Microbiology (2006), **152**, 2049–2059 **DOI** 10.1099/mic.0.28937-0

Isolation of salt-sensitive mutants of *Sinorhizobium meliloti* strain Rm1021

Mark Miller-Williams, Peter C. Loewen and Ivan J. Oresnik

Department of Microbiology, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

Correspondence
Ivan J. Oresnik
oresniki@cc.umanitoba.ca

Received 20 February 2006
Revised 17 March 2006
Accepted 21 March 2006

The determinants necessary for adaptation to high NaCl concentrations and competition for nodule occupancy in *Sinorhizobium meliloti* were investigated genetically. Mutations in *fabG* as well as *smc02909* (transmembrane transglycosylase), trigger factor (*tig*) and *smc00717* (probably *ftsE*) gave rise to strains that were unable to tolerate high salt and were uncompetitive for nodule occupancy relative to the wild-type. Moreover *exoF1*, *exoA* and *pgm* determinants were determined to be necessary for strain Rm1021 to survive high NaCl and/or $MgCl_2$ concentrations. The introduction of an *expR*$^+$ allele was capable of suppressing the Mg^{2+} sensitivity associated with the *exoF1*, but not the *exoA*, mutation in a manner independent of exopolysaccharide II (EPS II)-associated mucoidy. The results also show that the EPS II-associated mucoid phenotype was affected by either Mg^{2+} or K^+, but not by Li^+, Ca^{2+}, or high osmolarity.

INTRODUCTION

Sinorhizobium meliloti is a Gram-negative soil microbe that is capable of either living as a saprophytic organism or entering into an endosymbiotic association with leguminous plants, in which it reduces nitrogen gas to ammonia, which is then supplied to its host. Although a good deal is understood about the steps of nodulation (Spaink, 2000) relatively little is known of the determinants that are necessary for the bacterium to switch from a saprophytic to a symbiotic existence.

The rhizosphere has been defined as the volume of soil surrounding the root influenced by the exudation of plant-derived compounds (Bowen & Rovira, 1976). To enter into an endosymbiotic relationship, a bacterium must first be able to survive in the rhizosphere and to colonize the roots of the host. In addition to the exudation of compounds into the rhizosphere, the plant also influences the rhizosphere environment by actively removing nutrients such as water and other trace elements that are essential for its own survival, and excluding others such as sodium which are deleterious to the plant's survival. It has been predicted that this exclusion may salinize the rhizosphere and lead to an osmolality within the rhizosphere that is higher than that found in bulk soil (Miller & Wood, 1996).

S. meliloti is more halotolerant than many other rhizobia (Botsford, 1984; Breedveld *et al.*, 1990). A number of studies have shown that osmoadaptation in *Rhizobium* appears to be atypical compared to that found in many enteric bacteria (Miller & Wood, 1996). Whereas enteric bacteria often synthesize glycine betaines as osmoprotectants, *S. meliloti* responds by accumulating glutamate, the novel dipeptide *N*-acetylglutaminyl glutamine amide (NAGGN) and potassium (Botsford & Lewis, 1990; Smith & Smith, 1989). In response to extreme osmotic shock, trehalose accumulation occurs (Smith *et al.*, 1994). In addition, a number of compounds, including disaccharides, are capable of acting as non-accumulated osmoprotectants by *S. meliloti* (Gouffi *et al.*, 1999, 2000; Le Rudulier & Bernard, 1986).

A number of studies have shown that *S. meliloti* polysaccharide determinants are altered with changes to either media or salt concentrations (Breedveld *et al.*, 1990; Lloret *et al.*, 1995, 1998). *S. meliloti* has the ability to synthesize two different exopolysaccharides (EPS), succinoglycan (EPS I), which when bound to Calcofluor causes it to fluoresce under long-wave UV light, and galactoglucan (EPS II), which confers a mucoid colony phenotype when it is synthesized (Glazebrook & Walker, 1989; Leigh *et al.*, 1985). *S. meliloti* strain Rm1021 normally only produces EPS I (Zhan *et al.*, 1991). Inability to produce EPS I has been correlated with an inability to establish a functional symbiotic association (Keller *et al.*, 1988; Leigh *et al.*, 1985). The necessity for EPS I in symbiosis can be replaced by the presence of low-molecular-mass EPS II or a symbiotically active form of a capsular polysaccharide termed K antigen or KPS (González *et al.*, 1996a; Pellock *et al.*, 2000; Reuhs *et al.*, 1995).

Regulation of EPS II is complex and is in part dependent upon the presence of the *expR*$^+$ allele (Glazebrook & Walker, 1989; Pellock *et al.*, 2002). The wild-type, strain Rm1021, contains an insertion element (ISRm2011-1) within the *expR* open reading frame and only synthesizes EPS II under low-phosphate conditions (Mendrygal & González, 2000; Rüberg *et al.*, 1999; Zhan *et al.*, 1991). In the halotolerant *S. meliloti* isolate EFB1 (growth unaffected

Abbreviation: EPS, exopolysaccharide.

by the addition of 300 mM NaCl), EPS II production is decreased in response to NaCl (Lloret *et al.*, 1998). The mechanism by which this occurs has not been investigated. Microarray analysis of *expR*$^+$-dependent gene expression in Rm1021 has shown that a large number of genes influencing many cellular processes are affected, including some involved in low-molecular-mass EPS I biosynthesis (Hoang *et al.*, 2004).

To determine if mutants unable to tolerate high salt concentrations are less efficient in competition for nodule occupancy, *S. meliloti* Rm1021 was mutagenized, and the mutants screened for salt sensitivity and their ability to compete for nodule occupancy. The results show some of the strategies used by *S. meliloti* in dealing with high solute regimes.

METHODS

Bacterial strains and plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. Complex (LB) and defined (VMM) media were as previously described (Miller, 1972; Vincent, 1970). Antibiotics were used as necessary at the following concentrations: streptomycin (Sm), 200 μg ml^{-1}; neomycin (Nm), 200 μg ml^{-1}; tetracycline (Tc), 5 μg ml^{-1}; chloramphenicol (Cm), 20 μg ml^{-1}; gentamicin (Gm), 20 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}; rifampicin, 100 μg ml^{-1}. To assay for ion sensitivity, LB base medium containing 10 g tryptone and 5 g yeast extract per litre was amended with the appropriate concentration of salt (NaCl, $MgCl_2$, KCl, $CaCl_2$, LiCl) as indicated.

Genetic techniques, DNA manipulations and sequencing. Bacterial matings and transposon mutagenesis were carried out essentially as previously described (Finan *et al.*, 1988). Phage transductions using ϕM12 were carried out as previously described (Finan *et al.*, 1984). Standard procedures were used for plasmid isolation, restriction endonuclease digestions, ligations, transformations, gel electrophoresis and Southern transfers (Sambrook *et al.*, 1989).

The point of insertion for Tn*5* mutants was determined by a modification of an arbitrary PCR protocol (Caetano-Anollés, 1993; Raffa & Raivio, 2002). Strains containing Tn*5*, or Tn*5*-233, inserts were purified and the genomic DNA was used as a template. A low-stringency PCR amplification (45 °C) using the primers IS50(1) (5′-CACGATGAAGAGCAGAAG-3′) and DGEN(1) (5′-GGCCACGCGTCGACTAGTCAGNNNNNNNNNACGCC-3′) was carried out. The products of this reaction were subsequently amplified with a higher-stringency PCR amplification (60 °C) using the primers IS50(2) (5′-TAGGAGGTCACATGGAAGTCAGAT-3′) and DGEN(2) (5′-GGCCACGCGTCGACTAGTCAG-3′). The primers IS50(2) and IS50(1) are found within the IS*50* elements of Tn*5* such that the IS50(2) primer is nested between primer IS50(1) and the sequence flanking the transposon. The DGEN(2) primer was designed to be complementary to the 3′ end of the amplification product produced by using the IS50(1) primer [i.e. identical to the 5′ end of DGEN(1)]. The products of the second PCR were gel isolated and sequenced using the IS50(2) primer as previously described (Oresnik *et al.*, 1998). Sequencing was performed at the University of Calgary Core DNA facilities. Sequence data were trimmed of IS*50* sequences and database searches were done with BLASTX (Altschul *et al.*, 1997) against the *S. meliloti* database at http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/ (Barnett *et al.*, 2001; Finan *et al.*, 2001; Galibert *et al.*, 2001).

