



Published in final edited form as:

*Mol Microbiol.* 2009 July ; 73(1): 32–42. doi:10.1111/j.1365-2958.2009.06730.x.

## Dimerization of the Quorum Sensing Transcription Factor TraR Enhances Resistance to Cytoplasmic Proteolysis

Uelinton M Pinto and Stephen C Winans\*

Department of Microbiology Cornell University Ithaca, New York 14853

### Summary

TraR is a LuxR-type quorum sensing protein encoded by the Ti plasmid of *Agrobacterium tumefaciens*. TraR requires the pheromone 3-oxooctanoylhomoserine lactone (OOHL) for biological activity, and is dimeric both in solution and when bound to DNA. Dimerization is mediated primarily by two alpha helices, one in the N-terminal OOHL binding domain, and the other in the C-terminal DNA binding domain. Each of these helices forms a parallel coiled coil with the identical helix of the opposite subunit. We have previously shown that OOHL is essential for resistance to proteolysis, and here we asked whether dimerization is also required for protease resistance. We constructed a series of site-directed mutations at the dimer interface, and tested these mutants for activity *in vivo*. Alteration of residues A149, A150, A153, A222 and I229 completely abolished activity, while alteration of three other residues also caused significant defects. All mutants were tested for dimerization as well as for specific DNA binding. The cellular abundance of these proteins in *A. tumefaciens* was measured using western immunoblots and OOHL-sequestration, while the half-life was measured by pulse-chase radiolabelling. We found a correlation between defects in *in vivo* activity, *in vitro* dimerization, DNA binding, protein half-life. We conclude that dimerization of TraR enhances resistance to cellular proteases.

### Introduction

The TraR protein of *Agrobacterium tumefaciens* is a member of the LuxR family of transcription factors, which are intracellular receptors of *N*-acylhomoserine lactones (AHLs), that act as pheromones in intercellular communication {Waters, 2005 #14}. AHLs are generally synthesized by a cognate LuxI-type protein (Pappas *et al.*, 2004). These pheromones accumulate in a population density-dependent manner and form complexes with their receptors when they reach a threshold concentration. LuxR/LuxI-type regulatory systems therefore allow bacteria to monitor their cell population densities, and to coordinate their behavior in a process referred to as quorum sensing. This phenomenon was first discovered in the marine bacterium *Vibrio fischeri*, whose LuxR and LuxI proteins regulate genes required for bioluminescence. Homologous systems have also been identified in many proteobacteria where they regulate diverse activities, including pathogenesis, biofilm formation, horizontal DNA transfer and the production of secondary metabolites (Waters and Bassler, 2005).

TraR regulates genes that are required for vegetative replication (*rep* genes) and conjugal transfer (*tra* and *trb* genes) of the tumour-inducing (Ti) plasmid (Fuqua and Winans, 1994; Li and Farrand, 2000; Pappas and Winans, 2003). TraR activity requires *N*-3-oxooctanoyl-L-homoserine lactone (OOHL), which is synthesized by the Ti plasmid-encoded TraI protein. TraR subunits form dimers that bind to dyad-symmetrical DNA sequences. It has

two domains, an N-terminal OOHL-binding domain and a C-terminal DNA binding domain (Pappas *et al.*, 2004). OOHL is required for TraR to become resistant to proteolysis *in vivo*, and is thought to act as a scaffold for TraR folding (Zhu and Winans, 1999, 2001).

The structure of TraR bound to OOHL and *tra* box DNA has been solved by X-ray crystallography (Vannini *et al.*, 2002; Zhang *et al.*, 2002). The N-terminal and C-terminal domains both contribute to protein dimerization. The N-terminal domain dimerizes chiefly through  $\nabla$ -helix 9 of each subunit (the longest  $\nabla$ -helix in the protein), forming a coiled-coil (Figure 1A). The C-terminal domain dimerizes through  $\nabla$ -helix 13 of each subunit, which form a second coiled-coil (Fig. 1A). Additional contacts include ionic interactions between the C-terminal carboxylate of each subunit and Arg230 of the opposite subunit.

Previous studies have shown that OOHL-mediated folding is essential for resistance to cytoplasmic proteases (Zhu and Winans, 1999, 2001). However, it was not known whether fully folded TraR monomers were protease resistant, or whether dimerization was also required. Cellular proteases are generally thought to detect denatured proteins by virtue of hydrophobic residues that would lie within the hydrophobic core of a properly folded protein (Wickner *et al.*, 1999). The interface between TraR monomers is quite hydrophobic, and dimers are stabilized in large part by hydrophobic interactions. TraR monomers would therefore probably present a large hydrophobic surface, which might be recognized by proteases, and lead to proteolysis. Dimerization would sequester these hydrophobic patches, and could thereby block proteolysis. It therefore seemed plausible that protection from proteolysis might require not only binding of OOHL, but also dimerization of TraR monomers. If so, then mutations that block dimerization would be predicted to be more susceptible to proteolysis. In this study, TraR alleles were isolated that block biological activity, protein dimerization and DNA binding *in vitro*. These mutants showed an increased rate of turnover compared to wild type TraR.

## Results

### Structure-informed mutagenesis of the TraR dimerization interface

The goals of this study were to test amino acid residues that lie at the dimerization interface for their role in dimer formation and to determine whether defects in dimerization affect the rate at which these proteins are eliminated from the cells by proteolysis. Residues facing the dimerization interface and potentially mediating hydrophobic interactions were chosen for site-directed mutagenesis (Fig. 1B). In addition, we altered residues K119, and N122 because they interact with residues D6 and D10 of the opposite monomer (Zhang *et al.*, 2002) and could potentially aid in dimerization. Residues I155 and S160 were chosen based on a previous study that showed their contribution to dimerization (Luo *et al.*, 2003). Three other mutations were included as controls that were unlikely to have a role in dimerization. Of these, alteration of residue R206 confers a DNA binding defective phenotype (White and Winans, 2007), while alteration of residue G123 confers a positive control phenotype (Luo and Farrand, 1999), and alteration of residue L5 is phenotypically silent (this work). Wherever possible, mutations were made that preserve interactions with neighboring amino acid residues.

### Transcriptional activation

Each TraR mutant was tested for its ability to activate a *PtraI-lacZ* fusion *in vivo* using a broad range of OOHL concentrations (Table 1). Mutations A149E, A149V, A150E, A150V, G153E, Q154E, A222D, and I229Y caused strong defects in TraR activation, with transcriptional levels less than 15% of the wild type for all OOHL concentrations tested. Mutation of other residues had weaker effects on the transcription activity, especially at

higher concentrations of OOHL (Table 1). Residues A149 and A150, which are located on the alpha-helix 9, were especially critical for activity, as even conservative mutations strongly impaired transcriptional activity.

