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Detection of loci in the *leu* region of *Rhizobium meliloti* chromosome

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Abstract. A multi-marked strain of *Rhizobium meliloti* was developed by the co-mutation method and employed to contribute to the genetic map of *R. meliloti* chromosome. Seven loci were placed at 5 sites in the *leu* region in the order *man-aba*, *fix*, *leu-cro-azt*, *ost-thi*.

Keywords. *Rhizobium meliloti*; *Rhizobium*–legume symbiosis; *Rhizobium* chromosome; *leu* region.

1. Introduction

Soil bacteria of genus *Rhizobium* induce nitrogen fixing root nodules on leguminous plants (Bauer 1981). The association between legumes and rhizobia is symbiotic and of considerable agronomic importance (Paau 1989). Genetic investigations are in progress to dissect the complex series of interactions involved in *Rhizobium*–legume symbiosis (Long 1989). *Rhizobium meliloti* bacteria and their legume hosts are the most extensively investigated systems for these interactions. In most of the studies, strains 102F34, 1021 and AK631 have been employed as the wild type *R. meliloti* (Ditta *et al.* 1980; Long *et al.* 1982; Banfalvi *et al.* 1985). A large number of mutations affected in biosynthesis of building blocks and symbiotic response have been mapped (Kondorosi *et al.* 1980; DeVos *et al.* 1986; Finan *et al.* 1986; Long *et al.* 1988; Kerppola and Kahn 1988). The genetic and molecular maps of the chromosomes and symbiotic plasmids *a* and *b* that are emerging for *R. meliloti* 1021, AK631 and 102F34 are very similar (Beringer *et al.* 1984; Keller *et al.* 1988; Glazebrook and Walker 1989; Charles and Finan 1990; Dylan *et al.* 1990 *a*, *b*; Sharma and Signer 1990; Mertinez *et al.* 1990; Reed and Walker 1991; Charles *et al.* 1991; Sobral *et al.* 1991). Here we detect and arrange several new markers adjacent to the *leu* locus of *R. meliloti* AK631.

2. Materials and methods

2.1 *Bacterial, plasmid and phage strains*

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

2.2 *Media and growth conditions*

Various complex and minimal media used for *R. meliloti* and *Escherichia coli* have been described earlier (Kumar 1976; Khanuja and Kumar 1989). Antibiotics were used at the following concentrations (μg/ml): ampicillin, 250; kanamycin, 50;

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Table 1. Bacterial, plasmid and phage strains

Strain	Relevant characteristics	Source or reference
Rhizobium meliloti		
Rmd201	Wild type: spontaneous streptomycin resistant derivative of AK631; colonies stain red in congo red growth medium and remain unstained on aniline blue medium; grows on solid medium containing 20μg/ml NaN_3 and 0.5 M NaCl	Khanuja and Kumar (1988, 1989)
Rmd1001	Nitrosoguanidine (NG) induced mutant of Rmd 201; leucine auxotroph (*leu*); nitrogen fixation deficient (*fix*); unable to utilize mannose (*man*); colonies stain blue on aniline blue medium (*aba*) and remain unstained on congo red medium (*cro*): sensitive to NaCl (*ost*)	This study
Rm1021	Wild type	Meade *et al.* (1982)
Rm102F34	Wild type	Ditta (1986)
Rmd1002	NG–induced thiamine auxotroph (*thi*) of Rm 102F34nal	This study
Rm6085	*exoB*:: Tn5 of Rm1021	DeVos *et al.* (1986)
Rm6086	*exoA*:: Tn5 of Rm1021	
Escherichia coli		
HB101	F *ara xyl lac mtl met pro leu thi supE rpsL hsdM hsdR recA*	Boyer and Roulland-Dussoix (1969)
CA8000	Hfr *thi relA1 min*	Kumar (1976)
KG33	Hfr *thiA*	Kawasaki *et al.* (1968)
KG1673	Hfr *thiB*	
KG6593	Hfr *thiC*	
CV512	*leuA*	Somers *et al.* (1973)
Plasmids		
pRK290	Wide host range cloning vector, Tc^R, *Ori (RK2)*	Ditta *et al.* (1980)
pRK290::1–1200	pRK290 with cloned segments of genomic DNA of *R. meliloti* Rm102F34	Ditta *et al.* (1980)
pSP676	pRK290:: *sxfC*$^+$	Khanuja *et al* (1991)
pRK2013	Helper plasmid for mobilization of pRK 290 and pLAFR1; *tra* (RK2), *Ori* (Col E1) Km^R	Figurski and Helinski (1979)
pD2	pLAFR1::*exoB*$^+$	Long *et al.* (1988)
pD34	pLAFR1::*thi*$^+$ – *exoH*$^+$ (including *exoA*$^+$)	
pD56	pLAFR1::*exoJ*$^+$– *exoF*$^+$ (including *exoB*$^+$)	
pRmS1	pRK290:: *leu*$^+$	This study
pRmS2	pRK290:: *leu*$^+$*thi*$^+$	
pRmS3	pRK290:: *thi*$^+$	
pGR1	pJB3JI – prime carrying all the known *nif*, *fix* and *nod* genes	Kondorosi *et al.* (1984)
Phages		
26,36,38,50,52, 79,86,90 and 145	Capable of growing on *R. meliloti* strains Rmd201, Rm1021, Rm4013 and Rm102F34	Khanuja and Kumar (1988)
61,64,67,80, 85 and 88	Fail to plaque on *R. meliloti* strains Rm1021, Rm4013 and Rm102F34	

