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TtsI regulates symbiotic genes in *Rhizobium* species NGR234 by binding to *tts* boxes

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Summary

Infection of legumes by *Rhizobium* sp. NGR234 and subsequent development of nitrogen-fixing nodules are dependent on the coordinated actions of Nod factors, proteins secreted by a type III secretion system (T3SS) and modifications to surface polysaccharides. The production of these signal molecules is dependent on plant flavonoids which trigger a regulatory cascade controlled by the transcriptional activators NodD1, NodD2, SyrM2 and TtsI. TtsI is known to control the genes responsible for T3SS function and synthesis of a symbiotically important rhamnose-rich lipo-polysaccharide, most probably by binding to *cis* elements termed *tts* boxes. Eleven *tts* boxes were identified in the promoter regions of target genes on the symbiotic plasmid of NGR234. Expression profiles of *lacZ* fusions to these *tts* boxes showed that they are part of a TtsI-dependent regulon induced by plant-derived flavonoids. TtsI was purified and demonstrated to bind directly to two of these *tts* boxes. DNase I footprinting revealed that TtsI occupied not only the *tts* box consensus sequence, but also upstream and downstream regions in a concentration-dependent manner. Highly conserved bases of the consensus *tts* box were mutated and, although TtsI binding was still observed *in vitro*, *gfp* fusions were no longer transcribed *in vivo*. Random mutagenesis of a *tts* box-containing promoter revealed more nucleotides critical for transcriptional activity outside of the consensus.

Introduction

Symbioses between legumes and rhizobia which result in the formation of nitrogen-fixing root nodules are the result of a complex signal exchange between both partners. Initially, flavonoids exuded by the plant trigger synthesis of Nod factors (NF) that are secreted from the bacteria and are critical for rhizobial infection (Broughton *et al*., 2000; Perret *et al*., 2000). Nevertheless, establishment of functional nitrogen-fixing nodules requires other bacterial signals such as surface polysaccharides and secreted proteins (Fraysse *et al*., 2003; Broughton *et al*., 2006; Soto *et al*., 2006; Jones *et al*., 2007). *Rhizobium* sp. NGR234 (hereafter NGR234) is the most

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promiscuous known microsymbiont, capable of establishing symbioses with more than 112 genera of legumes (Pueppke and Broughton, 1999). Its genome is partitioned into three replicons: the chromosome and two large plasmids, pNGR234*a* and pNGR234*b* (Viprey *et al*., 2000). pNGR234*a* is also called the symbiotic plasmid as it contains all genes necessary for NF synthesis and nitrogen fixation. Sequencing of this 536-kb plasmid showed that it also contains orthologues of a type III protein secretion system (T3SS) (Freiberg *et al*., 1997). Until this discovery, such secretion systems were thought to be characteristic of pathogenic bacteria, where they play important roles in host infection. T3SS form an apparatus that injects bacterial proteins directly into eukaryotic cells to disrupt normal functioning of the cell, facilitating infection (Hueck, 1998). The T3SS of NGR234 is capable of secreting Nops (nodulation outer proteins), and is an important determinant of host range (Viprey *et al*., 1998).

Production of NF requires flavonoids and the LysR-type regulator NodD1, which binds to conserved motifs (termed *nod* boxes) found in the promoter regions of genes/operons responsible for NF synthesis. Nop secretion also requires flavonoids, NodD1 and another regulatory protein TtsI (Viprey *et al*., 1998; Marie *et al*., 2004). A *nod* box is located in the promoter region of *ttsI*, and NodD1 is thought to activate TtsI which in turn initiates transcription of T3SS genes (Kobayashi *et al*., 2004). TtsI shares characteristics with the DNA-binding response regulators of two-component regulatory systems (Viprey *et al*., 1998; Marie *et al*., 2004). Usually, such regulators are activated by their partner sensor, histidine protein kinases, which auto-phosphorylate at a histidine residue upon sensing an environmental signal. The phosphoryl group is subsequently transferred to an aspartate residue in the response regulator, inducing a conformational change that leads to its activation. Once phosphorylated, response regulators act as transcriptional activators by binding to *cis* elements in the promoters of genes required to process the initial environmental signal. TtsI, however, has a glutamate residue instead of the conserved aspartate. In other bacterial response regulators, such a substitution leads to constitutive activation, bypassing the requirement for the sensor kinase partner. It is thus possible that TtsI functions as a transcriptional activator independent of phosphorylation and a sensor kinase partner. Instead, transcription of *ttsI* and therefore function (s) regulated by TtsI, are modulated by NodD1 in a flavonoid-dependent manner (Kobayashi *et al*., 2004; Marie *et al*., 2004).

Sequencing numerous rhizobial genomes has revealed that T3SS and TtsI control are relatively common. A comparison of the promoter regions of T3SS genes from several rhizobia identified a putative *cis*-regulatory element termed a *tts* box (TB). TtsI is thought to bind TB and stimulate transcription of downstream genes (Krause *et al*., 2002). Using this consensus sequence, 11 putative TBs (TB1–TB11) were identified on pNGR234*a* (Marie *et al*., 2004). Five of the TBs are located upstream of genes/operons involved in the assembly of the type III secretion machine; others precede genes encoding possible secreted proteins. TBs are also found in the promoters of genes encoding proteins not directly related to T3SS functions (Fig S1). Thus TtsI potentially regulates more than the T3SS, and indeed mutation of *ttsI* leads to different symbiotic phenotypes compared to a T3SS mutant alone (Viprey *et al*., 1998). As well as being impaired in protein secretion, the *ttsI* mutant failed to produce a rhamnose-rich lipo-polysaccharide (LPS) known to be important for successful nodulation (Marie *et al*., 2004; Reuhs *et al*., 2005; Broughton *et al*., 2006). A flavonoid-inducible operon encoding enzymes responsible for rhamnose synthesis was shown to require TtsI for activation and to contain a TB in its promoter region. Evidence that the TB is essential for the activity of this operon was obtained by deleting a small region containing the TB which abolished TtsI-mediated induction (Marie *et al*., 2004).

In this work, we determined whether the promoter regions containing the 11 predicted TB are inducible in a TtsI- and flavonoid-dependent manner. We then tested if TtsI could physically bind to TB-containing promoters and mapped the actual binding site *in vitro* by DNaseI

footprinting. We also mutated key residues in the TB consensus sequence and randomly mutated a TB-containing promoter to identify further important residues. It seems likely that the TtsI/TB regulatory system is a basic feature of rhizobial T3SS as *Bradyrhizobium japonicum* USDA110, *Mesorhizobium loti* MAFF303099 and *Sinorhizobium fredii* USDA257 all possess T3SS, TtsI homologues, as well as predicted TB sequences (Krause *et al*., 2002; Krishnan *et al*., 2003; Hubber *et al*., 2004). Thus our findings are applicable to multiple genera of rhizobia.

Results

NGRΔ*ttsI*, a non-polar deletion mutant of *ttsI*

Polar mutation of *ttsI* (NGRΩ*ttsI*) demonstrated that TtsI is required for Nop secretion and synthesis of a rhamnose-rich polysaccharide, as well as the transcriptional activation of two of the 11 TBs, TB2 and TB8. Although introduction of a plasmid-born copy of *ttsI* into NGRΩ*ttsI* allowed complementation of rhamnan synthesis, it failed to restore Nop secretion (Viprey *et al*., 1998; Marie *et al*., 2004). As *ttsI*, *rhcC*2 and y4xK are predicted to form an operon (Perret *et al*., 2003), the insertion of an Ω cassette into *ttsI* most probably blocked transcription of these downstream genes. In other bacteria, *rhcC*2 encodes an essential component of the T3SS: its addition *in trans* (possibly y4xK as well) would thus be required for complementation of Nop secretion by NGRΩ*ttsI*. Additional regulatory controls of T3SS gene expression have been shown in some bacteria: if the machinery fails to assemble correctly (because of mutation of a key gene for example), then expression of genes encoding secreted proteins is suppressed (Wei *et al*., 2000). It is thus possible that the block in TB expression in NGRΩ*ttsI* is due to the absence of RhcC2, causing the T3SS assembly machine to fail. In this case, TB expression was probably suppressed by another regulator. For this reason, we constructed a non-polar deletion mutant of *ttsI* (NGRΔ*ttsI*) to maintain transcription of *rhcC*2 and y4xK from *nod* box 18 and thus avoid this possibility (Fig. 1). To characterize the new mutant, secreted proteins and surface polysaccharides were isolated from NGRΔ*ttsI* and NGR234. Nop secretion and *de novo* synthesis of rhamnose-rich LPS were blocked in NGRΔ*ttsI* (Fig. S2), consistent with our previous observations of the polar mutant NGRΩ*ttsI* (Marie *et al*., 2004). To complement NGRΔ*ttsI*, *ttsI* with its own promoter region was subcloned into pRG960 (Van den Eede *et al*., 1992), giving rise to p*ttsI*-2. Introduction of p*ttsI*-2 into NGRΔ*ttsI* restored both Nop secretion and production of rhamnose-rich LPS. Thus NGRΔ*ttsI* does not appear to dramatically affect transcription of *rhcC*2 (or y4xK) and this mutant was used in all subsequent work.

