The Antiactivator TraM Interferes with the Autoinducer-dependent Binding of TraR to DNA by Interacting with the C-terminal Region of the Quorum-sensing Activator*

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Conjugal transfer of Agrobacterium tumefaciens Ti plasmids is regulated by quorum sensing via the transcriptional activator TraR and the acyl-homoserine lactone Agrobacterium autoinducer (AAI). Unique to this system, the activity of TraR is negatively modulated by an antiactivator called TraM. Analyses from yeast two-hybrid studies suggest that TraM directly interacts with the activator, but the conditions under which these components interact and the region of TraR responsible for this interaction are not known. Induction of traM in a strain in which TraR was activating transcription of a reporter system led to rapid cessation of gene expression. As assessed by a genetic assay that measures AAI-dependent DNA binding, TraM inhibited TraR function before and after the transcription factor had bound to its DNA recognition site. Consistent with this observation, in gel retardation assays, purified TraM abolished the DNA binding activity of TraR in a concentration-dependent manner. Such inhibition occurred independent of the order of addition of the reactants. As assessed by far Western analyses TraM interacts with TraR by directly binding the activator. TraM in its native form interacted with native TraR and also with heat-treated TraR but only when SDS was included with the denatured protein. TraM interacted with TraR on blots prepared with total lysates of cells grown in the presence and absence of AAI. Far Western analysis of N- and C-terminal deletion mutants localized a domain of TraR contributing to TraM binding to the C-terminal portion of the activator protein. Random mutagenesis by hydroxylamine treatment and error-prone polymerase chain reaction identified several residues in this region of TraR important for interacting with TraM as well as for transcriptional activation of or DNA binding. We conclude that TraM inhibits TraR by binding to the activator at a domain within or close to the helix-turn-helix motif located at the C terminus of the protein.

Many bacteria control the expression of certain gene sets by quorum sensing, a regulatory strategy that ties gene expression to the size of the bacterial population. Quorum sensing is dependent upon a diffusible signal molecule called the autoinducer, which in Gram-negative bacteria often is an acylated homoserine lactone (acyl-HSL1). The signal, which the bacteria produce themselves, accumulates in the environment as the cells grow (1, 2). At a certain threshold concentration that corresponds to a critical cell density, the autoinducer interacts with its cognate receptor, resulting in expression of the target genes.

In Agrobacterium tumefaciens, the transcriptional activator TraR and its ligand Agrobacterium autoinducer (AAI, N-3-oxooctanoyl-3-homoserine lactone) regulate in a quorum-dependent manner the expression of the three operons responsible for conjugational transfer of the Ti plasmids (3–6). This system, in turn, is controlled by a hierarchical cascade in which the expression of traR itself is regulated by opines, metabolites produced by the plant tumors induced by the bacteria (4, 7, 8). In the nopaline-type Ti plasmid pTiC58, traR is part of the arc operon, a group of five genes that is regulated by AccR, the repressor that responds to agrocinopines A and B (8, 9). Purified TraR from the octopine-type Ti plasmid pTiR10 binds specifically to a cis-acting recognition site, the 18-base pair tra box, adjacent to promoters of genes regulated by quorum sensing (10). That such activity was dependent upon growth of the cells with AAI suggested that the signal is required for TraR to bind DNA. Consistent with this, in a genetic assay that measures DNA binding, TraR from the nopaline-type Ti plasmid pTiC58 bound the tra box only when the cells were cultured with AAI (11).

In addition to opine regulation, transcriptional activation by TraR is negatively modulated by the product of traM (12, 13), a component critical for quorum sensing but not for TraR-mediated autoinduction of the tra and trb operons (14). All identified traM genes encode an 11.2-kDa protein with a highly hydrophobic region located at the C terminus. Null mutations in traM result in constitutive conjugation even at low population density (14).

Addition of excess exogenous AAI does not overcome the inhibitory effect of TraM on TraR function under normal conditions (12, 15). Rather, inhibition of TraR activity by TraM is dependent upon the relative levels of these two proteins (12). In addition, TraM does not directly affect expression of traR or the tra and trb genes (12). These observations led Hwang et al. (12) to propose that TraM exerts its effect by directly interacting with TraR. Genetic analyses using the yeast two-hybrid system are consistent with this model (16).

Taken together, these results indicate that TraM functions

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as an antiactivator, a class of regulatory proteins that exert their inhibitory effect by interacting with and deactivating their corresponding transcription factor. However, several aspects concerning the interactions between TraM and TraR remain to be determined. First, there is no physical evidence supporting the hypothesis that these two proteins interact with each other. Second, it is not known whether TraM exerts its inhibitory effect before TraR binds DNA or if it can actively interfere with transcriptional initiation. Third, the direct consequence on TraR upon interaction with TraM remains obscure. Finally, it is not clear what role, if any, AAI plays in the interaction between TraR and TraM. In this paper, we have combined genetic analyses with biochemical tests to assess the influence of TraM on TraR activity and to analyze interactions between TraR and this antiactivator. We show that TraM negatively influences the DNA binding activity of TraR and that the antiactivator exerts its influence irrespective of whether TraR has bound to its target DNA site. Furthermore, by far Western analysis, we show that TraM binds TraR and that binding is independent of AAI. Finally, using a combination of genetic and biochemical analyses, we show that TraM binds the C terminus of TraR and that amino acid residues of TraR involved in interacting with TraM are critical for DNA binding and perhaps also for transcriptional activation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**

