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Transcriptome Profiling Reveals the Importance of Plasmid pSymB for Osmoadaptation of *Sinorhizobium meliloti*▽†

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In this work, DNA microarrays were used to investigate genome-wide transcriptional responses of *Sinorhizobium meliloti* to a sudden increase in external osmolarity elicited by addition of either NaCl or sucrose to exponentially growing cultures. A time course of the response within the first 4 h after the osmotic shock was established. We found that there was a general redundancy in the differentially expressed genes after NaCl or sucrose addition. Both kinds of stress resulted in induction of a large number of genes having unknown functions and in repression of many genes coding for proteins with known functions. There was a strong replicon bias in the pattern of the osmotic stress response; whereas 64% of the upregulated genes had a plasmid localization, 85% of the downregulated genes were chromosomal. Among the pSymB osmoresponsive genes, 83% were upregulated, suggesting the importance of this plasmid for *S. meliloti* osmoadaptation. Indeed, we identified a 200-kb region in pSymB needed for adaptation to saline shock which has a high density of osmoregulated genes.

Salinity is one of the most serious factors limiting the productivity of agricultural crops (48). Nearly 40% of the world's land surface can be categorized as having potential salinity problems (66). Rhizobia are aerobic gram-negative soil bacteria that are able to establish nitrogen-fixing symbioses with leguminous plants under nitrogen deprivation conditions. During this process, an exchange of molecular signals between the two partners occurs (32, 57), leading to the formation of root nodules, where biological nitrogen fixation takes place (44, 56).

The *Rhizobium*-legume symbiosis is more sensitive to salt or osmotic stress than the free-living rhizobia are (12, 65). Salt stress may inhibit the initial steps of the symbiosis (root colonization and infection and nodule development), but it also has a depressive effect on nitrogen fixation (66). Nevertheless, rhizobial tolerance to salinity is also important for the symbiosis. High salt concentrations may have a detrimental effect on rhizobial populations as a result of direct toxicity, as well as through osmotic stress. Furthermore, it has been shown that *Rhizobium* mutants whose adaptation to high salinity is affected have deficiencies in their symbiotic capacity (41). These results emphasize the importance of studying the mechanisms of adaptation of rhizobia to changes in the osmotic conditions of the environment.

An increase in the external salinity and osmolarity triggers an outflow of water from the cell, resulting in a reduction in turgor and dehydration of the cytoplasm, which causes a decrease in the cytoplasm volume and thus an increase in the ion concentration in the cytosol (53). Rhizobia may use distinct mechanisms for osmotic adaptation when they are exposed to salt stress, such as intracellular accumulation of low-molecular-weight organic solutes (osmolytes), including amino acids, sugars and polyamines, or accumulation of ions (i.e., K^+) (37, 66). Other salt-induced responses in rhizobia are changes in cell morphology and size and modifications in the pattern of extracellular polysaccharides (30, 31, 54, 66). The latter responses may have an impact on the symbiotic interaction because exopolysaccharides and lipopolysaccharides (LPS) are very important for the development of root nodules. Nevertheless, response and adaptation to environmental stresses are probably complex phenomena involving many physiological and biochemical processes that likely reflect changes in gene expression and in the activity of enzymes and transport proteins (10, 60).

DNA microarrays have been used to examine gene expression in response to various abiotic stresses in *Sinorhizobium meliloti* (29, 50). Rüberg et al. (50) determined that prolonged exposure of *S. meliloti* 1021 to 380 mM NaCl resulted in inhibition of amino acid biosynthesis, iron uptake, motility, and chemotaxis and activation of genes related to polysaccharide biosynthesis and transport of small molecules (amino acids, amines, peptides, anions, and alcohols).

The terms "salinity stress" and "hyperosmotic stress" have often been used in a confusing manner. Although these stresses are similar, it has been shown that they can be recognized as different environmental stimuli by some organisms (20, 27). On the other hand, the response to any stress condition can be divided into two phases: the initial reaction to sudden exposure and the subsequent cellular adaptation to prolonged growth under that condition. Consequently, it is interesting to monitor not only the persistent changes in the transcriptional profile of cells continuously cultured at high salinity but also the changes in the gene expression pattern on a time-resolved scale following a shock.

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics[a]	Reference
Rm1021	SU47 *str-21* Str^r (wild type)	36
Rm5378	ΔΩ5020-5011::Tn*5-oriT*	13
Rm5408	ΔΩ5033-5007::Tn*5*-233 Gm^r-Sp^r Nm^r	7
RmF117	ΔΩ5060-5033::Tn*5*-233 Gm^r-Sp^r Nm^r	7
RmF514	ΔΩ5061-5047::Tn*5*-11 Gm^r-Sp^r Nm^r	7
RmF638	ΔΩ5145-5061::Tn*5*-233 Gm^r-Sp^r Nm^r	7
RmF666	ΔΩ5146-5111::Tn*5*-233 Gm^r-Sp^r Nm^r	7
RmF680	ΔΩ5085-5061::Tn*5*-233 Gm^r-Sp^r Nm^r	7
RmF693	ΔΩ5085-5142::Tn*5*-233, Gm^r-Sp^r Nm^r	7
RmF728	ΔΩ5177-*lac-56*::Tn*5* Gm^r-Sp^r Nm^r	7
RmF909	ΔΩ5085-5047::Tn*V* Gm^r-Sp^r Nm^r	7
RmG506	Ω5040::Tn*5*-233 ΔG506 Gm^r-Sp^r Nm^r	7

[a] Gm^r, gentamicin resistance; Sp^r, spectinomycin resistance; Nm^r, neomycin resistance; *lac*, lactose utilization genes; *oriT*, origin of transfer.

