

# Plasmids That Insert into the Rhamnose Utilization Locus, *rha*: A Versatile Tool for Genetic Studies in *Sinorhizobium meliloti*

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## Key Words

*Sinorhizobium meliloti* · Chromosomal insertion · Mutant complementation · Reporter strain

## Abstract

Described is a suite of plasmids that can be used to deliver DNA into a specific site in the chromosome of *Sinorhizobium meliloti* with a minimal impact in the physiology of the organism. This allows stable, single-copy, insertions of DNA while maintaining a constant chromosomal context. The plasmids integrate into *rhaS*, one of a group of genes encoding proteins for rhamnose utilization, enabling a simple screening method for recombinants, while leaving other cellular processes unaffected by the insertion. Construction of plasmids for *gfp* labeling, mutant complementation, gene expression and protein over-expression studies are outlined. The *rha* insertion plasmids constitute a flexible and easy to use collection of cloning vectors that can be efficiently delivered by conjugation or transformation, and that could be used in genetic and physiology studies of *S. meliloti*, and, with minor modifications, in other bacterial species.

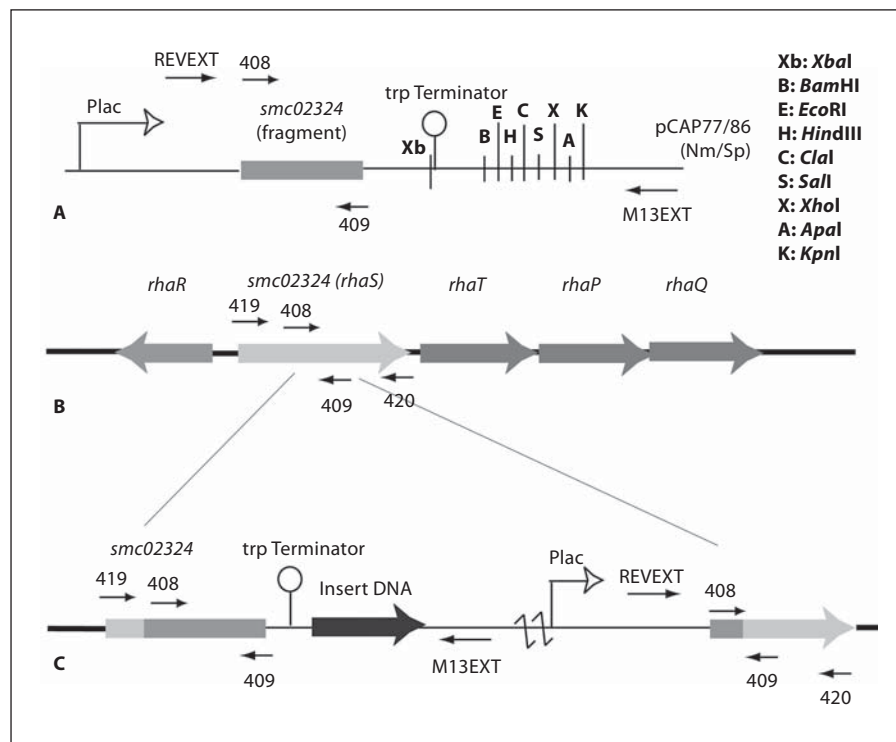
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## Introduction

Integration of DNA into the genomes of bacteria is a frequently used genetic tool that allows for a variety of genetic experiments, such as creating gene disruptions or deletions, generating promoter-reporter gene fusions for the study of gene expression, or generating protein-GFP fusions to study protein localization in the cell [Gao and Teplitski, 2008; Kalogeraki and Winans, 1997; Quandt and Hynes, 1993]. Other applications include the delivery of heterologous, or mutated, genes in order to study gene function.

Transposons are frequently used to deliver DNA into the genomes and can be very convenient, however, transposons often insert randomly into the genome, potentially creating mutations that may affect the phenomenon being studied. Another method of DNA delivery utilizes a plasmid with a conditionally defective origin of replication. The plasmid, unable to replicate in the recipient, can recombine in the host genome if there are regions of high DNA similarity between the plasmid and the genome. The plasmid will preferentially recombine into those regions, and the frequency of recombination will depend on the length of the similar regions. This method is especially useful for creating gene disruptions and gene re-

**Fig. 1. A** Schematic of plasmids pCAP77 and pCAP86. **B** Rhamnose operon, *rhaS* (*smc02324*) is the target of *rha* insertion plasmids. **C** Rhamnose operon with recombined *rha* insertion plasmid carrying a cloned DNA fragment. Arrows indicate primer-binding sites. Figures not to scale.



placements. Insertion of DNA into a predictable and neutral site in the genome can be achieved by using site-specific transposons or phages, like Tn7 and bacteriophage  $\lambda$ . Tn7 has been used in *Bradhyrizzobium japonicum*, *Sinorhizobium meliloti*, and *Rhizobium leguminosarum*, although specific-site insertion was reported only for *S. meliloti* and *R. leguminosarum* [Bolton et al., 1984; Liu et al., 1989; Miksch and Lentzsch, 1987]. A set of plasmids to introduce Tn7 into gram-negative bacteria was reported to be functional in many species [Choi et al., 2005], but did not work with *S. meliloti*, under the conditions in our laboratory [Garcia, Arango Pinedo and Gage, unpubl.].

We here describe a set of plasmids that can be used to deliver DNA into a specific site in the chromosome of *S. meliloti*, allowing stable, single-copy, insertions of DNA while maintaining a constant chromosomal context. The plasmids can be easily modified for use in other bacterial species.