nalidixic acid, 10; streptomycin, 100; and tetracycline, 10. Sodium azide (NaN_3) was added at 10 to 50 μg/ml and NaCl at 0·2 to 0·7 M. Calcofluor white, congo red and aniline blue dyes were used at 200, 100 and 50 μg/ml, respectively. Sugars were added at 1 mg/ml. Incubation temperatures for *R. meliloti* and *E. coli* were 30 and 37°C, respectively (Sathyanarayana 1989).

2.3 *Bacterial and nodulation procedures*

The procedures described by Khanuja and Kumar (1988, 1989) were used.

3. Results and discussions

Nitrosoguanidine (NG) is known to cause mutations coincident with the movement of the replication fork along DNA. Consequently, treatment of bacteria with NG can result in induction of mutations at many closely linked loci (co-mutations; Oeschger and Berlyn 1974). This property of NG mutagenesis was employed to produce sub-strains of *R. meliloti* Rmd201 affected at several chromosomal loci. A culture grown in complete medium was exposed to NG at 200 μg/ml for 30 min and then ampicillin-enriched for auxotrophs by the procedure described earlier (Singh *et al.* 1984). Screening of colony-forming units from this culture led to isolation of several different auxotrophs. These mutants were compared with Rmd201 for sensitivity towards different phages, antibiotics, NaN_3 and NaCl–hyperosmolarity and symbiotic response on alfalfa, sugar utilization and stainability with dyes. One of the mutant strains designated Rmd1001 that was observed to require leucine and differ from its parent prototroph in several additional characteristics was used to reveal the local arrangement of affected loci with reference to the *leu* locus.

The observed phenotypic differences between Rmd201 and Rmd1001 are shown in the tables 2, 3 and 4. The Leu^- Rmd1001 was incapable of utilizing mannose as the sole carbon source (Man^-, table 2). It was symbiotically defective and induced nodules on alfalfa that could not reduce acetylene to ethylene efficiently (Fix^-, tables 2 and 4). Rmd1001 was relatively more azide sensitive (Azt^-) and osmosensitive (Ost^-). Whereas Rmd201 tolerated upto 20 μg/ml NaN_3 and 0·5 M NaCl, Rmd1001 tolerated 10 μg/ml NaN_3 and 0·3 M NaCl (table 3). The stainability of Rmd1001 by congo red and aniline blue dyes was opposite to that of Rmd201 (table 2). While aniline blue stained Rmd201 but not Rmd1001 (Aba^-), congo red was absorbed by Rmd1001 (Cro^-) and Rmd201 was opaque to it. Congo red and aniline blue are known to be the indicators of cellulose and curdlan types of polysaccharides, respectively (Hisamatsu *et al.* 1977; Keller *et al.* 1988). The Cro^+ Aba^+ phenotype of Rmd201 indicates that these bacteria synthesize cellulose but not a curdlan type of polysaccharide. The Cro^- Aba^- Rmd1001 bacteria possess genetic elements for synthesizing curdlan polysaccharides and not for cellulose.

Are *aba*, *azt*, *cro*, *fix*, *leu*, *man* and *ost* in Rmd1001 co-mutations? If these are indeed co-mutations then receipt of a wild type DNA fragment corresponding to the affected region should be able to restore the wild type phenotype in Rmd1001. By screening 1200 colonies of the gene bank of *R. meliloti* 102F34, a colony was isolated which restored Aba^+ Azt^+ Cro^+ Fix^+ Leu^+ Man^+ Ost^+ phenotype to Rmd1001. When the concerned DNA clone pRmSl was carried, Rmd1001 had

Table 2. Some properties of *R. meliloti* Rmd1001, with and without the recombinant plasmids pRmS1, pRmS2 and pRmS3 from *R. meliloti* clone bank.

