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N- and C-terminal Regions of the Quorum Sensing Activator TraR Cooperate in Interactions with the Alpha and Sigma-70 Components of RNA Polymerase

Yinping Qin, Carrie Keenan, and Stephen K. Farrand*

Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801

Summary

Positive control mutants defining 20 residues of the quorum-sensing activator TraR were isolated that bind DNA but show defects in activating transcription from Class I, Class II, or both types of promoters. These PC residues, located in both the N- and C-terminal regions, combine to form three patches, one on the top (II) and two near the DNA binding domain on both lateral faces of the dimer (I and III). Patches I and II, but not Patch III involve residues from both protomers and are essential for activation. TraR-mediated activation in *E. coli* requires expression of the α -subunit of *Agrobacterium* (α_{At}). We report that TraR also activates a Class II promoter in *E. coli* when co-expressed with σ^{70}_{At} . Analyses in *E. coli* expressing α_{At} , σ^{70}_{At} , or both subunits indicate that most of the PC residues are important for interactions with α_{At} and that these interactions are predominant for activation of Class II promoters. Using the *E. coli* system we identified nine residues in the C-terminal domain of α_{At} that are required for stimulating TraR-mediated activation. We conclude that N- and C-terminal residues of TraR from both protomers cooperate to define regions of the protein important for interactions with RNAP.

Keywords

Quorum-sensing; TraR; α -subunit; σ^{70} -subunit; Transcriptional Activation; *Agrobacterium tumefaciens*

Introduction

TraR is a member of the LuxR family of quorum-sensing regulators and controls conjugative transfer and copy number of the Ti plasmid of the plant pathogen, *Agrobacterium tumefaciens* (Piper *et al.*, 1993; Li and Farrand, 2000; Pappas and Winans, 2003). Most members of the LuxR family are transcriptional activators and require as ligands acyl homoserine lactone (acyl-HSL) signals to bind DNA and activate transcription (Miller and Bassler, 2001; Whitehead *et al.*, 2001; Fuqua and Greenberg, 2002; Nasser and Reverchon, 2007). Upon binding N-3-(oxo-octanoyl)-L-HSL (3-oxo-C8-HSL), TraR forms active dimers and becomes more resistant to proteolysis (Zhang *et al.*, 1993; Zhu and Winans, 1999; Qin *et al.*, 2000; Pinto and Winans, 2009). Each protomer of the dimeric activator is composed of two functional domains joined by a 12-residue flexible linker (Vannini *et al.*, 2002; Zhang *et al.*, 2002; Luo *et al.*, 2003; White and Winans, 2007). The N-terminal 162-residue segment contains the acyl-HSL binding site and the primary dimerization domain while the C-terminal 60-residue segment contains secondary

*To whom correspondence should be addressed: Department of Microbiology University of Illinois at Urbana-Champaign B103 CLSL 601 S. Goodwin Ave. Urbana, IL 61801 Tel: 217-333-1524 Fax: 217-244-6697 stephenf@illinois.edu.

dimerization regions and a helix-turn-helix-type (HTH) DNA binding domain. Based on crystal structure of a TraR dimer from an octopine-type Ti plasmid bound to its DNA recognition element, both of the two C-terminal domains and the two N-terminal domains display an axis of two-fold symmetry. However, these two axes of symmetry are aligned at approximately a 90° angle to one another, leading to high asymmetry of the overall structure of this dimeric activator. As a result of this asymmetry, one protomer is compact while the other is somewhat extended (See Fig.1) (Zhang *et al.*, 2002; Vannini *et al.*, 2002).

Activators such as CAP, BMRR, and CooA that bind to a two-fold symmetric DNA recognition element, function as two-fold symmetrical dimers (Busby and Ebright, 1997; 1999; Zheleznova-Heldwein and Brennan, 2001; Komori *et al.*, 2007). Among the most well-studied activators, dimeric CAP binds to DNA, recognizing a 22-bp inverted repeat consensus site, and in several cases it activates bi-directional promoters (Busby and Ebright, 1999; Benoff *et al.*, 2002). CAP-dependent promoters can be classified into two groups depending upon the location of the DNA binding site. In Class I promoters, the activator binding site is centered approximately 65 bp upstream of the +1 site. At such promoters, CAP is believed to recruit RNAP by interacting specifically with the C-terminal domain of the α -subunit (Lawson *et al.*, 2004). In Class II promoters, the DNA binding site is centered adjacent to the -35 promoter element, and makes multiple contacts with RNAP including those with α and σ^{70} subunits (Niu *et al.*, 1996; Rhodius *et al.*, 1997; Busby and Ebright, 1997; 1999). TraR also activates from binding sites, called the *tra* box, showing two-fold symmetry. Based on the location of this 18-bp inverted repeat, five of the seven known TraR-dependent promoters, including $P_{traAFBH}$, P_{traCDG} , $P_{traItrb}$ and P_{repA1} and P_{repA3} are Class II-type in nature (Farrand *et al.*, 1996; Fuqua and Winans, 1996; Li and Farrand, 2000; Pappas and Winans, 2003), while two, P_{traM} and P_{repA2} are Class I-type promoters (Hwang *et al.*, 1995; Fuqua *et al.*, 1995; White and Winans, 2005). Moreover, two of these Class II promoters, $P_{traAFBH}$ and P_{traCDG} are divergently oriented and are activated by TraR bound to a single, centrally located *tra* box (Farrand *et al.*, 1996; Fuqua and Winans, 1996). Given its asymmetry, however, it is very likely that TraR behaves differently with respect to its interaction with RNAP as compared to highly symmetric dimeric activators like CAP. As such, studies of the interactions between TraR and RNA polymerase could serve as a prototype to understand the mechanisms by which this large family of LuxR-type AHL-dependent quorum-sensing activators interacts with and recruits RNAP to their promoters.

Our previous studies of the closely related TraR from the nopaline-type Ti plasmid pTiC58 showed that alterations at two N-terminal residues, Asp-10 and Gly-123, yield a strong positive control (PC) phenotype; both mutant proteins retain wild-type levels of DNA binding activity but fail to activate transcription (Luo and Farrand, 1999; Qin *et al.*, 2004a). When co-expressed with the wild-type protein, these mutants exhibit dominant negativity; they strongly inhibit activation of the reporter by wild-type TraR (Luo and Farrand, 1999; Qin *et al.*, 2004a). However, when co-expressed as pairs, TraRD10A and TraRG123A, or TraRD10A and TraRG123R complemented one another. Together, these results suggest that the regions of the two protomers defined by these two residues interact with each other in the dimer (Qin *et al.*, 2004a). Consistent with this hypothesis, Asp-10 of one protomer and Gly-123 of the other lie adjacent to each other in the active dimers and could contribute to an activating surface patch. In addition, because of its asymmetry, the two patches are located on different surfaces of the dimer: one patch lies on the lateral face of dimer close to the bound DNA, while the other is located on the top face of the dimer (Fig.1B, 1C) (Zhang *et al.*, 2002; Vannini *et al.*, 2002).

Several lines of genetic evidence suggest that LuxR is an ambidextrous activator, and as such makes contacts with the α - and σ^{70} - subunits of RNAP (Stevens *et al.*, 1994; 1999; Finney *et al.*, 2002; Trott and Stevens, 2001; Johnson *et al.*, 2003). Given the overall

relatedness of TraR and LuxR and the location of the *tra* box within target promoters, TraR also is likely to be an ambidextrous activator, interacting with the α -subunit and with σ^{70} of RNAP during initiation of transcription from Class II promoters. In this regard, two lines of evidence indicate that TraR directly interacts with the α -subunit of RNAP. First, TraR requires co-expression of the α -subunit from *A. tumefaciens* (α_{At}) to activate transcription from a Class II promoter in *E. coli* (Qin *et al.*, 2004a). Second, as assessed by *in vitro* assays, TraR directly interacts with the C-terminal domain of the α -subunit (α CTD) from *A. tumefaciens*, and this interaction is dependent upon Asp-10 and Gly-123; substitution mutations at one or the other of these residues of the activator abolish α -subunit binding (Qin *et al.*, 2004a).

