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Biochemistry

TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid *N*-acylhomoserine lactone autoinducer

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ABSTRACT **Conjugal transfer of the nopaline-type *Agrobacterium* Ti plasmid pTiC58 is regulated by a transcriptional activator, TraR, and a diffusible signal molecule, conjugation factor (CF). CF is a member of a family of substituted homoserine lactones (HSLs) that act as coinducers for regulating gene expression in diverse Gram-negative bacteria by a mechanism called autoinduction. In *Vibrio fischeri* HSL production is conferred by the *luxI* gene. Homologues of this gene are responsible for HSL production by other Gram-negative bacteria. A gene that we call *traI*, conferring production of material with CF activity, was localized to a 1-kb region at the upstream end of *tra3* of pTiC58. Spectroscopy showed that the activity was authentic CF. Sequence analysis showed that *traI* could encode a protein of 211 amino acids, TraI, that is related to the proteins responsible for HSL production by other bacteria. A second, partial open reading frame immediately downstream of *traI* could encode a protein related to TrbB of plasmid RP4, which is required for conjugal transfer. Transcription of *traI* and of the downstream *tra3* genes requires TraR and CF and initiates from the *traI* promoter. The results show that *traI* is responsible for CF production, that it is the first gene of the *tra3* operon, and that expression of this operon is regulated by autoinduction.**

Conjugal transfer of *Agrobacterium* Ti plasmids is activated by small gall-specific carbon compounds, called opines, exuded by the plant tumor cells (1). These compounds, which also can be catabolized by the agrobacteria, induce the gene systems required for their utilization, as well as for conjugal transfer. Agrocinopines A and B induce transfer of the nopaline-type Ti plasmid pTiC58 (2). Primary regulation of the transfer system and of the agrocinopine catabolism locus, *acc*, is mediated by a repressor, AccR (3), and mutations in the gene encoding this regulator result in Ti plasmids that constitutively express both functions (3).

Conjugal transfer is further regulated by a system involving cell–cell signaling. Donor bacteria induced by the conjugal opines release into the medium a diffusible signal molecule, conjugation factor (CF), that enhances the frequency at which the Ti plasmids transfer (4). Strains harboring Tra^c Ti plasmids produce CF constitutively (4), suggesting that synthesis of this signal is under control of the opine regulon.

The CF-dependent regulatory system involves a transcriptional activator called TraR (5) and is closely related to a family of autoregulatory systems present in other bacteria, including *Vibrio fischeri* (6), *Pseudomonas aeruginosa* (7), *Erwinia carotovora* (8, 9), and *Enterobacter agglomerans* (9). TraR is a homologue of LuxR, LasR, and ExpR, the autoinducing transcriptional activators of the first three organisms respectively (5, 8, 10, 11). In turn, each of these activators requires a cognate substituted homoserine lactone (HSL) (12–14). The HSLs, also called autoinducers (AIs), are synthesized and released into the medium by the bacteria themselves.

CF is *N*-(β-ketooctanoyl)-L-HSL (15) and is a homologue of the other known HSL AIs. These HSL messengers differ mainly in the length of their alkyl side chains. Synthesis of AIs by *V. fischeri* requires *luxI* (16). Genes responsible for HSL synthesis in *P. aeruginosa*, *Erwinia*, and *Enterobacter* are related, and form a conserved family with *luxI* (7–9). In this work we show that an *Agrobacterium* gene, *traI*, is sufficient to confer CF production. The predicted gene product of *traI* is related to each of the other known proteins involved in AI biosynthesis. Furthermore, we show that *traI* is the first gene of a gene cluster on the Ti plasmid required for conjugal transfer and that its transcription and that of downstream *tra* genes is regulated by TraR and CF.‖

MATERIALS AND METHODS

Strains and Plasmids. *Agrobacterium tumefaciens* strains used were C58, NT1(pTiC58Δ*accR*), which harbors an *acc*^{c}/*tra*^{c} Ti plasmid (3); NT1, which lacks a Ti plasmid; and NT1(pJM749, pSVB33), the indicator strain for detecting CF (5). Media and culture conditions were described previously (3, 17). Broad-host-range vectors were pRK415 (18), pRK415K (19), pLAFR6 (20), and pDSK519 (18).

