medium (10, 13). These observations indicate that the response to desiccation in conjunction with the presence of NaCl is physiological in origin (4, 13) and that the underlying genetic mechanisms are important for the survival of the cell during desiccation. The *in situ* relevance of this physiological effect is further demonstrated by the fact that NaCl and the availability of osmoprotectants affect survival of rhizobia in soil (23, 24) as well as in seed inocula (25).

Examples of genetic mechanisms involved in survival during desiccation have been identified previously and include the operon *agl* for trehalose-maltose and sucrose uptake (*smb03060* to *smb03065*) (26) and the trehalose-maltose-sucrose operon (*thu*; *smb20324* to *smb20330*) identified by Jensen et al. (27). The *thu* operon is expressed to a higher level during an osmotic upshift (28), and McIntyre et al. (29) showed that loci involved in trehalose metabolism affect survival during desiccation. The above-described uptake systems involve osmoprotectants, which accumulate during salt stress, thus potentially affecting NaCl-mediated survival during desiccation.

In addition to trehalose uptake, a target of desiccation responses is the cell wall, which is also affected by the presence of NaCl (15, 30, 31). These responses include exopolysaccharide production (32, 33, 34), which is assumed to affect desiccation survival (4). Furthermore, Wei et al. (35) and Miller-Williams et al. (36) have shown that responses to NaCl and osmotic stress affect genes encoding proteins potentially involved in central me-

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tabolism such as elongation factors, DNA ligases, chaperones, and cell division proteins. It is well known that DNA is a target for desiccation stress, and recently Humann (37) confirmed this for rhizobia.

In this study, the hypothesis that certain NaCl-responsive loci are involved in survival during desiccation was further tested. We identified Tn*5luxAB*-tagged loci that are responsive to increased concentrations of NaCl. Some of these mutants were also tested for their ability to survive desiccation, and NaCl-responsive loci involved in survival during desiccation were characterized for their response to water stress. Finally, we also tested their response to β-lactam antibiotics to test for potential involvement in cell wall function.

MATERIALS AND METHODS

Materials. *S. meliloti* 1021 was obtained from our strain collection (38). An *Escherichia coli* strain containing plasmid pRK2013 (39) was obtained from T. Lessie (University of Massachusetts) and used for triparental matings. Phage ΦM12, employed to reconstruct Tn*5luxAB* transcriptional fusions, was supplied by Graham Walker (40). A Tn*5luxAB* transcriptional fusion mutant bank was created for *S. meliloti* strain 1021 as described previously (41). *S. meliloti* strains Sce1 and Sce12 were identified and characterized in this study. *S. meliloti* strain CV2, which serves as the positive control for NaCl-dependent luciferase expression, was previously described by Milcamps et al. (41). A negative control for NaCl-dependent luciferase expression (strain 1D1) was randomly chosen from the mutant bank. *E. coli* DH5α- and JM109-competent cells were obtained from Invitrogen or prepared following standard protocols (42). All strains were maintained on tryptone-yeast extract (TY) plates with the appropriate antibiotics. Media used were TY (43), GTS (41), LB (42), and YMB and PMM (5, 13, 44). Alfalfa seeds were obtained from the seed company Outsidepride (BS-ALFALFA-5; lot no. A2N-1769-3; Outsidepride, Salem, OR). According to the manufacturer, these seeds had not been treated with any chemicals.

Induction studies using Tn*5luxAB* transcriptional fusions. The screening of the Tn*5luxAB* transcriptional fusion mutant bank was carried out using a photonic camera (Hamamatsu C1966-20 [45]) as described previously by Milcamps et al. (41) and adapted to screen for luciferase fusions induced during exposure to NaCl as follows. The induction screenings were performed on PMM plates (PMM with 15 g/liter agar) containing 400 mM NaCl, and luciferase expression was measured after 4 and 8 h of incubation. Strains were selected based on an increase in luciferase expression compared to the same strain not exposed to NaCl. Those strains with increased luciferase expression in four replicate screenings were considered further. The luciferase activity assays using a luminometer were performed as described by Phillips et al. (46), with the following modification: PMM was employed. One culture (optical density [OD] = ~0.2) was split into six culture tubes (23 mm in diameter, 5 ml of culture per tube) and diluted with the same amount (1:1) of PMM or PMM with 800 mM NaCl, resulting in PMM media containing 0 and 400 mM NaCl in triplicate. Light emission was measured using a TD 20/20 luminometer by adding a 100-μl subsample to a 10-μl bovine serum albumin (BSA) (Sigma Co.) (2%)–aldehyde (n-decanal) (Sigma Co.) (0.2%) solution. Strains with a positive response during 8 h in three replicate experiments were explored further.