In this study, we identified and characterized a series of N- and C-terminal PC mutants of TraR from pTiC58. We also expanded our *E. coli* system (Qin *et al.*, 2004A) to measure the influence of σ^{70}_{At} on activation by TraR in this enteric host. Using these tools we found that maximum expression from a Class II promoter is dependent upon coexpressing both α and σ^{70} subunits from *Agrobacterium*. Moreover, using this system coupled with assays using a TraR-dependent Class I promoter reporter, we distinguished between PC residues of TraR required for interaction with α and those influencing interaction with σ^{70} . Finally, we identified residues in the C-terminal domain (CTD) of the α -subunit from *A. tumefaciens* that are required for TraR-mediated activation.

Results

Identification of N-terminal mutations with a positive control phenotype

In our previous studies we identified four PC mutants of TraR from the nopaline-type Ti plasmid pTiC58 located at Asp-10 and Gly-123: D10N, D10A, G123R and G123A (Luo and Farrand, 1999; Qin *et al.*, 2004a). These mutant proteins still bind *tra* box DNA as well as the wild-type protein, but fail to activate transcription. Since the glycine at position 123 does not have a side group, we made alanine substitutions at positions Asn-122 and Ser-124 to test if residues around Gly-123 could play any role in activation. As shown in Table 1, Asn122→Ala did not significantly affect the activation activity from our Class II reporter while Ser124→Ala completely lost both activation and DNA binding activity, suggesting a structural role for Ser-124 and Gly-123.

We then employed random error-prone PCR mutagenesis to identify additional PC mutants. In this screen we isolated two mutants, D6G and D6R, each of which retained full DNA binding activity as measured by both repressor activity and gel retardation assays but lost over 98% of their activation activity (Table 1). The Asp-6 residue is surface-exposed, lies close to Asp-10, and these two residues in one protomer combine with Gly-123 from the other protomer to form the two D6-D10/G123 patches in the TraR dimer (Fig. 1B, 1C).

To further explore the region, we converted to alanines the surface-exposed residues near the two D6-D10/G123 patches in the N-terminal segment of the dimer. Of the nine alanine substitution mutants, at positions 45, 46, 74, 75, 77, 78, 79, 80 and 119, all retained more than 80% DNA binding activity as assessed by our repression assay and all but four activated transcription at levels of 80% or greater (Table 1). The four activation-defective mutants, H46A, R75A, K77A and S78A, showed activities ranging from 45 to 77% that of wild-type TraR. Setting as a cutoff a loss of 20% or greater in activation activity (Niu *et al.*, 1993), we retained these four N-terminal mutants as candidate PC alleles (Fig. 1 and Table 1). As assayed by Western analysis, all of these mutant proteins were detectable in cells at levels indistinguishable from wild-type TraR (data not shown).

Identification of C-terminal mutations with a positive control phenotype

Considering that the α and σ^{70} subunits bind at or near promoters and often contact activators around the DNA-binding domain, we used three strategies to screen for PC mutants of TraR with alterations in the C-terminal DNA interaction region. In the first, reasoning that residues involved in contacting components of RNAP would be conserved among orthologs of *traR* from plasmids in related α -proteobacteria such as *A. rhizogenes* (Moriguchi *et al.*, 2001), *Sinorhizobium meliloti* (Marketon and Gonzalez, 2002) and *Rhizobium* spp (Danino *et al.*, 2003; He *et al.*, 2003; Tun-Garrido *et al.*, 2003), we mutagenized the 14 C-terminal residues that are conserved in all of the TraR proteins known to be functional. In the second, in screens for mutants of TraR that do not interact with TraM, we had isolated three mutants with single amino acid substitutions in the C-terminal region (Luo *et al.*, 2000; Qin *et al.*, 2004b; Qin *et al.*, 2007). In the third approach, we mutagenized alanines C-terminal residues of TraR that correspond to residues identified as exhibiting PC properties in LuxR (Egland and Greenberg, 2001) and in TraR from otopine-type Ti plasmids (White and Winans, 2005).

Of the total of 20 new C-terminal mutants, four of them, M189A, E192A, S204A and A212V retained more than 85% activity in both repression and activation assays. Six of the remaining 16 mutants, L174A, G188A, T190I, A195V, V205A and R215A, exhibited less than 15% activity in activation and repression assays, whereas two mutants (G199A, V200A) were almost equally affected in repression and activation. The Leu-209→Ala substitution lost virtually all activator function, and 70% of its repression activity. Leu-209 is located between two adjacent residues in the recognition helix that make specific contacts with *tra* box nucleotides (Vannini *et al.*, 2002; White and Winans, 2007; Zhang *et al.*, 2002), suggesting that the L209A mutation affects DNA binding rather than activation *per se*. The remaining seven mutants (W184A, E193A, D196A, V197A, E198A, K201A, and E211K) are more defective in activation than in repression, and thus were retained as PC mutants (colored in magenta in Fig.1 and highlighted in bold in Table 2).

In addition to the *in vivo* repression assay, we quantified the DNA binding properties of each of the PC mutants using gel retardation assays. All but two of these mutants displayed similar relative binding affinities in these two assays (Table 1, Table 2, and Fig.2). TraR D196A lost about 45% repression activity *in vivo* but displayed levels of affinity for the *tra* box similar to that of the wild-type protein *in vitro*, while TraR K201A acted in an opposite fashion; this mutant showed near-wild type repression activity *in vivo* but over a hundred-fold reduced binding affinity *in vitro* (Table 2, Fig.2), suggesting that some factors *in vivo* may modulate the interactions between TraR and *tra* box DNA. All of these mutant proteins were produced in soluble form in cells grown with 3-oxo-C8-HSL and are detectable in cells at amounts equivalent to that of the wild-type protein independently expressed from the same vector (Qin *et al.*, 2007; data not shown).

Relative activation activities of TraR mutants at Class I and Class II promoters

To differentiate residues involved in interacting with the α -subunit from those that interact with σ^{70} , we compared the activation properties of all of the TraR mutants on Class I and Class II promoter reporters. We reasoned that PC mutants showing a reduced activity on both promoters or only on the Class I promoter would represent proteins affected in interactions with the α -subunit, while those showing a reduced activity on Class II but not on Class I promoters, would correspond to activators defective in interaction with σ^{70} or with α NTD but not with α CTD (William *et al.*, 1997; Zhou *et al.*, 1994; Niu *et al.*, 1996; Lawson *et al.*, 2004).

Of the 26 mutants tested, 16 including D6G(R), D10A, R45A, R75A, R119A, G123A, S124A, W184A, E193A, A195V, D196A, V197A, E198A, S204A and L209A exhibited similar activation properties on Class I and Class II promoters (Fig. 3 and Table S1). Ten mutants displayed differences of more than 20% in relative activation on the two types promoters. Seven of these, including H46A, K74A, K77A, R79A, K80A, N122A and G199A, showed levels of activity at least 20% higher on Class II as compared to Class I promoters while the other three, S78A, K201A, and E211K, displayed levels of activity at least 20% higher on Class I as compared to Class II promoters (Fig. 3 and Table S1).