Unless otherwise specified, *Escherichia coli* strains DH5α, BL21(DE3)(pLysS) (Novagen), and their derivatives were grown at 37 °C in L broth (LB), on L agar plates, or in A medium (17). *A. tumefaciens* strain NT1 and its derivatives were grown at 28 °C in LB, MG/L (18), or ABM minimal medium (19). Plasmids were maintained by including the appropriate antibiotics in the media at concentrations specified previously (20). When necessary, cell growth was monitored by Klett colorimetry (red filter) or by measuring optical density at 600 nm (OD 600) using a Spectronic 20. To induce expression from Polo, either Ppolo or Ppolo- arabinose or IPTG was added at a final concentration of 0.4% or 200 μM, respectively. Unless otherwise specified, synthetic AAI, prepared as described previously (21), was added to a final concentration of 25 μM. X-Gal was included in agar medium at 40 μM to detect β-galactosidase.

**Plasmid Construction**

Plasmids that allow independent induction of TraR and TraM were constructed by cloning the open reading frames of these two genes into expression vectors pKK38-1(22) and pLB4 (20) to generate pKKTR2-I and pDLB-M. Expression of these two genes is inducible by IPTG and arabinose, respectively. Precise deletions were constructed by PCR using *Pfu* DNA polymerase (Stratagene) and trara cloned in pLQR (11) as template. For N-terminal deletions, primer 5′-ATGCTCAGTTTACATGTGGTTACCTG-3′ that contains the native translational stop codon (underlined antiparallel strand) was coupled with N-terminal primers containing an in-frame ATG as part of an NdeI site to amplify fragments coding for versions of TraR initiating at successively later positions. For C-terminal deletions, primer 5′-CGTCTCAGTTTACATGTGGTTACCTG-3′ that contains the native translational ATG (italicized) in the form of an NdeI site (underlined) was coupled with C-terminal primers containing a TGA stop codon placed to give versions of TraR terminating at successively earlier positions. In all cases, PCR products were cloned into the expression vector pZLQ (11) as NdeI/EcoRI fragments, and the constructs were confirmed by DNA sequence analysis.

To reconstitute the N terminus of TraR-insensitive mutants of TraRN2−4, mutated regions of pZLQ derivatives were isolated by digesting with NotI, a restriction enzyme that recognizes two sites, one located in the middle of trar and the other in the vector distal to the 3′ end of the gene. The recovered DNA fragments were cloned into a vector harboring the 5′ end through the NotI site of wild-type trar. Clones with the 3′ mutant half of trar inserted in the correct orientation were identified by restriction analysis. A similar strategy was used to place the 3′-half of several C-terminal deletion and missense mutants of trar into traRN2−4.

**Mutagenesis**

The N-terminal deletion mutant trarRN2−4 (11) was subjected to chemical and error-prone PCR-mediated random mutagenesis according to previously described protocols (23, 24). PCR products were cloned into pZLQ as NdeI/EcoRI fragments.

Random 5′ deletion mutagenesis of trar was carried out on pDLR1, a derivative of pDSK519 (25) carrying a copy of trar downstream from several foreign sites suitable for conducting DNA-mediated unidirectional digestion (26). Deletions were produced using the Erase-a-Base system (Promega) following the manufacturer's instructions.

**Protein Purification**

Native active TraR was purified from *E. coli* BL21(DE3)(pLysS) harboring pETR, a derivative of pET17-b (Novagen) containing trar, the expression of which is under the control of the T7 promoter. This strain was grown at 28 °C in 1 liter of a medium (17) containing 100 μg AAI, 200 μg/ml ampicillin, and 34 μg/ml chloramphenicol. When the OD600 of the culture reached 0.3–0.4, expression of TraR was induced by adding IPTG to a final concentration of 200 μM. Cells were harvested following overnight induction and were stored at −80 °C. Approximately 4 g of cells were thawed, resuspended in 20 ml of TEDGT buffer (50 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 0.15 mM NaCl, 1 mM dithiothreitol, 5% glycerol, and 0.05% Tween 20) containing 10 units/ml DNase, and were broken by two passages through a French press at 10,000 pounds/square inch. Active TraR was purified from the lysate essentially as described by Zhu and Winans (10). Samples first were chromatographed on a heparin-Sepharose affinity column (Amersham Pharmacia Biotech, 16 mm × 20 cm). TraR was eluted from the column with 100 μl of a linear gradient of NaCl from 0.15 to 1.0 M in TEDGT buffer. Fractions containing TraR, as detected by anti-TraR antibodies raised against affinity-purified His-tagged activator protein, were pooled and desalted by ultrafiltration (Amicon). The protein was further purified by fast protein liquid chromatography using a Mono-S column (HR 5/5, Amersham Pharmacia Biotech). TraR was eluted from the column with 30 ml of a linear gradient of NaCl from 0.15 to 1.0 M in TEDGT buffer. (His)6-TraR was purified from BL21(DE3)(pLysS, pMA2) (16) according to the protocol of the Ni2+ resin manufacturer (Novagen). The two proteins were better than 95% pure as assessed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Protein concentration was determined by the Bradford method using the Coomassie Plus Protein Assay Reagent (Pierce). Purified TraR and (His)6-TraR were stored at −20 °C in TEDGT buffer containing 50% glycerol.