Promoter activities of TB-containing loci

To examine the function of all TBs, we subcloned the 11 predicted TBs into pMP220, a broad-host-range transcriptional-*lacZ* reporter system (Spaink *et al*., 1987), thus creating pMP-TB1 to pMP-TB11 (see Table 1). To assess the flavonoid and TtsI dependence on transcription of the TB, tri-parental matings were used to introduce each of the constructs into NGR234 and NGRΔ*ttsI*. Liquid cultures were grown to an OD_{600} of 0.1 in RMS and the flavonoid daidzein added at 2×10^{-7} M. At 1, 6 and 24 h post induction (hpi), β-galactosidase activities were monitored in transconjugants of NGR234 (red bars in Fig. 2) or at 1 and 24 hpi for NGRΔ*ttsI* transconjugants (pink bars in Fig. 2). In the absence of an inducer, and with the exception of pMP-TB5, only low promoter activities were observed with the different constructs (open bars in Fig. 2), but addition of daidzein caused significant increases in β-galactosidase activities 24 hpi (Fig. 2). At 24 hpi, TB8, which controls expression of the *nopBrhcJnolUVrhcNy4yJrhcQRSTU* operon, is the strongest promoter (2500 ± 130 Miller units), whereas the lowest activity was recorded with TB11 (157 ± 5 units). Although expression of TB11 appears to be low, it represents a sixfold induction over that found in the NGRΔ*ttsI* mutant (Fig. 2K). By mobilizing the different TB–*lacZ* fusions into the mutant strain

NGRΔ*ttsI*, the role of TtsI in the activation of each individual TB was assessed. All TBs lost flavonoid inducibility in NGRΔ*ttsI* (Fig. 2). Introduction of p*ttsI*-2 into four randomly selected NGRΔ*ttsI* (pMP-TB2, TB4, TB8 and TB10) transconjugants restored flavonoid-dependent induction (closed bars, Fig. S3).

TtsI binds to TB2 and TB8

TtsI contains an N-terminal conserved receiver domain and a C-terminal helix-turn-helix (HTH) domain, both typical of response regulators of two-component systems. The HTH domain usually interacts directly with a DNA binding site present in the promoters of genes controlled by these regulators. Although the HTH structure is highly conserved, it has the ability to recognize specific bases, and thus discriminate small modifications in a binding site (Stock *et al*., 2000). TtsI of NGR234 and other rhizobia has been shown to control the expression of several genes containing a TB in the promoter region, but direct contact had never been shown. To further characterize the binding of TtsI to the TB-containing promoter regions, we cloned the NGR234 *ttsI* coding sequence into the pETBlue2 vector, then overexpressed and purified a His-tagged TtsI protein. High levels of expression were obtained, but more than 50% of the TtsI-His was insoluble, which was also observed with other members of this family (Kumagai *et al*., 2006). To test the ability of TtsI-His to bind to TB sequences, we initially performed electrophoretic mobility shift assays using a 150-bp region of the *nopB* promoter that contains TB8. When increasing amounts of TtsI-His were added to the reaction, slower migrating bands were observed, consistent with DNA–protein interactions (Fig. 3). The slowest band appeared only at increasing concentrations of TtsI, which could be a result of oligomerization of TtsI-His upon binding to DNA, or the presence of more than one TB at different locations in the DNA. Inspection of the sequence did not reveal any other candidate region, suggesting that TtsI forms oligomers upon binding TB8. Addition of up to 50 μg ml^{-1} poly(dI-dC) as heterologous competitor DNA did not disrupt the DNA–protein complexes, suggesting that binding of TtsI to TB8 is specific (Fig. S4). This specificity was confirmed using a double-stranded oligonucleotide specific to TB8 which disrupted TtsI–*nopB* promoter complexes (data not shown). A second double-stranded oligonucleotide composed of the adjacent sequence upstream of TB8 (originally designed as a control) also disrupted the complexes, however. This unexpected effect suggested that the TtsI binding region extends outside of the TB consensus sequence and, for this reason, we mapped exactly where TtsI bound to the *nopB* promoter.

The identification of the precise interaction site of TtsI with the *nopB* promoter was determined by DNase I cleavage protection (footprinting) assays in which evidence of protein binding is seen as modifications of the cleavage pattern that result in both decreases and increases in the intensity of cleaved fragments. When the polymerase chain reaction (PCR) fragment containing the TB8 site used in the mobility shift assays described above was labelled and used as the probe, it clearly interacted with TtsI-His as revealed by the presence of protected and hyperreactive bands in the consensus sequence (Fig. 4A). Increasing concentrations of TtsI produced a clearer pattern of interaction, but did not reveal any new interaction sites. Furthermore, TtsI caused a modification to the cleavage pattern of bases located upstream and downstream of the TB, suggesting that it occupies a broad section of the target DNA (Fig. 4A). This result is consistent with oligomerization of TtsI upon binding to DNA, as suggested by the mobility shift assays. The hyperreactive bands observed are an indication that TtsI induces major distortions in the bound DNA, exposing these sites to DNase I cleavage.

To test whether such a broad footprint is specific to TB8, a TB2-containing portion of the *rmlB* promoter region was also used as a template in identical experiments. As seen with TB8, modified bases were also observed within and outside of the TB, and increasing the quantity of TtsI in the reactions sharpened the footprint (Fig. 4B). Thus TtsI has a broader than predicted

binding site on both promoter regions. Bases modified by TtsI binding in both TBs were mapped (Fig. 4C and D), but it was not possible to detect any trend of base cleavage modification which could pinpoint bases important for binding outside both TBs. Nevertheless, it is evident from the protection and hyper-reactivity of the highly conserved bases of the TB that they are clearly involved in direct interaction with TtsI. It should be noted that the modified cleavage pattern induced by TtsI towards the 5′ and 3′ ends of the templates may not necessarily indicate direct contact of these sequences with the protein, but could represent distortion of the DNA caused by the binding of the activator with the TB.

Site-directed mutagenesis of a TB

Specific point mutations were introduced to three highly conserved bases of the TB8 consensus sequence (specifically GTCAG to GAATG), and the ability of TtsI to bind and activate transcription was tested *in vitro* and *in vivo*. Mobility shift assays showed that TtsI binds *in vitro* to both wild-type and mutant TBs at similar concentrations (Fig. 5). The ability of the mutated promoter to activate transcription of a reporter gene was then tested using the green fluorescent protein (GFP) to facilitate reporter assays. First, the non-mutated TB8 promoter was sub-cloned upstream of a promoter-less *gfp* gene in plasmid pPROBE-GT′ (Miller *et al*., 2000) and then introduced into NGR234 as well as NGRΔ*ttsI* by tri-parental matings. Cultures were induced with flavonoids for 40 h and GFP production measured in terms of cellular fluorescence. As expected, a strong GFP signal was observed in NGR234 containing the TB8–*gfp* fusion, whereas no expression was observed from NGRΔ*ttsI* (Fig. 6). The mutated TB8 promoter was also subcloned upstream of *gfp* in plasmid pPROBE-GT (Miller *et al*., 2000). When the activity of this plasmid was assayed in NGR234 (under the same conditions of induction), no GFP signal was observed (Fig. 6). To answer the question of why the mutated TB8 promoter was inactive *in vivo* but apparently unaffected in its binding *in vitro* led us to test the ability of TtsI to bind to the GAATG TB8 by the more sensitive assay of DNase I footprinting. Using the same experimental conditions described earlier, differences between TB8 and GAATG TB8 were found, especially near the mutated bases, but also elsewhere in the TB8 consensus (Fig. 7). Thus although the GAATG TB8 mutation is clearly insufficient to block TtsI binding, it alters the nature of the binding which in turn may well lead to the inability of GAATG TB8 to be transcribed, as observed *in vivo*.