Five N-terminal mutants, K74A, R79A, K80A, R119A and N122A, while still active on the Class II promoter reporter, showed losses in activation 46%, 27%, 51%, 29% and 38% respectively on the Class I promoter (Fig. 3 and Table S1). In addition, the H46A, K77A and G199A mutants, while showing 26%, 55% and 45% loss of activation activity on the Class II promoter respectively, exhibited between 61 and 85% decrease of activity on the Class I promoter (Fig. 3 and Table S1). These results suggest that these residues play only a modest role in activation from a Class II promoter but may contribute to interactions with RNAP specific to activation from a Class I promoter. Based on this observation we call these eight mutants, H46A, K74A, K77A, R79A, K80A, R119A, N122A and G199A, Class I-specific PC mutants (Fig. 1 and Fig. 3). On the other hand, the three mutants S78A, K201A and E211A are more active on Class I than Class II promoters (Fig. 3) and therefore are Class II-specific PC mutants.

PC residues located in the N-terminal but not the C-terminal half display dominant negativity

Assessing dominant negativity allows us to identify residues required in both promoters as compared to those required in only one promoter of the TraR dimer (Luo *et al.*, 2003; Qin, *et al.*, 2004a). We co-expressed each new mutant identified in this study with wild-type TraR to examine its dominance properties.

On the Class II promoter, all of the C-terminal PC mutants including W184A, E193A, D196A, V197A, E198A, K201A and E211K were recessive, while among the N-terminal mutants, all but R75A and S78A, exhibited significant dominant negativity (Table 3). As expected, co-expressing the C-terminal DNA binding-defective mutants L174A, G188A, and A195V, resulted in moderate inhibition of wild-type TraR activity. The four other null mutants, T190I, V205A, L209A, and R215A, were recessive to wild-type TraR (Table 3).

We then tested the PC mutants for dominant-negativity on the Class I-promoter reporter. Most of these mutants displayed characteristics similar to their reaction on the Class II promoter (Table 3). All C-terminal PC mutants were recessive to wild-type TraR while the N-terminal mutants, D6G, D6R, D10A, K77A, S78A and G123A exhibited degrees of dominance over wild-type TraR (Table 3). However, the Class I-specific N-terminal PC mutants, including H46A, K74A, R79A, K80A and N122A, were recessive when co-expressed with wild-type TraR on the Class II promoter, but showed 26 to 38% dominant-negativity over wild-type TraR as assayed on the Class I promoter (Table 3), suggesting that those residues play a role in the interaction specifically with α during transcriptional activation from the Class I promoter.

TraR requires both α and σ^{70} from *Agrobacterium* to maximally activate transcription in *E. coli*

TraR fails to activate transcription of our Class II *traG::lacZ* reporter in *E. coli* unless co-expressed with the α -subunit from *Agrobacterium* (α_{At}) (Qin *et al.*, 2004a). This dependence results from our observation that TraR has a lower affinity for the α -subunit from *E. coli*

(α_{Ec}) than that from *A. tumefaciens* (Qin *et al.*, 2004a). Considering the possibility that TraR is an ambidextrous activator, we assessed the effect of coexpressing σ_{70} from *A. tumefaciens* (σ_{70}^{At}) on TraR activation activity in *E. coli*. When co-expressed in *E. coli* with σ_{70}^{At} alone, TraR activated the *traG::lacZ* reporter to levels 3-5 fold higher as compared to cells expressing only TraR (Fig. S1 Insert). However, when co-expressed with α_{At} and σ_{70}^{At} , TraR activated the reporter to levels more than 100-fold higher than that of the *E. coli* cells expressing only the activator (Fig. S1). These results suggest that full activity of TraR on a class II promoter requires proper interactions with both α_{At} and σ_{70}^{At} .

Genetic analysis of TraR PC mutants in interaction with α_{At} and σ_{70}^{At}

Using the *E. coli* genetic system described above we assessed whether our PC mutants are defective in interactions with α_{At} , with σ_{70}^{At} , or with both. To simplify the comparisons, activity for each mutant is expressed as percent of the background-corrected activity of wild-type TraR in each experimental set. To begin, we tested all of our activation-defective mutants of TraR in *E. coli* co-expressing α_{At} . As shown in Fig. 4A, TraR mutants D10A, K77A, G123A, W184A, A195V, D196A, V197A, E198A and L209A yielded levels of activation less than 10% of wild-type TraR; mutants K74A, V200A and E193A exhibited activities ranging between 10 and 25%; mutants R75A, R79A, K80A, N122A and G199A had activities between 25 and 50%; mutants H46A, S78A, S204A and E211K showed activities ranging from 50 to 75%; and mutants K45A, R119A and K201A showed activation levels at least 75% that of wild-type TraR.

We then tested all of the mutants in *E. coli* co-expressing σ_{70}^{At} . As shown in Figure 4B, mutants K77A, A195V, D196A and L209A displayed 0 to 25% activity; mutants D10A, R75A, S78A and E198A yielded activities between 25 and 50%; and mutants K45A, H46A, K74A, R79A, K80A, V197A and V200A retained 50 to 75% activity of wild-type TraR. Mutants R119A, G123A, E193A, K201A, S204A and E211K activated the reporter to levels between 75 and 100% of wild type TraR, while mutants N122A, W184A and G199A yielded levels of activity higher than that of the wild-type activator (Fig. 4B).

In comparing the response of the PC mutants to co-expression of α_{At} with their response when co-expressed with σ_{70}^{At} , the N-terminal PC residues between positions 10 and 80 and C-terminal residues between positions 196 to 201 are about equally required for stimulation by both α and σ_{70} , while residues between 119 and 193 are required for stimulation by α but not by σ_{70} . Strikingly, G199A, although not identified as a PC mutant in the screen against our Class II promoter (Table 2), activated the reporter to levels more than two-fold higher than wild-type TraR in cells co-expressing σ_{70}^{At} (Fig. 4B). This enhanced level of activation most likely accounts for the much higher levels of activity of this mutant on Class II than on Class I promoters (Fig. 3). Consistently, a second mutant, S78A, that shows a lower activity on Class II as compared to Class I promoters (Fig. 3), exhibited a greater defect in σ_{70}^{At} -dependent activation as assayed in this genetic system (Fig. 4A and 4B).

When tested in *E. coli* co-expressing both α_{At} and σ_{70}^{At} , each mutant activated the *traG::lacZ* Class II reporter to relative levels virtually indistinguishable from those observed when tested in *E. coli* co-expressing only α_{At} (Fig. 4C). Although mutants including N122A, W184A and G199A showed a somewhat higher activity in *E. coli* when co-expressed with σ_{70}^{At} only, co-expressing both subunits of RNAP did not result in higher levels of activation (Fig. 4C).

Residues of α CTD influencing TraR-mediated activation at Class II promoters

These results, combined with our previous studies (Qin *et al.*, 2004a) strongly suggest that contact between the CTD of RpoA_{At} and TraR is essential for activation, prompting us to

initiate an analysis of this region of the α -subunit. Of their shared residues, the CTDs of the α -subunits of *A. tumefaciens* and *E. coli* are 72% similar at the sequence level (Fig. 5A). However, RpoA_{At} contains an 8-residue extension at the C-terminus. Deleting between 2 and 8 residues of this extension resulted in no more than a 20% decrease in activation of the *traG::lacZ* reporter as tested in *E. coli* co-expressing σ^{70}_{At} (Fig. 5B).

We then randomly mutagenized the C-terminal domain of rpoA_{At} and used our *E. coli* system to screen for alleles that were altered in their ability to stimulate TraR-mediated expression of the *traG::lacZ* reporter, all as described in *Experimental procedures*. From this screen we isolated nine mutants with single amino acid substitutions (Fig. 5A). Two such mutants, S262L and G295S stimulated levels of TraR activation to levels about twice that of wild-type RpoA_{At} when tested in the *E. coli* system. The remaining eight mutants, including L269Q, L280P, I281N, T284I, E287V, R290H, G295D and A307P yielded levels of stimulation of TraR-mediated activation ranging from 0 to 40% that of wild-type RpoA_{At} (Fig. 5B).

