Cell–cell communication in the plant pathogen
Agrobacterium tumefaciens

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The plant pathogen Agrobacterium tumefaciens induces the formation of crown gall tumours at wound sites on host plants by directly transforming plant cells. This disease strategy benefits the bacteria as the infected plant tissue produces novel nutrients, called opines, that the colonizing bacteria can use as nutrients. Almost all of the genes that are required for virulence, and all of the opine uptake and utilization genes, are carried on large tumour-inducing (Ti) plasmids. The observation more than 25 years ago that specific opines are required for Ti plasmid conjugal transfer led to the discovery of a cell–cell signalling system on these plasmids that is similar to the LuxR–LuxI system first described in Vibrio fischeri. All Ti plasmids that have been described to date carry a functional LuxI-type N-acylhomoserine lactone synthase (TraI), and a LuxR-type signal receptor and transcriptional regulator called TraR. The traR genes are expressed only in the presence of specific opines called conjugal opines. The TraR–TraI system provides an important model for LuxR–LuxI-type systems, especially those found in the agriculturally important Rhizobiaceae family. In this review, we discuss current advances in the biochemistry and structural biology of the TraR–TraI system.

Keywords: Agrobacterium tumefaciens; tumour-inducing plasmids; signal receptor; conjugal opines

1. INTRODUCTION

A general interest in the soil bacterium Agrobacterium tumefaciens began with the observation, published in a series of papers almost 30 years ago, that wild-type strains of this organism have the unique ability to directly transform plant cells by transfer of discrete DNA fragments (Escobar & Dandekar 2003). This transformation causes the infected plant cells to overproduce phytohormones, causing cell proliferation, which results in the growth of tumours called crown galls. The transforming DNA (T-DNA) also encodes genes for the production of novel compounds called opines, which are sources of nutrients for the colonizing bacteria. By inducing plant cell growth and directing these cells to produce nutrients that only the colonizing bacteria can use, A. tumefaciens makes a novel niche for itself in its environment, giving itself a clear advantage over other plant-colonizing bacteria. Since these early discoveries, the possible utility of A. tumefaciens in plant genetic engineering certainly did not go unnoticed, and in fact plant transformation using A. tumefaciens is currently the most efficient method available for many types of plants (Gelvin 2003).

Almost all of the genes required for tumorigenesis are carried on large (approx. 200 kb) plasmids in pathogenic isolates of A. tumefaciens (Kerr 1969, 1971; Van Larebeke et al. 1974). These plasmids, called tumour-inducing (Ti) plasmids, have complete conjugation systems, yet conjugate only in the presence of specific opines (Genetello et al. 1977; Kerr et al. 1977). Therefore, Ti plasmid conjugation can only occur in the crown gall tumour environment, as these tumours are the only natural sources of these compounds. The type of opine required for conjugation depended on the type of Ti plasmid (Genetello et al. 1977; Kerr et al. 1977).

Since these discoveries, it has been shown that the Ti plasmids also carry a quorum-sensing system related to the LuxR–LuxI system of Vibrio fischeri (Engebrecht et al. 1983; Engebrecht & Silverman 1984, 1987). The basic components of the system include the LuxI-type protein TraI, which synthesizes N-3-oxooctanoyl-L-homoserine lactone (3-oxo-C8 HSL; Fuqua & Winans 1994; Hwang et al. 1994; More et al. 1996), and the receptor of 3-oxo-C8 HSL, the LuxR-type protein TraR (Piper et al. 1993; Zhang et al. 1993; Fuqua & Winans 1994). All known LuxI proteins synthesize an N-acylhomoserine lactone (AHL), which is detected by the cognate LuxR homologue. At high population densities, 3-oxo-C8 HSL accumulates to some threshold level, and TraR-3-oxo-C8 HSL complexes then activate transcription of target genes on the Ti plasmid, including those required for Ti plasmid conjugation (Fuqua & Winans 1996a). Therefore, inducing concentrations of 3-oxo-C8 HSL are required for conjugation. It was later shown that the expression of traR genes requires specific opines called conjugal opines (Beck von Bodman et al. 1992; Fuqua & Winans 1996b). This explained the previous observation that conjugation requires opines.

Since the initial discovery of TraR and TraI, a series of papers have been published on quorum sensing in A. tumefaciens, including many of the first biochemical and structural studies of LuxR–LuxI-type systems. The regulation of TraR activity is rather complex, and we certainly do not have a complete picture of how this system works. However, our current model and available data do prompt many exciting questions about how this...
system works. In this review, we first present the background information relevant to quorum sensing in *A. tumefaciens* and then focus on our current knowledge of the molecular biology of the TraR–TraI system.

2. AGROBACTERIUM TUMEFACIENS, THE TUMOUR INDUCING PLASMIDS AND CROWN GALL DISEASE

*Agrobacterium tumefaciens* is a member of the Rhizobiaceae, which is in the α-subgroup of the proteobacteria. All members of this family are soil bacteria, and are traditionally divided into two groups. The rhizobia, which include members of the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium*, are all nitrogen-fixing symbionts of host legumes. The agrobacteria include the plant pathogens *A. tumefaciens*, *Agrobacterium rubi*, *Agrobacterium vitis* and *Agrobacterium rhizogenes*, as well as the non-pathogenic *Agrobacterium* radiobacter. *Agrobacterium tumefaciens*, *A. rubi* and *A. vitis* all induce the formation of crown galls through plant transformation as described previously (Binns & Costantino 1998). In addition, *A. vitis*, which is restricted to grape, causes tumours on the crown of grape vines as well as necrotic lesions on grape roots (Burr & Otten 1999). *Agrobacterium rhizogenes* also causes disease by transforming plant cells, but induces the formation of ‘hairy roots’ rather than galls (Binns & Costantino 1998). Recently, it was proposed that the genus *Agrobacterium* be abandoned and that all species be placed in the genus *Rhizobium* (Young et al. 2003).