Random mutagenesis of a *TB*

As TtsI bound to regions of TB-containing promoters outside of the TB, we used a random mutagenesis approach to identify other potential bases important for transcriptional control by TtsI. Error-prone PCR was performed on the *nopB* promoter, the products cloned into pGEM-T, and then sequenced. Because of the mutagenic conditions used, several bases were often changed in each mutant. A number of these PCR products were selected according to the location of the mutations and subcloned upstream of the promoter-less *gfp* gene and mobilized into NGR234. Most of the mutants analysed had no activity, some had partial activity when induced by apigenin, but none had constitutive activity in the absence of flavonoid (Fig. 8). Three mutants had no mutations within the TB (A62, A117 and A74) and had zero, 6% and 18% of the wild-type TB8 activity, suggesting that either the consensus sequence is longer than predicted (in agreement with the large footprint), or the mutated bases altered binding of other components of the transcriptional machinery. Interestingly, one mutant exhibited higher activities than the wild type upon induction. Attempts to delimit the *nopB* promoter by footprinting assays using the holoenzyme *Escherichia coli* RNA polymerase were not successful, possibly as a result of differences in the housekeeping sigma factors of both species. However, the *nopB* promoter region of *S. fredii* USDA257 is identical to NGR234, and the exact position of the start of transcription was mapped to a cytidine residue located at the −30 position from the ATG (Kovács *et al*., 1995). Inspection of the upstream sequence using the proposed consensus sequence for rhizobial promoters (MacLellan *et al*., 2006) allowed the

identification of a compatible −10 RNA polymerase binding site (Fig. 9). Although the −10 sequence is fairly well conserved (four out of six bases), the −35 region does not resemble the consensus, and indeed overlaps the highly conserved GTCAG bases of the TB. The majority of inactive mutants had mutations either in the TB or the putative −10 site (Fig. 9). On the other hand, the three mutants that remained active (B1127) or partially active (A2D andA3G) did not have mutations in the TB consensus sequence or the putative −10 promoter. Interestingly, the A518 mutant possessed four mutations, none of which mapped to either of binding sites, but it still exhibited very low transcriptional activity. This indicates that bases outside of the TB are important for *nopB* transcription, presumably by modulating binding of TtsI.

Discussion

Phytopathogenic bacteria use T3SS to facilitate infection or conversely to trigger plant defences (Mudgett, 2005; Grant *et al*., 2006). Many different effector proteins can be secreted by such T3SS and they have been catalogued based upon homology searches or by assessing the potential for co-regulation with other T3SS genes (Cunnac *et al*., 2004; Lindeberg *et al*., 2006). Specific plant compounds that induce expression of T3SS genes are not known and, as a consequence, the proteins used as sensors by phytopathogens have not been identified. Nevertheless, many players in the intermediate stages have been identified through mutagenesis and two broad classes of gene regulation are now recognized (Mole *et al*., 2006; Tang *et al*., 2006): in group I (represented by *Erwinia* spp., *Pantoea* spp. and *Pseudomonas syringae*), the activator is a sigma factor, termed HrpL which is thought to bind directly to a *cis* element (*hrp* boxes) in promoter regions of T3SS genes. In group II, the direct activators are members of the AraC family of transcriptional regulators, called HrpX in *Xanthomonas* spp. and HrpB in *Ralstonia solanacearum*, both of which are proposed to bind to *cis* elements in the promoter regions of T3SS genes. HrpL and AraC-type transcriptional activators are encoded by genes in the T3SS loci.

In contrast, flavonoids trigger induction of T3SS (along with other symbiotic genes) in rhizobia (Krause *et al*., 2002; Krishnan *et al*., 2003; Kobayashi *et al*., 2004; Viprey *et al*., 1998). Members of the NodD/LysR family of transcriptional regulators act at the top of this cascade, and by binding to *nod* boxes activate both genes encoding the synthesis of symbiotic signalling molecules along with other regulatory proteins (Schlaman *et al*., 1998). The gene encoding one such regulator, TtsI, is found within the T3SS loci of several rhizobia and in all cases is preceded by a *nod* box. *ttsI* is the only gene encoding a transcriptional regulator in all T3SS loci and, when translated, is proposed to recognize a further *cis* element, the TB, found in the promoters of T3SS-related genes. The TtsI/TB regulatory system is found in for all rhizobia possessing T3SS (Krause *et al*., 2002; Krishnan *et al*., 2003; Marie *et al*., 2004). Thus the demonstration that the response regulator transcriptional activator TtsI binds directly to TB suggests that the regulation of rhizobial T3SS is different from that found in phytopathogens.

TtsI binding is not specific to the TB consensus sequence, however, and the DNase I footprint extended up to 100 bp up- and downstream of the TB. Furthermore, a double-stranded oligonucleotide (located from −111 to −79) paired to sequences adjacent to the TB and competed with TtsI binding to the *nopB* promoter region. It should be noted that other members of the response regulator family of transcriptional activators also demonstrate large footprints despite specific consensus sequence predictions (Cullen *et al*., 1996). It is possible that other proteins bind TtsI and, in doing so, increase specificity of the interaction with TB. Mixing the soluble protein fraction of cell extracts with TtsI and TB-containing promoter regions had no effect on binding. Furthermore, TtsI did not bind to promoter regions lacking TB, nor did it bind to its own promoter (containing a *nod* box – data not shown), demonstrating that TtsI recognizes specific DNA sequences and that auto-regulation is absent.

Site-specific mutation of TB8 blocked promoter activity but did not abolish TtsI binding. A similar result was observed with HrpL of *Pantoea agglomerans* where direct binding was unaffected by mutations of the *hrp* box consensus sequence that did not permit transcription (Nissan *et al*., 2005). Given that TtsI has a large footprint, it may well bind to or interact with a relatively large stretch of DNA, and thus site-directed mutants would not be expected to prevent binding. However, application of the more sensitive DNase I cleavage protection assay to promoter regions containing the site-specific mutations revealed subtle differences compared with the non-mutated promoter. Modulation of cleavage protection was observed at the sites of mutation, but also at a second location in the TB, suggesting that the interaction between TtsI and the TB is altered and transcription thus blocked. It should also be noted that the nature of the mutations may have indirectly affected the ability of DNase I to digest the target DNA as this ability is very dependent on the nature of the sequence (Herrera and Chaires, 1994).

Random mutagenesis of the *nopB* promoter region identified further bases that are important for TtsI-dependent transcriptional activity. In one case, transcriptional activity was higher than wild-type levels, although flavonoids and TtsI were always required. No constitutively active mutations were generated. The mutants A117, A518 and A74, in which activity was blocked, were particularly revealing as none of the mutated residues were within the TB consensus sequence. As the *nopB* promoter regions of *S. fredii* USDA257 and NGR234 have identical DNA sequence, we used the experimentally proven transcription start of USDA257 to identify a putative rhizobial RNA polymerase binding site. Although the *nopB* promoter is not highly similar to the consensus sequences of *S. meliloti* (MacLellan *et al*., 2006), the −10 box is a good match, whereas the −35 region is poorly conserved. However the sequence encompassing the −35 region overlaps with numerous highly conserved residues of the TB. It is possible that the high activity of the *nopB* promoter may be due to the transition from a normally inactive state to one in which the changed conformation upon TtsI binding, exposes the promoter to RNA polymerase. Indeed, other transcriptional activators are known to interact with RNA polymerase and bind to sites located very close to the promoter (Browning and Busby, 2004), some by binding to sites which partially overlap the −35 region of the promoter and interact directly with the σ^{70} subunit of RNA polymerase (Dhiman and Schleif, 2000; Wickstrum and Egan, 2004). Transcriptional control of genes by the response regulator PhoB, where the conserved −35 hexamer in the RNA polymerase binding site is absent and replaced by a *pho* box to which PhoB binds (Makino *et al*., 1993), might be a good model for the TtsI/TB system. In these promoters, RNA polymerase is only able to bind in the presence of phosphorylated PhoB.

Following comparison of promoter regions of T3SS genes from several rhizobial species, Krause *et al*. (2002) predicted a consensus TB sequence. Using this sequence to search the symbiotic plasmid of NGR234, we identified 11 TBs, the majority of which are involved in regulating T3SS functions. Although we had previously demonstrated that TB2 and TB8 are active (Marie *et al*., 2004), it is now clear that 10 of the 11 known TB loci of pNGR234*a* are functional flavonoid- and TtsI-dependent promoters. TB3, which is upstream of y4gJ, an open reading frame (ORF) without obvious homologues (but part of a cluster of genes involved in LPS modification, some of which are controlled by TB2), is also induced to relatively high levels in a TtsI-dependent manner. Taken together, these data suggest that genes downstream of TB3 (e.g. y4gJ which is involved in modifying LPS) may also have a role in symbiosis.