When the response to water activity and osmotic stress was tested, media with double the amount of the final concentration of NaCl were mixed 1:1 with the growing cell culture. Except in the screening in which luciferase expression is reported as relative light units (RLU)/ml/min, all of the study results are reported as RLU/ml/min/OD at 595 nm (OD_{595}). Concentrations mimicking a reduction in water activity were calculated from empirically obtained data presented by Leistner and Rodel (47) and by Brown (48). The addition to PMM of 400 mM NaCl, 520 mM glycerol, 222 mM sucrose, or 780 mM polyethylene glycol 200 (PEG 200) results in a final water activity value (A_w) of 0.986.

Growth experiments. Initial growth experiments were performed as follows. Five milliliters of PMM and PMM amended with 400 mM NaCl in culture tubes was inoculated with 50 μl of a 3-day-old TY culture and incubated at 28°C with shaking at 220 rpm. Over the course of 5 days, growth was checked twice daily and compared to that of *S. meliloti* 1021. Growth curves were generated using 30 ml media in 250-ml flasks, inoculated with 1/100 (vol/vol) 3-day-old TY culture, and incubated at 28°C and agitated at 220 rpm. Antibiotics were used at the following concentrations: kanamycin at 25 μg/ml, streptomycin at 25 μg/ml, spectinomycin at 25 μg/ml, and chloramphenicol at 10 μg/ml. For the amino acid complementation studies, 5 ml of PMM–400 mM NaCl in culture tubes was inoculated with 50 μl of full-density, 3-day-old TY cultures. Amino acids were added at a final concentration of 50 μg/ml. Cultures were incubated at 28°C and agitated at 220 rpm.

Molecular methods. All molecular procedures were based on protocols of Sambrook and Russell (42) or performed as previously described by Wolk et al. (45) and Milcamps et al. (41). The copy number of Tn*5luxAB* was determined using Southern hybridization, and the rescue, sequencing, and insertion site determinations were performed as described by Milcamps et al. (41) and Wolk et al. (45). Fragments resulting from BglII and EcoRI restriction digests were separated on 0.7% agarose gels and transferred to nitrocellulose filters using Southern blotting. EcoRI-digested, digoxigenin (DIG)-labeled pRL1063a served as the probe (Boehringer Mannheim, Richfield, CT). The insertion site was determined by sequencing outward from the insertion sequence using primers as previously described by Milcamps et al. (41) and comparing the sequences to the *S. meliloti* 1021 database (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi/). The Tn*5luxAB* insertions were transduced using ΦM12 to reconstruct the same mutation in a new genetic background to reduce the possibility of secondary mutations (40). Transductants were selected on GTS with kanamycin and streptomycin, and the copy number of Tn*5luxAB* was determined as described above.

Molecular sequence analysis. The amino acid sequences from the loci tagged in strain Sce10 and Sce11 were searched using BLAST and the cDart and the conserved domain database at NCBI (http://www.ncbi.nlm.nih.gov). The promoter prediction programs employed were (i) Neural Networks (http://www.fruitfly.org/seq_tools/promoter.html; 49), (ii) Sequence Alignment Kernel (50), and (iii) Virtual Footprint (http://prodoric.tu-bs.de/vfp/vfp_promoter.php; 51).

Antibiotic susceptibility. A full-density TY culture was diluted in sterile water to OD_{595} = 0.05. A volume of 100 μl of diluted cells was spread on PMM plates, and a filter paper with a defined amount of antibiotics was placed on the plate surface. Antibiotic discs were generated from filter paper or supplied by Benton-Dickinson (Sparks, MD). Plates were incubated for 3 days at 28°C, and growth was checked twice daily. The antibiotics tested were streptomycin, kanamycin, bacitracin, cycloserine, penicillin, and vancomycin. Inhibition with lysozyme was also tested by dropping 5 μl of a 50 mg/ml solution onto a lawn of cells. Plates were checked daily for inhibition of the lawn.

Survival during desiccation. This method was previously described by Vriezen et al. (13) and used with the following modifications. Three culture tubes containing 5 ml liquid TY medium with the appropriate antibiotics were inoculated with one isolated colony from fresh TY plates and grown to full OD at 28°C and agitated at 220 rpm. A 50-μl volume of these cultures were transferred to three culture tubes containing 5 ml PMM–400 mM NaCl and antibiotics and incubated until OD_{595} values of 0.2 to 0.4 were reached. Equal amounts of cells, estimated using the OD_{595} values, were concentrated in a microcentifuge (13,000 × *g* for 2 min), the supernatant was removed, and the pellets were washed in 1 ml of PMM–400 mM NaCl. Suspensions (100 μl) were pipetted onto a membrane filter in a microcentrifuge tube. Six tubes were stored in a 450-ml glass jar containing 100 ml oversaturated KCl solution, which resulted in a relative humidity in the air phase of 22%. After a storage time of at least 3 days at

