Amino-Terminal Protein Fusions to the TraR Quorum-Sensing Transcription Factor Enhance Protein Stability and Autoinducer-Independent Activity

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TraR of *Agrobacterium tumefaciens* is a member of the LuxR family of quorum-sensing transcription factors and regulates genes required for conjugation and vegetative replication of the tumor-inducing (Ti) plasmid in the presence of the autoinducer 3-oxooctanoyl-homoserine lactone (OOHL). In the absence of OOHL, TraR is rapidly destroyed by proteolysis, suggesting that this ligand is required for TraR folding. To date, no TraR variant has been found that is active in the absence of OOHL. In this study, we conducted whole-cell and plasmid mutagenesis experiments to search for constitutive mutations of *traR* and identified two constitutive alleles. Surprisingly, neither contained a point mutation within the *traR* gene, but rather, both encoded fusion proteins between TraR and the N-terminal domain of an aminoglycoside *N*-acetyltransferase, encoded by a plasmid-borne antibiotic resistance gene present in the original strain. Data from Western immunoblot assays, pulse-chase assays, and immunoprecipitation assays show that these fusion proteins are far more stable to proteolysis than native apo-TraR. We also constructed a library of *traR* alleles encoding random amino-terminal fusions and selected for constitutive TraR activity. Five independent fusion proteins were identified by this approach. These fusion proteins accumulated to far higher levels than wild-type TraR in the absence of OOHL. One of these fusions was overexpressed in *Escherichia coli* and showed detectable *tra* box binding in the absence of OOHL. These data suggest that the native amino terminus of TraR may signal proteolysis and that fusing it to other proteins might sequester it from intracellular proteases.

Acyl-homoserine lactone (AHL)-based quorum-sensing systems are widespread cell-cell communication systems found throughout the proteobacteria. These systems control diverse sets of genes in a population density-dependent manner and regulate diverse biological functions, including bioluminescence, virulence, the formation of biofilms, exopolysaccharide production, and plasmid conjugation (10, 21, 35). These systems involve two major components. First, they have an AHL synthase, which usually resembles the LuxI protein of *Vibrio fischeri* and which synthesizes chemical signals that diffuse across the cell membrane. The other component is an AHL signal receptor and transcription factor that resembles the *V. fischeri* LuxR protein. These transcription factors consist of two domains, an N-terminal AHL binding domain and a C-terminal DNA binding domain (8, 14). These transcription factors are thought to bind to *cis*-acting operator sequences located in the promoter region of target genes (36).

Although a large number of putative LuxR family members have been identified, only a few of them have been extensively studied genetically, biochemically, and structurally. When overexpressed in *Escherichia coli*, several LuxR-type proteins accumulate only in an insoluble form in the absence of their cognate autoinducers but are highly soluble in their presence. These proteins include LuxR from *V. fischeri* (32), TraR from *Agrobacterium tumefaciens* (42), and CepR from *Burkholderia cenocepacia* (34). These data suggest that these proteins require AHLs for proper folding into a native conformation. In

the case of TraR, AHLs were further shown to be required for cotranslational folding of the nascent protein into a protease-resistant conformation (43). This indicates that AHLs may function for this and related proteins as scaffolds for correct protein folding. In three studies, a highly restricted set of AHLs acted as agonists for a particular LuxR-type protein, and in two of these studies, several heterologous AHLs acted as rather potent antagonists (28, 34, 40).

The TraR protein of *A. tumefaciens* is one of the best studied members of the LuxR family. TraR requires its cognate AHL, 3-oxooctanoyl-homoserine lactone (OOHL), for folding, for protease resistance, and for dimerization. In the absence of OOHL, the nascent TraR protein is rapidly destroyed by proteolysis in *A. tumefaciens* and in *E. coli* (42, 43). When TraR was expressed at native levels, it was easily detected by Western immunoblotting when OOHL was added but was undetectable in the absence of OOHL due to rapid proteolysis (3). This is further supported by the X-ray crystal structures of TraR-OOHL-DNA complexes, in which OOHL is buried deeply within the hydrophobic core of the protein and has no significant contact with the aqueous environment outside the protein (33, 39). Genetic, biochemical, and structural studies of several LuxR-type proteins indicate that a number of conserved residues in the N-terminal domain are important for AHL binding and that mutation of these residues often causes dramatically impaired AHL binding (3, 16, 18). TraR binds OOHL virtually irreversibly under native conditions, though it can be slowly released by using large amounts of nonionic detergents (43).

