

The expression of a novel antisense gene mediates incompatibility within the large *repABC* family of α -proteobacterial plasmids

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Summary

Large extrachromosomal replicons in many members of the α -proteobacteria encode genes that are required for plant or animal pathogenesis or symbiosis. Most of these replicons encode *repABC* genes that control their replication and faithful segregation during cell division. In addition to its chromosome, the plant endosymbiont *Sinorhizobium meliloti* also maintains the 1.4 Mb pSymA and 1.7 Mb pSymB symbiotic megaplasmids both of which are *repABC*-type replicons. In all *repABC* loci that have been characterized, an apparently untranslated intergenic region between the *repB* and *repC* genes encodes a strong incompatibility determinant (referred to as *inca*). Here we report the isolation of mutations within the *inca* regions of pSymA and pSymB that eliminate incompatibility. These mutations map to and inactivate a promoter in the intergenic region that drives the expression of an approximately 56 nucleotide untranslated RNA molecule that mediates incompatibility. This gene, that we have named *incaA*, is transcribed antisense to the *repABC* genes. Our analysis suggests that the *incaA* gene is conserved in *repABC* loci from a diverse spectrum of bacteria.

Introduction

repABC loci control the replication, segregation and copy number of many plasmids and second chromosomes in the alpha subgroup of proteobacteria. The *repA* and *repB* genes encode homologues of the ParAB family of replicon partitioning proteins (Bignell and Thomas, 2001) and *repC* is believed to encode the replication initiation (replicase) protein because its expression is sufficient and required

for autonomous replication of *repABC* basic replicons (Bartosik *et al.*, 1998; Ramirez-Romero *et al.*, 2001). These basic replicons have generated significant interest for three main reasons: (i) their distribution within the alpha subgroup of proteobacteria is widespread where they control the maintenance in cell populations of important extrachromosomal elements such as the symbiotic megaplasmids in nitrogen-fixing plant endosymbionts (Galibert *et al.*, 2001), the tumour-inducing plasmids in *Agrobacterium* spp. (Goodner *et al.*, 2001), and the second chromosomes in the animal pathogen, *Brucella* (Paulsen *et al.*, 2002); (ii) at least in some cases *repABC* expression, and therefore plasmid copy number control, is subject to a TraR-mediated quorum sensing system (Pappas and Winans, 2003) and (iii) *repABC* genes are unusual amongst plasmid partitioning/replication systems because the partitioning cassette (*repAB*) and replication cassette (*repC*) are encoded in a direct series raising the possibility that the locus is encoded as a single transcriptional unit (Ramirez-Romero *et al.*, 2000) and therefore may have arisen from an operonic fusion event between plasmid *par* and *rep* genetic loci that in other plasmids are encoded as separate transcriptional units.

In every *repABC* replicon that has been genetically characterized a non-translated intergenic region between the *repB* and *repC* gene encodes a strong incompatibility determinant called *inca* (Ramirez-Romero *et al.*, 2000). However, the molecular basis of this incompatibility has never been elucidated. Here, we show that the analogous regions from pSymA and pSymB, the *repABC*-type symbiotic megaplasmids of *Sinorhizobium meliloti*, mediate incompatibility in a parental replicon-specific manner. We also report the isolation of point mutations in the pSymB *inca* region that eliminate incompatibility and show that they map to, and inactivate, a promoter upstream of and antisense to *repC*. This promoter is responsible for the expression of a novel gene that we have named *incaA*. We further show that *incaA* encodes a small RNA that is transcribed in an orientation opposite to the transcription of the *repABC* operon. Through the convergence of various genetic and biochemical data we show that its expression mediates the strong incompatibility that is characteristic of the *repB*–*repC* intergenic region in pSymB. Our data suggest that this gene is not unique to pSymB but probably is ubiquitously encoded

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within *repABC* operons from otherwise unrelated DNA replicons.

Results

repB–repC intergenic regions mediate incompatibility

In every case where it has been examined, the untranslated intergenic region between the *repB* and *repC* genes in *repABC* replicons (see Fig. 1) when isolated upon a broad-host range (bhr) plasmid mediates incompatibility against the replicon from which it was derived. When we subcloned the intergenic regions from either pSymB (*inca1*) or pSymA (*inca2*) into the bhr plasmid pBBR1MCS-3 (generating plasmids pTH1505 and pTH1491, respectively), we found that neither construct could be conjugatively transferred into *recA*-deficient *S. meliloti* cells. In contrast, both constructs could be transferred and maintained in *Agrobacterium tumefaciens* cells. We inferred that replicon-specific incompatibility was responsible for this phenotype in *S. meliloti* and that either our test plasmids could not supplant the symbiotic megaplasmids from the *S. meliloti* cell or that incompatibility-mediated megaplasmid supplantation is lethal. To formally test whether this incompatibility effect was replicon specific, we conjugatively transferred test plasmids carrying either *inca1* (from pSymB) and *inca2* (from pSymA) into *recA* *S. meliloti* (strain Rm5004), *A. tumefaciens* (strain At9023), and strain At9023 already possessing either pSymA (strain At128) or pSymB (strain At125). The results in Table 3 indicate that the pSymA *inca2* test plasmid cannot be maintained in Rm5004 or in strain At128 (already containing pSymA) but can be maintained in the parental *A. tumefaciens* strain At9023 and a derivative already containing pSymB (At125). Likewise, plasmid pTH1505 (the pSymB *inca1* test plasmid) cannot be maintained in cells that already possess pSymB but replicate quite normally in cells devoid of pSymB. Thus, in accord with previously published accounts of the behaviour of the *repB–repC* intergenic region in *repABC* replicons (Turner and Young, 1995; Bartosik *et al.*, 1998; Ramirez-Romero

et al., 2000), our data show that the analogous regions from both pSymA and pSymB mediate incompatibility in a replicon-specific manner, as would be expected for a genuine incompatibility phenomenon. Because all of our plasmids replicate as expected in *A. tumefaciens* strain At9023 and because *A. tumefaciens* and *S. meliloti* are quite closely related, we utilize strain At9023 as a surrogate host species for experiments where incompatibility precludes us from using *S. meliloti* itself.

Identification of point mutations that eliminate incompatibility

The genetic organization of a *repABC* locus is indicated in Fig. 1. In an effort to begin to define the molecular basis of incompatibility that is displayed by the *repB–repC* intergenic (*inca1*) region of pSymB and other *repABC* replicons, we generated mutations in *inca1* using either a mutD strain of *Escherichia coli* or by error-prone polymerase chain reaction (PCR). A bhr plasmid such as pOT1 will generally conjugate into *S. meliloti* with a frequency of 10^{-3} – 10^{-1} . When pOT1 contains the wild type 244 bp *inca1* sequence, however, no transconjugant colonies arise because of the incompatibility effect. Therefore, conjugation into *S. meliloti* provides a powerful selection mechanism for identifying mutant plasmids that have lost incompatibility as a result of PCR- or mutator strain-generated mutations. Using this strategy, we isolated a number of incompatibility mutants, the majority of which possessed single-point mutations. A description of these mutations and their location are documented in Table 1 and mapped onto the nucleotide sequence in Fig. 4C. A multiple alignment of *inca* regions (see Fig. 4C for a partial alignment) revealed that the mutated nucleotides that eliminate incompatibility are highly conserved amongst loci from several *repABC*-type plasmids including pSymA from *S. meliloti*, the linear chromosome, pTiC58, and pATC58 from *A. tumefaciens*, and p42d from *Rhizobium etli*. All of these mutations have been isolated in *inca1* from pSymB but when the single-point mutation in mutant E9 (a T to C transition) was imparted into the pSymA *inca* region (*inca2*) by site-directed mutagenesis, it also eliminated the incompatibility normally displayed from this sequence (not shown). These data and the nucleotide sequence conservation within these discrete regions lead us to believe that the mutations we have identified would have similar consequences in other *repABC* replicons.

