Quorum-sensing-regulated transcriptional initiation of plasmid transfer and replication genes in *Rhizobium leguminosarum* biovar *viciae*

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Transfer of the *Rhizobium leguminosarum* biovar *viciae* symbiosis plasmid pRL1JI is regulated by a cascade of gene induction involving three LuxR-type quorum-sensing regulators, TraR, BisR and CinR. TraR induces the plasmid transfer *traI-trb* operon in a population-density-dependent manner in response to *N*-acylhomoserine lactones (AHLs) made by TraI. Expression of the *traR* gene is primarily induced by BisR in response to AHLs made by CinI, and expression of *cinI* is induced by CinR and repressed by BisR. Analysis of transcription initiation of *cinI*, *traR* and *traI* identified potential regulatory domains recognized by the CinR, BisR and TraR regulators. Deletion and mutation of the *cinI* promoter identified potential recognition motifs for activation by CinR and repression by BisR. Analysis of the DNA sequence upstream of *traI* and expression of transcriptional gene fusions revealed a predicted TraR-binding (*tra*-box) domain. Two transcript initiation sites were identified upstream of the plasmid replication gene *repA*, which is divergently transcribed from *traI*; one of these *repA* transcripts requires the quorum-sensing cascade mediated via BisR and TraR, showing that the pRL1JI plasmid replication genes are co-regulated with the plasmid transfer genes.

INTRODUCTION

Many plant-associated bacteria produce different *N*-acylhomoserine lactones (AHLs) that are used to regulate gene expression in a population-density-dependent manner (Cha *et al.*, 1998; d'Angelo-Picard *et al.*, 2005). Such 'quorum-sensing' regulation involves the accumulation of the AHLs; as the concentration of AHLs rises, the levels of AHLs within the cells also rise, inducing gene expression by activating regulators that induce expression from specific promoters (Swift *et al.*, 2001). Various cellular processes are regulated in this manner (Barnard *et al.*, 2007; Swift *et al.*, 2001). Many of the regulators that recognize AHLs belong to the LuxR class of DNA-binding proteins, so-named because LuxR regulates the production of light by bioluminescent bacteria (Fuqua *et al.*, 2001). The AHLs recognized by LuxR are synthesized by the AHL synthase LuxI, and a large family of related bacterial AHL synthases has been identified (Gray & Garey, 2001).

One of the quorum-sensing regulatory systems controls the regulation of plasmid transfer by strains of *Agrobacterium tumefaciens*, which causes tumours in plants by directly transforming plant cells (Fuqua *et al.*, 1994; Hwang *et al.*, 1994; Piper *et al.*, 1993). Such strains contain plasmids that carry genes required for plant pathogenicity, and the transfer of these plasmids between agrobacteria requires both an appropriate population density and the presence of a specialized carbon source (opines) (Fuqua & Winans, 1996; Piper *et al.*, 1999). The DNA transferred to the plant includes genes for promoting cell proliferation and for synthesizing secondary metabolites called opines (Escobar & Dandekar, 2003). These plant-made opines are secreted from transformed plants and induce the expression of specialized uptake and catabolism genes in *A. tumefaciens*. One of the genes co-induced is *traR*, the product of which regulates the transfer of the pathogenesis plasmid. TraR dimers bind to the AHL 3-oxo-octanoyl-L-homoserine lactone (3-O-C_8-HSL) and this induces the expression of plasmid-transfer genes, including the *traI-trb* operon. The *traI* gene product synthesizes 3-O-C_8-HSL, resulting in positive autoregulation. The structure of TraR bound to

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Abbreviations: 3-O-C_8-HSL, 3-oxo-octanoyl-L-homoserine lactone; 3-OH-$C_{14:1}$-HSL, *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone; AHL, *N*-acylhomoserine lactone.

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3-O-C_8-HSL has been resolved (Vannini *et al.*, 2002; Zhang *et al.*, 2002) and a consensus TraR-binding sequence (the *tra*-box) has been defined (Zhu *et al.*, 2000).

Rhizobium leguminosarum is closely related to *A. tumefaciens* and many of the genes required for the nitrogen-fixing symbioses between *R. leguminosarum* and legumes are present on plasmids (Downie *et al.*, 1983; Young *et al.*, 2006). The transfer of one of these plasmids (pRL1JI) between different rhizobia can be induced by potential recipient strains of *Rhizobium* in a population-density-dependent manner (Danino *et al.*, 2003). As in *A. tumefaciens*, there is a TraR regulator that induces the expression of plasmid transfer genes, including a *traI-trb* operon; this requires the accumulation of 3-O-C_8-HSL, which is synthesized by TraI in a population-density-dependent manner. Expression of *traR*, however, is under the direct control of a second LuxR-type regulator encoded by *bisR*, which is adjacent to *traR* on pRL1JI (Fig. 1). BisR induces *traR* expression in response to *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone (3-OH-$C_{14:1}$-HSL), which is produced by the chromosomally encoded *cinI* gene product (Lithgow *et al.*, 2000). The *cinI* gene is so-called because in bacteriocin-type assays it is required for growth inhibition of some strains of *R. leguminosarum* (Lithgow *et al.*, 2000); sensitive strains include those carrying pRL1JI and their sensitivity to CinI-made 3-OH-$C_{14:1}$-HSL is related to the activation of TraR, because mutations affecting *traR* or *traI* expression cause a loss of the sensitivity phenotype. *R. leguminosarum* strains carrying pRL1JI do not make much 3-OH-$C_{14:1}$-HSL, because BisR represses *cinI* expression (Wilkinson *et al.*, 2002). Such strains carrying pRL1JI are poised to detect 3-OH-$C_{14:1}$-HSL made by potential *Rhizobium* recipients, which lack *bisR* (and therefore pRL1JI) and so are not repressed for 3-OH-$C_{14:1}$-HSL synthesis. Thus, when a strain of *R. leguminosarum* (donor) carrying pRL1JI is growing near a population of a strain lacking pRL1JI (potential recipient), 3-OH-$C_{14:1}$-HSL made by the recipient strain activates BisR to induce *traR* expression in the donors (Fig. 1). Then, as the population density of the donors increases, TraR induces the plasmid-transfer genes and conjugal transfer of pRL1JI is induced (Danino *et al.*, 2003). The sequenced *R. leguminosarum* bv. *viciae* strain 3841 lacks *bisR* (Young *et al.*, 2006) and so this strain is one such potential recipient.