Given our previous data showing that wild-type TraR requires OOHL for correct folding and protease resistance, it

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
DH5 α	<i>E. coli</i> , α complementation	Stratagene
XL1-Red	<i>E. coli</i> mutator strain, <i>mutS mutD mutT</i>	Stratagene
KYC55	<i>A. tumefaciens</i> R10, pTi-less, <i>arcB::Tn5 gusA7 Km^r</i>	5
Plasmids		
pJZ301	pMal-c2 backbone, <i>malE-traR</i> translational fusion, Amp ^r	4
pJZ304	<i>traA-traC</i> intergenic region cloned into pBend3	41
pJZ335	1-kb EcoRI fragment from pTiR10 containing <i>traR</i> cloned into the EcoRI site of pPZP201, Sp ^r	40
pJZ372	<i>PtraI-lacZ</i> fusion cloned into pSW213	40
pJZ407	<i>PtraI-ocd</i> fusion cloned into pBBR1MCS5	Unpublished data (J. Zhu and S. Winans)
pYC336	<i>traR</i> from pJZ301 cloned into the BamHI and HindIII sites of pPZP201	This study
pYCM101	<i>aacC1-traR</i> fusion in pPZP201, containing 576 nucleotides of <i>aacC1</i>	This study
pYCM107	<i>aacC1</i> from pYCM101 and <i>traR</i> from pJZ301 cloned into pPZP201	This study
pYCM110	<i>aacC1-traR</i> fusion in pPZP201, containing 195 nucleotides of <i>aacC1</i>	This study

seemed worthwhile to try to isolate mutant alleles of *traR* that activate a target promoter in the absence of OOH_L. We know of only one LuxR-type protein, LuxR itself, in which such constitutive mutations have been found. In two studies, a total of eight point mutations showed an elevated level of activity in the absence of autoinducer (25, 31). Surprisingly, these mutant genes encode sequences spanning the length of the LuxR protein including the C-terminal DNA binding domain. In addition, deletion mutations of LuxR and of the *Pseudomonas aeruginosa* LasR protein that remove the entire N-terminal domain also result in constitutive activity (6, 16). In the case of LuxR, it was proposed that the N-terminal domain inhibits the C-terminal domain, that autoinducer neutralizes this inhibition in the wild-type protein, and that inhibition can be relieved either by removing the N-terminal domain or by point mutations that block transduction of this inhibitory signal. However, several genetic, biochemical, and structural studies indicate that the N-terminal domains of LuxR and TraR play a positive role in protein function, both by mediating dimerization and by contacting RNA polymerase (4, 7, 18, 41). This complicates the interpretation of these data and underscores the need to isolate and study constitutive alleles encoding TraR, a protein that has been exceptionally amenable to biochemical analysis.

MATERIALS AND METHODS

Media and reagents. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37°C. *A. tumefaciens* strains were cultured at 28°C in AT minimal medium (2). Antibiotics were added at the following concentrations: spectinomycin at 100 μ g ml⁻¹, gentamicin at 100 μ g ml⁻¹ for *A. tumefaciens* and 10 μ g ml⁻¹ for *E. coli*, and tetracycline at 2 μ g ml⁻¹. X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was included in the medium at 40 μ g ml⁻¹, and IPTG (isopropyl- β -D-thiogalactopyranoside) was added at 500 μ M. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was purchased from Sigma. Restriction enzymes and other DNA modification enzymes were purchased from New England Biolabs. OOH_L was generously provided by Anatol Eberhard and had been prepared as previously described (40). DNA automatic sequencing was performed at the Cornell Biotechnology Center.

Mutagenesis of whole cells and of plasmids. Strain KYC55(pJZ335)(pJZ372)(pJZ407) was used for whole-cell mutagenesis. A 100-ml culture was grown at 28°C in AT minimal medium, and cells were harvested at mid-log growth phase. Approximately 5×10^{10} cells were washed with 50 ml of 100 mM citrate buffer (pH 5.5) twice and resuspended in 10 ml of the same buffer (1). MNNG was

added to the cell resuspension at a final concentration of 200 μ g ml⁻¹, followed by incubation at 28°C for 30 min with gentle shaking. Cells were centrifuged, washed twice with 10 ml of 100 mM phosphate buffer (pH 7.4), and resuspended in 1 ml of the same buffer (27). Resuspensions were diluted 20-fold into LB broth, distributed in 2-ml portions into test tubes, and grown at 28°C overnight with vigorous shaking. The next day, cells were harvested, washed with phosphate buffer, and plated on solid medium containing AT salts supplemented with appropriate antibiotics, IPTG, X-Gal, and 800 μ g of arginine (added as the sole carbon source) ml⁻¹, and incubated at 28°C for 10 days. Blue colonies were streaked onto fresh AT minimal agar plates containing glucose and X-Gal to confirm elevated β -galactosidase activities. Derivatives of pJZ335 were recovered by purifying all three plasmids, introducing them into DH5 α by transformation, and selecting for Sp^r. To ensure that these plasmids contained mutant *traR* alleles, they were retransformed into KYC55(pJZ372) and screened for TraR activity with X-Gal. Plasmids containing mutant *traR* alleles were analyzed by agarose gel electrophoresis and by automated DNA sequencing.