repB–repC intergenic regions encode a promoter

As a prelude to isolating mutations that eliminate incompatibility in *inca1*, we cloned a 244 bp PCR amplified DNA fragment encompassing the *repB–repC* intergenic region

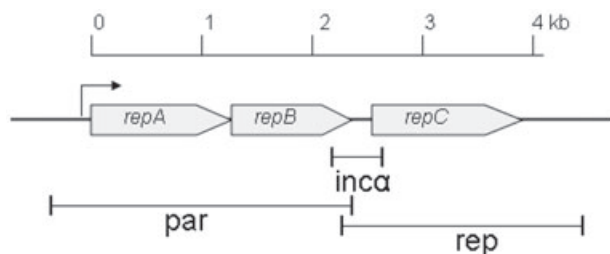


Fig. 1. Schematic depiction of general genetic organization of *repABC* loci. Functional designations indicating partitioning (par), replication (rep), and incompatibility (inc) from published data and from our own unpublished observations. Scale is approximate.

Table 1. Bacterial strains and plasmids used in study. Nucleotide positions are from GenBank Accession Number NC_003078.

Strain or plasmid	Characteristic or genotype	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Strain collection
LE30	mutD (mutator strain)	Strain collection
<i>S. meliloti</i>		
Rm5004	Rm1021 (<i>recA::Tn5</i>) (Sm ^r Nm ^r)	Strain collection
<i>A. tumefaciens</i>		
C58	wt	Gift from C. Baron (McMaster University)
At9023	pATC58 deletion derivative of strain C58 (Rif ^r Sm ^r)	Strain collection
At125	At9023 (pSymB) (Rif ^r Sm ^r Nm ^r)	Finan <i>et al.</i> (1986)
At128	At9023 (pSymA) (Rif ^r Sm ^r Gm ^r Sp ^r)	Finan <i>et al.</i> (1986)
Plasmids		
pUCP30T	Suicide vector (Gm ^r)	Schweizer (2001)
pBBR1MCS-5	Broad-host range vector (bhr) (Gm ^r)	Kovach <i>et al.</i> (1995)
pBBR1MCS-3	bhr vector (Tc ^r)	Kovach <i>et al.</i> (1995)
pOT1	bhr <i>gfpUV</i> transcriptional reporter plasmid (Gm ^r)	Finan <i>et al.</i> (1986)
pTH883	242 bp PCR product (AB25808-AB25810) encompassing <i>inca1</i> region cloned into pBBR1MCS-5	This study
pTH1015	4.9 kb PCR product (AB27695/AB24732) encompassing entire pSymB <i>repABC</i> region cloned into pUCP30T	This study
pTH1414	244 bp PCR product (ML1564/ML1565) encompassing pSymB <i>inca1</i> region in pOT1 (reverse orientation to <i>repABC</i>)	This study
pTH1415	244 bp PCR product (ML1562/ML1563) encompassing pSymB <i>inca1</i> region in pOT1 (same orientation as <i>repABC</i>)	This study
pTH1491	269 bp PCR product (AB29541-AB29542) encompassing pSymA <i>inca2</i> region cloned into pBBR1MCS-3	This study
pTH1496	258 bp PCR product (ML3099/ML3100) encompassing pSymA <i>inca2</i> region in pOT1 (reverse orientation to <i>repABC</i>)	This study
pTH1497	260 bp PCR product (ML3097/ML3098) encompassing pTiC58 <i>inca</i> region in pOT1 (reverse orientation to <i>repABC</i>)	This study
pTH1505	same insert as pTH883 cloned using flanking XbaI and XhoI sites in pBBR1MCS-5 into pBBR1MCS-3	This study
pTH1530	Site-directed T to C mutation (ML3787/ML3788) (same mutation as E9 isolate) in pTH1496	This study
pTH1560	162 bp PCR product (ML1565/ML4487) encompassing <i>incA</i> promoter and ending 6 nt before T-rich motif	This study
pTH1561	199 bp PCR product (ML1565/ML4488) encompassing <i>incA</i> promoter and ending 21 nt after T-rich motif	This study
E2	Error-prone PCR generated <i>inc</i> mutant of pTH1414 (multiple mutations)	This study
E3	Error-prone PCR generated <i>inc</i> mutant of pTH1414 (multiple mutations)	This study
E9	Error-prone PCR generated <i>inc</i> mutant of pTH1414 (T to C transition at nt position 56459)	This study
E11	<i>E. coli</i> mutator strain (LE30) generated <i>inc</i> mutant of pTH1414 (G deletion at nt position 56482)	This study
E12	<i>E. coli</i> mutator strain (LE30) generated <i>inc</i> mutant of pTH1414 (T to C transition at nt position 56487)	This study
E14	<i>E. coli</i> mutator strain (LE30) generated <i>inc</i> mutant of pTH1414 (A to G transition at nt position 56483)	This study
E15	<i>E. coli</i> mutator strain (LE30) generated <i>inc</i> mutant of pTH1414 (T to C transition at nt position 56460)	This study
E16	<i>E. coli</i> mutator strain (LE30) generated <i>inc</i> mutant of pTH1414 (G to A transition at nt position 56461)	This study
E17	<i>E. coli</i> mutator strain (LE30) generated <i>inc</i> mutant of pTH1414 (G insertion after nt position 56475)	This study

wt, wild type.

from pSymB into the *gfpUV* transcriptional reporter vector, pOT1 (Allaway *et al.*, 2001). We chose pOT1 to use as a general cloning vector for our mutational analysis primarily because it lacks the endogenous promoter activity reading through the polylinker that typifies many other bhr cloning vectors and that might influence our mutational analysis. In addition, it would allow us to detect promoter activity within the *inca* sequence, a region not previously known

to exhibit such activity. We found that incompatibility was displayed by the *inca* region regardless of its orientation in pOT1 and the plasmid therefore could not be maintained in *S. meliloti* (see *Experimental procedures*) but would replicate as expected in our surrogate host species, *A. tumefaciens* strain At9023. When colonies of At9023 possessing the plasmids were viewed over a UV transilluminator we also found that apparent promoter activity

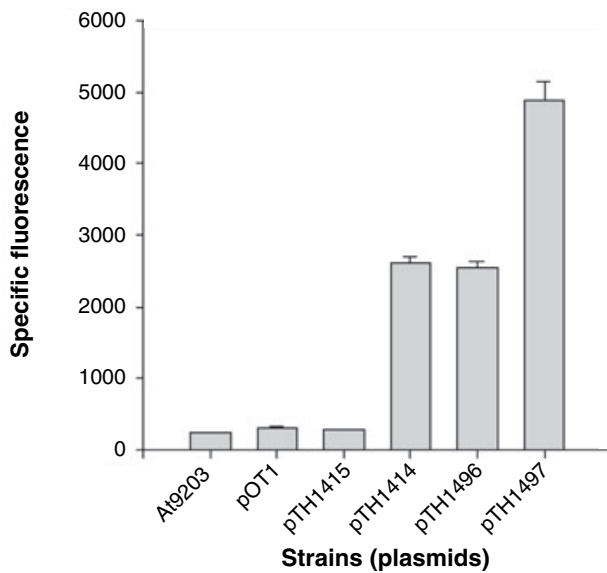


Fig. 2. Quantification of promoter activity emanating from *inc α* sequences estimated from specific GfpUV fluorescence. Plasmids pTH1415 and pTH1414 contain the pSymB *inc α 1* subsequence in the forward (relative to *repABC* transcription) and reverse orientations, respectively. Plasmids pTH1496 and pTH1497 contain the analogous subsequences cloned in the reverse orientation from pSymA and *A. tumefaciens* pTiC58, respectively.

