Inhibition of the Agrobacterium tumefaciens TraR Quorum-sensing Regulator

INTERACTIONS WITH THE TraM ANTI-ACTIVATOR*

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The Agrobacterium tumefaciens quorum-sensing transcriptional regulator TraR and its inducing ligand 3-oxo-octanoyl-L-homoserine lactone control conjugal transfer of the tumor-inducing plasmid, the primary virulence factor responsible for crown gall disease of plants. This regulatory system enables A. tumefaciens to express its conjugal transfer regulon preferentially at high population densities. TraR activity is antagonized by a second tumor-inducing plasmid-encoded protein designated TraM. TraM and TraR are thought to form an anti-activation complex that prevents TraR from recognizing its target DNA-binding sites. The formation and inhibitory function of the TraM-TraR anti-activation complex was analyzed using several different assays for protein-protein interaction, including surface plasmon resonance. The TraR-TraM complex forms readily in solution and is extremely stable (Kd of 1–4 × 10⁻⁹ m). Directed mutational analysis of TraM identified a number of amino acids that play important roles in the inhibition of TraR, clustering in two regions of the protein. Interestingly, several mutants were identified that proficiently bound TraR but were unable to inhibit its activity. This observation suggests a mechanistic separation between the initial assembly of the complex and conversion of TraR to an inactive form.

Conjugation of the Agrobacterium tumefaciens tumor-inducing (Ti) plasmid is regulated by crown gall tumor-released compounds called opines and by the acylated homoserine lactone (acyl-HSL) 3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL; see Refs. 1 and 2). Acyl-HSLs are diffusible pheromones produced by a variety of Gram-negative bacteria. At low population densities, acyl-HSLs are rapidly dissipated by diffusion into the surrounding environment. The contribution of each signal-producing cell is additive, and if population density increases, the relative acyl-HSL concentration is elevated, eventually reaching an inducing concentration and stimulating a programmed set of adaptive responses. The release and subsequent detection of acyl-HSLs therefore enable bacteria to monitor their own population density, a process often referred to as quorum sensing (3, 4). Synthesis of acyl-HSLs in most cases is mediated by proteins that resemble the LuxI protein of Vibrio fischeri, whereas most of the receptors of these signals are acyl-HSL-dependent transcriptional regulators and resemble the V. fischeri LuxR protein.

A simplified model of acyl-HSL quorum sensing in A. tumefaciens is presented in Fig. 1. The TraI protein, encoded on the Ti plasmid, directs synthesis of 3-oxo-C8-HSL (5–9). Ti plasmid conjugal transfer (tra) genes are activated by TraR in response to the pheromone (6, 10). Complexes of TraR with 3-oxo-C8-HSL bind to promoter elements, called tra boxes, upstream of at least five different tra operons on the Ti plasmid (11–13). By comparison to LuxR itself, TraR can be divided into two functional domains (4). A region in the amino-terminal half of the protein has conserved residues known to be required for 3-oxo-hexanoyl-homoserine lactone binding by LuxR (14). The carboxyl-terminal region of the protein contains a helix-turn-helix motif (position 193–211) that has been implicated in DNA binding for both TraR and LuxR (17, 18). One acyl-HSL molecule binds per monomer of TraR in an extremely stable complex that is partially dissociable with detergent treatment (19).

In contrast to most other LuxR-type proteins, the activity of TraR is directly influenced by several other regulatory proteins (Fig. 1). The TrlR protein (previously designated TraS) is a truncated homologue of TraR that contains sequences necessary for dimerization but lacks the helix-turn-helix DNA-binding motif (20, 21). TrlR inhibits TraR through formation of inactive heterodimers (22). The activity of TraR is also under the influence of the Ti plasmid-encoded TraM regulator, a small protein thus far only identified in A. tumefaciens and other members of the family Rhizobiaceae (23–25). TraM inhibits the activity of the TraR protein, and this inhibition is absolutely required for the normal operation of the A. tumefaciens quorum sensor.

Mutants in traM are hyperconjugal and, in contrast to wild type, will transfer the Ti plasmid to recipient cells at high
efficiency even at low densities of conjugal donors (6, 23, 26). In effect, TraM acts to set the threshold level of acyl-HSL-TraR complex required to activate the expression of Ti plasmid tra genes. Therefore, TraM plays a crucial role in determining what constitutes a bacterial quorum and when Ti plasmid conjugal transfer is initiated. Recent studies (27, 28) of the TraM protein encoded by the nopaline-type Ti plasmid suggested that it inhibits activation of tra gene expression through formation of a putative anti-activation complex with TraR. Yeast two-hybrid studies and far-Western analysis of TraM binding to immobilized TraR suggest that TraM associates with a region in the carboxyl-terminal half of the transcription factor (27, 28). Mutational analysis of the nopaline-type Ti plasmid (pTiC58) traM coding sequence identified two amino acid residues in the carboxyl terminus of the protein, where alterations resulted in reduced inhibitory function (27).

Our findings analyzing TraM from the octopine-type Ti plasmid pTiR10 are consistent with the formation of a TraR-TraM anti-activation complex. We expand on this previous work, analyze TraM inhibition at additional TraR-binding sites, and demonstrate TraR-TraM complex formation in solution. Moreover, kinetic analysis of the interaction between these two proteins using SPR reveals that the anti-activation complex assembles slowly but once formed is highly stable. Addition of TraM as an affinity tag of pRSETA, under the control of the T7 promoter (pT7), and using the oligonucleotides 5'-GGCGGTACCCTTGATATTACGAGAAAGAGATGAAACACCGAGAAGATGACG (KpnI cleavage site, Shine-Delgarno sequence and start codon are underlined) and 5'-GGATCCATCATTCCPTGC (The 1027-bp amplification product, with a region in the carboxyl-terminal half of the transcription factor ATG) resulted in reduced inhibitory function (27, 28). Mutational analysis of the nopaline-type Ti plasmid (pTiC58) traM coding sequence identified two amino acid residues in the carboxyl terminus of the protein, where alterations resulted in reduced inhibitory function (27).