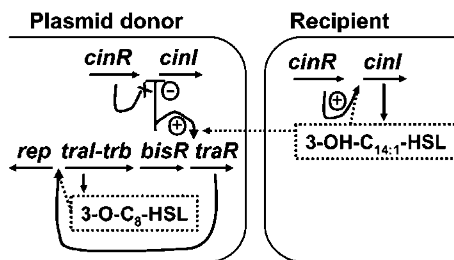

Fig. 1. Model of transfer of plasmid pRL1JI in *R. leguminosarum*. BisR induces *traR* expression in response to 3-OH-$C_{14:1}$-HSL, which is normally produced by CinI under quorum-sensing regulation by CinR. However, BisR also represses *cinI* expression and so very little 3-OH-$C_{14:1}$-HSL is made by donor cells, thereby allowing them to detect this AHL made by potential recipients. The 3-OH-$C_{14:1}$-HSL made by recipients is detected by BisR, which then induces *traR* expression. This induction allows TraR to induce the *traI-trb* operon under quorum-sensing regulation in response to TraI-made 3-O-C_8-HSL.

Although DNA-binding motifs have been identified for many of the LuxR-type regulators (Egland & Greenberg, 1999; Fuqua *et al.*, 2001), there is no obvious recognition motif upstream of the genes regulated by the LuxR-type regulators BisR and CinR (Danino *et al.*, 2003; Lithgow *et al.*, 2000; Wilkinson *et al.*, 2002). In this work, we have mapped the transcript start sites of genes regulated by CinR, BisR and TraR and identified conserved regions of dyad symmetry that may represent the respective DNA-binding sites.

METHODS

Microbiological techniques. *Rhizobium* and *Agrobacterium* strains were grown at 28 °C in TY medium (Beringer, 1974). Antibiotics were added at the following final concentrations (μg ml^{-1}): streptomycin, 400; kanamycin, 40; spectinomycin, 200; tetracycline, 5. Bacterial growth was monitored at 600 nm using a Perkin Elmer MBA2000 spectrophotometer. Green fluorescent protein (GFP) fluorescence was measured using a Tecan SAFIRE microplate reader with an excitation wavelength of 488 nm and an emission wavelength of 510 nm, and for the GFP experiments bacterial growth was monitored at an absorbance of 600 nm in the same plate reader. Units of *β*-galactosidase activity were measured as described by Miller (1972) using a Titertek Multiscan spectrophotometer. Cultures were grown for 2 days in TY medium containing the appropriate antibiotics then diluted 1 : 100 in AMS (acid minimal salts) pyruvate minimal medium (Poole *et al.*, 1994) for GFP assays or TY medium for *lacZ* assays. Where required, AHLs were added at the time of dilution to the final concentrations stated. Bacteriocin growth-inhibition tests were done as described previously (Wilkinson *et al.*, 2002).

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. A924 (*cinR3* : : Tn*5*) was isolated from a Tn*5* insertion library of strain 3841, and the Tn*5* insertion was found by DNA sequencing to be located 205 bp from the translation start of *cinR*. Plasmid pIJ9718 was made by amplifying the *cinI-cinR* intergenic region from genomic DNA of strain 3841 (using *Taq* polymerase and the 5′ primer 5′-TGCTCGTTTCAAACTCGGCTG-3′ and the 3′ primer 5′-GCGAGCGAATCGTAGCTGTC-3′), generating a 388 bp fragment which was ligated into pGEM-T Easy (Promega) following the manufacturer's instructions. All other plasmids were constructed using DNA from strain A34. The *cinI–gfp* transcriptional fusion plasmid pIJ9611 was made by amplifying a 450 bp *Hin*dIII DNA fragment (5′ primer 5′-CTCGGCAAAGCTTACAAGGAT-ATTTC-3′; 3′ primer 5′-CAGAGCGTATCGGCGAAAAGCTTCT-TGC-3′) containing 136 bp of *cinR* coding region, the *cinRI* intergenic region and 70 bp of *cinI* coding region, followed by cloning the DNA fragment into the *Hin*dIII site of pRU1156. The *traR–gfp* transcriptional fusion plasmid pIJ9612 consists of a PCR-amplified 217 bp *Hin*dIII DNA fragment (5′ primer