Plant assays. Plant symbiotic assays were carried out as previously described (Oresnik *et al.*, 1994). Alfalfa (*Medicago sativa* cv. Rangelander) seeds were surface sterilized by treating the seeds for 1 min with 70 % ethanol followed by 10 min in a 1/5 dilution of bleach (approx. 1 % hypochlorite). The seeds were then extensively washed with sterile distilled water and germinated on 1·5 % water agar plates. Seedlings were transferred after about 2–3 days to sterile Leonard jar assemblies. Plants were grown in a quartz sand : vermiculite mixture, and nitrogen-free Jensen's nutrient solution and water were added as required (Vincent, 1970). Plants were inoculated by growing *S. meliloti* cultures overnight in LB, diluting the cultures 1/100 in sterile distilled water and inoculating each Leonard jar assembly with 10 ml. Plants were harvested between 28 and 35 days after inoculation. The plants were unearthed and the shoots (above the cotyledon) were dried to determine plant dry weight. The nodules on the root systems were counted and representative nodules were crushed and the bacteria isolated. Bacteria that were isolated from nodules were streaked onto LB agar plates and single colonies were checked for relevant phenotypes. Plant competition assays were carried out as previously described (Oresnik *et al.*, 1998). Data from the competition experiments were evaluated for statistical significance using both binomial probability and a Fisher's least significance difference (LSD) assuming that a P value of less than 0·05 indicated a difference in strain competitiveness.

RESULTS

Isolation of NaCl-sensitive mutants

Between 11 and 89 % of *S. meliloti* strains are able to grow on media containing 2 % NaCl (about 70 mM), whereas >90 % of all *R. leguminosarum* strains cannot (Jordan, 1984). LB generally contains about 86 mM NaCl. A brief survey of the *S. meliloti* strains showed that strains Rm1021, Rm2011, AK631, Rm102F34, Rm102F21, RCR2012 and CC2013 were able to grow on LB whereas strain 104A14 could not (data not shown).

To identify mutants unable to tolerate elevated concentrations of NaCl we first determined what concentration of NaCl could be tolerated by strain Rm1021 when grown on LB medium. Initial experiments determined that Rm1021 could grow on LB containing a final concentration of 300 mM NaCl, whereas growth was very poor on 400 mM and sparse on 500 mM (data not shown). Transposon-induced mutants were therefore screened for the ability to grow on LB agar containing 100 mM NaCl and an inability to grow on LB containing either 300 or 350 mM NaCl. Approximately 5000 Tn*5* mutants were screened. To ensure that the transposon insert was the cause of the salt-sensitive phenotype, the insert was transduced into Rm1021 and screened for cotransduction of the antibiotic-resistance marker associated with the transposon and the salt-sensitive phenotype. In all cases the insertion and the phenotype were 100 % linked by transduction (typically 50–100 colonies screened), strongly suggesting that the transposon insertion was the cause of the salt sensitivity. From these screens, approximately 20 putative mutants were isolated and further tested.

Based on their growth characteristics on various media, eight of the putative mutants appeared to be salt sensitive without any other notable growth defects. One of these,

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype*	Reference or source
Sinorhizobium meliloti		
Rm1021	SU47 derivative, Sm^R wild-type	Meade *et al.* (1982)
Rm2011	SU47 derivative, Sm^R wild-type	M. Hynes, University of Calgary
AK631	Rm41 *exoB631*	Banfalvi *et al.* (1981)
RCR2012	*S. meliloti* wild-type	T. Charles, University of Waterloo
102F21	*S. meliloti* wild-type	T. Charles
102F34	*S. meliloti* wild-type	T. Charles
104A14	*S. meliloti* wild-type	T. Charles
CC2013	*S. meliloti* wild-type	T. Charles
Rm7055	Rm1021 *exoF55*::Tn*5*	Leigh *et al.* (1985)
RmG182	Rm1021 Ω317::Tn*5*-233, *expR*$^+$	J. Glazebrook via T. Finan, McMaster University
Rm5439	Rm1021 *pckA1*::Tn*V*	Finan *et al.* (1988)
SRmA239	Rm1021 s*mb20192*::Tn*5*	This work
SRmA251	Rm1021 *fabG1*::Tn*5*-233	This work
SRmA258	Rm1021 *exoF1*::Tn*5*	This work
SRmA259	Rm1021 *pgm-1*::Tn*5*	This work
SRmA262	Rm1021 Smc02909::Tn*5*	This work
SRmA267	Rm1021 *tig*::Tn*5*	This work
SRmA282	Rm1021 Smc00717::Tn*5*	This work
SRmA284	Rm1021 Smb20056::Tn*5*	This work
SRmA287	Rm1021 *rpoH2*::Tn*5*-233, (Ω317::Tn*5*-233)	This work
SRmA305	SRmA258 transduced into Rm1021	This work
SRmA309	Rm1021 *expR*$^+$, Ω317::Tn*5*-233, *pckA1*::Tn*V*	This work
SRmA316	Rm1021 *expR*$^+$, *pckA1*::Tn*V*	This work
SRmA321	Rm1021 *exoF1*::Tn*5*, *expR*$^+$, Ω317::Tn*5*-233	This work
SRmA322	Rm1021 *exoF1*::Tn*5*, Ω317::Tn*5*-233	This work
SRmA337	Rm1021 *pgm-2*::Tn*5*	This work
SRmA339	Rm1021 *exoA1*::Tn*5*	This work
SRmA363	Rm1021 *expR*$^+$	This work
SRmA457	SRmA363 *exoF1*::Tn*5*	This work
SRmA458	SRmA363 *exoA1*::Tn*5*	This work
Escherichia coli		
MM294A	*pro-82 thi-1 hsdR17 supE44*	T. Finan
MT607	MM294A *recA56*	Finan *et al.* (1986)
MT616	MT607 (pRK600)	Finan *et al.* (1985)
Plasmids		
pRK600	pRK2013 *npt*::Tn*9* Cm^r Nm-Km^s	Finan *et al.* (1985)
pRK602	pRK600::Tn*5*	Finan *et al.* (1986)
pRK607	pRK2013::Tn*5*-233	de Vos *et al.* (1986)

*Abbreviations for antibiotics are as follows: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Tc, tetracycline.

strain SRmA259, was subsequently identified as carrying a *pgm* (phosphoglucomutase) mutation (see below). Since this mutation is pleiotropic, SRmA259 was characterized in a limited manner. Growth rates were determined on the remaining mutants to ensure that they could grow in LB broth containing 100 mM NaCl at rates comparable to the wild-type and to show that they were compromised in their ability to grow in LB broth containing elevated salt (Table 2).