Strain	Growth requirement	Symbiotic behaviour on alfalfa	Ability to utilize mannose	Ability to be stained by congo red	Ability to be stained by aniline blue
Rmd201	Nil	Nod^+Fix^+	+	+	−
Rmd1001	Leucine	Nod^+Fix^-	−	−	+
Rmd1001(pRmS1)	Nil	Nod^+Fix^+	+	+	−
Rmd1001(pRmS2)	Nil	Nod^+Fix^+	−	+	ND
Rmd1001(pRmS3)	Leucine	Nod^+Fix^-	−	+	+
Rmd1001(pGR1)	Leucine	Nod^+Fix^-	ND	ND	ND

+ = ability present; − = ability absent; ND = not done.

Table 3. Sensitivity of *R. meliloti* Rmd1001 towards NaN_3 and NaCl and complementation of defect by the recombinant plasmids pRmS1, pRmS2 and pRmS3

Strain	Highest concentration of NaN_3 (μg/ml) tolerated	Highest concentration of NaCl (M) tolerated
Rmd201	20	0.5
Rmd1001	10	0.3
Rmd1001(pRmS1)	40	0.6
Rmd1001(pRmS2)	40	0.6
Rmd1001(pRmS3)	40	0.6
Rmd201(pRmS1)	40	0.6
Rmd201(pRmS2)	50	0.6
Rmd201(pRmS3)	40	0.6
Rmd201(pRK290)	20	0.5
Rmd201(pRmSP676)	20	0.5
Rmd1001(pRK290)	10	0.3
Rmd1001(pRmSP676)	10	0.3

Table 4. Complementation of the Fix^- phenotype of *R. meliloti* Rmd1001 by the recombinant plasmids pRmS1 and pRmS3

Inoculated bacterial strain	Shoot: Colour	Shoot: Dry weight (mg)	Root nodules: Colour	Root nodules: Shape	Root nodules: Acetylene reduction ability (nmoles/h/mg)
Rmd201	Green	3.0	Pink	Club	37.0
Rmd1001	Yellow	0.8	White	Sphere	0.9
Rmd1001(pRmS1)[a]	Green	3.5	Pink	Club	30.8
Rmd1001(pRmS3)[b]	Yellow	0.4	White	Sphere	0.8

(Column headings span: Nodulation related characteristics of inoculated alfalfa seedlings.)

a = Rmd1001(pRmS2) were Fix^+ like these;
b = Rmd1001(PGR1) were Fix^- like these.

about the same phenotype as Rmd201 (tables 2, 3 and 4). These observations showed that the above 7 markers (loci) must be located close together on the genome of *R. meliloti*. Close association between Leu and Fix phenotypes had been reported earlier also (Scherrer and Denarie 1971; Truchet *et al.* 1980; Kondorosi *et al.* 1980). The *fix-1* locus had been placed next to *leu* locus on the chromosome of *R. meliloti* (Beringer *et al.* 1984).

R. meliloti gene bank clones overlapping with pRmSl were required for positioning the various markers of Rmd1001 with respect to each other. Some of the clones that had been isolated on account of their being complementary to different auxotrophic markers were transferred to Rmd1001 to see if any of them complemented its *leu* marker. A clone called pRmS2 which had been isolated as complementary to the *thi* marker of Rmd1002 was found to be also complementary to the *leu* marker of Rmd1001. However, another clone called pRmS3 which complemented *thi* mutation of Rmd1002 did not complement *leu* of Rmd1001. Likewise pRmSl did not complement *thi* of Rmd1002 (table 2). These observations showed the existence of linkage between *leu* and *thi* loci in *R. meliloti* and also that pRmS1, pRmS2 and pRmS3 must possess overlapping DNA fragments from the *R. meliloti* genome.

In earlier studies, based on their properties, the thiamine auxotrophs of *R. meliloti* had been divided into 2 groups (Finan *et al.* 1986). A group of *thi* mutants (class-2) was mapped on the pSym-b plasmid, amidst *exo* genes. The class-1 mutants were found to be complemented by genomic clones that also complemented *thiA*, *thiB* and *thiC* mutants of *E. coli* (Bachmann 1987). The class-1 mutants did not map on pSym plasmids. Instead, the concerned mutations were thought to be located on the main chromosome of *R. meliloti* like in *E. coli*. The *thi* mutation of Rmd1002 was considered akin to the already known class-1 *thi* mutations for the reasons given below. (1) pSym-b DNA complementary to class-2 *thi* mutants did not suppress Thi$^-$ phenotype of Rmd 1002 (table 5). (2) pRmS2 and pRmS3 complemented *thiA*, *thiB* and *thiC* mutants of *E. coli* (table 5).