### Assays for TraR dimerization *in vitro*

The assays described above measured the ability of TraR mutants to activate transcription *in vivo*, but did not directly address the role of the mutated residues in dimerization. We therefore developed assays that directly evaluate the ability of these mutant proteins to form dimers. These assays involved the construction of fusions between these mutants and the maltose binding protein (MBP) of *E. coli*. It was previously shown that native TraR can form heterodimers with MBP-TraR fusions (Zhu and Winans, 2001). Clarified lysates containing MBP-TraR fusion proteins were combined with lysates containing wild type TraR. After allowing sufficient time for heterodimer formation, MBP-TraR fusion proteins were purified using an amylose-agarose affinity resin. The bound fractions were eluted using maltose and examined by SDS-PAGE gels for the presence of native TraR. In these assays, only the fusion protein contains the point mutation of interest, while the native TraR has a wild type sequence. In contrast, in the *in vivo* assays described above, both subunits of TraR dimers contained identical mutations.

As expected, a fusion protein containing wild-type TraR sequences efficiently bound native TraR protein (Fig. 2). As expected, native TraR was not detectably bound by MBP lacking TraR sequences (data not shown). In contrast, most of the fusion proteins containing TraR mutations were defective in binding native TraR (Fig. 2, Table 2). In many cases, a correlation was found between defects in *in vivo* activity and heterodimer formation (Table 2). Residues A149, A150, G153, Q154, and I229 were especially strongly defective in both assays. A few mutant proteins did not follow this pattern, in that they were strongly defective in activity, yet had only subtle defects in dimerization (see Table 1 and 2, mutants G153R, R206Q, and T225S). These mutants were judged to be defective in some other TraR property such as DNA binding or the ability to recruit RNA polymerase to the adjacent promoter.

### Assays for binding to DNA fragments containing TraR binding sites

It is well established that TraR heterodimers containing just one DNA binding domain fail to bind to *tra* box DNA sequences (Chai *et al.*, 2001; Oger *et al.*, 1998; Zhu and Winans, 1998). This indicates that TraR monomers do not bind DNA with high affinity and that dimerization is essential for high affinity DNA binding. To test whether defects in dimerization lead to defects in DNA binding, we conducted electrophoretic mobility shift assays (EMSA) using wild type or mutant proteins and a DNA fragment that contains a consensus *tra* box. As expected, virtually all mutants that had strong defects in transcription activation and dimerization were defective in DNA binding (Fig. 3, Table 2), while mutants with subtle phenotypes in the former assays were proficient in DNA binding. An exception to this pattern was found in mutant N122A, which was defective in *in vivo* activity, yet showed wild-type affinity for *tra* box DNA. We conclude that this mutant is defective in positive control. We have identified other residues that lie close to N122 that are also required for positive control (E. Costa, H. Cho, and S. C. Winans, manuscript submitted).

### *In vivo* accumulation and stability of dimerization mutants

The central question addressed in this study is whether dimerization of TraR is required for resistance to proteolysis. We addressed this question in three ways, two of which involve measurements of TraR abundance, and one of which directly measures the half-life of these mutant proteins. TraR abundance was assayed, first, by measuring the ability of whole *A. tumefaciens* cells expressing wild type or mutant protein to sequester exogenously provided

OOHL. TraR mutants that are rapidly degraded or that cannot fold are defective in this assay (Chai and Winans, 2004). All dimerization mutants were proficient in binding OOHL, though the levels of OOHL binding were in many cases lower than with wild type TraR (Table 2).

We also assayed for the intracellular abundance of each mutant protein in *A. tumefaciens* by semi-quantitative western immunoblots. All mutant TraR proteins were readily detected immunologically, even those having very strong defects in transcription activation, dimerization, and DNA binding (Fig. 4, Table 2). These data, taken at face value, would tend to support the idea that dimerization does not play a critical role in resistance to proteolysis. However, subtle effects on protein stability could be hard to measure in this semi-quantitative assay of protein abundance.

We also used pulse-chase experiments in *E. coli* to evaluate the rate at which each mutant is degraded *in vivo*, as previously done using wild type protein (Zhu and Winans, 2001). As a negative control, a strain growing in the absence of OOHL showed very little accumulation of soluble TraR (Fig. 5), and the detectable TraR was rapidly degraded. The same strain cultivated in the presence of OOHL showed little or no TraR degradation. Mutant TraR proteins that have strong defects in dimerization, DNA binding, and activity were degraded more rapidly than the wild type protein in the presence of OOHL, but far more slowly than the wild type in the absence of OOHL. We conclude that blocks in dimerization lead to decreases in protease resistance, but that this effect is more subtle than the requirement of OOHL.

## Discussion

The crystal structure of TraR-OOHL-DNA ternary complexes has enabled a series of studies about the roles of particular amino acid residues in binding of OOHL, in decoding of *tra* box DNA, in binding to RNA polymerase (Chai and Winans, 2004; White and Winans, 2005, 2007), and now in the dimerization of the TraR subunits. Each of these studies provides an ever-sharper picture of how TraR subunits interact with their molecular environment. The results obtained in this work corroborate a model (Fig. 6) in which OOHL binds to and stabilizes TraR, allowing time for monomers to form dimers. Dimerization further enhances resistance to degradation.

By altering all residues at the subunit interface, we were able to evaluate the contribution of each to dimer formation and overall function. Residues A149, A150, and G153, all from helix 9, were critical for dimerization, as the mutations A149V, A149E, A150V, A150E, and G153E caused very strong defects in dimer formation (Fig. 2, Table 2). This could be due to the larger size of the mutant residues, and to the lack of available unoccupied space in this region of the dimer interface. Elsewhere in helix 9, mutations V146A, V146E, A157V and A157E caused relatively mild defects, even though the wild type residues make direct contacts with the opposite subunit. The structure suggests sufficient space to accommodate these bulkier residues. Helix 13 must also play a role in dimerization, as mutations A222D and I229Y caused severe defects in activity, DNA binding, and heterodimer formation (Tables 1 and 2), though more conservative mutations had milder defects. Overall, these results indicate that positions *a* and *d* on the helical wheel projection (which corresponds to residues 149, 153, 225, and 229 on TraR, Fig. 1) show specific defects in protein dimerization (Lupas, 1996). A study with the *E. coli* FNR transcription factor also showed that residues located at similar positions on the helical wheel projection were the main ones directly involved in FNR dimerization (Moore and Kiley, 2001).