For the rhizobia and agrobacteria, intimate associations with host plants can be achieved only if the bacteria carry large self-transmissible genetic elements, either plasmids or integrating conjugative elements that include almost all of the determinants for either symbiosis or disease. Tumorigenic strains of *Agrobacterium* contain either Ti or root inducing plasmids. Functional LuxR–LuxI-type systems have been described for a number of the rhizobial symbiosis plasmids (Wisniewski-Dye & Downie 2002; Gonzalez & Marketon 2003). However, the TraR–TraI system of the Ti plasmids remains the only well-studied quorum-sensing system of this agriculturally important family.

The complete genome sequence of *A. tumefaciens* strain C58 has been published by two groups (Goodner et al. 2001; Wood et al. 2001). The genome is unusual in that it has both a circular and a linear chromosome. These two replicons carry all of the essential and housekeeping genes of the organism, although the linear chromosome has fewer of these per megabase than the circular chromosome. This and other evidence suggest that the linear chromosome may have actually evolved from a plasmid (Goodner et al. 2001; Wood et al. 2001). C58 also carries two large circular plasmids, pAtC58 (also called the ‘cryptic’ plasmid) and the Ti plasmid pTiC58. The *traR* and *traI* genes of the quorum-sensing system are both located on pTiC58, while a few additional *luxR* homologues are found in the genome sequence of C58. It is not yet clear whether these are functional as AHL receptors or not. Curiously, there are no corresponding *luxI*-type genes found anywhere on the genome.

Ti plasmids from different isolates of *A. tumefaciens* are traditionally classified by opine type. However, a variety of different opine synthases and their corresponding catabolism genes are encoded by each isolate (Zhu et al. 2000). In addition, there is often an overlap in the types of opines associated with different plasmids. The best-studied are the nopaline-type (including pTi37 and pTiC58) and the octopine-type (including A6, B6, Ach5, 15966 and R10) Ti plasmids. However, these octopine-type Ti plasmids are all virtually identical and a composite DNA sequence of these plasmids has been published (Zhu et al. 2000). The genes required for virulence (the *vir* genes), conjugation (*tra* and *trb* genes) and vegetative replication (*rep*) are highly conserved between the octopine-type and the nopaline-type Ti plasmids.

The *vir* genes are arranged in a number of operons, most of which are essential for plant cell transformation. The expression of these genes is induced only in response to signals released from plant wounds (phenolics, sugars and a pH range of 5.0–5.5; Johnson & Das 1998). These signals are detected by VirA, a transmembrane sensor kinase, which then phosphorylates its cognate response regulator, VirG (Stachel & Zambryski 1986; Winans et al. 1988). Phospho-VirG then activates the *vir* genes, which direct T-DNA processing from the Ti plasmid and its transfer into plant host cells (Gelvin 2003). In the host cell cytoplasm, the single-stranded T-DNA is targeted to the nucleus and integrated into host genomic DNA. T-DNA transfer requires a direct contact between the bacterium and the plant cell, and sequence analysis shows that the transfer apparatus has evolved from a bacterial conjugation system (Lessl et al. 1992; Kado 1994; Lessl & Langa 1994; Pohlman et al. 1994). However, the *vir* system and the *tra* and *trb* genes required for Ti plasmid conjugation are not closely related. T-DNA transfer, which is an example of a type IV secretion system, and T-DNA integration into plant genomic DNA have been the subjects of intense study, but are beyond the scope of this review. We refer the reader to recent reviews of these fields (Escobar & Dandekar 2003; Gelvin 2003).

As alluded to previously, T-DNA-encoded genes expressed in the plant host cell direct phytohormone overproduction and opine synthesis (Morris 1990; Dessaux et al. 1992). The opines are taken up by the agrobacteria via dedicated ABC-type uptake systems, and used as sources of energy, carbon and, in some cases, nitrogen or phosphorus (Dessaux et al. 1998). The expression of opine uptake and catabolic genes is positively regulated by the cognate opines (Dessaux et al. 1992).

3. A MODEL OF QUORUM SENSING IN *A. TUMEFACIENS*

The regulation of TraR activity is quite complex and occurs at the levels of transcription, protein folding, resistance to proteolysis and the formation of quaternary structures with other TraR subunits and with other proteins. In this section, we will provide an overview of how the system works and the many factors involved in regulation. The model presented in figure 1 is a
transcription of the T-DNA and vir genes are shown for reference.

**Figure 1. A model of the quorum-sensing system in octopine-type Ti plasmids.** The traR gene is transcribed in response to octopine and the apo-protein binds to 3-oxo-C8 HSL, the quorum-sensing signal produced by TraI. TraR–3-oxo-C8 HSL dimers activate transcription of traM and the tra, trb and rep operons of the Ti plasmid. TraR–3-oxo-C8 HSL complexes can be inactivated through direct interactions with TraM or TrlR. Transcription of trlR is activated by MocR in response to mannopine. The approximate locations of the T-DNA and vir genes are shown for reference.

summary of the information presented here for octopine-type Ti plasmids. In §4, we will discuss current advances in the biochemical and structural biology of some of the individual proteins involved in quorum-sensing regulation in *A. tumefaciens*.