EXPERIMENTAL PROCEDURES

Reagents, Strains, and Plasmids—Strains and plasmids used in this study are detailed in Table I. Buffers, antibiotics, and microbiological media were obtained from Fisher and Sigma. All plasmids were introduced into A. tumefaciens via electroporation (29). Unless otherwise stated, all DNA manipulations were performed by standard protocols (30) with enzymes purchased from New England Biolabs. Fast-Link T4 DNA ligase was purchased from Epicentre Technologies (Madison, WI). RGS-His antibody was obtained from Qiagen Inc. (Valencia, CA). Rabbit anti-TraM antiserum was raised against purified hexahistidinyl-tagged TraM (H6TraM) by Cocalico Biologicals Inc. (Reamstown, PA).

Construction of an Affinity-tagged traM Derivative—The H6TraM derivative was obtained by PCR amplification of the traM coding sequence using synthetic oligonucleotides 5'-GGCGGTACCGAAGACTG-GAAAGTACGAAAAAGTACGACG (which contains an NheI site (underlined)) and 5'-GGATCCATCATTCCPTGC. The 1027-bp amplification product, digested with NheI and Bsm HI (Bsm HI cleaves the fragment at an internal site downstream of the traM coding sequence to generate a 592-bp fragment), was ligated with NheI-Bsm HI-digested pRSETA (Invitrogen Corp, Carlsbad, CA) to create pMB107. In pMB107 the amino-terminal end of the traM-coding sequence is fused to the RGS(H)6 affinity tag of pRSETA, under the control of the T7 promoter (pT7). Site-directed Mutagenesis—Oligonucleotide-directed mutagenesis was used to construct mutations in the A. tumefaciens traM gene on pMB107. Mutations were introduced using the Quick-change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA), and mutated plasmids were checked with automated DNA sequencing by the Indiana Molecular Biology Institute. Following sequence confirmation the mutated traM alleles were PCR-amplified with Pfu Polymerase (Stratagene) using the oligonucleotides 5'-GGCGGTACCTTGATATTACGAGAAAGAGATGAAACACCGAGAAGATGACG (KpnI cleavage site, Shine-Delgarno sequence and start codon are underlined) and the pRSETA reverse primer 5'-TAGTTATTTGTCAGCGCG (The PCr product was digested with KpnI and Bam HI (which cleaves within the pRSETA multiple cloning site internal to the 5' primer), followed by ligation to the Bsm HI traR expression vector pBBR1-MCS5, also cleaved with KpnI and Bam HI (32). These derivatives expressed the traM mutant alleles from the E. coli Pbac promoter on pBBR1-MCS5. Translation of the lacZα peptide was terminated at two tandem stop codons (in bold type) just downstream from the KpnI site, and translation of the native traM gene was initiated at its own ATG codon further downstream. The complete traM-coding sequence was determined for each BHR construct prior to phenotypic analysis.

In Vivo Activity of traM Alleles—The BHR plasmids described above were introduced into A. tumefaciens NT-L4 (a strain cured of the Ti plasmid), harboring a plasmid carrying a PtraI-lacZ fusion (pCF372) and plasmid expressing traR either strongly as a PtraI-trac traR fusion (pCF218) or moderately as a PtraI-trac traR fusion (pCF346). The PCR product expressing traR also carry the E. coli lacZα repressor gene, which allows for controlled expression of the PtraI-trac transcriptional fusions (33). Expression of PtraI-lacZ in cultures grown in ATGN minimal media with a variety of supplements was monitored by β-galactosidase assays (33). For the traM mutant alleles, the ratio of traI-lacZ expression (Miller units of β-galactosidase activity) in the presence of the PtraI-trac inducer IPTG relative to traI-lacZ expression in the absence of IPTG was calculated. This activity ratio was subtracted from 1 to obtain inhibitory activity. Percent inhibitory activity was determined as the ratio of inhibitory activities of mutant traM alleles to wild type traM.

Purification of H6TraM Proteins and TraR—E. coli BL21/ADE3 harboring pMB107 and several of its mutant derivatives were used to overproduce H6TraM fusion proteins. For purification, 250 ml of cells were incubated at 37 °C with vigorous shaking in LB medium supplemented with 100 mg of kanamycin and 25 mg of chloramphenicol to an absorbance at 600 nm (A600) of 0.6. IPTG was added to a final concentration of 0.5 mM to induce expression of the PtraI-H6TraM gene. After 3 h of additional incubation, cells were collected by centrifugation (11,760 × g, 10 min, 4 °C) and suspended in Extraction Buffer (300 mM NaCl and 50 mM NaH2PO4, pH 7.0). Cells were disrupted by passage through a French pressure cell (Amino, Urbana, IL) at 18,000 pounds/square inch. Unlysed cells and

| Bacteria/plasmid | Relevant features | Ref. |
|------------------|------------------|-----|
| E. coli DH5αF' | Standard cloning host | 50 |
| E. coli BL21/ADE3 | T7 expression host | 51 |
| A. tumefaciens NT-L4 | Ti plasmidless derivative, C58 chromosomal background | 52 |