In NGR234, at least 20 genes are thought to be involved in T3SS, including those that encode NopA, NopB, NopC, NopL, NopP and NopX (Viprey *et al*., 1998; Marie *et al*., 2003; Ausmees *et al*., 2004; Deakin *et al*., 2005; Saad *et al*., 2005). NopA, B and X are associated with pilus-like cell surface appendages, and are therefore thought to be a part of extracellular component of the T3SS machine (Krishnan *et al*., 2003; Deakin *et al*., 2005; Saad *et al*., 2005). NopL and

NopP are known effectors, which are probably injected into the cytoplasm of host cells (Bartsev *et al*., 2003; 2004; Marie *et al*., 2003; Ausmees *et al*., 2004; Skorpil *et al*., 2005). Generally, genes involved in the formation of the membrane-spanning secretion apparatus, such as *nopBrhcJnolUVrhcNy4yJrhcQRSTU* (TB8), *nopX* (TB7) and *nopCAy4yQrhcVy4yS* (TB10), are under the control of moderate to strong promoters. In contrast, TB6 and TB9 that control transcription of two effector proteins (NopL and NopP) are only weakly induced (threefold to 12-fold induction as compared with non-induced levels) 24 hpi (Fig. 2F and I). Furthermore, the ORFs y4fR (TB1), y4lO (TB4) and y4zC (TB11) are all homologous to known T3SS effector proteins (Marie *et al*., 2001) and their expression profiles are lower than TB controlling the machinery (sixfold, 13-fold and sixfold respectively). That these promoters are active in a TtsI-dependent manner and after flavonoid induction suggests that they may well be upstream of genes encoding functional effector proteins. Generally, promoters upstream of potential effector proteins have variable expression levels. As an example, the expression profile of pMP-TB5 differs significantly from those of other constructs. Although a slight induction (1.2-fold) by daidzein was observed 24 hpi, TB5 had basal transcriptional activities as high as 3440 ± 190 Miller units even under non-induced conditions. As a null mutation in *ttsI* had no affect on its basal activity, TB5 is probably under additional regulatory control. ORF downstream of TB5 are not obviously related to T3SS or LPS functions, and seem to be involved in stabilization of pNGR234*a* (Dombrecht *et al*., 2001).

Use of the TB consensus sequence in a bio-informatics screen to identify TtsI-regulated genes on pNGR234*a* was largely successful as 10 of the 11 predicted TBs are active. Indeed, extending such screens to the other replicons of NGR234 as well as other rhizobia should be feasible, although the predictions will need to be verified on a case-by-case base as the example of TB5 shows. Differences in the TtsI regulon could be specific to T3SS function, as rhizobia possess and may well secrete different effector proteins, which TB-based searches can help identify. Alternatively, production of diverse signalling molecules could also be controlled by TtsI, such as the rhamnose-rich LPS synthesized by NGR234 but not by *B. japonicum* USDA110 or *S. fredii* USDA257(Marie *et al*., 2004). Control of rhamnose synthesis is brought about by genes recruited into the TtsI regulon out of temporal necessity, as all are induced relatively late, or as a functional requirement linked to T3SS activity. Over 30 predicted TB sequences are present in the *B. japonicum* USDA110 genome (Suss *et al*., 2006). Completion of more rhizobial genomes, particularly those with T3SS and TB, will allow ever more powerful comparative studies into the roles of flavonoid-inducible genes in symbiosis.

Conclusions

We have extended the inventory of members of the flavonoid-regulatory cascade of NGR234 which bring about the exceptionally broad host range of this *Rhizobium*. Methods used here to analyse the TtsI regulon of NGR234 are applicable to other TB-possessing rhizobia, although it is very probable that genes modulated by TtsI will vary considerably. What remains is to test TB expression levels *in planta*, as the potential cocktail of inducing compounds and environments may well reveal more mechanisms of regulatory control.

Experimental procedures

Microbiological techniques

Escherichia coli recombinants were grown at 37°C on Luria–Bertani medium (Sambrook *et al*., 1989). NGR234 and its derivatives were raised at 28°C in *Rhizobium* minimal medium containing succinate as the carbon source (RMS) (Broughton *et al*., 1986) or TY (Beringer, 1974). Ampicillin (for Bluescript/pGem), gentamycin, kanamycin (for pRK2013), rifampicin, spectinomycin and tetracycline were added at concentrations of 50, 20, 50, 100, 50 and 15 μg

ml^{-1} respectively. The flavonoids, apigenein or daidzein were used to induce *ttsI*, as both have been shown to induce NB18 (Viprey *et al*., 1998; Kobayashi *et al*., 2004; Marie *et al*., 2004).

Construction of NGRΔ*ttsI* and p*ttsI*-2

To obtain a mutant with a deletion in *ttsI*, the 2.4-kb PstI fragment from pUC::*ttsI* (Marie *et al*., 2004), which carries *ttsI* as well as its flanking region (Fig. 1), was digested with SacII and NheI, purified by electrophoresis, treated with the Klenow fragment, and self-ligated. Then, the remaining 0.8-kb PstI fragment, lacking *ttsI*, was purified and sub-cloned into the PstI site of pJQ200-mp18 (Quandt and Hynes, 1993). The resulting plasmid was mobilized into NGR234 by tri-parental matings using the pRK2013 helper plasmid (Ditta *et al*., 1980). Marker exchange in NGRΔ*ttsI* was confirmed by Southern hybridization. To complement NGRΔ*ttsI*, *ttsI* along with its own *nod* box-containing promoter region, the 2.4-kb PstI fragment of pUC::*ttsI* (Fig. 1), were subcloned into the PstI site of pRG960 (Van den Eede *et al*., 1992), a vector which is compatible with pMP220-based *lacZ* fusion constructs described below, giving rise to p*ttsI*-2.

Extraction and analysis of Nops and LPS

After 40-h induction with 10^{-6} M apigenin, secreted proteins and LPS were recovered from the various NGR234 strains as described in Viprey *et al*. (1998) and Marie *et al*. (2004) respectively. Aliquots of purified proteins were separated on SDS-polyacrylamide (PAGE) gels and stained with silver (Ausubel *et al*., 1991) or, for immuno-detection, separated proteins were transferred to PVDF Immobilon-P membranes (Millipore Corporation, Bedford, Massachusetts, USA) and probed with antibodies against NopL, NopP and NopX. Horseradish peroxidase-labelled goat anti-rabbit immunoglobulin antibodies of the ECL kit (GE Life-sciences Amersham Pharmacia Biotech, Uppsala, Sweden) were used as secondary antibodies. Reactions were visualized by enhanced chemi-luminescence. Extracted LPS samples were separated on 16% deoxycholic acid (DOC)-PAGE and stained with silver nitrate (Tsai and Frasch, 1982).

Cloning of the 11 TBs in pMP220

Predicted promoter regions containing TB1, TB3–TB11 were amplified by PCR using the primer pairs listed in Table 1. The amplified products were subcloned into pBluescript KS^+ (Stratagene, La Jolla, California) and verified by sequencing the inserts. Then, the inserts were excised and cloned into pMP220. pMP220 constructs containing TB2 (pMP-TB2) and TB8 (pMP-TB8) correspond to plasmids pMP220-*rmlB* and pMP220B (Marie *et al*., 2004) respectively. Promoter constructs cloned into the broad-host-range reporter vector pMP220 (Spaink *et al*., 1987) were mobilized into NGR234 and its derivatives by tri-parental matings using pRK2013 as the helper plasmid (Ditta *et al*., 1980). Flavonoid induction was performed as described previously (Kobayashi *et al*., 2004): rhizobial cultures grown to a density of $^{1cm}OD_{600}$ 0.5–0.6 were diluted to $^{1cm}OD_{600}$ 0.1 in RMS medium and induced with 2×10^{-7} M daidzein or 1×10^{-6} M apigenin. β-Galactosidase activity was assayed according to Miller (1972).

DNase I footprinting

DNA fragments were amplified with polynucleotide kinase-end-labelled primers. The *rmlB* and *nopB* promoter regions were amplified with the following primers (respectively): *rmlB*-F and *rmlB*-R, and *nopB* for and *nopB* rev (Table 1). DNase I footprinting assays were performed in a total volume of 50 μl of TAP buffer without polyethyleneglycol (50 mM Tris-acetate pH 8.0, 100 mM potassium acetate, 8 mM magnesium acetate, 27 mM ammonium acetate, 1 mM dithiothreitol). Labelled fragments were added at a final concentration of approximately 5 nM and incubated with the proteins for 30 min at room temperature. After incubation, 0.05 U of

freshly diluted DNase I (Invitrogen, Carlsbad, CA) was added, and the reaction was allowed to run for 2 min at room temperature. Then, the samples were extracted with phenol, precipitated with ethanol, washed, re-suspended in loading buffer and loaded on a 6% DNA sequencing gel. Images were obtained by a Cyclone imager (Packard Institute, Downers Grove, Illinois) after exposing the dried gel to a Phosphoimager screen or by exposing the gels to X-ray film and scanning. Sequence ladders were prepared using the Sequenase Quick Denature Plasmid DNA Sequencing Kit as described by the manufacturer (USB, Cleveland, Ohio).