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	References
R. leguminosarum		
3841	Str^R derivative of *R. leguminosarum* 300	Johnston & Beringer (1975)
8401	Str^R derivative of *R. leguminosarum* 8400; $pSym^-$	Lamb *et al.* (1982)
A34	*R. leguminosarum* 8401 carrying pRL1JI; Str^R	Downie *et al.* (1983)
A549	A34 *bisR1*::Tn*5*	Wilkinson *et al.* (2002)
A552	8401 *cinR1*::Tn*5*	Lithgow *et al.* (2000)
A568	8401 *cinI2*::Tn*5*	Lithgow *et al.* (2000)
A627	A34 *traR1*::Tn*5*	Wilkinson *et al.* (2002)
A741	8401 *cinR2*Ωspec	Lithgow *et al.* (2000)
A924	3841 *cinR3*::Tn*5*	This work
A. tumefaciens		
C58.00	*A. tumefaciens* lacking pTi and pAT plasmids. Makes no detectable AHLs	Vaudequin-Dransart *et al.* (1995)
A677	C58.00 Str^R	Danino *et al.* (2003)
Plasmids		
pBBR1-MCS2	Kan^R broad-host-range vector	Kovach *et al.* (1995)
pKT230	Kan^R, Str^R broad-host-range cloning vector	Bagdasarian *et al.* (1981)
pRU1156	Tet^R *gusA* and *gfpmut3.1* transcriptional fusion vector	Karunakaran *et al.* (2005)
pMP220	Tet^R *lacZ* transcriptional fusion vector	Spaink *et al.* (1987)
pIJ7630	*trb, bisR, traR* region in pUC18	Wilkinson *et al.* (2002)
pIJ7749	*cinRI* region in pUC19	Lithgow *et al.* (2000)
pIJ7867	2.2 kb *Sac*I–*Eco*RI fragment carrying *bisR* and *traR* in pKT230	Wilkinson *et al.* (2002)
pIJ9036	*repABC-traI* region in pUC18	Wilkinson *et al.* (2002)
pIJ9577	*traR* in pBBR1-MCS2	Danino *et al.* (2003)
pIJ9581	*bisR* in pBBR1-MCS2	Danino *et al.* (2003)
pIJ9611	*cinI–gfp* fusion in pRU1156 (starts 360 nt upstream from transcript start)	This work
pIJ9612	*traR–gfp* fusion in pRU1156 (starts 83 nt upstream from transcript start)	This work
pIJ9753	*repA–lacZ* fusion in pMP220	This work
pIJ9718	*cinIR* intergenic region from 3841	This work
pIJ9884	*cinI–gfp* fusion in pRU1156 (starts 79 nt upstream from transcript start)	This work
pIJ9991	*traR–gfp* fusion in pRU1156 (starts 53 nt upstream from transcript start)	This work

5′-GTACATTTGAAGCTTATCACTCCCCAC-3′, 3′ primer 5′-GAAGCTTTTCAGGGCACTCTTG-3′) containing 26 bp of *bisR* coding region, the *bisR-traR* intergenic region and 82 bp of *traR* coding region cloned into the *Hin*dIII site of pRU1156. The *repA–lacZ* plasmid pIJ9753 was made by subcloning a 930 bp *Xba*I/*Kpn*I fragment from pIJ9278 (Danino *et al.*, 2003) into pMP220. The *cinI–gfp* plasmid, pIJ9884, was made by PCR amplification (5′ primer 5′-CTCGGCAAAGCTTACAAGGATATTTC-3′; 3′ primer 5′-CAGAGC-GTATCGGCGAAAAGCTTCTTGC-3′) followed by *Hin*dIII digestion and ligation into pRU1156, producing a plasmid containing 180 bp DNA fragment, which has the same 3′ end as pIJ9611, but is shorter by 270 bp at the 5′ end. The *traR–gfp* plasmid, pIJ9991, was made as pIJ9612 except the 5′ primer was 5′-GAGTAACCCA-AGCTTGGGTATCGGTTTG-3′, generating an insert size of 188 bp.

Primer extensions. Total RNA was isolated from *R. leguminosarum* using an RNeasy kit from Qiagen. Primer extension analysis and DNA sequencing were done as previously described (Sawers & Böck, 1989; Wexler *et al.*, 2001). The oligonucleotides used in these studies are shown in the legends to the respective figures.