| Plasmids | Relevant features | Ref. |
|----------|------------------|-----|
| pRSETA | N-terminal hexahistidinyl fusion vector, Ap | Invitrogen |
| pBBR1-MCS5 | BHR PtraI expression vector, Gm | 32 |
| pCF430 | BHR IncP PBAD expression plasmid, Tc | 38 |
| pCF218 | BHR IncP traR expression plasmid, Te | 6 |
| pCF372 | BHR IncW traI-lacZ expression plasmid, Sp | 11 |
| pCF361 | pBSK + derivative carrying tra box II and III, Ap | 11 |
| pJZ304 | pBend + derivative carrying tra box I, Ap | 19 |
| pJZ355 | PtraI-trac, pRSETA derivative | 19 |
| pCF436 | BHR IncP PBAD-traR, pCF430 derivative | This study |
| pMB107 | PtraI-H6traM, pRSETA derivative | This study |
| pCF444 | PtraI-H6traM, PtraI-trac, pRSETA derivative | This study |
debris were removed by centrifugation (38,000 x g, 30 min, 4 °C). Particulate material was removed by ultracentrifugation of extracts (287,000 x g, 1 h, 22 °C). H6TraM was purified by affinity chromatography on an Acta-FPLC (AP Biotech, Fiscataway, NJ) using a XK-16 column containing 13 ml of Talon cobalt affinity resin (CLONTECH Inc, Palo Alto, CA) and a flow rate of 1 ml/min. Following washing in Extraction Buffer plus 5 mM imidazole, the bound H6TraM was eluted from the column using a linear gradient of 0-500 mM imidazole in Extraction Buffer and was obtained as a single large peak at ~200 mM imidazole. Active TraR was purified from cultures of E. coli BL21/DE3 (pJZ358) cultured in the presence of 0-300 mM-CHS as described previously (19). Protein concentrations were determined by the technique of Gill and von Hippel (34) and confirmed using the Micro-BCA assay (Pierce).

**Gel Mobility Shift Assays of TraR DNA Binding Activity and TraM Inhibition**—Plasmids pJZ304 and pCF361 were cleaved with the appropriate restriction endonuclease (see text) and end-labeled using [γ-32P]dCTP and the Klenow fragment of DNA polymerase I by standard techniques. Gel mobility shift assays were performed essentially as described in Zhu and Winans (19). Briefly, a range of TraR concentrations was added to the labeled DNA (2,500 cpm) in reaction buffer (12 mM HEPES-NaOH, 4 mM Tris-Cl, 60 mM potassium glutamate, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol) and allowed to incubate for 30 min at room temperature. The reactions were loaded directly on 8% polyacrylamide (80:1 acrylamide/bisacrylamide) gels and electrophoresed. The gels were dried and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Unless otherwise indicated, TraR and H6TraM derivatives were obtained by a standard in vivo expression system. E. coli BL21(DE3) harboring plasmids containing traR or tra genes expressed from the T7 promoter were induced to grow on-stick at a concentration of 0.5 IPTG was added (0.5 mM). After incubation for 30 min, rifampicin was added to inhibit the E. coli RNA polymerase. After 30 min, 50 μCi of [35S]Met (5000 Ci/mmol) was added to the cultures, and incubation was continued for 3 h. Cells were harvested by centrifugation (11,760 x g, 10 min, 4 °C) and lysed using a French pressure cell as described above.

**Gel Filtration Chromatography of TraR-TraM Complexes**—To analyze complexes between the two regulatory proteins in vitro, E. coli lysates containing radiolabeled H6TraM, radiolabeled TraR, or both were prepared in TTEDG buffer (50 mM Tris-Cl, pH 7.9, 0.1% Tween 20, 0.5 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 200 mM NaCl) as described above. The lysates were mixed in equal proportions and incubated for 15 h at room temperature to allow complete complex formation. Following incubation, these reactions were applied to a pre-equilibrated 15-ml Superdex 75 gel filtration column in a mobile phase of TTEDG at a flow rate of 0.2 ml/min. Fractions (0.2 ml) were analyzed by SDS-PAGE. Radioactivity was quantitated using a PhosphorImager. [35S]His monoclonal antibodies (Qiagen Inc.) were used to confirm the identity of the band representing presumptive H6TraM.

**Assays of TraM Interactions with Immobilized TraR**—We prepared [35S]Met-labeled wild type and mutant H6TraM proteins from E. coli BL21/DE3 as described above. Whole cell lysates containing the radiolabeled H6TraM were generated using B-PER nonionic lysis reagent (Pierce) following the supplier's recommendations. Several dilutions of each lysate were fractionated on SDS-PAGE gels, and the dried gels were analyzed using a PhosphorImager. The amount of labeled H6TraM in each lysate was determined, and they were normalized for the concentration of radiolabeled H6TraM.

Lysates of E. coli BL21/DE3 (pJZ358) were fractionated by SDS-PAGE and electrotransferred to nitrocellulose membranes. The membrane was washed extensively in Tris-buffered saline plus 0.01% Tween 20 (TTBS) and blocked with TTBS + 5% skim milk (Blotto). The blocked membranes were incubated with separate BPER lysates containing different [35S]Met-H6TraM proteins on a rocking platform for 17 h at room temperature. Following incubation, the membranes were washed five times in TTBS, air-dried, and analyzed using a PhosphorImager.

**Surface Plasmon Resonance (SPR) Assays**—The BIACORE 2000 system (BIA-CORE Inc., Upsalla, Sweden) was used to perform SPR analysis of TraR-TraM interactions. TraR was immobilized via primary amine cross-linking to the CM5 fiber of a CM5 research grade chip. Carboxyl groups on the CM5 fibers were first activated by injection of a mixture of N-hydroxysuccinimide and N-ethyl-N’-(dimethylaminopropyl)-carbodiimide. TraR (50 μg) was purified as a complex with 3-oxo-C8-HSL was injected in maleate buffer (5 mM, pH 7.0) into the flow cell at 2 μl/min to allow covalent bonding to the activated CM5 fibers. Unreacted CM5 fibers were titrated using acidified ethanolamine (pH 8.5), and the response units (RU) of TraM immobilized on the chip surface were determined. H6TraM was bound to a CM5 chip by conjugating the protein via a thiol linkage to its single dispensable Cys residue (see Results) using maleimide coupling chemistry. To do this, a CM5 chip was activated using the N-hydroxysuccinimide/N-ethyl-N’-(dimethylaminopropyl)-carbodiimide mixture described above, followed by ethylenediamine treatment. The heterobifunctional cross-linker Sulfo-MMBS (Pierce) was injected and conjugated to the CM5 fibers on the sensor chip surface. H6TraM (650 μg) was injected in maleate buffer and reacted with the immobilized cross-linker via its thiol side chain. Remaining active maleimide residues were titrated using 100 mM l-cysteine.