To obtain more insight into the response of *S. meliloti* to an osmotic upshift, a transcriptional profiling approach was utilized to investigate the initial reaction to a sudden increase in osmolarity elicited by the addition of NaCl or sucrose to exponentially growing cultures and to determine the time course of the response at the transcriptional level. We obtained clear evidence that salt stress and hyperosmotic stress have similar effects on gene transcription in *S. meliloti*, provoking induction of a large number of genes (mainly on plasmids) having unknown functions and repression of many chromosomal genes coding for proteins with known functions. Consistent with these results, a region in plasmid pSymB was identified as important for adaptation to saline conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are shown in Table 1. *S. meliloti* strains were grown at 30°C in TY complex medium (3) or in minimal medium (MM) containing glutamate and mannitol as nitrogen and carbon sources, respectively (22).

To induce osmotic or salt stress, cells were cultured in MM to exponential phase (A_{600}, 0.4), and then the appropriate amount of NaCl or sucrose was added to the culture in order to obtain the desired concentration of the compound. An equivalent amount of fresh MM was added to the control, unstressed cultures. Cells were then incubated under standard growth conditions (30°C, 200 rpm), and aliquots were collected after 15, 30, or 60 min or 4 h. At all times the cultures were growing exponentially. Bacteria from control and stressed cultures were briefly centrifuged, and the cell pellets were immediately frozen in liquid nitrogen and conserved at −80°C until RNA was isolated.

RNA isolation and synthesis of labeled cDNA. For microarray hybridization, RNA was isolated after mechanical disruption of the cells as described previously (50). Cy3- and Cy5-labeled cDNAs were prepared by the method of DeRisi et al. (9) from 10 μg of total RNA. Three slide hybridizations were performed using the labeled cDNA synthesized from each of the RNA preparations from three independent bacterial cultures.

For real-time reverse transcription (RT)-PCR, RNA was isolated using the TRI REAGENT LS procedure of Molecular Research Center, Inc., and the residual DNA was removed with RNase-free DNase I (Roche).

Hybridization and image acquisition. Hybridization, image acquisition, and data analysis were performed as described previously (2, 29, 50). Mean signal and mean local background intensities were determined for each spot on the microarray images using the ImaGene 5.5 software for spot detection, image segmentation, and signal quantification (Biodiscovery Inc., Los Angeles, Calif.). The $\log_2$ value of the ratio of intensities was calculated for each spot using the formula $M_i = \log_2(R_i/G_i)$, where $R_i = I_{ch1i} - Bg_{ch1i}$ and $G_i = I_{ch2i} - Bg_{ch2i}$ (where I_{ch1i} and I_{ch2i} are the intensities of spots in channel 1 and channel 2, respectively, and Bg_{ch1i} and Bg_{ch2i} are the background intensities of spots in channel 1 and channel 2, respectively). The mean intensity (A_i) was calculated for each spot as follows: $A_i = \log_2(R_iG_i)^{0.5}$ (50). A normalization method based on local regression that accounts for intensity and spatial dependence in dye biases was used (62). Normalization and t-statistics were carried out using the EMMA 1.1 microarray data analysis software developed at the Bioinformatics Resource Facility, Center for Biotechnology, Bielefeld University (http://www.genetik.uni-bielefeld.de/EMMA/) (11). Genes were considered differentially expressed if they were obtained for at least five of the nine replicate spots and if the confidence indicator (P) was ≤0.05, the mean intensity (A) was ≥9, and the $\log_2$ of the expression ratio (M) was ≥2 or ≤−2 (i.e., if there was at least a fourfold difference between the two experimental conditions) in at least 2 of the 16 experiments performed.

For cluster analysis, differentially expressed genes were selected for which P was ≤0.05 and A was ≥9 in at least 80% of the 16 experiments performed. Complete linkage clustering using uncentered correlation was performed using the Cluster 3.0 software, and for visualization the Java Tree View software was utilized.

Real-time RT-PCR. Real-time RT-PCR was utilized to determine the relative transcription of selected genes 60 min after a 0.4 M NaCl shock. Reverse transcription was carried out with 1 to 3 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) and random hexamers (Roche) as primers. Control reaction mixtures lacking reverse transcriptase were also used to confirm the absence of contaminating genomic DNA. Gene-specific primers were designed using the VectorNTI Suite 8.0 software (Table 2). The efficiency for each primer pair (E) was determined using the following formula: $E = [10^{(1/a)} - 1] \times 100$, where a is the slope of the standard curve.

Real-time PCR was carried out by using a Bio-Rad I-Cycler with the SYBR green I nucleic acid gel stain (Molecular Probes) in 96-well plates, and a melting curve analysis was conducted to ensure that there was amplification of a single product. Two replicates of every reaction were performed. The relative expression of each gene was normalized to the expression of 16S rRNA, and the results were analyzed using the comparative critical threshold method (45).