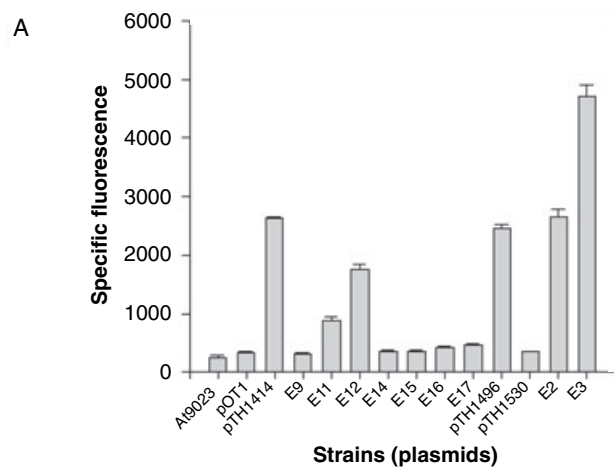
(i.e. fluorescence) was exhibited by the fragment when cloned into pOT1 in the reverse orientation relative to *repABC* transcription, but not when cloned in the same orientation as *repABC* transcription (relative to the promoterless *gfpUV* gene) (not shown). This apparent promoter activity was also displayed in *E. coli* indicating that this putative *S. meliloti* promoter is recognized in *E. coli*. In order to quantify this promoter activity in the *A. tumefaciens* background, we measured specific fluorescence in liquid culture (Fig. 2). When the *inc α 1* region is cloned into pOT1 in the same orientation as *repABC* transcription (plasmid pTH1415), low specific fluorescence similar in magnitude to that from At9203 cells containing only pOT1, was exhibited. However, the fragment cloned in the opposite orientation (plasmid pTH1414), exhibited over eight-fold higher activity.

In order to ascertain whether this putative promoter activity was peculiar to pSymB or, alternatively, whether it might be a feature of *inc α* regions in general, we PCR amplified DNA from the analogous regions of the *S. meliloti* pSymA megaplasmid and the tumour-inducing plasmid of *A. tumefaciens*, pTiC58, and cloned them into pOT1 in an orientation opposite to their respective *repABC* genes. In both cases, fluorescence was again detected in *A. tumefaciens* host cells [Fig. 2, plasmids pTH1496 (pSymA) and pTH1497 (pTiC58)] and in *E. coli* (not shown). Perhaps because we are using *A. tumefaciens* host cells, the *A. tumefaciens* (pTiC58) *inc α* region

promoter exhibited nearly twice the activity of those from either pSymB or pSymA. Based on these data, we had tentatively identified a previously undetected promoter that is active in a direction that is antisense with respect to *repABC* transcription and is present in three different RepABC replicons from two different species.

Mutations that eliminate incompatibility map to inc α antisense promoter

In order to isolate the *inc* mutants that are described in Table 1 and Fig. 3B, we had cloned the pSymB *inc α* region into pOT1 in an orientation that was antisense with respect to *repABC* transcription. Thus, our detection of apparent promoter activity was more or less coincident with our attempts to isolate mutations that eliminated



B

mutant	genotype	phenotype (inc/promoter activity)
<i>Class I</i>		
E9	T→C	-/-
E11	G deletion	-/partial
E12	T→C	-/partial
E14	A→G	-/-
E15	T→C	-/-
E16	G→A	-/-
E17	G Insertion	-/-
<i>Class II</i>		
E2	multiple	-/+
E3	multiple	-/+

Fig. 3. A. Promoter activities from wild type and mutant (compatible) *inc α* sequences in *A. tumefaciens* cells. B. Genotypes and phenotypes of incompatibility mutants (also see text and in Table 1).

incompatibility. We noticed that, with only extremely rare exceptions (see later) all *inc* mutations that we obtained had also lost promoter activity, as indicated by the loss of *gfpUV* fluorescence exhibited by the colonies when viewed over a UV transilluminator. Most of the *inc* mutants indicated in Table 1 possess single-point mutations and we now define this type of mutation as Class I. Thus, with rare exception, single-point mutations eliminated both incompatibility and the promoter activity endogenous to *inca1*. We quantified the effects of the point mutations that eliminate incompatibility on the promoter activity exhibited by *inca1* (Fig. 3A) in *A. tumefaciens* cells. In contrast to the wild type sequence (plasmid pTH1414), most of the *inc* mutant sequences possess only basal activity (see mutants E9, E14, E15, E16 and E17). Interestingly, two mutants (E11 and E12) possess lower but still significant activities.

We reported above that when the T to C transition mutation in mutant E9 (Table 1) was imparted into the pSymA *inca2* region, incompatibility was eliminated. To assess the effect of this mutation on the pSymA *inc* region, we measured the wild type *inca2* promoter activity (plasmid pTH1496) and that of its mutant derivative (plasmid pTH1530). As is the case for mutant E9, this mutation also eliminated the endogenous promoter activity from pSymA *inca2*. A simple model to explain these results is that point mutations that eliminate incompatibility do so by inactivating a promoter that is required for the expression of a product that mediates incompatibility. Thus, Class I mutations result in impaired promoter function. Although all of the mutants appeared fluorescence-negative on agar plates, it is clear that some of the mutants retain some degree of promoter activity (e.g. mutants E11 and E12) but apparently not enough to result in incompatibility because our selection method depends upon the loss of incompatibility.

With an extremely low frequency (about 1/10 000 *inc* mutant colonies) we also isolated a second class of *inc* mutant (Class II) that still displayed obvious promoter activity in our qualitative plate-based screen and in our quantitative fluorescence assay (Fig. 3A, see Class II mutants E2 and E3). One mutant (mutant E3) has almost twice the activity of wild type in the *A. tumefaciens* background and may therefore possess a promoter-up mutation. When sequenced, these Class II mutants (all of which arose from using error-prone PCR mutagenesis) possessed multiple-point mutations (mutant E2 possesses two-point deletions and one transition substitution and mutant E3 possesses one-point deletion and three transition substitutions) none of which mapped to the regions in which the point mutations that eliminate both promoter activity and incompatibility occur (i.e. Class I mutations). To reconcile these observations with the model stated above, we hypothesize that this much rarer

class of mutants may possess mutations that attenuate the function of an expressed product or factor that mediates incompatibility, rather than eliminating its expression in the first place. This mutant class may be detected with very low frequency because the phenotype requires not only multiple mutations, but also that these mutations occur simultaneously within defined and distinct areas of the expressed incompatibility factor so as to knock out its function and therefore its incompatibility.