**a) Regulation of traR gene expression**

Two groups independently discovered that the traR gene is regulated by the ‘conjugal opines’ on both the nopaline- and the octopine-type Ti plasmids. On nopaline-type Ti plasmids, traR is the fourth gene in the five-gene arc operon, which is divergently transcribed from the acc operon (Beck von Bodman et al. 1992). The transcriptional regulator AccR is encoded by the first gene of the acc operon, the other members of which are required for catabolism of the opines agrocinopine A and B. In the presence of agrocinopine A or B, repression of both the arc and the acc operons by AccR is relieved, resulting in the transcription of all of the genes of these operons, including traR (Beck von Bodman et al. 1992; Piper et al. 1999).

In octopine-type Ti plasmids, the conjugal opine is octopine (figure 1; Genetello et al. 1977; Kerr et al. 1977). Octopine binds to its intracellular target, the transcriptional regulator OccR, resulting in activation of the occ operon (Fuqua & Winans 1996b). The traR gene is at the distal end of this operon, while many of the genes upstream of traR are required for uptake and catabolism of octopine (Fuqua & Winans 1996b). When traR is expressed from a constitutive promoter, conjugation no longer requires octopine. Therefore, regulation of traR expression by OccR completely explains the requirement of octopine for Ti plasmid conjugation.

Control of traR expression by opines therefore has evolved independently in these two types of Ti plasmids. The genes of the arc operon and occ operon are not similar, except for traR. Furthermore, traR is the only gene in each of these operons that is required for conjugation (Fuqua & Winans 1996b; Piper et al. 1999). The regulators of the occ and the arc operons are also dissimilar. OccR is a LysR-type transcriptional activator, which binds to promoter DNA both in the presence and in the absence of the inducing signal (Wang et al. 1992). Ligand binding to LysR-type proteins often results in a shift from a high-angle bend in the DNA to a low-angle bend, allowing RNA polymerase to bind to the promoter (Schell 1993; McFall et al. 1998). A direct binding of OccR to occ promoter DNA and a change in OccR-DNA conformation in response to octopine have been demonstrated in vitro (Wang et al. 1992). In contrast, AccR is similar to the Lac repressor (Beck von Bodman et al. 1992). Binding of the inducing signal to these proteins results in a conformational change that disrupts DNA binding, resulting in derepression (Lewis 2005).

Control of traR expression by opines is a feature of all Ti plasmids that have been studied to date (figure 2). Regulation of traR expression on pTiChry5 is also thought to be through an AccR homologue, although in this case derepression occurs in response to agrocinopines C and D, and traR is in a two-gene operon (also called arc; Oger & Farrand 2001). Agrocinopines C and D are also known to induce conjugal transfer of
There is convincing evidence that TraR is a so-called intrinsically unstructured protein, as it requires 3-oxo-C8 HSL for stability and accumulation in vivo. When TraR is strongly overexpressed in either Escherichia coli or A. tumefaciens it does accumulate, but forms insoluble inclusion bodies. This led one group to suggest that apo-TraR is sequestered in the cellular membrane, and is released (and thus able to bind to DNA) only upon association with 3-oxo-C8 HSL (Qin et al. 2000). However, if the protein is only mildly expressed, then it does not accumulate in the absence of 3-oxo-C8 HSL but is in fact degraded by the cytoplasmic proteases (Zhu & Winans 2001). This led to the alternative suggestion that TraR requires 3-oxo-C8 HSL for stabilization against proteolysis. Therefore, 3-oxo-C8 HSL must trigger some conformational change in the protein upon binding. Furthermore, in the same study it was demonstrated with pulse-chase experiments that the apo-protein was targeted for proteolysis extremely rapidly. This led to the suggestion that 3-oxo-C8 HSL must stabilize TraR during translation, and therefore is likely to be part of the protein folding process itself. It was also demonstrated that purified apo-TraR is rapidly degraded by trypsin, whereas purified TraR-3-oxo-C8 HSL complexes were much more resistant to degradation (cleaving only at an inter-domain linker; Zhu & Winans 2001). This further supported the suggestion that the apo-protein is at least some degree unfolded and must undergo a disorder-to-order transition upon 3-oxo-C8 HSL binding.

The data described above suggested that if inducing concentrations of 3-oxo-C8 HSL are present, TraR is stabilized in the cell and can activate transcription of target genes (Zhu & Winans 1999, 2001). However, the activity of stable TraR-3-oxo-C8 HSL complexes can still be directly inhibited by two different proteins, TraM and TriR. On the octopine-type Ti plasmid, the...
The traM gene is adjacent to traR, just beyond the end of the occ operon (figure 1; Fuqua et al. 1995; Hwang et al. 1995). The fact that traR and traM are linked also suggests that traM and its function in the LuxR-LuxI system may be conserved. In fact, it has been shown that traM is even associated with these systems on the symbiosis megaplasmids of the rhizobia (He et al. 2003). For both the nopaline and the octopine-type Ti plasmids, it was shown that a null mutation in traM results in responses to lower concentrations of 3-oxo-C8 HSL than wild-type, and causes a hyperconjugal phenotype, while traM overexpression has the opposite effect (Fuqua et al. 1995; Hwang et al. 1995). Thus, it is clear that the traM gene product somehow attenuates TraR activity. Subsequent studies showed that the anti-activation effect of TraM is through direct protein–protein interactions with TraR-3-oxo-C8 HSL complexes (Hwang et al. 1999; Swiderska et al. 2001).