Electrophoretic mobility shift assays

^{32}P-labelled PCR fragments were incubated for 30 min in TAP buffer (50 mM Tris-acetate, 100 mM potassium acetate, 8 mM magnesium acetate, 3.5% polyethylene glycol 8000, 1 mM DTT, pH 7.9) with the indicated concentrations of TtsI and reactions loaded into a 4% native PAGE gel. Running buffer contained 50 mM Tris, 400 mM glycine, 2 mM EDTA, 8 mM $MgSO_4$ at pH 8.5. Dried gels were exposed to X-ray films and images obtained by scanning the film.

Site-directed mutagenesis of TB8

The non-mutated TB8 promoter was excised from pMP-TB8 as a HindIII fragment and subcloned upstream of a promoter-less *gfp* gene in plasmid pPROBE-GT′. Point mutations in TB8 were introduced by PCR using a pair of primers that replace the highly conserved triplet TCA for AAT. Two independent PCR reactions were carried out using primers TB8-F mut (5′-ggccggtagaatgcgtgtcgtcagctcgcctc-3′) versus *nopB* rev (5′-ggactcgattacttaactctttgac-3′) and TB8-R mut (5′-ggaggcgagctgacgacacgcattctaccggc-3′) versus *nopB* for (5′-ctcgtcttgataaaccaaatctgaa-3′). PCR products were pooled, amplified, cloned into pGEM-T (Promega, Madison, Wisconsin) and sequenced. The mutated promoters were subsequently transferred into pPROBE-GT as an EcoRI insert and orientation was checked. The constructs were mobilized into NGR234 or NGRΔ*ttsI* as described previously.

Random mutagenesis of TB8

Mutagenesis of the *nopB* promoter region was accomplished using mutagenic PCR as described by Vartanian *et al*. (1996). PCR reactions contained 1× *taq* buffer, 2.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 50 μM dATP and dCTP, 1 mM dGTP and dTTP, 2.5 μM each primer, template DNA and *taq* polymerase (Eppendorf). PCR fragments were cloned into pGEM-T (Promega), sequenced and transferred to pPROBE-GT as an EcoRI insert and orientation was checked.

Assay of GFP intensity

Rhizobial cultures were grown to an OD_{600} of 0.5, diluted to an OD_{600} of 0.1 in RMS medium and induced with 1×10^{-6} M apigenin. Both optical density (OD_{595}) and fluorescence (excitation filter at 485 nm and emission filter at 535 nm) were measured on 100 μl of cultures 40 hpi using a Plate Chameleon Multilabel Detection Platform (Hidex Oy, Turku, Finland). Optical densities and fluorescence were corrected to background levels using un-inoculated media, and the results represent the means of at least three independent experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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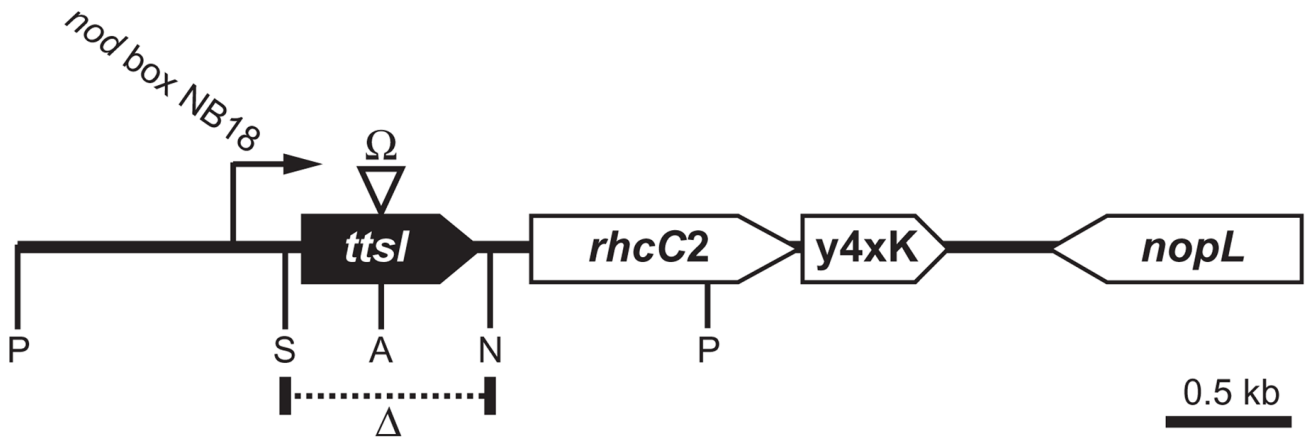


Fig. 1.

Physical map of the *ttsI* locus of pNGR234*a*. Restriction sites are as follows: ApaI (A); NheI (N); PstI (P); SacII (S). Location of the deleted region in NGRΔ*ttsI* is shown by Δ and the site of the omega cassette insertion in NGRΩ*ttsI* shown by Ω.

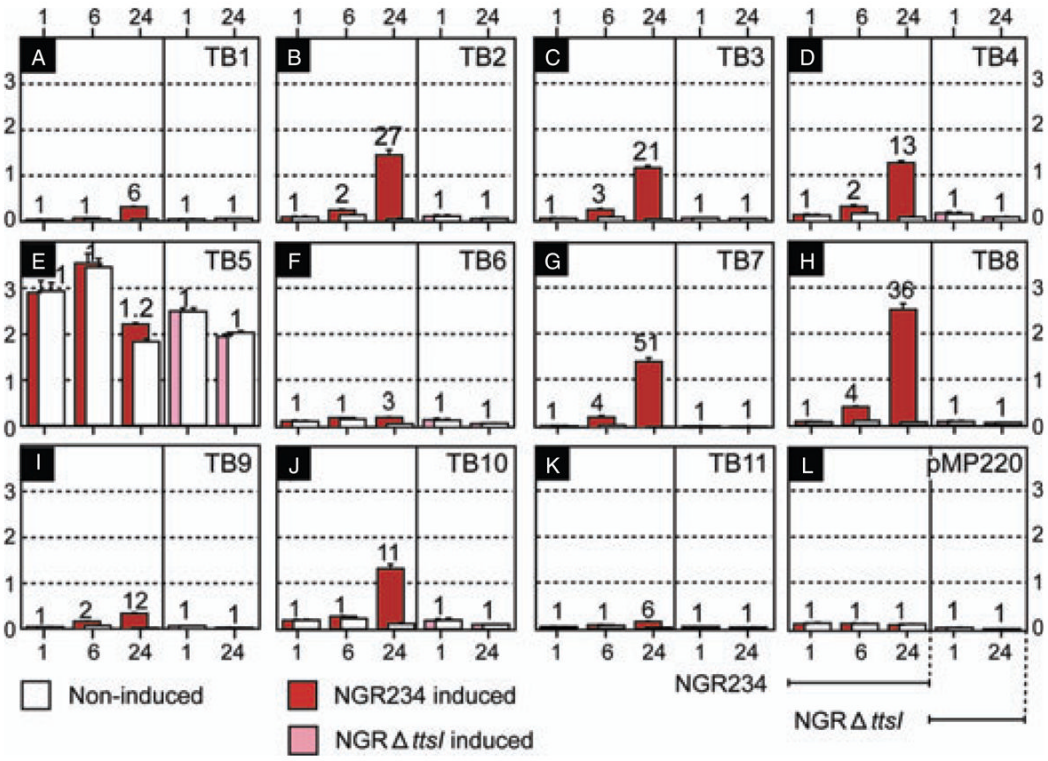


Fig. 2.

Expression analyses of TBs in pNGR234*a*. Flavonoid inducibility of TBs in the wild-type and NGRΔ*ttsI* backgrounds. A–L represent the levels of β-galactosidase activity (× 10^3 Miller's units). Activity of the vector pMP220 devoid of an insert is reported in L. Assays were performed 1, 6 and 24 hpi with 2×10^{-7} M daidzein. In the absence of an inducer, basal levels of β-galactosidase activity are shown as open bars. Values obtained with induced transconjugants are coded as: NGR234 (red bars); NGRΔ*ttsI* (pink bars). The values reported represent the means of three independent experiments. Error bars are shown on top of each column. Numbers on top of each bar represent the relative increase in activity of induced- as compared with non-induced cultures.