Though this study focused on the use of TraR mutants, we hope to make inferences about the wild type protein. Does wild type TraR ever exist as a monomer, and if so, what are its properties? At the earliest stages of quorum sensing induction, some cells could have as few as one TraR molecule, obviously a monomer. Even in cells with more than one monomer, newly synthesized TraR monomers must require a short time interval to dimerize, and even then, dimers exist in a dynamic equilibrium with monomers (Chai *et al.*, 2001; Oger *et al.*, 1998; Zhu and Winans, 1998). Would such monomers be protease-sensitive? For several reasons, we believe that the mutants we have described are a good approximation of wild type monomers. First, wherever possible, mutations were chosen that preserve interactions with neighboring amino acid residues and change only the surface of the protein. This was done to try to minimize defects in tertiary structure. Second, most of the mutations described here did not cause large decreases in the sequestration of OOHL *in vivo*, suggesting that their overall tertiary structure was intact. Third, a general pattern was observed, in which mutants with severe defects in dimerization tended to have severe defects in protein instability, and conversely (Fig. 2 and Fig. 5). These data strongly suggest that blocks in dimerization lead directly to decreased half-life, and that these mutants are informative about the properties of wild type monomeric protein.

TraR monomers would likely have a considerable hydrophobic surface exposed to solvent, which could in principle target the protein for proteolysis. It is conceivable however, that the hydrophobic patches of the two domains could interact with each other, possibly forming an antiparallel coiled coil. If so, this might shield both domains from proteases, and possibly increase the solubility of the protein. If that is true, the formation of a TraR dimer would require the dissociation of the N-terminal and C-terminal domains of each monomer before they could interact with their counterparts of the opposite subunit.

Dimerization of transcription factors is often mediated by parallel or antiparallel helices (Lupas, 1996). For instance, the catabolite activator protein (CAP) of *E. coli* dimerizes via two parallel  $\nabla$ -helices that extend from residues 108 to 137 of each subunit (Joung *et al.*, 1995; Schultz *et al.*, 1991). The *E. coli* protein FNR (a CAP homolog), has been mutagenized in the corresponding dimerization interface, and the resulting mutants invariably showed defects in dimerization and in DNA binding (Moore and Kiley, 2001). FNR dimerization occurs only in anaerobic conditions, in which a  $(4\text{Fe-4S})_2^+$  cluster in each subunit is reduced to a  $(2\text{Fe-2S})_2^+$  form (Kiley and Beinert, 1998). The former is thought to distort the dimerization helix, thereby blocking dimer formation (Kiley and Beinert, 1998). The receiver domain of the *E. coli* PhoB protein has been resolved in the active and inactive conformations (Bachhawat *et al.*, 2005). Dimerization of the inactive form is mediated primarily by  $\nabla$ helix 1 of each subunit, while dimerization of the active form is mediated by  $\nabla$ -helix 4,  $\exists$ -strand 5, and  $\nabla$ -helix 5 of each subunit. In the former state, the two DNA binding domains of the dimer cannot associate, while in the latter state, they are brought into close proximity, allowing them to bind DNA. The transcription factor BmrR of *Bacillus subtilis* consists of an N-terminal DNA binding domain and a C-terminal ligand binding domain that are connected by a long  $\nabla$ helix (residues 77–119). This helix forms a long antiparallel coiled coil with its counterpart from the opposite subunit (Heldwein and Brennan, 2001). This structure is thought to be conserved among other members of the MerR family. Two dimers of *E. coli* Lac repressor associate via four C-terminal  $\nabla$ -helices, each 20 amino acids in length, with two antiparallel helices contributed from each dimer (Lewis *et al.*, 1996). Each of these helices is connected to the rest of the subunit by a flexible linker, allowing considerable conformational freedom between the two dimers. The LysR-type regulator CbnR of *Ralstonia eutropha* consists of a N-terminal DNA binding domain fused to a C-terminal ligand binding domain via a long  $\nabla$  helix that forms a parallel coiled coil with the opposite subunit (Muraoka *et al.*, 2003). In contrast, some transcription factors, including Lrp of *E. coli* and PrgX of *Enterococcus faecalis*, dimerize

by other sorts of secondary structures (de los Rios and Perona, 2007; Shi *et al.*, 2005). We have not seen other reports showing that multimerization of any of these proteins plays a role in their stability.

## Experimental Procedures

### Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 3. *E. coli* strains were cultured in Luria broth (LB) or solid medium at 37°C (Miller, 1972). *A. tumefaciens* strains were cultured in AT minimal medium at 28°C (Cangelosi *et al.*, 1991). Synthetic OOHL was provided by Dr. Anatol Eberhard (Cornell University). Antibiotics were added at the following concentrations: 100 µg ml<sup>-1</sup> spectinomycin; 100 µg ml<sup>-1</sup> kanamycin, and 100 µg ml<sup>-1</sup> ampicillin. IPTG was added at 500 µM.

### DNA manipulations

Recombinant DNA techniques were performed using established procedures (Sambrook and Russel, 2001). Plasmid DNA was isolated using QIAprep spin miniprep kits (Qiagen). DNA fragments generated by PCR or restriction digestion were gel purified using QIAquick Gel Extraction Kit (Qiagen). Restriction endonucleases were obtained from New England Biolabs and used according to methods described by the manufacturers. Plasmid DNA was introduced into *E. coli* and *A. tumefaciens* by electroporation (Cangelosi *et al.*, 1991).

### Site-directed mutagenesis of *traR* and cloning into different vectors

Site-directed mutagenesis of *traR* was performed by using a synthetic overlap extension PCR and two complementary mutagenic primers (Sambrook and Russel, 2001). For mutations on the N-terminal domain of TraR, a 978 bp fragment of pYC335 (Chai and Winans, 2004) was amplified using *Pfx* DNA Polymerase (Invitrogen) to include a unique *EcoRI* site (located upstream of *traR*) and a native *SacII* site (near codon 168 of *traR*). For mutations on the C-terminal domain of TraR, a 303 bp fragment of pYC335 was amplified as above to include a native *SacII* site (near codon 168 of TraR) and a unique *MfeI* site (just downstream of the stop codon of TraR). All oligonucleotides used in this study are listed in Table 4 and were obtained from Integrated DNA Technologies (Coralville, IA). The flanking primers were used in separate reactions with two different mutagenic primers that overlap at the mutation, using pYC335 as the template. These two PCR products were then combined and used as the template in a second round of PCR with the same flanking primers to generate the complete fragment. The second set of PCR products was digested with *EcoRI* and *SacII* (for N-terminal domain mutations) or *SacII* and *MfeI* (for C-terminal domain mutations), and ligated to pYC335 digested with the same enzymes. Mutant sequences were confirmed by automated DNA sequencing.