Molecular and Genetic Techniques. Plasmid DNA was isolated as described (17). DNA sequences, determined as described (3) were analyzed using the DNA^{STAR} program (DNAstar, Madison, WI). Related sequences in the data bases were identified with the BLAST protocol (21) and aligned by using the Genetics Computer Group package (University of Wisconsin).

Mutant and Reporter Constructions. Plasmid pPLE2 was mutagenized with Tn*3*HoHo1 as described (3). The *traI*::*lacZ* reporter fusion was constructed by cloning a 1.6-kb *Eco*RI–*Hin*dIII fragment encoding the 5′ untranslated region and the first 17 aa of *traI* from pCF1 into pLKC482 (22), generating pKP14. This produced a fusion of the amino terminus of TraI and the amino end of LacZ encoded by pLKC482. A cassette containing the 5′ untranslated region of *traI* and the *traI*::*lacZ* fusion was recloned as a *Bgl* II fragment into pLAFR6 to generate pKP19.

Abbreviations: CF, conjugation factor; HSL, homoserine lactone; AI, autoinducer; ORF, open reading frame.

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‖The sequence reported in this paper (956-bp insert in pCL1) has been deposited in the GenBank data base (accession no. L22207).

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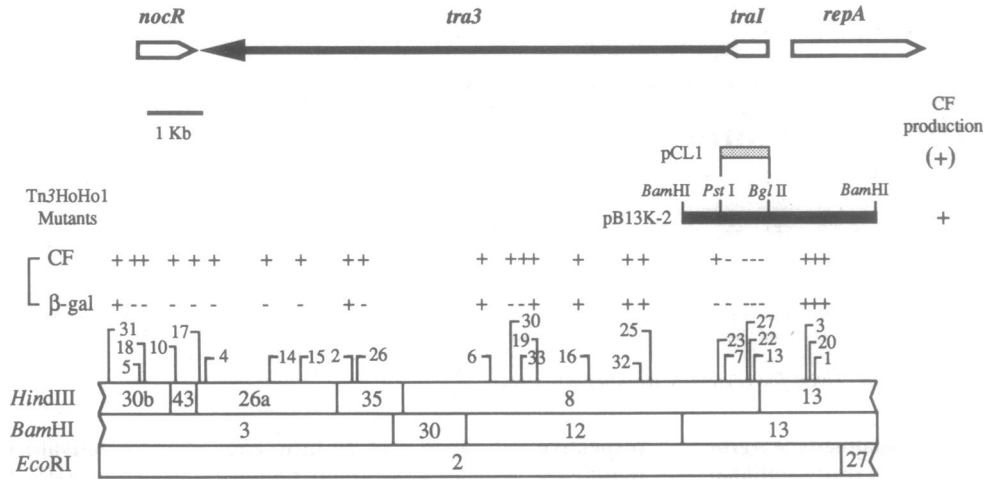

FIG. 1. Localizing and subcloning the *traI* gene from pTiC58. Map shows region of pTiC58 between the nopaline catabolism operon (*nocR*) and the replication region (*repA*). Vertical lines show sites of Tn*3*HoHo1 insertions in pPLE2. Crossbars indicate the direction of transcription reported by transposon *lacZ* gene fusions. + and − represent production of CF and β-galactosidase (β-gal) by *Agrobacterium* strains harboring each of the insertion mutants. Small filled bars represent subclones of pPLE2. (+) indicates that NT1(pCL1) produces barely detectable amounts of CF.

Conjugation Factor Assay. A plate of AB mannitol agar (17) containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside was overlaid with 3 ml of melted 0.7% agar seeded with $\approx 10^8$ cells of the CF indicator strain. Colonies of strains to be tested were patched onto the surface and the plates were incubated overnight. Production of CF was indicated by a diffuse blue zone surrounding the tested colony.