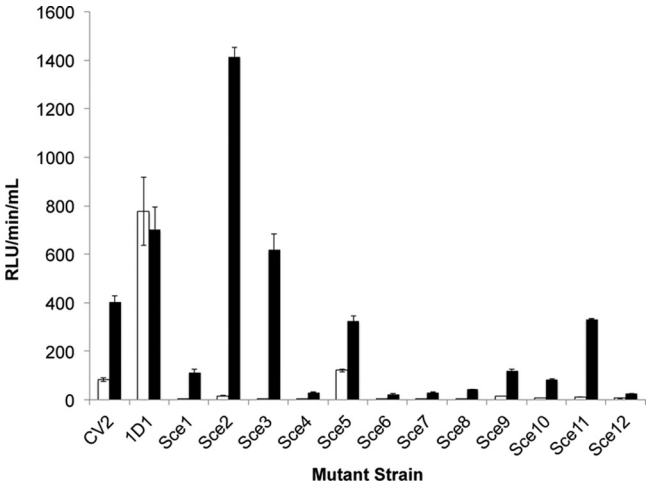

FIG 1 Identification of *S. meliloti* 1021 mutants carrying NaCl-responsive Tn*5luxAB* transcriptional fusions after 8 h of exposure to 400 mM NaCl. RLU, relative light units; white bar, PMM; black bar, PMM–400 mM NaCl; Sce, sodium chloride expressed. Error bars represent standard errors of the means (SEM); $n = 9$ except for CV2 ($n = 6$) and 1D1 ($n = 12$). Using the one-sided *t* test with equal variances, all luciferase fusions were significantly expressed on the $P < 0.001$ level.

room temperature in the dark, samples were removed and exposed to 100% relative humidity (RH) for 1 h. YMB (1 ml) was added to resuspend the cells, and surviving CFU were established. The relative survival rates were calculated using the CFU at $T = 0$ as 100%.

Symbiotic phenotypes. The mutants selected were tested for their symbiotic phenotypes as described by Milcamps et al. (41). Inoculated seedlings were incubated for 4 weeks at 20°C with a 12-h light and dark cycle. Alfalfa roots were checked for the occurrence of nodules.

RESULTS

Identification of a NaCl-responsive putative operon. The goal of this work was to identify genetic loci in *S. meliloti* 1021 involved in survival during desiccation. Previous studies have shown that survival during desiccation of *S. meliloti* increases in the presence of NaCl (10, 13). Therefore, a *S. meliloti* Tn*5luxAB* transcriptional fusion mutant bank (41) was screened for an increase in luciferase expression during exposure to 400 mM NaCl followed by a screen for loci affecting the desiccation phenotype of mutant strains. After 8 h of exposure to NaCl, 12 mutants whose expression was significantly ($P < 0.001$) induced in the presence of NaCl were identified (Fig. 1) that had a single copy of Tn*5luxAB* integrated (data not shown). These strains were annotated sodium chloride expressed-strains, abbreviated as Sce1 to Sce12. Except for Sce5 and Sce12, all strains showed a significant increase in luciferase expression after 3 h of incubation in the presence of 400 mM NaCl (fold increase ≥ 2) ($P < 0.001$; $n \geq 6$). The genomic sequence of regions immediately adjacent to Tn*5luxAB* was determined for 10 strains and was used to identify the tagged loci in *S. meliloti* 1021 (Table 1). Expression of Sce10 (*asnO*) in response to NaCl was confirmed using reverse transcription-PCR (RT-PCR) (data not shown).

Four of the 10 Tn*5luxAB*-tagged loci identified in strains Sce2, Sce3, Sce10, and Sce11 mapped to the same part of the genome and had inserted into what appears to be one operon (Fig. 2A). The first four genes are involved in dipeptide uptake, the fifth is a potential regulator of this operon, and the last two are hypothetically involved in the synthesis of NAGGN, a compatible solute known to result in growth at increased osmolarity upon osmotic shock (52, 53).

Ngg (N-acetylglutaminylglutamine synthetase)::Tn*5luxAB* affects the ability to respond to water stress. To test if the tagged mutants are indeed affected in their ability to grow on increased concentrations of NaCl, eight mutants (Sce1, Sce2, Sce3, Sce4, Sce6, Sce9, Sce10, and Sce11) were grown in PMM and in PMM amended with 400 mM NaCl. No mutant showed differences in growth compared to the reference strain in PMM, indicating the absence of auxotrophs under this condition. However, in the presence of 400 mM NaCl, the ability of strains Sce10 and Sce11 to grow was reduced (Fig. 3A). The Tn*5* mutations in these two

TABLE 1 Selected NaCl responsive Tn*5lux*AB transcriptional fusions in *S. meliloti* 1021 after 8 h of incubation