Of the nine randomly-generated mutants of RpoA_{At}, three represent residues that are variant in the *E. coli* ortholog (Fig. 5A). We then introduced by site-directed mutation substitutions in an additional 13 positions of the CTD of RpoA_{At} that are not conserved in RpoA_{Ec} (Fig. 5A). Eleven of the substitutions, including N271A, N273A, N300T, M309A, H312A, E316A, P318A, A319N, E323A, E326A and L328A had no significant effect on RpoA_{At}-mediated stimulation of TraR activity in *E. coli* (Fig. 5B). The V275A substitution increased the level of TraR-mediated activation about 1.6-fold while the A286V allele resulted in a close to 85% decrease in RpoA_{At} activity (Fig. 5B). The A286V substitution converts this residue to the corresponding Val-286 of the *E. coli* ortholog (Fig. 5A) raising the possibility that the failure of the *E. coli* α -subunit to support activation by TraR is linked to valine at this position. However, mutating Val-287 to alanine in RpoA_{Ec} did not confer on the *E. coli* α -subunit the ability to stimulate TraR-mediated activation (data not shown).

Discussion

In this work we have screened mutant alleles of TraR with alterations at 34 residues in the N- and C-terminal regions for those that have lost activation function but retain DNA binding activity. In these screens we used two assays to measure DNA binding activity: *in vivo* repression and *in vitro* gel retardation, and employed both Class I and Class II promoter-based reporters. From the current screen and previous studies (Qin, *et al.* 2004a) we have catalogued 22 mutants defining 20 residues with PC properties. Of these, 12 residues, D6, D10, H46, K74, R75, K77, S78, R79, K80, R119, N122, and G123 are located in the N-terminal domain while eight, W184, E193, D196, V197, E198, G199, K201 and E211 map to the C-terminal region of the protein. Mutations at 13 of these residues show PC properties in the closely related TraR protein (TraR_{O_{ct}}) from pTiR10 (White and Winans, 2000; Costa, *et al.*, 2009). However the two TraR orthologs exhibit some differences; four N-terminal PC residues of TraR_{O_{ct}}, Lys-7, Ala-13, Glu-15 and Ile-20 were not identified in TraR from pTiC58 while our studies identified two N-terminal residues, His-46 and Arg-119 not reported in TraR_{O_{ct}}. Similarly, we identified five C-terminal PC residues of TraR from pTiC58, Asp-196, Glu-198, Gly-199, Lys-201 and Glu-211 that were not reported to exhibit such properties in TraR_{O_{ct}}. Conversely, two C-terminal PC residues of TraR_{O_{ct}}, Lys-187 and Lys-189 (White and Winans, 2005; Costa, *et al.*, 2009) were not identified in our study of TraR from the nopaline-type Ti plasmid. Of the three residues of LuxR described as exhibiting PC properties, Met-189, Glu-192 and Val-197 (Egland and Greenberg, 2001), a substitution at only Val-197 of TraR_{nop} resulted in a PC phenotype (Table 2).

The positive control residues combine to form three distinct patches on the dimer structure of TraR

Several of the PC residues combine to form three surface patches on the dimer structure of TraR. As viewed down the DNA axis, Patch II is located on the top of the dimer, and Patches I and III are located near the DNA binding domain on the two lateral faces, left and right, of the dimer (Fig. 1C). Patch I, located in the left face of the dimer, is composed of Asp-6, Asp-10, Trp-184, Glu-193, Asp-196, Val-197, Glu-198 and Gly-199 from the compact protomer, and Asn-122 and Gly-123 as well as the other N-terminal residues from the extended protomer (Fig.1B) (Vannini *et al.*, 2002; Zhang *et al.*, 2002). Patch II, located on the top of the dimer, is made up of the N-terminal PC residues Asp-6 and Asp-10 from the extended protomer, and Asn-122 and Gly-123 as well as the other N-terminal PC residues from the compact protomer. Patch III, an isolated face on the right side of the dimer, is composed only of the C-terminal PC residues Trp-184, Glu-193, Asp-196, Val-197, Glu-198 and Gly-199 of the extended protomer (Fig.1B and 1C).

We previously reported that the D10A and G123A substitution mutants are strongly dominant-negative over wild-type TraR and that when co-expressed the two mutant alleles complement one another (Qin *et al.*, 2004a), suggesting that both Patch I and Patch II are essential for activation. Consistent with this hypothesis, substitution mutations at all of the N-terminal PC residues contributing to the formation of these two patches exhibit dominant negativity (Table 3). Conversely, all of the C-terminal PC mutants were recessive when co-expressed with the wild-type TraR. These results suggest that Patches I and II, partially formed and exclusively formed by N-terminal PC residues respectively, are important for activation. However, the data suggest that Patch III, which is formed exclusively by C-terminal PC residues from the extended protomer, is not required for activation (Fig. 1C; Fig. 6A-3).

Clearly, the N-terminal domain of TraR is indispensable for activation function, a conclusion consistent with our analysis of N-terminal deletion derivatives (Qin *et al.*, 2000; Luo *et al.*, 2003) as well as a co-published study of TraR_{O_{ct}} (Costa *et al.*, 2009). The requirement for specific N-terminal residues of both protomers for activation could explain our inability to isolate N-terminal truncated mutants of TraR that retain activity (Luo and Farrand, 1999). Given that such N-terminal deletion mutants of both LuxR and LasR retain the ability to activate (Choi and Greenberg, 1991; Kiratisin *et al.*, 2002), our results suggest that TraR and other members of the LuxR family may differ in how they interact with RNA polymerase to activate transcription (Nasser and Reverchon, 2007). Alternatively, the N-terminal deletion mutants of LuxR and LasR may activate transcription by a mechanism different from that of the full-sized proteins (see Finney *et al.*, 2002).

Discrimination between residues important for interaction of TraR with α from those important for interaction with σ^{70}

Twelve of the 20 mutants with PC properties, including D6A, D10A, H46A, R75A, K77A, G123A, W184A, E193A, D196A, V197A, E198A, and G199 show significant (<80% of wild-type activity) defects on both Class I and Class II promoters, suggesting that these mutants are compromised in their interactions with the α -subunit (Fig. 3). Consistent with this hypothesis, these mutants showed reduced activity from our Class II promoter in *E. coli* co-expressing α_{At} (Fig. 4A). However, when co-expressed with σ^{70}_{At} only seven of these 12 mutants, including D10A, H46, R75A, K77A, D196A, V197A and E198A, showed decreased levels of activity as compared to that of wild-type TraR. Three mutants, G123A, W184A and E193A, showed no significant defect in cells co-expressing σ^{70}_{At} only while one mutant, G199A was hyperstimulated by co-expressed σ^{70}_{At} (Fig. 4B). We propose that the seven mutants define residues important for interactions with both α_{At} and σ^{70}_{At} while

the remaining four mutants define residues that contribute to interactions exclusively with α_{At} on Class II promoters. Three additional mutants, S78A, K201A and E211K show significant defects on Class II but less so on Class I promoters (Fig. 3). We suggest that these three residues are important for interactions between TraR and σ^{70}_{At} and perhaps also α_{At} . Of the remaining five mutants, three, K74A, R79A and K80A show defects only on the Class I promoter (Fig. 3) and were stimulated by co-expressed σ^{70}_{At} but not α_{At} (Fig. 4A and B) suggesting that these three residues contribute to interactions with RpoA but not Sigma-70. The last two PC mutants, R119A and N122A show similar levels of defects on both promoters (Fig. 3). The R119A mutant is strongly stimulated by both RNAP subunits while the N122A mutant is stimulated strongly by σ^{70}_{At} but not by α_{At} (Fig. 4A and B). In summary, of the 20 PC mutants, seven, H46A, K74A, R79A, K80A, R119A N122A, and G199A are specific to the Class I promoter. Ten of the remaining mutants define residues that are important for activation at both Class I and Class II promoters, while three mutants, S78A, K201A and E211K identify residues that are disproportionately important for activation of a Class II promoter.