RESULTS

Transcription of *cinI*

To identify the promoters involved in the quorum-sensing-regulated plasmid-transfer system in *R. leguminosarum*, we first identified the transcription initiation site of *cinI*. The autoinduction of *cinI* in the potential recipient is the first step of the regulatory cascade leading to pRL1JI transfer (Fig. 1). Primer extensions were performed (Fig. 2a) on RNA extracted from *R. leguminosarum* strain 8401 (lacking pRL1JI), which induces *cinI*, and from an isogenic *cinR* mutant (*R. leguminosarum* strain A741), which does not (Lithgow *et al.*, 2000). Transcription of *cinI* initiated from a single site 27 bp upstream of the predicted *cinI* translational start. As expected (Lithgow *et al.*, 2000), the appearance of this transcript required *cinR* (Fig. 2a). We also identified the transcription initiation site of *cinI* in *R.*

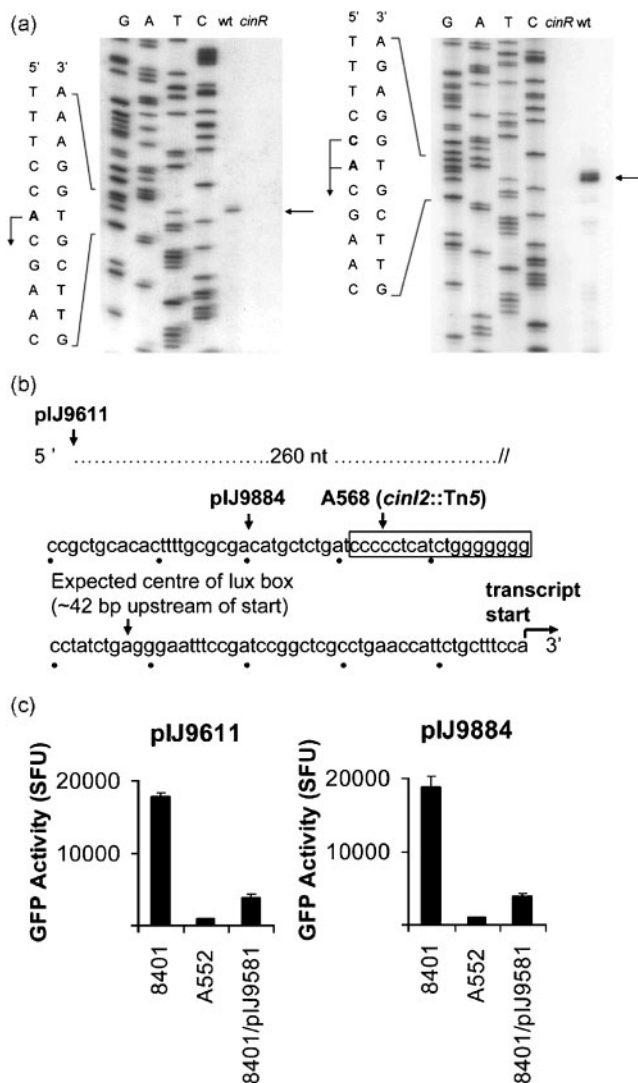

Fig. 2. Transcription start and expression analyses using *cinI* promoter fragments. (a) Primer extension analysis of the *cinI* transcript in *R. leguminosarum* strains 8401 (left) and 3841 (right). Total RNA was harvested from 8401 (wt, left) or 3841 (wt, right) and their isogenic *cinR* mutants A741 and A942 after growth on TY medium. Primer extensions and DNA sequencing reactions were done using the primer 5′-AACATCTGGTCGAGTACGGC-AGC-3′, which extends from bp +62 to +39 relative to the predicted translation initiation codon of *cinI*. pIJ7749 and pIJ9718 were used as the DNA sequencing templates for 8401 and 3841, respectively. The locations of the transcriptional start sites and flanking sequences are indicated. (b) The DNA sequence upstream of the 8401 transcription start site shows the position 42 bp upstream of the transcription start site and the observed 18 nt region of dyad symmetry (region boxed centred 60 bp upstream of the transcription start site), predicted to include the CinR-binding site. Also shown is the location of the Tn*5* insertion in mutant A568 and the 5′ ends of the constructs pIJ9884 and pIJ9611 used for assays of promoter activity. (c) Expression of *cinI–gfp* fusion derivative was measured with plasmids pIJ9611 or pIJ9884 in *R. leguminosarum* strains 8401 (wt) and A552 (*cinR*::Tn*5*) in the presence and absence of *bisR* on pIJ9581. GFP activities are expressed as specific fluorescence units (SFU) and are means of at least three independent assays ±SEM.

leguminosarum strain 3841 (Fig. 2b), whose genome has been sequenced (Young *et al.*, 2006). Strain 3841 lacks *bisR* and induces *cinI* expression, based on measurements of 3-OH-$C_{14:1}$-HSL accumulation, which inhibits the growth of *R. leguminosarum* strain A34 (Fig. 3a). An isogenic *cinR* mutant (*R. leguminosarum* strain A924) did not produce 3-OH-$C_{14:1}$-HSL (Fig. 3a). A *cinR*-dependent transcript was identified also, starting 27 bp upstream of the *cinI* translation initiation codon in this strain (Fig. 2b). In neither strain was there an obvious recognition sequence similar to a *lux* or *tra* box at the anticipated location, usually centred about 42–45 bp upstream of the transcription start. However, we did identify a region of dyad symmetry centred 60 bp upstream of the *cinI* transcription start site (Fig. 2b); a similar gap (65 bp) between a transcript start and quorum-sensing regulator (TraR) binding site has been observed previously (Pappas & Winans, 2003).