## Results and Discussion

### The *rha* Insertion Suicide Vectors

We developed a set of suicide plasmids that recombine into the *rhaS* gene (*smc02324*) in the chromosome of

*S. meliloti*. The basic delivery plasmids (pCAP77 and pCAP86) carry the ColE1 origin of replication, which does not allow replication in *S. meliloti*. Thus, to be retained, the plasmids must integrate into the chromosome by recombination. To make the recombination a site-specific event, the plasmids also carry a 465-bp internal fragment of *rhaS* (*smc02324*), the first gene in an operon devoted to rhamnose utilization [Mauchline et al., 2006]. In the simplest of the *rha* insertion plasmids, a multiple cloning site is located downstream of the *rhaS* fragment, and is preceded by a *trp* terminator to help ensure that transcription of the cloned DNA from the *rhaS* promoter region is minimized (fig. 1A). *rha* insertion plasmids that lack the terminator are also available (table 1).

The *rha*-insertion plasmids carry neomycin or spectinomycin antibiotic resistance markers for ease of selection, and possess *oriT*, an origin of transfer that allows for conjugal transfer. All these characteristics give the plasmids great flexibility, and they have been engineered for uses that include: labeling of cells with fluorescent proteins, delivery of complementing genes, construction of reporters for gene expression, study of mutant proteins, and overexpression studies. Here, we report the construction of these plasmids, characteristics of strains carrying the inserted plasmids and examples of their application.

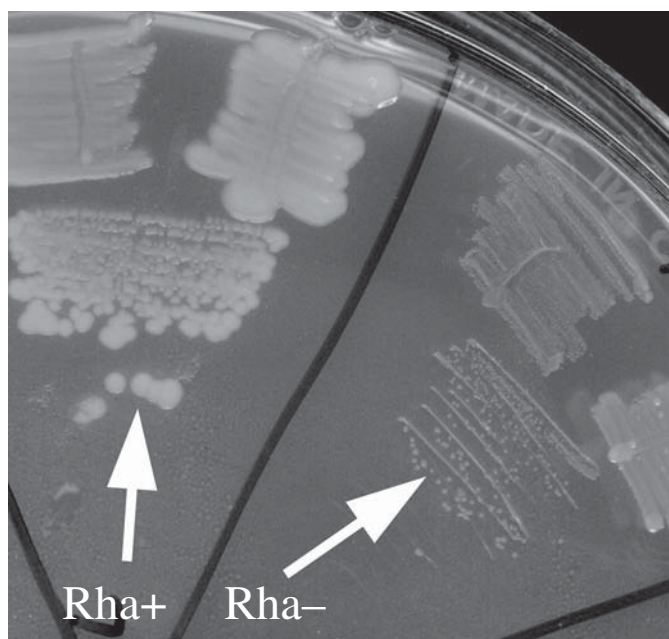
**Table 1.** Strains, plasmids, and primers

Strain	Genotype	Reference
Rm1021	wild-type <i>S. meliloti</i>	Meade et al., 1982
XL1B MRF <sup>+</sup>		Stratagene (11011 N. Torrey Pines Rd, LaJolla, Calif., USA)
RmTJ170	<i>nodC</i> ::Tn5	Jacobs et al., 1985
AG01	Rm1021 <i>dctA</i> ::Tn5	A. Garg and D. Gage, unpublished
RB33	<i>lacR</i> ::Tn5-233	Bringhurst and Gage, 2002
CAP43	Rm1021, <i>rhaS</i> ::pCAP77	Pinedo et al., 2008
CAP57	Rm1021, <i>rhaS</i> ::pCAP95	this study
CAP74	AG01, <i>rhaS</i> ::pCAP108	this study
CAP69	1021, <i>rhaS</i> ::pCAP99	this study
CAP89	RmTJ170, <i>rhaS</i> ::pCAP107	this study
CAP90	RB33, <i>rhaS</i> ::pCAP106	this study
Plasmid	Relevant genotype <sup>a</sup>	Reference
pGEMT-Easy	Amp	Promega (2800 Woods Hollow Rd., Madison, Wisc., USA)
pMB438	<i>oriT</i> Nm	Barnett et al., 2000
pMB439	<i>oriT</i> Sp	Barnett et al., 2000
pZKH2	P <sub>nptII</sub>	Herron, Gage and Cardon, unpublished
pTB1G	P <sub>trp</sub> :: <i>gfpS56T</i>	Gage et al., 1996
pTB93F	P <sub>trp</sub> :: <i>gfpS56T</i>	Gage et al., 1996
pDG71	P <sub>trp</sub> :: <i>gfp-mut3b</i>	Gage, 2002
pDG101	<i>gfp-mut3b</i>	this lab
pCAP11	P <sub>melA</sub> :: <i>gfpS56T</i>	Pinedo et al., 2008
pCAP75	pMB438, <i>trp</i> terminator	this study
pPG60	pGEMT-Easy::P <sub>sma0113</sub>	Garcia and Gage, unpublished
<i>rha</i> -insertion plasmids	Genotype/resistance marker <sup>a</sup>	Reference
pCAP76	pMB438, <i>rhaS</i> Nm	this study
pCAP77	PCAP75, <i>rhaS</i> Nm	Pinedo et al., 2008
pCAP84	pCAP77, P <sub>sma0113</sub> Nm	this study
pCAP85	pMB439, <i>rhaS</i> Sp	this study
pCAP86	pCAP85, <i>trp</i> terminator Sp	this study
pCAP87	pCAP77, P <sub>trp</sub> Nm	this study
pCAP88	pCAP77, P <sub>nptII</sub> Nm	this study
pCAP89	pCAP77, P <sub>sma0113</sub> :: <i>gfpS65T</i> Nm	this study
pCAP90	pCAP77, P <sub>nptII</sub> :: <i>gfpS65T</i> Nm	this study
pCAP91	pCAP86, P <sub>nptII</sub> Sp	this study
pCAP92	pCAP77, P <sub>trp</sub> :: <i>gfpS65T</i> Nm	this study
pCAP93	pCAP77, P <sub>sma0113</sub> :: <i>gfp-mut3b</i> Nm	this study
pCAP94	pCAP77, P <sub>nptII</sub> :: <i>gfp-mut3b</i> Nm	this study
pCAP95	pCAP77, P <sub>trp</sub> :: <i>gfp-mut3b</i> Nm	this study
pCAP96	pCAP86, P <sub>sma0113</sub> Sp	this study
pCAP97	pCAP86, P <sub>trp</sub> Sp	this study
pCAP98	pCAP77, <i>gfp-mut3b</i> Nm	this study
pCAP99	pCAP77, P <sub>melA</sub> :: <i>gfpS65T</i> Nm	this study
pCAP100	pCAP86, <i>gfp-mut3b</i> Sp	this study
pCAP106	pCAP77, P <sub>trp</sub> :: <i>lacR</i> Nm	this study
pCAP107	PCAP86, P <sub>trp</sub> :: <i>nodC</i> Sp	this study
pCAP108	pCAP77, P <sub>dctA</sub> :: <i>dctA</i> Nm	this study
pCAP127	pCAP86, P <sub>nptII</sub> :: <i>gfp-mut3b</i> Sp	this study
Primer	Sequence (5' to 3')	Comment
280	<u>CTAGATCCCGAGCCCGCTAATGAGCGGGCTTTTTTTGGTC</u>	<i>trp</i> terminator, overhangs underlined
281	<u>CTAGGACCAAAAAAAGCCCGCTCATTAGGCGGGCTGGGGAT</u>	<i>trp</i> terminator, overhangs underlined
408	<u>GCGGCCGCGCGGCATCAAGGTCATCTCC</u>	<i>NotI</i> site underlined
409	<u>GCGGCCGCTCGGATGGGACTGGATGA</u>	<i>NotI</i> site underlined
419	CATTGATGGTGACGGCCGCC	N-terminal-coding end of <i>rhaS</i>
420	GAGGCGCCGACTTCACGTGT	C-terminal-coding end of <i>rhaS</i>
M13EXT	ACGACGTTGTAAAACGACGGCCAGT	

