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Symbiotic and galactose utilization properties of phage RMP64-resistant mutants affecting three complementation groups in *Rhizobium meliloti*

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Abstract. Random Tn*5* insertional mutants were induced in *Rhizobium meliloti* Rmd201, a streptomycin-resistant mutant of AK631 (which is itself a compact colony morphology mutant of the wild-type strain Rm41), and screened for sensitivity to a set of 16 phages. Out of 3000 mutants 240 were found to be phage-resistant. The phage-resistant mutants were separable into six groups on the basis of their sensitivity pattern against test phages. Nodulation tests on alfalfa showed that although all the phage-resistant mutants induced root nodules, 7 mutants out of 12 of a class resistant to phage RMP64 (Sxf^-) induced atypical nodules that were ineffective in nitrogen fixation (Fix^-). The aberrant nodules were small, white, contained only a few bacteria and no bacteroids, and phenotypically resembled nodules elicited by already known *exoB*, *exoH*, *ndvA* and *ndvB* mutants of *R. meliloti*. Spontaneous mutants selected for resistance to RMP64 also fell into two groups: Fix^+ and Fix^-. Genetic complementation tests between the Sxf^- mutants defined three genes *sxfA*, *sxfB* and *sxfC*, of which *sxfA* and *sxfB* comprise an operon. These also demonstrated that *sxfA*, *sxfB* and *sxfC* must be located on the same replicon. All the Sxf^- mutants were Calcofluor-positive, like their parent strains Rmd201 and AK631. Characterization of carbohydrate metabolism of the mutants revealed that while the *sxfA* (Fix^-) and *sxfB* (Fix^+) mutants utilized galactose as sole carbon source, *sxfC* (Fix^-) mutants did not. It has been concluded that *sxfA*, *sxfB* and *sxfC* are new genetic loci and that *sxfA* and *sxfC* have roles in nodule invasion and development.

Keywords. *Rhizobium meliloti*; symbiotic genes; *sxf* genes; phage resistance markers; nodulation mutants.

1. Introduction

Gram-negative soil bacteria of the species *Rhizobium meliloti* invade roots of many leguminous plants specific to them, including alfalfa (*Medicago sativa*), and induce the formation of root nodules (Dart 1977; Vincent 1980; Bauer 1981). In the nodules the rhizobia use substrates from the host plant to obtain energy and convert atmospheric nitrogen into a form utilizable by the plant (Duncan 1981; Stowers 1985; Renalier *et al.* 1987). The nitrogen-fixing nodules arise by a sequence of events including the following: positive chemotactic movement of bacteria leading to their adherence to root hairs, aberrant root hair growth at the point of bacterial attachment, penetration of root hair by bacteria, formation of tubular infection thread in which bacteria multiply at the expense of the host, invasion of root cortex by the growing infection thread, commencement of meristematic growth in root cortex, organogenesis of nodule and accompanied ramification by infection thread, release of bacteria from infection thread into host cell cytoplasm, and differentiation

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of bacteria into nitrogen-fixing bacteroids surrounded by peribacteroid membrane (Truchet *et al.* 1980; Beveridge 1981; Newcomb 1981; Hirsch *et al.* 1982, 1983; Long 1984; Parke *et al.* 1985; Vasse and Truchet 1985; Noel *et al.* 1986; Dudley *et al.* 1987). The *R. meliloti* symbiotic genes have been defined by isolation and characterization of bacterial mutants that either form no nodules at all or form nodules that fail to fix nitrogen (Long *et al.* 1982; Ruvkun *et al.* 1982; Corbin *et al.* 1983; Kondorosi *et al.* 1984; Szeto *et al.* 1984). These studies have indicated that nodulation determinants comprise two sets of genes: (i) *nodABC* and *nodD* (nodulation genes common to the genus *Rhizobium*), and (ii) *nodFE*, *nodG* and *nodH* (host-specific nodulation genes) (Jacobs *et al.* 1985; Debellé *et al.* 1986; Horvath *et al.* 1986; Renalier *et al.* 1987). The biochemical roles of *nod* genes are not fully understood. It is known that NodD protein positively regulates *nodABC*, *nodFE*, *nodG* and *nodH* genes (Honma and Ausubel 1987; Horvath *et al.* 1987; Spaink *et al.* 1987); NodA and NodB are cytosol proteins (Schmidt *et al.* 1988); and NodC is an outer membrane protein (John *et al.* 1985). Further it has been speculated that NodA and NodB are involved in the synthesis of cytokinin-like substances (Schmidt *et al.* 1988) and that NodC may be serving as a transducer of bacterial signal to plant cells. The genetic determinants of nodulation proficiency in nitrogen fixation fall into three groups: (i) *nifHDKE*, *nifB*, *nifA*, *nifN*, *ntrC* and *ntrA* genes, which are functionally analogous to the identically named genes characterized in *Klebsiella pneumoniae* (Corbin *et al.* 1983; Buikema *et al.* 1987; Ronson *et al.* 1987; Szeto *et al.* 1987); (ii) *fixABCX* and *fixF* genes responsible for as yet undefined functions that are unique to *Rhizobium* (Aguilar *et al.* 1985; Batut *et al.* 1985; Weber *et al.* 1985; Earl *et al.* 1987), and *fixLJ* genes that regulate all the nitrogen fixation genes (David *et al.* 1988); and (iii) *exoA*, *exoB*, *exoC*, *exoD*, *exoF*, *exoH*, *exoL*, *exoM*, *exoP* and *exoQ*, and *ndvAB* genes required for massive bacterial growth in the infection thread and accompanying nodule growth, mediated by synthesis of specific surface polysaccharides (Leigh *et al.* 1985; Finan *et al.* 1986; Doherty *et al.* 1988; Long *et al.* 1988; Muller *et al.* 1988). While knowledge about the known symbiotic genes is far from complete, few data are available on the genes remaining to be identified (Batut *et al.* 1985; Renalier *et al.* 1987; Long *et al.* 1988; Putnoky *et al.* 1988).

The already known characteristics of symbiotic association between *M. sativa* and *R. meliloti* indicate that different surface functions of *R. meliloti* must be involved in the various stages of nodule development on *M. sativa* roots, especially in the stages relating to chemotaxis, adherence, uptake of substrates for growth and of signal substances for differentiation from host, and export of structural constituents of glycocalyx and infection thread wall and of signals for plant cells for nodule organogenesis. Although, with the isolation of mutants in *exo* and *ndv* genes, the outer surface of *R. meliloti* has been directly implicated in infection thread formation, the genetics of the entire range of surface functions involved in the development of nodules remains to be investigated.

