Lactose utilization and enzymes encoded by megaplasms in *Rhizobium meliloti* SU47: implications for population studies

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Cell extracts prepared from *Agrobacterium tumefaciens* GMI9023 derivatives carrying megaplasms from *Rhizobium meliloti* strain SU47 were examined by non-denaturing polyacrylamide gel electrophoresis and subsequent specific enzyme staining. The results demonstrated that an unidentified or 'nothing' dehydrogenase is encoded by the *nod-nif* megaplasmid pRmeSU47a, and a β -galactosidase activity that is required for growth on lactose as sole carbon source is encoded by the *exo-thi* megaplasmid pRmeSU47b. These results are of particular importance regarding the choice of markers used in bacterial population genetic studies.

Introduction

Enzyme electrophoresis is commonly used in bacterial population genetics (Selander *et al.*, 1986), and in *Rhizobium* it has been used in the study of indigenous soil populations (Young, 1985). A recent survey (Young, 1985) of isolates from lucerne nodules assigned *R. meliloti* strains to two main groups, a major distinguishing factor being the presence of two β -galactosidase activities in members of one group, and only one β -galactosidase activity in members of the other. Two β -galactosidase activities, one constitutive and one inducible, have previously been reported in the *R. meliloti* SU47 derivative Rm2011 (Niel *et al.*, 1977). Only the inducible enzyme activity could support growth on lactose, therefore mutants lacking the inducible enzyme could not use lactose as sole carbon source although the constitutive β -galactosidase activity of the second enzyme remained.

Most strains of *R. meliloti* are known to carry two megaplasms, each of which exceeds 1000 kb in size (Banfalvi *et al.*, 1985; Bromfield *et al.*, 1987; Burkhardt *et al.*, 1987; Hynes *et al.*, 1986; Rosenberg *et al.*, 1981, 1982). In *R. meliloti* SU47 the two megaplasms have been designated pRmeSU47a and pRmeSU47b. Among

the limited number of genes known to be carried on the megaplasms are nodulation (*nod*) and nitrogen fixation (*nif*) genes on pRmeSU47a (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981), and C₄-dicarboxylate transport (*dct*), exopolysaccharide and thiamin biosynthesis genes on pRmeSU47b (Finan *et al.*, 1986; Hynes *et al.*, 1986; Watson *et al.*, 1988). In the course of a survey of the genetic structure of *R. meliloti* strains using enzyme electrophoresis, we included an *R. meliloti* SU47 derivative and *Agrobacterium tumefaciens* strains carrying megaplasmid pRmeSU47a or pRmeSU47b, or no plasmid. Thus we were able to differentiate between chromosomal and megaplasmid-encoded enzymes. Here we present evidence that a β -galactosidase enzyme is encoded by genes carried on the *R. meliloti* *exo-thi-dct* megaplasmid pRmeSU47b, and determine the position of this gene on the recently constructed genetic map of pRmeSU47b (Charles & Finan, 1990). In addition, we show that an unidentified or 'nothing' dehydrogenase activity (Selander *et al.*, 1986) is encoded by the *nod-nif* megaplasmid pRmeSU47a.

Methods

Bacterial strains. These are shown in Table 1.

Media, antibiotics and growth conditions. Bacteria were cultured in LB (Miller, 1972), LBmc (Finan *et al.*, 1984) or M9 salts (Miller, 1972) supplemented with 0.25 mM-CaCl₂, 1 mM-MgSO₄, 15 mM-glucose or

Abbreviations: MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; ONPG, *o*-nitrophenyl β -D-galactopyranoside; PMS, phenazine methosulphate.

Table 1. *Bacterial strains*

Strain	Relevant characteristics*	Source or reference
<i>Rhizobium meliloti</i>		
RM1021	SU47 <i>str</i> -21	Meade <i>et al.</i> (1982)
RmF416	Rm1021, <i>lac</i> -416::Tn5	This work
RmF417	Rm1021, <i>lac</i> -417::Tn5	This work
Rm5455	Rm1021, <i>lac</i> -56::Tn5	Charles & Finan (1990)
GMI214	SU47 deleted of the <i>nod-nif</i> region of pRmeSU47a	J. Denariet†
<i>Agrobacterium tumefaciens</i>		
GMI9023	C58 cured of pTiC58 and pATC58, Sm ^r Rf ^r	Rosenberg & Huguet (1984)
At125	GMI9023, pRmeSU47bΩ5007::Tn5- <i>oriT</i>	Finan <i>et al.</i> (1986)
At128	GMI9023, pRmeSU47aΩ30::Tn5-11	Finan <i>et al.</i> (1986)
<i>Escherichia coli</i>		
YMC9	<i>pro</i> -82 <i>thi</i> -1 <i>hsdR</i> 17 <i>supE</i> 44 Δ(<i>lac</i> - <i>pro</i>)	De Vos <i>et al.</i> (1986)

* Abbreviations for antibiotics are as follows: Rf, rifampin; Sm, streptomycin.