As well as showing that the mutants all grew at rates comparable to strain Rm1021 in LB broth culture, the data show that the mutations do not have identical effects at elevated salt concentrations. Strains SRmA251 and SRmA267 seem to be the least affected whereas strain SRmA258 appears to be most debilitated (Table 2). The growth rate of SRmA251 and SRmA267 correlated well with their growth on agar (some growth could be seen) whereas when SRmA258 was streaked on LB agar containing

Table 2. Growth rate of salt-sensitive mutants grown on either 100 mM or 350 mM NaCl

Strain	Relevant genotype	Generation time (h)* on	
		100 mM NaCl	350 mM NaCl
Rm1021	Wild-type	2·9±0·1	3·5±0·3
SRmA239	*smb20192*	3·3±0·3	11·8±1·3
SRmA251	*fabG*	3·1±0·3	5·4±0·1
SRmA258	*exoF1*	3·3±0·3	31·4±6
SRmA262	*smc02909*	3·5±0·1	8·4±1·8
SRmA267	*tig*	3·1±0·2	5·5±0·6
SRmA282	*smc00717*	3·3±0·2	11·5±0·9
SRmA284	*smb20056*	3·4±0·3	12·7±0·2

*Generation times are expressed as hours per generation calculated from OD_{600} measurements on cultures that were in exponential phase of growth.

350 mM NaCl it was not uncommon to see some isolated colonies. A number of these were shown to have acquired second-site mutations that were transductionally unlinked to the original mutation (M. Miller-Williams & I. J. Oresnik, unpublished).

Identification of mutations

The point of insertion of the transposon in each mutant was identified and the annotated name or number as well as a brief description of the gene are presented in Table 3. The mutations break down into three broad classes: mutations that directly affect the cell structure (*fabG*, *exoF1*, *pgm*, Smc02909), uptake of metabolite(s) (Smb20056, Smb20057, Smb20058) as well as a number of mutations that are more likely to have an indirect or pleiotropic effect (*ftsEX*, *tig*, Smb20912).

Since Tn*5* generates polar mutations, it has also been noted whether the mutation may be affecting the expression of other genes (Table 3). For example, in strain SRmA258, the insertion in *exoF1* affects other genes in succinoglycan production; this has been shown by others experimentally (González *et al.*, 1996b). The Tn*5* insertions in strains SRmA284 and SRmA239 are likely to have polar effects on downstream genes since the inserts are in the first genes of operons and there appears not to be enough space for a downstream promoter. The insertion in strain SRmA282 is within an ATP-binding cassette protein with similarity to *ftsE* and is likely polar on a gene that is related to *ftsX* (Smc00716) since the two genes have a 4 bp overlap. It is unclear whether the insertion could have a polar effect on Smc00715 and Smc00714 since the spacing between these genes is 109 and 105 bp, respectively, yet they are transcribed in the same orientation as Smc00717. Whereas Smc00715 is predicted to be a gene that encodes a hypothetical protein, Smc00714 is predicted to encode a putative 1-acyl-*sn*-glycerol-3-phosphate acyltransferase. It is also worth pointing out that *ftsEX* mutations in *Escherichia coli* also have an ion-related phenotype (de Leeuw *et al.*, 1999; Schmidt *et al.*, 2004).

The inserts in strains SRmA262 and SRmA267 do not have polar effects since the next annotated genes are transcribed in the opposite orientation to the genes interrupted by the Tn*5* insertion. The insert in strain SRmA251 is within *fabG*. Although the next gene downstream is transcribed in the same orientation, there is a gap of 471 bp between these genes, making it unlikely that they are cotranscribed. The insert in strain SRmA259 is within *pgm*. The space between *pgm* and the next gene, *glgX1*, which is transcribed in the same orientation is 233 bp and it is unclear whether the Tn*5* has a polar effect. However, *Rhizobium tropici* contains an identical arrangement of genes and *glgX* has been shown to transcribed independently of *pgm* (Marroquí *et al.*, 2001)

Ion phenotypes of salt-sensitive mutants

PEG has been used experimentally to generate high osmotic environments because it is excluded from cells,

Table 3. Identification of Tn*5* mutations isolated as sensitive to NaCl

Strain	Gene number/ name	Replicon	Name/function	Polar	Gene(s) affected
SRmA239	Smb20912	pSymB	ATP-dependent DNA ligase	Yes	Smb20911
SRmA251	*fabG*	Chromosome	Probable 3-oxoacyl-acyl-carrier protein reductase	No	
SRmA258	*exoF1*	pSymB	Succinoglycan production	Yes	*exoQ*
SRmA259	*pgm*	Chromosome	Phosphoglucomutase	?	*glgX1*
SRmA262	Smc02909	Chromosome	Transmembrane transglycosylase	No	
SRmA267	*tig*	Chromosome	Probable trigger factor, protein secretion	No	
SRmA282	Smc00717/*ftsE*	Chromosome	Cell division ABC protein	Yes	Cell division *ftsX*
SRmA284	Smb20056	pSymB	ABC transporter, ATP-binding protein	Yes	Smb20057, Smb20058, permeases with ABC transporter

non-metabolizable and osmotically active. The mutants we had isolated were streaked onto LB agar plates containing 5 % PEG 8000 and 100 mM NaCl. The results showed that the mutants were not sensitive to high osmolarity under these conditions (data not shown).

To determine if the mutants were specifically sensitive to Na^+, their ability to tolerate high concentrations of other cations was determined. Since strains SRmA258 and SRmA259 were shown to carry mutations that affect EPS synthesis (Leigh *et al.*, 1985), the other mutants were also screened for their ability to fluoresce on Calcofluor (Table 4). Of the eight mutants analysed, SRmA251 (*fabG*::Tn*5*) was the only one that appeared to be sensitive only to NaCl. It is noteworthy to point out that of all the mutants isolated with a salt-sensitivity phenotype, SRmA251 appeared to be the least sensitive (Table 2, Table 4). The rest of the mutants tended to show some degree of sensitivity to the other ions that were tested.

The most notable phenotypes observed were those associated with strains SRmA258 and SRmA259. The insertion in SRmA258 was localized within the *exoF1* gene of *S. meliloti* and it was the only strain that showed a marked sensitivity to Mg^{2+}. SRmA259 carries a lesion in *pgm* and it is also incapable of producing EPS (Leigh & Coplin, 1992). Consistent with this, neither strain was fluorescent on Calcofluor (Table 4). However, unlike SRmA258, which was sensitive to Mg^{2+}, SRmA259 was not sensitive to Mg^{2+}, but it was very sensitive to Ca^{2+}. The addition of as little as 3·5 mM $CaCl_2$ to an LB base medium containing 100 mM NaCl gave an observable salt-sensitive phenotype (data not shown).

EPS I mutations and salt sensitivity

The original genetic screens yielded two mutants that were first reported as *exo* mutations: *pgm* (*exoC*) and *exoF* (Leigh *et al.*, 1985). Mutations in *pgm* are pleiotropic, and affect the synthesis of both succinoglycan and LPS. To our knowledge, the inability to synthesize succinoglycan has not previously been shown to be related to a salt-sensitive phenotype in *S. meliloti*. We note that cultures of strain SRmA258 that were grown from inocula taken from older plates had a tendency to lose their salt-sensitivity phenotype, and cultures of SRmA258 also generated a high number of isolated colonies if they were left on LB base medium with 50 mM $MgCl_2$.

Strain Rm7055, which carries an *exoF* (Leigh *et al.*, 1985) mutation, in our hands was not sensitive to salt and did not show any of the phenotypes that were associated with either strain SRmA259 (*pgm*) or strain SRmA305 (*exoF*). Since we had seen a number of second-site mutations arise in SRmA305, we hypothesized that this may have also occurred in Rm7055. The transposons from both Rm7055 and SRmA305 were transduced into strain Rm1021. Transductants from both crosses were 100 % linked with their respective salt phenotypes, suggesting that either both phenotypes were correct, or a second-site mutation had arisen that was tightly linked with one of the *exoF* alleles. The position of the Tn*5* insertion in Rm7055 was determined by generating an arbitrary PCR fragment from the genomic DNA flanking the Tn*5*. Nucleotide sequencing of the PCR fragment clearly showed that the *exoF55* allele in Rm7055 was not within the *exoF* gene as previously reported but within the *exoY* gene, which is predicted to be responsible

Table 4. Sensitivity of salt-sensitive mutants to other ions

Strains were streaked onto various media and scored for growth. Designations are as follows: ++, like wild-type; +, poor growth; +/−, some growth on primary streak; −, no growth.