For plasmid mutagenesis, pJZ335 was introduced into *E. coli* XL-1 Red competent cells (Stratagene) by transformation. Ten transformants were inoculated individually into LB broth supplemented with 100 μ g of spectinomycin ml⁻¹ and cultured at 37°C with vigorous shaking. Cultures were diluted 1,000-fold into fresh LB broth every 10 generations. Plasmid DNA was prepared from the cultures after 20 and 40 generations, using a Qiaquick Spin column (QIAGEN), and were introduced into KYC55(pJZ372) by electroporation. Cells were plated on AT minimal agar plates containing 0.5% glucose, appropriate antibiotics, IPTG, and X-Gal and were incubated at 28°C. Blue colonies were analyzed as described above.

Assays of TraR-dependent activities in vivo. Bioassays for TraR activity were conducted with derivatives of *A. tumefaciens* strain KYC55(pJZ372) containing a second plasmid expressing either wild-type TraR or a TraR variant. These strains were cultured in AT minimal medium supplemented with 500 μ M IPTG and 250 nM OOH_L, grown with vigorous aeration for 12 h at 28°C, and assayed for β -galactosidase activity (20). All data represent the averages of two independent experiments, and error bars represent standard deviations.

Immunological assays of TraR accumulation in vivo. *A. tumefaciens* strain KYC55(pJZ372) containing a second plasmid expressing either wild-type TraR or a TraR variant was cultured at 28°C in AT minimal medium supplemented with antibiotics, 500 μ M IPTG, and 250 nM OOH_L. Cells were harvested at late exponential growth phase, resuspended in 1 ml of TEDG buffer (4), and disrupted with a French pressure minicell (10,000 lb/in²). The resulting lysates were separated into soluble and pellet fractions by ultracentrifugation (65,000 $\times g$ for 30 min) and were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred by electrophoresis to a nitrocellulose membrane (Bio-Rad) and were immunodetected with affinity-purified polyclonal anti-TraR rabbit antiserum as described previously (3).

Immunoprecipitation assays of pulse-labeled TraR. *A. tumefaciens* strains expressing TraR were cultured at 28°C in AT minimal medium supplemented with appropriate antibiotics and 500 μ M IPTG until exponential growth phase. [³⁵S]methionine was added to the cultures to a final concentration of 5 μ Ci ml⁻¹,

and after an interval of 5 min, nonlabeled methionine was added to the cultures at a final concentration of 2 mM. Cells were withdrawn at various intervals, washed with cold Slota buffer (3), and immediately frozen at -80°C . Cell pellets were resuspended in 450 μl of an ice-cold lysis buffer (50 mM Tris-HCl [pH 7.9], 200 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 1 mM β -mercaptoethanol, 0.1% protease inhibitor cocktails, 1% NP-40, 500 μg of freshly prepared lysozyme ml^{-1}) and were incubated on ice for 30 min. The resulting lysates were ultracentrifuged and were subjected to immunoprecipitation by using prepurified TraR antibody and protein A-agarose following a published protocol (15). Results were analyzed by using a Storm B840 PhosphorImager (Molecular Dynamics).

Screening of TraR random fusions. Plasmid pYC336 was constructed by cloning a *traR* fragment lacking its start codon from pJZ301 (4) between the BamHI and HindIII sites of pPZP201 (13). Chromosomal DNA of *E. coli* DH5 α was prepared as described previously (11). Chromosomal DNA and pYC336 were subjected to restriction enzyme digestions, purified by using Qiaquick PCR purification kits (QIAGEN), and ligated overnight at 16°C . Ligated DNA was introduced into strain KYC55(pJZ372) by electroporation. Cells were plated on AT minimal agar plates with appropriate antibiotics, 500 μM IPTG, and 40 μg of X-Gal ml^{-1} and were incubated at 28°C . Blue colonies were analyzed by methods described above.