**Table 1** (continued)

Strain	Genotype	Reference
REVEXT	ATTTCACACAGGAAACAGCTATGACCA	
452	<u>GGTACCCTCGCCCGTTCCAGTCA</u>	Amplification of <i>nodC</i> , <i>KpnI</i> site underlined
453	<u>CTCGAGCCTCCGCATCACTGAACA</u>	Amplification of <i>nodC</i> , <i>XhoI</i> site underlined
454	<u>ATCGATGGCAGAAGGACGGATGGA</u>	Amplification of <i>lacR</i> , <i>ClaI</i> site underlined
455	<u>GGTACC</u> GGCAGGGCTCGAATTGAT	Amplification of <i>lacR</i> , <i>KpnI</i> site underlined
456	<u>CTCGAG</u> GAAGTTTGACCATGCGGA	Amplification of <i>dctA</i> and native promoter, <i>XhoI</i> site underlined
457	<u>GGTACC</u> TTATTTCGGCGGGCTGGAC	Amplification of <i>dctA</i> and native promoter, <i>KpnI</i> site underlined

<sup>a</sup> Amp = Ampicillin; Nm = neomycin; Sp = spectinomycin.



**Fig. 2.** Rhamnose utilization is affected by interruption of *rhaS*. Shown are colonies of wild-type strain Rm1021 (intact *rhaS*, Rha+) and *rha* insertion mutant strain CAP43(*rhaS*::pCAP77, Rha-) grown on M9 plates with rhamnose (0.2%) as the sole carbon source.

#### *Interruption of rhaS Affects Growth on Rhamnose but Does Not Significantly Affect Other Cellular Processes*

*rhaS* is the first ORF in a *S. meliloti* operon induced by rhamnose and encodes a putative rhamnose-binding protein (fig. 1B) [Mauchline et al., 2006]. Rhamnose is not known to induce any other genes encoding sugar transport proteins which suggested that *rhaS* was necessary for rhamnose utilization [Mauchline et al., 2006].

**Table 2.** Growth rate  $k$  (1/h) of the wild-type strain Rm1021 (intact *rhaS*) and the *rha*-insertion mutant CAP43 (*rhaS*::pCAP77) in M9 medium with different carbon sources (average  $\pm$  SE)