Recommended internet resources. We recommend the following internet resources: for the *S. meliloti* genome, http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/index.html and http://www.cebitec.uni-bielefeld.de

TABLE 2. Gene-specific primers used for real-time RT-PCR

Gene identification no.	Gene name	Forward primer	Reverse primer
SMa0233	*otsA*	CAGTCTCTATGCCGATTG	ACCATTTGATCCCTCTCG
SMb20249		ACGTGCTCGTCTCCTATCTT	ATTGAGCTGGTAGGTCCGAT
SMb20345		GACGATGAGGACGATGAT	ATGGCAAAGGGCTTCAAC
SMb20346		TTACCTGGTTCTTCGCCT	AAGCCTGGTGACATTCTC
SMb20347		CCACGACGACCATTTCAT	GCCAACATCTATCGCTTC
SMb20537		ACACCGTCGAAGCGTAAT	TCCTCAAGGTCGCAAATG
SMb20651		ATGAAAGCCGAAATCCGC	AAGGTCAAGGGCATTCGT
SMb21406		AGGAAGATCAGGTCAAGG	CGGTGTCAGATAATAGGG
SMb21448		AACGACGATTGCCTCTCT	TGGTGAGATAGCATTCCG
SMb21484	*rpoE5*	TCGCACGCAGTACAAGATTC	CCTTATAGCTCATTCCGAACC
SMc02163	*pgi*	AATGACATCGGTTCCCTG	CGGCTTCTACAATCGCAA
SMc03224	16S rRNA	TCTACGGAATAACGCAGG	GTGTCTCAGTCCCAATGT

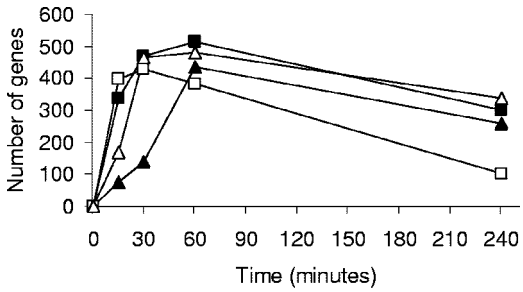

FIG. 1. Numbers of differentially expressed genes at different times after addition of 0.3 M NaCl (■), 0.4 M NaCl (▲), 0.5 M sucrose (□), and 0.7 M sucrose (△).

/groups/nwt/sinogate; for the Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/; for the Cluster 3.0 software, http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster; for the Java Tree View software, http://genome-www.stanford.edu/~alok/TreeView; and for the ArrayExpress database, http://www.ebi.ac.uk/arrayexpress.

Microarray data accession number. The microarray data have been deposited in the ArrayExpress database under accession no. E-MEXP-785.

RESULTS AND DISCUSSION

To study changes in gene expression of *S. meliloti* cells in response to salinity and hyperosmotic stress, Sm6kOligo DNA microarrays were used (29). First we performed an internal control experiment in which Cy3- and Cy5-labeled cDNAs were synthesized from total RNA that had been extracted from unstressed cells. All but three data points were between the two reference lines, which corresponded to *M* values of 2.0 and −2.0. This control experiment suggested that data points that appeared above the upper reference line or below the lower reference line could be regarded as representing genes whose expression was induced or repressed, respectively.

We used NaCl (0.3 M or 0.4 M) and sucrose (0.5 M or 0.7 M) to induce high-salinity and hyperosmotic stress, respectively. For each of the four conditions examined, changes in the mRNA levels were monitored 15, 30, 60, and 240 min after the shock. A total of 1,003 genes (16% of the annotated genes in the *S. meliloti* 1021 genome) appeared to be differentially expressed (see the supplemental material). The number of differentially expressed genes in the different conditions tested increased with time, reaching a maximum after 30 to 60 min and declining after this (Fig. 1). The same behavior was observed for the mean induction and repression values so that many genes showed a transient response to the osmotic upshifts.

Of the 1,003 genes regarded as differentially expressed, 539 were identified as upregulated and 444 were identified as downregulated. The remaining 20 genes showed variable responses and were catalogued as induced or repressed at different times. More than 40% of the differentially expressed genes formed part of presumptive operons in which two or more genes appeared to be osmoregulated under our experimental conditions.

Real-time RT-PCR was used to confirm microarray data. Ten genes were chosen based on their genomic locations and transcriptional changes (Table 3). Transcriptional induction of another gene, SMb20345, was tested by RT-PCR in order to verify the induction of the complete operon that this gene presumptively forms together with SMb20346 and SMb20347, both of which appeared to be osmotically inducible. Unexpectedly, RT-PCR confirmed the inducibility of the SMb20345 and SMb20347 genes, but the mRNA of SMb20346 was undetectable under both control and stress conditions. The SMb21484, SMb20249, and SMb21406 genes were confirmed to be induced, but their induction ratios could not be quantified since they were consistently expressed under the stress conditions and not in the control cultures. Confirmation of the microarray data was obtained for all but one of the remaining genes tested. The SMb20651 gene was induced twofold in our real-time RT-PCR experiments, although it appeared to be a repressed gene in the microarray experiments. This gene was also considered to be inhibited after long-term growth under saline stress conditions (50).

There was a strong replicon bias in the pattern of the osmotic stress response (Fig. 2); as many as 346 (64%) of the upregulated genes had a plasmid localization, which was 1.4-fold more than expected, since 46% of all predicted protein-encoding genes are located on the megaplasmids and 54% are located on the chromosome. On the other hand, 378 (85%) of the downregulated genes were chromosomal, which was 1.6-fold more than expected. This replicon distribution is similar to that found for differentially expressed genes in *S. meliloti* bacteroids or in free-living cells overexpressing *nodD3* (1).