The adaptive significance of TraM is not entirely clear. In both nopaline- and octopine-type plasmids, it has been demonstrated that traM is activated by TraR in the presence of 3-oxo-C8 HSL, thus forming a negative feedback loop (Fuqua et al. 1995; Hwang et al. 1995). The results of a recent gene expression study using microarrays suggest that traM is also modestly induced in the presence of plant-released phenolics (remember that these compounds activate the vir genes of the Ti plasmid; Cho & Winans 2005). Perhaps this is a mechanism to avoid concurrent expression and activity of the vir and conjugation systems. Both have a type IV secretion system that is required for DNA transfer. It is possible that these two systems could interfere with each other if expressed at the same time in the cell.

The TraI inhibitor of TraR activity is only known to be associated with octopine-type Ti plasmids. The trlR gene is near the distal end of the mot operon, which includes the genes required for mannopine uptake (figure 1; Oger et al. 1998; Zhu & Winans 1998). The expression of this operon is activated only in the presence of mannopine, possibly via the MocR protein (Oger et al. 1998; Zhu & Winans 1998). Mannopine has been shown to attenuate conjugation in octopine-type Ti plasmids, while conjugation can be restored by disrupting trlR (Oger et al. 1998; Zhu & Winans 1998). In addition, expression of trlR from a constitutive promoter also results in a decrease in conjugation (Chai et al. 2001). These experiments demonstrate that the negative effect of mannopine on conjugation is specifically through activation of trlR gene expression. Interestingly, it was also found that favoured catabolites, including succinate, glutamine and tryptone, block trlR expression (Chai et al. 2001). This led to the speculation that TraI functions to attenuate the energetically expensive process of conjugation when nutrients are limiting.

4. BIOCHEMICAL AND STRUCTURAL STUDIES OF THE TRA–TRA SYSTEM

(a) TraI: the autoinducer synthase

In 1991, it was predicted that the LuxI-type autoinducer synthases would use S-adenosylmethionine (SAM) and either acyl-ACP or acyl-CoA as the precursors for the homoserine lactone and fatty acid tail of the AHLs, respectively (Eberhard et al. 1991). This prediction was later confirmed using the purified histidine-tagged TraI protein (More et al. 1996). To define the exact substrates for 3-oxo-C8 HSL synthesis, the purified protein was mixed with a variety of potential substrates. It was determined that 3-oxoocanoyl-ACP was the sole fatty acid donor and that SAM was the sole precursor of the homoserine lactone group. This was the first demonstration of the activity of any LuxI-type protein in vitro in a purified system with defined substrates. Subsequent studies with other LuxI-type proteins, including LuxI and Rhl, suggest that all of these proteins use similar substrates and have very similar reaction mechanisms (Val & Cronan 1998; Parssek et al. 1999).

High-resolution crystal structures are available for two LuxI-type proteins, EsaI of Pantoea stewartii and LasI of Pseudomonas aeruginosa (Watson et al. 2002; Gould et al. 2004). The reaction mechanisms of all of the proteins in this family are expected be quite similar, and a number of residues in the active site of the proteins are conserved. A discussion of these proteins and AHL synthesis is beyond the scope of this review, and we refer the reader to another recent review (Pappas et al. 2004).

(b) TraR: the autoinducer receptor

The realization that TraR requires 3-oxo-C8 HSL for stability allowed overexpression and purification of stable TraR-3-oxo-C8 HSL complexes from E. coli for biochemical and structural studies. It was demonstrated in vitro with purified TraR-3-oxo-C8 HSL complexes that 3-oxo-C8 HSL binds to TraR monomers in a 1:1 mole ratio and that these complexes form homodimers in solution (Qin et al. 2000; Zhu & Winans 2001). It was also shown, with gel shift and footprint assays, that TraR-3-oxo-C8 HSL complexes bind, as dimers, to 18 bp sequences (called tra boxes) on the Ti plasmid with high affinity and specificity (Zhu & Winans 1999).

Two high-resolution crystal structures of TraR-3-oxo-C8 HSL complexes bound to tra box DNA were published in the same year, and to date they are the only structures of LuxR-type proteins available (Vannini et al. 2002; Zhang et al. 2002b). As one would expect, the structures are nearly identical, except for differences in the orientation of a few side chains at the surface of the protein. These structures of TraR confirmed earlier predictions that the protein binds to DNA as a dimer and that each monomer has two domains, an N-terminal 3-oxo-C8 HSL-binding domain and a C-terminal DNA-binding domain (figure 3a). The N-terminal domain has an α–β–α sandwich structure, with one molecule of 3-oxo-C8 HSL embedded between the β-sheet and a layer of α-helix (Vannini et al. 2002; Zhang et al. 2002b). In fact, the ligand is completely engulfed in the core of the protein and protected from solvent. Therefore, it is quite likely that the pheromone is incorporated during the protein folding process itself as previously predicted, or that major structural rearrangements must occur in TraR upon 3-oxo-C8 HSL binding. The contacts themselves between TraR and 3-oxo-C8 HSL are extensive, and include hydrophobic packing interactions between residues of TraR and the acyl chain. The polar groups

of the 3-oxo-C8 HSL make hydrogen bonds with residues in the binding pocket, such that the partial charges do not disrupt the integrity of the domain core (Vannini et al. 2002; Zhang et al. 2002b). These intimate and extensive contacts further support the model that 3-oxo-C8 HSL stabilizes the TraR protein in vivo by participating in the protein folding process itself.