Previously, in A568 a Tn*5* insertion was identified between *cinI* and *cinR*, causing greatly reduced 3-OH-$C_{14:1}$-HSL production (Fig. 3) (Lithgow *et al.*, 2000). The Tn*5* is inserted 65 bp upstream of the 5′ end of the *cinI* transcript and so there is not an intact promoter within 65 bp of the *cinI* transcript start (Fig. 2b). To define the 5′ end of the *cinI* promoter, we amplified *cinI* promoter regions starting 79 bp and 360 bp upstream of the transcription start site and cloned the DNA products into a GFP transcriptional fusion vector to make pIJ9884 and pIJ9611, respectively. The results obtained with these promoter fusions (Fig. 2c) showed that an intact and functional CinR-dependent *cinI* promoter is within the 79 bp upstream of the transcription start site (Fig. 2c). We conclude that some of the 14 bp of the *cinI* upstream region between the 5′ end of pIJ9884 (position −79) and the Tn*5* insertion in A568 (position −65) must be required for normal *cinI* promoter activity. The Tn*5* in A568 is within a region of dyad symmetry and it is possible that CinR may bind to this region interrupted by the Tn*5* insertion in A568.

The expression of *cinI* is repressed by BisR (Wilkinson *et al.*, 2002), although it is not known if this is due to repression by binding to the promoter or some other mechanism, such as the formation of inactive heterodimers with CinR. Cloned *bisR* repressed *cinI* expression in pIJ9611 and pIJ9884 (Fig. 2d). To identify whether BisR-mediated repression is retained in A568 (containing Tn*5* in the promoter region of *cinI*), we made use of the observation that a weak promoter reads out from Tn*5* (Berg *et al.*, 1980). To determine if this could be observed with A568, we used a strain of *R. leguminosarum* whose growth is extremely sensitive to inhibition by the CinI-made 3-OH-$C_{14:1}$-HSL (Wilkinson *et al.*, 2002). A568 does cause some growth inhibition detectable by this strain (Fig. 3b) and we

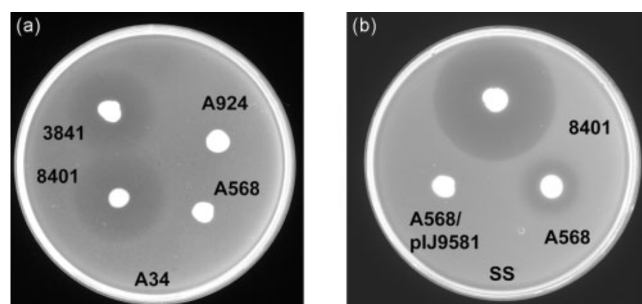

Fig. 3. Measurement of 3-OH-$C_{14:1}$-HSL levels using the small bacteriocin test. (a) A lawn of *R. leguminosarum* A34 was used to test growth inhibition (haloes) induced by strains 8401, 3841, A924 (*cinR*) and A568 (*cinI2*::Tn5). (b) Strain A34 carrying the cloned *bisR* and *traR* genes on pIJ7867 was used as a very sensitive indicator lawn to test growth inhibition (haloes) induced by strains 8401, A568 (*cinI2*::Tn*5*) and A568 (*cinI2*::Tn*5*) carrying *bisR* cloned on pIJ9581.

conclude that this is probably due to *cinI* expression from the weak Tn*5* promoter. The growth inhibition by A568 was abolished by introducing the *bisR* gene, cloned on pIJ9581, into strain A568 (Fig. 3b), showing that BisR-mediated repression is retained even though normal *cinI* induction by CinR is lost in A568.

Transcription of *traR*

The next step in the induction of pRL1JI transfer is BisR-mediated induction of *traR* in response to 3-OH-$C_{14:1}$-HSL (Fig. 1) (Danino *et al.*, 2003). BisR shows 59 % overall identity to CinR (Wilkinson *et al.*, 2002); in the predicted DNA contact regions (equivalent to residues 191–221 in TraR; Zhang *et al.*, 2002) of BisR (residues 195–225) and CinR (residues 196–206) the identity is 68 % (21 identities in 31 residues). Database searches with residues 195–225 of BisR returned the highest significant score with CinR (with the exception of BisR itself and the likely orthologue of BisR in *Rhizobium etli*). This high similarity between the predicted DNA-binding domains of BisR and CinR from *R. leguminosarum* suggested that promoters regulated by BisR and CinR might share some sequence similarity. The transcription initiation site of *traR* was determined (Fig. 4a) using RNA isolated from *R. leguminosarum* A34 carrying pRL1JI and an isogenic *bisR* mutant (A549), both grown in the presence of 3-OH-$C_{14:1}$-HSL to activate *traR* induction by BisR. A *bisR*-dependent transcript was observed starting 53 bp upstream from the predicted *traR* translation start. The region between *bisR* and *traR* is short and so the transcription start site of *traR* is only 52 bp downstream from the translation stop of *bisR*. No typical *lux*- or *tra*-box-like elements could be identified upstream of the transcription start site. We constructed two *traR* promoter fusion constructs (Fig. 4b), pIJ9612 starting 83 bp upstream of the transcription start site (within the end of