What is the relationship between *leu* mutation in Rmd1001 and the various *leu* markers known and mapped in *E. coli*? The *R. meliloti*-derived plasmids pRmS1 and pRmS2 were found to complement the *leuA* mutants of *E. coli*. The above findings altogether suggest that *leu* and *thi* loci are located near each other on the chromosome in *R. meliloti*.

Table 5. Complementation of the phenotype of *R. meliloti* Rmd1002 and of *E. coli thiA*, *thiB* and *thiC* mutants by pRmS2 and pRmS3.

	Thi phenotype of plasmid carrying			
	R. meliloti	*E. coli*		
Plasmid	Rmd1001 *thi*	KG33 *thiA*	KG1673 *thiB*	KG6593 *thiC*
pRmS2	+	+	+	+
pRmS3	+	+	+	+
pRmS1	−	−	−	−
pD2	−	−	−	−
pD34	−	−	−	−
pD56	−	−	−	−

What are the relative positions of the genetic markers identified in Rmd1001? To define the arrangement of *aba*, *azt*, *cro*, *fix*, *leu*, *man* and *ost* markers, the phenotypes of Rmd1001, Rmd1001(pRmS1), Rmd1001(pRmS2) and Rmd1001(pRmS3) were compared (tables 2, 3 and 4). The map that emerged is shown in figure 1. The following was defined as the sequence of markers in the *leu* region of *R. meliloti* chromosome: *man-aba*, *fix*, *leu-cro-azt*, *ost-thi*. Further work will be required to determine the relative positions of *aba*, *fix* and *leu* on the one hand and *azt* and *ost* on the other hand. It remains to be determined whether $Aba^-Fix^-Leu^-$ phenotypes are a result of a pleiotropic mutation in Rmd1001.

Table 3 shows that the Rmd1001 bacteria carrying the recombinant plasmids pRmS1, pRmS2 or pRmS3 were more azide- and osmo-tolerant than Rmd201. Presence of the recombinant plasmids also enhanced the azide- and osmo- tolerance of Rmd201. This property of *R. meliloti* clones was specific to them as it was not shared by the vector plasmid pRK290 and clones possessing unrelated and distant loci. It is possible that the *R. meliloti* carriers of pRmS1, pRmS2 or pRmS3 may be more tolerant to azide and salt due to the presence of multiple copies of plasmids in them. A strategy for improving the salt tolerance of *R. meliloti* and possibly other rhizobia is indicated by these results.

Briefly, in this study seven markers have been placed in a chromosome region of *R. meliloti* in the order (*man*)-(*aba*, *fix*, *leu*)-(*cro*)-(*azt*, *ost*)-(*thi*).

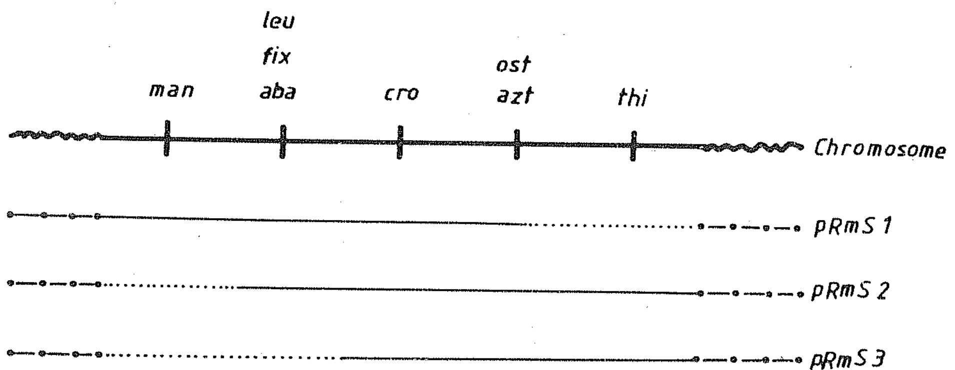

Figure 1. Map of the *leu* region of *Rhizobium meliloti* chromosome. ——, chromosomal DNA present in the recombinant plasmids pRmS1, pRmS2 and pRmS3; ····· DNA not present in the plasmids; —·—·— vector DNA.

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