An increase in external osmolarity triggers an efflux of water from cells that causes a decrease in the cytoplasm volume and thus an increase in the ion concentration in the cytosol (53). A similar hypertonic environment is created by addition of a high concentration of NaCl to the growth medium. Therefore, high-salinity stress and hyperosmotic stress were expected to have similar effects on gene transcription. This was confirmed in this study as transcription of most genes was induced or repressed by both stresses. Only 105 of the 1,003 genes responded exclusively to the addition of NaCl (meaning that they were not considered induced or repressed in any of the experiments performed using sucrose), whereas 112 genes appeared to be exclusively responsive to the addition of high sucrose concentrations. However, 38 (18%) of the 217 genes apparently displaying an exclusive response to one stress compound or the

TABLE 3. Quantitative real-time RT-PCR analysis of relative transcript abundance in NaCl-shocked cultures (0.4 M NaCl) with respect to control cultures after 1 h

Gene identification no.	Ratio[a]	SD	Detected change	Microarray *M* value
SMa0233	17.67	6.59	Induction	4.59
SMb21484			Induction	3.91
SMb20249			Induction	7.21
SMb20345	5.94	3.16	Induction	
SMb20346			ND[b]	3.43
SMb20347	3.74	2.20	Induction	3.30
SMb21406			Induction	2.42
SMb20651	2.09	0.89	Induction	−1.74
SMb21448	1.61	0.48	Induction	2.94
SMb20537	18.74	5.46	Induction	4.25
SMc02163	0.35	0.09	Repression	−2.46

[a] Average for at least two biological replicates with two technical replicates each.
[b] ND, not detectable.

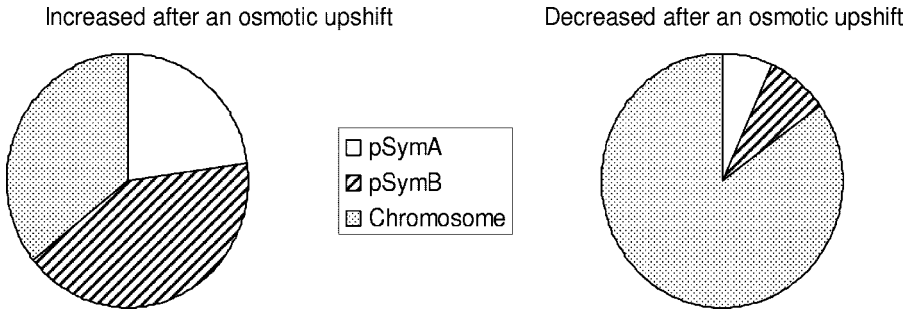

FIG. 2. Distribution of genes upregulated (left diagram) or downregulated (right diagram) after an osmotic upshift for the three *S. meliloti* 1021 replicons (chromosome, pSymA, and pSymB).

other formed likely operons with genes differentially expressed in both NaCl and sucrose shock experiments.

According to the genome sequence annotations provided by the *S. meliloti* Genome Project (http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/index.html), a large fraction of the upregulated genes are annotated as either having unknown functions or exhibiting partial or global homology to genes deposited in the databases (Fig. 3). Of the 428 genes induced by both stresses, 278 (65%) had unknown or hypothetical functions. It is remarkable that 29 of these genes were nonclassified regulators. Such a large proportion of proteins with undefined functions indicates that a significant part of the cellular and physiological reactions to salinity or hyperosmotic growth conditions is still unexplored. In contrast to the stress-induced genes, most downregulated genes fall into defined functional categories (Fig. 3). Most of the changes are related to the lower growth rate and less intense protein biosynthesis that are characteristic of stressed cells.

Carbon and energy metabolism. When the osmolarity of a culture of *Escherichia coli* growing in minimal medium is abruptly and substantially increased, the cells almost instantaneously lose water by plasmolysis, the synthesis of macromolecules is inhibited, and the rate of respiration decreases (23). We found that there was inhibition of many functions of the central metabolism and energy production systems. The tricarboxylic acid cycle was clearly repressed (*sdhBD*, *mdh*, *sucABCD*, *pckA*), along with some glucolytic enzymes (*cbbA*, *pdhB*, *pgi*). The repression of genes involved in the uptake (*smoEFGK*) and metabolism (*smoS*, *mtlK*, *cbbA*, *xylA*) of mannitol, the carbon source provided in our experiments, indicated that the general metabolism slowed down. Along with this, repression of the following different complexes in the respiratory chain and associated functions was observed: *nuoA1C1D1E1G1IJK1LMN*, *fbcBC*, *ctaBDE*, *rrpP*, *ppa*, and *atpABDF2GH*.

In many bacteria under growth-limiting conditions, carbon sources accumulate in the form of glycogen. This glucose-containing polysaccharide may be utilized as a source of energy during stationary phase to prolong viability. On the other hand, induction of the operon responsible for glycogen accumulation in *Yersinia pestis* by hyperosmotic stress led to the proposal that the accumulation of glycogen could assist in restoring the cytoplasmic volume after an osmotic shock (20). We found that there was a high level of induction of *glgA2*, *glgB2*, and *glgX2*, three genes involved in glycogen metabolism located on the pSymB plasmid of *S. meliloti*; however, the corresponding chromosomal alleles were not affected.

Many genes related to iron uptake were repressed after an osmotic shock. Several *sit* genes (*sitB*, *sitC*, and *sitD*) were downregulated after sucrose addition, and the first gene of the operon (*sitA*) was found to be repressed by both sucrose and NaCl. We also observed repression of genes involved in the synthesis and regulation of the siderophore rhizobactin 1021 (*rhbA*, *rhbB*, *rhbC*, *rhbE*, *rhbF*, and *rhtA* [34], as well as SMa2339), genes associated with siderophore-type iron transporters (*exbB* and *exbD*), genes encoding a heme compound transporter (*hmuS* and *hmuT*), and other genes related to iron uptake (SMa1746, SMc00784, SMc02726, and SMb21432). Most of these genes were previously found to be repressed in *S. meliloti* after prolonged growth under high-salinity conditions (50).