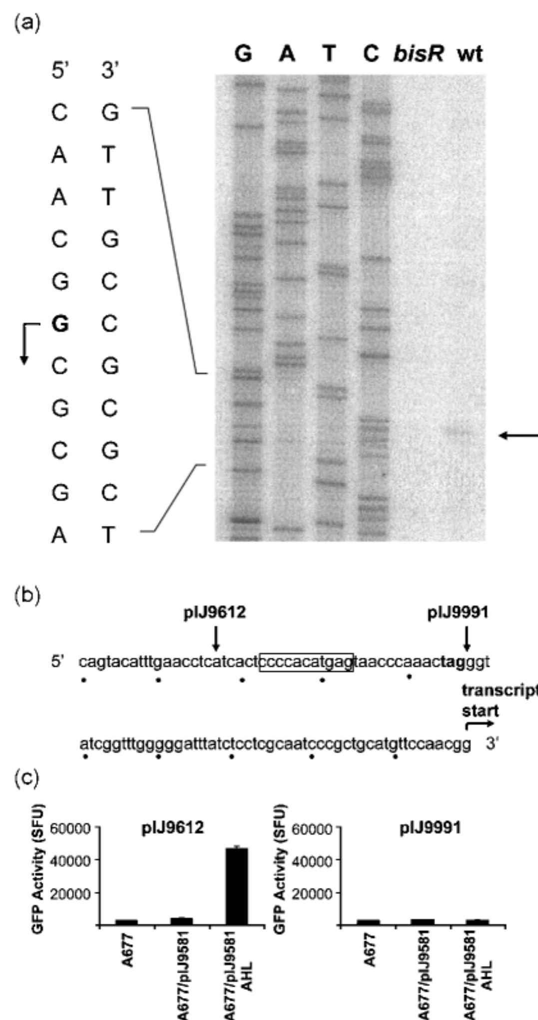

Fig. 4. Transcription start site and expression analysis using *traR* promoter fragments. (a) The *traR* transcription start site was determined using primer extension with total RNA isolated from A34 (wt) and A549 (*bisR*::Tn*5*) after growth on TY medium containing 10 nM 3-OH-$C_{14:1}$-HSL. The primer 5′-CACTC-TTGATCATCCGCTCATTGTGC-3′, binding within the coding region of the *traR* gene (nucleotides +52 to +27, relative to the predicted translation start codon), was also used for DNA sequencing with pIJ7630 as template. The location of the transcription start site and flanking sequences are indicated. (b) The DNA sequence upstream of the transcription start site shows the *bisR* translation stop codon in bold and the 5′ ends of the fragments cloned in pIJ9612 and pIJ991 (arrowed). The predicted BisR-binding site is boxed. (c) The expression levels of the *traR*–*gfp* fusion on pIJ9612 or pIJ9991 in *A. tumefaciens* A677 are shown, revealing the effects on expression of cloned *bisR* on pIJ9581 and of 3-OH-$C_{14:1}$-HSL. GFP activities are expressed as specific fluorescence units and are means of at least three independent assays ±SEM.

bisR) and pIJ9991 starting 53 bp upstream of the *traR* transcription start site (at the translational stop of *bisR*). Induction of the *traR* promoter was assayed as described

previously using strain A677 (Danino *et al.*, 2003), which is a derivative of *A. tumefaciens* that makes no detectable AHLs (Vaudequin-Dransart *et al.*, 1995). Normal induction of *traR–gfp* by 3-OH-$C_{14:1}$-HSL was seen with pIJ9612, but there was no induction with pIJ9991 (Fig. 4c), demonstrating that the induction probably occurs following BisR binding to DNA included within the 83–53 bp region upstream of the *traR* transcript start. We compared the *traR* promoter region with the *cinI* promoter region to try to identify potential BisR- and CinR-binding motifs. Two regions of similarity were noted. The sequence TGAGGGAATTT, centred 41 bp upstream of the *cinI* transcript start (Fig. 2b), is similar to the sequence TGGGGGATTT, 41 bp upstream of the *traR* transcript start (Fig. 4b). However, these sequences are fully retained in the *cinI2*::Tn*5* mutant A568 (Fig. 2b) and the *traR* promoter fusion plasmid pIJ9991 (Fig. 4b), neither of which allows expression of the downstream genes (Figs 3a and 4c). Therefore, these sequences alone seem very unlikely to be the CinR- and BisR-binding motifs. The other region of similarity seen was that the sequence CCCCACATGAG, starting 78 bp upstream of the *traR* transcription start site (Fig. 4b), which is present in the opposite orientation in the *cinI* promoter region (as CTCATCTGGGG, starting 64 bp upstream of the *cinI* transcription start site; Fig. 2b). This motif is fully retained in the inducible *traR* promoter fusion in pIJ9612 (Fig. 4b, c) and in the *cinI*::Tn*5* promoter mutant A568, in which BisR-mediated repression of *cinI* is retained (A568/pIJ9581 in Fig. 3b) and overlaps with the predicted CinR-binding site (Fig. 2b). Therefore the CTCATSTGGGG sequence might act as (part of) a BisR-binding site that could allow induction of *traR*, but prevent CinR binding to the *cinI* promoter. Clearly DNA footprinting experiments with BisR and CinR will be required to exactly define their DNA-binding sites.