Strain	Glc*	NaCl† 100	NaCl‡ 350	LiCl§	KCl§	$MgCl_2$§	$CaCl_2$§	Calc‖
Rm1021	++	++	+	+	++	++	++	+
SRmA239	++	++	−	+/−	+/−	++	++	+
SRmA251	++	++	+/−	+	++	++	++	+
SRmA258	++	++	−	−	−	+/−	++	−
SRmA259	+	++	+/−	+/−	+/−	++	−	−
SRmA262	++	++	−	+/−	+	++	++	Dim
SRmA267	++	++	−	−	+	++	++	+
SRmA282	++	++	−	−	+/−	++	++	+
SRmA284	++	++	−	−	−	++	+	+

*VMM with 15 mM glucose as a sole carbon source.

†LB base medium with 100 mM NaCl.

‡LB base medium with 350 mM NaCl.

§Salts were added to LB base medium such that the final concentrations were as follows: LiCl, 350 mM; KCl, 350 mM; $MgCl_2$, 50 mM; $CaCl_2$, 50 mM.

‖LB with 0·02 % Calcofluor: +, bright; −, dark.

for the transfer of the initial galactose onto a carrier as the first step in succinoglycan biosynthesis (Reuber & Walker, 1993).

It is a common practice to grow strain Rm1021 on LB that contains Mg^{2+} and Ca^{2+} (Glazebrook & Walker, 1991). Since we had found a difference in the salt sensitivity of strains SRmA305 (*exoF1*) and Rm7055 (*exoY*), and we had seen that 3·5 mM $CaCl_2$ could have an effect on a strain's sensitivity to salt, we wanted to know if the difference between the strains could be attributed to the genetic screens that had been employed or the media on which they were purified.

Because of the simplicity of the screen, an additional 2000 Tn*5* mutants were visually screened on LB that contained Calcofluor (*exo* mutants show a dark phenotype on LB Calcofluor plates). Two dark mutants were isolated, purified and designated SRmA337and SRmA339. Both mutants were sensitive to 350 mM salt, and arbitrary PCR analysis of these mutants showed that Tn*5* in SRmA337 was located in *pgm* whereas the Tn*5* insertion in SRmA339 was in *exoA*. Consistent with the previous data, the strain carrying the *pgm* mutation was sensitive to Ca^{2+}, and the strain carrying the *exoA* mutation was sensitive to Mg^{2+}. The isolation of these mutations further supports the hypothesis that EPS I plays a role in protecting the cell against high ion concentrations.

The Mg^{2+} sensitivity phenotype of *exoF* is suppressed by *expR*$^+$

The ability of *expR*$^+$ to suppress EPS-associated symbiotic phenotypes has been previously reported (Glazebrook & Walker, 1989). To determine if *expR*$^+$ also affects the level of sensitivity of *exoF* mutants to NaCl and $MgCl_2$, the *exoF* allele was transduced from strain SRmA305 into RmG182. Because RmG182 also contains the insert Ω317::Tn*5*-233 marker linked in transduction to the *expR*$^+$ allele, it was also transduced from RmG182 into Rm1021 to make strain SRmA287 so that all possible combinations of markers could be tested. The presence of the *expR*$^+$ allele did not affect the salt sensitivity associated with the *exoF* allele but it did alleviate the associated sensitivity to Mg^{2+} (Table 5). Surprisingly, RmG182 was found to be sensitive to NaCl (Table 5), but SRmA287 was not (Table 5).

These data suggest that either the *expR*$^+$ allele confers sensitivity to NaCl or *expR*$^+$ in an SRmA287 background was responsible for the observed salt phenotype. To address the latter of the two possibilities, it was reasoned that knowing the nature of the mutation caused by the insertion Ω317::Tn*5*-233 in SRmA287 might be helpful. The insertion was located 23 aa from the C terminus of RpoH2, placing the insert approximately 25 kb from *expR*$^+$, which correlates well with the 65 % cotransduction frequency (data not shown). *rpoH2* is known to affect EPS synthesis in *Rhizobium* sp. strain TAL1145 (Kaufusi *et al.*, 2004). Expression of *rpoH2* in a TAL1145 *rpoH2* background resulted in functional complementation (Kaufusi *et al.*, 2004).

It was deemed prudent to construct an *expR*$^+$ strain that was completely devoid of transposons. A strategy that employed a Tn*5* insert that was unable to grow on defined media and was linked in transduction was needed. The *pckA* gene is located at approximately 140 kb from the *expR*$^+$ allele, whereas the *rpoH2*::Tn*5*-233 is about 165 kb from *pckA*. Thus these two markers flank the *expR*$^+$ gene. Utilizing strain Rm5439, which contains *pckA*::Tn*V* [unable to use succinate as a sole carbon source (Suc^-)] (Finan *et al.*, 1988), a lysate was grown on this strain and used to transduce strain RmG182 to Nm^R. The resultant transductants were Muc^+, Gm^RSp^R and Suc^-. One colony was purified and designated strain SRmA309. A lysate was grown on SRmA309 and the *pckA*::Tn*V* marker from this strain was transduced into strain Rm1021. All of these transductants were Gm^SSp^S and 1 % of the transductants were both Nm^R (unable to use

Table 5. Effect of *expR*$^+$ on Na^+ and Mg^{2+} sensitivity

Designations are as follows: ++, like wild-type; +, poor growth; −, no growth; muc, mucoid colony morphology. Where a colony morphology designation has not been made, the colony morphology is dry.

Strain	Relevant genotype	LB	LB + 350 mM NaCl	LB + 100 mM $MgCl_2$
Rm1021	Wild-type	++	++	++
SRmA305	*exoF1*	++	−	−
SRmA339	*exoA1*	++	−	−
SRmA287	Ω317::Tn*5*-233	++	++	++
SRmA321	*exoF1 expR*$^+$ Ω317::Tn*5*-233	++^{muc}	−	++^{dry}
SRmA322	*exoF1 expR*$^-$ Ω317::Tn*5*-233	++	−	−
RmG182	*expR*$^+$ Ω317::Tn*5*-233	++^{muc}	−	++^{dry}
SRmA363	*expR*$^+$	++^{muc}	−	++^{dry}
SRmA457	*expR*$^+$ *exoF1*	++^{muc}	−	++^{dry}
SRmA458	*expR*$^+$ *exoA1*	++^{muc}	−	−

succinate) and mucoid, suggesting that the *expR*$^+$ allele and the *pckA* allele were cotransduced into Rm1021. One of these was purified and designated strain SRmA316. To eliminate the *pckA*::Tn*V* mutation, a wild-type lysate was used to transduce SRmA316 to prototrophy. The resultant strain was purified and designated SRmA363. Utilizing the published primer sequences that flank *expR*$^+$ (Pellock *et al.*, 2002) a 0·9 kb PCR fragment was generated by using SRmA363 genomic DNA as template. Subsequent gel isolation and nucleotide sequencing of both strands of the PCR fragment showed that SRmA363 contained the *expR*$^+$ gene (data not shown).