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5 mM-lactose as carbon source, and 0.3 mg of biotin per litre. Thiamin was used at 5 µM and proline was used at 2 mM when required. Antibiotics were used at the following concentrations for *R. meliloti* (µg ml⁻¹): gentamicin, 20; neomycin, 200; oxytetracycline, 0.3–0.5; spectinomycin, 100; streptomycin, 200; tetracycline, 10. *R. meliloti* was grown at 30 °C; *E. coli* was grown at 30 °C or 37 °C.

Enzyme electrophoresis. Cell-free extracts were prepared from cultures by sonication in sonication buffer (20 mM-Tris/HCl pH 7.4, 1 mM-MgCl₂, 0.1 mM-dithiothreitol) followed by centrifugation to remove cell debris. The extracts were electrophoresed at 4 °C in 7% (w/v) native acrylamide gels using Tris/borate/EDTA buffer, pH 8.9 (81 mM-Tris, 21 mM-boric acid, 1.5 mM-disodium EDTA) at 150–300 V for 4–5 h at 4 °C, then stained for β-galactosidase or dehydrogenase activity. β-Galactosidase activity was detected after incubation in a 0.17 mg ml⁻¹ solution of 4-*O*-methylumbelliferyl β-D-galactoside in citrate/phosphate buffer, pH 4.5. The citrate/phosphate buffer contained 5.13 g citric acid and 12.5 g dibasic sodium phosphate heptahydrate l⁻¹ (Harris & Hopkinson, 1976). Fluorescent bands were visualized under short-wavelength UV on a transilluminator. Dehydrogenase activity was detected by incubating the gel in 0.1 M-Tris/HCl pH 7.4 containing 3 mM-MgCl₂, 50 mM-KCl, 0.3 mg NAD or NADP ml⁻¹, 0.3 mg 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) ml⁻¹ and 0.04 mg phenazine methosulphate (PMS) ml⁻¹, plus substrate (100 mM-sodium L-malate for malic enzyme) if required (Harris & Hopkinson, 1976).

β-Galactosidase assay. Cultures were grown overnight in LBmc, then subcultured 1:5 into LBmc or LBmc supplemented with 5 mM-lactose and incubated for 4 h with aeration at 30 °C. Cells were washed and resuspended in 2 ml Z buffer (Miller, 1972), then permeabilized by treatment with one drop of SDS (0.1%) and two drops of chloroform. β-Galactosidase activity was assayed at 30 °C according to Miller (1972) using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate.

Genetic techniques. Transduction, plasmid conjugation and transposon Tn5 mutagenesis using pRK602 were performed as described previously (Finan *et al.*, 1984, 1986). Recombinant cosmids which complemented the lactose mutations were isolated from the pLAFRI clone bank of Rm1021 (Friedman *et al.*, 1982) as described previously (Finan *et al.*, 1988) except that selection was on M9 medium containing 5 mM-lactose.

Results and Discussion

Megaplasmid-encoded β-galactosidase

When proteins from *R. meliloti* Rm1021 cells grown in LBmc medium were examined by polyacrylamide gel electrophoresis, two β-galactosidase activities were detected. Presumably these correspond to the 'inducible' and 'constitutive' β-galactosidase activities previously described in this strain (Niel *et al.*, 1977). Extracts from cells grown in TY, a medium commonly used in *Rhizobium* population genetic studies (e.g. Eardly *et al.*, 1990), also showed the two enzyme activities. For gels run with extracts from lactose-induced cells the intensity of staining of the lactose-inducible β-galactosidase was greater than that of the constitutive enzyme. Consequently photographs of such gels often showed only the inducible β-galactosidase band. Thus Fig. 1 shows that for lactose-grown cells of wild-type *R. meliloti* (Rm1021 = SU47 Sm^r) and *A. tumefaciens* GMI9023 there were single β-galactosidase bands of characteristic electrophoretic mobility. These bands correspond to the inducible enzyme. For *A. tumefaciens* At125 (and not At128), however, two bands corresponding in mobility to the *R. meliloti* and *A. tumefaciens* enzymes were present. This indicated that the inducible β-galactosidase of *R. meliloti* was encoded by pRmeSU47b and was expressed in *A. tumefaciens*.