**In Vivo Assay of TraR Activities**

*E. coli* strain DH5α(pPBL1, pKKTR2-I, pDLB4-M) was constructed to analyze the effect of TraR on AAI-dependent DNA binding activity of TraR. Plasmid pPBL1 reports the AAI-dependent repressor activity of TraR (11), and pKKTR2-I and pDLB4-M harbor trar and traM inducible by IPTG and arabinose, respectively. An overnight culture was diluted 1:200 into fresh A medium, and the culture was incubated at 37 °C for 1 h. Synthetic AAI was added after taking the first sample (t0), and the culture was incubated as above. After the OD600 of the culture reached about 0.05, the culture was divided into the necessary number of subcultures, and inducers for trar and traM expression were added at appropriate times. Cultures were incubated as before; growth was monitored, and samples were taken at different time intervals following addition of the inducers.

*A. tumefaciens* strain NT1(pPKHLH4I41, pDLB4-M) was used to examine the effect of TraR on activation of transcription by TraR. Plasmid pPKHLH4I41 contains a copy of constitutively expressed wild-type trar and a trar::lacZ fusion, the expression of which is dependent upon TraR and exogenous AAI (11). An overnight culture of this strain was diluted 1:200 into ABM medium, and the new culture was incubated at 28 °C with shaking. When the culture density reached an OD600 of about 0.05 (t0), a sample was removed, and synthetic AAI was added to a final concentration of 25 μM. The culture was divided into the appropriate number of subcultures; incubation was continued as above, and arabinose was added at the indicated times to induce expression of traM from pDLB4-M. Samples were withdrawn at set time intervals, and the expression level of the trar::lacZ reporter was determined.

**Gel Retardation Assays**

The 251-base pair trara-traC intergenic region of pTiC58 (27) was amplified from pZLB251 (11) by PCR using primers 5′-GGGTGATCA-GAGGCTCGTCTGCTGCCGGAGGCTGGAGG-3′ and 5′-TACCAGGGT-CGCAATCCCTCGGAAATCCCTGCGCC-3′. This region contains the entire TraR-AAI dependent divergent tra promoter system (27, 28).
Purified PCR product was labeled at the 3' ends with digoxigenin-11-ddUTP and terminal transferase using protocols provided by the supplier (Roche Molecular Biochemicals). For the DNA binding reaction, purified TraR was incubated with the digoxigenin-3'-end-labeled DNA fragment (2 ng) in a buffer containing 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 60 mM KCl, 30 μg/ml salmon sperm DNA, 20 μg/ml BSA, 0.05% Tween 20, and 10% glycerol in a total volume of 20 μl. Reactions were incubated at room temperature for 20 min. After adding 5 μl of loading buffer, the samples were subjected to electrophoresis at 4 °C on a native 6% polyacrylamide gel in 0.25× TBE buffer (1× TBE: 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). Following electrophoresis, DNA and protein-DNA complexes were electroblotted onto positively charged nitrocellulose membranes and visualized by chemiluminescence detection as described by the manufacturer's protocols (Roche Molecular Biochemicals). Three approaches were used to test the effect ofTraM on the DNA binding activity of TraR. As described above, all reactions were performed in a volume of 20 μl. In the first, labeled DNA was added to reaction mixtures containing 1 pmol of TraR (final concentration of 50 nM) and 10–240 pmol of TraM (final concentration of 1–12 μM TraM) and incubated for 20 min. In the second, DNA was added to reaction mixtures containing 100 pmol of TraR (final concentration of 5 μM) and 0.5 pmol of TraM (final concentration of 25 nM) that had been coincubated for 1–20 min. In the third, 100 pmol of TraR (final concentration of 5 μM) and DNA had been coincubated for 1–20 min. All reactions were incubated at room temperature for an additional 20 min, subjected to electrophoresis, and analyzed by electroblotting and chemiluminescence as described above.

**Western Blots**

Immunoblots were carried out using murine anti-(His)_6-TraR antibodies. To prepare cell lysates for such assays, overnight cultures in M9/L medium (18) were diluted 1:15 into fresh M9/L, and the cultures were incubated at the appropriate temperature with shaking. When the OD_600 of the culture reached about 0.3, IPTG was added; incubation was continued, and the cells were harvested when the OD_600 reached about 1.0. When needed, AAI was added at the same time as IPTG. Cells were resuspended in one-tenth of the culture volume of SDS loading buffer (29), and the cell suspension was boiled for 5 min. Cell debris was removed by centrifugation (10 min, 4 °C) in an Eppendorf microcentrifuge (Centrifuge 5402), and 18 μl of supernatant was loaded onto a 15% SDS-PAGE gel. Following electrophoresis, proteins were electroblotted onto nitrocellulose membranes. After 1 h blocking using PBS buffer (29) containing 5% non-fat milk, membranes were incubated for 2 h in the same buffer containing anti-TraR antibodies. Antibody-protein complexes were visualized using alkaline phosphatase-coupled anti-murine second antibody (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine salt and nitro blue tetrazolium chloride (Life Technologies, Inc.) as substrates.