Transcription of *traI*

RNA from A34, carrying the *traI-trb* operon on pRL1JI, and from an isogenic *traR* mutant (A627) was used to identify the 5′ end of the *traI* transcript. Cultures were grown in the presence or absence of 3-OH-$C_{14:1}$-HSL to induce *traR* by BisR, thereby allowing TraR to induce the *traI-trb* operon (Danino *et al.*, 2003). The *traI* gene was transcribed from a single promoter, which required both TraR and 3-OH-$C_{14:1}$-HSL (Fig. 5a and data not shown). In *A. tumefaciens* and *Rhizobium* sp. NGR234, transcription of *traI* requires a conserved TraR-binding site with dyad symmetry (*tra*-box) centred 42–45 bp upstream of the transcription start site (Fuqua *et al.*, 2001; He *et al.*, 2003). A predicted *tra*-box sequence, similar to the *tra*-box identified upstream of *traI* from pNGR234a (pSym) in *Rhizobium* NGR234, was identified centred 44 bp upstream of the *traI* transcript start on pRL1JI (Fig. 5b). This predicted TraR-binding site on pRL1JI shows some similarity (Fig. 5b) to the *tra*-box upstream of *traI* in *A. tumefaciens* and to other *tra*-boxes (Fuqua & Winans, 1996; Li & Farrand, 2000; Zhu *et al.*, 2000).

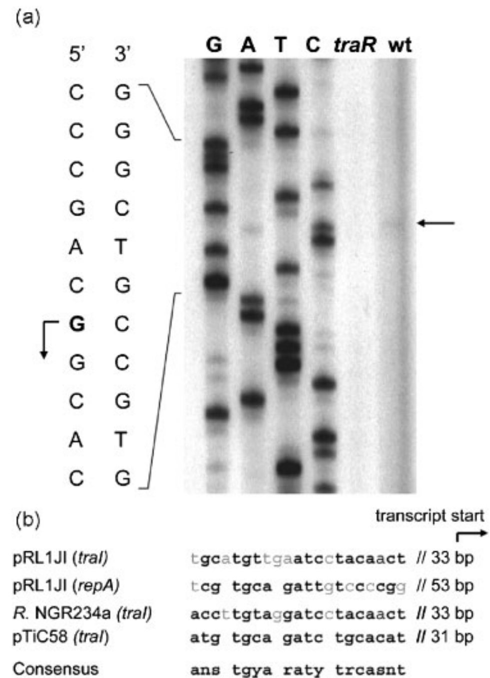

Fig. 5. Transcription initiation of *traI* and alignment of predicted *tra*-boxes. (a) The transcription start site of *traI* was determined by primer extension using total RNA isolated from A34 (wt) and A627 (*traR*::Tn*5*) after growth on TY medium containing 10 nM 3-OH-$C_{14:1}$-HSL. The primer 5′-GCGTCAACGAGCTGTCTTTCGT-GC-3′ hybridized within the coding region of *traI* (nucleotides +35 to +23 from the predicted translation start site) was also used for DNA sequencing, with pIJ9036 as template. The location of the transcriptional start and flanking sequences are indicated. (b) The predicted *tra*-boxes centred 43 bp and 63 bp upstream of the identified transcript starts of *traI* and *repA* respectively are compared with *tra*-boxes upstream of *traI* from *Rhizobium* sp. NGR234a (*R.* NGR234a), *A. tumefaciens* (pTiC58) and with a consensus *tra*-box sequence (Li & Farrand, 2000). Nucleotides conforming to the consensus are shown in bold. In the consensus n, any nt; s, g or c; y, t or c; r, a or g.

Transcription of *repA*

The expression of the *repABC* plasmid replication operon of *A. tumefaciens* virulence plasmids is regulated in response to quorum-sensing signals, with *repA* expression being enhanced by TraR and its cognate AHL (Li & Farrand, 2000; Pappas & Winans, 2003). Using RNA from A34 and the *traR* mutant A627 grown with added 3-OH-$C_{14:1}$-HSL to induce *traR* gene expression, the pRL1JI *repA* gene was found to be transcribed from two start sites, only one of which required *traR* (Fig. 6). The *traR*-independent promoter is likely to be constitutive because expression of *repA* will be necessary throughout growth.

To confirm that *repA* expression is enhanced by *traR* and its cognate AHL, a *repA–lacZ* fusion (pIJ9753) was introduced into A677 carrying *traR* (on pIJ9577) or lacking

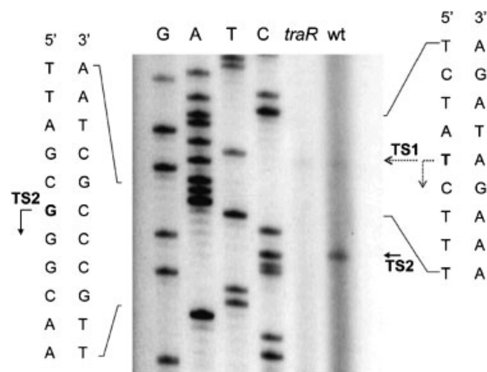

Fig. 6. Transcription initiation of *repA*. The transcription start site of *repA* was determined by primer extension using total RNA isolated from A34 (wt) and A627 (*traR*::Tn*5*) after growth on TY medium containing 10 nM 3-OH-$C_{14:1}$-HSL. The primer 5′-TTCTACTCGGTTTGCTATCGCCA-3′ from within *repA* (+63 to +40 bp relative to the predicted translation start codon) was also used for DNA sequencing, with pIJ9036 as template. The *traR*-independent and the *traR*-dependent transcript starts TS1 and TS2, respectively, are shown along with their flanking DNA sequences.