Protein overexpression and gel mobility shift assays. Strains DH5 α (pE3A7) and DH5 α (pJZ335) were cultured in 100 ml of LB medium containing 100 μg of spectinomycin ml^{-1} and 500 μM IPTG in the presence or absence of OOHl at 37°C . Cells were harvested at exponential growth phase, resuspended in 2 ml of TEDG buffer, and disrupted with a French pressure minicell (10,000 lb/in 2). Cell debris was removed by ultracentrifugation (65,000 $\times g$ for 30 min), and cleared lysates were diluted and used for gel mobility shift assays. A 240-bp fragment containing the *traA-traC* intergenic region was cut from pJZ304 by digestion with XbaI and HindIII and was end labeled by using [α - ^{32}P]dATP and the Klenow fragment (3' $\rightarrow$ 5' *exo* $^{-}$) of DNA polymerase I. Binding reaction mixtures containing 10^{-12} M DNA and cleared lysates in the amounts indicated were used at 4°C in a buffer containing 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 30 μg of calf thymus DNA ml^{-1} , 20 μg of bovine serum albumin ml^{-1} , and 10% glycerol. Samples were incubated for 20 min and size fractionated at 4°C , using 5% polyacrylamide gels in 0.5 $\times$ TBE buffer (42). Gels were analyzed with a Storm PhosphorImager.

RESULTS

Screening and selection systems for TraR constitutive mutations. To search for constitutive mutations of TraR, we mutagenized both whole cells and plasmids. For whole-cell mutagenesis (Fig. 1A), three plasmids, pJZ335 (*Plac-traR* fusion for TraR overexpression), pJZ372 (*PtraI-lacZ* fusion), and pJZ407 (*PtraI-ocd* fusion), were introduced into the Ti plasmid-less strain KYC55. The *ocd* gene encodes an ornithine cyclodeaminase for conversion of ornithine to proline, which is required for proline utilization in *A. tumefaciens* (5). In *A. tumefaciens* R10, there are two genes that encode this enzyme, one is the *ocd* gene on the Ti plasmid and the other is the *arcB* gene on the chromosome (5). Since both of these genes are absent in KYC55, the *PtraI-ocd* fusion can be used to select for elevated *PtraI* expression by growing cells in ornithine (or arginine) as the sole carbon source. The *PtraI-lacZ* fusion was added to provide a simultaneous screen for elevated TraR activity. We reasoned that either fusion could be upregulated by a *cis*-acting mutation but that only a *trans*-acting mutation in *traR* would upregulate both fusions, leading to a blue colony. In control experiments, this strain grew vigorously on this medium in the presence of OOHl, forming blue colonies in 3 to 5 days. We mutagenized 300 ml of cells (approximately 1.5×10^{11} cells) in 30 individual pools and obtained approximately 200 independent colonies after selective growth for 10 to 12 days.

For plasmid mutagenesis (Fig. 1B), we introduced plasmid

FIG. 1. Construction of the strains used for whole-cell mutagenesis and plasmid mutagenesis. (A) Three plasmids were introduced into strain KYC55, which lacks a Ti plasmid. Plasmid pJZ335 contains a *Plac-traR* fusion for TraR expression; plasmid pJZ372 contains a *PtraI-lacZ* fusion for screening of expression of the *PtraI* promoter; plasmid pJZ407 contains a *PtraI-ocd* fusion for selection of *PtraI* expression. (B) pJZ335 was mutagenized by continued growth in strain XL1-Red and then introduced into KYC55(pJZ372) by electroporation. Up-regulation of the *PtraI* promoter was screened by using X-Gal minimal medium.

pJZ335 into an *E. coli* XL-1 Red mutator strain. Plasmid DNA was purified from 10 independent pools of these cultures after 20 and 40 generations of cell growth. We then introduced mutagenized plasmid DNA into KYC55(pJZ372) by electroporation and screened for transformants that formed blue colonies on AT minimal agar plates containing glucose, X-Gal, and IPTG. Since glucose was provided as the carbon source, this approach provided a screen for constitutive mutants rather than a genetic selection. Approximately 30,000 independent colonies were screened.

Characterization of two constitutive mutants of TraR. Twelve constitutive candidate mutants were identified as a result of the whole-cell mutagenesis described above, while no mutants were found by plasmid mutagenesis. Of the 12 candidates, 10 were found to have wild-type *traR* genes (data not shown), suggesting that these strains had *cis*-acting mutations in the reporter fusions. The two remaining candidates showed TraR-dependent elevated activity of the *traI-lacZ* reporter. We recovered pJZ335 plasmid derivatives (designated pYCM101 and pYCM110) from those two strains. We introduced these plasmids into strain KYC55(pJZ372) by transformation, cultured the resulting strains in the presence or absence of OOHl and IPTG, and measured β -galactosidase specific activity. Plasmids pYCM101 and pYCM110 activated the *traI-lacZ* fusion at 30 and 110 Miller units, respectively (Fig. 2A), a much higher level than that observed for TraR from the wild-type pJZ335 (less than 2 Miller units). In the presence of OOHl, they activated the *lacZ* reporter slightly more strongly than did the

FIG. 2. (A) Assays of β -galactosidase specific activities of derivatives of KYC55(pJZ372) containing the wild-type plasmid pJZ335, pYCM101, or pYCM110 in the presence or absence of 250 nM OOHl and 500 μ M IPTG. (B) *traR* fusions found in pYCM101 and pYCM110 and the wild-type *traR* in the parent plasmid pJZ335. (C) Alignment of the first 20 residues of the amino termini of AAC in pJZ407 and DHFR from *S. enterica*.

wild type. Unlike activity from the wild-type strain, β -galactosidase activity from strains containing pYCM101 or pYCM110 was not significantly enhanced by IPTG (Fig. 2A).