In gram-negative bacteria, the outer cell surface consists of the outer membrane and appendages borne on it—flagella for motility, fimbriae for adherence, and sex pili for conjugation (Reeves 1979; Braun and Hantke 1981; Costerton *et al.* 1985). The outer membrane consists of a phospholipid bilayer embedded with lipopolysaccharide and other polysaccharides, and a variety of proteins, including porins (Braun and Hantke 1977; Koshland 1979; Hancock 1987). The proteins in the outer membrane are involved in active and passive transport of nutrients

(Wayne and Neilands 1975; Braun and Hantke 1977; Costerton *et al.* 1985; Hancock 1987). Outer membrane macromolecules and appendages commonly serve as specific receptors for different phages, besides performing their other functions (Wayne and Neilands 1975; Braun and Hantke 1977, 1981; Lindberg 1977; Reeves 1979; Handelsman *et al.* 1984). Thus mutant bacteria resistant to different phages are usually affected in distinct cell surface macromolecules and often have pleiotropic phenotypes (Kumar 1976).

With this background, in order to begin to define those surface function genes of *R. meliloti* that are required for induction of nitrogen-fixing root nodules by it on alfalfa, random mutants resistant to different phages were isolated in a wild-type strain of *R. meliloti* and tested for nodulation properties. It was found that some mutants resistant to certain phages had also become symbiotically defective. On alfalfa, these mutants formed atypical nodules that were incapable of fixing nitrogen. The colonies of the mutants on solid medium were stainable with Calcofluor, suggesting that they were extracellular acidic polysaccharide-positive. Genetic complementation tests revealed that the mutants defined two new symbiotic genes. This paper reports these experiments.

2. Materials and methods

2.1 *Bacterial strains, plasmids and phages*

Table 1 lists the bacterial strains, plasmids and phages used.

2.2 *Media and growth conditions*

Complex media used were TB and MacConkey for *E. coli* (Kumar 1976), and TY, MSY (mannitol, 10 g; yeast extract, 200 mg; K_2HPO_4, 200 mg; KH_2PO_4, 200 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; $CaCl_2 \cdot 2H_2O$, 50 mg; H_2O, 1 litre) and GSY (mannitol in MSY replaced with 0·2% glucose) for *R. meliloti* (Sikka and Kumar 1984; Khanuja and Kumar 1988). The minimal medium used for *R. meliloti* has been described previously (Sikka and Kumar 1984). It was supplemented with glucose or other carbon sources at 2 mg/ml. Media for pouring plates contained 1·6% agar. Top agar medium contained 0·8% agar. Both *E. coli* and *R. meliloti* were grown at 30°C. Calcofluor White was used at 0·02% (w/v) in MSY medium. Sodium deoxycholate was used at 2% (w/v) in TY medium. Antibiotics were routinely used at the following concentrations (in μg/ml). For *R. meliloti*: chloramphenicol (Cm), 20; gentamycin (Gm), 20; kanamycin (Km), 50; streptomycin (Sm), 100; tetracycline (Tc), 10; trimethoprim (Tm), 100. For *E. coli*: ampicillin (Ap), 200; Cm, 20; Gm, 20; Km, 20; Tc, 10. Antibiotics were added to agar-containing media just before pouring plates.

2.3 *Phage methods*

Phage lysates were made in *R. meliloti* AK631 or Rmd201. Methods used for preparation of phage lysates and for making phage sensitivity tests on *R. meliloti* strains were as described elsewhere (Khanuja and Kumar 1988).

Table 1. Bacterial strains, plasmids and phages used.

Strain, plasmid or phage	Relevant characteristics	Source or reference
Rhizobium meliloti		
AK631	Spontaneous compact colony-forming and relatively less mucilage-producing derivative of Rm41	Kondorosi *et al.* 1984
Rmd201	Spontaneous streptomycin-resistant derivative of AK631	Khanuja and Kumar 1988
Rmd202	Rmd201 *sxfA-1* Nod$^+$ Fix$^-$ Gal$^+$	Present work
Rmd203	Rmd201 *sxfA-2* Nod$^+$ Fix$^-$ Gal$^+$	
Rmd204	Rmd201 *sxfB-1* Nod$^+$ Fix$^+$ Gal$^+$	
Rmd205	Rmd201 *sxfB-2* Nod$^+$ Fix$^+$ Gal$^+$	
Rmd432	Rmd201 *sxfA*::Tn5-35 Nod$^+$ Fix$^-$ Gal$^+$	
Rmd436	Rmd201 *sxfA*::Tn5-2873 Nod$^+$ Fix$^-$ Gal$^+$	
Rmd441	Rmd201 *sxfA*::Tn5-2231 Nod$^+$ Fix$^-$ Gal$^+$	
Rmd443	Rmd201 *sxfA*::Tn5-2720 Nod$^+$ Fix$^-$ Gal$^+$	
Rmd433	Rmd201 *sxfB*::Tn5-129 Nod$^+$ Fix$^+$ Gal$^+$	
Rmd435	Rmd201 *sxfB*::Tn5-2872 Nod$^+$ Fix$^+$ Gal$^+$	
Rmd437	Rmd201 *sxfB*::Tn5-53 Nod$^+$ Fix$^+$ Gal$^+$	
Rmd439	Rmd201 *sxfB*::Tn5-2229 Nod$^+$ Fix$^+$ Gal$^+$	
Rmd442	Rmd201 *sxfB*::Tn5-2563 Nod$^+$ Fix$^+$ Gal$^+$	
Rmd434	Rmd201 *sxfC*::Tn5-2203 Nod$^+$ Fix$^-$ Gal$^-$	
Rmd438	Rmd201 *sxfC*::Tn5-861 Nod$^+$ Fix$^-$ Gal$^-$	
Rmd440	Rmd201 *sxfC*::Tn5-2226 Nod$^+$ Fix$^-$ Gal$^-$	
Rmd601	Rmd201 *exo*::Tn5-1 Calcofluor-dark Nod$^+$ Fix$^-$; pJB3JI-prime carrying this mutation complements the *exoA32* but not the *exoB13* mutation in Rm1021	S. Subbarao, this laboratory
Rmd611	Nitrosoguanidine-induced mutant of Rmd201 unable to utilize succinate as carbon source	A. Pandey, this laboratory
Rmd612	As above but isolated independently of Rmd611	
AK635	Derivative of Rm41 carrying a deletion covering *nodABCD* and *nifN* genes, produces more Calcofluor-binding exopolysaccharides than parent	Banfalvi *et al.* 1985
ZB129	AK631 derivative carrying a deletion covering *nifHDK*, *nifE hsnABCD*, *nodABCD* and *nifN* genes	
AK1212	AK631 carrying a deletion covering genes lost in ZB129 plus *nifA*, *nifB* and *fixABCX*	
ZB138	AK631 in which deletion covers genes lost in AK1212 plus *efn* plus a region covering about 400 kilobases (kb) 5′ to *nod* gene cluster	
Rm1021	SU47 *str-21*	Meade *et al.* 1982
4013	Wild type	Sikka and Kumar 1984
102F34	Wild type	Ditta *et al.* 1980
Escherichia coli		
HB101	F$^-$ *ara xyl lac mtl met pro leu thi supE rpsL* (Sm^R) *hsdM hsdR recA*	Boyer and Roulland–Dussoix 1969