traR (containing the vector pBBR1). Expression of *repA–lacZ* on pIJ9573 in A677/pIJ9577 (carrying *traR*) was 4692±111 Miller units and was enhanced to 11910±487 units by the addition of 3-O-C_8-HSL, which activates transcription by TraR. In A677/pBBR1 (lacking *traR*), the level of *repA–lacZ* expression was 4721±303 units and was unaffected by the addition of 3-O-C_8-HSL (4644±274 units). We conclude that the AHL-independent *repA–lacZ* expression of around 4500 units is probably the result of constitutive expression of the gene from the upstream promoter (Fig. 6), and the increased expression observed when both *traR* and 3-O-C_8-HSL are present is due to induction of the downstream TraR-dependent promoter. There is a potential *tra*-box centred 63 bp upstream of the TraR-dependent transcription start site of *repA* (Fig. 5), but it does not show strong dyad symmetry, typical of many other *lux*- and *tra*-boxes.

DISCUSSION

The conjugal transfer of pRL1JI involves the consecutive action of the three LuxR-type regulators CinR, BisR and TraR, the latter ultimately inducing the expression of genes required for mating bridge formation and conjugation (Danino *et al.*, 2003). The reason for this complex cascade of regulation, compared with the relatively simple TraR-mediated gene induction in other rhizobia and agrobacteria (He & Fuqua, 2006), is presumably related to the unusual mechanism required to regulate recipient-induced plasmid transfer. This involves the induction of *traR* gene expression by BisR in donors in response to 3-OH-$C_{14:1}$-HSL made by potential recipients. The consequent induction of *traR* expression in donors leads to the induction of the *traI-trb* operon expression, and presumably the *traAFBH* and *traCDG* operons (Farrand, 1998; Zhu *et al.*, 2000), as in agrobacteria and other rhizobia.

Upstream of the *traI-trbBCDEJKLFGHI* operon on pRL1JI are two conserved *tra*-boxes similar to those identified in rhizobia and agrobacteria (Zhu *et al.*, 2000). One is centred 43 bp upstream of the transcription start site of *traI* and the other is centred 63 bp upstream of the inducible transcription start site of the divergently transcribed *repABC* genes. There are two transcript starts upstream of *repA*; one, presumably constitutively expressed, is 12 bp upstream of the predicted translation initiation codon and the other, 5 bp upstream of the translation start, is regulated by TraR in response to AHLs. Thus, as has been observed in other rhizobia and agrobacteria, induction of conjugal transfer may be coupled to an increase in plasmid copy number.

Expression of *traR* is induced by BisR in response to 3-OH-$C_{14:1}$-HSL, and this AHL is also recognized by CinR. BisR and CinR are 59 % identical at the amino acid sequence level (Wilkinson *et al.*, 2002) and therefore BisR may have arisen as a duplication of CinR, retaining similar AHL-binding characteristics. However, despite significant conservation in the DNA-binding domains, their promoter specificities must be different, because BisR cannot induce expression of *cinI* and CinR cannot directly induce expression of *traR* (Danino *et al.*, 2003; Lithgow *et al.*, 2000; Wilkinson *et al.*, 2002). In fact, BisR represses *cinI* expression (Wilkinson *et al.*, 2002). Within the *traR* promoter region, we identified a possible BisR-binding site that is conserved in the opposite orientation in the *cinI* promoter region and so this is a possible site of repression, possibly by BisR preventing access of CinR to the *cinI* promoter. Evidence of direct binding of BisR to the *cinI* promoter came from the inhibition by BisR of the low levels of *cinI* expression in the mutant A568, which has Tn*5* inserted in the *cinI* promoter. This mutant has greatly reduced *cinI* expression, probably associated with a weak promoter within Tn*5*. The *bisR* gene could repress this expression, and in this situation the simplest explanation is that the BisR-binding site is retained in this mutant. Therefore, a BisR-binding motif is predicted to be present in the *cinI* promoter downstream of the site of Tn*5* insertion. The most likely location seems to be the conserved sequence overlapping with the predicted CinR-binding site, but we cannot exclude the possibility that BisR may bind to the conserved sequence centred about 40 bp upstream of the *cinI* transcript start. The precise location of BisR binding will require DNA footprinting experiments with purified BisR.

The mechanism by which BisR can act as an inducer of *traR* but a repressor of *cinI* expression is not clear. It seems likely that the BisR repression function can occur in the absence of 3-OH-$C_{14:1}$-HSL, because BisR prevents the formation of this AHL. However, BisR-dependent

induction of *traR* absolutely requires 3-OH-$C_{14:1}$-HSL (Danino *et al.*, 2003). Possibly the binding of 3-OH-$C_{14:1}$-HSL can change the affinity of BisR for the *traR* promoter, such that in the presence of 3-OH-$C_{14:1}$-HSL BisR binds more strongly to the *traR*-type promoter.

Although the precise promoter-binding regions for BisR and CinR have yet to be defined, our data have allowed us to delimit the regions at which they are likely to interact and provide new insights into the complex mechanisms controlling plasmid replication and maintenance in rhizobia.

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