**Far Western Analysis**

**Dot Blots—**All incubations and reactions were conducted at room temperature. Native or heat-denatured (95 °C, 5 min) TraR in TEDGT buffer was spotted onto nitrocellulose membranes, and the samples were allowed to dry in air. After blocking for 30 min with PBS buffer (29) containing 5% non-fat milk, the membranes were incubated for 20 min in blocking buffer containing native or heat-denatured (His)_6-TraR at a final concentration of 45 nM. Following three washes with PBS buffer (1 ml/cm², 10 min each), the membranes were incubated for 2 h in blocking buffer containing anti-TraM antibodies (16). Reacting complexes were visualized using alkaline phosphatase-coupled anti-murine second antibody (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine salt and nitro blue tetrazolium chloride (Life Technologies, Inc.) as substrates.

**β-Galactosidase Assay**

β-Galactosidase activity, expressed as units/ml culture or as units/10^9 colony-forming units (cfu) was measured as described previously (12, 17).

**DNA Sequence Analysis**

Mutations were identified by double strand DNA sequence analysis conducted by the Genetic Engineering Facility at the University of Illinois.

**RESULTS**

**TraM Abolishes the Repressor and Activator Activities of TraR—**By using a genetic assay that measures gene repression as an indicator of AAI-dependent DNA binding activity of TraR (11), we examined the characteristics and in vivo kinetics of TraM inhibition of TraR activity. With the reporter strain DH5α(pPBL1, pKKTR2-I, pDLB4-M), expression of traR is inducible by IPTG, whereas expression of traM is inducible by arabinose. In a subculture to which no inducer was added, the reporter gene expressed constitutively, leading to the accumulation of β-galactosidase (Fig. 1, panel A). When only traR was induced, the expression of the reporter was strongly repressed (Fig. 1, panel A). However, when traR and traM were induced simultaneously or when traM was induced before traR, no repression was observed (Fig. 1, panel A). Moreover, in a culture in which traR had been induced previously, induction of traM led to the derepression of the reporter gene within 30 min (Fig. 1, panel A). Induction of traR alone had no effect on the expression of the reporter gene, indicating that the antiactivator has no repressor activity of its own (Fig. 1, panel A). The ability of TraM to abolish repression by TraR suggested that in an activation assay induction of TraM would prevent further expression of genes activated by TraR. Such was the case; in a strain that contains a traG3a::lacZ reporter fusion, and a plasmid coding for constitutively expressed traR and traM expressed from P_BAD, the antiactivator significantly inhibited the TraR-dependent expression of the reporter gene within 1 h after induction by addition of arabinose (Fig. 1, panel B).

**TraM Interferes with DNA Binding by TraR—Results from these in vivo tests predicted that TraM could abolish binding between TraR and its DNA recognition site. We examined this hypothesis by determining the effect of TraM on the DNA binding activity of TraR using a gel retardation assay. As shown in Fig. 2, panel A, when mixed with target DNA, TraR caused a retardation in the mobility of the DNA probe presumably by forming a protein-DNA complex. Addition of TraM to the binding reactions prevented the activator from forming these complexes. Moreover, the inhibitory effect of TraM on the formation of such complexes was dependent upon the relative amounts of these two proteins. Under our experimental conditions, a ratio of TraM to TraR of 20:1 detectably reduced the formation of TraR-DNA complexes (Fig. 2, panel A). As the ratio was increased to 80:1, TraR-DNA complexes became undetectable (Fig. 2, panel A). In reactions in which labeled DNA was added into mixtures containing TraR and TraM that had been coincubated for 1–20 min, no detectable TraR-DNA complex was observed (Fig. 2, panel B). Furthermore, similar to observations made in our in vivo analysis in which TraM relieved repression of the reporter gene (Fig. 1, panel A), addition of TraM to reactions in which TraR and DNA had been coincubated for periods ranging from 1 to 20 min disrupted the TraR-DNA complexes (Fig. 2, panel B). Incubation of TraM alone with the same DNA probe gave no complexes detectable by gel retardation analysis (data not shown).

**TraM Interacts with TraR by Direct Binding—**We employed far Western analysis to assess the capacity of TraM to bind TraR in vitro. Blocked nitrocellulose membranes onto which solutions with decreasing amounts of purified active TraR had been spotted were incubated with TraM protein and then with anti-TraM antiserum. Under such conditions, TraM strongly bound to TraR on these dot blots but not to BSA or to any protein species in lysates of A. tumefaciens NT1 (Fig. 3 panels).
cells were assayed for 

Samples were removed at the indicated times from all cultures, and the 

containing AAI, and all of the cultures were incubated in parallel. 

(0.4%) to induce TraM expression was established from the culture 

f 

3 M), and incubation of both was continued. At 0- ( 

DH5 

TraM inhibits repression by TraR. The reporter strain 

Panel A, TraM inhibits repression by TraR. The reporter strain 

and data not shown). The intensity of binding varied 

II, and data not shown). The intensity of binding varied with the amount of TraR loaded on the filter (Fig. 3, panel II). 

Under the conditions tested TraM routinely gave a positive signal with spots containing as little as 15 fmol of native TraR. Identiﬁcal blots probed with a control solution lacking TraM gave no detectable signal when challenged with the anti-TraM antibodies, indicating that the signal was not due to cross-recognition of TraR by anti-TraM antibodies (data not shown). Similar results were obtained when TraR was used to detect 

TraM fixed on the membrane (data not shown). As interactions between proteins often require that the partners be properly folded, we tested preparations of TraR, TraM, or both proteins that had been denatured by heat treatment. Heat-denatured TraM failed to bind to detectable levels to blots containing heat-denatured TraR (Fig. 3, panel I, D). However, heat-denatured TraR was bound, although poorly, by native TraR (Fig. 3, panel I, C). Interaction between heat-denatured TraM and native TraR also was detectable, but the signal was very weak (Fig. 3, panel I, B). Incubating membranes spotted with heat-denatured TraR under conditions that could allow protein renaturation (30) did not restore binding by native TraM (data not shown).