The smaller C-terminal domain of each monomer is a four-helix bundle (figure 3a; Vannini et al. 2002; Zhang et al. 2002b). The second and third helices of this domain are a typical bacterial helix-turn-helix (HTH) DNA-binding motif (Nelson 1995). In fact, the amino acid sequence and the structure of this domain are highly conserved and place the LuxR family within the larger NarL–FixJ superfamily of bacterial transcription regulators (Fuqua & Greenberg 2002). All of these proteins have similar DNA-binding domains, but differ in their signal transduction domains. The high-resolution structures of only two additional proteins in this superfamily have been determined. One is the structure of the NarL DNA-binding domain, also crystallized with its DNA-binding site, and the other is the smaller protein GerE of Bacillus subtilis, which lacks a signal-binding domain altogether (Ducros et al. 2001; Maris et al. 2002). The DNA-binding domains of the otherwise distantly related TraR, NarL and GerE proteins superimpose extremely well with very little deviation.

There are two dimerization interfaces in the TraR-3-oxo-C8 HSL dimer (figure 3a). The most extensive of these is composed of a long α-helix in the N-terminal domain of each subunit that is parallel with the same helix of the opposite subunit (Vannini et al. 2002; Zhang et al. 2002b). A number of residues buried at this interface appear to contribute to dimer formation, a finding confirmed by mutational analysis (Luo et al. 2003). A less extensive dimerization interface occurs between the C-terminal helices of the DNA-binding domain of each monomer. The N- and C-terminal domains of each monomer are connected by a 12-residue unstructured linker. Owing to this flexible linker and the fact that the dimerization interface is not continuous between the two monomers, there is extensive flexibility of the N-terminal domains relative to the C-terminal domains of the dimer. This flexibility resulted in a pronounced asymmetry in the crystal structure. The C-terminal domains of each dimer have a twofold axis of symmetry, and the N-terminal domains also have an axis of twofold symmetry, but these two axes actually lie at a 90° angle to each other (figure 3b). This flexibility has been proposed to play a role at divergently transcribed promoters (described in §5), although this remains to be determined.

In both of the structures the protein was crystallized with the 18 bp consensus tra box, which has a perfect dyad symmetry and is the strongest binding site for TraR (Vannini et al. 2002; Zhang et al. 2002b). The binding site is a regular B-form DNA, but with a smooth 30° bend towards the protein. Although this bend is modest when compared with that of other transcription factors, it still results in an increase in the buried surface area between the protein and the DNA (Jones et al. 1999). The protein–DNA interface is quite extensive, however, the only sequence-specific contacts are mediated by three amino acid residues of the recognition helix (the second helix of the HTH) and the edges of only four bases in the major groove of each tra box half site (figure 4; Vannini et al. 2002; Zhang et al. 2002b). However, many non-specific contacts between TraR and the DNA backbone also occur on either side of the major groove of each half site, and a number of these individual interactions are critical for binding affinity (White & Winans in press). At the centre of the tra box, the minor groove (separating the major groove of each half site) faces the protein and is not contacted by TraR. However, the bases of this ‘central spacer’ are still important for the protein–DNA interactions, most probably through their effects on DNA structure and flexibility (White & Winans in press).

There is significant interest in understanding the specificity of the interactions between LuxR-type receptors and their cognate AHLs, and how AHL binding converts these receptors from inactive to active forms. As described above, 3-oxo-C8 HSL is required for TraR stability against proteolysis (Zhu & Winans 2001). In addition it has been shown that when TraR is expressed at native levels in vivo, it binds to 3-oxo-C8 HSL with very high specificity (Zhu et al. 1998). In other words, TraR is able to distinguish its cognate ligand from a variety of other AHLs, even those that have very similar structures to 3-oxo-C8 HSL. The polar contacts in the binding pocket include four hydrogen bonds between the residues of TraR and the polar groups of 3-oxo-C8 HSL (figure 5). Two mutational studies of TraR confirmed that these hydrogen bonds are critical to 3-oxo-C8 HSL binding and stabilization of the protein (Luo et al. 2003; Chai & Winans 2004). One of these studies
further considered the role of the 3-oxo group (which is not common to all AHLs) in specificity of binding (Chai & Winans 2004). This group makes a water-mediated hydrogen bond to a threonine residue in the binding pocket of TraR. Point mutations of the threonine residue were constructed in an attempt to increase hydrophobicity, which was predicted to exclude the water molecule from the binding pocket and therefore alter specificity for a 3-unsubstituted AHL (C8-acyl homoserine lactone). These mutations did result in an observable effect, although this was to broaden rather than alter the specificity of binding. In the same study, point mutations were also constructed in an attempt to change the preference of binding to AHLs with fatty acid tails shorter than eight carbons, by increasing the hydrophobic bulk in the binding pocket. A number of these mutations did result in altered specificity, however the stability of these mutants was also decreased. Although strongly altered specificity mutants were not identified in this study, the results predicted that the residues of the binding pocket play dual roles in ligand binding and stability or folding of the N-terminal domain. This really comes as no surprise, as these residues are buried in the hydrophobic core of the protein and therefore must be important for protein folding and stabilization.