Considering the activity of the PC mutants in response to the co-expression of α_{At} or σ^{70}_{At} in *E. coli*, we mapped the residues important for interactions with α or with σ^{70} on the crystal structure of the dimeric DNA-bound activator. As shown in Fig. 6A-1, TraR residues that are important for α_{At} -dependent activation in *E. coli* combine to form two surface patches that co-localize with Patch I and Patch II in Figure 1. Those residues that are important for σ^{70}_{At} -dependent activation form two Ler patches that overlap with the α -patches (Fig. 6B-1).

Based on these results alone, we cannot discriminate between effects arising from direct molecular interactions and indirect effects due to conformational changes in the mutant proteins. Several of the PC residues may play roles in maintaining the structure of either the monomers or the dimeric form of TraR. For example, there are at least two direct contacts made by PC residues within the N-terminal domains between the two protomers; a salt bridge between Asp-6 and Lys-119 and a hydrogen bond between Asp-10 and the amide of Asn-122 (Vannini *et al.*, 2002; Zhang *et al.*, 2002, Fig. 6). In addition, several residues participate in contacts between the N- and C-terminal domains within the compact monomer of the TraR dimer including two salt bridges (Lys-7 and Glu-198, Asp-27 and Lys-177) and two hydrogen bonds (His-31 to the carbonyl of Gly-199, and Arg-183 to the carbonyl of Ala-13) (Figs. 1 and 6, Vannini *et al.*, 2002; Zhang *et al.*, 2002). This structural information suggests that certain PC residues including Asp-6, Asp-10, Asn-122, Glu-198 and Gly-199 and those very close to these structural residues including Gly-123, Trp-184, Asp-196 and Val-197 may play roles in maintaining the interactions between the N- and C-terminal domains (Fig. 6A-2, 3 and 6B-2).

The C-terminal region of TraR forms an eared helix with the scaffolding helices protruding from the main dimeric structure (Fig. 1C). Again due to its strong asymmetry, one such ear is juxtaposed next to the left lateral face formed by the N-terminal domain of the compact protomer (Fig. 6A-2, 4), while the other ear contributes to the C-terminal apex of the extended protomer on the right face of the dimer (Fig. 1C, Fig. 6A-3). Five residues with PC properties, Trp-184, Glu-193, Asp-196, Val-197 and Glu-198 all contribute to the structure of the ears (Fig. 1B and C, Fig. 6A-4). Mutants at D196A and E198A completely lost activation functions on both Class I and Class II promoters (Fig. 3) suggesting that these two residues play a structural role (Fig. 6A-2; 6B-2). That Glu-198 participates in a salt bridge with Lys-7 is consistent with this interpretation. The remaining four residues, in conjunction with the patch formed by the N-terminal PC residues form the lip of a pocket on the lateral face of the dimer (Fig. 1; Fig. 6). We propose that this pocket represents one site at which α - or σ^{70} - subunits interact with the dimeric activator. Given that all five mutants are recessive

to wild-type TraR, only one ear appears essential. We propose that the second ear in close juxtaposition to Patch III, located at the lower apex of the extended protomer, does not participate in interactions with RNA polymerase (Fig. 1C; Fig. 6A-3).

Excluding the residues that may play a conformational role in TraR as well as those that contribute to the C-terminal ear structure within the compact protomer, only three PC residues in the N-terminal region, R75, K77 and S78, remain which are required for TraR-mediated activation from both Class I and II promoters (Fig. 3). We suggest that these three residues contribute, in part, to the interaction interface on TraR for RNAP (Fig. 6A-5, 6; 6B-3). The R75A mutant showed similar levels of defect on both promoters (67% on Class I and 77% on Class II, see Fig. 3) as well as a similar decrease of activation in *E. coli* expressing either α_{At} or σ^{70}_{At} (38% for α_{At} and 37% for σ^{70}_{At} , see Fig. 4). The K77A mutant showed decreased levels of activation on both promoters but showed the greatest defect on the Class I promoter (15% as compared to 45% on Class II; see Fig. 3). This mutant also consistently showed a lower level of response when co-expressed with α_{At} than σ^{70}_{At} in *E. coli* (Fig. 4). The alanine substitution at S78 affected activation activities on both reporters, but showed a considerably greater effect on Class II (59%) as compared to Class I (87%) promoters (Fig. 3). This mutant also consistently showed a greater defect in activation when co-expressed with σ^{70}_{At} as compared to α_{At} (Fig. 4). Based on these results, we propose that the two nearly identical patches formed by R75, K77 and S78 on the two protomers play two roles in the interaction between TraR and RNAP (Fig. 6A-5, 6 and 6B-3). We predict that during activation from Class II promoters, the R75-K77-S78 patch located on the top of the TraR dimer is required for interaction with the CTD of one copy of α -subunit (Fig. 6A-6), while the other patch, located on the front face of the TraR dimer within Patch I is important for interaction with σ^{70} (Fig. 6B-3). However, both patches in conjunction with the flanking Class I promoter-specific PC residues may contribute to interactions with the C-terminal domains of both α -subunits during activation from Class I promoters (Fig. 6A-5, 6).

Under conditions in which only α_{At} or only σ^{70}_{At} is co-expressed, TraR weakly activates a Class II promoter in *E. coli* (Fig. S1). However, co-expressing both subunits from *A. tumefaciens*, results in levels of activation much higher than the sum of those obtained when only one or the other subunit is co-expressed (Fig. S1). This observation suggests that the TraR-mediated activation at Class II promoters resembles that of FNR or CAP, requiring interactions with both α and σ^{70} -subunits (Bell and Busby, 1994; Rhodius and Busby, 2000; Li *et al.*, 1998; Lawson *et al.*, 2004). Consistent with this hypothesis, purified DNA-bound TraR complexes interact directly with α - (Qin *et al.*, 2004a) and with σ^{70} -subunits respectively (Qin and Farrand, unpublished data). However, while the relative activation levels of each TraR PC mutant in *E. coli* co-expressing both α_{At} and σ^{70}_{At} are virtually identical to those observed in cells co-expressing only α_{At} , there was little correlation with levels of activation in cells co-expressing only σ^{70}_{At} (Fig. 4B). These results imply that, although interactions of TraR with α_{At} and with σ^{70}_{At} are both important, the interaction with α_{At} is the predominant determinant required for activation of Class II promoters by TraR, a conclusion consistent with studies of TraR_{O_{ct}} (Costa, *et al.*, 2009).