S. meliloti strain	Sequencing and BLAST result(s)			Tn*5luxAB* location[c]	Reference(s) or source
	Description[a]	Locus	Gene/locus name		
1021					38
Sce1	Hypothetical protein with SH3 region	*smc01590*		256–265	This study
Sce2	Putative dipeptide transporter permease and ATP binding protein	*smb20478*		572–580	This study
Sce3	Putative dipeptide ABC transporter ATP binding protein	*smb20479*		124–133	This study
Sce4	Putative nutrient deprivation induced	*smb20227*	*ndiA1*	299–307	67, 68
Sce5	Hypothetical protein HAD superfamily	*smc04299*	*loe3*[b]	−1–7	56
Sce6	Conserved hypothetical protein	*smc01445*		4–12	This study
Sce7	NS				This study
Sce8	Putative methyl transferase	*smb20238*	*loe2*[b]/C1	17–26	41, 56
Sce9	NS				This study
Sce10	Asparagine synthase	*smb20481*	*loe4*[b]/*asnO*	979–987	44, 53, 55, 56, 69, 70
Sce11	GCN5-related N-acetyltransferase	*smb20482*	*loe6*[b]/*ngg*	1058–1066	44, 53, 56, 69, 70
Sce12	Short-chain alcohol dehydrogenase	*smb20073*		273–281	This study

[a] HAD, haloacid dehalogenase; NS, not sequenced.
[b] Expressed under conditions of low oxygen.
[c] Location of the Tn*5luxAB* insertion relative to upstream ATG.

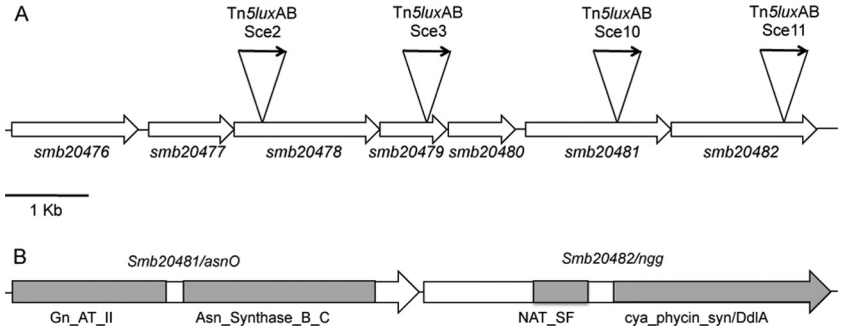

FIG 2 Physical map of 4 of the 12 identified NaCl-responsive Tn*5luxAB*-tagged loci, which may form one continuous operon. (A) Large arrows indicate an identified open reading frame (ORF); the *smb* numbers designate the *Sinorhizobium meliloti* SymB ORF reference numbers. The insertion sites of the Tn*5luxAB* in the respective strains are indicated. Locations are approximate, but sizes are to scale. Smb20476 to Smb20479 are involved in ABC transport of dipeptides. Smb20476 is a putative periplasmic dipeptide binding protein, Smb20477 a putative permease, Smb20478 the putative ATP binding protein of the permease, Smb20479 a putative ATP binding protein, Smb20480 a putative transcriptional regulator, presumable *cis*-acting, Smb20481 asparagin synthase, and Smb20482 a hypothetical protein. (B) Domain structure of AsnO and Ngg. Only domains with the highest BLAST scores are given. Abbreviations for the different domains are as follows: Gn_At_II for glutamine aminotransferase, Asn_Synthase_B_C for asparagine synthase, NAT_SF for A-acetyltransferase, cya_phycin_syn for cyanophycin synthase, and DdlA for dAla-dAla ligase.