(c) The anti-activators: TvlR and TraM

Previously, we introduced TraM as an inhibitor of TraR activity (Fuqua et al. 1995; Hwang et al. 1995). A number of studies have been published on the activity of TraM, including two crystal structures of the protein (Chen et al. 2004; Vannini et al. 2004). Prior to the structural studies, TraM had been demonstrated using yeast two-hybrid assays and far western immunoblots to interact directly with TraR (Hwang et al. 1999). The same study used deletion and point mutations to show that the protein–protein interactions occurred at the C-terminal regions of both proteins. In an in vitro system with purified TraR and histidine-tagged TraM, it was shown that TraM can both block binding of TraR to tra box DNA and also disrupt preformed TraR–DNA complexes (Luo et al. 2000). These data led to the suggestion that TraM binds directly to the DNA-binding surface of TraR, making it inaccessible to DNA. In support of this argument, TraR–TraM complexes form with high affinity (in the nanomolar range) and are quite stable (Swiderska et al. 2001).
However, the ratio of TraR monomers to TraM monomers in TraR–TraM complexes was not so clear. Two different studies both used gel filtration chromatography to determine the mass of TraR–TraM complexes. The results from one study (Chen et al. 2004) suggested that one TraR monomer binds to one or two TraM monomers, while the results of the other study (Vannini et al. 2004) were consistent with two TraR dimers binding to two TraM dimers. These discrepancies must be due to differences in the experimental conditions. Another intriguing addition to the possible mechanism of TraR inhibition by TraM is that binding and inactivation may occur in sequential steps, as determined from studies using several TraM point mutants (Swiderska et al. 2002b).

In both of the structural studies published, TraM was crystallized in the absence of TraR (Chen et al. 2004; Vannini et al. 2004). TraM crystallized as a dimer, with each subunit consisting largely of two antiparallel α-helices. A significant hydrophobic interface is buried at the interface between the two subunits. This extensive dimerization interface had also been confirmed in a study using deletion mutants of TraM (Qin et al. 2004b). The two groups that crystallized TraM published different models of how TraR–TraM interactions might disrupt TraR–DNA interactions. One model suggests that TraR and TraM homodimers may dissociate upon binding to each other so that the TraR–TraM anti-activation complex can form (Chen et al. 2004). This is consistent with mutational studies that showed that residues of TraM that are important for initial binding to TraR are different from those required for TraR inactivation (Swiderska et al. 2001). Furthermore, when the two subunits of TraM dimers are covalently cross-linked to each other, the altered dimers are still able to bind TraR but are not able to disrupt DNA binding (Chen et al. 2004). A different model proposed that two dimers of TraM bind to two dimers of TraR, such that the TraM dimers are ‘clamped’ between the TraR dimers (Vannini et al. 2004). This complex would be expected to physically inhibit TraR dimers from binding to tra box DNA. Structural studies of TraR–TraM complexes may resolve the issue of how TraM inactivates TraR. However, static structures are not expected to provide a complete picture, as TraM binding to TraR and inactivation of TraR are expected to occur in sequential steps rather than simultaneously.

The other anti-activator of TraR activity, TrlR, has a much less complicated mechanism of TraR inhibition. The trlR gene is thought to have originated from a gene duplication event of traR itself (Oger et al. 1998). This gene is virtually identical to traR except for a single frame-shift difference upstream of the C-terminal DNA-binding domain. Although TrlR cannot activate tra genes, correction of the frame-shift mutation by site-directed mutagenesis results in an active protein (Zhu & Winans 1998). The similarity of TrlR to the TraR N-terminal domain led to the suggestion that TrlR inhibits TraR activity directly through formation of inactive heterodimers (which would have only one functional DNA-binding domain rather than two). Like TraR, TrlR requires 3-oxo-C8 HSL for solubility, and inactive TrlR–TraR heterodimers were shown to form in vitro using purified proteins (Chai et al. 2001). However, genes identical to trlR have been identified on a number of octopine-type Ti plasmids, indicating that the truncation is not a laboratory artefact (Zhu & Winans 1998).

**4 Regulation through signal depletion:**

3-oxo-C8 HSL turnover

In addition to inhibition of TraR activity through TraM and TrlR, the products of the attKLM operon are also thought to inhibit the quorum-sensing system, although through an entirely different mechanism. The attKLM genes are located on the ‘cryptic’ plasmid pAtC58 and are the only known genes that are involved in the quorum-sensing system that are not on the Ti plasmid (Goodner et al. 2001; Wood et al. 2001). Interest in attKLM stemmed from a recent study in which the AttM-like protein AiiA of Bacillus cereus was shown to hydrolyze the lactone ring of AHLs (Dong et al. 2001). This lactonase activity results in the formation of N-acyl homoserines, which are inactive as signal molecules. AttM of *A. tumefaciens* was shown by two different groups to have the same lactonase activity on AHLs (Zhang et al. 2002a; Carlier et al. 2003).