To develop an assay suitable for examining the interaction between TraM and mutants of TraR that cannot be puriﬁed in active form by current procedures (see “Experimental Procedures”), we assessed the capacity of TraM to bind to TraR transferred to nitrocellulose membranes following SDS-PAGE. TraM gave a detectable signal with as little as 0.1 pmol (26 ng) of TraR by this SDS-PAGE-based far Western analysis (Fig. 4, panel B). However, no comparable signals were detected when TraR was used as the challenging protein to detect similarly resolved TraM (data not shown). Furthermore, TraM binds to TraR present in lysates of cells following electrophoresis and
Native TraM interacts strongly with heat-denatured TraR following SDS-PAGE, but only weakly when examined by dot blot analysis (Fig. 4 and Fig. 3, panel I, C). However, when SDS was included in the TEDGT buffer at 0.1%, whether before or after denaturation, heat-treated TraR dot-spotted to membranes was bound by TraM at levels even higher than that of native TraR (compare rows A and B of Fig. 3, panel II).

**Interaction between TraM and TraR Does Not Require AAI**—As active TraR is tightly associated with AAI (10), it was unclear whether binding this acyl-HSL signal molecule is a prerequisite for interaction with TraM. The ability of TraM to bind TraR in cell lysates provided us with a simple and reliable way to dissect the interactions between these two proteins without the need to purify the activator. We tested for dependence of TraM binding on the autoinducer by probing for TraR present in cells grown with or without AAI. TraR present in lysates of both cultures interacted with TraM with indistinguishable intensities using both dot blot and gel blot analyses (Fig. 4, panel C, and data not shown).

**TraM Binds to the C-terminal Region of TraR**—We localized the domain of TraR to which TraM binds by subjecting a series of N- and C-terminal deletion mutants of the activator to far Western analysis. Deletion mutants of TraR lacking as few as 4 amino acids from the N terminus no longer repress or activate appropriate reporter fusions (11). Furthermore, these mutants are recessive to the wild-type TraR (i.e. they do not affect the function of the wild-type protein) (11). However, some of these mutants block the effect of TraM in a strain in which the activity of wild-type TraR is inhibited by the antiactivator (16). When examined by far Western analysis, N-terminal deletion mutants of TraR lacking 4–104 residues are bound by TraM (Fig. 5, panel A). These mutants all exerted dominant interfering activity (Table I and Ref. 16). Again, no signal was detected in a strain containing the empty expression vector or when the filter was not challenged with TraM protein (Fig. 5, panel A, and data not shown).

N-terminal deletion mutants of TraR shortened for more than 104 amino acids failed to give any measurable phenotypes (Ref. 16 and data not shown). Furthermore, we were unable to detect proteins encoded by these mutants by either Western or far Western analysis (data not shown), suggesting that these mutant proteins are unstable. Thus, we designed a genetic screen to isolate mutants of TraR with large N-terminal deletions that still interact with TraM. A clone coding for traR was treated with EcoIII as described under “Experimental Procedures,” and the subsequent ligation products were introduced into the reporter strain NT1(pRMLH4I41) (16). Among a number of candidates, we identified two mutants with relatively long 5’ deletions that exhibited dominant interfering activity against wild-type TraR encoded by pRMLH4I41 (16). Sequence analysis revealed that these mutants, traRN41 and traRN68 (Table I), code for derivatives of the activator that retain the C-terminal 104 and 93 amino acids of TraR, respec-
Fig. 5. Interaction between TraM and N-terminal deletion mutants of TraR. Equal amounts of protein from total lysates of A. tumefaciens harboring plasmids coding for wild-type and N-terminal deletion mutants of traR were subjected to SDS-PAGE, and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked and reacted with (His)_6-TraM, and the complexes formed were detected using anti-TraM antiserum all as described under “Experimental Procedures.” Panel A, lanes contain lysates from cells expressing the following: lane 2, wild-type TraR; lane 3, TraRΔN2–4; lane 4, TraRΔN2–9; lane 5, TraRΔN2–49; lane 6, TraRΔN2–69; lane 7, TraRΔN2–89; lane 8, TraRΔN2–104; lane 9, vector control. Panel B, lanes contain lysates from cells expressing the following: lane 2, wild-type TraR; lane 3, TraRΔN2–89; and the N-terminal protected mutants as follows: lane 4, TraRΔN41 (Δ1–130); lane 5, TraRΔN68 (Δ1–141). Lanes 1 contain protein standards of known molecular sizes in kilodaltons as indicated to the left of each panel.

Protein-Protein Interactions in Quorum Sensing

We assessed the biological consequence of these deletion mutations by transferring the C-terminal portions of some of these mutants into TraRΔN2–4. All but one of these C-terminal deletion derivatives of TraRΔN2–4 conferred a dominant interfering activity consistent with the signals observed from the far Western analysis. For example, C-terminal deletions of up to 20 residues exerted a weak but detectable dominant interfering activity and bound TraM at very low but detectable levels (Table I). TraM no longer bound TraRΔN2–89 that no longer are able to interact with TraM at detectable levels (Fig. 6, panel B).