To confirm that the inducible β-galactosidase gene was located on megaplasmid pRmeSU47b, two independent mutants, RmF416 and RmF417, unable to grow on M9 salts agar with lactose as sole carbon source were identified following Tn5 mutagenesis of *R. meliloti* Rm1021. In transduction from both strains, the Lac⁻ phenotype was 100% cotransducible with the neomycin

Fig. 1. Extracts of *R. meliloti* and *A. tumefaciens* strains separated on 7% acrylamide gels and stained for β -galactosidase. Upper and lower arrows indicate the inducible *R. meliloti* (Rm), and *A. tumefaciens* (At) β -galactosidase (β GAL) bands respectively.

resistance encoded by Tn5, indicating that the Lac⁻ phenotype resulted from the Tn5 insertion in both cases. The two mutations, designated *lac-416::Tn5* and *lac-417::Tn5*, were mapped by three-factor ϕ M12 transductional cross to transposon markers on pRmeSU47b and were found to lie between *thi-502* and Ω 5079 and to be 48% and 37% linked, respectively, to *thi-502::Tn5*-11 (gentamicin-spectinomycin resistant; see Charles & Finan, 1990, Fig. 2 and data not shown).

Fig. 2. Genetic map of part of pRmeSU47b showing the positions of *det*, *thi* and *lac* loci. The numbers below the arrows represent ϕ M12 cotransductional frequencies (%), with the arrow tail indicating the selected marker and the arrow head indicating the unselected marker. The order and locations of transposon insertions (designated by Ω) and *det* and *thi* loci were previously determined (Charles & Finan, 1990).

Table 2. β -Galactosidase activities of *R. meliloti* *lac* mutants

β -Galactosidase activities were determined, using ONPG as substrate, on cells grown in LBmc with or without supplemental lactose (5 mM) as described in the text. Specific activities are expressed as Miller units (Miller, 1972). Values are the means of triplicate assays $\pm$ standard error of the mean. Values with the same superscript letter are not significantly different at the 95% confidence level using Student's *t*-test.

Strain	β -Galactosidase specific activity	
	Plus lactose	Minus lactose
RmF416	5.93 $\pm$ 0.34 ^b	5.69 $\pm$ 0.31 ^b
RmF417	4.80 $\pm$ 0.11 ^a	5.01 $\pm$ 0.10 ^a
Rm5455	4.60 $\pm$ 0.54 ^a	4.98 $\pm$ 0.26 ^a
Rm1021	7.18 $\pm$ 0.68 ^c	10.33 $\pm$ 0.69 ^d

Examination of the recently constructed megaplasmid genetic map (Charles & Finan, 1990) revealed that the previously isolated insertion, Ω 5056::Tn5, mapped in the *lac* region (Fig. 2). We subsequently found that strains carrying Ω 5056::Tn5 were Lac⁻ (Table 2, Rm5455 = Rm1021 Ω 5056::Tn5). Insertion Ω 5056 was designated as *lac* allele *lac-56::Tn5*.

Results of β -galactosidase assays performed on the wild-type and the three mutants are presented in Table 2. For LBmc-grown cells, β -galactosidase activities of these mutants were significantly less than that of wild-type Rm1021, and no induction was seen in the presence of 5 mM-lactose. The β -galactosidase activity of Rm1021 in LBmc + 5 mM-lactose was about twice that of the mutants grown in the same medium, and about 50%

greater than that of Rm1021 grown in LBmc alone. Presumably the background activity of about 5 Miller units is due to the second, non-inducible, β -galactosidase, which does not allow growth on lactose. Similar data were previously obtained by Niel *et al.* (1977). Our results also suggest that the inducible β -galactosidase is expressed in LBmc medium even in the absence of added lactose. The significance of this is not clear as we have not done a systematic analysis of β -galactosidase expression in defined media.

Recombinant clones that restored lactose utilization ability to the mutants were isolated from a pLAFR1 cosmid bank of Rm1021 DNA (Friedman *et al.*, 1982). Restriction analysis identified three different plasmids, each of which carried common 5 and 7 kb *Eco*RI fragments (not shown). One of these, designated pTH45, was examined further. When extracts of *A. tumefaciens* GMI9023 containing pTH45 were examined by polyacrylamide gel electrophoresis a second β -galactosidase activity in addition to the *Agrobacterium* enzyme was detected (Fig. 1). The second band of activity had the same electrophoretic mobility as the inducible Rm1021 enzyme. Thus pTH45 carries the *R. meliloti* β -galactosidase enzyme structural gene. pTH45 failed to complement the *Escherichia coli* *lac* deletion mutant YMC9 for growth on M9 salts with lactose as sole carbon source, indicating that the inducible *R. meliloti* β -galactosidase is not expressed in *E. coli*. This is similar to the result recently reported by Fanning & O'Gara (1988) for the cloned β -galactosidase-encoding gene of *R. meliloti* 102F34.