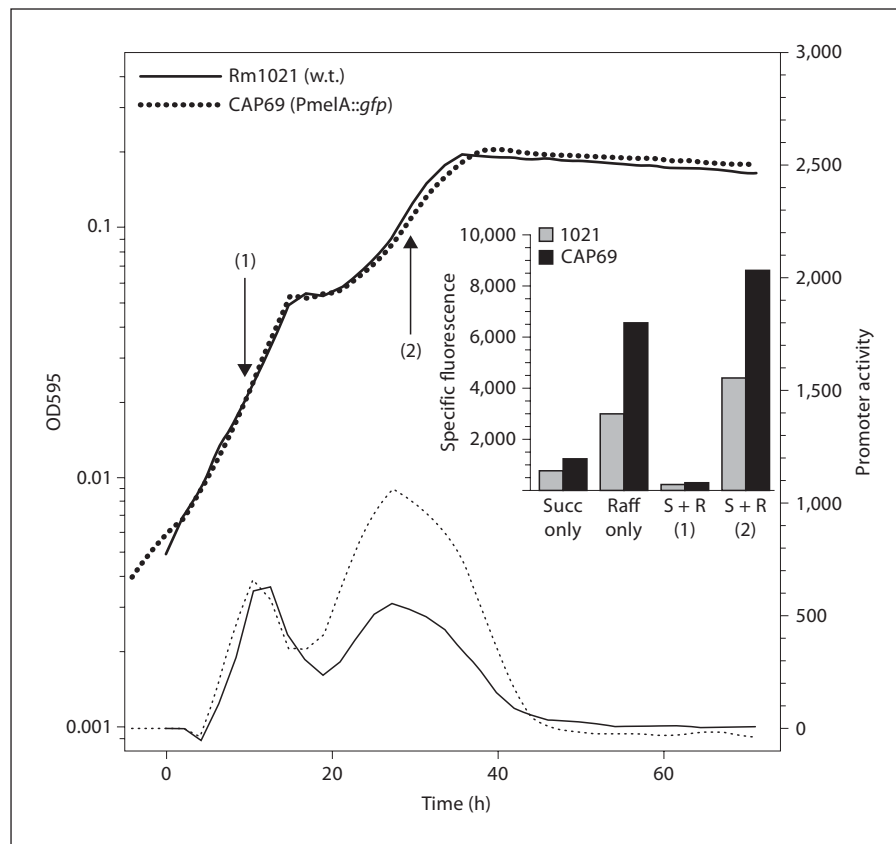
Carbon source	Rm1021 (intact <i>rhaS</i> )	CAP43 ( <i>rhaS</i> ::pCAP77)
Succinate	0.19 $\pm$ 0.017	0.18 $\pm$ 0.029
Raffinose	0.12 $\pm$ 0.005	0.12 $\pm$ 0.001
Lactose	0.071 $\pm$ 0.018	0.071 $\pm$ 0.056
Rhamnose	0.067 $\pm$ 0.003	0.004 $\pm$ 0.001

To confirm this, the basic delivery plasmid pCAP77 was introduced into *S. meliloti* strain Rm1021 (wild type). Insertion of the plasmid into *rhaS* was confirmed by neomycin resistance and PCR screening. The strain, CAP43(*rhaS*::pCAP77), was tested for rhamnose utilization on minimal medium M9 plates with rhamnose as the sole carbon source. Growth was substantially reduced in strain CAP43(*rhaS*::pCAP77), while the wild-type strain exhibited abundant growth (fig. 2). The insertion into *rhaS* in strain CAP43(*rhaS*::pCAP77) did not affect growth rates on succinate, raffinose, or lactose (table 2). Nor did it affect succinate-mediated catabolite repression (SMCR) with raffinose, maltose or lactose as secondary carbon sources (data not shown). Previous studies showed that interruption of *rhaS* did not affect the symbiotic abilities of *S. meliloti* [Pinedo and Gage, 2009].

#### *The Inability to Use Rhamnose Is a Useful Screening Tool*

The initial isolation of recombinants that carry the delivery plasmids can be done by selecting for antibiotic resistance (neomycin or spectinomycin). However, if the

**Fig. 3.** Characterization of *mela-agp* expression using a  $P_{mela}::gfpS56T$  fusion inserted into the *rhaS* gene (CAP69 (*rhaS*::pCAP99)). Cells were grown in M9-minimal medium with succinate (0.05%) plus raffinose (0.1%) as carbon sources. Thick lines show growth; thin lines show  $P_{mela}$  promoter activity as reported by the fusion. Promoter activity was calculated as  $[\Delta\text{fluorescence}/\Delta t]/\text{OD}$  for 5 time points flanking each OD. Inset shows specific fluorescence values for midexponential cultures grown in: M9 succinate, M9 raffinose or M9 succinate plus raffinose. M9 succinate plus raffinose values were obtained at early (1) or late (2) time points during the growth, as indicated by the arrows in the growth curve.



delivery plasmids are carrying another fragment of DNA that is also present in the *S. meliloti* genome, homologous recombination can happen at two different locations in the genome: at the sequence of interest, or at the *rhaS* site. Strains carrying insertions into *rhaS* can be identified by their inability to grow on rhamnose as the sole carbon source. Insertion can be confirmed by PCR screening using a primer that sits at the N-terminal coding end of *rhaS*, pointing into the recombined plasmid (primer 419; table 1) and a primer that sits elsewhere in the recombined plasmid (e.g. primer M13EXT; table 1). Successful amplification indicates recombination into *rhaS* (fig. 1C).

#### Constructing Constitutively Labeled, and Reporter Strains

Plasmids based on pCAP77/PCAP86 can be used to construct reporter strains if a gene for a detectable protein, such as *gfp*, *lacZ* or *inaZ*, is placed under the control of a promoter. Strains with a reporter gene under the control of an inducible, or otherwise regulated, promoter are frequently used for gene expression studies as well as for

answering questions in microbial ecology [Bringhurst et al., 2001; Gage et al., 2008]. We constructed a strain (CAP69) that expresses GFP in response to  $\alpha$ -galactosides by fusing the *S. meliloti mela* promoter region to *gfpS65T* ( $P_{mela}::gfpS56T$ ). Strain CAP69( $P_{mela}::gfpS56T$ ), grown in M9 minimal liquid medium with succinate plus raffinose as carbon sources, exhibited green fluorescence and elevated promoter activity once succinate was exhausted which indicated that induction of the *mela* promoter was repressed by succinate (fig. 3). The single-copy reporter in strain CAP69( $P_{mela}::gfpS56T$ ) yielded a 5- to 20-fold increase of specific fluorescence (induced/uninduced) (fig. 3, inset), while a 50-fold increase was observed for Rm1021 carrying the multi-copy reporter plasmid pCAP11 (data not shown). However, the presence of a single copy of a promoter, as opposed to the many copies present when cells carry a multi-copy plasmid, can be advantageous. For example, in cases in which availability of transcriptional regulators controlling expression from the promoter is low, titration of the regulator by multiple copies of the promoter could potentially affect regulation and its phenotypic manifestation.