The resultant strain, SRmA363, was used to construct strains carrying either *exoA*, *expR*$^+$ or *exoF*, *expR*$^+$. These strains were designated SRmA458 and SRmA457, respectively, and tested for NaCl and $MgCl_2$ sensitivity (Table 5). The results showed that there is a strong correlation between the presence of *expR*$^+$ and salt sensitivity. Moreover, *expR*$^+$ was clearly capable of reversing the sensitivity to $MgCl_2$ associated with *exoF1*, but not *exoA*, and the addition of 100 mM $MgCl_2$ appeared to reverse the mucoid phenotype associated with the *expR*$^+$ allele, suggesting that the presence of mucoidy (and presumably EPS II) was not the determining factor in reversing the $MgCl_2$ phenotype associated with *exoF1*.

EPS II is affected by ions and not osmolarity

The basis for the original isolation of *expR*$^+$ was the exhibition of a mucoid phenotype (Glazebrook & Walker, 1989). Because $MgCl_2$ appears to reverse the mucoid phenotype, and *expR*$^+$ appears to confer sensitivity to NaCl, the effects of other ions and osmolarity were investigated. Streaking of either strain SRmA363 or strain RmG182 onto LB containing different ions revealed that either 50 mM Mg^{2+} or 350 mM K^+ in LB could reverse the mucoid phenotype conferred by the presence of *expR*$^+$ (Fig. 1). The strains did not appear to grow slower than those streaked onto LB not containing the ions. To ensure that the ions did not delay the onset of mucoidy, plates were incubated for extended periods. Delayed mucoidy was not observed. The mucoid colony phenotype was not reversed by Li^+, Ca^{2+}, or the presence of high osmolarity induced by the presence of 5 % PEG 8000.

Some salt-sensitive mutants are not competitive for nodule occupancy

Prior to testing our hypothesis regarding competition in the rhizosphere and salt sensitivity, the eight mutants were first tested for symbiotic proficiency. Consistent with previously published data, *exoF*, *exoA* and *pgm* mutants were unable to form normal nitrogen-fixing nodules (Finan *et al.*, 1985; Leigh *et al.*, 1985). The six remaining strains were symbiotically proficient and not different from the wild-type with respect to nitrogen fixation, nodule number and morphology (data not shown).

Each strain's ability to compete for nodule occupancy was tested by inoculating approximately equal amounts of wild-type and mutant onto alfalfa seedlings. Nodules were harvested and the bacteria were reisolated and tested for phenotype. The results showed that of the six strains tested, two, SRmA239 and SRmA284, occupied a percentage of nodules not significantly different from the proportion of the strain found in the inoculum (Table 6). The remaining four strains, SRmA262, SRmA282, SRmA251 and SRmA267, were all found to occupy a significantly lower proportion of nodules than would be expected from the proportion of each strain found in the inoculum, suggesting that these strains were less competitive for nodule occupancy than the wild-type (Table 6).

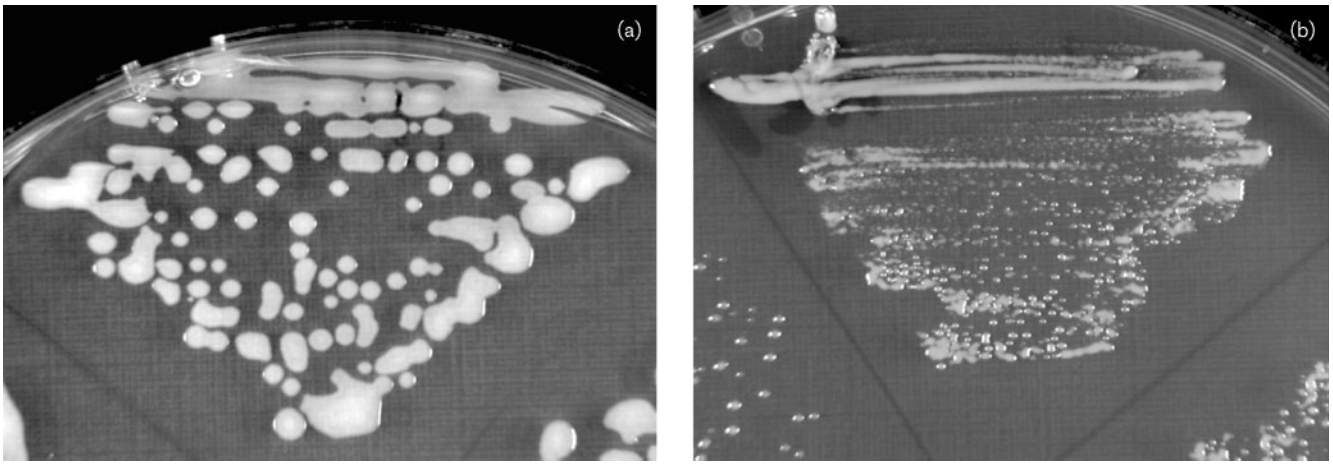

Fig. 1. The *expR*$^+$ mucoidy is ion dependent. LB agar plates of strain RmG182 showing colony morphology when grown without (a) or with (b) a supplement of 350 mM KCl.

Table 6. Nodulation competition assays of salt-sensitive mutants with Rm1021 as an indicator strain

One representative set of data representing at least 50 nodules is shown. This experiment was replicated three times with identical results.

Strain*	Relevant genotype	Inoculum (%)	Nodules (%)	*P* value†
SRmA239	Smb20192	68	45	NS
SRmA251	*fabG*	88	21	$<1\times10^{-4}$
SRmA262	Smc02909	66	24	$<1\times10^{-4}$
SRmA267	*tig*	48	20	1×10^{-3}
SRmA282‡	Smc00717	30	4	2×10^{-4}
SRmA284	Smb20056	78	86	NS

*Strains were assayed against Rm1021.

†*P* values are shown for treatments that were statistically significant. NS, not significant.

‡Data from a single replicate. See text for details.

DISCUSSION

This work was carried out to identify candidate genes that play a role in determining what events occur within a high-solute rhizosphere. It is particularly striking that so few of the mutations we isolated correspond to genes that have been identified as having increased or decreased expression by microarray analysis (Rüberg *et al.*, 2003), or previous genetic studies that focused on salt effects and *Rhizobium* (Barra *et al.*, 2003; Nogales *et al.*, 2002). The mutations isolated appear to be diverse, suggesting that many loci affect the ability of *S. meliloti* to grow at elevated salt concentrations. Although it is unlikely that the concentration of salt in the rhizosphere is equivalent to that which was used to screen for mutants, it is pertinent to consider that elevated concentrations of salt also increase the osmotic stress encountered by the bacteria. It has been suggested that bacteria may need to adapt to changes in osmolarity during symbiosis (Gore & Miller, 1993; Spaink, 2000; Vedam *et al.*, 2003). Therefore, the use of this screen, followed by assaying the mutants for a subtle phenotype (competition for nodule occupancy), has the potential to unveil not only genes that affect salt sensitivity but also those that affect growth in the rhizosphere or during symbiosome development.

Genes Smb20192 and Smb20056 (represented by strains SRmA239 and SRmA284, respectively) do not appear to play pivotal roles in competition within the rhizosphere. The nodulation competition data suggest that mutants *fabG* (strain SRmA251), Smc02909 (strain SRmA262), *tig* (strain SRmA267) and Smc00717 (strain SRmA282) are significantly affected in competing against the wild-type for nodule occupancy (Table 6). Proteomic studies have identified trigger factor (Tig) as a protein that is increased in nodule bacteria or during stress (Djordjevic *et al.*, 2003). In *E. coli*, trigger factor is responsible for the correct targeting of some secreted proteins to either the cytoplasmic membrane or the periplasm (Beck *et al.*, 2000). Our data showing that SRmA267 (*tig*::Tn*5*) has a reduced ability to grow in LB with elevated salt as well as being unable to compete against the wild-type for nodule occupancy corroborate this finding (Table 2, Table 6). Strain SRmA267 can, however, form effective nodules; therefore *tig* is dispensable for normal nodule formation and nitrogen fixation.