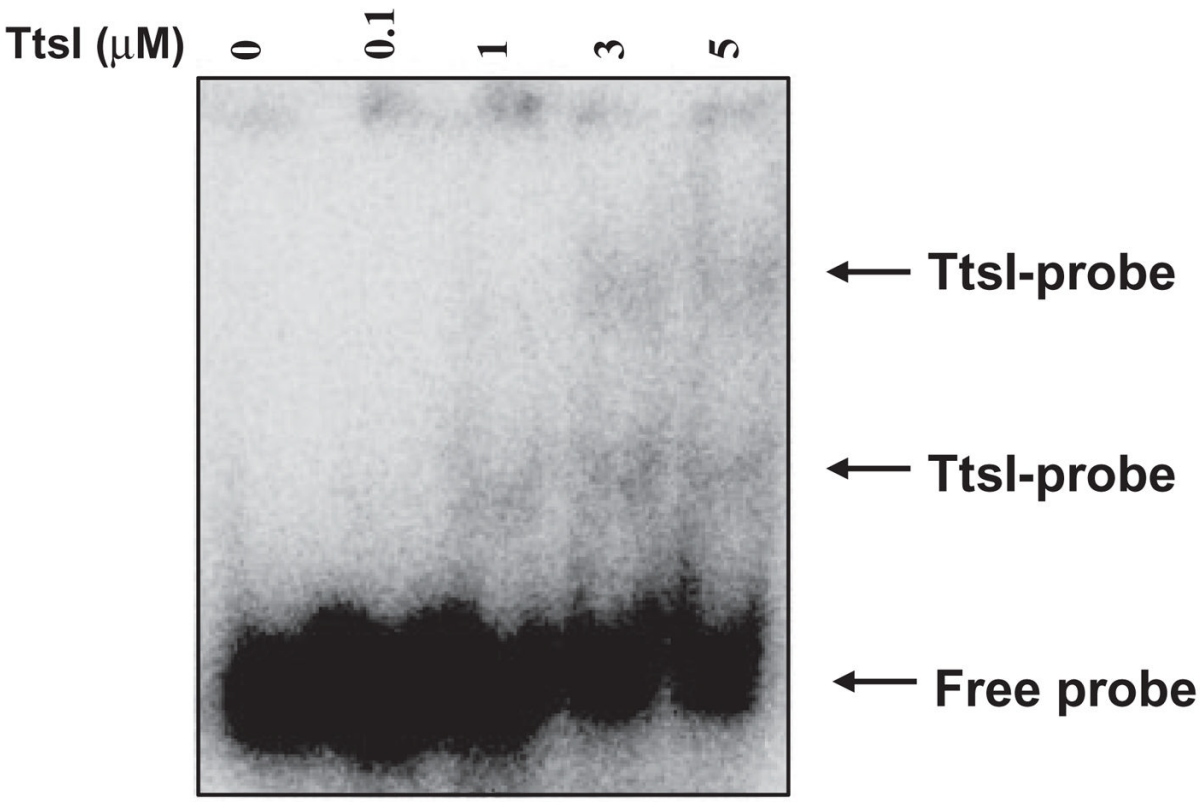
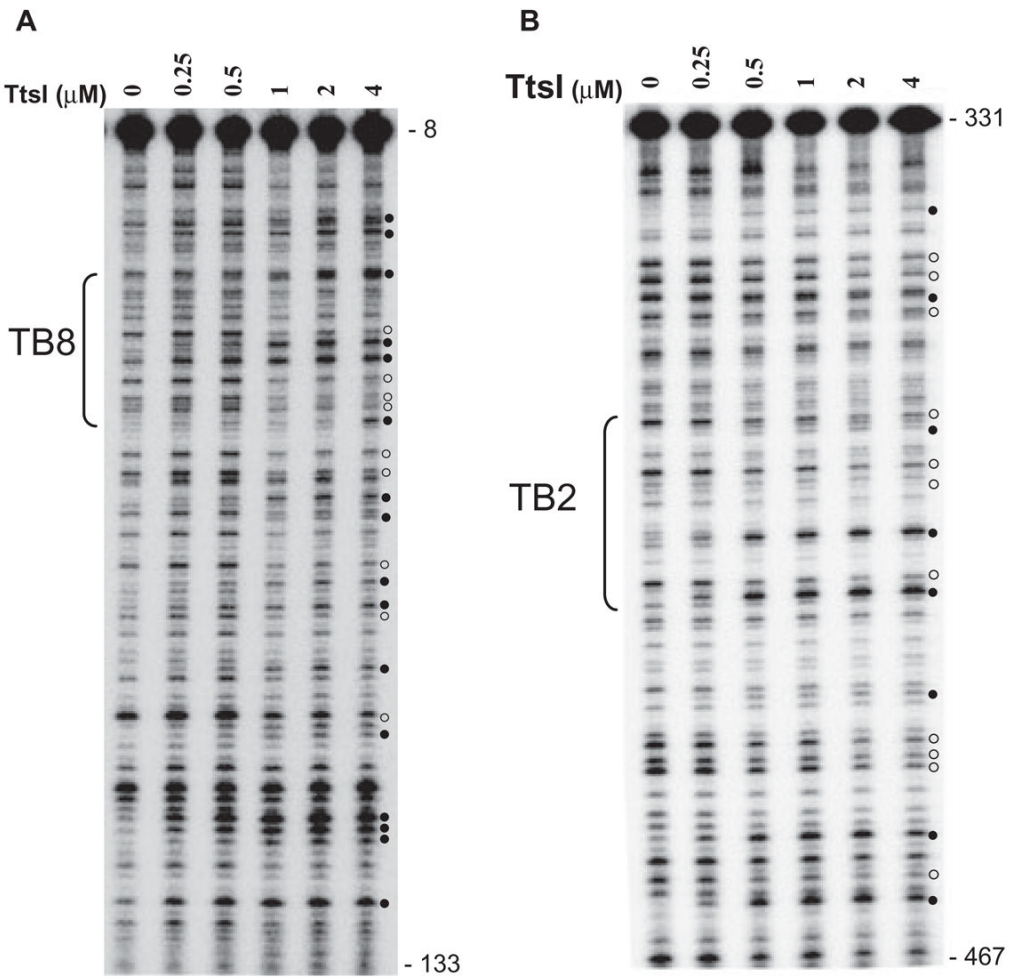


Fig. 3.

TtsI binding to the *nopB* (TB8) promoter. Mobility shift assay using a ^{32}P-labelled 150-bp PCR fragment containing the *nopB* promoter region. Purified TtsI-His was added at increasing concentrations, incubated for 20 min and subject to electrophoresis under native conditions.



C

-133 aatctCccat gcgGTTcaac t*cg*ACt*a*aCa TcactctTca AtgggcaAgc gacgctgccg

tts box (TB8)

gTa**g*tcag***cg tgtc**g**Tc**ag**c tc*g*cctcgCt agagttccAc gTcaaagagt taagtaatcg

D

tts box (TB2)

-467 t*a*cccgTc*g*C *c*tAtggat*gt* *cc*t*a*aaT*a*gg agagt*t*c***gTc ag***ctttTc***g***a a**ag**ctcagcC

gaaTa*g*cagT g*gg*aaggcCa agaCc*g*acC*a* acc*t*acctga attcagaaaT gg

Fig. 4.

DNase I footprints of TtsI bound to the *nopB* (TB8) and *rmlB* (TB2) promoter regions. ^{32}P-labelled PCR fragments were incubated with different concentrations of TtsI and digested using limiting concentrations of DNase I. After purification, DNA fragments were separated in a 6% sequencing gel, dried, exposed to Phosphoimager screens and scanned. The TB8- and TB2-containing PCR fragments are shown in A and B respectively. Numbers on the right refer to the DNA position compared with ATG of the downstream gene. Brackets indicate the limits of the TB. Open and closed circles pinpoint DNase I protected and hyperreactive bands respectively. Below the panels, the DNA sequences show the organization of the *nopB* (C) and *rmlB* (D) promoter regions and their reactivity to DNase I cleavage. Underlined bases delineate

the TBs with the highly conserved bases in bold font. Capital letters represent DNase I hyperreactive bases and italics show protected bases. Numbers refer to positions upstream of the ATG of the downstream gene.