The *traR* point mutants were also cloned into vectors pAFM02 and pJZ358. For the former plasmid, mutants that lie between codons 85 and 168 were cloned by digesting pYC335 derivatives with *BstBI* and *SacII* enzymes. The digestion of pYC335 derivatives with the above enzymes generated four fragments of distinct sizes. The one of 306 bp contained the mutated codons that were ligated in pAFM02 digested with the same enzymes. For other mutants located downstream of the codon 168, the unique sites, in both plasmids, *SacII* and *HindIII* were used. For cloning into pJZ358, digestion was performed with *HindIII* and *BbsI* enzymes. A fragment of 867 bp originating from pYC335 derivatives starting at codon 24 (*BbsI* site) of *traR* and going 227 bp downstream of its stop codon (*HindIII* site) was used to replace a fragment of 922 bp from pJZ358 digested with the same enzymes.

### Measurement of TraR activity *in vivo*

Bioassays of TraR activity were performed with each TraR mutant using NTL4(pCEW260) (pYC335) or derivatives of pYC335 carrying each of the *traR* mutants. Strains were cultured in AT minimal medium at 28°C to an OD<sub>600</sub> of 0.3–0.4. Each culture was then diluted 20-fold into AT medium containing concentrations of 0.1, 1, 10 or 100 nM OOHL, incubated with vigorous aeration at 28°C for 8 h, and assayed for  $\beta$ -galactosidase activity (Miller, 1972). Experiments were performed in triplicate using three different isolates for each strain.

### Heterodimer formation assay

To measure the ability of TraR to form heterodimers with MBP-TraR fusions, clarified extracts containing MBP-TraR fusions expressed from *E. coli* BL21/DE3(pAFM02) or pAFM02 derivatives carrying each TraR mutant were combined with equal volumes of clarified extracts containing native TraR protein expressed from BL21/DE3(pJZ358). Combined supernatants were incubated for 2 h at 28°C to allow subunit exchange, and were applied to an amylose affinity chromatography column (New England Biolabs). Proteins were step eluted with buffer containing 10 mM maltose. Purified proteins were size-fractionated using 12% SDS-PAGE gels and band intensities were quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>). Cells expressing only TraR or only MBP-TraR respectively were used as negative and positive controls.

### Electrophoretic mobility shift assays

To perform gel shift assays, clarified cell extracts were prepared from *E. coli* strain KY2347 (a *clp*, *lon* mutant) carrying pYC335 or its derivatives with each of the *traR* point mutants. Strains were cultured in LB broth containing 100  $\mu\text{g mL}^{-1}$  of spectinomycin in the presence of 200 nM OOHL at 28°C. When cultures reached OD<sub>600</sub> of 0.2, IPTG was added to a final concentration of 500  $\mu\text{M}$  to induce TraR expression, and incubation was followed by an additional 6 h. TraR abundance in the soluble fraction was estimated using Western immunoblots as previously described (White and Winans, 2005). A 247 nucleotide PCR fragment containing a consensus *tra* box was obtained from plasmid pCEW250 using primers Ptra-box For and Ptra-box Rev (Table 4). A negative control fragment of 211 nucleotides in length was PCR amplified from plasmid pCEW250 with primers Pcontrol-gel-shift-For and Pcontrol-gel-shift-Rev. Both fragments were end-labeled with [<sup>32</sup>P]-dATP (Pelkin Elmer) using T4 polynucleotide kinase (New England Biolabs). Binding reactions were performed as previously described (White and Winans, 2005), with equivalent amounts of soluble TraR in each reaction. Gel shift assays were performed with independent clarified lysates in duplicate for each strain, and data were analyzed using a Storm B840 Phosphorimager.

### Measurements of TraR abundance in *A. tumefaciens*

The abundance of each TraR allele was determined using strain NTL4(pCEW160)(pYC335) or derivatives of pYC335 carrying each of the *traR* mutants. Strains were cultured in AT at 28°C with 10 nM OOHL. Cells were harvested at mid-log phase, and westerns were performed as described previously (White and Winans, 2005). Westerns were prepared with independent cell lysates at least twice for each strain. Data were analyzed using IMAGEJ and normalized against cross-reacting material in each lane.

### OOHL sequestration assay by TraR in whole cells

*A. tumefaciens* strain NTL4(pCEW260)(pYC335) or derivatives of pYC335 carrying each TraR mutant allele were used for OOHL sequestration assays as previously described (Chai *et al.*, 2001). All assays were performed twice with independent cultures.

## TraR stability in *E. coli*

The measurement of TraR turnover for key TraR point mutants was determined using strain BL21/DE3(pJZ358) (Zhu and Winans, 1999) or derivatives of pJZ358 carrying *traR* mutants. The experiments were performed as described previously (Zhu and Winans, 2001) except that OOHl was added 10 min before the addition of radiolabel.

## Acknowledgments

The authors gratefully acknowledge Dr Anatol Eberhard (Cornell University) for the synthesis and purification of OOHl used in this study, and Ana Lidia Flores-Mireles for construction of pAFM02 plasmid. We also thank all members of our laboratory for helpful discussions and critical review of the manuscript. Funding for this study was provided by the National Institute of General Medical Sciences (GM41892). UMP acknowledges the financial support of the Brazilian government through a fellowship grant from the Coordenacao de Aperfeicoamento de Pessoal de Nivel Superior (Capes).