(*continued*)

Table 1. (*continued*)

WA803	F⁻ *met thi*	Selvaraj and Iyer 1983
Plasmids		
pRK290	Wide host range cloning vector; Tc^R *ori*(RK2)	Ditta *et al.* 1980
pRK2901·1–1200	pRK290 with cloned segments of genomic DNA of *R. meliloti* 102F34	
pRK2013	Helper plasmid for mobilization of pRK290 derivatives; *tra*(RK2) *ori*(ColEl) Km^R	Figurski and Helinski 1979
pJB3JI	Km^S derivative of pR68·45 capable of mobilizing genomic segments of its host; Tc^R Ap^R (Ap^R is expressed in *E. coli* but not in *R. meliloti* Rmd201)	Brewin *et al.* 1980
pGR1	pJB3JI-prime carrying all the known *nif, fix* and *nod* genes	Kondorosi *et al.* 1984
pPH1JI	Incompatible to pRK290 and pJB3JI; Gm^R	Beringer *et al.* 1978
pGS9	N incompatibility group plasmid::Tn5 (wild type Km^R) suicidal in rhizobia; Cm^R	Selvaraj and Iyer 1983
RMP phages		
38, 26, 36, 46, 50, 52, 79, 86, 90 and 145	Capable of growing on *R. meliloti* wild-type strains AK631, Rm1021, 4013 and 102F34 (broad host range or BHR phages)	Khanuja and Kumar 1988
64 (Sxf), 61, 67 80, 85 and 88	Fail to plaque on *R. meliloti* wild-type strains Rm1021, 4013 and 102F34 (narrow host range or NHR phages)	
M12	Transducing phage that plaques on AK631, Rm1021, 4013 and 102F34	Finan *et al.* 1984

2.4 *Bacterial mating conditions*

Donor and recipient strains were grown in TY broth, mixed in equal volumes, patched on TY agar plate, and incubated. The time of incubation was 8 h to recover Tn5 mutants. Otherwise patch was incubated overnight. Cells were scraped from the patch, suspended in MSY and plated on selective media. Transconjugants were purified two times by single colony isolation.

2.5 *Tn5 mutagenesis*

Mutagenesis of *R. meliloti* Rmd201 with wild-type Tn5 was done by conjugational transfer of suicide plasmid pGS9 from *E. coli* WA803 (pGS9) and plating on TY medium containing Km and Sm. A large number of matings were done and only 1–5 transconjugants were picked from a mating. Scoring for presence of Tn5 marker and absence of plasmid marker among purified transconjugants was done by spotting on TY agar containing Km and Cm singly and in combination. A total of 3000 Tn5 mutants were collected from over 700 matings.

2.6 *Complementation studies*

For complementation tests, first pJB3JI-primes (R's) carrying genomic sites of different Tn5 insertions were derived *in vivo*. For this purpose specific Tn5 mutants

(Km^R) of Rmd201 were introduced with pJB3JI (Tc^R). Each such *R. meliloti* derivative (Km^RTc^R) was then used as donor in matings with *E. coli* HB101, and $Ap^RKm^RTc^R$ transconjugants were selected on minimal medium. At least two transconjugants were purified from each mating. Construction of a pJB3JI-prime was considered positive if *R. meliloti* Rmd201 homogenotized for the Tn*5*-mutated *Rhizobium* genomic sequence borne on pJB3JI-prime had the same phenotype(s) as that of the Tn*5* mutant of Rmd201 from which the specific genomic sequence::Tn*5* got originally cloned into pJB3JI vector. Homogenotization was mediated by simultaneous selection for Gm^RKm^R after transfer of pPH1JI to *R. meliloti* Rmd201 already carrying pJB3JI-prime. Derivation of homogenotes was confirmed by scoring for Tc^S.

Later, merodiploids of *R. meliloti* strains for complementation were constructed by allowing conjugation of Tn*5*-containing clones (in pJB3JI-primes, Km^RTc^R) with *R. meliloti* Rmd201 carrying genomic Tn*5* mutations. The purified Tc^RKm^R transconjugants were characterized for their phenotype(s).

2.7 *Isolation of clones for the region determining nodulation and phage sensitivity*

The gene bank of *R. meliloti* 102F34 (Ditta *et al.* 1980) was employed. The pRK290 random insert population in *E. coli* HB101 was plated and 1200 individual colonies were patched on selective medium (30 per plate) and incubated. Insert in each patch was then mobilized by triparental crosses with helper *E. coli* HB101 (pRK2013) into *R. meliloti* Rmd438 recipient carrying a Tn*5*-induced mutation giving phage resistance phenotype. To carry out triparental matings, cells from cultures of HB101 (pRK2013) and Rmd438 were mixed and spread on TY agar. The double-seeded plate was replica-plated with growth of 30 donor patches and incubated for 36 h. The growth on triparental plates was replica-plated on minimal medium plates containing glucose as sole carbon source and Tc. The replicas were incubated for 1 week. The Tc^R *R. meliloti* transconjugant colonies from each of 1200 crosses were inoculated into TY broth individually for further growth. The resulting cultures were used for testing phage growth patterns. Cells from cultures in which the phage resistance phenotype of *R. meliloti* Rmd438 had been complemented were purified for detailed characterization of their phenotype(s).

2.8 *Plant test*

Alfalfa (*Medicago sativa* cv T9) seeds were sterilized with 95% (v/v) ethanol for 3 min and with 0·1% (w/v) mercuric chloride for 5 min. The seeds were then washed with sterile distilled water five times and germinated under sterile conditions on filter paper bridges in Jensen's medium in test tubes (18 by 50 mm). Two seeds were sown in each test tube. Two-to-four-day-old seedlings were inoculated with rhizobia. For inoculation, bacteria of each strain were grown on MSY agar medium and suspended in sterile water. About 10^8 colony forming units were added to each test tube. Each bacterial strain was tested on at least 4 plant tubes. Seedlings were observed for nodules and for nitrogenase activity by the acetylene reduction assay after 4 and 6 weeks of growth at $28 \pm 2°C$ under 2·5 klx fluorescent light in a 16-h day 8-h night cycle. Plant nodulation tests were repeated twice. Four-week-old nodules were fixed in 5·5% glutaraldehyde, 1% paraformaldehyde, 0·05% cacodylate

buffer, pH 7·4, for 4 h at room temperature. They were postfixed in 1% osmium tetroxide at 0°C for 2 h, then dehydrated in a graded acetone series, and fixed in spurr. Preparation of sections for viewing was as described by Hayat (1972).