Immobilized TraR and immobilized H6TraM surfaces were used to analyze TraR-TraM interactions. The analyte, TraR or H6TraM, was injected in a buffer containing HEPES-buffered saline (10 mM HEPES, pH 7.4, 150 mM NaCl) with 3 mM EDTA and 0.005% polysorbate 20 (HBS-EP). Binding was monitored as an increase in RU. The dissociation of the complex was monitored as a decrease in RU following termination of analyte injection and continued mobile phase flow. The surface was regenerated with a short pulse of 6 x guanidine hydrochloride (GdnHCl) for 1 min at a flow rate of 10 μl/min as described by Schuster et al. (35). HBS-EP buffer was applied at 25 μl/min for 5 min to remove GdnHCl. Regeneration was checked by repeated injections of analyte. Determination of kinetic constants was performed at low ligand surface densities over a range of protein concentration in a randomized series with injections performed in triplicate for each concentration. Reported values are based on averaged data for each analyte concentration in triplicate.

**RESULTS**

**TraM Inhibits TraR-DNA Complex Formation in Vitro**—To test the anti-activator function of TraM, gel mobility shift assays were performed using a DNA fragment containing tra box I, a TraR-binding site located between divergent pTIR10 tra operons (Fig. 1) as before (Refs. 11 and 19). Addition of TraR (10 nM) resulted in quantitative formation of TraR-DNA complexes.
complex. The unbound fragment containing traI sequences that resemble traI. The region upstream of traI, expressed in E. coli extracts, contains two sequence elements upstream of traI. The promoter proximal element (tra box II) is required for transcriptional activation of traI, and, as with tra box I, is centered at ~42.5 nucleotides upstream of the transcription start site (11). Another element (tra box III) is located at position ~167 relative to traI but is not required for traI expression (11, 36). A plasmid carrying both tra box II and III (pCF361) was cleaved to separate the two elements from each other, generating fragments II and III (Fig. 3, panel A). Gel mobility shift assays with TraR using this DNA preparation resulted in two distinct complexes (Fig. 3, panels B and C). To determine which fragment was present in each complex, the two fragments were gel-purified and used separately in gel mobility shift assays. As expected, the faster migrating complex contained fragment III, whereas the slower migrating complex contained fragment II (Fig. 3, panel C). To test whether TraM could inhibit binding of TraR to these sites, we preincubated H6TraM with TraR, and we tested TraR for DNA binding activity. H6TraM inhibited the binding of each site with the same potency as observed with tra box I (Fig. 3, panel D).

**Gel Filtration Chromatography of the TraR-TraM Complex—**Luo et al. (28) demonstrated that TraR will bind to TraM that has been strongly expressed in E. coli extracts and immobilized on nitrocellulose membranes. To examine the presumptive complex between TraM and TraR in solution, we used gel filtration chromatography on mixtures of E. coli extracts containing TraR and H6TraM (both radiolabeled). TraR purified from cells grown in the presence of 3-oxo-C8-HSL elutes with an approximate molecular size of 52 kDa (Fig. 4, panels A and D), whereas TraR produced in the absence of the pheromone elutes as 28-kDa monomer (17). H6TraM in E. coli extracts elutes as a species having a molecular mass of 36 kDa, which is slightly less than 3 times its monomer molecular mass of 12.8 kDa, suggesting that it migrates as a trimer or perhaps a rapidly eluting dimer (Fig. 4, panel B). Co-incubation of these two extracts resulted in co-elution of all the labeled TraR and a portion of the H6TraM at an approximate molecular size of 60 kDa (Fig. 4, panel C). The mass of this complex suggests that it may consist of two TraR monomers and one TraM monomer, or of one TraR monomer and several TraM monomers. When higher protein concentrations were used, both proteins eluted as higher molecular mass complexes (data not shown). Simi-
However, these treatments did not effectively remove H6TraM after each injection, usually with high salt or mild detergents. To test the reversibility of the SPR format and generate a more homogeneous surface, we immobilized H6TraM by its single dispensable cysteine (Cys-64, see below) on the surface of TraR. To test the reversibility of the SPR format, the initial binding event is relatively slow, but it is not certain that 3-oxo-C8-HSL bound to TraR on the CM5 chip is retained after exposure to the denaturant, in solution chips not treated with denaturant (data not shown). Although it is not certain that 3-oxo-C8-HSL, bound to TraR on the CM5 chip is retained after exposure to the denaturant, in solution 3-oxo-C8-HSL is not removed from TraR by GdnHCl treatment (17). Kinetic analysis of H6TraM interaction was performed over a range of concentrations from 9 to 300 nM (Fig. 5, panel A). The results obtained were fit to a Langmuir binding model using BIA-Evaluation 3.0 software ($\chi^2$ value of 7). The association and dissociation rates calculated from these data provide a reasonable estimate of the interaction (Table II). Binding of H6TraM to the TraR surface proceeds at 6.98 $\times$ 10^4 binding events $\mu$M$^{-1}$ s$^{-1}$, whereas dissociation of bound H6TraM occurs at 6.5 $\times$ 10$^{-5}$ dissociations s$^{-1}$. This suggests that for this assay format, the initial binding event is relatively slow, but once formed the H6TraM-TraR complex is highly stable.

The population of TraR proteins immobilized on the CM5 chip described above is by definition heterogeneous, cross-linked at a fraction of the potential primary amines exposed on the surface of TraR. To test the reversibility of the SPR format and generate a more homogeneous surface, we immobilized H6TraM by its single dispensable cysteine (Cys-64, see below) at 25 $\mu$l/min (to stabilize TraR prior to the next injection. Chips treated in this way bound H6TraM with the same kinetics as chips not treated with denaturant (data not shown). Although it is not certain that 3-oxo-C8-HSL, bound to TraR on the CM5 chip is retained after exposure to the denaturant, in solution 3-oxo-C8-HSL is not removed from TraR by GdnHCl treatment.
using maleimide cross-linking chemistry. This presumably generates a homogeneous surface with each His6TraM liganded identically. Kinetic analysis with a low density (140 RU) His6TraM surface and TraR as the analyte results in binding that is ~2-fold more rapid than when TraR is the ligand (1.2 × 10^6 events s^-1), Table II and Fig. 5, panel B). However, the complex also dissociates ~2.5-fold more rapidly (4.9 × 10^-4 events s^-1). Overall, the liganded TraR bound to the His6TraM analyte is 4-fold more stable than the liganded His6TraM-TraR analyte complex (estimated K_d values of 9.3 × 10^-10 and 4.1 × 10^-10, respectively, Table II).