Protein metabolism. Of the 105 genes considered to respond exclusively to the addition of NaCl, 56 were downregulated after the shock. Strikingly, 25 of the 56 salt-repressed genes

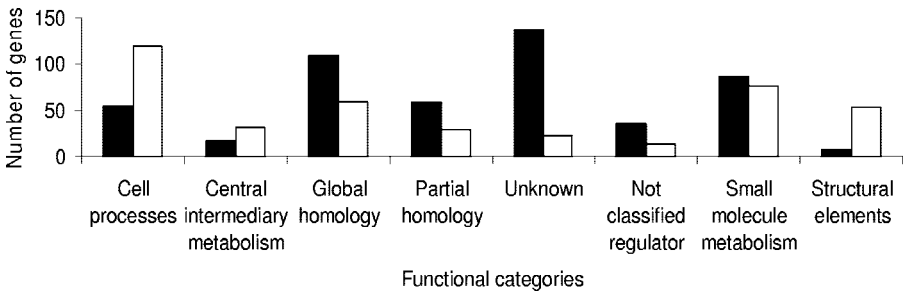

FIG. 3. Distribution of upregulated (solid bars) and downregulated (open bars) genes in functional categories.

code for ribosomal proteins, along with two genes in one of the ribosomal operons (*tufA* and *fusA1*) coding for probable elongation factors. Six additional ribosomal genes and another elongation factor gene (*tufB*) were repressed by both sucrose and NaCl stresses. On the other hand, we observed induction by sucrose of *rbfA* coding for a ribosome binding factor that functions in assisting 16S rRNA maturation (61). Expression of *ftsJ* was enhanced by the addition of sucrose and NaCl. FtsJ is a methyltransferase for 23S rRNA whose mutation results in decreased 70S ribosome stability and decreased translational rates in *E. coli* (6). We also observed induction of *hemK1* by both compounds, which is involved in the methylation of release factors and thus plays a role in translational termination. *E. coli hemK* mutants display severe growth defects and an enhanced rate of read-through of nonsense codons (40). These data suggest that although protein synthesis is inhibited after an osmotic shock, translational accuracy must be kept optimal.

Surprisingly, *tig* (trigger factor gene) was repressed by NaCl addition. Tig is a ribosome-bound protein that combines catalysis of peptidyl-prolyl isomerization and chaperone-like activities in *E. coli*. This factor was shown to cooperate with the DnaK (Hsp70) chaperone machinery in the folding of newly synthesized proteins (for a review, see reference 21), and proteomic studies have identified Tig as a protein whose level increases during stress (10). The chaperone genes *groESL1* and *groESL2* were repressed by both osmotic and hypersaline stresses. The chaperones encoded by these genes are known to be heat shock proteins that help to refold denatured proteins, but they are also involved in facilitating the folding of newly synthesized proteins both during and after translation and in protein secretion. Repression of chaperone genes may correlate with a reduction in protein synthesis. In fact, several other chaperones that do not function in association with the ribosome were induced by NaCl and sucrose, including ClpB, DegP, and IbpA. ClpB is a protease that dissolves inactive protein aggregates that accumulate during stress (16, 67), and it is also upregulated in *Synechocystis* under high-salinity or osmotic stress conditions (27); DegP appears to confer a capacity for intracellular survival and resistance to oxidative stress and elevated temperatures in several infectious microbes (see reference 46 for a review); and IbpA is a small heat shock protein that, in association with IbpB, stabilizes thermally aggregated proteins in a disaggregation-competent state in *E. coli* (33).

Cell division and cell envelope. Induction of the *maf* gene after NaCl shock was observed. This gene was also found to be upregulated by salinity but not by sorbitol-induced osmotic stress in *Synechocystis* (27). In *Bacillus subtilis* overexpression of *maf* results in inhibition of septation, leading to extensive filamentation (5). The induction of *maf* suggests inhibition of cell division probably associated with a general osmotic stress response since repression of *ftsZ1* after both NaCl and sucrose shocks was also detected. Furthermore, we observed inhibition of genes related to chromosomal replication or segregation after sucrose addition (*smc*, *ftsK*) or in response to both stress compounds (*dnaN*, *dnaA*, *ruvA*).

Rüberg et al. (50) observed that in contrast to genes related to the formation of other surface structures, the osmoadaptive response of *S. meliloti* included the induction of genes for succinoglycan production. Among these genes, we observed early induction of *exoHK* after an osmotic upshift. *S. meliloti* has the capacity to produce two exopolysaccharides, EPSI (succinoglycan) and EPSII (galactoglycan). *S. meliloti* produces succinoglycan in low-molecular-weight and high-molecular-weight forms. The *S. meliloti exoK* gene encodes an endoglycanase that specifically depolymerizes nascent succinoglycan but not succinoglycan that has accumulated in culture supernatants, yielding low-molecular-weight succinoglycan. In addition, we also observed induction of the *eglC* gene encoding an endoglycanase which contributes to depolymerization of succinoglycan (52). Succinylation of EPSI, which involves ExoH activity, seems to be a requirement for glycanase action (64). On the other hand, repression of *mucR* was observed. MucR is a transcriptional regulator that is essential for the production of high-molecular-weight succinoglycan (4). MucR also represses *exp* gene transcription and therefore galactoglucan production in *S. meliloti* (4). These data suggest that as an initial response to an osmotic shock, the pattern of synthesis of extracellular polysaccharides is modified, resulting in induction of low-molecular-weight succinoglycan and galactoglucan production.