Reporter strains have been used in complex environments such as the rhizosphere, on plants, or under other conditions where maintaining antibiotic pressure to ensure plasmid presence may be difficult [Cardon and Gage, 2006; Gage et al., 2008]. Loss of labeling can result in significant underestimation of bacterial numbers, which may be inadmissible for certain applications. To investigate if chromosome-based labeling would be more advantageous than plasmid-based labeling, we compared the frequency of label loss between strains carrying *gfpS56T*, under the control of the *trp* promoter in a high-copy plasmid (pTB93F), a low-copy number plasmid with *parD/parE*-conferred stability (pDG71), and as a single-copy insertion in the *rhaS* gene (strain CAP57). The strains were grown overnight in TY medium without selection for antibiotic resistance. The cultures were enumerated on plates, and colonies that had lost *gfp* labeling were identified by their lack of fluorescence. After overnight growth, strain CAP57 exhibited a frequency of excision of the integrated plasmid (growth on rhamnose and loss of *gfp* label) of  $5 \times 10^{-6}$ , while Rm1021/pDG71 exhibited no detectable loss of the plasmid (detection limit was 1 loss event per  $10^5$  cells) and strain Rm1021/pTB93F exhibited a frequency of loss of 10%. Although strain Rm1021/pDG71 exhibited satisfactory retention of the plasmid in the absence of selective pressure, the increased stability in plasmids with a toxin/antitoxin system like ParD/ParE comes with an extra cost to the population, where the net growth may be lower than for a strain not carrying such a plasmid [Cooper and Heineemann, 2000].

#### Complementation of Mutants

*S. meliloti* can live in symbiosis with alfalfa plants, in root nodules where it fixes nitrogen for its host. The ability of pCAP77, and related plasmids, to complement mutations in *trans* was investigated using the symbiotically deficient strains RmTJ170(*nodC*::Tn5) and AG01(*dctA*::Tn5). Strain RmTJ170(*nodC*::Tn5) does not induce nodules on alfalfa plants, while strain AG01(*dctA*::Tn5) elicits Fix<sup>-</sup> nodules. Additionally, strain AG01(*dctA*::Tn5) cannot grow on succinate as the sole carbon source [Engelke et al., 1989; Long et al., 1988].

An intact copy of the *nodC* gene placed under the control of the *trp* promoter, or an intact copy of the *dctA* gene with its native promoter, were delivered into *rhaS* using the *rha* insertion plasmids. Complementation of *nodC* and *dctA* was evaluated by inoculating alfalfa seedlings with mutant, gene-supplemented or wild-type strains. Plant size and color were recorded, and nodules count-

**Table 3.** Symbiotic abilities of mutant and complemented strains inoculated on alfalfa (*Medicago sativa*) plants (average  $\pm$  SE)

Inoculated strain	Genotype	Number of white nodules	Percentage of pink nodules
Rm 1021	wild type	5.3 $\pm$ 0.6	82 $\pm$ 6
RmTJ170	<i>nodC</i> -	0.2 $\pm$ 0.2 <sup>a</sup>	NA
CAP89	<i>nodC</i> -P <sub>trp</sub> :: <i>nodC</i>	4.8 $\pm$ 0.7	61 $\pm$ 8
AG01	<i>dctA</i> -	7.4 $\pm$ 0.9	12 $\pm$ 9 <sup>a</sup>
CAP74	<i>dctA</i> -P <sub>dctA</sub> :: <i>dctA</i>	7.3 $\pm$ 0.7	60 $\pm$ 6
No inoculum		0 <sup>a</sup>	NA

NA = Not applicable.

<sup>a</sup> Significantly lower than the wild type, n = 20, p < 10<sup>-12</sup>.

ed, 30 days after inoculation (table 3). Plants inoculated with mutant strains AG01(DctA<sup>-</sup>) and RmTJ170(NodC<sup>-</sup>) were small and yellow, suggesting nitrogen deficiency, and exhibited only white nodules (DctA<sup>-</sup>) or no nodules at all (NodC<sup>-</sup>). In contrast, plants inoculated with strains Rm1021(wild type), CAP89(P<sub>trp</sub>::*nodC*) or CAP74(P<sub>dctA</sub>::*dctA*) were large, with green and abundant leaves, looked healthy, and exhibited pink (Fix<sup>+</sup>) nodules, indicating that the *nodC* and *dctA* mutations had been complemented with the inserted genes, and that overexpression of *nodC* (with the constitutive P<sub>trp</sub>) did not interfere with symbiotic abilities. The succinate utilization defect of strain AG01(DctA<sup>-</sup>) was also complemented by introduction of *dctA*, as evidenced by growth of strain CAP74 (P<sub>dctA</sub>::*dctA*) on M9-succinate plates (not shown).

#### Construction of a Strain That Overexpresses the Repressor LacR

In *S. meliloti*, LacR represses the *lac* operon in the absence of lactose. In strain RB33(*lacR*::Tn5-233), expression of the *lac* genes is constitutive and is not sensitive to repression by succinate [Bringhurst and Gage, 2002]. To determine if the *rha* insertion plasmids could be used to study the effects of overexpression of a protein when its gene was present in single copy, the *lacR* gene was cloned in plasmid pCAP87, under the control of the strong, constitutive, *trp* promoter. The plasmid was delivered into the *rhaS* site of the LacR-mutant RB33(*lacR*::Tn5-233), creating the LacR overexpressing strain CAP90(*lacR*::Tn5-233, P<sub>trp</sub>::*lacR*).