Isolation, Purification, and Identification of CF. The cells from 6 liters of a 48-hr culture in AT mannitol medium were removed and CF was extracted from the culture supernatant

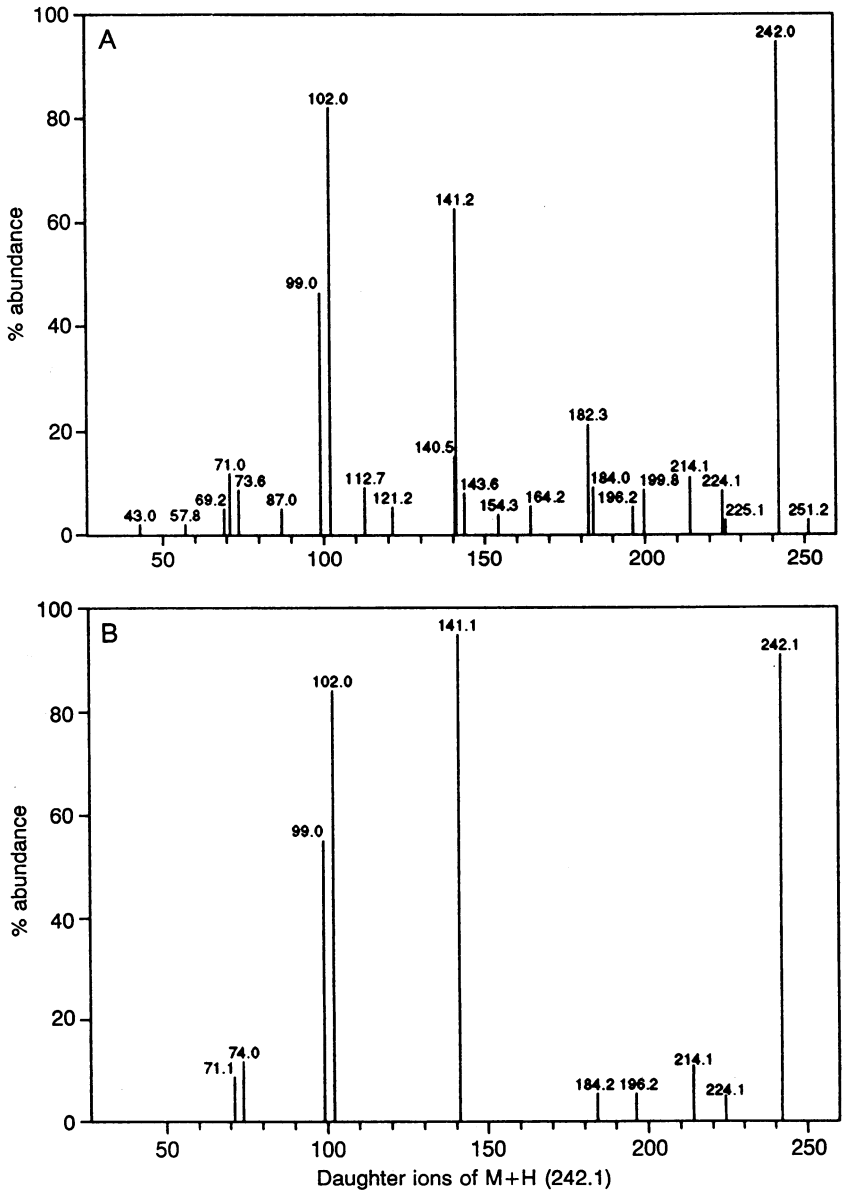

FIG. 2. MS of the CF activity produced by strain NT1(pCL1, pSVB33). (*A*) Material with CF activity isolated from the culture supernatant. (*B*) Synthetic *N*-(β-ketooctanoyl)-L-HSL (15).

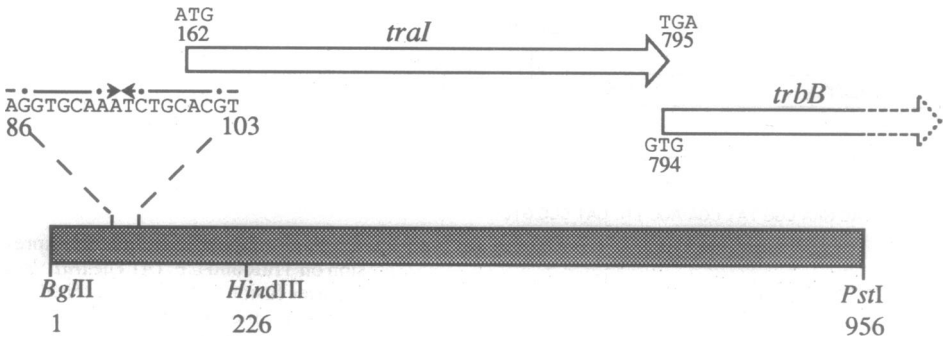

FIG. 3. Organization of open reading frames (ORFs) within the insert of pCL1. The sequence spans from the *Bgl* II site to the *Pst* I site shown in Fig. 1. The *Hin*dIII site is that which was used for the *lacZ* reporter constructs pKP19 and pHM25.