3. Results

3.1 *Isolation of nodulation-defective phage-resistant mutants*

The *R. meliloti* strain Rmd201 is a streptomycin-resistant derivative of the compact colony morphology mutant AK631 of the natural isolate Rm41. This strain, used as wild type here, has been shown to carry genetic information for normal nodulation response on alfalfa plants and for sensitivity to a variety of phages including the broad host range (BHR) phages RMP 26, 36, 38, 46, 50, 52, 79, 86, 90 and 145 and the narrow host range (NHR) phages RMP 61, 64, 67, 80, 85 and 88, all test phages in the present experiments (Khanuja and Kumar 1988). Three thousand Tn*5* mutants of Rmd201 were isolated using the suicidal plasmid pGS9 as the donor of wild-type Tn*5*. Each of the random Tn*5* insertional mutants was screened for sensitivity to the 16 test phages. A total of 240 mutants proved to be resistant to one or more of the test phages. Table 2 shows that the 240 phage-resistant mutants fell into six distinct phenotypic classes. All the phage-resistant mutants were tested for nodulation of alfalfa. Table 3 shows that among the 240 mutants, only 8 were

Table 2. Behaviour of 3000 random Tn*5* mutants of *Rhizobium meliloti* towards phages RMP 26, 36, 38, 46, 50, 52, 61, 64, 67, 79, 80, 85, 86, 88, 90 and 145[a].

Phenotypic group	RMP phages towards which *R. meliloti* mutants were resistant	Number of mutants
I	64, 61, 67, 80, 85 and 88	12[b]
II	26, 36, 46 and 50	8
III	38, 52, 79 and 145	156
IV	26, 36, 38, 46, 50, 52, 79 and 145	60
V	26, 36, 38, 46, 50, 52, 61, 64, 67, 79, 80, 85, 86, 88, 90 and 145	3
VI	38, 52, 61, 64, 67, 79, 80, 85, 88, 90 and 145	1

[a]A total of 2760 mutants were found to be sensitive to all the test RMP phages.
[b]These mutants were designated Sxf$^-$ for their resistance to phage RMP64.

Table 3. Symbiotic behaviour on alfalfa of Tn*5* mutants of *Rhizobium meliloti* Rmd201 with phage resistance phenotypes.

Phage resistance phenotype (as given in table 2)	Total number of mutants	Symbiotic phenotype	
		Nod$^+$ Fix$^+$	Nod$^+$ Fix$^-$
I	12	5	7
II	8	8	0
III	156	155	1
IV	60	60	0
V	3	3	0
VI	1	1	0

found to be altered in the nodulation process in that they formed nodules that were unable to fix nitrogen (Nod^+ Fix^-). Seven of these Nod^+ Fix^- mutants belonged to the class of 12 mutants resistant to NHR phages (group A) RMP 61, 64, 67, 80 and 85 but sensitive to all BHR phages (Sxf^-, for resistant to phage RMP64, a representative of NHR phages).

The *R. meliloti* strains Rmd202, Rmd203, Rmd204 and Rmd205, isolated as spontaneous derivatives of the wild-type Rmd201 resistant to the NHR RMP phages 64, 65, 67 and 85 respectively, were observed to be resistant to RMP phages 61, 64, 67, 80, 85 and 88 and sensitive to all the BHR phages. Thus it can be concluded that they are Sxf^- like the group A phage-resistant Tn*5* mutants. Tests for nodulation against alfalfa on the four spontaneous Sxf^- mutants revealed that Rmd202 and Rmd203 were Nod^+ Fix^- and Rmd204 and Rmd205 had the wild-type nodulation response. These results demonstrated that among both Tn*5* and spontaneous Sxf^- mutants of Rmd201, some were symbiotically defective.

The parental strain Rmd201 formed 2–5 pink cylindrical nodules that contained infection threads and were full of bacteroids. In contrast the nodules induced by Sxf^- Fix^- mutants were more numerous (2–12), relatively smaller, and white. These contained neither bacteroids nor infection threads, although some bacteria were visualized in intercellular spaces of the peripheral cell layers.

The question was now asked whether mutations in any of the already reported symbiotic genes of *R. meliloti* AK631 blocked sensitivity to RMP64. The observations summarized in table 4 show that RMP64 plated normally on mutants from which known *nif*, *fix*, *hsn* and/or *nod* genes had been deleted. Further, presence of pGR1, a plasmid that carries all the known *nif*, *fix*, *hsn* and *nod* genes, failed to affect the Sxf^- Fix^- phenotype of the mutants. Several Exo^- mutants of Rmd201 including Rmd601 were also found to be sensitive to RMP64, like the wild type. The above observations indicated that Sxf^- mutants were perhaps novel and required further characterization.

3.2 *Calcofluor fluorescence properties of Sxf mutants*

Synthesis of acidic exopolysaccharide has been shown to be a requirement for

Table 4. Sensitivity of symbiotic mutants of *Rhizobium meliloti* AK631 to phages RMP64, RMP38 and M12.

	Ability to plate		
Bacterial strain	RMP64	RMP38	M12
Rmd201	+	+	+
Rmd432	−	+	+
Rmd438	−	+	+
Rmd601	+	+	+
Rmd611	+	+	+
AK631	+	+	+
AK635	+	+	+
ZB129	+	+	+
AK1212	+	+	+
ZB138	+	NT	+

NT, Not tested.

Nod$^+$ Fix$^+$ phenotype in *R. meliloti*. Colonies of *R. meliloti* mutants deficient in synthesis of acidic exopolysaccharide (Exo$^-$) fail to fluoresce under UV light when grown on medium containing Calcofluor. Since some of the Sxf$^-$ mutants were Fix$^-$, the Sxf$^-$ mutants were screened for Calcofluor fluorescence for detecting in them deficiency in exopolysaccharide synthesis. The AK631 strain of *R. meliloti* synthesizes rather low amounts of Calcofluor-binding exopolysaccharide and therefore its colonies on Calcofluor medium are dimly fluorescent such that they lack fluorescent zones (halos) around them. Calcofluor fluorescence properties of Rmd201 and its Sxf$^-$ mutants were identical to those of AK631. This experiment demonstrated that Sxf$^-$ mutants synthesized acidic exopolysaccharide in amounts about equal to that in Rmd201.

3.3 *Sodium deoxycholate sensitivity of Sxf$^-$ mutants*

In *R. phaseoli* a class of symbiotic mutants are known to be defective in their lipopolysaccharide (LPS) moieties (Carlson *et al.* 1987). On the other hand, LPS mutants of *R. meliloti* are known to become phage-resistant (Keiber *et al.* 1987). Since sensitivity to sodium deoxycholate (DOC) is a general indicator of LPS alterations in bacteria, Sxf$^-$ mutants were compared with wild type for their sensitivity to DOC. It was found that the colony-forming ability of Rmd201 was completely inhibited by 2% DOC. None of the Sxf$^-$ mutants was either more or less sensitive to DOC than Rmd201.