Site-directed Mutagenesis of Residues Conserved between TraM Homologues—To identify TraM residues likely to be important for function, we aligned the protein sequences of octopine-type and nopaline-type TraM proteins as well as a functional TraM orthologue found on the plasmid pNGR234a from Rhizobium sp. NGR234 (24). This alignment was basically conserved residues (Fig. 6, panel A). Site-specific mutagenesis was used to generate derivatives of pMB107 containing single mutations in each conserved residue.

All conserved residues except alanine itself were converted to alanine, whereas conserved alanines were converted to glycine. A single nonconserved cysteine at position 64 was also mutated to alanine, whereas conserved alanines were converted to glycine.

The TraM proteins that exhibited reduced activity were used to direct bind to immobilized TraR in vitro (see Experimental Procedures). Extracts from BL21/D3(pJZ358) (which overexpresses TraR) were fractionated by SDS-PAGE and electrotransferred to nitrocellulose membranes. Extracts containing radiolabeled mutant or wild type His6TraM were

![Fig. 6. Mutation of conserved TraM residues and analysis of stability. Panel A, positions of the TraM mutations generated in the current study. Panel B, Western blot of lysates from A. tumefaciens cells harboring pCF372 (P_paac-lacZ), pCF218 (P_paac-TraR), and each of the pBBR1-MCS5 derivatives expressing traM alleles from P_paac. Lysates prepared from cultures grown in the presence of 0.5 mM IPTG, electrophoresed on SDS-PAGE, transferred to nitrocellulose, and probed with anti-TraM antisera. Antibody binding was detected using horseradish peroxidase-conjugated goat anti-rabbit antibodies and SuperSignal PicoWest chemiluminescence substrate, followed by exposure to x-ray film.

### Table II

| Ligand | Analyte | Apparent k_a (10^k s^-1) | Apparent k_d (10^k s^-1) | Apparent k_d / k_a (10^k) | x^2 |
|--------|---------|-------------------------|--------------------------|----------------------------|-----|
| TraR   | His6TraM| 6.98 × 10^5             | 6.32 × 10^-5             | 9.41 × 10^-10              | 7.05|
| His6TraM| TraR    | 1.20 × 10^7             | 4.90 × 10^-4             | 4.08 × 10^-9               | 4.10|
| TraR   | His6TraM| 4.17 × 10^4             | 2.01 × 10^-5             | 5.04 × 10^-10              | 52.47|
| TraR   | His6TraM| 5.06 × 10^4             | 2.32 × 10^-4             | 4.56 × 10^-9               | 34.47|
| TraR   | His6TraM| 1.35 × 10^4             | 6.33 × 10^-4             | 4.72 × 10^-8               | 2.02|
| TraR   | His6TraM| 1.33 × 10^4             | 4.49 × 10^-4             | 3.42 × 10^-9               | 4.95|
| TraR   | His6TraM| 1.05 × 10^4             | 4.42 × 10^-4             | 4.42 × 10^-8               | 25.67|
| TraR   | His6TraM| 7.12 × 10^3             | 4.14 × 10^-5             | 6.05 × 10^-9               | 0.62|

a All experiments with TraR as ligand were performed on the same CM5 chip with TraR conjugated by primary amines (576 RU of immobilized TraR). His6TraM ligand experiment was performed with CM5 chip and protein-immobilized by cross-linking the single cysteine residue (Cys64) (140 RU of His6TraM immobilized). b All values were calculated as averages of SPR assays performed in triplicate with BIA Evaluation 3.0 software.

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used to probe the individual nitrocellulose membranes. Specific labeling of a band corresponding to TraR in the immobilized lysates was observed (Fig. 7). Identical lysates without TraR were not labeled, and probing with equivalent lysates lacking H₇ TraM also did not label TraR in the extracts (data not shown). A second, more weakly labeled protein significantly smaller than TraR was also observed and may represent a degradation product. Comparison of labeling between the mutants revealed that TraMH₄₀₄₄, R₄₁₄, L₅₄₄, G₉₄₄, and P₉₇₄ also did not label TraR in the extracts (data not shown). A second, more weakly labeled protein significantly smaller than TraR was also observed and may represent a degradation product. Comparison of labeling between the mutants revealed that TraMH₄₀₄₄, R₄₁₄, L₅₄₄, G₉₄₄, and P₉₇₄ also did not label TraR in the extracts (data not shown).

**TABLE III**

**In vitro inhibition assay for TraM activity**

| Allele | High [traR] | Low [traR] | % Inhibition |
|--------|-------------|------------|--------------|
| **WT traM** | 2348<sup>d</sup> | 87<sup>d</sup> | (100)<sup>f</sup> |
| E134 | 2286 | 488 | 82 | 32 | <0.5<sup>e</sup> | 100 |
| L29A | 2638 | 2417 | 9 | 49 | 25 | 49 |
| Q30A | 2319 | 610 | 77 | 40 | <1<sup>e</sup> | 99 |
| A36G | 2392 | 2614 | 10 | 24 | 4 | 87 |
| L37A | 2422 | 2386 | 15 | 33 | 29 | 12 |
| R38A | 1661 | 224 | 90 | 32 | <1<sup>e</sup> | 99 |
| H40A | 2402 | 2499 | <1 | 27 | 62 | <1<sup>e</sup> |
| A41A | 1337 | 2563 | <1 | 47 | 54 | <1<sup>e</sup> |
| A42A | 2386 | 125 | 98 | 32 | <1<sup>e</sup> | 99 |
| L43A | 2329 | 1923 | 18 | 25 | 5 | 82 |
| L54A | 2820 | 2259 | 21 | 33 | 63 | <1<sup>e</sup> |
| C64S | 2446 | 108 | 99 | 24 | <1<sup>e</sup> | 98 |
| Q68A | 2185 | 149 | 97 | 26 | <1<sup>e</sup> | 98 |
| Y72A | 2441 | 1334 | 47 | 29 | 1 | 97 |
| A81G | 2286 | 1683 | 27 | 31 | 2 | 94 |
| Q82A | 2408 | 2503 | <1 | 32 | 63 | <1<sup>e</sup> |
| L93A | 2396 | 2337 | 3 | 36 | 16 | 55 |
| G94A | 2341 | 2454 | <1 | 30 | 36 | <1<sup>e</sup> |
| P97A | 2202 | 2227 | <1 | 40 | 86 | <1<sup>e</sup> |