In order to establish that the promoter we had identified in *inca1* was genuine and to define a transcriptional start site for the expressed RNA transcript, we performed primer extension on RNA extracted from our *recA* derivative of *S. meliloti* 1021 (Rm5004), Rm5004 carrying pOT1, and from Rm5004 cells maintaining a Class 1 mutant (mutant E9) and the Class 2 mutant that displayed wild type levels of promoter activity (mutant E2). Figure 4A shows the results of these primer extension experiments using the primer ML3826 (described in Table 2). For *S. meliloti* strain Rm5004, strain Rm5004 carrying empty pOT1, and the Class 1 mutant plasmid E9, one major and one minor extension product is visible and the amount of product is similar in all three cases (Fig. 4A, lanes 1–3). We propose that the amount of extension product accumulated reflects the basal wild type amounts of target RNA in the cell and that this amount is not influenced by the presence of empty pOT1 nor by the presence of the transcriptionally silent *inca* DNA of mutant E9. In the cases of the Class 2 mutant, a much greater amount of extension product accumulated with a slightly different pattern for the major and minor transcriptional start sites for an RNA transcript. Of the multiple-point mutations possessed by mutant E2, a point deletion occurs adjacent to the –10 region and it may be this mutation that alters the start site choice in mutant E2 because the deletion decreases the distance between the –10 promoter and transcriptional start sites. For mutant E2, we propose that the mutationally inactivated target RNA transcript is over-expressed (relative to wild type) because the functional *inca* promoter is carried upon the multicopy pOT1 plasmid. Thus, the amount of extension product generated from mutant E2 RNA results from the background levels of specific transcript plus the inactive transcript expressed from the multicopy plasmid. We are currently assessing the consequences of the individual mutations in the Class II mutants particularly with regards to their ability to (over)express an inactive incompatibility factor. Because a clear and major transcriptional start site was indicated by the reaction with Rm5004 RNA, we report this as the most likely *in vivo* transcriptional start site. In Fig. 4C, we indicate this major start site (arrow) and also the weaker secondary start site on the nucleotide sequence (oriented 5' to 3') that would correspond to the bottom strand of double-stranded *repABC* DNA if indicating the *repABC*

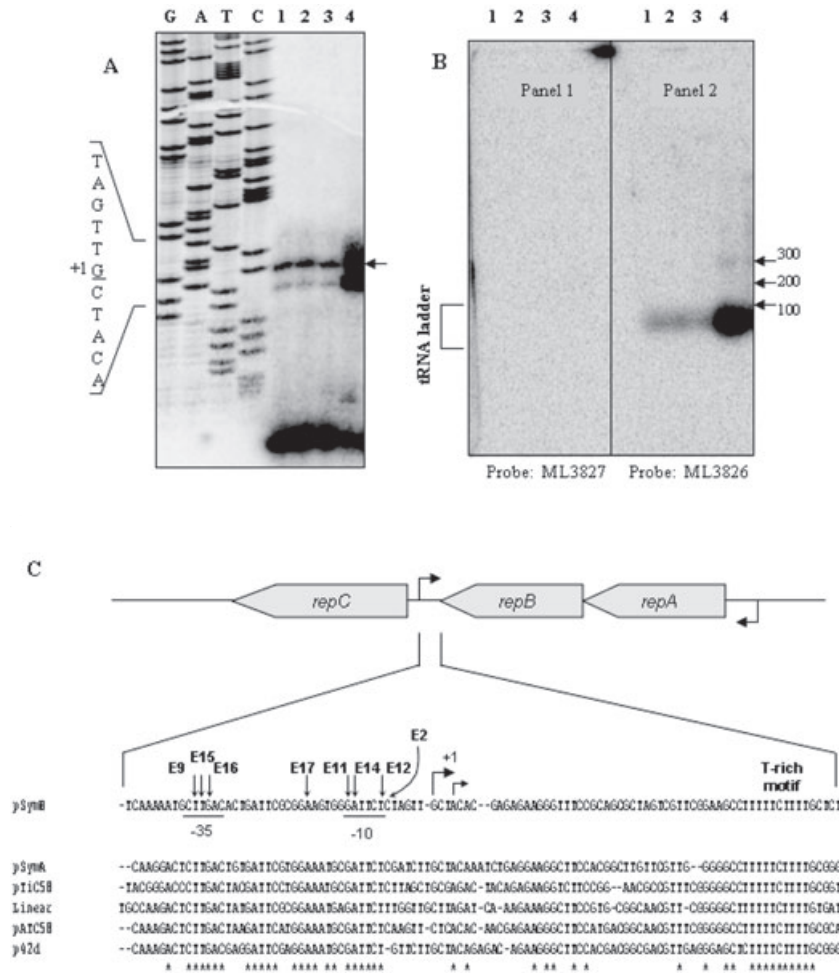


Fig. 4. Detection of a novel transcript expressed from the pSymB *incα1* region. **A.** Primer extension was conducted on total RNA (40 µg per reaction) extracted from the following strains: lane 1, *S. meliloti* strain Rm5004; lane 2, Rm5004 carrying pOT1; lane 3, Rm5004 carrying the E9 class I mutant plasmid; and lane 4, Rm5004 carrying the E2 class II mutant plasmid. The major product in lane 1 (see arrow) was used to infer the transcriptional start site of the transcript. **B.** Northern hybridization using total RNA (20 µg each lane) extracted from strains as indicated for lanes 1–4 of (A). Panel 2 membrane was probed with same primer used for primer extension analysis (ML3826) after end-labelling with ³²P. Panel 1 was probed with a labelled oligonucleotide (ML3827) that is exactly complementary to ML3826. The approximate positions of bands from the commercial RNA ladder employed and the *S. meliloti* tRNA ladder visible after ethidium bromide staining are indicated for size estimation of the bound probe. tRNAs in *S. meliloti* range from about 40–90 nucleotides in length. **C.** Schematic depiction of *repABC* locus in reverse orientation and partial nucleotide sequence of pSymB *incα1* sequence in alignment (ClustalW) with analogous regions from *S. meliloti* pSymA, *A. tumefaciens* pTiC58, *A. tumefaciens* linear chromosome, *A. tumefaciens* pAtC58, and p42d from *R. etli*. Based on primer extension analysis, the major transcriptional start site (large angled arrow) and the minor transcriptional start site (small angled arrow) and inferred –10 and –35 promoter regions are indicated as are the class I mutations (vertical arrows) described in Table 1, Fig. 3B and in the text that eliminate or reduce promoter activity. Also indicated is the point deletion (nucleotide C adjacent to –10) in mutant E2 that likely alters the transcriptional start site from this test plasmid (see text). The conserved ‘T-rich’ motif that likely constitutes the end of the *incA* transcript and other nucleotides that are conserved are indicated by asterisks below the alignment. pSymB sequence depicted starts 48 nucleotides from *repC* start codon and ends two nucleotides before *repB* stop codon.

genes in the normal reading fashion from left to right. Also in Fig. 4C, we show the location of Class 1 mutations that eliminate both incompatibility and apparent promoter activity. Strikingly, six of the seven Class I mutations fall in nucleotide regions that correspond nicely to the –10 and –35 region nucleotides upstream of the experimentally deduced transcriptional start site and that are strongly conserved in the analogous sequences from

pSymA, three different *A. tumefaciens* replicon sequences, and from the *R. etli* plasmid, p42d (Fig. 4C). While E17 is the lone example of a mutation that eliminates both incompatibility and promoter activity but which falls outside of the –10 or –35 regions, this single nucleotide insertion mutation increases the spacing between the –10 and –35 regions and reduces promoter activity to near basal levels (Fig. 4C, Table 3). On the basis of our

Table 2. Oligonucleotides used in the study.