3.4 *Carbohydrate and dicarboxylate utilization properties of Sxf$^-$ mutants*

Since bacterial surface alterations leading to phage insensitivity are known to affect uptake and/or utilization of carbohydrates or dicarboxylates as well (Kumar 1976; Schwartz 1987), the Sxf$^-$ mutants of Rmd201 were examined for utilization of a variety of carbohydrates (table 5) and succinate (table 6). All the Sxf$^-$ Fix$^+$ mutants, like the wild type, utilized all the sugars for which tests were performed: arabinose, galactose, glucose, lactose, maltose, mannitol, sucrose and xylose. The Sxf$^-$ Fix$^-$ mutants fell into two classes with respect to their carbohydrate utilization properties. One class of mutants among them were like the wild type. The second group of Sxf$^-$ Fix$^-$ mutants failed to utilize galactose as sole carbon source although they utilized the other sugars. All the Sxf$^-$ mutants utilized succinate. These results

Table 5. Carbohydrate utilization properties of Sxf$^-$ mutants of *Rhizobium meliloti* Rmd201.

			Capability to utilize	
Class of Sxf$^-$ mutants	Sxf$^-$ mutant strains	Symbiotic behaviour on alfalfa	Galactose	Arabinose, glucose, lactose, mannitol, sucrose, xylose
A	Rmd202, 203, 432, 436, 441 and 443	Nod$^+$ Fix$^-$	+	+
B	Rmd204, 205, 433, 435, 437, 439 and 442	Nod$^+$ Fix$^+$	+	+
C	Rmd434, 438 and 440	Nod$^+$ Fix$^-$	−	+

Table 6. Succinate utilization properties of Sxf⁻ mutants and other derivatives of *Rhizobium meliloti* AK631.

Strains	Capability to utilize succinate as sole carbon source
AK631, Rmd201 Rmd202, Rmd204 Rmd432, Rmd433 Rmd434 and ZB138	+
Rmd611 and Rmd612	−

resolved the Sxf⁻ mutants of Rmd201 into three categories on the basis of their phenotypes: Sxf⁻ Fix⁺ Gal⁺, Sxf⁻ Fix⁻ Gal⁺, and Sxf⁻ Fix⁻ Gal⁻.

3.5 *Complementation between Sxf⁻ mutants*

For defining *sxf* genes, merodiploids containing pairs of *sxf* mutations were constructed. To a mutant carrying a spontaneous *sxf* mutation or an *sxf*::Tn*5* insert, an *sxf*::Tn*5* insert, on the plasmid pJB3JI, was introduced as a second mutation. Merodiploids were scored for phage sensitivity, galactose utilization and nodulation phenotypes. Table 7 shows that the available Sxf⁻ mutants most likely defined three genes. Since the spontaneous and Tn*5* mutants with the phenotype Sxf⁻ Gal⁺ Fix⁻ were complemented by Tn*5* mutants possessing different phenotypes but not by mutants that have the same phenotype, they belonged to a class with mutation in the same gene, which was given the name *sxfA*. The mutants that had the Sxf⁻ Gal⁻ Fix⁻ phenotype were complemented by Sxf⁻ Gal⁺ Fix⁺ mutants on one hand and by Sxf⁻ Gal⁺ Fix⁻ mutants on the other, but they were not complemented by each other. Therefore they together defined another gene, called *sxfC*. The Sxf⁻ Gal⁺ Fix⁺ mutants failed to be complemented either by *sxfA* mutants or mutants internally from their group, although they were complemented by *sxfC* mutants. It was concluded that these mutants defined a third gene, which was

Table 7. Sxf, Fix and Gal phenotypes of *sxf* mutants of *Rhizobium meliloti* and their merodiploids.

Sxf⁻ mutant strains to which pJB3JI-primes carrying different *sxf*::Tn*5* inserts were transferred to derive merodiploids	Phenotype	*sxf*::Tn*5* insert mutations borne on pJB3JI-primes		
		sxf::Tn*5*-35 or *sxf*::Tn*5*-2873	*sxf*::Tn*5*-129 or *sxf*::Tn*5*-2229	*sxf*::Tn*5*-861 or *sxf*::Tn*5*-2226
Rmd202, 203, 432, 436,	Sxf	−	+	+
441, 443, the *sxfA*	Gal	+	+	+
class of mutants	Fix	−	+	+
Rmd204, 205, 433, 435,	Sxf	−	−	+
437, 439, 442, the *sxfB*	Gal	+	+	+
class of mutants	Fix	+	+	+
Rmd434, 438, 440, the	Sxf	+	+	−
sxfC class of mutants	Gal	+	+	−
	Fix	+	+	−

designated *sxfB*. These results also suggest that (a) *sxfA* and *sxfB* belong to the same transcriptional unit (operon), *sxfA* being the promoter-proximal gene of this operon; (b) *sxfC* is a part of a distinct operon; and (c) that *sxfAB* and *sxfC* must be on the same replicon.

3.6 *Cloning of sxfB gene*

By screening 1200 colonies of the gene bank of *R. meliloti* 102F34, three colonies were isolated which restored Sxf^+ Fix^+ Gal^+ phenotype to *R. meliloti* Rmd438, which harbours an *sxfC*::Tn*5* mutation.

4. Discussion

In this paper we have reported the isolation of mutants in the genes *sxfA*, *sxfB* and *sxfC* of *R. meliloti* Rmd201 that are resistant to phage RMP64. The mutants in genes *sxfA* and *sxfC* are symbiotically defective in that they evoke on alfalfa formation of root nodules that are small, white, bacteroid-less and non-nitrogen-fixing. The discussion below will show that *sxfA* and *sxfC* are new genetic loci with roles in nodule invasion and development.

In *R. meliloti* the property of eliciting bacteroid-free nodules on alfalfa is not unique to *sxfA* and *sxfC* mutants. It is also characteristic of mutants in *exo* genes, which affect production, succinylation or pyruvylation of acidic surface polysaccharides (Leigh *et al.* 1985, 1987; Muller *et al.* 1988), and mutants in *ndv* genes, which affect synthesis and transport of cyclic (1→2)-β-D-glucans (Dylan *et al.* 1986; Geremia *et al.* 1987). Thus it appears that mutants in the genes *exoB*, *exoH*, *ndvA*, *ndvB*, *sxfA* and *sxfC* elicit nodules that are similarly arrested in development. However, other properties of these mutants and their parent strains, listed below, distinguish *sxfA* and *sxfC* mutants from the other four. (i) A Calcofluor-dark *exoB* mutant of the strain Rmd201 proved sensitive to phage RMP64, unlike *sxfA* and *sxfC* mutants. (ii) Colonies of the strains AK631 and Rmd201 and the latter carrying *sxfA* or *sxfC* mutation gave equally dim fluorescence on Calcofluor plates. (iii) The wild-type strain SU47 or RCR2011 and its derivative Rm1021, which are the parents of all well-characterized *exo* mutants, and strain 102F34, the parent of *ndvA* and *ndvB* mutants, are all inherently resistant to phage RMP64 (Khanuja and Kumar 1988). (iv) The pleiotropic Ndv^- phenotype of *ndvA* and *ndvB* mutants includes inability to plate phage RMP38 (T. Dylan, personal communication), to which *sxfA* and *sxfC* mutants are sensitive. Therefore *sxfA* and *sxfC* must be different from the known *exo* and *ndv* loci.