Plasmids pYCM101 and pYCM110 were analyzed by agarose gel electrophoresis and by automated DNA sequencing. Surprisingly, restriction enzyme digestions of the two candidates showed quite different patterns from those of the parent plasmid, indicating that a gross DNA rearrangement had occurred (data not shown). A series of subclones of these two candidates were constructed to identify the fragments with TraR-dependent constitutive activities. Plasmids pYCM101 and pYCM110 each arose from a nonhomologous recombination event between the parental plasmids pJZ335 and pJZ407. In both cases, the *traR* structural gene was intact but separated from the *Plac* promoter by integration of the entire pJZ407 plasmid. In both cases, these fusions resulted in protein fusions between the products encoded by *traR* and the *aacC1* gene of pJZ407, which encodes aminoglycoside acetyltransferase [AAC(3)-I, here abbreviated AAC] and confers resistance to gentamicin (30). In pYCM101, *traR* was fused to a 576-bp

sequence of the *aacC1* structural gene, while in pYCM110, it was fused to a 195-bp sequence of the same gene. Both of the fusion proteins were expressed from the *aacC1* promoter (while the *traR* gene of pJZ335 was fused to the *Plac* promoter), fully explaining why β -galactosidase activity was not stimulated by IPTG. Sequence analysis suggested that these fusion genes could express both AAC-TraR fusion protein and native TraR protein from the same mRNA (Fig. 2B). We also used BLAST to search for proteins similar in sequence to the N-terminal domain of the ACC protein and found an almost perfect match between the N-terminal residues of AAC and a dihydrofolate reductase (DHFR) of *Salmonella enterica* (Fig. 2C). The related DHFR protein of *E. coli* (sharing 33% identity and 58% similarity with *S. enterica* DHFR) was recently proposed to contain an intramolecular chaperone-like sequence at its N terminus (12, 22, 24).

apo-AAC-TraR and apo-TraR produced from pYCM101 accumulated in vivo due to reduced protein turnover. In the absence of OOHl, TraR is rapidly destroyed by proteolysis in both *E. coli* and *A. tumefaciens* (42, 43) and did not accumulate to significant levels when mildly overproduced in strain KYC55(pJZ335) (3). We reasoned that the constitutive activities of the two mutants might be attributable to their proteolytic stability. Using the candidate with the stronger activity (pYCM101), we performed Western immunoblot assays to compare the production and stability of the AAC-TraR fusion protein with that of wild-type TraR. We found two major protein bands, one corresponding in size to native TraR and the other corresponding in size to the AAC-TraR fusion protein (Fig. 3A, lanes 5 to 8). Approximately half of both proteins was in the supernatant fraction after ultracentrifugation (Fig. 3, compare lanes 5 and 6). Both the apo-AAC-TraR fusion protein and the native-length apo-TraR protein expressed from pYCM101 accumulated to much higher levels than did the apo-TraR protein expressed from pJZ335 (Fig. 3A, compare lanes 2 and 6). In the presence of OOHl, the TraR and AAC-TraR fusion proteins from pYCM101 were both produced at higher levels than TraR from pJZ335 (Fig. 3A, compare lanes 3 and 4 to 7 and 8), possibly due to differences between the *aacC1* promoter of the mutant and the *lac* promoter of the wild type. Surprisingly, much of the TraR and AAC-TraR proteins were in the soluble fraction (Fig. 3A, lane 6). We also conclude that the AAC-TraR fusion protein somehow protects the coexpressed native TraR protein from proteolysis.

Since both the apo-TraR and apo-AAC-TraR fusion proteins were produced from pYCM101 and both accumulated more abundantly in the cell, we wondered which one (or both) accounted for the constitutive activities. We therefore fused a *traR* fragment lacking its ATG start codon to the same *aacC1* fragment as that found in pYCM101, resulting in a fusion that expressed only the AAC-TraR fusion protein and not the native TraR protein (see Materials and Methods). We introduced the resulting plasmid, pYCM107, into KYC55(pJZ372) and measured β -galactosidase activities in the presence or absence of OOHl. The strain containing pYCM107 expressed the reporter at an elevated level, although lower than that of pYCM101 (Fig. 3B). We also performed Western immunoblot assays and found that the strain containing pYCM101 and that containing pYCM107 accumulated apo-AAC-TraR to compa-