We also assessed the effect of TraM on these mutants and tested them with our anti-TraR antisera in Western analysis (Ref. 11 and data not shown). However, when assessed by far Western analysis, both mutants produced detectable proteins of the anticipated sizes when probed with TraM and challenged with our anti-TraM antisera (Fig. 5, panel B).

We also assessed a series of C-terminal deletion mutants of TraR to far Western analysis. Interactions between these mutants and TraM cannot be assessed genetically as most exert a strong dominant-negative effect on the activity of wild-type TraR (11). All such mutants encode a stable polypeptide detectable by anti-TraR antibodies (Fig. 6, panel A). TraM mutants lacking as few as 2 or as many as 20 amino acids from the C terminus gave a very weak but detectable signal. Deletions removing 25 or more amino acids resulted in polypeptides that no longer are bound by TraM at detectable levels (Fig. 6, panel B).

We assessed the biological consequence of these deletion mutations by transferring the C-terminal portions of some of these mutants into TraRΔN2–4. All but one of these C-terminal deletion derivatives of TraRΔN2–4 conferred a dominant interfering activity consistent with the signals observed from the far Western analysis. For example, C-terminal deletions of up to 20 residues exerted a weak but detectable dominant interfering activity and bound TraM at very low but detectable levels (Table I and Fig. 6, panel B). However, TraRΔN2–4/C-25, while showing weak dominant interference (Table I), did not detectably bind the antiactivator (Fig. 6, panel B). TraRΔN2–4/ΔC-30, on the other hand, exhibited no dominant interfering activity and did not bind TraM at a detectable level (Table I and Fig. 6, panel B).

Substitution Mutations in the C Terminus of TraR Affect Binding by TraM—To identify residues of TraR that are critical for interaction with TraM, we first tested four previously isolated TraR substitution mutants for their ability to bind the antiactivator. Two of these mutants, which were isolated in a screen for alleles of TraR that maintain activator activity but no longer are inhibited by TraM, contain leucine or serine substitutions for the proline at position 176 (Ref. 16; Table I). As assessed by far Western analysis, although detectable, the ability of TraM to bind to these two mutants was severely reduced (Fig. 7). The two additional mutants, traR111 and traR112, were identified as being unable to either repress or activate appropriate reporter constructs (11). These mutants produce proteins with substitutions in or near the putative helix-turn-helix (H-T-H) motif located in the C terminus (Ref. 11; Table I). TraM no longer bound TraR111 (Fig. 7). However, the antiactivator interacted with TraR112 at a level indistinguishable from that of the wild-type protein (Fig. 7).

That TraR111 fails to bind TraM suggests that the antiactivator interacts with residues of TraR that are important for DNA binding and/or activator activity. Mutants of TraR with such phenotypes certainly will escape any genetic screen based on the protein maintaining its activator activity. Thus, we designed a more unbiased screen for TraR-insensitive mutants of TraR by searching for variants of traRΔN2–4 that fail to exert dominant interfering activity in strain NT1(pRMLH4141). DNA was mutagenized by hydroxylamine treatment or by error-prone PCR and was introduced into this reporter strain. Following incubation, white colonies appearing on agar medium supplemented with AAI and X-gal were selected as harboring mutants of TraRΔN2–4 that no longer are able to interfere with TraM. All hydroxylamine-induced mutants were recloned into a fresh vector to avoid any effect derived from mutations in the cloning vehicle.

Eleven independent mutants that completely or partially lost dominant interfering activity but encode polypeptides with sizes indistinguishable from that of traRΔN2–4 were obtained from the screen. Sequence analysis identified one frameshift, three early termination mutations, and seven missense mutations, all affecting the C terminus of the protein (Fig. 8 and data not shown). Since we already had analyzed a series of C-terminal deletion mutants, we retained only the missense mutants for further studies. The seven mutants fell into two classes. In one, represented by three independent mutants, leucine at position 182 is changed to phenylalanine (L182F) (Fig. 8). In the second, represented by four independent mutants, alanine at position 195 within the putative H-T-H motif is changed into either threonine (A195T) or valine (A195V) (Fig. 8). Each of the seven mutants produced a stable protein of the expected size as judged by Coomassie Blue staining and Western analysis with anti-TraR antiserum (data not shown). However, all seven mutants failed to exert dominant interfering activity against TraM-mediated inhibition of TraR activity at levels comparable to that of the parent, TraRΔN2–4 (Table I). When subjected to far Western analysis, these seven mutants showed a greatly decreased ability to interact with TraM (Fig. 7, panel A).
Since TraRΔN2–4, the parent of these mutants, lacks both activator and repressor activities, we could not determine how these substitution mutations affect the activity of wild-type TraR. Thus, we replaced the N-terminal portion of these mutants with that of wild-type TraR to generate full size TraR proteins harboring the substitution mutations at leucine 182 and alanine 195. All such derivatives failed to induce expression of a traG::lacZ reporter in the presence of AAI (Table I).