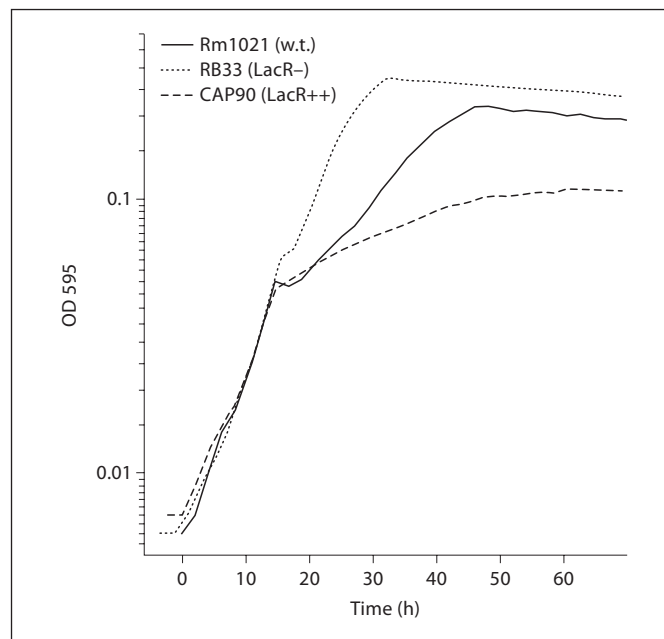
Over-expression of LacR in strain CAP90(*lacR*::Tn5-233, P<sub>trp</sub>::*lacR*) prevented normal growth on lactose as the sole carbon source on M9 minimal plates, while strains

Rm1021 (wild type) and RB33(*lacR::Tn5-233*) exhibited abundant growth (data not shown). When strain CAP90(*lacR::Tn5-233*,  $P_{trp}::lacR$ ) was plated on M9 minimal medium with succinate plus lactose as carbon sources and XGal, over-expression of LacR enhanced SMCR of lactose utilization. Colonies of Rm1021 (w.t.) were initially white but later turned blue (indicating normal SMCR), colonies of strain RB33 (*lacR::Tn5-233*) exhibited a dark blue coloration early on (indicating weak SMCR) and the LacR over-expressing strain exhibited very pale blue coloration which did not darken (data not shown). A similar effect was observed in diauxic curves when the strains were grown in liquid M9 medium with succinate plus lactose as carbon sources (fig. 4). Enhanced SMCR in strain CAP90(*lacR::Tn5-233*,  $P_{trp}::lacR$ ) was probably a consequence of high concentrations of the LacR repressor in the cell, resulting from strong expression of  $P_{trp}::lacR$ .

#### *The rha Insertion Plasmids Can Be Successfully Delivered by Mating or by Electroporation*

Both electroporation and mating were used successfully to deliver the *rha*-insertion plasmids into *S. meliloti*. The combined transformation-recombination efficiency obtained by electroporation was between 150 and 890 transformants/ $\mu$ g of DNA, and frequency ranged from  $1 \times 10^{-9}$  to  $2 \times 10^{-6}$  transformants/recipient. The wide range of the transformation-recombination frequency was probably a result of differences in electrocompetence of cell batches. Although electroporation efficiencies as high as  $10^8$  transformants/ $\mu$ g of DNA have been reported for *R. leguminosarum* [Garg et al., 1999], a study of electro-transformation of plasmids into rhizobial species reported an efficiency under 1,000 transformants/ $\mu$ g DNA for *S. meliloti* and  $10^4$ – $10^5$  transformants/ $\mu$ g DNA for two different strains of *R. leguminosarum* [Hayashi et al., 2000]. Since the efficiency values for *rha*-insertion plasmids in this report are combined for transformation and recombination, they compare favorably with efficiencies expected in *S. meliloti*.

Because the delivery plasmids carry the *oriT* origin of transfer, they are mobilizable using a helper plasmid with transfer functions. We successfully delivered plasmids pCAP94( $P_{nptII}::gfp$ -mut3b) and pCAP95( $P_{trp}::gfp$ -mut3b) into *S. meliloti* strains using triparental matings. The combined conjugation-recombination frequency was evaluated for donor to recipient ratios of 0.05 and 0.01 (helper to recipient ratio fixed at 1:10). The frequencies obtained were on the order of  $10^{-10}$  to  $10^{-11}$  recombinant transconjugants per recipient.

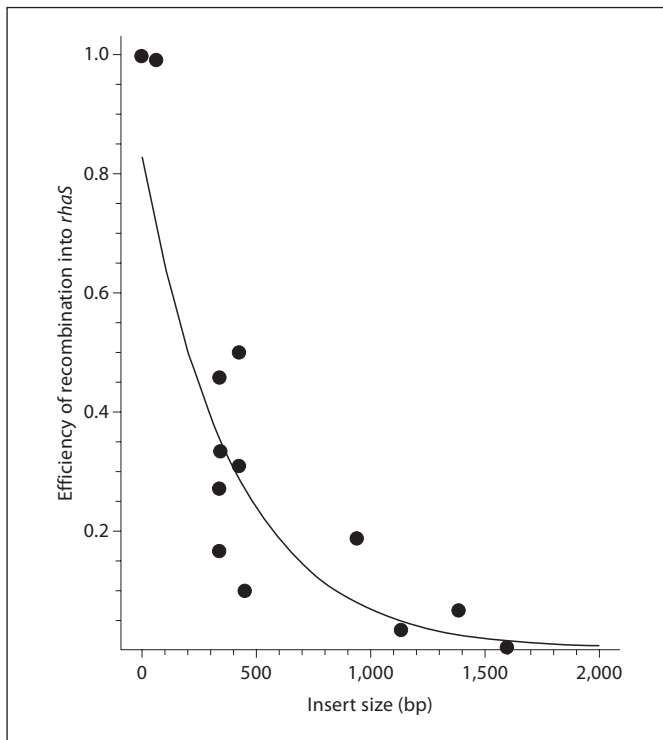


**Fig. 4.** Effect of over expression of transcriptional repressor LacR in *S. meliloti*. Diauxic growth of strains Rm1021 (w.t.), RB33 (LacR<sup>-</sup>) and CAP90(LacR<sup>++</sup>) in minimal medium with succinate (0.05%) plus lactose (0.1%) as carbon sources was monitored. Overexpression of LacR resulted in intensified catabolite repression of lactose utilization when succinate was present.