The *rkpA* gene was repressed by sucrose addition, and the *rkpK* gene was repressed by both sucrose and NaCl. These genes are involved in production of the capsular polysaccharide that can replace exopolysaccharides in the infection process (49). The *rkpA-J* and *rkpK* genes are involved in the synthesis of capsular polysaccharide (28), but *rkpK* is also required for the formation of UDP-galacturonic acid, which is used during LPS synthesis. Although early and transient induction of the *wzy* gene (for synthesis of O antigen) after an osmotic shock was observed, we also found repression of the Tol import system (*tolBCQR* and *pal* genes), which is involved in internalization of certain molecules (together with the Exb system, which was repressed as well) and is required for correct surface expression of O antigens that are assembled in a *wzy* (polymerase)-dependent manner. TolA and possibly Pal could have a role in modulating the surface expression of O antigen by involvement in the processing of the O-antigen subunits, either during membrane translocation of O antigen or during subsequent stages of LPS assembly in the periplasm. It is known that the absence of TolA and Pal elicits a sustained extracytoplasmic stress response that in turn reduces O-antigen polymerization without affecting the stability of the Wzy polymerase (58). The repression of *tol* and *pal* genes may be in consonance with the repression of the genes for many transport systems, like the *smoEFGK* genes for sorbitol and mannitol uptake, *appJMPQ* and *livFHK* for amino acid transport, and genes for iron transport (see above) or polyamine uptake (see below). Despite *wzy* induction, LPS biosynthesis is probably slowed after an osmotic upshift, as we also observed downregulation by sucrose of *uppS*, which has been implicated in undecaprenyl pyrophosphate biosynthesis. Undecaprenyl pyrophosphate is required as a lipid carrier of glycosyl transfer in the biosynthesis of a variety of cell wall polysaccharides in bacteria, including LPS. This gene is also downregulated by hyperosmotic stress but not by NaCl in *Y. pestis* (20).

Also related to the cellular envelope, we observed repression of genes involved in the synthesis of peptidoglycan (*murACG*) and in the transport of periplasmic cyclic beta-glucans (*ndvA*), which are known to accumulate in response to a decrease in

external osmolarity (see reference 4 for a review), and induction of cyclopropane fatty acyl phospholipid synthase (*cfa2*). The main modification in *Lactococcus lactis* membrane fatty acid composition in response to high osmolality is the increase in cyclopropane fatty acid (19). In *E. coli*, cyclopropanation is thought to be involved in long-term survival of nongrowing cells and is often associated with environmental stresses (for a review, see reference 18).

As many as 27 genes involved in chemotaxis and cell motility were downregulated after an osmotic upshift. These genes included genes related to flagellar biosynthesis (*flaAB*, *flhA*, *fliEMP*, *flgBDEFGEKL*) and the regulatory gene *flbT*, as well as the flagellar motor genes *motABCD*. The chemotaxis genes included *cheABDR* and *mcpTUXZ*. These results are consistent with previous data on the repression of motility and chemotaxis genes in *S. meliloti* by prolonged salt stress conditions (50) and a wide range of environmental stress factors that influence motility and chemotaxis in bacteria (for a review, see reference 55).

Stress-related metabolism. Several of the upregulated genes have been described previously as stress-responsive genes. This category includes *ndi* genes (*ndiA1*, *ndiA2*, and *ndiB*), which are known to be induced in *S. meliloti* by oxygen, nitrogen, or carbon deprivation, by osmotic stress, and during entry into the postexponential stationary phase (8). We also observed induction of genes related to oxidative stress, such as a putative dioxygenase gene (SMa1814), *sodC* (encoding a Cu,Zn superoxide dismutase), and *catC* (catalase). Genes probably related to virulence have also been observed to be induced in response to an osmotic upshift. Some examples are *attA1* and *attA2*, which are involved in attachment in *Agrobacterium tumefaciens*, and two open reading frames identified as genes that encode putative virulence-associated protein homologues that may constitute an operon (SMc04408 and SMc04881). Several upregulated genes might be involved in DNA repair processes; these genes include genes encoding two putative DNA ligases (SMb20912 and SMb20008), a putative DNA polymerase-related protein (SMb21448), a putative DNA invertase (SMc02287), and a probable exodeoxyribonuclease III (*xthA1*).

Slight induction of the *rpo*-like gene (SMb20592) was observed after sucrose shock, but this sigma factor was not the only RNA polymerase sigma factor to be induced after an osmotic shock. In fact, there was more consistent induction of the *rpoE2*, *rpoE5*, and *rpoH2* genes by both salt and sucrose. Fourteen putative sigma factor genes have been identified by genome sequence analysis of *S. meliloti* (15). Two of these genes code for σ^{32}-like proteins: *rpoH1* and *rpoH2* (42, 43). There are as many as six copies of the *rpoE* gene in the *S. meliloti* genome. Each of these copies probably has specific functions since we observed induction of just two of them (*rpoE2* and *rpoE5*) and repression of another (*rpoE4*) upon an osmotic upshift. RpoE is a sigma factor belonging to the extracytoplasmic function family and might be even more important for survival of *E. coli* in the stationary phase than the starvation sigma factor RpoS is (63). Sigma factors belonging to the extracytoplasmic function family are part of the bacterial stress response regulon (38). They react to stress signals outside the cytoplasmic membrane by transcriptional activation of genes encoding products involved in defense or repair processes (47).