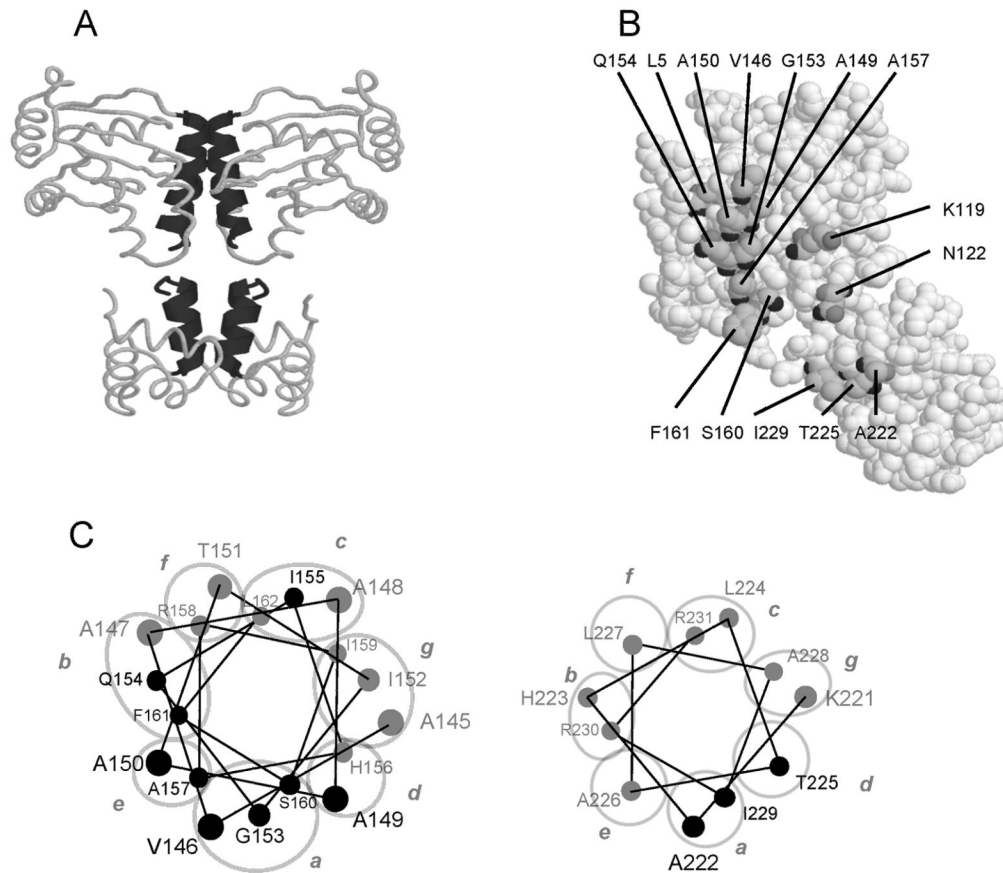
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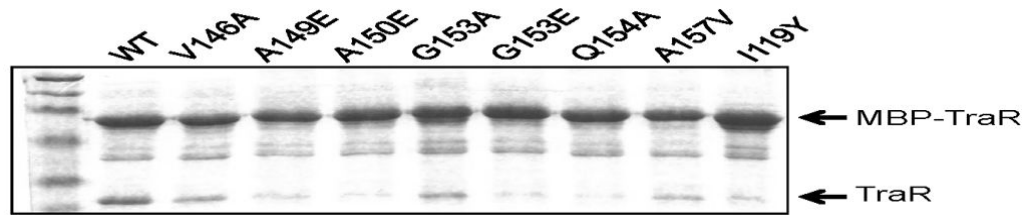


**Fig. 1. Dimerization interface of TraR**

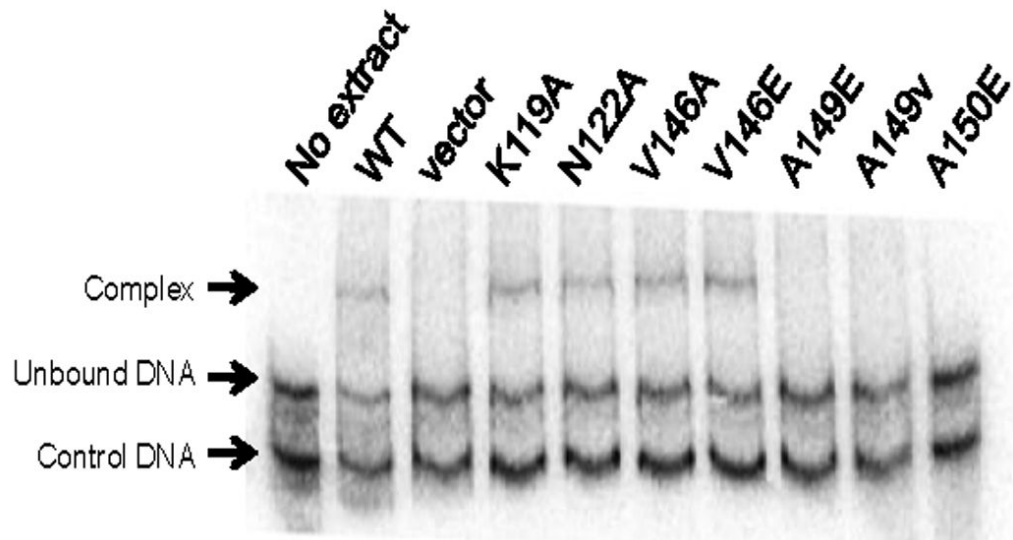
A. Two alpha helices in the N-terminal domain form a coiled coil, and two additional alpha helices in the C-terminal domain form a second coiled coil. These two coiled coils provide the majority of the dimerization interface.

B. A monomer of TraR with the amino acid residues that were mutated in this study.

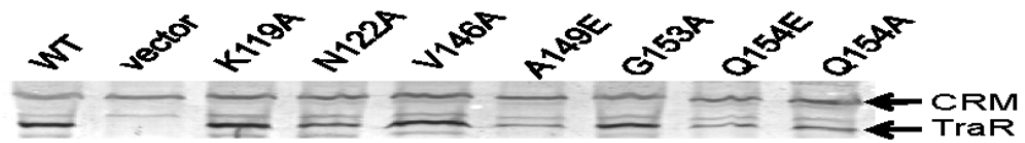
C. Helical wheel projection of alpha helix 9 and alpha helix 13. Residues depicted in black were altered by site-directed mutagenesis in this study. Positions *a* to *g* of the helical wheel projection are encircled and labeled with lower case letters.



**Fig. 2.** Dimerization of wild type TraR with MBP-TraR fusions containing point mutations at the subunit interface. Cell supernatants containing native TraR and various fusion proteins were combined and allowed to form heterodimers, then purified by amylose affinity chromatography. MBP-TraR fusions having wild type TraR sequence retain native TraR on the column, while some MBP-TraR fusions having mutant TraR sequences fail to retain TraR.