The *sxfA* and *sxfC* loci must also be different from all the other known symbiotic genes, because (i) the *R. meliloti* AK631 derivatives from which all the known *nod*, *fix* and *nif* genes had been deleted (Banfalvi *et al.* 1985; Putnoky and Kondorosi 1986) propagated phage RMP64, and (ii) the pGR1 plasmid carrying all the known *nif*, *fix* and *nod* genes (Kondorosi *et al.* 1984) failed to complement the *sxfA* and *sxfC* mutants. Thus it can be suggested that *sxfA* and *sxfC* might be new genes involved in nodule development.

Previous work has led to the suggestion that nodule induction in alfalfa roots by *R. meliloti* can occur by one of two known modes (Debellé *et al.* 1988; Klein *et al.* 1988a, b). The invasion of root tissue takes place either by the infection pathway

involving tubular infection threads which initiate within root hairs and penetrate deep inside through the cortex, or by entry through spaces at intercellular junctions of root epidermal cells, remaining intercellular in peripheral layers of the cortex. While the first mode of infection is normal the latter is atypical. The wild type *R. meliloti* and mutants in various *nif* genes and the *fixABCX* genes have been reported to induce nodules by the root hair infection method (Aguilar *et al.* 1985; Hirsch and Smith 1987). Normal-size nodules result from such infection. These nodules are ramified, with rhizobia-containing infection threads. A large number of host cells of the nodules carry bacteroids, the differentiated forms of rhizobia released from infection threads, in massive numbers.

The atypical intercellular mode of infection has been implicated in the induction of abnormal nodules by *R. meliloti* mutated in the loci *exoB*, *exoH*, *ndvA* and *ndvB*, and by *R. trifolii* and *Agrobacterium tumefaciens* carrying certain segments of *R. meliloti* pSymA plasmid (Truchet *et al.* 1984; Leigh *et al.* 1985, 1987; Dylan *et al.* 1986; Debellé *et al.* 1988). In these situations the bacteria are incapable of eliciting normal infection threads that can penetrate the root cortex. The resulting nodules are of rudimentary nature, lacking tubular infection threads and bacteroids, and instead having intercellular rhizobia in small numbers in the peripheral cell layers. This phenotype indicates that in the alfalfa root nodule, a network of *R. meliloti*-containing infection threads is a requirement for its continued development from early to mature stages and for delivery of rhizobia to host cells for differentiation into bacteroids. In this study nodules induced by *sxfA* and *sxfC* mutants have been found to generally resemble those elicited by *exoB*, *exoH*, *ndvA* and *ndvB* mutants. These observations lead to the suggestion that in *R. meliloti* SxfA and SxfC functions belong to a class that includes Exo and Ndv functions. This class of functions is required early for formation of the network of infection threads in nodules induced on alfalfa and for the associated normal development of the nodules initiated.

The nodulation-defective *sxfA* and *sxfC* mutants described in this paper were originally detected because of another phenotype, that of resistance to the phage RMP64. One simple explanation of their symbiotic properties is that, due to failure to produce phage receptor(s) that might also be acting as receptor(s), porin(s) or porin components, the *sxfA* and *sxfC* mutants may be deficient in transport system(s) for plant-supplied precursors or signals, essential for bacterial multiplication and growth of infection thread. Alternatively, or in addition, they may be lacking transporter(s) of signals/precursors to host cells required for the latters' response in providing accommodation for the growing infection thread and in eliciting growth and morphogenesis. Additional work will be required to characterize the roles of SxfA and SxfC and to reveal whether *sxfA* and *sxfC* mutants carry structural defects in regulatory functions. It is also possible that mutations in *sxfA* and *sxfC* genes might affect levels of expression of nearby genes by being polar.

A difference between the *sxfA* and *sxfC* mutants is that *sxfC*::Tn*5* mutants fail to utilize galactose whereas spontaneous and Tn*5*-insertional *sxfA* mutants can do so. It is possible that the *sxfC* gene product is either involved directly in utilization of galactose from the medium or acts by regulating other gene(s) that are actually involved in transport of galactose. Its role may be analogous to the *lamB* product of *E. coli* (Schwartz 1987). The relationships of *sxfA*, *sxfC* and *sxfB* to their action on each other remain to be elucidated, since their products must all be associated with the same cell surface.

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References

Aguilar O. M., Kapp D. and Pühler A. 1985 Characterization of a *Rhizobium meliloti* gene (*fixF*) located near the common nodulation region. *J. Bacteriol.* 164: 245–254

Banfalvi Z., Kondorosi E. and Kondorosi A. 1985 *Rhizobium meliloti* carries two megaplasmids. *Plasmid* 13: 129–138

Batut J., Terzaghi B., Gherardi M., Huguet M., Terzaghi E., Garnerone A. M., Boistard P. and Huguet T. 1985 Localization of a symbiotic *fix* region on *Rhizobium meliloti* pSym megaplasmid more than 200 kilobases from the *nod–nif* region. *Mol. Gen. Genet.* 199: 232–239

Bauer W. D. 1981 Infection of legumes by rhizobia. *Annu. Rev. Plant Physiol.* 32: 407–409

Beringer J. E., Beynon J. L., Buchanan-Wollaston A. V. and Johnston A. W. B. 1978 Transfer of the drug resistance transposon Tn5 to *Rhizobium. Nature* (*London*) 276: 633–634

Beveridge T. J. 1981 Ultrastructure, chemistry and function of bacterial wall. *Int. Rev. Cytol.* 72: 229–317

Boyer H. S. and Roulland-Dussoix D. 1969 A complementation analysis of the restriction and modification of DNA in *Escherichia coli. J. Mol. Biol.* 41: 459–472

Braun V. and Hantke K. 1977 Bacterial receptors for phages and colicins as constituents of specific transport systems. In *Receptors and recognition* (ed.) J. L. Reissig (London: Chapman and Hall) series B, vol. 3, pp. 101–130

Braun V. and Hantke K. 1981 Bacterial cell surface receptors. In *Organization of prokaryotic cell membranes* (ed.) B. K. Ghosh (Boca Raton, Florida: CRC Press) vol. 2, pp. 1–73

Brewin N. J., Beringer J. E. and Johnston A. W. B. 1980 Plasmid-mediated transfer of host range specificity. *J. Gen. Microbiol.* 120: 413–420

Buikema W. J., Klingensmith J. A., Gibbons S. L. and Ausubel F. M. 1987 Conservation of structure and location of *Rhizobium meliloti* and *Klebsiella pneumoniae nifB* genes. *J. Bacteriol.* 169: 1120–1126

Carlson R. W., Kalembasa S., Turowaki D., Pachori P. and Noel K. D. 1987 Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. *J. Bacteriol.* 169: 4923–4928