| Allele of traR | Mutation | Activation | Repression | Activity Fold interference |
|---------------|----------|------------|------------|---------------------------|
| Wild type     | None     | 136        | 13         | NA/NA                     |
| traRΔN2–4     | Δ2–4     | 3          | 122        | 116/58                     |
| traRΔN2–9     | Δ2–9     | 1          | 137        | 124/62                     |
| traRΔN2–49*   | Δ2–49    | 2          | 134        | 116/58                     |
| traRΔN2–69    | Δ2–69    | 3          | 124        | 104/57                     |
| traRΔN2–89    | Δ2–89    | 1          | 140        | 99/49                      |
| traRΔN2–104b  | Δ2–104   | 2          | 127        | 14/7                       |
| traRΔN41t     | Δ1–130   | 1          | 134        | 91/45                      |
| traRΔN68s     | Δ1–141   | 1          | 138        | 93/46                      |
| traRΔ111      | M213I, R215H | 2    | 151        | NA/NA                     |
| traRΔ112      | M191I, R206H | 2    | 130        | NA/NA                     |
| traRΔ26       | P176L    | 101        | 26         | NA/NA                     |
| traRΔ28       | P176S    | 89         | 87         | NA/NA                     |
| traRΔC–2      | Δ252-Term| 2          | 154        | NA/NA                     |
| traRΔC–8      | Δ226-Term| 2          | 137        | NA/NA                     |
| traRΔC–13     | Δ221-Term| 1          | 143        | NA/NA                     |
| traRΔC–20     | Δ214-Term| 2          | 131        | NA/NA                     |
| traRΔC–25     | Δ209-Term| 2          | 127        | NA/NA                     |
| traRΔC–30     | Δ204-Term| 2          | 122        | NA/NA                     |
| traRΔC–35     | Δ199-Term| 1          | 124        | NA/NA                     |
| traRΔC–45     | Δ189-Term| 2          | 134        | NA/NA                     |
| traRΔ171      | L182F    | 2          | 125        | NA/NA                     |
| traRΔ11       | A195T    | 1          | 137        | NA/NA                     |
| traRΔ91       | A195V    | 2          | 139        | NA/NA                     |
| traRΔN2–4/R111| Δ2–4; M213I, R215H | 2 | NA | 1 | None |
| traRΔN2–4/R112| Δ2–4; M191I, R206H | 2 | NA | 97 | 48 |
| traRΔN2–4/R26 | Δ2–4; P176L | 1 | NA | 34 | 17 |
| traRΔN2–4/R28 | Δ2–4; P176S | 2 | NA | 23 | 12 |
| traRΔN2–4/C–2 | Δ2–4; 232-Term | 1 | NA | 25 | 12 |
| traRΔN2–4/C–8 | Δ2–4; 226-Term | 2 | NA | 17 | 8 |
| traRΔN2–4/C–20| Δ2–4; Δ214-Term | 2 | NA | 19 | 9 |
| traRΔN2–4/C–25| Δ2–4; Δ209-Term | 4 | NA | 15 | 7 |
| traRΔN2–4/C–30| Δ2–4; Δ204-Term | 3 | NA | 1 | None |
| traRΔN2–4/R171| Δ2–4; L182F | 2 | NA | 34 | 17 |
| traRΔN2–4/R11 | Δ2–4; A195T | 1 | NA | 21 | 10 |
| traRΔN2–4/R91 | Δ2–4; A195V | 2 | NA | 31 | 15 |

a) Expressed as units per 10⁹ colony-forming units.
b) Assessed in A. tumefaciens NT1(pRMLH4141) (16) in which the activity of TraR is inhibited by TraM. Mutants of traR were cloned in pZLQ (11) and assays were carried out as described above for dominant negativity.
c) Measured as the repression of expression of the promoter-tra box-lacZ complex in DH5α(pPBL1) (11) in the presence of 25 nm AAI.
d) All values are averages of three independent experiments with variations less than 15%.

Two hybrid analysis

Since TraRΔN2–4, the parent of these mutants, lacks both activator and repressor activities, we could not determine how these substitution mutations affect the activity of wild-type TraR. Thus, we replaced the N-terminal portion of these mutants with that of wild-type TraR to generate full size TraR proteins harboring the substitution mutations at leucine 182 and alanine 195. All such derivatives failed to induce expression of a traG::lacZ reporter in the presence of AAI (Table I). These mutants also were unable to repress expression of the reporter in pPBL1 (11) (Table I). Furthermore, in a manner similar to other alleles of TraR bearing mutations near or within the helix-turn-helix motif (11), these mutants exerted a strongly dominant-negative effect over the wild-type activator (data not shown). When assessed by far Western analysis, like their parents, these mutants interacted with TraM at severely decreased levels (Fig. 7, panel C).

DISCUSSION

Consistent with yeast two-hybrid studies (16), our analyses using far Western blots demonstrate that TraM can bind TraR. TraM apparently binds specifically and selectively to TraR; the antiactivator did not bind detectably to any other proteins present in lysates of A. tumefaciens. Moreover, as indicated by the observation that TraM can prevent TraR from forming complexes with its DNA recognition site in vitro, the antiactivator can bind the activator in solution. The fact that binding, as measured by far Western analysis, correlates well with the biological activity of TraR mutants in vivo validates the far Western assay as a method useful for assessing interaction between pure preparations of TraM and TraR.

Functional TraR exists in dimer form and dimerization is dependent upon binding AAI.2 Given that purified TraM binds efficiently to TraR present in lysates of cells not exposed to AAI, we conclude that the antiactivator can bind the inactive, monomer form of the activator. This conclusion also is consistent with our two-hybrid analysis; yeast strains expressing the traM-bait and TraR-prey fusions display strong interaction phenotypes in the absence of AAI (16). However, as shown by dot blot far Western analysis using purified proteins (Fig. 3), TraM also binds the transcriptionally active, dimer form of the activator.