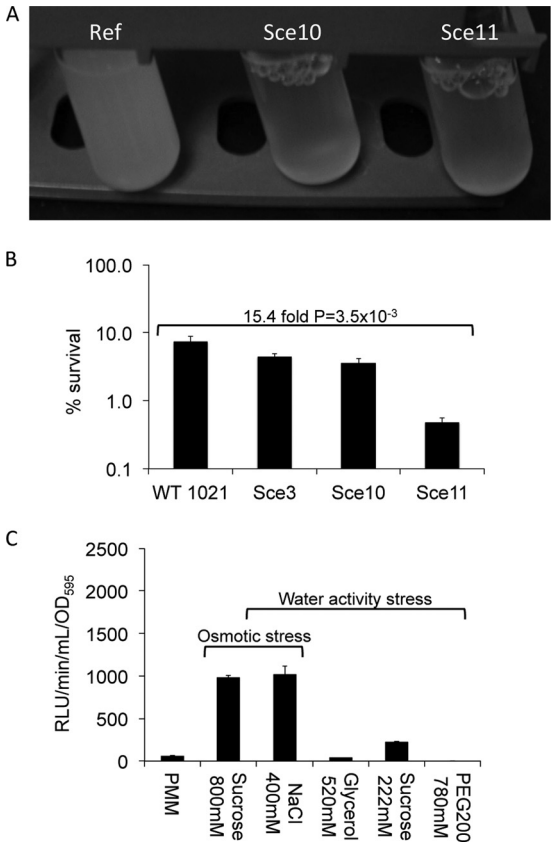

FIG 3 Responses of strains *S. meliloti* 1021, Sce3, Sce10, Sce11, and *ngg*::Tn*5luxAB* to water stresses. (A) Growth of *S. meliloti* 1021 (Ref [reference strain]), Sce10, and Sce11 in the presence of 400 mM NaCl. (B) Survival during desiccation of strains *S. meliloti* 1021, Sce3, Sce10, and Sce11. The error bars represent standard errors of the means (SEM) measured twice in three independent incubations ($n = 6$ for all cases except wild-type [WT] 1021, for which $n = 12$). (C) Responses of *ngg*::Tn*5luxAB* to a decrease in water activity (A_w = 0.986; 400 mM NaCl, 520 mM glycerol, 222 mM sucrose, and 780 mM PEG 200) and osmotic stress (0.800 osmol, 400 mM NaCl, and 800 mM sucrose). Luciferase activity was measured after 3 h of incubation. The error bars represent standard errors of the means (SEM) of the result determined for one independent incubation measured trice ($n = 3$).

strains were reconstructed in the reference strain and their geno- and phenotypes confirmed. Furthermore, complementation by a plasmid borne *asnO* in Sce10 was verified (data not shown).

To determine if loci *asnO* and *ngg* also affect the ability of *S. meliloti* 1021 to survive desiccation, strain Sce10 and Sce11 were dried at 22% RH for 3 days in PMM in the presence of 400 mM NaCl. As controls, the survival rates of *S. meliloti* 1021 and strain Sce3 were also determined. As the data indicate, strains Sce3 and Sce10 were able to survive desiccation to a degree similar to that seen with the reference strain ($P > 0.05$), while survival of strain Sce11 was 15.4-fold reduced relative to that of the reference strain ($P = 3.5 \times 10^{-3}$; Fig. 3B). The ability of strains Sce3 and Sce10 to survive desiccation shows that the presence of the transposon, the expression of luciferase in the presence of NaCl, the ability to grow in the presence of NaCl, the presence of NAGGN, or polar effects cannot account for the reduced ability of strain Sce11 to survive desiccation.

To determine if a reduction in water activity affects expression of *ngg*::Tn*5luxAB*, strain Sce11 was exposed to several compounds in concentrations that lead to the same reduction in water activity (A_w). The presence of 400 mM NaCl, 520 mM glycerol, 222 mM sucrose, or 780 mM PEG 200 in PMM results in an A_w of 0.986 (47, 48). The results depicted in Fig. 3C indicate that a reduction in the A_w does not affect expression of *ngg*::Tn*5luxAB* since in that case luciferase expression levels would be similar under all four conditions. Although *ngg*::Tn*5luxAB* does not respond to a reduction in water activity, the responses to the same osmotic stress caused by 400 mM NaCl or 800 mM sucrose, a nonaccumulating osmoprotectant in rhizobia (54), are similar under both conditions. This indicates that *ngg*::Tn*5luxAB* responds to osmotic stress and that the response to NaCl is more likely caused by osmotic stress than by ionic stress. Thus, a reduction in the A_w and an increase in ionic stress do not affect expression of *ngg*::Tn*5luxAB*.

Glutamine and glutamate complementation of growth in the presence of NaCl. *asnO* and *ngg* are genetically linked in a diverse range of microorganisms and are involved in the production of NAGGN, a dipeptide that accumulates during osmotic stress (13, 44, 52, 53). The possibility that strains Sce10 and Sce11 are limited in their response to grow in the presence of glutamine under inducing and growth-limiting conditions was tested since biosyn-

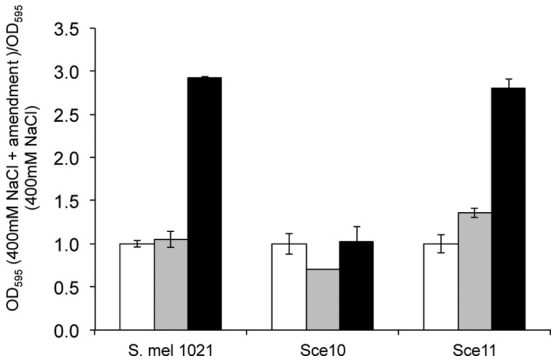

FIG 4 Growth of *S. meliloti* 1021, Sce10, and Sce11 in the presence of 400 mM NaCl with and without amino acid supplementation. Data are expressed as the ratio of the OD_{595} in PMM–400 mM NaCl. Error bars represent the SEM of three growing cultures measured once ($n = 3$). White bars, PMM–400 mM NaCl; gray bars, PMM–400 mM NaCl–glutamate; black bars, PMM–400 mM NaCl–glutamine.