Oligonucleotide used	Sequence
Site-directed mutagenesis	
ML3787	CGGTGCCAAGGACTCCTGACTGTGATTCGTG
ML3788	CACGAATCACAGTCAGGAGTCCTTGGCACCG
Primer extension/northern hybridization	
ML3826	GGCTTCCGAACGACTAGCGCTGCGG
ML3827	CCGACGCGCTAGTCGTTCCGGAAGCC
PCR	
AB25808	AAGAATTCGGTGAGGCAGAGGCTCGAAGC
AB25810	CGTGGATCCAAAGGGCGTCGTCACACTTCC
AB27695	AGAGGATCCTAGCCGAGGGTCATTCCGCC
AB24732	ATCGGATCCGCGGCGTTTCGTTCTGCATTTCCGCC
AB29541	CCGAATTCTCGAAAGCCTGCACGACC
AB29542	AGAGGATCCACAGGGCAAGCGTCATCG
ML1562	GCGAAGCTTCGTGAGGCAGAGGCTCGAAGC
ML1563	CGTCTAGAAAAGGGCGTCGTCACACTTCC
ML1564	CGGTCTAGACGTGAGGCAGAGGCTCGAAGC
ML1565	GCGAAGCTTAAAGGGCGTCGTCACACTTCC
ML3097	CGGTCTAGAGCTCCGCTCGACTGAGATGG
ML3098	GCGAAGCTTCGTTGAGATATGCGTCTGC
ML3099	CGGTCTAGACTGCACGACCAGTTCATGC
ML3100	GCGAAGCTTGGGCAAGCGTCATCGACC
ML4487	CGCTCTAGACGAACGACTAGCGCTGCGG
ML4488	CGCTCTAGAAAACAGGAGACTAACAGAGC

primer extension analysis, and the genotypic and phenotypic results of our mutational analysis of incompatibility and promoter activity, we conclude that the pSymB *inca* region encodes a genuine promoter that is required for the expression of an RNA product that either directly or indirectly mediates incompatibility. Class I mutations eliminate incompatibility by eliminating or reducing the expression of a gene required for incompatibility. It is worthwhile to note that (with the exception of mutant E17) we have not obtained dual inc/promoter mutants that lack mutations in either the -10 or -35 nucleotide regions. Class II mutations do not eliminate promoter activity, but may eliminate incompatibility by inactivating the incompatibility factor itself.

In order to establish an approximate size for the inc transcript, we performed Northern hybridization on the same RNA samples used for primer extension (Fig. 4A) after their separation through a formaldehyde-agarose gel (Fig. 4B). Our end-labelled probe (the same primer used

for primer extension) hybridized with a transcript that was smaller than the 100 nucleotide RNA marker we employed (panel 2). When we used an oligonucleotide probe (ML3827) from the opposite strand (Fig. 4B, panel 1), no signal was generated indicating the strand specificity of the detected RNA product. Theoretically, probe ML3827 (panel 1) could anneal to a mRNA produced from the *repABC* locus itself, however, the separation conditions were designed to detect a small RNA molecule and we assume that RNA greater than 3000 nucleotides would not appreciably enter the gel.

The bands visible in panel 2 comigrated with the tRNA ladder that was visible after separation on the 2% agarose gel followed by ethidium bromide staining. Based on the range of sizes of tRNA molecules in *S. meliloti*, we estimated the approximate size of the RNA transcript to be about 50–70 nucleotides in length. Consistent with the primer extension analysis, the transcript was expressed at basal levels in *S. meliloti* strain Rm5004, and this strain

Table 3. Replicon specificity of incompatibility phenotypes displayed by pSymB *inca1* and pSymA *inca2* sequences.

Test plasmid ^a	Conjugation frequency ^b using following recipient strains			
	Rm5004	At9023	At128 (pSymA)	At125 (pSymB)
pBBR1MCS-3	10 ⁻²	10 ⁻³	10 ⁻³	10 ⁻³
<i>inca2</i> (from pSymA) (pTH1491)	<10 ⁻⁸	10 ⁻³	10 ⁻⁸	10 ⁻³
<i>inca1</i> (from pSymB) (pTH1505)	<10 ⁻⁸	10 ⁻³	10 ⁻³	10 ⁻⁸

Test plasmids (the broad-host range plasmid pBBR1MCS-3 with and without the *inca* sequences from pSymA and pSymB) were transferred into *S. meliloti* strain Rm5004, *A. tumefaciens* strain At9023 and this latter strain carrying either pSymA or pSymB.

a. Test plasmids are either the bhr pBBR1MCS-3 or with indicated inc sequences cloned into its multiple cloning site.

b. Conjugation frequency is reported as number of transconjugant colony forming units per recipient colony forming unit. The data in this table are rounded values from one experiment and are representative and typical of the results of at least three experiments.

carrying either pOT1 or the E9 mutant plasmid while it was overexpressed relative to wild type in the Class II mutant (mutant E2) – a consequence of multicopy representation in the cell.

On the basis of our genetic analysis, and primer extension and Northern hybridization experiments, we propose the existence of a previously undetected gene encoded in antisense to the *repABC* genes between the *repB* and *repC* genes. The expression of this gene mediates the incompatibility characteristic of the *incA* regions from pSymB and many other *repABC* replicons. We have named this gene *incA* (for *intergenic nucleotide* sequence that influences plasmid *compatibility gene A*).

Features of a novel gene, *incA*, required for incompatibility

We have identified a novel antisense gene that we have named *incA* and that encodes a product approximately 50–70 nt in length. A multiple alignment of the pSymB *incA* gene with the analogous regions from other *repABC* replicons (Fig. 4C) reveals that centred approximately 51 nucleotides downstream of the transcriptional start site of *incA* an unusual motif (TTTTCTTTTG) is conserved in all of the replicons. This region of low complexity is reminiscent of the T-rich region that follows a stem-loop structure in rho-independent transcriptional terminator signals. In pSymB, no obvious stem-loop precedes the T-rich motif but an inspection of some of the other sequences in the alignment reveals that some do encode putative stem-loop generating sequences. In order to ascertain whether the T-rich motif on pSymB encodes a transcriptional terminator, we cloned DNA fragments encompassing the *incA* gene but ending either just before (pTH1560) or just after (pTH1561) the putative terminator sequence into the reporter vector pOT1 (Fig. 5A) and compared their activities with the original 244 bp fragment from which we originally detected promoter activity (pTH1414). As indicated in Fig. 5B, the activity of pTH1414 (that ends about 70 bp downstream of the T-rich motif) is indistinguishable from the activity of pTH1561 (that ends about 20 bp downstream of motif). However, these activities are about 10% that of pTH1560 (that ends about 20 bp upstream of the T-rich motif). Thus, the intervening sequence that encompasses the T-rich motif results in termination with about 90% efficiency. Interestingly, this means that the original promoter activity that we detected (from pTH1414) was because of a small inefficiency in termination (about 10% of the actual promoter activity), but this residual readthrough was fortunately high enough for us to detect above the background fluorescence we routinely encounter. Because the *incA* transcript likely ends somewhere around the end of the T-rich terminator sequence, we report *incA* as having a size of approximately 56 nucleotides.