Within the genome there are eight genes annotated as transmembrane glycosylases, one of which, Smc02909, is mutated in strain SRmA262. A conserved-domain search recognizes this gene to encode a lytic transmembrane transglycosylase that belongs to COG 2951 and is 90 % aligned with MltB (membrane-bound lytic murein transglycosylase B), an enzyme that is capable of both hydrolysing and synthesizing β-1,4-glycosidic linkages in peptidoglycan. The most straightforward interpretation of the salt and competition phenotype associated with this mutation is that growth and/or peptidoglycan remodelling occurs during both these processes. Although this suggests that the mutation could be pleiotropic, we note that the ability of this strain to grow on standard laboratory media does not seem compromised and it does appear normal with respect to its ability to nodulate and fix nitrogen.

The insert in *fabG* is intriguing because FabG reduces the β-ketoester from the condensation of malonyl-CoA with a growing acyl chain (Cronan & Rock, 1996). In *E. coli* and *Salmonella enterica* this gene has been shown to be essential for viability, with only temperature-sensitive mutants having been isolated (Lai & Cronan, 2004; Zhang & Cronan, 1998). In strain Rm1021 *nodG* appears to be a duplication of *fabG* (López-Lara & Geiger, 2001) and expression of *nodG* is regulated by NodD (Maillet *et al.*, 1990), suggesting that *nodG* should not be transcribed under free-living conditions. The fact that the mutation in strain SRmA251 is not lethal suggests that either there is expression of *nodG* or there is a third copy of a gene encoding a FabG-like protein. The latter possibility is unlikely since the entire genome has been sequenced. It has been shown that expression of *nodG* does increase in the presence of *nodD3* in the absence of inducer, but this effect is not seen with *nodE*,

nodH, *nodA* or *nodD3* fusions (Maillet *et al.*, 1990). These data suggest that it may be possible to have some *nodG* expressed under free-living conditions and this may allow a *fabG* strain to be viable in *S. meliloti*.

The reason for the inability of strain SRmA251 to compete for nodule occupancy is unknown but may involve a change in membrane composition in response to high ion concentrations. Fatty acid synthesis is involved in providing the acyl portion of acylated homoserine lactones in *Pseudomonas aeruginosa* (Hoang *et al.*, 2002) and in its potential interaction with the synthesis of acylated lipochito-oligosaccharides in rhizobia (Spaink, 2000). Therefore, mechanisms involving either of these processes in compromising a strain carrying a *fabG* mutation from effectively competing may be proposed, but further work is necessary.

Strain SRmA282 carries a mutation in *ftsE* and appears to be less competitive for nodule occupancy than strain Rm1021 (Table 6). These results should be viewed with caution. The results presented represent a single dataset. Although the strain was assayed multiple times, and consistently showed a decreased ratio of nodules recovered containing the mutant when compared to the inoculation ratio, we noted that upon dilution of SRmA282, we would continually attain inoculation ratios far below the anticipated ratios (data not presented). The results we present may represent a pleiotropic effect reflecting the fitness of the strain rather than an inability to compete against the wild-type.

The finding that EPS mutants were sensitive to NaCl should not have been unexpected. A number of reports have shown that either polysaccharide expression or composition are altered with the addition of NaCl (Breedveld *et al.*, 1990; Lloret *et al.*, 1998; Rüberg *et al.*, 2003). We have isolated two strains unable to synthesize EPS I using two different genetic screens and both had similar ion sensitivities (Table 4). Testing of a previously characterized strain, Rm7055, was not consistent with our findings.

The site of the mutation in strain Rm7055 is in *exoY* and not within *exoF* as has been previously reported (Leigh *et al.*, 1985). It is postulated that ExoF and ExoY are necessary for the initial addition of galactose during EPS I biosynthesis (Reuber & Walker, 1993). The representative *exoF* allele used in the study was the *exoF55* allele from Rm7055 (Reuber & Walker, 1993). Effectively this means that ExoF more than likely does not play a role in the initiation of EPS I synthesis, and based on the annotation within the *S. meliloti* database, it is likely to be an outer-membrane protein involved in polysaccharide export (http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/). Thus we would predict that a strain carrying an *exoF1* mutation should produce a polysaccharide equivalent to that of a strain carrying an *exoQ* mutation. *exoQ* mutants have been shown to produce complete succinoglycan subunits and are believed to be unable to make high-molecular-mass polysaccharide (Reuber & Walker, 1993). It is therefore possible that the difference in the sensitivity to NaCl between Rm7055 and the mutations we isolated may be due to the ability to initiate EPS I synthesis. Further work will be necessary to resolve these differences.

The sensitivity to Mg^{2+} exhibited by a strain carrying an *exoF1* mutation was suppressed by *expR*$^+$, but *exoA* was unaffected by *expR*$^+$ (Table 5). The suppression of the *exoF* phenotype was not due to the production of EPS II because both strain SRmA321 (*exoF expR*$^+$ *rpoH2*) and strain SRmA457 (*exoF expR*$^+$) were non-mucoid on media containing Mg^{2+}. This suggests that *expR*$^+$ does not suppress the *exoF*-associated phenotype by the production of EPS II. It also shows that EPS II production is somehow regulated by the presence of Mg^{2+}. Recent microarray studies demonstrate that ExpR affects many genes (Hoang *et al.*, 2004). It is not clear which of the ExpR target gene(s) may be responsible for the suppression of Mg^{2+} sensitivity.

The reversal of the mucoid phenotype associated with *expR*$^+$ prompted us to try different ions. We found that both Mg^{2+} and K^+ had the same effect. Presumably the lack of mucoidy is due to a lack of EPS II biosynthesis. Although EPS II is not normally synthesized by strain Rm1021, it can be synthesized under low-phosphate conditions (Mendrygal & González, 2000; Rüberg *et al.*, 1999; Zhan *et al.*, 1991). Mutations affecting phosphate uptake show an osmolarity-dependent EPS II synthesis that is reversed by the addition of NaCl, $MgCl_2$ or KCl (Oresnik *et al.*, 1994). Taken together, the data suggest that in addition to being regulated by ExpR and phosphate, EPS II synthesis may also be regulated in an ion-dependent manner independent of ExpR.

EPS II is regulated by NaCl in a *S. meliloti* strain that was isolated as being halotolerant (Lloret *et al.*, 1998). Since the presence of ExpR in strain Rm1021 is correlated with increased salt sensitivity (Table 5), the effect of NaCl on the mucoid phenotype could not be determined directly.

The finding that strain Rm1021 derivatives carrying *expR*$^+$ were sensitive to salt was unexpected. It appears that in addition to regulating EPS II biosynthesis, ExpR must also regulate genes that play a role in either osmotic adaptation or salt tolerance. Using published microarray data, it is clear that ExpR affects the expression of EPS I biosynthesis genes, as well as *ndvA* regulation (Hoang *et al.*, 2004). Our data show that strains carrying some EPS I biosynthesis mutations are sensitive on media containing salt (Table 3, Table 4). Previous work has also shown that strains carrying *ndvA* mutations are compromised under hypo-osmotic conditions (Dylan *et al.*, 1990). Interestingly, *ndv* osmotic pseudorevertants were shown to be suppressed by the presence of loci corresponding to EPS I biosynthesis (Nagpal *et al.*, 1992). Taking these findings together, it might not be unexpected that changes in either salt or osmotic sensitivities may be seen in an *expR*$^+$ background.