Regions of the CTD of RpoA from *A. tumefaciens* required for activation by TraR

Studies with CAP have identified three domains of RpoA_{Ec} important for activation. The 265 domain (Savery, *et al.*, 1998; reviewed in Rodius and Busby, 1998), composed of nine residues, is 100% conserved in RpoA_{At} while the 261 and 287 domains of the two RpoA proteins share 3 of 4 and 5 of 9 residues in common (Fig. 5A). Of the nine mutants of RpoA_{At} defective in stimulating TraR-mediated activation in *E. coli* (Fig. 5B), four, T284I, A286V, E287V and R290H are surfaced-exposed (Fig. S2) and map to the 287 determinant. Of these, Thr-284, Ala-286 and Glu-287 correspond to three of the six residues of RpoA_{Ec}

that make direct contacts with CAP (Benoff, *et al.*, 2002). On the other hand, only one of our mutants, G295D, corresponds to the six residues of RpoA_{Ec} required for transcriptional activation by LuxR (Finney *et al.*, 2002). Overall, these results suggest that, as in the case of the CAP-RpoA_{Ec} interaction, the 287 region of RpoA_{At} is important for making contact with TraR. However, an alanine substitution at Glu-316, corresponding to a fourth CAP-interaction residue in the 287 region of RpoA_{Ec} had no effect on stimulation of TraR-mediated activation. Remarkably, substitutions at three sites in RpoA_{At}, Ser-262, Val-275 and Gly-295, resulted in stimulation of TraR activity to levels 1.6 to 3 times higher than wild-type in *E. coli* (Fig. 5B). Gly-295 is of particular interest; an aspartate substitution results in a mutant of RpoA_{At} unable to stimulate transcription by TraR while a serine substitution yields a mutant that supports higher than wild-type activation. Two of these residues lie close to or within the 265 domain, a region of the α -subunit of *E. coli* involved in interactions with DNA (Busby and Ebright, 1999; Lawson *et al.*, 2004). These results are consistent with our conclusions that interactions with RpoA are critical for TraR-mediated induction of transcription and further suggest that TraR may mediate interactions between the α -subunit and the promoter regions of TraR-dependent genes that facilitate activation of transcription.

Experimental procedures

Bacterial strains and media

A. tumefaciens strain NTL4 (Luo *et al.*, 2001) and *Escherichia coli* strains DH5 α and S17-1 were used for all constructions and most experiments. *E. coli* strain BL21(DE3) (pLysS), harboring the appropriate plasmids, was used for overexpression and purification of native TraR and its mutants. DH5 α was used to monitor TraR activation activities in *E. coli* co-expressing α_{At} , σ^{70}_{At} or both.

E. coli strains were grown in Luria broth (Invitrogen) at 37 °C or in minimal medium A containing glucose as sole carbon source at 28°C (Miller, 1972). *A. tumefaciens* strains were grown at 28 °C on nutrient agar (Difco), in MG/L medium (Cangelosi *et al.*, 1991), or in AB minimal medium supplemented with 0.2% mannitol (ABM medium) as sole carbon source (Chilton *et al.*, 1974). When required, antibiotics were added to the media at the concentrations described previously (Farrand *et al.*, 1996). X-Gal (Sigma) was included in the media at 40 μ g/ml to monitor expression of *lacZ* reporter genes. IPTG (Sigma) was added at 100 μ M to induce expression of *traR* when necessary. Synthetic 3-oxo-C8-HSL [N-(3-oxo-octanoyl)-L-homoserine lactone] from Sigma was added to cultures at concentrations as indicated in the text.

Reporter plasmids

Plasmid pH4I41, a derivative of pCP13/B which harbors a *traG::lacZ* transcriptional fusion (Luo and Farrand, 1999), was used as the Class II-promoter reporter for TraR activation. Plasmid pRKLH4I41, a derivative of pRK415 that contains *traR* and the *traG::lacZ* transcriptional fusion (Luo *et al.*, 2003), was used as the Class II-promoter reporter for assays of dominant negativity. Plasmids pQKK-*lacZ*10II and pQKKR-*lacZ*10II, two derivatives of pQKK harboring a *traM::lacZ* transcriptional fusion, were used as the Class I-promoter reporters for TraR activation and for dominant negativity assays respectively. pQKK-*lacZ*10II and pQKKR-*lacZ*10II were constructed by cloning into pQKK (Qin *et al.*, 2004a) and pQKKR (Qin *et al.*, 2004a) a HindIII fragment obtained from pMP1 (Hwang *et al.*, 1995), which harbors an insertion of Tn3HoHo1 at the start of the *traM* gene. The repression reporter pPBL1, in which the 18bp *tra box* is centered at the -10 region of a promoter driving expression of *lacZ* in *E. coli*, was used to measure the DNA binding activity of TraR and its mutant proteins *in vivo* (Luo *et al.*, 1999).

Construction of plasmids expressing α_{At} , σ^{70}_{At} , or both α_{At} and σ^{70}_{At}

Plasmid pZLQrpoA expressing α_{At} was constructed by cloning into pZLQ an NdeI-BamHI fragment encoding RpoA_{At} as described previously (Qin *et al.*, 2004a). Plasmids expressing σ^{70}_{At} , or both α_{At} and σ^{70}_{At} were constructed as follows. *Agrobacterium rpoD* (*rpoD*_{At}, Atu2187) was amplified by PCR using *Pfu* polymerase and *Agrobacterium* genomic DNA as template. The resulting fragment was engineered to contain an NdeI site within the start codon and a BamHI site just distal to the stop codon of the *rpoD*_{At} gene. This coding region of *rpoD*_{At} was then cloned between the NdeI and BamHI sites of pZLQ to generate pZLQatrpoD expressing σ^{70}_{At} . In addition, a new BamHI-BamHI fragment of *rpoA*_{At} containing a BamHI site 5' to the start codon was generated by PCR using pZLQrpoA as template. This new *rpoA* fragment was cloned into pZLQatrpoD at the BamHI site to generate pZLQrpoDA that expresses both σ^{70}_{At} and α_{At} .

DNA manipulations

Recombinant DNA techniques were performed as described by Sambrook *et al* (2001). Plasmid DNA was isolated by alkaline lysis or using a miniprep kit from QIAGEN Inc. according to the manufacturer's instructions. Digestions with restriction endonucleases were carried out as described by the manufacturers of the enzymes. Plasmid DNA was introduced into *E. coli* by calcium chloride-mediated transformation and into *A. tumefaciens* by electroporation or by biparental matings from *E. coli* S17-1 (Mesereau *et al.*, 1990; Dessaux *et al.*, 1989).

Mutagenesis of traR

traR D6G and D6R were generated by error-prone PCR. The other substitution mutations in *traR* were introduced by site-directed mutagenesis using the QuickChange Kit (Stratagene) or generated by chemical treatment with hydroxylamine as described previously (Luo *et al.*, 2000). The validity of all mutant constructs was confirmed by DNA sequence analysis performed by the Center of Core Sequencing Facility at the University of Illinois at Urbana-Champaign.

Mutagenesis of α CTD

A DNA fragment encoding the C-terminal 81 amino acids (255-336) of α_{At} and engineered to contain a SacI site at the 5'-end and an XbaI site at the 3'-end was amplified by PCR using *Pfu* DNA polymerase. This α CTD_{At} fragment was cloned into pBlueScript(SK) to generate pSK α CTD. We also made a similar construct called pSKEC α CTD that harbors the C-terminal 73 amino acids (256-328 aa) of α_{Ec} . We then used pSK α CTD or pSKEC α CTD as template to perform error-prone PCR and site-directed mutagenesis to produce mutated fragments called α CTD*. These mutant fragments were digested with SacI and XbaI, and cloned into a trapper plasmid pQESHrpoDA-CTD, a derivative of pZLQrpoDA in which nucleotides encoding the C-terminal 81 amino acids of α_{At} were deleted and replaced with the SacI-HindIII MCS region of pUC18. The resulting constructs, named pQESHrpoDA-CTD*, were screened for mutant phenotypes following electroporation into the reporter strain NTL4(pQKKR-I41). The validity of all mutants was confirmed by DNA sequence analysis.