The attKLM operon is repressed by the product of the divergently transcribed attJ gene. In an attJ null mutant, 3-oxo-C8 HSL did not accumulate to appreciable levels (Zhang et al. 2002a). However, it was not clear whether AttM was selected for this role. In one study, the attKLM operon in strain A6 was induced only at high population densities, and a later study by the same group suggested that carbon or nitrogen starvation and the ppGpp stress response are involved in attKLM regulation (Zhang et al. 2002a, 2004). However, in studies with *A. tumefaciens* strain C58,
a population-density effect on attKLM induction was not detected (Carlier et al. 2004; Y. Chai & S. C. Winans 2005, unpublished data). The difference between these studies could be due to a difference in the strains used.

As attM had been shown to be important for 3-oxo-C8 HSL depletion (Zhang et al. 2002a), a number of different groups tested AHLs for activation of the attKLM operon, expecting that these compounds might inactivate AttJ (Zhang et al. 2002a; Carlier et al. 2004; Chai et al. in press). However, none of the AHLs that were tested had any effect on attKLM induction. This finding suggested that perhaps AHLs are not the natural substrates of AttKLM.

In a recent study, a number of different γ-butyrobetonic acids (GBLs) were tested as possible AttKLM substrates, as AttM was known to be a γ-butyrobetonic acid hydrolase (Carlier et al. 2004). Only one of the compounds tested, GBL, could be used as the sole carbon source for growth of A. tumefaciens strain C58, and this growth was dependent on a functional attKLM operon. The predicted intermediates of GBL degradation via AttKLM were γ-hydroxybutyrate (GHB), from activity of AttM in opening the lactone ring, and succinate semialdehyde (SSA) and succinic acid (an intermediate of the tricarboxylic acid cycle) from activities of AttL and AttK, respectively (figure 6). A wild-type strain of A. tumefaciens C58 was able to grow on both GBL and GHB as the sole carbon source, while the attKLM null mutant could not (Carlier et al. 2004). In the same study, it was also reported that GBL and the intermediates GHB and SSA were all efficient inducers of an attC: lacZ fusion in A. tumefaciens. Growth of the wild-type C58 strain on a medium supplemented with the attKLM inducers (GBL, GHB or SSA) inhibited accumulation of 3-oxo-C8 HSL, while growth without these inducers did not. A direct and negative effect of GBL on the affinity of purified AttJ for attKLM promoter DNA has also been demonstrated recently (Chai et al. in press). Furthermore, in vitro experiments with purified proteins and defined substrates confirmed the predicted pathway of GBL degradation via AttKLM as described previously, and also demonstrated that the 3-oxo-C8 HSL degradation pathway is probably similar (figure 6; Chai et al. in press). These experiments strongly suggest that GBL is the substrate for which this catabolic operon was selected.

The adaptive significance of this pathway to A. tumefaciens is not clear. It is possible that A. tumefaciens may be using GBL or very similar compounds released by other soil bacteria as nutrients. If this were the case, then AttKLM could degrade AHLs produced by other nearby soil bacteria, but this would not affect the quorum-sensing system of A. tumefaciens itself, as traR is induced only in the presence of opines in or near infected plant tissue. On the other hand, it has been reported that GBL can be detected in at least some plants, and that the AttK and AttM proteins accumulate when A. tumefaciens is exposed to roots of tomato (Lee & Shibamoto 2000; Rosen et al. 2003). Further studies to assess the role of ‘quorum-quenching’ in the TraR-TraI system during plant colonization and growth near crown gall tumours will be quite interesting.

5. TraR AS AN ACTIVATOR OF TRANSCRIPTION

As mentioned previously, all of the known TraR-regulated genes occur only on the Ti plasmids. These include the tra and trb genes, which are involved in conjugation, the rep genes, which are required for vegetative replication and plasmid partitioning into daughter cells, and traM. The operon structure, promoters and tra boxes for activation of these genes have all been described on the octopine-type Ti plasmid (figure 7). The tra genes, required for DNA transfer and replication, are arranged in two operons: traAFBH and traCDG-yci (Fuqua & Winans 1996a). These operons are divergently transcribed from each other, and the origin of replication is predicted to be in the intergenic region between them. The trb genes of the traI-trb operon are involved in mating pair formation and the type IV secretion system for DNA transfer (Fuqua & Winans 1996a). Note that the 3-oxo-C8 HSL synthase, traI, is the first gene of this operon. The activation of traI expression by TraR-3-oxo-C8 HSL complexes represents a positive feedback loop, as has been described for a number of other LuxR-LuxI-type systems (Whitehead et al. 2001). The traI-trb operon is divergently transcribed from the repABC operon (Fuqua & Winans 1996a; Pappas & Winans 2003a; C. Fuqua 2005, personal communication). Both tra operons are divergently transcribed from one tra box, called tra box I (which is also the consensus sequence). At both of these promoters the tra box is centred approximately 45 nucleotides upstream from the transcription start site and overlaps the −35 element of each promoter. This type of promoter structure, with...
At both the transcrip-
tion start site, but on the
same face of the DNA as
RNAP (Busby & Ebright
1999). At both the
traM promoter, activated from
tra box IV, and the
repAP2, activated from tra box II, the tra box is centred at –66
nucleotides from the transcription start
site. The tra boxes themselves are highly conserved.

The affinities of TraR–3-oxo-C8 HSL complexes for tra boxes I, II and III are highly similar and in the nanomolar range (Zhu & Winans 1999; Pappas & Winans 2003a). The tra box IV is the least conserved of all four tra boxes, perhaps explaining the observation that the traM promoter is expressed more weakly than any other (White & Winans 2005; C. Fuqua 2005, personal communication).