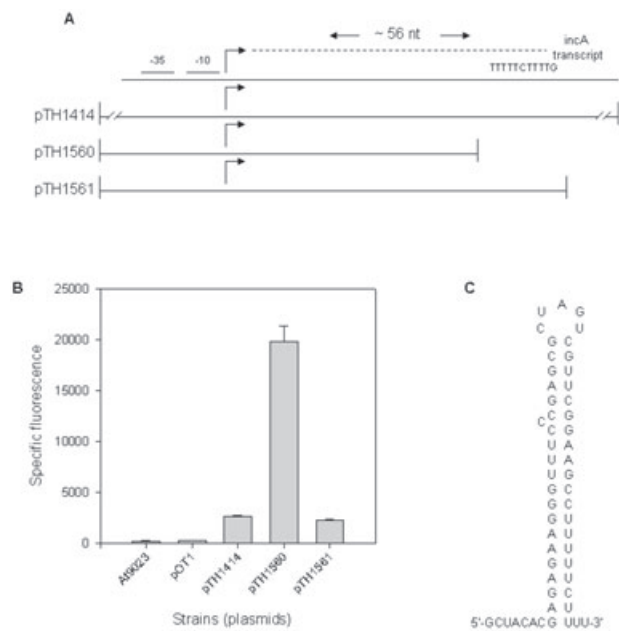


Fig. 5. Identification of a transcription termination signal in *incA1*. A. Schematic depiction of subclones used to deduce the influence of the 'T-rich' motif on transcriptional processivity into the reporter gene, *gfpUV* where the cloned sequences in pTH1414 and pTH1561 end beyond the T-rich motif while the sequence in pTH1560 ends before the motif. See text for details, not drawn to scale. B. Promoter activity exhibited by subclones depicted in (A). C. Schematic depiction of predicted structure for inferred pSymB *incA* RNA (see text). Redrawn from output of mfold (v.3.1).

Within the 56 nucleotide *incA* gene there are no putative start codons (ATG, TTG, GTG) in frame with putative stop codons and no obvious translation initiation signals are evident in the sequence. On this basis and from the fact that many plasmids encode incompatibility factors that are untranslated regulatory RNAs (see *Discussion*) we suspect that *incA* encodes a small untranslated RNA. Small RNAs in bacteria often adopt secondary structures consisting of double-stranded stem structures and one or more single stranded loops that play integral roles in mediating interactions with other single stranded regions often in target mRNA molecules. These single stranded loops often include a so-called U-turn motif (YUNR) (Franch *et al.*, 1999) that is thought to facilitate base pairing between cognate RNAs. We applied an RNA folding program (mfold v.3.1) (Zuker, 2003) to the pSymB *incA* sequence and the other sequences shown in Fig. 4C in an initial attempt to predict secondary structure in these molecules. The analysis suggests that *incA* from pSymB might adopt a single stem structure that ends with a five nucleotide single-stranded loop (Fig. 5C). The predicted loop includes a YUNR (CUAG) motif. Our attempts to predict the structures of the *incA* transcripts encoded on other *repABC* plasmids almost always yields single stem-loop structures, but with variably sized lateral bulges and

terminal loops that do not always include a YUNR motif. These data are a starting point for an *in vitro* chemical or enzymatic determination of *incA* transcript structure.

Discussion

The *repABC* genes constitute a relatively well-conserved family of loci that govern the replication and maintenance of DNA replicons throughout the alpha subgroup of proteobacteria. Notable amongst these replicons are the symbiotic megaplasmids in *S. meliloti* (Chain *et al.*, 2000) and other rhizobial species, the tumour-inducing plasmid (Li and Farrand, 2000) and linear chromosome of *A. tumefaciens*, and the second chromosomes within pathogenic *Brucella* biovars (DelVecchio *et al.*, 2002). Effectively then, *repABC* genes are in large part responsible for the maintenance in microbial cell populations of other genes that either directly or indirectly mediate endosymbiotic or pathogenic interactions with both plants and animals. Common features among RepABC replicons, and of plasmids in general, are sequences that mediate incompatibility phenotypes, and in *repABC* there exists a strong incompatibility determinant encoded within a non-translated intergenic region between *repB* and *repC*. In this report, we provide genetic and biochemical evidence that this incompatibility is a consequence of the expression of a novel gene, *incA*, that is encoded within the intergenic region of the *repABC* operon in a direction that is antisense with respect to the transcription of the *repABC* genes. An examination of the *incA* sequence has not revealed typical or obvious translation initiation features raising the possibility that *incA* is an untranslated RNA.

In most plasmids, incompatibility is linked either to partitioning systems or to one of two general mechanisms that exist to regulate replication initiation and copy number control within the cell. The first such mechanism is based on the presence of an array of short direct repeats, or iterons, usually upstream of a replication initiation gene that are bound by replicase proteins and mediate the so-called handcuffing of distinct replicons within a cell (Papp *et al.*, 1994). In doing so, protein-bound iterons regulate replication initiation and also mediate incompatibility under the appropriate conditions. The second general mechanism involves the expression of one or more non-translated small RNAs that act either directly or indirectly as an antisense regulator of plasmid replication initiation (del Solar *et al.*, 1998; Brantl, 2002). Amongst this latter plasmid class, the mechanism of this regulation differs. For example, in the Gram positive plasmids pT181 (Novick *et al.*, 1989) and pIP501 (Brantl *et al.*, 1993), the antisense RNA binds to its cognate mRNA target and mediates transcriptional attenuation within the untranslated mRNA leader sequence of the endogenous replicase gene. In other plasmids (e.g. the pMV158 family), the

antisense RNA is cognate to a sequence that encompasses the ribosome-binding site upon a replicase gene mRNA and acts to occlude this sequence from ribosome binding (del Solar and Espinosa, 2000). In the *E. coli* plasmid Collb-P9, two encoded antisense RNAs participate in regulating the formation of an RNA pseudoknot structure that ultimately regulates replicase translation (Asano *et al.*, 1991). Thus, in all of these cases, plasmid replication control is mediated by control of expression of a replicase protein that is required for replication initiation. In contrast, in ColEI-related plasmids, a small RNA acts as a primer for the initiation of DNA replication (del Solar *et al.*, 1998). Within all of these diverse mechanisms, however, the small regulatory RNA also acts as an incompatibility determinant by indirectly mediating copy number control.