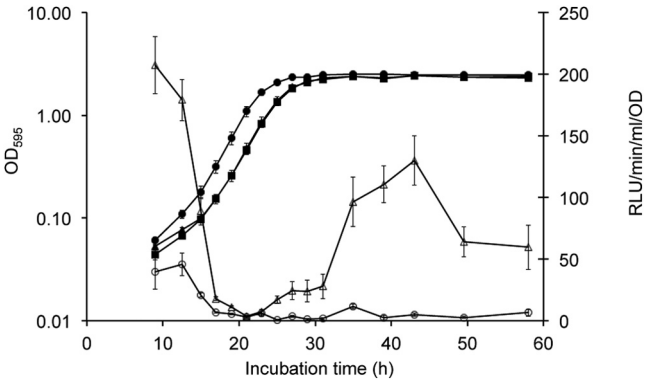

FIG 5 (A) Response of *asnO*::Tn*5luxAB* and *ngg*::Tn*5luxAB* during growth of strains *S. meliloti* 1021, Sce10, and Sce11 in liquid PMM. Error bars for OD measurements represent standard errors of the means (SEM) determined from three independent cultures ($n = 3$). RLU error bars represent the SEM of the results of three independent cultures performed in duplicate ($n = 6$). Black squares, growth of *S. meliloti* 1021 expressed in optical density (OD_{595}) units; black circles, growth of Sce11 expressed in OD units; black triangles, growth of Sce10 expressed in OD units; open circles, luciferase activity of *ngg*::Tn*5luxAB* expressed in units of RLU/min/ml/OD; open triangles, luciferase activity of *asnO*::Tn*5luxAB* expressed in units of RLU/min/ml/OD.

thesis of NAGGN uses glutamine as the substrate. The response of strain Sce11 resembles that of the reference strain, indicating that disruption of *ngg* does not lead to a malfunction in the response to NaCl in the presence of glutamine (Fig. 4). However, strain Sce10 had lost its ability to grow on 400 mM NaCl in the presence of glutamine, suggesting that disruption of *asnO* leads to the inability to use glutamine in a mechanism involved in the response to increased NaCl concentrations. Although the addition of glutamate did not affect growth in *S. meliloti* 1021 ($P = 0.32$), it stimulated growth slightly in Sce11 and reduced growth slightly in Sce10 ($P = 0.02$ and $P = 0.04$, respectively).

***asnO*::Tn*5luxAB* and *ngg*::Tn*5luxAB* are differentially expressed during the stationary phase.** Previous work indicated that the survival of *S. meliloti* increases 2.9-fold in the stationary phase compared to the exponential phase (13). To test if *asnO*::Tn*5luxAB* and *ngg*::Tn*5luxAB* are expressed during the stationary phase, luciferase activity levels were measured over the course of a growth curve (Fig. 5). When strains Sce10 and Sce11 grew in PMM, the growth curves were similar to those of *S. meliloti* 1021, suggesting the absence of auxotrophs under permissive conditions, although exponential growth started earlier in Sce11. When luciferase induction levels were measured during the course of growth, differential responses of *asnO*::Tn*5luxAB* and *ngg*::Tn*5luxAB* were found during the lag, exponential, and stationary phases. Expression of *asnO*::Tn*5luxAB* drastically decreased during exponential growth of strain Sce10 and increased again in the stationary phase. If the organism was responsive at all, expression of *ngg*::Tn*5luxAB* slightly increased very early during exponential growth; however, expression of *asnO*::Tn*5luxAB* showed a decrease in luciferase activity in the early stages of growth, while activity increased again during the stationary phase.

The domain structures of AsnO and Ngg relate to antibiotic resistance. A BLAST analysis of the amino acid sequence against the cDart and the conserved domain database at NCBI revealed that $AsnO_{S.\ meliloti\ 1021}$ contains a GnAT domain between amino acids 2 and 224 (Evalue = $6e^{-32}$) and is associated with a conserved Asn synthase domain located between amino acids 246 and 524 (Evalue = $1.7e^{-55}$) (Fig. 2B). $AsnO_{S.\ meliloti\ 1021}$ is in a class of sequences most closely related to $AsnB_{E.\ coli}$ (Evalue = $5.6e^{-118}$), a glutamine-hydrolyzing asparagine synthase, as reported earlier (53, 55, 56). More diverse members of this group of proteins include $AsnO_{Bacillus\ subtilis}$ and $LtsA_{Corynebacterium\ glutamicum}$ (55). Although previously not appreciated, $AsnO_{S.\ meliloti}$ also has similarity to β-$LS_{Streptomyces\ clavuligerus}$, a protein with the ability to synthesize clavulanic acid, an inhibitor of β-lactamases (57).

An amino acid sequence comparison of Ngg to entries in cDart and the conserved domain databases revealed two conserved domains. The first is an acetyltransferase domain between amino acids 170 and 240 (Evalue = $9e^{-6}$). The second domain has highest sequence similarity to RimK RelE ligase (Evalue = $5.2e^{-7}$), cyanophycin synthase (CphA; Evalue = $1.6e^{-56}$), and D-alanine-D-alanine (dAla-dAla) ligase (Ddl domain; peptidoglycan synthesis) between amino acids 287 and 595 (Evalue = $4.2e^{-47}$) (Fig. 2B). Potential involvement in dAla-dAla ligase activity and peptidoglycan synthesis suggests that strain Sce11 is cycloserine sensitive (58, 59) and/or penicillin sensitive (60).