FIG. 3. (A) Western immunoblot assays to detect TraR and AAC-TraR fusion proteins prepared from strains KYC55(pJZ372) (pYCM101) and KYC55(pJZ372)(pJZ335) cultured in the presence or absence of 250 nM OOHL. Lanes 1, 3, 5, and 7 represent samples from total lysates, while lanes 2, 4, 6, and 8 represent samples from soluble fractions after ultracentrifugation. (B) β -Galactosidase specific activities of KYC55(pJZ372)(pYCM107) and its parent strain cultured in the presence or absence of OOHL. The numbers in parentheses represent standard deviations. (C) Western immunoblot assays to detect putative AAC-TraR fusion proteins in the soluble fractions produced from KYC55(pJZ372)(pYCM107) cells cultured in the presence or absence of 250 nM OOHL.

able levels (Fig. 3A and C). We conclude that both the apo-AAC-TraR fusion protein and the cotranslated apo-TraR may contribute to the TraR-dependent constitutive activities in pYCM101.

To measure the stabilities of the apo-TraR and apo-AAC-TraR fusion proteins produced from pYCM101, we immunoprecipitated these proteins at various time intervals after pulse-labeling them with radiolabeled methionine. We cultured strains containing either pYCM101 or pJZ335 in AT minimal medium in the absence of OOHL. Both the apo-AAC-TraR fusion protein and the cotranslated apo-TraR produced from pYCM101 had a much longer half-life (16 and 12 min, respectively; Fig. 4) than that of apo-TraR produced from pJZ335 (2 min).

A library of fusions between *E. coli* genes and *traR*. Since an N-terminal fusion between the AAC protein and TraR caused constitutive TraR activity, we decided to test whether other N-terminal fusions had the same effect. We therefore constructed a library of random DNA fragments from the chromosome of *E. coli* fused to a *traR* gene whose ATG start codon had been removed. This library was introduced into KYC55(pJZ372), and scored for blue colonies on medium containing X-Gal but lacking OOHL.

We identified six new alleles of *traR* with elevated activities (Table 2). Two of these contain fragments of the *E. coli* *ahpC* gene, which encodes an alkyl hydroperoxide reductase subunit

FIG. 4. (A) Pulse-chase experiment to compare the protein stabilities of AAC-TraR fusion protein and the cotranslated TraR produced from KYC55(pJZ372)(pYCM101) to that of TraR produced from KYC55(pJZ372)(pJZ335) in the absence of OOHL. Arrows point to the immunoprecipitated apo-TraR and apo-AAC-TraR fusion protein that had been radiolabeled with [35 S]methionine and then treated with excess unlabeled methionine for the time intervals indicated above each lane. (B) Half-lives of TraR and AAC-TraR shown in panel A were quantitated with a Storm PhosphorImager.

C subunit. These two fusions had identical amounts of the *aphC* gene (19 codons) but differing amounts of upstream DNA. Of the remaining four fusions, one is from the *ascF* gene, encoding phosphotransferase system enzyme IIBC. Another is from a gene designated b2430, which encodes a putative beta-lactamase. Another is from an *ilvC* gene, which encodes a ketol-acid reductoisomerase. The last fusion is from the *yafO* gene, which encodes a hypothetical protein. We did not find significant similarities in size or primary amino acid sequence among these candidates, although all of them have high isoelectric points (between 9 and 10; Table 2).

apo-AhpC-TraR is more soluble than wild-type apo-TraR and weakly bound *tra*-box DNA. We overexpressed the AhpC-TraR fusion protein of pE3A7 in *E. coli* and size fractionated the proteins by SDS-PAGE. In the presence of OOHL, the

TABLE 2. Results of random fusion screening^a

Candidate	β -Galactosidase activity with:		Description of <i>traR</i> fusion	Isoelectric point	Net charge at pH 7.0
	250 nM OOHL	No OOHL			
pYC336	48	1.5	No fusion		
pB2D2	1,020	21	Codon 1–67 of <i>yafO</i>	9.31	3.405
pB2D3	860	26	Codon 1–106 of <i>ascF</i>	9.43	4.015
pE2G1	1,832	58	Codon 1–19 of <i>ahpC</i>	10.48	3.905
pE3A7	2,212	125	Codon 1–19 of <i>ahpC</i>	10.48	3.905
pE3A8	2,005	18	Codon 1–28 of <i>ilvC</i>	9.17	1.880
pE3C4	2,139	56	Codon 1–43 of b2430	10.04	2.074

^a KYC55(pJZ372) strains containing each of the seven candidate plasmids were cultured in the presence or absence of OOHL, and β -galactosidase activity (Miller units) was determined. Isoelectric points were calculated by using the DNASTAR program (Lasergene).