The *S. meliloti* pSymB *incA* gene that is described herein possesses a number of features consistent with the forementioned regulatory antisense RNA examples: (i) expression of *incA* mediates incompatibility, (ii) *incA* lacks obvious translation signals, (iii) the *incA* transcript is antisense to *repABC* mRNA and (iv) *incA* is encoded within an untranslated region that lies directly upstream of *repC*, the presumed replication initiation (replicase) gene that is required (and sufficient) for replication of mini-derivatives of RepABC replicons. It is also important to note that RepABC replicons do not seem to possess direct repeats that are characteristic of iterons. Northern hybridization experiments indicate that the *incA* transcript is between 50 and 70 nucleotides in length. Based on our sequence analysis of a number of *repABC incA* regions and on our genetic analysis of transcriptional termination, we believe that the transcript-encoding portion of the *incA* gene ends at the conserved T-rich motif (TTTTTCTTTTG) resulting in an RNA transcript approximately 56 nucleotides in length. Direct mapping of the 3' end of the transcript will be part of a future analysis of the overall secondary structure that this RNA molecule may adopt *in vivo*.

Based upon the behaviour of small RNA molecules that regulate replication in other plasmid systems and upon the results of preliminary experiments in our laboratory, we think it likely that the product of the *incA* gene regulates megaplasmid replication by negatively regulating the synthesis of the replicase protein, RepC. Like most small RNAs from plasmids, it might do this by interacting *in cis* with the mRNA sequence upstream of the replicase (*repC*)-coding sequence (i.e. with the mRNA sequence to which it is exactly complementary). *incA*-mediated repression of RepC expression could subsequently occur either at the level of transcription, translation, or even mRNA degradation. Because RepC is presumed to play an essential role in the initiation of replication of the host replicon, fluctuations in the expression of RepC expression should also influence replicon copy number in the cell

and this is testable. Preliminary results in our laboratory have suggested that uncontrolled RepC expression (i.e. overexpression) from a constitutive promoter is lethal in cells and that lethal overexpression can be attenuated by the expression of *incA* in *cis* or from a separate plasmid in *trans*. These observations are consistent with a model wherein *incA* RNA has the ability to negatively regulate (attenuate) the expression of RepC in the cell. pSymB *incA* expression might attenuate *repC* mRNA transcription because a promoter point mutation that eliminates *incA* expression in the context of the *repABC* locus increases transcriptional readthrough into *repC*, as deduced from transcriptional fusions to a reporter enzyme gene (S.R. MacLellan, unpubl. obs.). This possibility is intriguing because RNA-mediated termination of replicase gene transcription is a regulatory mechanism currently limited to a small group of Gram-positive bacterial plasmids.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in LB broth. *S. meliloti* and *A. tumefaciens* strains were grown at 30°C in LB broth supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ or on LB solidified with agar (16 g l⁻¹).

Incompatibility assays

The *repB–repC* intergenic region (*inca*) encodes a strong incompatibility determinant in the *S. meliloti* symbiotic megaplasmid, pSymB. Any *repABC* construct containing the *inca* region and when *inca* itself is isolated as a nucleotide sequence on a ~200 bp fragment and cloned into a bhr plasmid, displays incompatibility and results in a situation where the recombinant plasmid is unable to be maintained in *S. meliloti* cells. Thus, after a conjugation mating experiment, transconjugant colonies containing both pSymB and the incoming *inc* plasmid do not arise on media that is selective for the incoming *inc* plasmid. Perhaps because pSymB is essential to cellular viability, the *inc* plasmid cannot displace pSymB. Therefore, the manifestation of incompatibility in our system is the absence of transconjugant colony formation after conjugation. In contrast, an *inc* plasmid can be maintained in the closely related species, *A. tumefaciens* and in these cells conjugation results in a high (10⁻³–10⁻¹) frequency of transconjugant colony formation per recipient cell. These differential results form the basis of our incompatibility assay: plasmids that are incompatible conjugate with low frequency (<10⁻⁸) into *S. meliloti* recipient cells but with high frequency (typically 10⁻³–10⁻¹) into *A. tumefaciens* recipient cells. Plasmids that are not incompatible conjugate with high frequency into both *S. meliloti* and *A. tumefaciens* cells.

Test plasmids (bhr plasmids with or without *inc* sequences derived from the *S. meliloti* symbiotic megaplasmids) were mobilized from *E. coli* DH5α (donor) cells into *S. meliloti* or *A. tumefaciens* (recipient) cells in tri-parental matings that

included *E. coli* MT616, a helper strain that carries transfer function genes on pRK600. The *E. coli* donor and helper strain cultures used in each mating were adjusted to have twice the cell density as the recipient cells. Equal volumes of cells from cultures grown overnight were washed in saline and spotted onto an LB agar plate and incubated overnight at 30°C. The mating spot was resuspended in 0.85% saline and serially diluted and plated onto LB plates containing streptomycin and an antibiotic to select for the transferred test plasmid.

Generation of incompatibility mutants

Under reaction conditions predicted to lower the fidelity of DNA polymerases (Fromant *et al.*, 1995; Vartanian *et al.*, 1996), the PCR was used to amplify a 244 bp nucleotide sequence that encompasses the 161 bp *repB1–repC1* intergenic region (*inca1*) from pSymB (PCR primers ML1564 and ML1565). Platinum *Taq* DNA polymerase (Invitrogen) was used with reaction conditions as recommended by the manufacturer with the following exceptions: MgCl₂ was used at a concentration of either 5 or 6 mM; 2.5 U of Platinum *Taq* DNA polymerase was used per 50 µl reaction; and the forcing nucleotide (dGTP) concentration was 0.6 mM with the other nucleotides present at a concentration of 0.2 mM. Thermocycling proceeded with an extension time of 30 s for 25 cycles. The mutagenized amplification product was purified through a QIAquick PCR purification kit (Qiagen), cut with *Xba*I and *Hind*III and ligated overnight into the bhr plasmid pOT1 that had previously been linearized with *Xba*I and *Hind*III and purified by extraction from an agarose gel. The mutagenized DNA-pOT1 ligation mixture was transformed into *E. coli* DH5α. *E. coli* transformants were then used in conjugation experiments designed to select for incompatibility mutants (see below).

Alternatively, we made use of the mutagenic properties of an *E. coli* mutator strain, strain LE30. *E. coli* LE30 possesses the so-called *mutD* allele that results from a mutation(s) within the epsilon subunit of DNA polymerase III. In order to mutagenize *inca1* DNA, plasmid pTH1414 was transformed into competent LE30 cells and isolated transformants were grown overnight in liquid culture and used directly in tri-parental matings to select for incompatibility mutants (see below).

Selection for incompatibility mutants

Selection for recombinant plasmids that have lost the ability to mediate incompatibility proceeded as follows. Recombinant plasmids containing mutagenized *inca1* DNA (see above) were transformed into *E. coli* DH5α cells and plated onto selective LB agar plates. In a typical experiment, 500–750 *E. coli* transformant colonies were pooled by applying 0.5 ml of LB broth to the surface of the agar plate and by resuspending the colonies in the broth using a sterilized glass rod. The cell suspension was recovered, inoculated into 2 ml of LB broth containing gentamicin and allowed to incubate at 37°C for 30 min. Cells were recovered by centrifugation, resuspended in 0.5 ml of 0.85% saline and used with overnight cultures of *S. meliloti* Rm5004 recipient cells and *E. coli*

MT616 in a tri-parental mating, as described earlier. Because pTH1414 itself cannot support the formation of transconjugant colonies (as a result of incompatibility), only plasmids that have lost incompatibility as a result of PCR- or mutator strain-generated mutations will form colonies. Transconjugant *S. meliloti* colonies that did form were streak purified, plasmid DNA was recovered and used to transform *E. coli* DH5 α . Plasmid DNA purified from these transformants was sequenced and mutations were documented. Mutant phenotypes were confirmed by repeating the mating experiment using these latter *E. coli* transformants and fresh *S. meliloti* recipient cells.