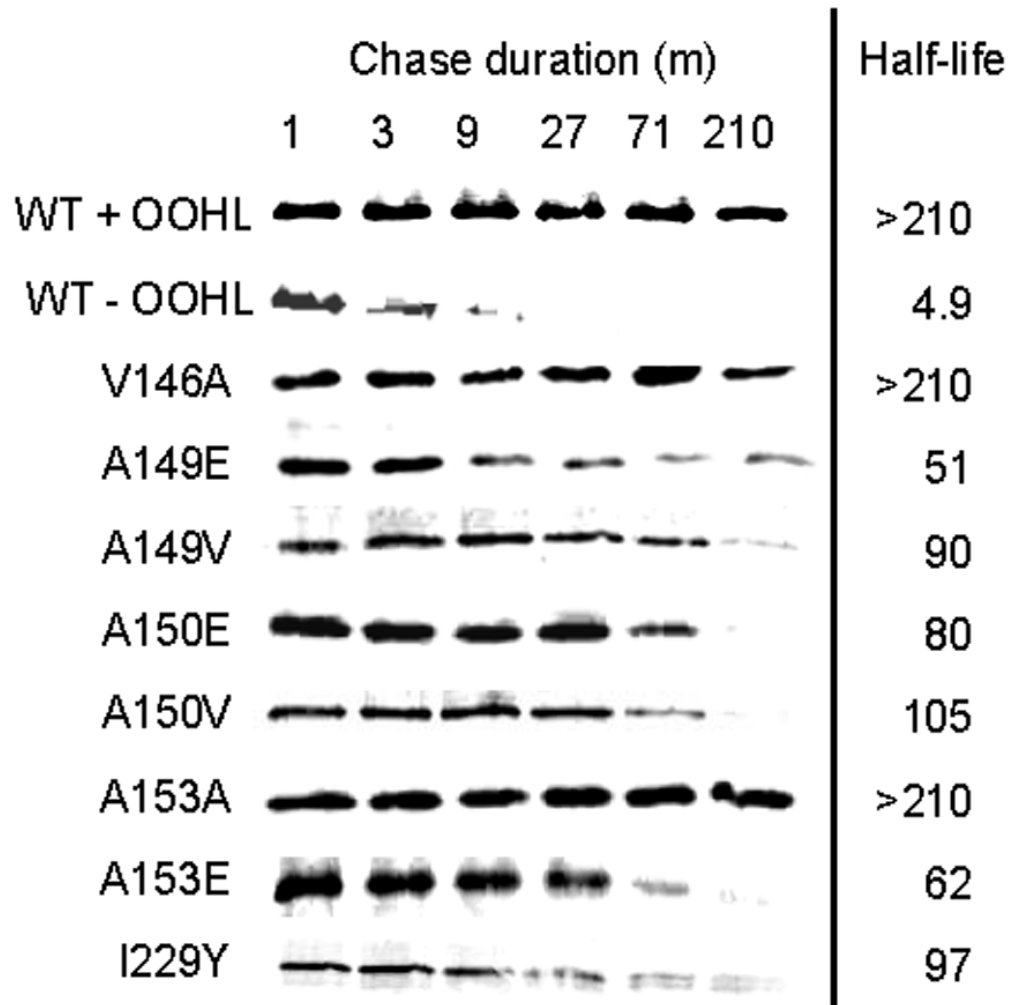


**Fig. 3.** Electrophoretic mobility shift assays using clarified cell extracts containing wild type or mutant TraR proteins and a DNA fragment containing a consensus TraR binding site. Control DNA lacks a binding site for TraR. The amount of cell extracts added was normalized for TraR abundance using Western immunoblots.

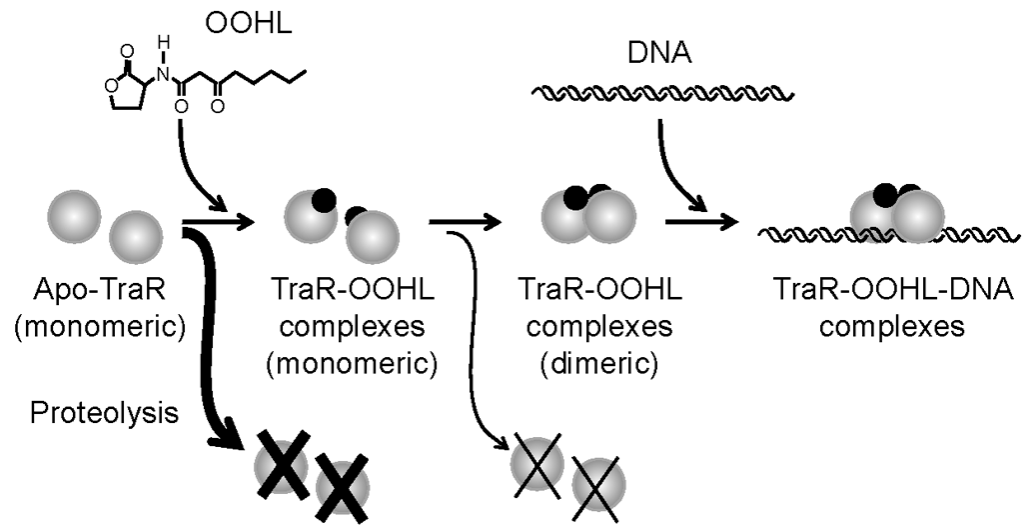


**Fig. 4.**

Western immunoblots showing the abundance of wild type and mutant TraR proteins in clarified cell extracts. Cells were cultured in the presence of 10 nM OOHL. CRM: cross-reacting material, which was used to ensure that equivalent amounts of total protein was added to each lane. A strain carrying pPZP201 vector was used as a negative control while pYC335 served as a positive control for TraR.

**Fig. 5.**

Pulse chase of TraR point mutants in *E. coli*. Cells expressing wild type or TraR point mutants from a phage T7 promoter were treated with rifampicin to block host transcription, then treated with [<sup>35</sup>S]methionine for 1 min, followed by addition of excess nonlabeled methionine. OOHl was provided prior to addition of radiolabel. At the indicated intervals after the addition of nonlabeled methionine, aliquots were frozen at -80°C to terminate proteolysis, then thawed and size-fractionated by SDS-PAGE. Radiolabel was quantitated using a Storm PhosphorImager. Calculated TraR half-lives are indicated at the right of each panel and represent the average of two independent experiments.



**Fig. 6.** TraR folding and dimerization model. TraR monomers that fail to bind OOHL after synthesis are immediately targeted for proteolysis (Zhu and Winans, 1999, 2001), while TraR-OOHL complexes that fail to dimerize are also targeted for proteolysis, though not as rapidly as apo-TraR. TraR-OOHL dimers are competent to bind *tra* box DNA with high affinity and specificity.



**Table 1**

*In vivo* activity at *PtraI-lacZ* fusion of all point mutants relative to wild-type TraR at different concentrations of OOHL.