Because of these similarities, we hypothesized that strains Sce10 and Sce11 are affected in their ability to deal with β-lactam antibiotics since β-lactam antibiotics target the cell wall (60). To put these hypotheses to the test, Kirby-Bauer experiments were performed (Table 2). Both strains show the expected sensitivities to several antibiotics, including streptomycin (all insensitive) and kanamycin (Tn*5luxAB* carries kanamycin resistance in Sce10 and Sce11, making them less sensitive to kanamycin). Sce10 is less sensitive to the β-lactam antibiotic penicillin and, in contrast, Sce11 is more sensitive to penicillin than reference strain *S. meliloti* 1021.

Symbiotic characterization. To further characterize the mutants, alfalfa seedlings were infected with the Tn*5luxAB* mutant strains and the ability to nodulate was recorded. All strains formed nodules; thus, no locus is essential for nodulation. When strains Sce10 and Sce11 were used to infect alfalfa roots, the strains formed pink nodules, indicating that they are nitrogen fixation proficient. Furthermore, alfalfa plants appeared the same when inoculated with *S. meliloti* 1021 or with Sce10 and Sce11 and did not appear nitrogen limited.

TABLE 2 Antibiotic sensitivity assay[a]

Antibiotic	Amt/disc	*S. meliloti* 1021		Sce10		Sce11	
		Diam (mm)	SEM	Diam (mm)	SEM	Diam (mm)	SEM
Streptomycin	10 μg	7.0	0.0	7.0	0.0	7.0	0.0
Kanamycin	30 μg	11.3	0.3	7.0*	0.0	7.0*	0.0
Bacitracin	2 IU	7.0	0.0	7.0	0.0	7.0	0.0
Lysozyme	250 μg	7.0	0.0	7.0	0.0	7.0	0.0
Cycloserine	100 μg	9.7	0.3	10	0.6	9.7	0.7
Penicillin	10 IU	14.3	0.9	7.7*	0.7	22.7*	1.5
Vancomycin	5 μg	21.3	1.2	19	0.0	22.7**	0.3

[a] The diameters of clearance around the disc (7 mm across) are presented together with the associated standard errors of the means (SEM). For each experiment, the average of the results determined for three plates is presented ($n = 3$). *, significant differences ($P < 0.01$; two-sided *t* test with equal variances) between the reference strain and strains Sce10 and Sce11; **, significant differences ($P < 0.01$; two-sided *t* test with equal variances) between strains Sce10 and Sce11.

DISCUSSION

To address our hypothesis that a genetic mechanism is in *S. meliloti* 1021 that is inducible by NaCl and affecting this organism's ability to survive desiccation (13), a genetic screen for NaCl-inducible loci tagged by Tn*5luxAB* was performed. Twelve mutants harboring single transcriptional fusions that express luciferase at higher levels with NaCl than without NaCl were found (Fig. 1). Four of these Tn*5luxAB*-tagged loci in strains Sce2, Sce3, Sce10, and Sce11 form what appears to be one operon (Fig. 2A). Parts of this operon were previously identified and described (53, 55, 56, 70). Genetic loci that are part of this operon are known to be responsive to a decrease in oxygen availability (56) as well as to an increase in NaCl (61) and to PhoB-independent phosphate limitation (62).

Our hypothesis that certain NaCl-responsive loci are involved in survival during desiccation was supported by the results showing that the ability of two of the mutants (Sce10 and Sce11, with *asnO* and *ngg* tagged, respectively) to grow at increased NaCl concentrations was reduced (Fig. 3A) and that the *ngg* locus was also involved in NaCl-mediated survival during desiccation (Fig. 3B). Induction of this locus is mainly osmotic stress related and is not a consequence of a reduction in water activity or of ionic stress (Fig. 3C). Other loci in *S. meliloti* must exist that affect survival during desiccation, since *ngg*::Tn*5luxAB* is not predominantly responsive to NaCl. This conclusion is supported by the fact that survival of reference strain *S. meliloti* 1021 is mainly affected by the presence of the chloride anion rather that its cation (13). AsnO, however, is not involved in NaCl-mediated desiccation resistance.

Although the loci tagged in Sce2, Sce3, Sce10, and Sce11 may form one large operon, experimental data supporting the idea of one large transcriptional unit are still lacking. Our expression data do indicate different dynamics between *asnO* and *ngg* during the stationary phase. If the dipeptide uptake system and *asnO* and *ngg* do not form one operon, we would expect promoter sequences just upstream of *asnO*, which were not found. Differential expression of *asnO*::Tn*5luxAB* and *ngg*::Tn*5luxAB* also indicated regulatory sites between these two open reading frames (ORFs), which were not found either; neither were terminator sites found as indicated by Kingsford et al. (63). Therefore, we expect these genes to form one large operon unless unknown genetic elements are present in this locus.