In summary, this work has investigated the hypothesis that *S. meliloti* mutants that are affected in their ability to survive at elevated NaCl concentrations will also be unable to

compete against the wild-type for nodulation in the rhizosphere. The analysis has shown that of the six salt-sensitive mutants that were capable of a symbiotic association, four were compromised in their ability to compete against the wild-type for nodule occupancy. In addition, a role for EPS I contributing to the tolerance of *S. meliloti* to high NaCl or $MgCl_2$ concentrations has been shown. The sensitivity of an *exoF* mutation to Mg^{2+} is altered in an *expR*$^+$-dependent, EPS II-independent manner. Moreover, we have observed that *expR*$^+$-dependent mucoidy is regulated by the addition of K^+ or Mg^{2+}. To our knowledge, the role ions play in gene regulation has not been previously addressed in *S. meliloti*. It seems unlikely that only EPS determinant genes are affected by ions. In light of this, our work is currently focused on pursuing both how ions are perceived and what determinants are affected by changes in ion concentration.

ACKNOWLEDGEMENTS

This work was supported by NSERC discovery grants to I. J. O. (RG238421) and to P. C. L. (OGP9600) and the Canada Research Chair Program to P. C. L.

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J. H., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I. & Kondorsi, A. (1981). Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*. *Mol Gen Genet* **184**, 318–325.

Barnett, M. J., Fisher, R. F., Jones, T. & 23 other authors (2001). Nucleotide sequence and predicted functions of the entire *Sinorhizobium meliloti* pSymA megaplasmid. *Proc Natl Acad Sci U S A* **98**, 9883–9888.

Barra, L., Bowser, L., Pica, N., Gouffi, K., Walker, G. C., Blanco, C. & Trautwetter, A. (2003). Glucose 6-phosphate dehydrogenase is required for sucrose and trehalose to be efficient osmoprotectants in *Sinorhizobium meliloti*. *FEMS Microbiol Lett* **229**, 183–188.

Beck, K., Wu, L., Brunner, J. & Müller, M. (2000). Discrimination between SRP- and SecA/SecB-dependent substrates involves selective recognition of nascent chains by SRP and trigger factor. *EMBO J* **19**, 134–143.

Botsford, J. L. (1984). Osmoregulation in *Rhizobium meliloti*: inhibition of growth by salts. *Arch Microbiol* **137**, 124–127.

Botsford, J. L. & Lewis, T. A. (1990). Osmoregulation in *Rhizobium meliloti*: production of glutamic acid in response to osmotic stress. *Appl Environ Microbiol* **56**, 488–494.

Bowen, G. D. & Rovira, A. D. (1976). Microbial colonisation of plant roots. *Annu Rev Phytopathol* **14**, 121–144.

Breedveld, M. W., Zevenhuizen, L. P. T. M. & Zehnder, A. J. B. (1990). Osmotically induced oligo- and polysaccharide synthesis by *Rhizobium meliloti* SU-47. *J Gen Microbiol* **136**, 2511–2519.

Caetano-Anollés, G. (1993). Amplifying DNA with arbitrary oligonucleotide primers. *PCR Methods Appl* **3**, 85–94.

Cronan, J. E. & Rock, C. O. (1996). Biosynthesis of membrane lipids. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 612–636. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.

de Leeuw, E., Graham, B., Phillips, G. J., ten Hagen-Jongman, C. M., Oudega, B. & Luirink, J. (1999). Molecular characterization of *Escherichia coli* FtsE and FtsX. *Mol Microbiol* **31**, 983–993.

de Vos, G. F., Walker, G. C. & Signer, E. R. (1986). Genetic manipulations in *Rhizobium meliloti* utilizing two new transposon Tn*5* derivatives. *Mol Gen Genet* **204**, 485–491.

Djordjevic, M. A., Chen, H. C., Natera, S., Van Noorden, G., Menzel, C., Taylor, S., Renard, C., Geiger, O. & Weiller, G. F. (2003). A global analysis of protein expression profiles in *Sinorhizobium meliloti*: discovery of new genes for nodule occupancy and stress adaptation. *Mol Plant Microbe Interact* **2003**, 508–524.

Dylan, T., Helinski, D. R. & Ditta, G. S. (1990). Hypoosmotic adaptation in *Rhizobium meliloti* requires β-(1-2) glucan synthesis. *J Bacteriol* **172**, 1400–1408.

Finan, T. M., Hartwieg, E., Lemieux, K., Bergman, K., Walker, G. C. & Signer, E. R. (1984). General transduction in *Rhizobium meliloti*. *J Bacteriol* **159**, 120–124.

Finan, T. M., Hirsch, A. M., Leigh, J. A., Johansen, E., Kuldau, G. A., Deegan, S., Walker, G. C. & Signer, E. R. (1985). Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. *Cell* **40**, 869–877.

Finan, T. M., Kunkel, B., de Vos, G. F. & Signer, E. R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol* **167**, 66–72.

Finan, T. M., Oresnik, I. & Bottacin, A. (1988). Mutants of *Rhizobium meliloti* defective in succinate metabolism. *J Bacteriol* **170**, 3396–3403.

Finan, T. M., Weidner, S., Wong, K. & 9 other authors (2001). The complete sequence of the 1,683-kb pSymB megaplasmid from the N_2-fixing endosymbiont *S. meliloti*. *Proc Natl Acad Sci U S A* **98**, 9889–9894.

Galibert, F., Finan, T. M., Long, S. R. & 53 other authors (2001). The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* **293**, 668–672.

Glazebrook, J. & Walker, G. C. (1989). A novel exopolysaccharide can function in place of calcofluor-binding exopolysaccharide in nodulation on alfalfa. *Cell* **56**, 661–672.

Glazebrook, J. & Walker, G. C. (1991). Genetic techniques in *Rhizobium meliloti*. *Methods Enzymol* **204**, 398–418.

González, J. E., Reuhs, B. L. & Walker, G. C. (1996a). Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. *Proc Natl Acad Sci U S A* **93**, 8636–8641.

González, J. E., York, G. M. & Walker, G. C. (1996b). *Rhizobium meliloti* exopolysaccharides: synthesis and symbiotic function. *Gene* **179**, 141–146.

Gore, R. S. & Miller, J. M. (1993). Cyclic β-1,6-1,3 glucans are synthesized by *Bradyrhizobium japonicum* bacteroids within soybean (*Glycine max*) root nodules. *Plant Physiol* **102**, 191–194.

Gouffi, K., Pica, N., Pichereau, V. & Blanco, C. (1999). Disaccharides as a new class of nonaccumulated osmoprotectants for *Sinorhizobium meliloti*. *Appl Environ Microbiol* **65**, 1491–1500.

Gouffi, K., Bernard, T. & Blanco, C. (2000). Osmoprotection by pipecolic acid in *Sinorhizobium meliloti*: specific effects of D and L isomers. *Appl Environ Microbiol* **66**, 2358–2364.

Hoang, H. H., Becker, A. & González, J. E. (2004). The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. *J Bacteriol* **186**, 5460–5472.

Hoang, T. T., Sullivan, S. A., Cusick, J. K. & Schweizer, H. P. (2002). Beta-ketoacyl acyl carrier protein reductase (FabG) activity of the

fatty acid biosynthetic pathway is a determining factor of 3-oxo-homoserine lactone acyl chain lengths. *Microbiology* **148**, 3849–3856.

Jordan, D. C. (1984). *Rhizobiaceae*. In *Bergey's Manual of Systematic Bacteriology*, pp. 234–241. Edited by N. R. Kreig. Baltimore: Williams & Wilkins.

Kaufusi, P. H., Forsberg, L. S., Tittabutr, P. & Borthakur, D. (2004). Regulation of exopolysaccharide synthesis in *Rhizobium* sp. strain TAL1145 involves an alternative sigma factor gene, *rpoH2*. *Microbiology* **150**, 3473–3482.