FIG. 5. (A) SDS-PAGE of putative AhpC-TraR fusion proteins overproduced in DH5 α in the presence or absence of OOHl. The diagonal arrow indicates the AhpC-TraR proteins produced in the soluble fraction in the absence of OOHl. (B) Immunodetection of TraR proteins, comparing the accumulation of AhpC-TraR and that of wild-type TraR in KYC55(pJZ372) strains containing either pE3A7 or pJZ335 in the presence or absence of OOHl. (C) Gel mobility shift assays of native and AhpC-TraR, using cleared lysates (0.25, 0.5, or 1 μ l of extract per lane) made from strains cultured in the absence or presence of OOHl, as indicated.

majority of the AhpC-TraR fusion protein was soluble, while in the absence of OOHl, a portion of the fusion protein was still in the soluble fraction (Fig. 5A). This contrasts with the native TraR, which is insoluble under similar conditions (42). We also overexpressed AhpC-TraR fusion proteins by using strain KYC55(pJZ372)(pE3A7) cultured in the presence or absence of OOHl and performed Western immunoblot assays to detect the accumulation of AhpC-TraR fusion proteins in *A. tumefaciens*. In the absence of OOHl, the apo-AhpC-TraR fusion protein accumulated to a higher level than apo-TraR produced from KYC55(pJZ372)(pJZ335), while in the presence of OOHl, they accumulated at similar levels (Fig. 5B). We did gel mobility shift assays using a DNA fragment containing the *traA-traC* intergenic sequence and a cleared lysate from DH5 α (pE3A7) cultured in the absence of OOHl. This lysate detectably (though weakly) shifted the *tra* box-containing fragment (Fig. 5C), while cleared lysates from DH5 α (pJZ335) did not do so when cultured in the absence of OOHl (Fig. 5C). As expected, when DH5 α (pE3A7) or

DH5 α (pJZ335) (Fig. 5C) was cultured in the presence of OOHl the resulting lysates efficiently shifted the *tra* box-containing fragment.

DISCUSSION

TraR is one of the best studied members of the LuxR family and has been the subject of extensive biochemical and structural studies, as well as deletion and point mutagenesis (3, 18, 26). No constitutive TraR point mutant has ever been described. In the present study, we conducted both whole-cell mutagenesis and plasmid mutagenesis in an effort to isolate mutations either in *traR* or in other genes that may result in constitutive expression of a TraR-regulated promoter. It seemed quite plausible that mutations in chromosomal genes, for example, in cytoplasmic proteases, could enhance TraR function. However, we have so far not isolated mutations in chromosomal genes that confer this phenotype. More unexpectedly, we also did not obtain constitutive point mutations in *traR*. We note that the strain used for mutagenesis grew vigorously on the selective medium used when OOHl was added, so the strategy for obtaining mutants seems valid. We also note that the same selection previously yielded a wealth of constitutive single and double point mutations in the *A. tumefaciens occR* gene, which encodes a LysR-type protein that activates the *occ* operon in response to octopine (1). However, the only constitutive mutants that we isolated contained fusions between TraR and a foreign protein. Given the number of mutagenized cells that we used for this selection, we believe that it may be impossible to find single point mutations in *traR* that would result in constitutive activity.

For ligand-dependent transcription factors, the isolation of ligand-independent mutants is a commonly applied method to look for residues required for ligand binding or signal transduction. Our selection for constitutive mutants yielded only fusion proteins, despite the fact that such mutants should arise far less frequently than point mutants. This selection strategy was validated using another protein (OccR) (1). We conclude that it may be impossible to find point mutations in *traR* that lead to a constitutive phenotype.

As described above, the only mutants we obtained from the original selection were cointegrates of two of the plasmids in the parent strain. Additional N-terminal fusions were later obtained by creating a library of such fusions and selecting for OOHl-independent TraR activity. Though the fusion proteins had partially constitutive activity, activity was further enhanced by OOHl. Strikingly, these N-terminal fusions almost completely blocked the rapid proteolysis that characterizes the wild-type apo-TraR. We have argued in the past the OOHl somehow acts as a scaffold for TraR folding (42, 43), and it now appears that certain N-terminal fusions can suppress the need for this scaffold. However, not every N-terminal TraR fusion protein has constitutive activity, inasmuch as we previously described a maltose-binding protein (MBP)-TraR fusion that is fully soluble and stable *in vivo* in the absence of OOHl but which is transcriptionally inactive (43).