AsnO and Ngg are both involved in a pathway for the production of NAGGN, a powerful osmoprotectant. Its biochemical production involves two steps as reported by Sagot et al. (53). In this pathway, Ngg (N-acetylglutaminylglutamine synthetase) produces the dipeptide N-acetylglutaminylglutamine (NAGG) from glutamine. AsnO is a glutamine-dependent amidotransferase transferring the amide nitrogen from glutamine to N-acetylglutaminylglutamine to produce NAGGN. The disruption of one or both steps leads to slower growth in the presence of NaCl. However, the fact that mutant Sce11 shows the same response to glutamine addition in the presence of NaCl as the reference strain and mutant Sce10 does not suggests that even in the absence of functional Ngg, a substrate for AsnO is still present. Thus, (i) another pathway for NAGG production must be present in *S. meliloti* 1021 or (ii) enough NAGG is present to ensure production of NAGGN for at least a couple of generations. Although unlikely due to the reduced growth on NaCl and the postulated lack of NAGGN accumulation, a possible alternative is that (iii) the Ngg::Tn*5luxAB* fusion protein in Sce11 is only partially disrupted; e.g., the acetyltransferase domain may still function. The argument in interpretation ii has some merit since growth complementation takes place at OD < 0.6 but is reduced with higher ODs (data not shown). Regardless, potential accumulation of NAGG in the *asnO* mutant does not lead to increased growth and from the data it is clear that the postulated reduced levels of NAGGN do not explain the reduced ability to survive desiccation of strain Sce11. It is most likely that as-yet-unknown functions, e.g., involvement in cell wall metabolism for the possible reasons explained next, are associated with AsnO and Ngg.

The amino acid sequence of Ngg is most similar to that of cyanophycin synthase and contains two domains, a NAT (or GCN-5) domain and a Ddl domain. Ddl domains are involved in the synthesis of peptidoglycan, and cell wall integrity is one of the main factors affecting the ability to survive desiccation in rhizobia (15, 30, 31). Furthermore, Ddl domains are found in dAla-dAla ligases, targets for cycloserine and peptidoglycan cross-linking enzymes and for β-lactam antibiotics such as penicillin. Our observations are that strain Sce11 is indeed more sensitive to penicillin than the reference strain; however, it is not affected by cycloserine (Table 2). The reduced ability to survive desiccation of strain Sce11is explained by a weaker cell wall, which is less able to withstand the extreme hypo-osmotic stress upon rehydration. That this may occur was shown previously by Salema et al. (30) and by Bushby and Marshall (31), who found the cell wall and envelope to be a major target upon rehydration.

The amino acid sequence of AsnO is most similar to that of AsnB, or asparagine synthase, and contains a GnAT domain and an Asn domain (Fig. 2B). Previously not appreciated, the similarity of AsnO to β-LS allows the hypothesis that AsnO may be in-

volved in the production of a β-lactamase inhibitor. In this case, dysfunction of the production of inhibitor leads to an increased resistance to antibiotics such as penicillin. The data in Table 2 show exactly that: strain Sce10 is less sensitive to the β-lactam antibiotic.

Even though strains Sce10 and Sce11 form effective nodules on alfalfa, it is unclear if both strains are effective during competition under conditions more closely matching natural environments. Soil is a harsh environment, and cells are continuously exposed to challenging environmental conditions (64). Soils frequently undergo drying and rewetting cycles. During drying, salts accumulate, which further reduces the ability of microbes to grow. Also, many soil organisms produce antimicrobials, which may have negative effects on strains lacking the *ngg* locus. The plants may reduce the competitiveness of strains lacking locus *ngg* by excreting toxic secondary metabolites. On the other hand, a slight growth advantage of strain Sce11 may allow faster invasion of hair roots and have a positive effect of nodule occupancy since growth may be a primary factor during infection.

In conclusion, the function of AsnO and Ngg is not limited to the production and accumulation of NAGGN alone. First, NAGGN accumulation is not involved in survival during desiccation. Second, AsnO and Ngg may have functions such as the production of a β-lactamase inhibitor and involvement in cell wall metabolism. Third, they are involved in responses to β-lactam antibiotics, and fourth, a regulatory function has been attributed to AsnO during nodule development (55, 70), although the nature of the regulation remains obscure. Alternatively, it is interesting to speculate about physical location and other roles; e.g., the opposite effects of AsnO and Ngg on antibiotic resistance may indicate a physical interaction between the proteins with antagonistic effects on the cell wall such as changes in permeability.

Finally, identification of 4 of 10 loci that are responsive to NaCl and oxygen deprivation is at least curious and was mentioned before (65). A possible explanation may be the reduced solvability of oxygen in media high in salt. It may also be explained by being part of the environmental niche of this organism. In the rhizosphere, salts accumulate and available oxygen may be in short supply. Most interestingly, evidence that *asnO* and *ngg* have undergone lateral gene transfer has been presented (J. A. C. Vriezen, unpublished data, and reference 66). Nevertheless, we have identified a system that warrants further investigation on both the molecular and the ecological levels.

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