Corbin D., Barran L. and Ditta G. 1983 Organization and expression of *Rhizobium meliloti* nitrogen fixation genes. *Proc. Natl. Acad. Sci. USA* 80: 3005–3009

Costerton J. W., Merrie T. J. and Cheng K.-J. 1985 Phenomena of bacterial adhesion. In *Bacterial adhesion* (eds.) D. C. Savage and M. Fletcher (New York: Plenum Press)

Dart P. 1977 Infection and development of leguminous nodules. In *A treatise on dinitrogen fixation, Section III: Biology* (eds.) R. W. F. Hardy and W. S. Silver (New York: Wiley and Sons) pp. 367–472

David M., Daveran M.-L., Batut J., Dadieu A., Domerque O., Ghai J., Hertig C., Boistard P. and Kahn D. 1988 Cascade regulation of *nif* gene expression in *Rhizobium meliloti. Cell* 54: 671–683

Debellé F., Maillet F., Vasse J., Rosenberg C., deBilly F., Truchet G., Dénarié J. and Ausubel F. M. 1988 Interference between *Rhizobi um meliloti* and *Rhizobium trifolii* nodulation genes: genetic basis of *R. meliloti* dominance. *J. Bacteriol.* 170: 5718–5727

Debellé F., Rosenberg C., Vasse J., Maillet F., Martinez E., Dénarié J. and Truchet G. 1986 Assignment of symbiotic developmental phenotypes to common and specific nodulation (*nod*) genetic loci of *Rhizobium meliloti. J. Bacteriol.* 168: 1075–1086

Ditta G., Corbin D., Leong S., Barran L. and Helinski D. R. 1980 Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti. Proc. Natl. Acad. Sci. USA* 77: 7347–7351

Doherty D., Leigh J. A., Glazebrook J. and Walker G. C. 1988 *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic Calcofluor-binding exopolysaccharide. *J. Bacteriol.* 170: 4249–4256

Dudley M. E., Jacobs T. W. and Long S. R. 1987 Microscopic studies of cell divisions induced in alfalfa roots by *Rhizobium meliloti. Planta* 171: 289–301

Duncan M. J. 1981 Properties of Tn5-induced carbohydrate mutants in *Rhizobium meliloti. J. Gen. Microbiol.* 172: 61–67

Dylan T., Ielpi L., Stanfied S., Kashyap L., Douglas C., Yanofsky M., Nester E., Helinski D. R. and Ditta G. 1986 *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens. Proc. Natl. Acad. Sci. USA* 83: 4403–4407

Earl C. D., Ronson C. W. and Ausubel F. M. 1987 Genetic and structural analysis of *Rhizobium meliloti fixA, fixB, fixC* and *fixX* genes. *J. Bacteriol.* 169: 1127–1136

Figurski D. H. and Helinski D. R. 1979 Replication of an origin-containing derivative of plasmid pRK2 dependent on a plasmid function provided in *trans*. *Proc. Natl. Acad. Sci. USA* 76: 1648–1652

Finan T. M., Hartwieg E., LeMieux K., Bergman K., Walker G. C. and Signer E. R. 1984 General transduction in *Rhizobium meliloti*. *J. Bacteriol.* 159: 120–124

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

Geremia R. A., Cavaignac S., Zorregueta A., Toro N., Olivares J. and Ugalde R. A. 1987 A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form beta(1,2) glucan. *J. Bacteriol.* 169: 880–884

Hancock R. E. W. 1987 Role of porins in outer membrane permeability. *J. Bacteriol.* 169: 929–933

Handelsman J., Ugalde R. A. and Brill W. J. 1984 *Rhizobium meliloti* competitiveness and the alfalfa agglutinin. *J. Bacteriol.* 157: 703–707

Hayat M. A. 1972 *Basic electron microscopy techniques* (New York: Van Nostrand Reinhold)

Hirsch A. M., Bang M. and Ausubel F. M. 1983 Ultrastructural analysis of ineffective alfalfa nodules formed by *nif*::Tn*5* mutants of *Rhizobium meliloti*. *J. Bacteriol.* 155: 367–380

Hirsch A. M., Long S. R., Bang M., Haskins N. and Ausubel F. M. 1982 Structural studies of alfalfa roots infected with nodulation mutants of *Rhizobium meliloti*. *J. Bacteriol.* 151: 411–419

Hirsch A. M. and Smith C. A. 1987 Effects of *Rhizobium meliloti nif* and *fix* mutants on alfalfa root nodule development. *J. Bacteriol.* 169: 1137–1146

Honma M. A. and Ausubel F. M. 1987 *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. *Proc. Natl. Acad. Sci. USA* 84: 8558–8562

Horvath B., Bachem C. W. B., Schell J. and Kondorosi A. 1987 Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant signal interacting with the *nodD* gene product. *EMBO J.* 6: 841–848

Horvath B., Kondorosi E., John M., Schmidt J., Török I., Györgypal Z., Barabas, I., Wieneke U., Schell J. and Kondorosi A. 1986 Organization, structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. *Cell* 46: 335–343

Jacobs T. W., Egelhoff T. T. and Long S. R. 1985 Physical and genetic map of a *Rhizobium meliloti* nodulation gene region and nucleotide sequence of *nodC*. *J. Bacteriol.* 162: 469–476

John M., Schmidt J., Wieneke U., Kondorosi E., Kondorosi A. and Schell J. 1985 Expression of the nodulation gene *nodC* of *Rhizobium meliloti* in *E. coli*—Role of the NodC gene product in nodulation. *EMBO J.* 4: 2425–2430

Keiber J., Clover R., Finan T. M. and Signer E. R. 1987 Surface properties of *Rhizobium meliloti* associated with symbiosis, In *Molecular genetics of plant–microbe interactions* (eds.) D. P. S. Verma and N. Brisson (New York: Springer-Verlag) pp. 182–184

Khanuja S. P. S. and Kumar S. 1988 Isolation of phages for *Rhizobium meliloti* AK631. *Indian J. Exp. Biol.* 26: 665–667

Klein S., Hirsch A. M., Smith C. A. and Signer E. R. 1988a Interaction of *nod* and *exo Rhizobium meliloti* in alfalfa nodulation. *Mol. Plant-Micro. Interaction* 1: 94–100

Klein S., Walker G. C. and Signer E. R. 1988b All *nod* genes of *Rhizobium meliloti* are involved in alfalfa nodulation by *exo* mutants. *J. Bacteriol.* 170: 1003–1006

Kondorosi E., Banfalvi Z. and Kondorosi A. 1984 Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*: identification of nodulation genes. *Mol. Gen. Genet.* 193: 445–452

Koshland D. E. 1979 Bacterial chemotaxis. In *The bacteria* (ed.) I. C. Gunsalus (New York: Academic Press) vol. 7, pp. 111–166

Kumar S. 1976 Properties of adenyl cyclase and cyclic adenosine 3′,5′-monophosphate receptor protein-deficient mutants of *Escherichia coli*. *J. Bacteriol.* 125: 545–555