We have speculated in the past that the sole function of OOHl might conceivably be to block TraR proteolysis. The fusion proteins isolated in the present study were far more abundant and protease resistant than wild-type protein and

were partially constitutive, supporting this hypothesis. However, OOHl strongly enhanced the activity of these fusion proteins, while having only a modest effect on their abundance or solubility. This suggests that OOHl plays roles in TraR activity that go beyond its proteolytic stability. Similarly, in previous studies we described an MBP-TraR fusion that is proteolytically stable and abundant in the absence of OOHl, but is inactive. This finding also suggests that OOHl does more than simply block TraR proteolysis. We propose that maturation of TraR may occur in at least two sequential steps. According to this idea, TraR can first fold into a form that is soluble and protease resistant, but still not competent for transcriptional activation. An additional step appears to be required to convert TraR to a form that is competent for transcription. If this model is correct, then OOHl appears able to facilitate both maturation steps rapidly, while the fusions described in this study carry out the first step fully but only partially carry out the second step. The MBP-TraR fusion also carries out the first step but is unable to further mature into a transcription-competent form without OOHl.

The dimer form of TraR is the active form for transcriptional activation, and purified apo-TraR is a monomer (43). We were also very interested to know what is the active form of fusion proteins and whether the N-terminal fusions promote dimerization of the fusion proteins (43). Based on the results of gel shift assays (Fig. 5C), we think that the active form of the AhpC-TraR fusion protein is a dimer, and this could also be true for other fusion proteins. While we believe that these N-terminal fusions stabilize TraR and may mildly promote dimerization, OOHl still has a dramatic effect on the dimerization of the fusion proteins, as was shown for AhpC-TraR fusions (Fig. 5C).

Other interesting results from this work are that the apo-ACC-TraR fusion proteins appeared to protect the native apo-TraR protein when both were coexpressed (Fig. 3A, lanes 5 and 6) and that both may contribute to transcriptional activation (compare Fig. 2A and Fig. 3B). Though the reasons for this are unclear, one hypothesis is that monomers of the fusion protein may help protect apo-TraR from misfolding and proteolysis, possibly by forming heterodimers or multimers.

TraR has a hydrophobic core, which in the presence of OOHl is buried inside, where it makes extensive contact with OOHl. In the absence of OOHl, at least some of this hydrophobic core could be exposed to solvent and apo-TraR could thus be vulnerable to aggregation and cytoplasmic proteolysis (43). It seems possible that the N-terminal portions of the fusion proteins described here might interact with the vulnerable hydrophobic core in some way or mimic the role of OOHl as a folding scaffold and lead the TraR portion of the fusion to fold into a conformation that is not only more soluble and resistant to proteolysis but also transcriptionally active. All foreign fragments in these fusion proteins showed high isoelectric points and are therefore positively charged in vivo. The positive charge of fragments could also lead to enhanced solubility.

The N-terminal fusions described in this study, in addition to the MBP-TraR fusion previously described, can be thought of as protein solubility enhancers, which are a class of proteins that when fused with other proteins normally prone to aggregation, increase the solubility of the fusion proteins. Examples

such as MBP and the *E. coli* glutathione *S*-transferase have been used for this purpose in many studies (9, 17).

The first two fusion proteins described in this study contained residues 1 to 192 or 1 to 65 of the AAC protein. The structure of this protein has been resolved by X-ray crystallography (37). To try to understand why fusions to this protein can increase the stability of apo-TraR and cause constitutive activity, we used BLAST to search for proteins similar in sequence to the N-terminal domain of the AAC protein. We found an almost perfect match between the N-terminal residues of AAC and DHFR of *S. enterica* (Fig. 2C). Interestingly, it was recently proposed that a closely related *E. coli* DHFR contains an intramolecular chaperone-like sequence at its N terminus (12, 22, 24). Intramolecular chaperones are generally divided into two families: one family in which the chaperone region is proteolytically cleaved after the formation of the mature protein and the other family in which the chaperone region is not cleaved. Examples of the first group are the subtilisin from *Bacillus subtilis* and the α -lytic protease of *Lysobacter enzymogenes* (19), while an example of the second group is the DHFR of *E. coli* (29). It has been proposed that such fragments may exist in many proteins, though it is difficult to predict how many proteins have such sequences. The N-terminal fragment of DHFR acts as both an integral part of the catalytic core and also as an intramolecular chaperone (12, 22). The role of the N terminus of DHFR as a folding chaperone was tested in *trans* in protein fragment complementation studies (23, 24). Although few studies have been reported on the folding of AAC, we speculate that the N terminus of AAC might function as an intramolecular chaperone, allowing folding of TraR into a conformation that is more resistant to proteolysis. Other TraR fusion proteins such as AhpC-TraR may have similar properties. Although we did not see any significant pattern in the size and sequence similarity of the fused fragments, all had high isoelectric points, similar to other intramolecular chaperone-like sequences (19, 38).

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