with ethyl acetate (15). The extract was concentrated *in vacuo*, dissolved in ethyl acetate, and chromatographed on silica gel with ethyl acetate. Fractions showing CF activity were pooled and subjected to reverse-phase chromatography on a C_{18} column with a linear gradient of 30–45% methanol in water (15). Active fractions were pooled and analyzed by chemical ionization (isobutane) tandem MS and by IR spectrophotometry (15).

RESULTS

Localizing and Subcloning the Gene Encoding CF Production. Strain NT1 harboring pPLE2, which contains the 14-kb *Eco*RI fragment 2 (E2) of pTiC58, produced a small amount of CF activity (Fig. 1). Fragment E2 spans the entire *tra3* region from the right side of *noc* to the left side of *oriV/rep*. Twenty-five Tn*3*HoHo1 insertions distributed over the 14-kb insert in pPLE2 were mapped and characterized (Fig. 1). Four of these insertions abolished production of CF (Fig. 1). All insertions producing fusions with the *lacZ* reporter oriented in the anticlockwise direction showed weak β-galactosidase activity on medium containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Fig. 1). Strains with insertions oriented in the opposite direction did not produce detectable β-galactosidase.

The region defined by the CF^- insertion mutations was subcloned as a 3.8-kb *Bam*HI fragment into pRK415K to generate pB13K-2 (Fig. 1). Strain NT1(pB13K-2) produced small amounts of CF activity (Fig. 1). A 1-kb *Pst* I–*Bgl* II fragment, when subcloned from pB13K-2 into pRK415 to produce pCL1, conferred barely detectable levels of CF production on strain NT1 (Fig. 1).

Regulation of CF Production. That strains harboring pPLE2 and its subclones produce very small amounts of CF suggests that other functions encoded by the Ti plasmid are required for maximum production of this AI. When pSVB33, which expresses *traR* (5), was introduced into NT1 harboring pPLE2, pB13K-2, or pCL1, the resulting strains produced substantially larger amounts of CF (data not shown).

The Active Agent Is Authentic CF. An ethyl acetate extract from culture supernatants of NT1(pCL1, pSVB33) was fractionated by chromatography and analyzed by IR spectroscopy and tandem MS. The biologically active material produced a subset of MS peaks representing the parent ion and its daughter fragments that were indistinguishable from those of synthetic CF (Fig. 2). IR spectroscopy produced signals at 1780, 1714, 1646, and 1540 cm^{-1} characteristic of β-ketoacyl substituents (data not shown).

DNA Sequence Analysis. Sequence analysis showed the *Pst* I–*Bgl* II insert in pCL1 to be 956 bp long (Fig. 3). The fragment encodes a 633-bp ORF initiating at an ATG at bp 162 and terminating at a TGA at bp 795. The orientation of this ORF corresponds to an anticlockwise transcriptional direction on the Ti plasmid. The ORF is preceded by sequences similar to the canonical ribosomal binding site and by −10 and −35 promoter elements. An 18-bp inverted repeat showing almost perfect dyad symmetry is located 61 bp upstream from the putative start codon (Fig. 3).

The ORF, *traI*, could encode a 211-aa protein of 23,436 Da. Pairwise amino acid alignments indicate that the product of the ORF, TraI, is ≈30% identical and ≈50% similar to LuxI (10), LasI (7), ExpI (8), CarI (9), and EagI (9) (data not shown). Overall, the six proteins show ≈45% conservation (Fig. 4*A*).