Leigh J. A., Reed J. W., Hanks J. F., Hirsch A. M. and Walker G. C. 1987 *Rhizobium meliloti* mutants that fail to succinylate their Calcofluor-binding exopolysaccharide are defective in nodule invasion. *Cell* 51: 579–587

Leigh J. A., Signer E. R. and Walker G. C. 1985 Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proc. Natl. Acad. Sci. USA* 82: 6231–6235

Lindberg A. A. 1977 Bacterial surface carbohydrate and bacteriophage adsorption. In *Surface carbohydrate of the prokaryotic cell* (ed.) I. W. Sutherland (New York: Academic Press) pp. 289–356

Long S. R. 1984 Genetics of *Rhizobium* nodulation. In *Plant-microbe interactions* (eds.) T. Kosuge and E. W. Nester (New York: MacMillan) vol. 1, pp. 265–306

Long S. R., Buikema W. and Ausubel F. M. 1982 Cloning of *Rhizobium meliloti* nodulation genes by direct complementation of Nod⁻ mutants. *Nature* (*London*) 298: 485–488

Long S., McCune S. and Walker G. C. 1988a Symbiotic loci of *Rhizobium meliloti* identified by random Tn*phoA* mutagenesis. *J. Bacteriol.* 170: 4257–4265

Long S., Reed J. W., Himawan J. and Walker G. C. 1988b Genetic analysis of a cluster of genes required for synthesis of the calcofluor-binding exopolysaccharide of *Rhizobium meliloti*. *J. Bacteriol.* 170: 4239–4248

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

Muller P., Hynes M., Kapp D., Niehaus K. and Pühler A. 1988 Two classes of *Rhizobium meliloti* infection mutants differing in exopolysaccharide production and in coinoculation properties with nodulation mutants. *Mol. Gen. Genet.* 211: 17–26

Newcomb W. 1981 Nodule morphogenesis and differentiation, *Int. Rev. Cytol.* suppl. 12, pp. 247–298

Noel K. D., VandenBosch K. A. and Kulpaca B. 1986 Mutations in *Rhizobium phaseoli* that lead to arrested development of infection threads. *J. Bacteriol.* 168: 1392–1401

Parke D., Rivelli M. and Ornston L. N. 1985 Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*. *J. Bacteriol.* 163: 417–422

Putnoky P., Grosskopf E., Camtta D. T., Kiss G. B. and Kondorosi A. 1988 *Rhizobium fix* genes mediate at least two communication steps in symbiotic nodule development. *J. Cell Biol.* 106: 597–607

Putnoky P. and Kondorosi A. 1986 Two gene clusters of *Rhizobium meliloti* code for early essential nodulation functions and a third influences nodulation. *J. Bacteriol.* 167: 881–887

Reeves P. 1979 The genetics of outer membrane proteins. In *Bacterial outer membranes* (ed.) M. Inouye (New York: John Wiley and Sons) pp. 255–291

Renalier M., Daveran M.-H., Batut J., Ghai J., Terzaghi B., Gherardi M., David M., Garnevone A. M., Vasse J., Truchet G., Higuet T. and Boistard P. 1987 A new symbiotic cluster on the pSym megaplasmid of *Rhizobium meliloti* 2011 carries a functional *fix* gene repeat and a *nod* locus. *J. Bacteriol.* 169: 2231–2238

Ronson C. W., Nixon B. T., Albright L. M. and Ausubel F. M. 1987 *Rhizobium meliloti ntrA* (*rpoN*) gene is required for diverse metabolic functions. *J. Bacteriol.* 169: 2424–2431

Ruvkun G. B., Sundaresan V. and Ausubel F. M. 1982 Directed transposon Tn*5* mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. *Cell* 29: 551–559

Schmidt J., Wingender R., John M., Wieneke U. and Schell J. 1988 *Rhizobium meliloti nodA* and *nodB* genes are involved in generating compounds that stimulate mitosis of plant cells. *Proc. Natl. Acad. Sci. USA* 85: 8578–8582

Schwartz M. 1987 The maltose regulon. In *Escherichia coli and Salmonella typhimurium: Cellular and molecular biology*. (eds.) F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger (Washington, D.C.: American Society for Microbiology) vol. 2, pp. 1482–1502

Selvaraj G. and Iyer V. N. 1983 Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. *J. Bacteriol.* 156: 1292–1300

Sikka V. K. and Kumar S. 1984 Underexpression of Ap from R-plasmids in fast-growing *Rhizobium* species. *Appl. Environ. Microbiol.* 48: 1248–1250

Spaink H. P., Wijffelman C. A., Pees E., Okker R. J. H. and Lugtenberg B. J. J. 1987 *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. *Nature* (*London*) 328: 337–340

Stowers M. D. 1985 Carbon metabolism in *Rhizobium* species. *Annu. Rev. Microbiol.* 39: 89–108

Szeto W. W., Nixon B. T., Ronson C. W. and Ausubel F. M. 1987 Identification and characterization of the *Rhizobium meliloti ntrC* gene: *R. meliloti* has separate regulatory pathways for activating nitrogen fixation genes in free-living and symbiotic cells. *J. Bacteriol.* 169: 1423–1432

Szeto W. W., Zimmerman J. L., Sundaresan V. and Ausubel F. M. 1984 A *Rhizobium meliloti* symbiotic regulatory gene. *Cell* 36: 1035–1043

Truchet G., Michel M. and Dénarié J. 1980 Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotically defective mutants of *Rhizobium meliloti*. *Differentiation* 16: 163–173

Truchet G., Rosenberg C., Vasse J., Julliot J. S., Camut S. and Dénarié J. 1984 Transfer of *Rhizobium meliloti* Sym genes into *Agrobacterium tumefaciens*: host-specific nodulation by atypical infection. *J. Bacteriol.* 157: 134–142

Vasse J. M. and Truchet G. L. 1984 The *Rhizobium*–legume symbiosis: observation of root infection by bright-field microscopy after staining with methylene blue. *Planta* 161: 487–489

Vincent J. M. 1980 Factors controlling the legume–*Rhizobium* symbiosis. In *Nitrogen fixation, Vol. II: Symbiotic associations and cyanobacteria* (eds.) W. E. Newton and W. H. Orme Johnson (Baltimore: University Park Press) pp. 103–109

Wayne R. and Neilands J. B. 1975 Evidence for common binding sites for ferrichrome compounds and bacteriophage 80 in the cell envelope of *Escherichia coli*. *J. Bacteriol.* 121: 497–503

Weber G., Aguilar O. M., Gronemeier B., Reilander H. and Pühler A. 1985 Genetic analysis of symbiotic genes of *Rhizobium meliloti*: mapping and regulation of nitrogen fixation genes. In *Advances in the molecular genetics of the bacteria–plant interaction* (eds.) A. A. Szalay and R. P. Legocki (New York: Media Services, Cornell University) pp. 13–15