*Strongly expressed traR supplied from pCF218.*

| Allele | High [traR] | Low [traR] | % Inhibition |
|--------|-------------|------------|--------------|
| **WT traM** | 2348<sup>d</sup> | 87<sup>d</sup> | (100)<sup>f</sup> |
| E134 | 2286 | 488 | 82 | 32 | <0.5<sup>e</sup> | 100 |
| L29A | 2638 | 2417 | 9 | 49 | 25 | 49 |
| Q30A | 2319 | 610 | 77 | 40 | <1<sup>e</sup> | 99 |
| A36G | 2392 | 2614 | 10 | 24 | 4 | 87 |
| L37A | 2422 | 2386 | 15 | 33 | 29 | 12 |
| R38A | 1661 | 224 | 90 | 32 | <1<sup>e</sup> | 99 |
| H40A | 2402 | 2499 | <1 | 27 | 62 | <1<sup>e</sup> |
| A41A | 1337 | 2563 | <1 | 47 | 54 | <1<sup>e</sup> |
| A42A | 2386 | 125 | 98 | 32 | <1<sup>e</sup> | 99 |
| L43A | 2329 | 1923 | 18 | 25 | 5 | 82 |
| L54A | 2820 | 2259 | 21 | 33 | 63 | <1<sup>e</sup> |
| C64S | 2446 | 108 | 99 | 24 | <1<sup>e</sup> | 98 |
| Q68A | 2185 | 149 | 97 | 26 | <1<sup>e</sup> | 98 |
| Y72A | 2441 | 1334 | 47 | 29 | 1 | 97 |
| A81G | 2286 | 1683 | 27 | 31 | 2 | 94 |
| Q82A | 2408 | 2503 | <1 | 32 | 63 | <1<sup>e</sup> |
| L93A | 2396 | 2337 | 3 | 36 | 16 | 55 |
| G94A | 2341 | 2454 | <1 | 30 | 36 | <1<sup>e</sup> |
| P97A | 2202 | 2227 | <1 | 40 | 86 | <1<sup>e</sup> |

*Weakly expressed traR supplied from pCF436.*

*All traM alleles expressed as P₉₄₆-traM fusions cloned in pBBR1-MCS5.*

*Miller units.*

*100% inhibitory activity was determined for wild type traM as a comparison of β-galactosidase activity in cultures grown with and without IPTG (0.5 mM), of A. tumefaciens NTL4 harboring pCF372 (traI-lacZ), and either pCF218 or pCF436 and pGB200 (P₉₄₆-traM) in the presence of exogenous crude acyl-HSL (∼0.5 μM). Percent inhibitory activity calculated for each traM mutant allele was compared with the inhibitory activity of wild type traM carried on pGB200.

**FIG. 7.** Binding of wild type and mutant H₇ TraM to immobilized TraR. Lysates from E. coli BL21/ADE3 (pJZ38) induced for TraR expression were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Individual membranes were probed with lysates containing mutant or wild type [³⁵S]Met-H₇ TraM as described under “Experimental Procedures.” Dried membranes were analyzed using a PhosphorImager.
identically to the wild type proteins, and all proteins had equivalent solubility. These proteins were compared with the wild type for the ability to inhibit the binding of TraR to the tra box I element in vitro (Fig. 8). Several of the mutant proteins exhibited significant, yet reduced ability to inhibit TraR activity (A36G, I37A, and L93A). In contrast, the TraM H40A, R41A, and Q82A mutant proteins were severely debilitated in TraR inhibition. It is of note that the Q82A mutant protein, despite strong TraR interactions as assessed in the direct binding assay (Fig. 7), was unable to inhibit TraR activity at any concentration tested. No inhibition of TraR and no supershifted species (e.g., ternary complexes) were observed, even when higher concentrations of the Q82A mutant were tested (100 μM, data not shown).

**SPR Analysis of TraM Mutant Proteins**—Kinetic analysis of purified mutant and H6TraM derivatives over a range of concentrations (9–300 nm) was performed with a single TraR-CM5 sensor chip (Table II). All of the mutant proteins exhibited alterations in binding to TraR consistent with that observed in the direct binding assay (Fig. 7). Sensorgrams were generated with 150 nM of either wild type or mutant proteins (Fig. 9). The A36G mutant has binding interactions with TraR that are very similar, perhaps even more rapid than wild type TraM. The Q82A and I37A mutants are also comparable with wild type TraM in the rate of association but dissociate more rapidly. In contrast, TraM mutants H40A and R41A exhibited reduced rates of association and more rapid dissociation. The L93A mutant was dramatically reduced for association with TraR, but once the complex formed, it was as stable as that between TraR and the wild type protein. Interestingly, although most of the binding curves fit imperfectly to Langmuir binding kinetics (as with the wild type), the highly defective mutant H40A mutant was an extremely good fit (Fig. 9 and Table II).