OOHL concentration	0.1 nM	1nM	10nM	100nM
Wild type TraR	100.0 (15.0)	100.0 (15.6)	100.0 (15.9)	100.0 (9.9)
Vector Control	0.1 (<0.1)	0.1 (<0.1)	0.1 (<0.1)	0.1 (<0.1)
L5M	NT	100.0 (4.8)	100.0 (1.2)	100.0 (0.2)
K119A	28.6 (7.1)	56.7 (11.9)	40.2 (7.1)	61.1 (28.9)
N122A	0.1 (<0.1)	2.9 (2.5)	21.9 (8.2)	29.3 (9.9)
G123R	NT	0.1 (<0.1)	NT	0.1 (<0.1)
V146A	45.2 (9.5)	41.3 (12.4)	39.1 (14.6)	33.9 (5.4)
V146E	5.9 (2.1)	56.4 (6.1)	60.5 (10.7)	75.7 (16.8)
A149E	0.2 (<0.1)	0.1 (<0.1)	0.1 (<0.1)	0.1 (<0.1)
A149V	0.1 (<0.1)	0.3 (0.2)	1.1 (0.9)	1.6 (0.8)
A150E	0.1 (<0.1)	1.3 (0.8)	6.2 (2.4)	8.0 (2.3)
A150V	0.2 (<0.1)	0.1 (<0.1)	0.5 (0.4)	1.5 (0.2)
G153A	17.1 (3.1)	84.8 (20.9)	105.1 (12.2)	90.0 (17.8)
G153E	0.1 (<0.1)	0.1 (<0.1)	0.1 (<0.1)	0.1 (<0.1)
Q154A	0.6 (0.4)	7.9 (3.4)	56.0 (14.6)	40.3 (31.8)
Q154E	0.2 (<0.1)	2.1 (1.8)	6.9 (4.4)	14.3 (10.1)
I155F	NT	13.5 (1.7)	60.7 (12.2)	94.4 (5.1)
A157E	10.6 (6.0)	30.2 (5.7)	56.7 (12.4)	68.7 (12.0)
A157V	9.2 (0.6)	44.3 (8.9)	55.7 (15.7)	74.6 (8.6)
S160A	NT	92.0 (12.1)	99.0 (8.0)	89.0 (15.5)
S160F	NT	11.5 (1.4)	62.4 (14.2)	99.3 (2.0)
F161A	5.5 (2.0)	17.8 (6.1)	42.8 (19.9)	37.7 (18.5)
F161K	0.8 (0.3)	8.7 (1.7)	33.5 (5.3)	52.4 (13.9)
R206Q	NT	0.1 (<0.1)	NT	NT
A222D	0.2 (0.1)	0.3 (0.1)	0.8 (0.2)	0.8 (0.4)
A222S	29.9 (17.0)	59.9 (4.4)	52.5 (13.1)	83.5 (11.1)
T225S	1.0 (0.5)	10.1 (3.4)	17.5 (5.0)	22.8 (3.1)
I229A	NT	35.0 (5.1)	NT	NT
I229Y	0.3 (0.1)	0.1 (<0.1)	0.1 (<0.1)	0.1 (<0.1)

Activity was measured at *PtraI-lacZ* fusion in NTL4 (pCEW260) with vector control (pPZP201), wild-type *traR* (pYC335) or each point mutant. Data represent the average of at least 3 repetitions compared to wild type TraR values that were set to 100%. Wild type TraR expression values were about 700, 2100, 2600 and 3000 units of  $\beta$ -galactosidase activity at 0.1 nM, 1 nM, 10 nM and 100 nM of OOHL, respectively. NT - Not tested.

**Table 2**

Correlation between Accumulation, DNA binding, Dimerization, and OOHL Retention.

	Accumulation <sup>1</sup>	DNA binding <sup>2</sup>	Heterodimer Formation <sup>1</sup>	OOHL Retention <sup>1</sup>
Wild type TraR	100	Y	100	100
Vector Control	0	N	0	0
L5M	100 (11)	Y	99 (11)	100 (5)
K119A	82 (10)	Y	94 (10)	89 (15)
N122A	28 (5)	Y	28 (1)	35 (16)
G123R	51 (8)	Y	49 (10)	67 (10)
V146A	110 (20)	Y	61 (10)	104 (19)
V146E	65 (15)	Y	52 (9)	87 (11)
A149E	44 (12)	N	12 (6)	38 (9)
A149V	45 (15)	N	19 (5)	33 (6)
A150E	60 (6)	N	14 (1)	55 (10)
A150V	47 (13)	N	15 (2)	52 (6)
G153A	63 (11)	Y	61 (22)	66 (10)
G153E	33 (8)	N	19 (4)	39 (7)
Q154A	45 (4)	N	27 (7)	45 (10)
Q154E	36 (8)	N	25 (5)	28 (5)
I155F	43 (7)	Y	50 (7)	NT
A157E	48 (6)	Y	48 (4)	65 (10)
A157V	43 (8)	Y	50 (5)	75 (18)
S160A	100 (10)	Y	100 (6)	NT
S160F	55 (5)	Y	69 (4)	NT
F161A	82 (7)	Y	49 (7)	73 (20)
F161K	44 (6)	Y	39 (6)	57 (7)
R206Q	105 (6)	N	71 (14)	NT
A222D	38 (10)	N	29 (7)	32 (14)
A222S	66 (7)	Y	53 (11)	60 (14)
T225S	39 (16)	N	43 (6)	35 (4)
I229A	NT	Y	NT	33 (5)
I229Y	41 (8)	N	23 (7)	28 (10)

<sup>1</sup>Values represent the average of at least 3 repetitions. Wild type levels were set to 100%. NT - not tested. Accumulation was measured by semi-quantitative western blots using strain NTL4 (pCEW260)(pYC335) or its derivatives carrying each *traR* mutant, dimerization was determined by the ability of a MBP-TraR fusion carrying each point mutation to form heterodimers with native TraR, and OOHL retention assays was tested as previously described (Chai and Winans, 2004).

<sup>2</sup>DNA binding affinity of each TraR allele was evaluated by gel retardation assays using cleared cell lysates and *tra* box DNA repeated at least twice for each mutant. Y - TraR shifted DNA, N - TraR did not shift the DNA.

Table 3

Strains and plasmids.