Quantitative assays for activation, and dominant negativity properties

The activation properties of TraR and its mutants cloned into pZLQ were assessed using the Class II-promoter reporter strain NTL4 (pH4I41) (Luo and Farrand, 1999) and Class I-promoter strain NTL4 (pQKK-lacZ10II) respectively. The dominant-negative properties of TraR substitution mutants cloned into pZLQ were assessed using the Class II-promoter reporter NTL4 (pRKLH4I41) (Luo *et al.*, 2003) and the Class I-promoter reporter NTL4

(pQKKR-*lacZ*10II). In all cases, strains were cultured in MG/L medium with or without 50 nM 3-oxo-C8-HSL.

Quantitative assays for repression and activation activity in *E. coli*

The ability of the mutant alleles to bind DNA *in vivo* was assessed using a repression assay with DH5 α (pPBL1) (Luo and Farrand, 1999). In this strain, TraR and its mutants cloned in the vector pZLQ were used to repress expression of a *lacZ* fusion encoded by pPBL1 in the presence of 50 nM 3-oxo-C8-HSL (Luo and Farrand, 1999).

TraR and its mutant alleles were assessed for activation activities in *E. coli* DH5 α harboring the Class II-promoter reporter plasmid pQKKR-I41 (Qin *et al.*, 2004a) encoding *traR* or its mutant gene, and one of these pZLQ derivatives, pZLQrpoA, pZLQatrpoD or pZLQatrpoDA, which encodes α_{AT} , σ^{70}_{AT} , or both α_{AT} and σ^{70}_{AT} respectively.

β -Galactosidase assays

Production of β -galactosidase by *A. tumefaciens* and *E. coli* strains was quantified using a modification of the method of Miller (Miller, 1972) as previously described (Cook and Farrand, 1992). Activity is expressed as Miller units or units of enzyme/10⁹ colony-forming units. Samples were assayed in triplicate, and experiments were repeated at least twice. In the absence of error bars or values of variation, results from a single representative experiment are shown.

Western analysis

The stability and expression levels of TraR mutant proteins *in vivo* as well as the amount of purified TraR protein used in gel mobility shift assays were determined by Western analysis using anti-TraR antiserum as described previously (Qin *et al.*, 2004b).

Gel retardation assays

Purified dimeric TraR or its mutant derivatives from the cleared cell extracts, following normalization by western analysis, were prepared at a series of two-fold dilutions and incubated together with a DIG-labeled 251 bp DNA probe containing an intact *tra* box sequence (20 ng) at room temperature in 20 μ l of reaction buffer (10 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM DTT, 60 mM KCl, 30 μ g ml⁻¹ salmon sperm DNA, 20 μ g ml⁻¹ BSA, 0.05% Tween 20 and 10% glycerol) for 20 min (Qin *et al.*, 2004b). The samples were subjected to electrophoresis in 0.25 $\times$ TBE buffer (1 $\times$ TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) on 7.5% native polyacrylamide gels. The separated DNA fragments and DNA-protein complexes were electroblotted onto positively charged nylon membranes and visualized by chemiluminescence as described previously (Qin *et al.*, 2000).

Structural analyses

The three-dimensional structures of TraR and α CTDs were analyzed using PyMOL Version 10.2 on a Macintosh. The coordinates for the structure of TraR from pTiR10 used in these analyses are available in the Protein Data Bank under code IL3L (Zhang *et al.*, 2003). An *in silico* crystal structure of the C-terminal domain of RpoA_{At} was constructed by threading the sequence of residues 243-336 of RpoA_{At} to the crystal structure of the homologous region of RpoA_{Ec} available in the Protein Data Bank under accession number 1LB2 (Benoff, *et al.* 2002) using the Swiss Model Server (<http://swissmodel.expasy.org>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Locations of single amino acid substitutions on the structure of TraR that yield a PC phenotype

A, A linear depiction of TraR with the locations of PC substitutions indicated. B and C, three distinct patches formed by PC residues on the TraR dimer structure complexed with an 18 bp *tra box* DNA fragment presented in two orthological views. Purple protomer: the compact monomer in which the N- and C-terminal domains contact each other; Green protomer: the extended monomer within which there is no contact between the N- and C-terminal domains. Residues in red and magenta indicate sites at which substitutions in the N- and C-regions of TraR respectively give positive control phenotypes on a Class II promoter. Residues in yellow or pink indicate sites at which substitutions show positive control phenotypes only on Class I but not on Class II or only on Class II but not on Class I promoters.

Figure 2. DNA binding affinities of TraR mutants as determined by gel mobility shift assays
 Mobility shift assays of wild-type TraR and representative PC mutants were conducted as described in *Experimental procedures*, TraR DNA-binding constants were defined by the concentrations of TraR at which 50% of the total probe (7.5×10^{-10} M) was shifted as a TraR-DNA complex (indicated by arrows). TraR and its representative mutants were tested at concentrations of 0 (2 nM for D196A), 4, 8, 17, 34, 67, 125, 250 and 500 nM, loaded in lanes 1, 2, 3, 4, 5, 6, 7, 8, and 9, respectively.

Figure 3. Influence of single amino acid substitutions in TraR on activation of Class I and Class II promoters in *A. tumefaciens*

Wild-type TraR and a series of single amino acid substitution mutants were tested for activation of the *traM::lacZ* Class I reporter (closed bars) and the *traCDG::lacZ* Class II reporter (open bars) in *A. tumefaciens* as described in *Experimental procedures*. In all cases the cultures were supplemented with *N*-(3-oxo-octanoyl)-L-HSL at a final concentration of 50 nM. Alleles in boxes represent mutations that disproportionately affect activation at the Class I promoter while alleles in ovals represent mutations that have the greatest effect on activation at the Class II promoter. The data represent the averages of three repetitions and error bars indicate standard deviations.

Figure 4. Influence of components of *A. tumefaciens* RNA polymerase on activation in *E. coli* of a Class II promoter by TraR and its PC mutants

Wild-type TraR and its PC mutant alleles, cloned in pQKK-I41 were tested for activation of the Class II *traCDG::lacZ* reporter in *E. coli* DH5 α harboring either pZLQrpoA that expresses *rpoA_{At}*(A), pZLQatrhoD that expresses *rpoD_{At}* (B), or pZLQrpoDA that expresses both *rpoA_{At}* and *rpoD_{At}* (C). In all cases the cells were grown in the presence of 25 nM *N*-(3-oxo-octanoyl)-L-HSL as described in *Experimental procedures*. Activity for each of the mutants is expressed as percent of the background-corrected activity of wild-type *traR* in each experimental set. The data represent the averages of three repetitions and error bars indicate standard deviations.

Figure 5. Influence of single amino acid substitutions in the C-terminal domain of RpoA_{At} on TraR-mediated activation of transcription in *E. coli*

Mutant alleles of *rpoA*_{At}, isolated as described in *Experimental procedures* were tested for their ability to stimulate TraR-mediated expression of the *traCDG::lacZ* reporter in *E. coli*. The amino acid sequence of the C-terminus of RpoA_{At}, the locations of the mutant residues, and the alignment with the C-terminus of RpoA_{Ec} are shown in panel A. Alleles in bold font represent mutations that have significant positive or negative effects on RpoA_{At} activity. The ability of each allele to stimulate transcriptional activation from pQKKR-I41 was tested in *E. coli* DH5α also expressing RpoD_{At} (panel B). Values representing β-galactosidase activities relative to that of wild-type RpoA_{At} are shown. The data represent the average of three repetitions with a deviation of less than 10%.

Figure 6.