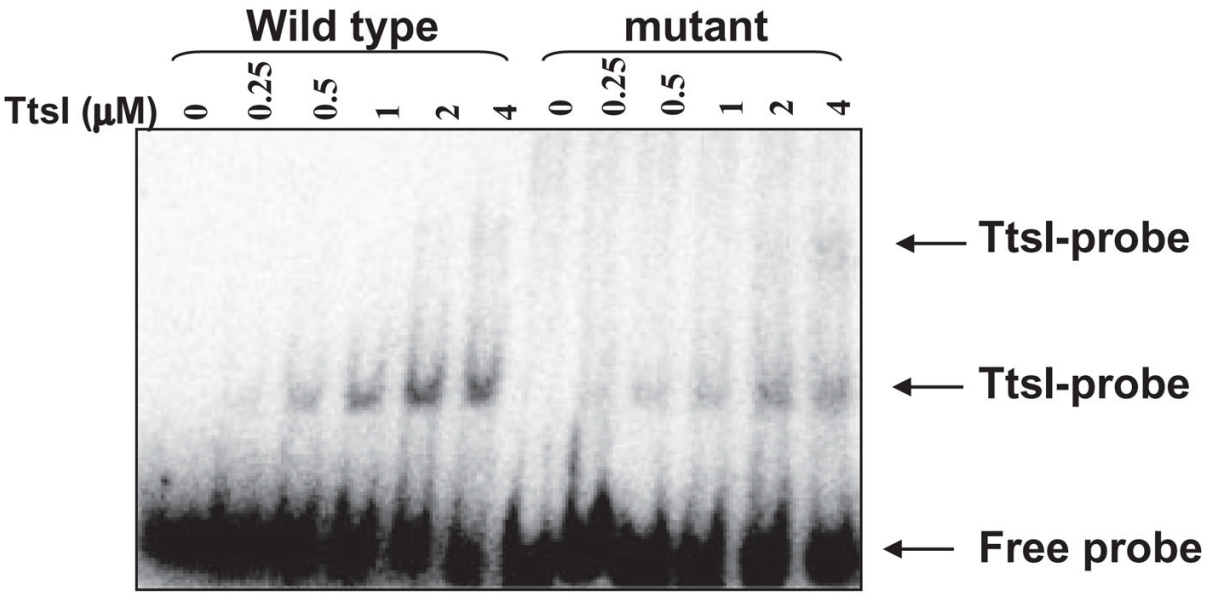


Fig. 5.
TtsI binds to the site-directed TB8 mutant. Mobility shift assays were performed as before, with ^{32}P-labelled 150-bp PCR fragments containing the *nopB* promoter regions.

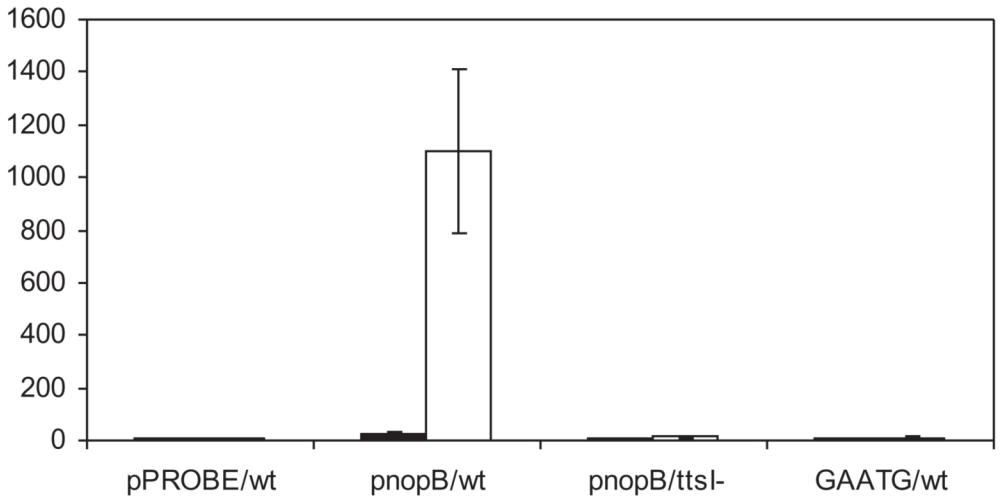


Fig. 6.

In vivo expression of the site-directed TB8 mutant. The mutated *nopB* promoter region was fused to a promoter-less *gfp* gene and assayed after 40-h induction with 2×10^{-6} M apigenin. Numbers shown ($\times 10^3$) are an average of at least three replicate experiments. pPROBE/wt, the vector control in NGR234; pnopB/ttsI-, the wild-type *nopB* promoter in NGRΔ*ttsI*; pnopB/wt, the wild-type *nopB* promoter in NGR234; GAATG/wt, the site-directed mutant *nopB* promoter in NGR234.

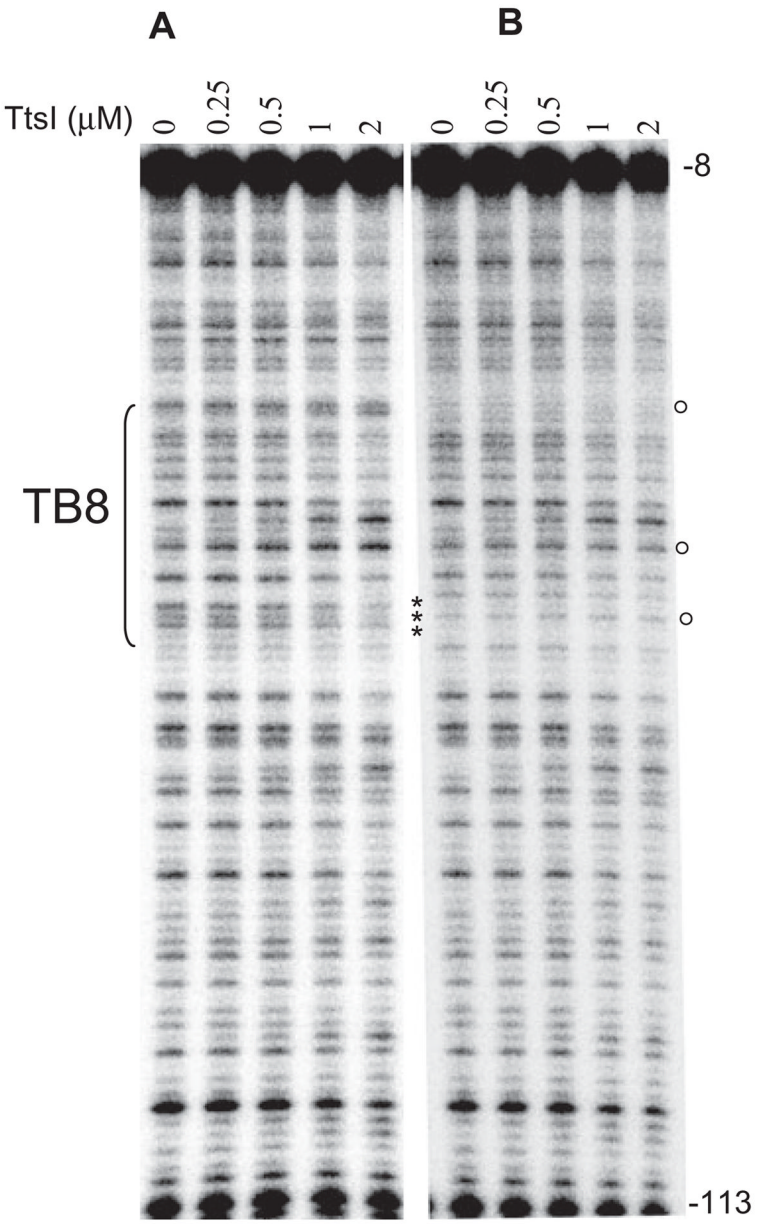


Fig. 7.

DNase I footprint of the site-directed TB8 mutant.^{32}P-labelled PCR fragments (A, wild-type and B, site-directed mutant) were incubated with increasing concentrations of TtsI and then digested with limiting concentration of DNase I. After purification, DNA fragments were separated in a 6% sequencing gel, dried, exposed to Phosphoimager screens and scanned. Numbers on the right refer to DNA positions compared with ATG of downstream genes. Asterisks show the mutated bases; open circles highlight the differences in DNase I activity observed (as compared with the wild-type TB8).