Successful establishment of the *rha* insertion plasmids in the cells requires not only plasmid introduction, but also recombination at the *rhaS* site. The frequency of recombination in the desired DNA region (*rhaS*) was calculated as the percentage of recombinant transformants that were unable to grow on rhamnose. For introduced DNA that had no identical counterpart in the genome other than the *rhaS* fragment, frequencies of recombination into *rhaS* were higher than 99% (i.e. plasmids pCAP77, pCAP94, pCAP95). The frequency decreased when additional *S. meliloti* DNA was cloned into the plasmids, and it declined with increasing length of the additional *S. meliloti* DNA, from 99% when the additional *S. meliloti* DNA was 60 base pairs long, to 6% when a *S. meliloti* DNA fragment of 1,595 basepairs was on the plasmid (fig. 5).

#### *The Complete Series of rha Insertion Plasmids Is a Versatile Collection for Cloning Applications in S. meliloti*

A series of plasmids that carry neomycin or spectinomycin resistance was derived from pCAP77 and pCAP86



**Fig. 5.** Frequency of recombination into *rhaS* as a function of the length of additional *S. meliloti* DNA sequences cloned into the *rha* insertion plasmid.

by including additional genetic components that add versatility to the collection (table 1). Plasmids with strong, constitutive promoters ( $P_{trp}$  and  $P_{nptII}$ ) can be used for complementation, labeling or over expression studies. Certain complementations where strong promoters would result in undesirable overexpression may use plasmids with the promoter region from the *sma0113-sma0114* operon of *S. meliloti*, which proved to be very weak [Garcia, Arango Pinedo and Gage, unpubl.]. Various plasmids with *gfpS65T* or *gfp-mut3b* under the control of strong promoters, as well as the weak  $P_{sma0113}$ , are available. Finally, plasmids with promoterless *gfp-mut3b* downstream of the multiple cloning site (pCAP98 and pCAP100) may prove useful for expression studies as a promoter of interest can be cloned in the MCS. Users can easily modify any of the plasmids for their own applications, for example by swapping *gfp* for *dsred*.

Mutant complementation in *S. meliloti* using the pCAP77-type plasmids by introduction of wild type genes inserted in *rhaS*, either under their native promoters, or under non-native promoters has been reported recently [Pinedo et al., 2008; Pinedo and Gage, 2009]. The

series of suicide plasmids has also been used to study the effects of site-directed mutations, when genes for site-directed mutants of HPr that were unphosphorylatable on the histidine-22 or the serine-53 residues were delivered into the *rha*-site of  $\Delta hpr$  mutant strains, revealing the importance of histidine-22 phosphorylation in SMCR in *S. meliloti* [Pinedo and Gage, 2009].

While it has not been confirmed yet, the plasmids have the potential to be used in other species of bacteria in which the *ColEI* origin of replication is not functional. This can be done by substituting another internal gene fragment for the *rhaS* fragment. *NotI* can be used to excise the *rhaS* fragment from the plasmid, before replacing it with a new internal fragment with *NotI* ends.

Overall, the *rha*-insertion plasmid series constitute a flexible and useful collection of cloning vectors that can be efficiently delivered by conjugation or transformation, and that can be used in genetic and physiology studies of *S. meliloti*, and, with minor modifications, in many other bacterial species.

## Experimental Procedures

### Strains and Media

All *S. meliloti* strains were grown in TY medium or in minimal M9 medium [Sambrook and Russell, 2001]. XL1B MRF' was the host for construction of plasmids, and was grown in LB medium [Sambrook and Russell, 2001]. When minimal medium was used, carbon sources were added to a final concentration of 0.4%, except in succinate plus lactose plates in which concentrations were 0.1 and 0.05% respectively, and for diauxic curves, where concentrations were 0.05% for succinate and 0.1% for the secondary carbon source. The following antibiotics were added as needed: streptomycin (500  $\mu\text{g/ml}$ ), spectinomycin (200  $\mu\text{g/ml}$ ), neomycin (200  $\mu\text{g/ml}$  for screening, 100  $\mu\text{g/ml}$  for maintenance of strains), kanamycin (25  $\mu\text{g/ml}$ ), ampicillin (100  $\mu\text{g/ml}$ ). X-Gal was added to succinate plus lactose plates as an indicator of  $\beta$ -galactosidase activity at 40  $\mu\text{g/ml}$  (SLX plates). All strains used in this study are described in table 1.

### Construction of the Base Delivery Vectors pCAP77 and pCAP86

The series of *rha*-insertion plasmids was based on suicide plasmids pMB438 and pMB439. A *trp* terminator to block transcription from the rhamnose operon promoter was constructed by annealing oligos 280 and 281. After annealing, the terminator, which had *XbaI*-compatible overhangs, was cloned into the *XbaI* site of pMB438, creating pCAP75. A 465-bp internal fragment of *rhaS*(*smc02324*) was amplified using primers 408 and 409 (table 1). The fragment was cloned into pGEM-T-Easy, cut out as a *NotI* fragment and cloned into pMB438 creating pCAP76. The same internal fragment was cloned as a *NotI* fragment in plasmid pCAP75, creating plasmid pCAP77. Plasmids pCAP76 and pCAP77 were digested with *SacI* and *KpnI* to excise the *rhaS*-

containing fragments (without and with the *trp* terminator, respectively), which were cloned into pMB438 to create plasmids pCAP85 and pCAP86.