An increase in the internal ion concentration above a certain level triggers the uptake or endogenous synthesis of neutral osmoprotectants which can accumulate to high intracellular concentrations without adversely affecting cellular processes (66). Trehalose is known to be a nonaccumulated sinorhizobial osmoprotectant (17). We detected salt stress induction of a putative maltooligosyl trehalose synthase gene, but two other trehalose synthesis mechanisms were induced by both salt and sucrose stress: a trehalose 6-phosphate synthase gene (*otsA*) and a putative trehalose synthase gene (SMb20099) together with five other genes in the same putative operon (SMb20095, SMb20096, SMb20097, SMb20098 and SMb20100). Induction of the *thuA*, *thuE*, and *thuG* genes was also observed upon an osmotic upshift. These genes are part of a cluster of six genes involved in trehalose transport and utilization (25, 26).

In *E. coli*, the response to an abrupt increase in external osmolarity is a rapid increase in the intracellular concentration of potassium. To maintain electroneutrality, intracellular K^+ must be balanced by anions. It is known that the increase in the K^+ concentration closely parallels accumulation of substantial amounts of glutamate (35). We observed induction of genes possibly involved in glutamate biosynthesis (SMc01813 and *asnO*), as well as repression of the catabolic genes *nodM*, *glmS*, and *gsh1*. On the other hand, three different putative putrescine (1,4-diaminobutane) transport systems were downregulated. We found that there was repression of *potD* and *potF*, which encode periplasmic substrate-binding proteins of the two ABC putrescine-specific and spermidine-preferential transporters identified in *E. coli* (for a review, see reference 24), *potB*, which belongs to the same operon as *potD*, and two genes of another likely operon also annotated as part of a spermidine/putrescine ABC transporter that were repressed by sucrose (SMc01965) or by both sucrose and NaCl (SMc01964). The level of the polyamine putrescine decreases in *E. coli* as the external osmolarity increases, and putrescine is replaced by potassium (51). Munro et al. (39) suggested that the physiological advantage of replacing divalent putrescine with monovalent potassium is that it allows an increase in cytoplasmic osmolarity with a less-than-proportionate increase in intracellular ionic strength.

Repression of a putative ornithine diaminopropane or arginine decarboxylase gene (SMc02983) could indicate that there is inhibition of putrescine biosynthesis, but it may also be associated with the general repression of genes involved in amino acid synthesis during adaptation to hyperosmotic conditions, which has been well documented previously (50, 59). Indeed, we observed repression of many amino acid biosynthetic genes (*thrA*, *serC*, *glyA1*, *gcvT*, *gcvP*, *metA*, *metH*, *ilvC*, *proC*, *argG*, *aroQ*, *aroE1*, etc.) but also induction of some catabolic genes, such as *hmgA*, *ald*, *bkdAa*, and SMc03211. The changes in expression of these genes most likely reflect the reduced growth rate of stressed cells. General inhibition of protein synthesis after an osmotic shock would make amino acid biosynthesis a waste of energy. Thus, the repression of other related processes, such as tRNA synthesis, was not a surprise (*hisS*, *proS*, *serS*).

Transcriptional profiles and gene clustering. An important aim of this study was to compare the transcriptional response to an increase in the external osmolarity and the transcriptional response to an increase in salinity. We obtained clear

evidence of the overlapping responses to the two kinds of stress. Nevertheless, the general redundancy found in the differentially expressed genes after sucrose or NaCl addition provided further validation of the results obtained. On the other hand, establishment of a time course for the adaptation response compared to prolonged growth under stress conditions was of great interest. The latter was studied by Rüberg et al. (50) using PCR-based Sm6kPCR microarrays to determine the transcriptomic response of cells growing in GMS medium in the presence of 380 mM NaCl.

A large number of genes (1,003 genes) were found to respond to an osmotic upshift, compared to the 137 genes that displayed significant changes in expression after prolonged growth on NaCl-containing medium (50). This obvious difference could have been due to various factors. First, the microarrays used in this work are probably more sensitive than those used by Rüberg and coworkers (50). Furthermore, Rüberg et al. studied only one time after long-term exposure of bacteria to a single stress condition, whereas the present study involved the early responses (at four times) of the cultures to four different insults (two concentrations of NaCl and two concentrations of sucrose). Most of the genes identified in this study exhibited a transient response to the addition of the stress compound, so after 4 h only 507 (51%) of the 1,003 genes remained differentially expressed in any of the four conditions tested. It is reasonable to think that the number of differentially regulated genes could be further reduced after longer exposure to the stress. Also, the intensity of the transcriptional changes decreased with time, and we observed stronger responses for many of the functional groups previously identified after prolonged exposition to salinity (50) (motility and chemotaxis, amino acid biosynthesis, iron uptake) along with new responses (energy metabolism, protein synthesis, cell division).

To investigate possible patterns in the expression profiles, a clustering analysis was performed with 615 differentially expressed genes. Cluster analysis resulted in definition of two main clusters; cluster A included 329 genes induced after an osmotic upshift, and cluster B included 286 repressed genes. Four groups were identified among the 329 induced genes. The 53 cluster A1 genes, most of which have unknown functions, displayed late induction and included 48 plasmid-borne genes, among which were *exoK*, *thuA*, and some *ndi* genes. Cluster A3 included 215 genes that were strongly and rapidly upregulated (between 15 and 60 min) and were only moderately induced after 240 min. Most of these cluster A3 genes had a plasmid localization. Cluster A4 included 56 genes (33 of which were chromosomal) that showed rapid induction but were slightly repressed after 240 min. Cluster A2 included five inducible genes that did not fall into any of the transcriptional profiles described above.