The promoter architecture of repABC is rather complex, and includes an additional promoter that is not TraR-dependent, ensuring that plasmid maintenance and partitioning still occur when TraR is inactive (Pappas & Winans 2003b). This promoter (repAP4) is the closest of all four to rep, and is negatively regulated by RepA and RepB (Pappas & Winans 2003b; Chai & Winans 2005b). These two proteins are involved in plasmid partitioning, but a recent study has shown that they also form a nucleoprotein complex that auto-represses P4 (Chai & Winans 2005b). RepA binds to
DNA at P4, RepB binds to DNA between the repA and
repB genes, and contacts also form between RepA and
RepB, possibly resulting in the formation of a repression
loop (Pappas & Winans 2003b; Chai & Winans 2005b). In addition, a small antisense RNA (repE, encoded between repB and repC) attenuates the expression of repC, most likely through forming a complex with the rep transcript to terminate transcription directly or by blocking translation (Chai & Winans 2005a). RepC is required for plasmid replication initiation (Ramirez-Romero et al. 1997).

Surprisingly, it was recently noticed in expression
studies using microarrays that the repABC operon is also induced in the presence of acetosyringone (AS; Cho & Winans 2005b). This compound is a plant-released phenolic that activates the vir genes via the VirA–VirG two-component system. AS-induced rep expression required activated VirG. The activation of rep via phospho-VirG enhances Ti plasmid copy number approximately fourfold (Cho & Winans 2005b), while activation via TraR–3-oxo-C8 HSL increases the copy number approximately eightfold (Pappas & Winans 2003a). A positive effect of TraR–3-oxo-C8 HSL complexes on rep transcription and Ti plasmid copy number in nopaline-type Ti plasmids has also been documented (Li & Farrand 2000). In fact, the genetic linkage of rep and traI–trb is strongly conserved in Ti plasmids, and even in some plasmids of *Rhizobium* sp. (Li & Farrand 2000; Zhu et al. 2000). Furthermore, all Ti plasmids studied have identifiable tra boxes in the intergenic region between traI–trb and rep. Therefore, it is expected that activation of Ti plasmid replication through the quorum-sensing system is a conserved feature.

The activation at TraR-dependent promoters on the octopine-type Ti plasmid requires only promoter DNA, TraR–3-oxo-C8 HSL complexes and σ70–RNAP

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Therefore, TraR is expected to make direct contacts with RNAP, and these contacts should serve to recruit polymerase to the promoter. At class II promoters, where an activator overlaps the –35 element, the activator is positioned to make multiple contacts with RNAP (Busby & Ebright 1999). However, at class I promoters, transcription activators are thought to recruit RNAP to the promoter solely through contacts with RNAP (Busby & Ebright 1999). However, class II promoters, where an activator overlaps the –35 site for the CTD of RNAP, based on its importance for activation at both class I and class II promoters.

It is unlikely that TraR would have more than one activating region that contacts the CTD of the α-subunit. However, due to the high degree of flexibility of the N-terminal domains of the TraR dimer relative to the C-terminal domains, it is possible that the two critical residues of the N-terminal domain can approach the activating region identified on the C-terminal domain. Both sets of residues could together form a single contact site for the αCTD of RNAP. This potential and novel activating region, formed from two distinct sets of residues from different regions of TraR, could be important in the activity of TraR at divergent promoters, which are common on the Ti plasmid as described previously. However, further studies are required to completely understand TraR–RNAP interactions at TraR-dependent promoters.

6. PERSPECTIVES AND FUTURE STUDIES

In this review we have discussed the regulation of the quorum-sensing system in A. tumefaciens. Although many advances have been made in the biochemical and structural biology of TraI, TraR, the anti-activators TraM and TrlR, and lactonase turnover by AttKLM, many questions still remain. The mechanism of TraR inactivation by TraM is not clearly understood, but may be clarified with future structural studies of TraR–TraM complexes. Future biophysical studies that focus on the dynamics of TraR–3-oxo-C8 HSL interactions will also be fascinating and lend understanding to the role that 3-oxo-C8 HSL plays in TraR stability, and possibly even in protein folding. These studies could also reveal why some AHLs and AHL analogues inhibit TraR activity (Zhu et al. 1998). Perhaps inhibitors are able to bind in the ligand pocket but do not induce the correct folding pathway to stabilize the protein against proteolysis. Structure determination of additional LuxR-type proteins will also allow for interesting comparative studies to understand the determinants of AHL specificity.

The recent studies described previously on AttKLM also prompt interesting questions about the substrates for which these enzymes were selected and about the significance of quorum-quenching by this pathway. 3-Oxo-C8 HSL degradation by AttKLM is ecologically important only if γ-butyrolactone and 3-oxo-C8 HSL are present at the same place and time. Only studies of the activity of both of these systems during plant colonization and pathogenesis will resolve these questions.

In this review, we have focussed on the molecular biology of the quorum-sensing system, but the adaptive significance of this system and the many regulators that are involved are not well understood. The quorum-sensing system of A. tumefaciens induces both vegetative replication and conjugal transfer of the Ti plasmid, but only in the presence of opines. This link between Ti plasmid replication, transfer and pathogenesis suggests that perhaps the role of TraR is to increase the copy number of the Ti plasmid in a population of plant-colonizing A. tumefaciens. Increased copy number could enhance both virulence and opine consumption, through a gene dosage effect. The recent evidence that the rep genes are also activated by phospho-VirG in response to ALS further supports this argument.

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