**DISCUSSION**

TraM is a potent inhibitor of the TraR transcriptional regulator (27, 28). In this study, we use several different approaches (gel filtration, direct binding to immobilized TraR, and SPR analysis) to demonstrate that TraR and TraM form a highly stable complex. In addition, we show that the formation of the TraM-TraR complex is coincident with inhibition of TraR DNA binding activity and that this is independent of the DNA sequences bound by TraR. These observations suggest that physical association with TraM promotes a general inactivation of the DNA binding activity of TraR. TraM therefore joins a growing list of prokaryotic regulatory proteins that exert their effects on gene expression by interacting directly with a transcription factor. Examples are well documented in the lytic cycles of bacteriophages P22 (Ant-c2) and P1 (cot-c1), in the sporulation of Bacillus subtilis (SinI-SinR), in genetic competence (ComS-ComK), and in maltose utilization in E. coli (MaY-MalT and MalK-MalT) (39–44). Additionally, several alternative σ factors in a variety of bacteria are regulated by anti-σ factors and anti-anti-σ factors that modulate and can even reverse RNAP holoenzyme formation by physically sequestering the σ factors (45–48).

Gel filtration analysis of complexes assembled by mixing whole cell lysates with 35S-labeled proteins suggests that the TraR-TraM complex formed under these conditions is slightly larger than the 52-kDa TraR dimer (Fig. 4, panel C). The most likely conformation of this complex is a dimer of TraR plus a monomer of TraM or a trimer of TraM plus a TraR monomer. Repeated attempts to determine the exact stoichiometry of TraR and TraM in the complex were confounded by differential labeling efficiencies for the two proteins. Additionally, we observed that the apparent molecular mass of this complex was
highly variable depending upon protein concentrations, further hindering our efforts at determining the ratio of TraR to TraM in the complex (data not shown). The physiological relevance of such higher order complexes is not clear.

The $K_D$ of the complex that forms between TraR and TraM, as estimated from SPR experiments using either TraR or H$_4$TraM as ligand, ranges from $1 \times 10^{-9}$ M to $4 \times 10^{-9}$ M (Table II). Our findings suggest that this complex does not form rapidly ($0.7-1 \times 10^3$ M$^{-1}$ s$^{-1}$) but dissociates slowly ($0.65-4.9 \times 10^{-4}$ s$^{-1}$). This \textit{in vitro} finding is consistent with the direct binding experiments with immobilized TraR, in which bound H$_4$TraM continued to increase for up to 17 h of incubation (Fig. 7 and data not shown). The relatively slow rate of association is also reflected in the large molar excess of TraM over TraR required to achieve full inhibition in our gel shift inhibition experiments (Figs. 2 and 3) as well as those reported previously (28). In these experiments, TraR and TraM were only briefly co-incubated (20 min to 1 h). The observation that nearly complete inhibition is achieved with longer incubation times (>15 h) at a 1:1 TraM to TraR molar ratio, but is incomplete below 1:1, suggests that the TraR-TraM interaction is noncatastic and relatively slow \textit{in vitro}. In contrast, Luo et al. (28) observed that induction of a $P_{BAG}$-\textit{traM} fusion by addition of L-arabinose to the culture resulted in relatively rapid inhibition (1–2 h) of TraR-controlled target gene expression. Inhibition in both experimental formats is clearly fostered at high TraM:TraR ratios, although the association of TraM may be less efficient \textit{in vitro}.

Stable complexes between TraM and TraR were detected using SPR over a range of protein concentration, with TraR as the ligand and TraM as the analyte or vice versa (Fig. 5). The calculated dissociation constant was about 4-fold smaller when TraR was the ligand than when TraM was the ligand (Table II and Fig. 5). This could be due to differences in the chemical cross-linking employed to bind the two proteins to the chip. Mutagenesis of Cys-64, the residue used to immobilize H$_4$TraM as the ligand, does not affect the inhibitory activity of \textit{traM in vivo} (Table IV). However, cross-linking to the CMD fiber at Cys-64 may modify the relative TraR-TraM interactions and result in the observed differences. Such effects would be averaged when TraR was the ligand as there are many different potential primary amines where cross-linking would occur (Fig. 5). In both formats, the kinetics of complex formation were best fit using Langmuir one-to-one binding. However, although the fit using the Langmuir model is reasonable, it is not ideal (see $\chi^2$ values in Table II), suggesting that the association of H$_4$TraM and TraR may be more complex than simple one-to-one binding or that additional interactions increase the overall complexity of the reaction. The association curves for both ligand configurations are slow to reach a steady-state plateau, even over long injection times (Figs. 5 and 9). This may reflect the stability of the TraR-TraM interaction or that TraM and TraR from the analyte phase continue to associate after formation of an initial one-to-one complex.

Effective regeneration of the stable TraR-TraM complex from the CM5 surface required treatment with 6 M GdnHCl. This is similar to the tenacious interactions between proteins of the bacterial chemotaxis machinery, which also required GdnHCl for effective regeneration in SPR analysis (35). The \textit{in vitro} inhibition of TraR activity by TraM is unaffected by 3-oxo-C8-HSL (this study and Ref. 28). Considering these observations, we propose that \textit{in vivo}, dissociation of TraR and TraM from the complex is not physiologically relevant and that this is a one-way, inhibitory process. This mechanism is similar to the proposed dead-end inhibitory complex formed between the SinI inhibitor and the SinR repressor of sporulation genes during initiation of endospor development in \textit{B. subtilis} and contrasts reversible regulatory complexes such as those typified by the MalK and MalY inhibitors of MalT (41, 42, 49).

Previous mutagenesis of the \textit{traM} gene from the nopaline-type Ti plasmid identified the two residues Val-86 and Pro-97, in the carboxyl-terminal region of the protein, as important for the inhibitory activity of TraM (27). Our directed mutagenesis approach has significantly expanded on these findings (Table IV). Of the 12 residues where mutations significantly affect TraM inhibitory activity, five (L29A, A36G, L43A, A81G, and L93A) demonstrated a more pronounced deficiency when TraR was overexpressed (Table IV). However, seven mutants, in two distinct clusters (position 37–41 and 82–97), had null phenotypes even when TraR was expressed at low levels, suggesting more severe mechanistic defects. Binding of TraM to TraR is clearly a prerequisite to inhibition of the protein. A number of the mutants (H40A, R41A, L54A, Y72A, G94A, and P97A) demonstrate substantial reductions in binding efficiency (Fig. 7 and Table II). Conserved residues within the region of TraM flanked by Leu-54 and Gln-82 (Gln-68, Tyr-72, Ala-81, and nonconserved Cys-64) do not appear to play important roles in the inhibition of TraR. Surprisingly, the L54A mutant manifests a more severe defect \textit{in vivo} with lower levels of TraR than with larger TraR pools (Table IV).