Although independent of the multimeric nature of TraR, the interaction between TraM and the activator is dependent upon

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the secondary structure of the two proteins. TraM must be in its native form. Thus, when denatured by heating, TraM failed to bind TraR, and binding could not be restored by simple renaturation protocols. Similarly, TraM bound heat-denatured TraR with considerably less affinity. However, if heated in the presence of SDS, or if treated with SDS after heating, denatured TraR was bound strongly by TraM. These observations suggest that denaturation by heating results in the occlusion of one or more sites of TraR required for recognition and/or binding by TraM. On the other hand, these sites must be available when the activator is associated with SDS. Although it is clear that heat-denatured TraR is not strongly bound by the activator, it remains to be determined if TraM can interact with naturally misfolded TraR.

Two lines of evidence indicate that TraM interacts with a domain located within the C terminus of TraR. First, N-terminal deletion derivatives lacking as many as 141 amino acids still were bound by TraM (Fig. 5). However, removing as few as two amino acids from the C terminus of TraR greatly decreased interaction with TraM, and removing 25 or more residues completely abolished binding by the activator in vitro (Fig. 6). Binding activity correlated well with the in vivo activity. N-terminal deletions extending up to 141 amino acids had little or no effect on dominant interfering activity (Table 1). However, deleting as few as two residues from the C terminus of TraRΔN2–4 led to almost complete loss of this activity in vivo. These results are consistent with our yeast two-hybrid analyses in which prey plasmid fusions containing as few as 50 C-terminal amino acids of TraR gave positive interaction phenotypes in strains expressing TraM as the bait fusion (16). These correlations between in vitro and in vivo activities also support our hypothesis that the dominant interfering activity exhibited by N-terminal deletion mutants of TraR results from the titration of available TraM thereby allowing the coexpressed wild-type activator to initiate transcription (16). Interestingly, while mutants deleted for 2–20 C-terminal residues still showed weak binding by TraM, deleting 25 residues from the C terminus of TraR abolished binding by the antiactivator as measured by far Western analysis (Fig. 6). This result suggests that interaction between TraM and TraR involves recognition of one portion of the activator followed by more stable binding to another region of the protein.

Second, all substitution mutations in TraR isolated by virtue of an altered interaction with TraM map to the C-terminal region of the activator. The single substitution at leucine 182 exhibited by N-terminal deletion mutants of TraR results from the titration of available TraM thereby allowing the coexpressed wild-type activator to initiate transcription (16). Interestingly, the C-terminal region of the activator is also required for binding of TraM as demonstrated by the loss of this activity when deletion derivatives lacking as many as 35 amino acids were tested (Table 1). Furthermore, these mutations strongly
decreased the dominant interfering activity of the parent protein, TraRΔN2–4 (Table I). The two independent substitutions at proline 176 of TraR decreased but did not abolish binding by TraM. This observation is consistent with our yeast two-hybrid analysis in which the P176S mutant of TraR gave a detectable although considerably diminished interaction phenotype when tested with wild-type TraM (16). Similarly, the two substitution mutations at position 176 resulted in a substantial but not complete loss of dominant interfering activity when inserted into the TraRΔN2–4 polypeptide (Table I). All three of these residues are conserved in TraR of the octopine-type Ti plasmid, pTiR10, which also is inhibited by TraM (13), but not in most other members of the LuxR family (data not shown).

Two additional substitution mutants, traR111 and traR112, present an informative contrast. TraR111, with substitutions at residues 213 and 215, is not detectably bound by TraM (Fig. 7). Furthermore, when inserted into the TraRΔN2–4 polypeptide, the traR111 substitutions resulted in the complete loss of dominant interfering activity (Table I). On the other hand, TraM bound to TraR112 in a manner indistinguishable from that of wild-type TraR (Fig. 7). When the TraR112 substitutions were inserted into the TraRΔN2–4 polypeptide, the resulting protein retained dominant interfering activity (Table I). Both alleles were isolated in a screen for mutants that abolished transcriptional activation by TraR (11). Moreover, as assessed in a repressor assay, neither mutant binds to its cis-acting promoter recognition element (11). Although both alleles contain two substitutions, in each both alterations are located within the C-terminal portion of the protein and map near or within the H-T-H domain (Fig. 8). Significantly, these residues also are conserved in TraR of pTiR10.

Residues identified as essential for binding TraM also are important for TraR activity. Thus, alterations at residue 176 decrease activator and repressor functions, whereas those at positions 182 and 195 completely abolish these activities (Table I). Similarly, the two substitutions in TraR111 at positions 213 and 215 result in the loss of activator and repressor activity. This correlation explains our earlier failure to isolate TraR mutants other than traR26 and traR289 that are unable to interact with TraM. The screen we used required that TraM retains activator function (16). Our alternative strategy, screening mutants of TraRΔN2–4 for loss of dominant interfering activity, circumvented this problem. However, not all residues important for DNA binding are required for TraM binding. Thus, the two substitutions in TraR112, while abolishing DNA binding and concomitant transcriptional activation, have virtually no effect on interaction with TraM (Table I and Fig. 7).

We showed by two-hybrid analysis that a fragment of TraR encompassing residues 121–185 interacts with TraM (16). Sim-
ensuring that conjugation is regulated in a quorum-dependent manner and that expression of the tra regulon in response to opine availability is maintained at a suitable level.

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