#### Construction of Labeling, Reporter and Reporter-Construction Vectors

Three different promoter regions were cloned into pCAP77 or pCAP86.  $P_{nptII}$  was excised from plasmid pZKH2 and cloned as an *EcoRI* fragment, to give pCAP88 or pCAP91.  $P_{trp}$  was excised from plasmid pTB1G and cloned as a *HindIII* fragment, resulting in pCAP87 or pCAP97.  $P_{sma0113}$  was cut from plasmid pPG60 and cloned as an *EcoRI* fragment, giving pCAP84 or pCAP96. *gfp*-mut3b was excised from plasmid pDG101 as a *SalI-KpnI* fragment and *gfp*S65T was excised from plasmid pTB1G as a *SalI* fragment. *gfp*S65T and *gfp*-mut3b were cloned into  $P_{sma0113}$ ,  $P_{nptII}$  or  $P_{trp}$ -carrying plasmids, resulting in plasmids pCAP89, pCAP90, and pCAP92 (*gfp*S65T, Nm); pCAP93, pCAP94 and pCAP95 (*gfp*-mut3b, Nm); and pCAP127 ( $P_{nptII}::gfp$ -mut3b, Sp).  $P_{melA}::gfp$ S65T was excised from plasmid pCAP11 and cloned as a *Clal-KpnI* fragment into promoterless plasmid pCAP77 to create pCAP99. Plasmids are listed in table 1.

#### Construction of Complementing and Overexpression Vectors

Plasmids for over expression of *lacR*, and complementation of *nodC* and *dctA* were constructed as follows. *nodC* and *lacR*, were amplified from Rm1021 genomic DNA using the Phusion High-Fidelity PCR kit (Finnzymes, Espoo, Finland) using primers 452 and 453 (*nodC*), and 454 and 455 (*lacR*) (table 1), A-tailed and cloned into pGEMT-Easy. Fragments were excised with *Clal-KpnI* or *XhoI-KpnI* and cloned into pCAP87 (*lacR*) or pCAP97 (*nodC*), creating plasmids pCAP106 ( $P_{trp}::lacR$ ) and pCAP107( $P_{trp}::nodC$ ). The complementing plasmid for *dctA* was constructed in a similar way, except that the amplified fragment, generated using primers 456 and 457, included the promoter region of *dctA*. The fragment was cloned into plasmid pCAP77 as a *XhoI-KpnI* fragment, creating plasmid pCAP108( $P_{dctA}::dctA$ ).

#### Plasmid Delivery

Plasmids were delivered by electroporation or by triple mating. Electroporations were conducted using an Ecoli Pulser (Bio-Rad, Hercules, Calif., USA). Electrocompetent *S. meliloti* were prepared based on a standard procedure. Electrocompetent cells were prepared in small batches (25 ml starting culture volume) or large batches (500 ml starting volume), by sequentially rinsing the cells in 1/2, 1/4, 1/10, 1/50 and 1/100 volumes of an ice-cold 15% glycerol solution and resuspended in the same solution to an  $OD_{595}$  of 1.0, then frozen at  $-80^{\circ}\text{C}$  in a modification of the protocol by Miller [1988]. Conditions for electroporation were: 40  $\mu\text{l}$  of cell suspension, 10–500 ng of DNA, and 23 kV/cm in 1-mm gap cuvettes. Following electroporation, 1 ml of SOC medium was added to the cell suspension, incubated at  $30^{\circ}\text{C}$  for 4 h and then plated on selective medium.

Triple matings used pRK600 as the helper plasmid. Strains were either thickly streaked together on a TY plate, or grown in appropriate broth, rinsed, pelleted, and the pellets mixed and spotted on a TY plate. After a 1-day incubation, the mating mixes were scraped, resuspended in M9 broth without carbon source, and spread on plates selective for transconjugants.

#### Screening for rhaS Insertions

To identify insertions into *rhaS*, colonies from selective plates (either from matings or electroporations) were patched on M9-rhamnose plates, and also on TY plates with selective antibiotics. After incubation, isolates that showed reduced growth on rhamnose were chosen. Insertion into *rhaS* was confirmed by PCR screening using primer 419 together with a downstream primer specific to the inserted DNA (fig. 1). Amplification of a product of the predicted size was an indication of insertion into *rhaS*. Further confirmation was obtained by using primers 419–420, which yielded a 760-bp product only if *rhaS* was intact. The PCR conditions were: 1 min of denaturation at  $96^{\circ}\text{C}$ , followed by 30 s at  $95^{\circ}\text{C}$ , annealing for 30 s at  $64^{\circ}\text{C}$ , and extension for 40 s at  $72^{\circ}\text{C}$  for 30 cycles, and a final extension of 5 min at  $72^{\circ}\text{C}$ .

#### Nodulation Assays

Nodulation was evaluated as described previously [Pinedo and Gage, 2009]. Rm1021 and derived strains were inoculated in tubes containing young alfalfa seedlings (2–3 days of sprouting) and incubated in a growth chamber for 4 weeks. Nodules were counted weekly.

#### Catabolite Repression Assays

Succinate-mediated catabolite repression was assessed as described [Pinedo et al., 2008; Pinedo and Gage, 2009]. Briefly, SLX plates were used for qualitative evaluation, while diauxic growth curves in an automatic plate reader Synergy HT (BioTek, Instruments, Inc., Winooski, Vt., USA) at  $OD_{595}$  were used for quantitative evaluation. When a  $P_{melA}::gfp$ S56T reporter construct was used, fluorescence was measured at 528/20 with excitation at 480/20, in the automatic plate reader in conjunction with cell density.

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