Consistent with the role for Pro-97 reported by Hwang et al. (27), our results implicate the carboxyl-terminal region of TraM in TraR inhibition and expand this region from Gln-82 to Pro-97. Comparison of the NGR234 and \textit{A. tumefaciens} TraM sequences reveals that Leu-93, Gly-94, and Pro-97 are located in a short 11-amino acid hydrophobic region of TraM shared by the two proteins. Individual mutations at all three positions in TraM cause significant deficiencies in TraR interactions, although the effect of the L93A mutation is most pronounced in the SPR experiments (Fig. 9 and Table II).

Interestingly, the Q82A mutation, just to the amino-terminal side of the conserved hydrophobic region, results in a protein that is only slightly reduced in binding to TraR (Table II and Figs. 7 and 9) but is completely unable to inhibit its activity \textit{in vivo} and \textit{in vitro} (Table IV and Fig. 8). The genetic background expressing lower levels of TraR provides no suppression of this defect (Table II), yet the Q82A mutant is highly proficient for simple binding interactions. This observation suggests that the Q82A mutant is affected for some other aspect of the productive interaction between TraR and TraM and that binding is insufficient to affect TraR activity. Similar results were obtained from studies of the inhibitory effect of the \textit{E. coli} MalK protein on MalT activity, in which mutations in either gene that abolished inhibition did not necessarily affect binding to MalT (41). We speculate that subsequent interactions between TraR and TraM that are facilitated by but distinct from the initial binding event act to reconfigure TraR into an inactive state and stabilize the complex. Analysis of deletion and substitution mutations in the \textit{pTiC58} \textit{traR} gene led Hwang et al. (28) to propose that TraM associates with a region between residues 142 and 176 of TraR and also residues closer to the carboxyl terminus of TraR. Our findings suggest that initial binding may not be sufficient for the anti-activation complex to form. It is intriguing to speculate that subsequent interactions between the far carboxyl terminus of TraR and other regions of TraM, requiring and perhaps including Gln-82, convert TraR to a stable, inactive form.

Our results suggest that in addition to the carboxyl-terminal sequences, the region between residues 29 and 54 plays an important role in the inhibition of TraR. Deletion analysis of the nopaline-type \textit{traM} gene suggested that loss of greater than 27 residues from the amino terminus significantly reduced the
interaction with TraR as assessed by a yeast two-hybrid assay (27). Our own deletion analysis of TraM suggests that the carboxyl terminus of the protein is necessary and sufficient for strong binding to TraR but that truncated proteins lacking the carboxyl terminus exhibit detectable binding to immobilized TraR. The H40A mutant exhibits the most severely deficient phenotype of mutants with alterations in this region. The observed complete deficiency of TraMH40A for TraR inhibition phenotype of mutants with alterations in this region. The observed complete deficiency of TraMH40A for TraR inhibition in vivo may be partially due to its instability when expressed in A. tumefaciens (Fig. 6). However, purified H6TraMH40A is unable to inhibit TraR activity in vitro (Fig. 8) and is severely reduced for binding to TraR (Fig. 7). SPR can detect weak interactions of TraMH40A with TraR, but this interaction is orders of magnitude weaker than that with wild type TraM (Fig. 9 and Table II). This suggests that residue H40A plays an important although perhaps structural role in the stable binding of TraM to TraR. The TraMH41A mutant exhibits a similar, yet less severe phenotype than the H40A mutant, suggesting a common deficiency and hence perhaps a related function for these adjacent residues. Our mutational analysis is consistent with the carboxyl terminus of TraM functioning in the initial binding of TraR but also suggests that stabilization of the complex requires interactions between other regions of the protein.

The observation of a major TraM degradation product, slightly smaller than full-length TraM (Fig. 6), in A. tumefaciens extracts may reflect proteolytic processing of the protein. In B. subtilis the ComS inhibitor of the primary competence transcription factor ComK is turned over through recruitment of MecA (a ClpP-type chaperone) and subsequent proteolysis via the ClpP protease (44). In fact, ComS-dependent titration of the MecA-ClpP proteolytic machinery is thought to play an important role in elevating ComK pool sizes during competence. TraR that has not bound its acyl-HSL ligand is rapidly turned over by proteolysis in A. tumefaciens, whereas the acyl-HSL bound form of TraR is more stable (17). It is plausible that the physical interaction of TraM with TraR stimulates the proteolysis of both proteins, augmenting the direct inhibitory effect of TraR association.

TraM clearly acts to prevent low levels of TraR expressed under noninducing conditions from activating tra gene expression. Our results demonstrate that the anti-activation complex that forms between TraR and TraM is likely to be irreversible. Furthermore, our findings suggest that formation of a fully stable complex may require interactions between the two proteins following the initial binding event, perhaps reconfiguring the TraR into a permanently inactive state. These observations parallel the recent finding that fully folded TraR cannot associate with 3-oxo-C8-HSL but rather must fold in the presence of the pharmacome to reach an active conformation (17). Therefore, in order for TraR to access its DNA targets and activate Ti plasmid conjugational transfer, it must be expressed in the presence of inducing levels of 3-oxo-C8-HSLs and attain levels sufficient to titrate the effect of the TraM inhibitor.

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15. Fag выводок, а также экономического развития, сказывают, что в эпоху, когда все еще был правописание, когда все еще была история, когда все еще был язык, когда все еще была наука, когда все еще была философия, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусства, когда все еще были музыка, когда все еще были литература, когда все еще были наука, когда все еще были искусств
Inhibition of the *Agrobacterium tumefaciens* TraR Quorum-sensing Regulator: INTERACTIONS WITH THE TraM ANTI-ACTIVATOR

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