Keller, M., Müller, P., Simon, R. & Pühler, A. (1988). *Rhizobium meliloti* genes for exopolysaccharide synthesis and nodule infection located on megaplasmid 2 are actively transcribed during symbiosis. *Mol Plant Microbe Interact* **1**, 267–274.

Lai, C. & Cronan, J. E. (2004). Isolation and characterization of β-ketoacyl carrier protein reductase (*fabG*) mutants of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **186**, 1869–1878.

Leigh, J. A. & Coplin, D. C. (1992). Exopolysaccharides in plant-bacterial interactions. *Annu Rev Microbiol* **46**, 307–346.

Leigh, J. A., Signer, E. R. & Walker, G. C. (1985). Exopolysaccharide deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proc Natl Acad Sci U S A* **82**, 6231–6235.

Le Rudulier, D. & Bernard, T. (1986). Salt tolerance in *Rhizobium*: a possible role for betaines. *FEMS Microbiol Rev* **39**, 67–72.

Lloret, J., Bolanos, L., Lucas, M. M., Peart, J. M., Brewin, N. J., Bonilla, I. & Rivilla, R. (1995). Ionic stress and osmotic pressure induce different alterations in the lipopolysaccharide of a *Rhizobium meliloti* strain. *Appl Environ Microbiol* **61**, 3701–3704.

Lloret, J., Wiulff, B. B. H., Rubio, J. M., Downie, J. A., Bonilla, I. & Rivilla, R. (1998). Exopolysaccharide II production is regulated by salt in the halotolerant strain *Rhizobium meliloti* EFB1. *Appl Environ Microbiol* **64**, 1024–1028.

López-Lara, I. M. & Geiger, O. (2001). The nodulation protein NodG shows the enzymatic activity of an 3-oxoacyl-acyl carrier protein reductase. *Mol Plant Microbe Interact* **14**, 349–357.

Maillet, F., Debellé, F. & Dénarie, J. (1990). Role of the *nodD* and *syrM* genes in the activation of the regulatory gene *nodD3*, and the common and host-specific *nod* genes of *Rhizobium meliloti*. *Mol Microbiol* **4**, 1975–1984.

Marroquí, S., Zorreguieta, A., Santamaría, C., Temprano, F., Soberón, M., Mégias, M. & Downie, J. A. (2001). Enhanced symbiotic performance by *Rhizobium tropici* glycogen synthase mutants. *J Bacteriol* **183**, 854–864.

Meade, H. M., Long, S. R., Ruvkin, G. B., Brown, S. E. & Ausubel, F. M. R. (1982). Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. *J Bacteriol* **149**, 114–122.

Mendrygal, K. & González, J. E. (2000). Environmental regulation of exopolysaccharide production in *Sinorhizobium meliloti*. *J Bacteriol* **182**, 599–606.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Miller, K. J. & Wood, J. M. (1996). Osmoadaptation by rhizosphere bacteria. *Annu Rev Microbiol* **50**, 101–136.

Nagpal, P., Khanuja, S. P. S. & Stanfield, S. W. (1992). Suppression of the *ndv* mutant phenotype of *Rhizobium meliloti* by cloned *exo* genes. *Mol Microbiol* **6**, 479–488.

Nogales, J., Campos, R., BenAbdelkhalek, H., Olivares, J., Lluch, C. & Sanjuan, J. (2002). *Rhizobium tropici* genes involved in free-living salt tolerance are required for the establishment of efficient nitrogen-fixing symbiosis with *Phaseolus vulgaris*. *Mol Plant Microbe Interact* **15**, 225–232.

Oresnik, I. J., Charles, T. C. & Finan, T. M. (1994). Second site mutations specifically suppress the Fix$^-$ phenotype of *Rhizobium meliloti ndvF* mutations on alfalfa: identification of a conditional *ndvF*-dependent mucoid colony phenotype. *Genetics* **136**, 1233–1243.

Oresnik, I. J., Pacarynuk, L. A., O'Brien, S. A. P., Yost, C. K. & Hynes, M. F. (1998). Plasmid encoded catabolic genes in *Rhizobium leguminosarum* bv. *trifolii*: evidence for a plant-inducible rhamnose locus involved in competition for nodulation. *Mol Plant Microbe Interact* **11**, 1175–1185.

Pellock, B. J., Cheng, H. & Walker, G. C. (2000). Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. *J Bacteriol* **182**, 4310–4318.

Pellock, B. J., Teplitski, M., Boinay, R. P., Bauer, W. D. & Walker, G. C. (2002). A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. *J Bacteriol* **184**, 5067–5076.

Raffa, G. R. & Raivio, T. L. (2002). A third envelope stress signal transduction pathway in *Escherichia coli*. *Mol Microbiol* **45**, 1599–1611.

Reuber, T. L. & Walker, G. C. (1993). Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. *Cell* **74**, 269–280.

Reuhs, B. L., Williams, M. N. V., Kim, J. S., Carlson, R. W. & Côté, F. (1995). Suppression of the Fix$^-$ phenotype of *Rhizobium meliloti exoB* mutants by *lpsZ* is correlated to modified expression of the K polysaccharide. *J Bacteriol* **177**, 4289–4296.

Rüberg, S., Pühler, A. & Becker, A. (1999). Biosynthesis of the exopolysaccharide galactoglucan in *Sinorhizobium meliloti* is subject to a complex control by the phosphate-dependent regulator PhoB and the proteins ExpG and MucR. *Microbiology* **145**, 603–611.

Rüberg, S., Tian, Z., Krol, E., Linke, B., Meyer, F., Wang, Y., Pühler, A., Weidner, S. & Becker, A. (2003). Construction and validation of a *Sinorhizobium meliloti* whole genome DNA microarray: genome-wide profiling of osmoadaptive gene expression. *J Biotechnol* **106**, 255–268.

Sambrook, J., Fritsch, E. F. & Maniatis, T. A. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schmidt, K. L., Peterson, N. D., Kustusch, R. J., Wissel, M. C., Graham, B., Phillips, G. J. & Weiss, D. S. (2004). A predicted ABC transporter, FtsEX, is needed for cell division in *Escherichia coli*. *J Bacteriol* **186**, 785–793.

Smith, L. T. & Smith, G. M. (1989). An osmoregulated dipeptide in stressed *Rhizobium meliloti*. *J Bacteriol* **171**, 4714–4717.

Smith, L. T., Smith, G. B., D'Souza, M., Pocard, J.-A., Le Rudulier, D. & Madkour, M. A. (1994). Osmoregulation in *Rhizobium meliloti*: mechanism and control by other environmental signals. *J Exp Zool* **268**, 162–165.

Spaink, H. P. (2000). Root nodulation and infection factors produced by rhizobial bacteria. *Annu Rev Microbiol* **54**, 257–288.

Vedam, V., Kannenberg, E. L., Haynes, J. G., Sherrier, D. J., Datta, A. & Carlson, R. W. (2003). A *Rhizobium leguminosarum* AcpXL mutant produces lipopolysaccharide lacking 27-hydroxyoctacosanoic acid. *J Bacteriol* **185**, 1841–1850.

Vincent, J. M. (1970). *A Manual for the Practical Study of Root-Nodule Bacteria*. Oxford, UK: Blackwell Scientific Publications.

Zhan, H. J., Lee, C. C. & Leigh, J. A. (1991). Induction of the second exopolysaccharide (EPSb) in *Rhizobium meliloti* SU47 by low phosphate concentrations. *J Bacteriol* **173**, 7391–7394.

Zhang, Y. & Cronan, J. E. (1998). Transcriptional analysis of essential genes of the *Escherichia coli* fatty acid biosynthesis gene cluster by functional replacement with the analogous *Salmonella typhimurium* gene cluster. *J Bacteriol* **180**, 3295–3303.