RNA extraction

Total RNA was extracted from *S. meliloti* cells using a hot phenol method based upon a previously published protocol (Chuang *et al.*, 1993). Briefly, overnight cultures of *S. meliloti* were used to inoculate 100 ml volumes of LB containing 2.5 mM MgSO₄ and 2.5 mM CaCl₂ and antibiotic where appropriate. Cultures were grown with shaking at 30°C to an O.D. of 0.6–0.7. Without delay, cultures were supplemented with a 1/10 volume of cell stop solution (5% unbuffered phenol in absolute ethanol) and immediately centrifuged to pellet cells. Cell pellets were flash frozen in liquid nitrogen and stored at –70°C until use. Thawed pellets from 50 ml of culture were resuspended in 960 μ l of RNase-free water, split into 3 vols of 480 μ l each. Cells were lysed by the addition of an equal volume of hot phenol buffer at RT. Hot phenol buffer consists of five parts buffer (20 mM Tris-HCl (pH 7.5), 400 mM NaCl, 40 mM EDTA, 1% SDS and 1% β -mercaptoethanol) and one part unbuffered phenol. After the addition of hot phenol buffer, the suspension was vortexed vigorously, and heated at 95°C for 1 min. The lysed cell suspension was vortexed at RT for 10 min at high speed to pellet debris and the aqueous supernatant was subjected to two phenol: chloroform extractions (using a 1:1 ratio of unbuffered phenol and chloroform) and one final chloroform extraction. Nucleic acids were precipitated with a 1/10 vol. of 3 M Na acetate (pH 5.2) and two volumes of isopropyl alcohol on ice. DNA in the sample was removed by digestion with RNase-free Dnase I and RNA was recovered after phenol/chloroform extraction by precipitation as described above. The concentration of RNA was estimated spectrophotometrically at 260 nm and the integrity of the nucleic acid was assessed by separating samples upon agarose/formaldehyde (1% agarose/6% formaldehyde) gels and visualizing the sharpness of the rRNA bands after staining with ethidium bromide.

Northern hybridization

Indicated amounts of total *S. meliloti* RNA were separated using either agarose/formaldehyde (2% agarose/6% formaldehyde) gels. Before running, samples were incubated in an equal volume of 2 \times loading buffer (95% formamide, 20 mM EDTA) with heating at 70°C. Size estimation was provided by running a low molecular weight range RNA marker (MBI Fermentas) as suggested by the manufacturer and by photographing the gel alongside a fluorescent ruler over a 312 nm UV transilluminator. Separated RNA was transferred

to positively charged nylon membrane by a descending passive transfer technique. Membranes were air-dried and RNA was fixed by exposing the membrane to UV light on a transilluminator for 60 s. Membranes were incubated in a prehybridization solution (250 mM sodium phosphate (pH 7.2), 7% (SDS) for 1 h at 50°C. Probes consisted of oligonucleotides end-labelled with γ -³²P-dATP and polynucleotide kinase (MBI Fermentas) and were added directly to the prehybridization buffer. After hybridization at 50°C overnight, membranes were washed at 60°C for 2 h with several changes of wash solution (2 \times SSC, 0.2% SDS) and a final RT wash with 0.2 \times SSC for 5 min. Air-dried blots were exposed to X-ray film for 1–3 days or to a phosphoimager screen for 5–24 h, as required. Phosphoimager signals were processed using the program ImageQuant v.5.1.

Primer extension

In a typical primer extension reaction, 40 μ g of total *S. meliloti* RNA (extracted as described above) in an 8 μ l volume was supplemented with: 1 \times 10⁵ cpm of end-labelled (γ -³²P-dATP) primer (ML3826), 4 μ l of 5 \times reverse transcriptase buffer, and 0.8 μ l of dNTP mixture containing all four nucleotides (25 mM each). The mixture was heated to 65°C in a water bath and allowed to cool to 35°C over 2 h. On ice, the annealed mixture was supplemented with 2 μ l of 100 mM DTT and 1 U of RNaseOUT (Invitrogen) and was incubated at 42°C for 2 min before supplementation with 2.5 U of M-MLV reverse transcriptase (Invitrogen) and a further incubation at 42°C for 50 min. After the reaction, the mixture was supplemented with an equal volume of 2 \times loading dye (95% formamide, 20 mM EDTA) and stored on ice or –20°C overnight. The primer extension product was separated on an 8% acrylamide (7 M urea) sequencing gel along with a sequencing reaction generated using the same primer and the Sequenase Version 2 DNA Sequencing kit (USB). Template DNA used for DNA sequencing was pTH1015, a plasmid containing a 4.9 kb insert that encompasses the entire *repABC1* operon.

Enzyme assays and other techniques

The *incA* promoter was first detected when a 244 bp nucleotide sequence (encompassing the *repB–repC* intergenic (*incA*) region) was cloned into the *gfpUV* transcriptional reporter vector, pOT1 and transformed or conjugated into *E. coli* or *A. tumefaciens*, respectively. To achieve maximal fluorescence, cultures were grown as isolated colonies or as patches upon LB plates in which the medium had been adjusted to have a pH of 8 using 1 M Tris base. The *A. tumefaciens* and *E. coli* DH5 α host strains were grown for 3–4 days at 30°C before assessing *gfp* fluorescence emanating from the colonies over a 312 nm UV transilluminator. Alternatively, the *E. coli* DH5 α host could be grown overnight at 37°C but the manifestation of fluorescence required a further 3–4 days incubation at 4°C. Quantitative measurements of *gfpUV* fluorescence was conducted on liquid cultures in a Tecan Safire Fluorimeter. Single bacterial colonies were inoculated (in triplicate for each strain) into 2 ml volumes of LB broth containing the appropriate antibiotic. The next day,

fresh 2 ml volumes of LB broth were inoculated to an optical density of 0.1 and grown to an O.D. of 0.5–0.7. Cells were collected by centrifugation, washed once in 0.85% saline, and resuspended in saline to the original culture volume. Two hundred microlitres volumes were applied to black solid bottom 96-well microtitre plates for fluorescence measurements and to clear polystyrene 96-well plates for absorbance readings conducted at 600 nm. Fluorescence was measured from the top of the plates using an excitation wavelength of 405 nm and emission wavelength of 505 nm with a slit width of 10 nm. Specific fluorescence was calculated by dividing each emission output with the respective absorbance. Data have been reported as the mean of three measurements plus or minus the standard error.

DNA sequencing and oligonucleotide synthesis was provided by MOBIXlab (McMaster University, Hamilton, Ontario). RNA structure prediction was carried out using the mfold v.3.1 nucleic acid folding server (Mathews *et al.*, 1999; Zuker, 2003).

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to T.M.F. and a Ontario Graduate Scholarship to S.R.M.

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