Regions of TraR involved in interactions with components of RNA polymerase. Views 1-5 show locations of PC residues on the lateral faces while View 6 shows locations of residues on the top surface of TraR that play roles in interaction with the α (A) and σ^{70} -subunits (B) of RNAP. Residues predicted to play structural roles, and those that form the ear structures in the C-terminal domains are shown in Views 2-4 of Panel A and View 2 of Panel B as indicated. In Panel A, View 3 is the back view of View 2 to indicate the difference of location of PC residues between the two protomers of a TraR dimer. The remaining PC residues colored in red and the Class I-specific PC residues colored in yellow, are shown in Views 5-6 of Panel A and View 3 of Panel B. Regions of TraR important for interactions with the C-terminal domain of RpoA_{At} at a Class I promoter, or with α CTD and RpoD_{At} at Class II promoters are circled and labeled as α CTD-Class I, α CTD-Class II and σ^{70} -Class II respectively. The compact protomer is shown in purple while the extended protomer is shown in green. For each protomer, N-terminal residues are shown in a darker hue.

Table 1

In vivo activation and repression activities and *in vitro* DNA binding affinities of TraR N-terminal mutants^a

<i>traR</i> allele	Activation ^b		Repression ^c		Ratio ^d	DNA Binding ^e
	Activity ^f	%activation ^g	Activity ^f	%inhibition ^h		
None	2	0	208	0	NA ⁱ	NA
Wild-type	704	100	7	100	1	8×10 ⁻⁹
D6G	13	1.5	7	100	67	8×10⁻⁹
D6R	12	1.4	8	100	71	4×10⁻⁹
D10A	2	0	12	98	>100	8×10⁻⁹
R45A	669	95	8	100	1.1	NT ^j
H46A	521	74	43	82	1.1	NT
K74A	706	100	7	100	1	NT
R75A	542	77	8	100	1.3	NT
K77A	317	45	9	99	2.2	NT
S78A	415	59	8	100	1.7	NT
R79A	669	95	8	100	1.1	NT
K80A	703	100	7	100	1	NT
R119A	591	84	8	100	1.2	NT
N122A	598	85	30	89	1	NT
G123A	11	1	7	100	100	8×10⁻⁹
S124A	2	0	220	0	NA	NT

^aMutants with PC properties are indicated by bold font.

^b*In vivo* activation by TraR and its mutant alleles was assessed in *A. tumefaciens* NTL4 harboring the reporter plasmid pH4141 and the pZLQ derivative expressing the indicated *traR* gene.

^c*In vivo* repression by TraR and its mutant alleles was assessed in *E. coli* DH5α harboring the reporter plasmid pPB1 and the pZLQ derivative expressing the indicated *traR* gene.

^dRepression/activation ratio was calculated as the fold repression value divided by the fold activation value.

^eDNA binding affinity of TraR and its mutant alleles was assessed by gel shift assays as described in *Experimental procedures*. The binding constant is expressed as the concentration of TraR (M) protein that resulted in 50% binding of the total probe DNA (7.5×10⁻¹⁰M).

^fExpressed as units of β-galactosidase activity /10⁹ cfu as described in *Experimental procedures*. Each allele of *traR* was tested a minimum of three times, and the results shown are from a representative experiment.

g %activation is calculated as $\{(\text{mutant-none})/(\text{wt-none})\} \times 100$, where mutant, none and wt are β -galactosidase activities of strains expressing mutant TraR, no TraR and wild-type TraR respectively.

h %repression is calculated as $\{(\text{none-mutant})/(\text{none-wt})\} \times 100$, where mutant, none and wt are β -galactosidase activities of strains expressing mutant TraR, no TraR and wild-type TraR respectively.

i NA=Not Applicable.

j NT=Not Tested

Table 2
In vivo activation and repression activities and *in vitro* DNA binding affinities of TraR C-terminal mutants^a

<i>traR</i> allele	Activation ^b		Repression ^c		Ratio ^d	DNA Binding ^e
	Activity ^f	%Activation ^g	Activity ^f	%Inhibition ^h		
None	2	0	208	0	NA ⁱ	NA
Wild-type	704	100	7	100	1	8×10 ⁻⁹
L174A	6	0.6	213	0	0	NT ^j
W184A	108	15	116	46	3	8×10⁻⁸
G188A	2	0	243	0	NA	NT
M189A	626	89	38	85	0.9	NT
T190I	92	13	199	4	0.3	NT
E192A	701	100	7	100	1	NT
E193A	380	42	38	85	2	8×10⁻⁸
A195V	3	0.1	216	0	0	NT
D196A	4	0.3	97	55	>100	2×10⁻⁹
V197A	263	37	22	93	2.5	1.7×10⁻⁸
E198A	4	0.3	106	51	>100	NT
G199A	391	55	124	42	0.8	NT
V200A	318	45	110	49	1.1	4×10 ⁻⁸
K201A	441	63	10	99	1.6	2.5×10⁻⁷
S204A	705	100	8	99	1	NT
V205A	18	2	199	4	2	NT
L209A	10	1	145	31	31	NT
E211K	416	59	13	97	1.6	NT
A212V	697	99	9	99	1	NT
R215A	81	11	196	6	0.5	NT

^a Assays and other information are as described in footnotes to Table 1.

Table 3

Dominant negativity of TraR mutants.

<i>TraR</i> allele ^a	Dominant negativity			
	Class II promoter ^b		Class I promoter ^c	
	Activity ^d	% Inhibition ^e	Activity ^d	% Inhibition ^e
None	156	0	201	0
Wild type	262	(68) ^f	241	(20)
D6G	61	61	34	83
D6R	61	61	86	57
D10A	3	98	86	57
R45A	179	(15)	175	13
H46A	204	(31)	149	26
R74A	187	(20)	125	38
R75A	172	(10)	143	29
K77A	92	41	127	37
S78A	142	9	143	29
R79A	187	(20)	129	36
K80A	165	(6)	129	36
R119A	134	14	187	7
N122A	162	(4)	133	34
G123A	5	97	82	59
S124A	125	20	165	18
L174A	117	25	NT ^g	
W184A	167	(7)	191	5
G188A	100	36	NT	
M189A	268	(72)	NT	
T190I	139	11	NT	
E192A	248	(59)	NT	
E193A	206	(32)	207	(3)
A195V	87	44	165	18
D196A	154	1	191	5
V197A	209	(34)	245	(22)
E198A	145	7	235	(17)
G199A	184	(18)	223	(11)
V200A	246	(58)	NT	
K201A	248	(59)	253	(26)
S204A	217	(39)	261	(30)
V205A	150	4	NT	
L209A	150	4	209	(4)
E211K	211	(35)	253	(26)
A212V	271	(74)	NT	

<i>TraR</i> allele ^a	Dominant negativity			
	Class II promoter ^b		Class I promoter ^c	
	Activity ^d	% Inhibition ^e	Activity ^d	% Inhibition ^e
R215A	189	(21)	NT	

^aThe second copy of *traR* or one of its mutant alleles expressed from derivatives of pZLQ constructed as described in *Experimental procedures*.

^bTested in *A. tumefaciens* NTL4 harboring pRKLH4I41, which codes for wild-type *traR* and the TraR-dependent *traG::lacZ* reporter, and a pZLQ derivative.

^cTested in *A. tumefaciens* NTL4 harboring pQKKR-*lacZ10II*, which codes for wild-type *traR* and the TraR-dependent *traM::lacZ* reporter, and a pZLQ derivative.

^dβ-galactosidase activity expressed as units per 10⁹ cfu as described in *Experimental procedures*. Each allele of *traR* was tested a minimum of three times, and the results shown are from a representative experiment.

^e% inhibition=100×{[wt+none]-[wt+mutant]}/[wt+none]. In this formula, [wt+none] and [wt+mutant] are levels of β-galactosidase activity in strains expressing only wild-type TraR or wild-type plus mutant TraR. The mutants that inhibited activation of the wild-type TraR by values greater than 20% are indicated in bold font.

^fValues in () indicate that the presence of the second *traR* allele results in levels of activation greater than wild-type *traR* alone.

^gNT= Not Tested.