Expression of *traI* Requires CF. pKP19 contains a chimeric reporter gene carrying the promoter region and a small portion of the *traI* structural gene translationally fused to *lacZ* (Fig. 5*A*). Strains NT1(pKP19) and NT1(pKP19, pSVB33) did not produce detectable CF activity, indicating that the fusion disrupts the *traI*-encoded function (results not

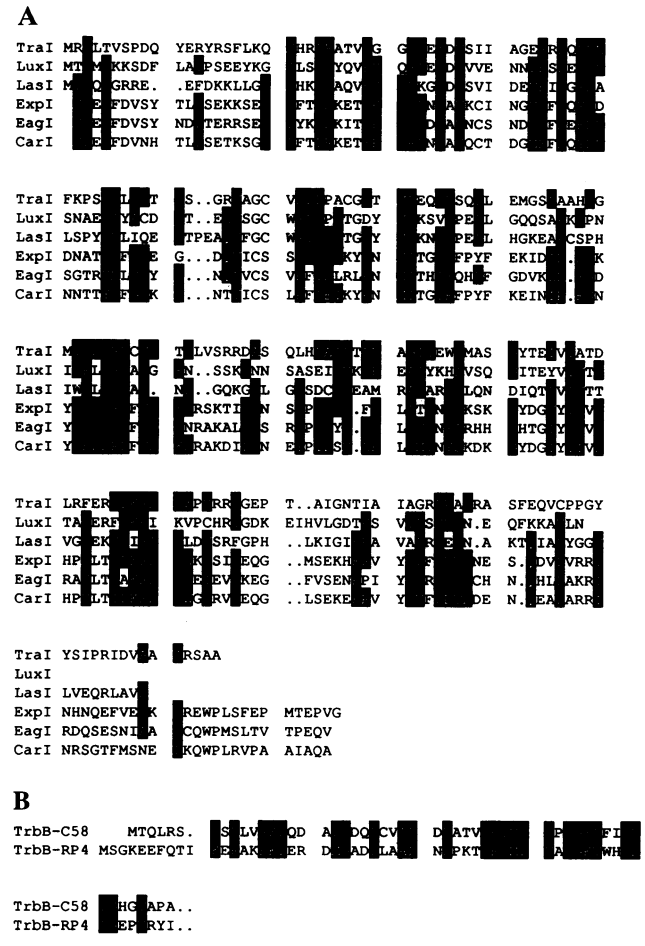

FIG. 4. Relatedness of proteins translatable from the ORFs encoded by the insert in pCL1. Identities and conservative changes are shown as white-on-black type. (*A*) TraI and the five known homologues. Conservations at a given position in at least four of the six proteins were considered significant. (*B*) TrbB of pTiC58 and TrbB of RP4.