Plasmid-encoded lactose utilization genes have been found in other bacterial species and in some strains the *lac* genes are part of transposable elements (Cornelis *et al.*, 1981). We have not examined the megaplasmid *lac* genes for the ability to transpose to other replicons. As plasmid-determined traits are generally considered to be unstable we note that in a previous survey eight out of eleven strains of *R. meliloti* examined could utilize lactose (Graham & Parker, 1964). More recently, 28 strains of *R. meliloti* were found to form two major electrophoretic types which differed in glucose-6-phosphate dehydrogenase enzyme patterns and in the presence of one (15 strains) or two (13 strains) β -galactosidase enzymes (Young, 1985). In another recent survey of 232 *R. meliloti* isolates two β -galactosidase isoenzymes were detected in 89% of the strains (Eardly *et al.*, 1990). Unfortunately it is unclear which of the two β -galactosidase alleles in the above studies corresponds to the megaplasmid β -galactosidase allele. The presence of only one β -galactosidase activity in some strains would suggest that one or other of the two genes has been lost from some strains. It is also possible that some strains of *R. meliloti* have never acquired the second β -galactosidase enzyme.

Fig. 3. Unidentified dehydrogenase activity associated with megaplasmid pRmeSU47a. Extracts of *R. meliloti* and *A. tumefaciens* strains were separated on 7% acrylamide gels and stained for malic enzyme, (a) in the presence of NADP using L-malate as substrate, or (b) with no added substrate and in the absence of NADP. The unidentified dehydrogenase band is indicated by the arrow.

Determination of these possibilities is important for understanding the genetic structure of natural populations of *R. meliloti*.

Megaplasmid-encoded unidentified dehydrogenase

While examining strains for various dehydrogenase activities by enzyme electrophoresis followed by specific enzyme staining in the presence of substrate, NAD or NADP, PMS and MTT, we observed that in addition to the specific enzyme activity, an additional band of characteristic mobility was present regardless what substrate was used. For example, when *R. meliloti* Rm1021 extract was stained for malic enzyme using L-malate as substrate, in addition to the malic enzyme band(s), there was also another band of greater electrophoretic mobility (Fig. 3a). This same enzyme activity was present in *A. tumefaciens* At128 (= GMI9023/pRmeSU47a) extract but was absent in *A. tumefaciens*

GMI9023 extract (Fig. 3a), suggesting that this second enzyme activity was encoded by pRmeSU47a. In other organisms, such activity is referred to as unidentified or 'nothing' dehydrogenase and has been used as an enzyme locus in population genetics studies (Selander *et al.*, 1986). In further experiments we have found that the *R. meliloti* 'unidentified dehydrogenase' did not require NAD or NADP but did require PMS for the reduction of MTT (Fig. 3b and not shown).

The unidentified dehydrogenase activity was present in extracts of the *R. meliloti* SU47 derivative GMI214, which is deleted of the entire pRmeSU47a *nod-nif* region (J. Denarie, personal communication). The activity was not detected in extracts of Rm1021 bacteroids (not shown). These data are consistent with the unidentified dehydrogenase not being directly involved in symbiosis, although they do not provide conclusive evidence for this.

Enzymes not detected on megaplasms

In addition to the enzymes discussed above, it is worth noting that in our survey, expression of the following *R. meliloti* enzymes was not detected from megaplasms in the *Agrobacterium*/megaplasms hybrid strains (data not shown): 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase, fumarase, phosphoglucose isomerase and tetrazolium oxidase.

Concluding statements

We have identified two enzyme activities associated with the megaplasms of *R. meliloti*, and both of these activities are and have been used as markers in analyses of bacterial population structures. In fast-growing *Rhizobium* strains plasmid DNA constitutes a large fraction of the genome. Our results suggest that in these bacteria caution should be exercised in assuming that markers are chromosomally located unless this has been shown. In particular, it may be necessary to re-evaluate *Rhizobium* population genetic studies such as that of Young & Wexler (1988) where β -galactosidase was used as a chromosomal marker.

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