Strains	Relevant features	References
DH5 $\nabla$	<i>E. coli</i> , $\nabla$ -complementation	Stratagene
NTL4	<i>A. tumefaciens</i> C58, pTi less	(Luo <i>et al.</i> , 2001)
BL21/DE3	<i>E. coli</i> B Plac-gene 1 of bacteriophage T7	(Studier <i>et al.</i> , 1990)
KY2347	<i>E. coli</i> MG1655, ( <i>clpPX-lon</i> ) 1196:: <i>cat</i>	(Herman <i>et al.</i> , 1998)
Plasmids		
pPZP201	Broad-host-range cloning vector, Spc <sup>R</sup>	(Hajdukiewicz <i>et al.</i> , 1994)
pMCSG9	PT7- <i>his6</i> -MBP-TEV, Amp <sup>R</sup>	(Donnelly <i>et al.</i> , 2006)
pJZ358	PT7- <i>traR</i> , Amp <sup>R</sup>	(Zhu and Winans, 1999)
pYC335	<i>Plac-traR</i> in pPZP201	(Chai and Winans, 2004)
pCEW250	pBluescriptSK+ with consensus <i>tra</i> box at <i>PtraI</i> , Km <sup>R</sup>	(White and Winans, 2007)
pCEW260	Consensus <i>PtraI lacZ</i> fusion, Km <sup>R</sup>	(White and Winans, 2007)
pAFM02	<i>traR</i> cloned into pMCSG9	This study
pUP-5M to 229Y	<i>traR</i> point mutants cloned into pYC335	This study
pUP2-5M to 229Y	<i>traR</i> point mutants cloned into pAFM02	This study
pUP3-5M to 229Y	<i>traR</i> point mutants cloned into pJZ358	This study

**Table 4**

Oligonucleotide primers used in this study.

Oligonucleotide Name	DNA Sequence
Flanking primers - N-terminal region	
PT1	5' - CTCACTCATTAGGCACCCAG - 3'
TraRb	5' - GTACAACGTGTAGGGCAACGC - 3'
Flanking primers - C-terminal region	
TraRa	5' - CATTCTTCGCACCACCC - 3'
TraRb	5' - GTACAACGTGTAGGGCAACGC - 3'
Mutagenic primers	
L5M-F	5' - CAGCACTGGATGGACAAGCTG - 3'
L5M-R	5'-CAGCTTGTCCATCCAGTGCTG - 3'
K119A-F	5' - AATACCCATCGCGACCGCCAACG - 3'
K119A-R	5' - CGTTGGCGGTCGCGATGGGTATT - 3'
N122A-F	5' - CAAGACCGCCGCGGCTTTATGT - 3'
N122A-R	5' - ACATAAAGCCGGCGCGGTCTTG - 3'
V146A-F	5' - GATCGATGCAGCCGAGCCGCTG - 3'
V146A-R	5' - CAGCGGCTGCGGCTGCATCGATC - 3'
V146E-F	5' - GATCGATGCAGAAGCAGCCGCTG - 3'
V146E-R	5' - CAGCGGCTGCTTCTGCATCGATC - 3'
A149E-F	5' - AGTCGCAGCCGAGGCAACCATCG - 3'
A149E-R	5' - CGATGGTTGCCTCGGCTGCGACT - 3'
A149V-F	5' - AGTCGCAGCCGTTGCAACCATCG - 3'
A149V-R	5' - CGATGGTTGCAACGGCTGCGACT - 3'
A150E-F	5' - CGCAGCCGCTGAAACCATCGGGC - 3'
A150E-R	5' - GCCCGATGGTTTCAGCGGCTGCG - 3'
A150V-F	5' - CGCAGCCGCTGTAACCATCGGGC - 3'
A150V-R	5' - GCCCGATGGTTACAGCGGCTGCG - 3'
G153A-F	5' - TGCAACCATCGCGCAGATCCATG - 3'
G153A-R	5' - CATGGATCTGCGCGATGGTTGCA - 3'
G153E-F	5' - TGCAACCATCGAGCAGATCCATG - 3'
G153E-R	5' - CATGGATCTGCTCGATGGTTGCA - 3'
Q154A-F	5' - AACCATCGGGGCGATCCATGCCC - 3'
Q154A-R	5' - GGGCATGGATCGCCCCGATGGTT - 3'
Q154E-F	5' - AACCATCGGGGAGATCCATGCCC - 3'
Q154E-R	5' - GGGCATGGATCTCCCCGATGGTT - 3'
I155F-F	5' - ATCGGGCAGTTCATGCCCGC - 3'
I155F-R	5' - GCGGGCATGGAAGTCCCGAT - 3'
A157E-F	5' - GCAGATCCATGAGCGCATCTCAT - 3'
A157E-R	5' - ATGAGATGCGCTCATGGATCTGC - 3'
A157V-F	5' - GCAGATCCATGTCCGCATCTCAT - 3'
A157V-R	5' - ATGAGATGCGGACATGGATCTGC - 3'

Oligonucleotide Name	DNA Sequence
S160A-F	5' - CCGCATCGCATTCTTCGCA - 3'
S160A-R	5' - TGCGAAGGAATGCGATGCGG - 3'
S160F-F	5' - GCCCGCATCTTCTTCCTTCGC - 3'
S160F-R	5' - GCGAAGGAAGAAGATGCGGGC - 3'
F161A-F	5' - CCGCATCTCAGCCCTTCGCACCA - 3'
F161A-R	5' - TGGTGCGAAGGGCTGAGATGCGG - 3'
F161K-F	5' - CCGCATCTCAAACTTCGCACCA - 3'
F161K-R	5' - TGGTGCGAAGTTTGTAGATGCGG - 3'
A222D-F	5' - GCAGCAAGGACCATCTTACC - 3'
A222D-R	5' - GGTAAGATGGTCTTGCTGC - 3'
A222S-F	5' - GCAGCAAGTCCCATCTTACC - 3'
A222S-R	5' - GGTAAGATGGGACTTGCTGC - 3'
T225S-F	5' - CCCATCTTCCGCGCTCGCC - 3'
T225S-R	5' - GGCGAGCGCGAAAGATGGG - 3'
I229Y-F	5' - GCTCGCTACCGCGGAAAC - 3'
I229Y-R	5' - GTTCCGCCGGTAGGCGAGC - 3'
<i>traR</i> cloning into pMCSG9	
<i>traR</i> -fusion-F	5' - TACTTCCAATCCAATATGCAGCACTGGCT - 3'
<i>traR</i> -fusion-R	5' - TTATCCACTTCCAATTCTCAGATGAGTTTCCG - 3'
To check correct cloning into pMCSG9	
pMCSG9-F	5' - ACGAGGAAGAGTTGGCGAAAGATC - 3'
pMCSG9-R	5' - TTAGAGGCCCAAGGGTTATGCTA - 3'
Oligonucleotides used in the gel shift assays	
Ptra-box For	5' - GAATTCTATGTGCAGATCTGCACATAGC - 3'
Ptra-box Rev	5' - GGATCAATACGACGAGCTCGAGGATCCAGC - 3'
Pcontrol gel shift For	5' - CCGCTACAGGGCGGTCC - 3'
Pcontrol gel shift rev	5' - CCAATTCGCCCTATAGTG - 3'