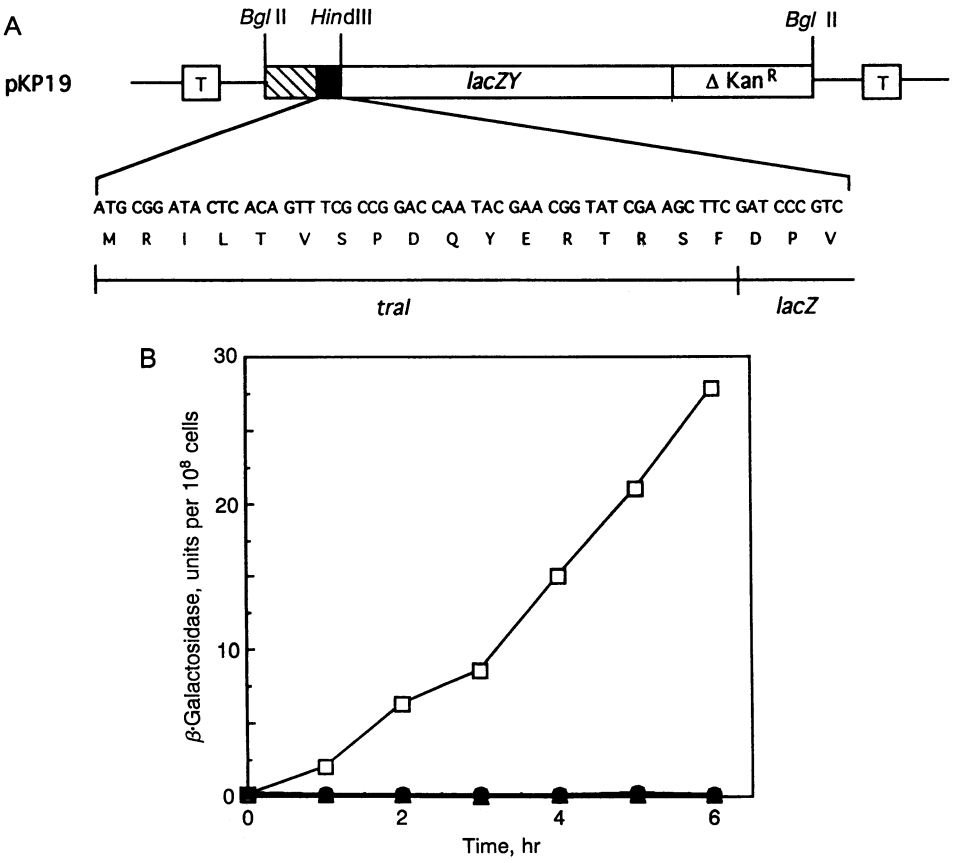

FIG. 5. Dependence of *traI* expression on TraR and CF. (*A*) The *traI*::*lacZ* reporter fusion in pKP19. Hatched box represents the 161 bp of the *traI* promoter region. Black box represents the first 17 codons of the *traI* structural gene. DNA and amino acid sequences of the fusion region are shown below the map. Boxes labeled T indicate transcriptional terminators flanking the cloning site in pLAFR6. (*B*) Induction of the *traI*::*lacZ* fusion in *Agrobacterium*. Cultures of NT1 harboring the indicated plasmids were grown to mid-exponential phase in AB mannitol medium (17). Each culture was split in half, and to one subculture was added a crude preparation of CF (5). Incubation was continued, samples were removed at intervals, and the cells were assayed for β-galactosidase (3). □, NT1(pSVB33, pKP19) plus CF; ◆, NT1(pSVB33, pKP19); ◇, NT1(pKP33ΔEc, pKP19); ■, NT1(pKP33ΔEc, pKP19) plus CF. The last three curves all cluster at the base line.

shown). These two strains also failed to produce detectable β-galactosidase activity (Fig. 5*B* and data not shown). Addition of CF to a culture of strain NT1(pKP19, pSVB33) resulted in rapid induction of the reporter gene (Fig. 5*B*). Added CF had no such stimulatory effect on reporter gene induction by strain NT1(pKP19, pKP33ΔEc), in which *traR* is inactivated by a deletion (5) (Fig. 5*B*).

***traI* Is Linked to the *tra3* Region.** An incomplete ORF with a GTG initiation codon at bp 794 is located immediately downstream from *traI* (Fig. 3). The putative protein encoded by this ORF is related to TrbB of plasmid RP4 (Fig. 4*B*; ref. 23). The two proteins show 39% amino acid identity and 48% similarity over the 54 residues for which sequence of the Ti plasmid gene is available.

This second ORF lacks recognizable 5′ transcriptional initiation signals and the GTG codon overlaps the *traI* translational termination codon, suggesting that this ORF and the downstream *tra3* genes are expressed from the *traI* promoter. To test this we examined the levels of β-galactosidase expressed from Tn*3*HoHo1 insertion 25 (Fig. 1). Two constructs were tested. The first, pPLE2-25, contained *traI* and its entire 5′ promoter region. The second, pHM25, was constructed by cloning *Hin*dIII fragment 8 from pPLE2-25 into pRK415. This fragment spans from codon 18 of *traI* to the middle of *tra3*, contains the entire Tn*3*HoHo1 insertion, but lacks the *traI* transcriptional regulatory region (Fig. 5*A*). pPLE2-25 expressed high levels of β-galactosidase activity when present in trans to pTiC58Δ*accR* (Table 1). It expressed only low levels of the enzyme when harbored by strain NT1 or when in trans to wild-type pTiC58. pHM25 did not express detectable β-galactosidase activity in any of the strains tested, indicating that it lacks the promoter that initiates transcription of the gene to which *lacZ* is fused (Table 1).

DISCUSSION

Our results show that a Ti plasmid-encoded gene, *traI*, is responsible for production of CF, the *Agrobacterium* conjugation signal (4). Moreover, the activity whose production is conferred by *traI* of the nopaline/agrocinopine-type plasmid pTiC58 is *N*-(β-ketooctanoyl)-L-HSL. This molecule is identical to the CF produced by *Agrobacterium* strains harboring octopine/mannityl opine-type Ti plasmids (15). Thus it is likely that the *traI* genes of the two Ti plasmid types are closely related. Consistent with this, *traI* of pTiC58 maps to a region of this Ti plasmid that exhibits strong homology with an octopine/mannityl opine-type Ti plasmid (24). From this we predict that the *traI* gene of the latter class of plasmids is located between the *moc* locus and the replication region.

traI is a member of a family of genes present in several Gram-negative bacteria that are responsible for production of structurally similar HSL signal molecules. Alignments of the amino acid sequences encoded by these genes indicate that TraI is no more related to one member of the family than it is to any of the others (Fig. 4*A*). All six proteins show about 45% relatedness (Fig. 4*A*), with the conserved regions distributed as discrete domains over most of the protein sequence.