A great majority of the 286 downregulated genes fell into cluster B3 (264 genes, mainly chromosomal), which displayed maximal repression at 60 min and were moderately repressed after this. Cluster B2 included 13 genes that exhibited strong repression only after 240 min, such as some chemotaxis genes (*mcpU*, *mcpZ*) and some *met* genes. Cluster B4 included only six genes with transient repression that was attenuated after 240 min. Cluster B1 included three genes that had variable responses (induced or repressed) at different times.

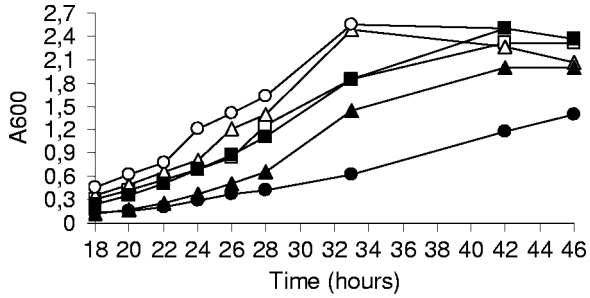

FIG. 4. Growth curves for Rm1021 (squares), RmF514 (circles), and RmF909 (triangles) in TY (open symbols) or TY containing 0.4 M NaCl (solid symbols). The data are from at least two replicate experiments. A600, optical density at 600 nm.

pSymB of *S. meliloti* is required for tolerance to high salinity. We found that there was an evident replicon bias in the pattern of the osmotic stress response. Upregulated genes were more likely to be carried on the symbiotic plasmids, whereas most repressed genes were chromosomal (Fig. 2). This fact was further supported by the differential responses of gene alleles located on different replicons, such as the *glg* genes (the plasmid alleles were induced, but the chromosomal alleles were unchanged) or the *nuo* genes (the chromosomal operon was repressed, but the plasmid operon was unchanged). Indeed, 83% of pSymB differentially expressed genes were upregulated. The preferential induction of plasmid genes, particularly genes in pSymB, led us to hypothesize that this plasmid could be required for adaptation of *S. meliloti* to hyperosmotic stress.

Plasmid pSymB (1.68 Mbp) is essential for cell viability, and thus *S. meliloti* cannot be cured of this plasmid. However, we used a collection of *S. meliloti* mutants carrying defined pSymB deletions (7) to test whether particular regions of this plasmid might be involved in the adaptation to high external osmolarity. Eleven of these pSymB deletion mutants were chosen in order to cover the entire plasmid. We found that all these strains exhibited reduced growth rates in minimal medium compared with the growth rate of the wild-type strain, which somewhat hampered the analysis under stress conditions. Moreover, only 6 of the 11 mutant strains (RmF693, RmF680, RmF666, RmF117, RmF909, and RmF514) had wild-type growth rates in rich TY medium and therefore could be tested for growth in high-osmolarity media. Altogether, the deletions carried by these strains spanned approximately one-half of pSymB. All six deletion mutant strains exhibited wild-type growth rates in TY supplemented with 0.7 M sucrose. However, strains RmF909 and RmF514 exhibited significantly delayed growth in TY with 0.4 M NaCl (Fig. 4) or 0.5 M NaCl (data not shown). Furthermore, these mutants also showed a reduced capacity for adaptation after a saline shock (Fig. 5), suggesting that the pSymB regions absent in these two strains are necessary for adaptation of *S. meliloti* to high salinity. Strikingly, the deleted pSymB regions in strains RmF909 and RmF514 overlap by about 200 kb (7). Therefore, this region must contain one or more genes important for *S. meliloti* salt tolerance. According to the annotation of the *S. meliloti* 1021 genome, there are 198 genes in this region (from SMb20485 to SMb21081), 72 of which (36%) appeared to be transcription-

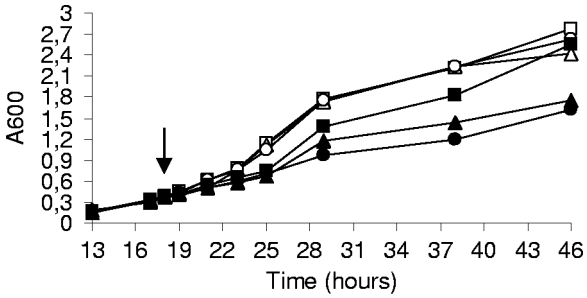

FIG. 5. Growth curves for Rm1021 (squares), RmF514 (circles), and RmF909 (triangles) in TY (open symbols) or TY containing 0.5 M NaCl (solid symbols). The arrow indicates the time of application of the osmotic upshift. The data are from at least two replicate experiments. A600, optical density at 600 nm.

ally responsive to salt shock and sucrose shock in our microarray experiments. Thus, this region has a 2.3-fold higher density of osmotically responsive genes than the whole genome has. Fifty-one of these genes were regarded as upregulated and 21 genes were regarded as downregulated after saline shock and sucrose shock in our microarray experiments. Intriguingly, 66 of these genes (92%) code for proteins having unknown or hypothetical functions. The induction of one of them, SMb20537 (encoding a putative transcriptional regulator), after a saline shock was verified by quantitative RT-PCR (Table 3). Detailed genetic characterization of this pSymB region is necessary to determine which of the osmotically regulated genes are actually important for *S. meliloti* adaptation to high external osmolarity.

The genetic data correlate well with the microarray results showing that pSymB contains a large number of genes upregulated after an osmotic upshift which may have an active role in the osmoadaptation of *S. meliloti*. The overall data also support the notion that pSymB plays a major role in the saprophytic competence of *S. meliloti* in the soil environment (14).

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