The HSL AIs act, along with their cognate activator proteins, as coinducers for transcription of autoinducible gene systems (5–8). In *V. fischeri*, expression of the *lux* AI biosynthesis gene *luxI* is dependent upon the activator protein LuxR (25). Similarly, expression of *traI* requires the Ti

Table 1. Expression of *tra3*::*lacZ* reporter fusions is dependent upon untranslated sequences upstream of *traI*

Reporter clone	Host strain	β-Galactosidase*
pPLE2-25	NT1	3
	NT1(pTiC58)	8
	NT1(pTiC58Δ*accR*)	469
pHM25	NT1	<1
	NT1(pTiC58)	<1
	NT1(pTiC58Δ*accR*)	<1

*Activity units per 10^9 cells.

plasmid-encoded activator protein TraR (Fig. 5). Furthermore, analogous to the *lux* system, TraR-dependent transcription of *traI* requires the *Agrobacterium* AI.

The *traI* gene in pCL1 is expressed at high levels only when provided with TraR and CF (Figs. 1 and 5). This indicates that the functional operator/promoter region for this gene is contained within the 161-bp upstream of the initiation codon present in the clone. This region contains an 18-bp inverted repeat (Fig. 3) that is almost identical to an inverted repeat located within the *oriT* region of this Ti plasmid (19). This latter sequence lies in the promoter region of two divergently transcribed *tra* operons that are regulated by TraR/CF (D.M.C., I.H., and S.K.F., unpublished work). The *lux* and *lasB* promoters each contain a 20-bp imperfect inverted repeat sequence showing dyad symmetry (26, 27) that is believed to be important for activator/AI-mediated induction. Interestingly, these inverted repeats and those in the two Ti plasmid *tra* regions share significant sequence similarities (Fig. 6).

Several lines of evidence indicate that *traI* is the first gene of an operon contained within the *tra3* region of the Ti plasmid. (*i*) An ORF is located immediately downstream from *traI* (Fig. 3) whose initiation codon overlaps the *traI* terminator, suggesting that the two genes are part of the same transcriptional unit. This is consistent with our observation that a *lacZ* fusion to a gene downstream from *traI* requires the *traI* promoter for expression (Table 1). (*ii*) The downstream ORF could encode a protein that is related to the product of *trbB*. This gene is required for conjugal transfer of the broad-host-range plasmid RP4 (23). (*iii*) Mutational analysis indicates that the entire 10-kb segment lying between *noc* and *oriV/rep* is required for Ti plasmid conjugal transfer (ref. 17; P.-L.L. and S.K.F., unpublished results). Expression from *lacZ* fusions predicts that the *tra* genes in this region all are transcribed in the anticlockwise direction and that they are regulated by TraR/CF-dependent autoinduction (Fig. 1; P.-L.L. and S.K.F., unpublished results).

The organization of the *traI–tra3* operon is similar to that of the *luxICDABEG* operon of *V. fischeri* (26). In both, the determinant conferring synthesis of the AI is the first gene of the expression unit. Moreover, in each case, expression of the operon is dependent upon the cognate activator and the HSL coinducer. Thus, in two unrelated organisms, there is conservation in the overall organization of two sets of autoinducible genes encoding very different functions. It may be that placement of the gene encoding synthesis of the AI at the 5′ end of the operon is important to the proper functioning of autoinducible gene systems.

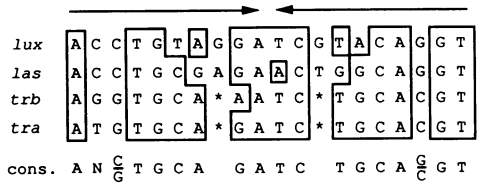

FIG. 6. Alignments of the inverted repeats present in the promoter regions of the *V. fischeri lux* operon, *P. aeruginosa lasB*, and the two *tra* regions of pTiC58. cons., Consensus.

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