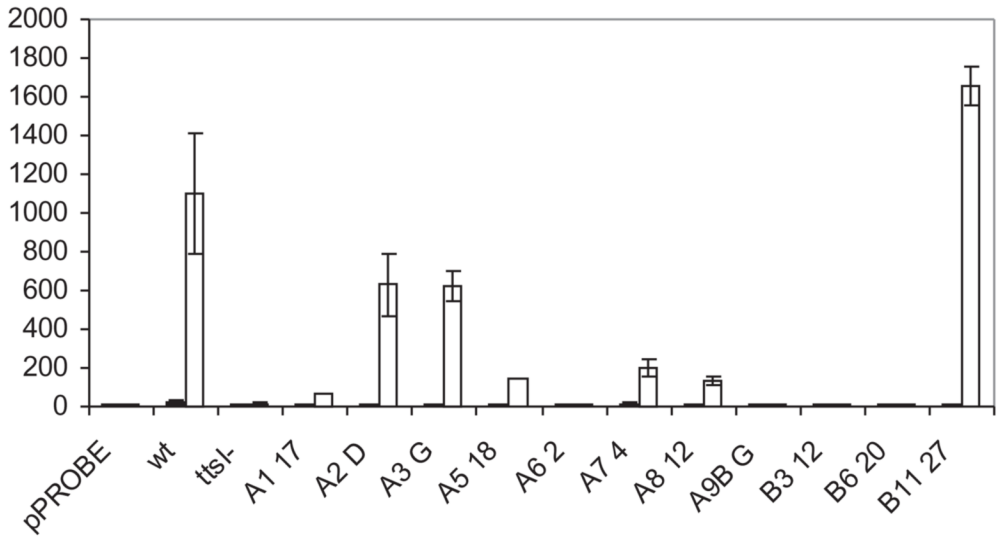


Fig. 8.

In vivo expression levels of the randomly mutated *nopB* promoter regions. Mutated *nopB* promoter regions were fused to promoter-less *gfp* genes, transferred to NGR234 and NGRΔ*ttsI* and assayed after 40-h induction with 2×10^{-6} M apigenin. The results ($\times 10^3$) are averages of at least three replicate experiments. pPROBE, vector only in NGR234; wt, the wild-type *nopB* promoter in NGR234; ttsI-, the wild type *nopB* promoter in NGRΔ*ttsI*; the code names refer to randomly mutated *nopB* promoters.

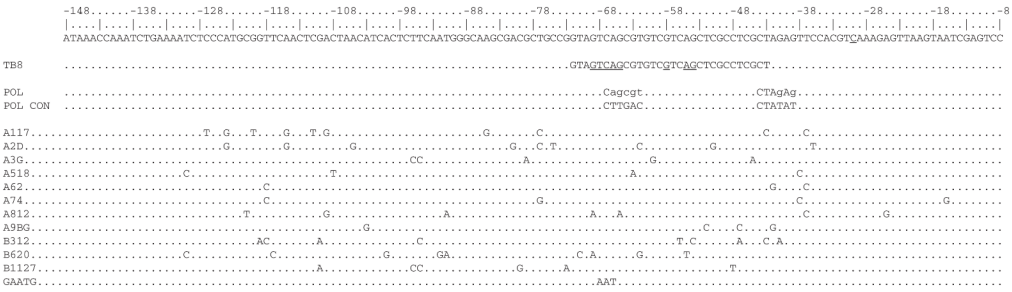


Fig. 9.

Positions of the mutated bases in the *nopB* promoter region. Top line of the DNA sequence shows the wild-type *nopB* promoter with the probable transcription start, TB8 underlined. The highly conserved bases of TB8 are also underlined. The putative −35/−10 promoter (labelled as POL) is shown, with bases not matching the consensus sequence (labelled as POL CON) in lower case. Dots represent unchanged bases, whereas mutated bases are lettered, code names are indicated at the left. Dotted lines below represent specific mutated *nopB* promoter regions (labelled with code names), mutated bases are lettered. Numbers refer to positions upstream of the *nopB* ATG.

Table 1

Bacterial strains and plasmids and primers.

	Relevant characteristics	Reference
Strains		
NGR234	Rif^R derivative of the isolate NGR234	Lewin *et al*. (1990)
NGRΔ*ttsI*	*ttsI* deletion mutant of NGR234 Rif^R	This work
NGRΔ*rmlB-wbgA*	Rhamnose synthesis mutant of NGR234, Rif^R, Km^R	Marie *et al*. (2004)
Plasmids		
pBluescript II	KS^+ Phage f1, *lacZ*, Ap^R	Stratagene
pUC::*ttsI*	pUC18 containing *ttsI* and its promoter as a 2.4-kb PstI fragment	Marie *et al*. (2004)
pJQ200mp18	Suicide vector used in directed mutagenesis, Gm^R	Quandt and Hynes (1993)
pJQΔ*ttsI*	As above, but carrying Δ*ttsI* fragment, subcloned into PstI site, Gm^R	This work
pRK2013	Tra^+ helper plasmid for mobilisation, Km^R	Ditta *et al*. (1980)
pRG960	IncQ vector containing promoter-less *uidA*, Sp^R	Van den Eede *et al*. (1992)
p*ttsI*-2	pRG960 containing *ttsI* and its promoter as a 2.4 kb PstI fragment	This work
pMP220	IncP vector containing promoter-less *lacZ*, Tet^R	Spaink *et al*. (1987)
pMP-TB1	TB1 cloned in pMP220 as a XbaI-PstI fragment	This work
pMP-TB2	Also called pMP220-*rmlB*; TB2 cloned as a BamHI fragment in pMP220	Marie *et al*. (2004)
pMP-TB3	TB3 cloned in pMP220 as a XbaI-PstI fragment	This work
pMP-TB4	TB4 cloned in pMP220 as a XbaI-PstI fragment	This work
pMP-TB5	TB5 cloned in pMP220 as a XbaI-PstI fragment	This work
pMP-TB6	TB6 cloned in pMP220 as a XbaI-PstI fragment	This work
pMP-TB7	TB7 cloned in pMP220 as a XbaI-PstI fragment	This work
pMP-TB8	Also called pMP220-B; TB8 cloned as a XbaI-PstI fragment in pMP220	Marie *et al*. (2004)
pMP-TB9	TB9 cloned in pMP220 as a XbaI-PstI fragment	This work
pMP-TB10	TB10 cloned in pMP220 as a XbaI-PstI fragment	This work
pMP-TB11	TB11 cloned in pMP220 as a XbaI-PstI fragment	This work
pPROBE-GT	pVS1/p15a vector, Gm^R	Miller, 2000
pPROBE-GT′	As above but with inverted MCS	Miller, 2000
pGT-*nopB*	TB8 region in pPROBE-GT′ as a HindIII fragment	This work
pGT-GAATG	Site-directed mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-A117	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-A2D	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-A3G	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-A518	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-A62	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-A74	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-A812	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-A9BG	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-B312	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-B620	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
pGT-B1127	Random mutation of TB8 region in pPROBE-GT as an EcoRI fragment	This work
Primers		
TB1-F	5′-tctcatctctagaccaatcggcg-3′	
TB1-R	5′-cctgcagcgatattgtctcctcg-3′	
TB3-F	5′-tctagagccgtcagtcatctttcg-3′	
TB3-R	5′-tgtcctgcagatcgttgatgagg-3′	
TB4-F	5′-tctagacgatggcgatgttgctc-3′	
TB4-R	5′-acgactgcaggccttcaagatgg-3′	
TB5-F	5′-tgctctagagcaccggaagc-3′	
TB5-R	5′-tcctgcaggggaaagtagcatc-3′	
TB6-F	5′-cctctagagcctgtcttttctcg-3′	
TB6-R	5′-gctgcaggcttgcgtttagtgg-3′	
TB7-F	5′-tcgtctagacgttgaacggtctac-3′	
TB7-R	5′-aaggctgcaggccgacattgtg-3′	
TB9-F	5′-tctctagatggcgtcaaatgctgc-3′	
TB9-R	5′-ttctgcagccatttgcttgctgg-3′	
TB10-F	5′-catctagactgagagagcttcacg-3′	
TB10-R	5′-acctgcagctactcctgccttag-3′	
TB11-F	5′-cgaatctagacgactttcgatcgc-3′	
TB11-R	5′-gactctgcagggcgttcgtttccc-3′	
rmlB-F	5′-aagcaccccgaaaactacct-3′	
rmlB-R	5′-gcattgcgaaatttggatgga-3′	
nopB-F	5′-ctcgtcttgataaaccaaatctgaa-3′	
nopB-R	5′-ggactcgattacttaactctttgac-3′	
TB8-F mut	5′-ggccggtagaatgcgtgtcgtcagctcgcctc-3′	
TB8-R mut	5′-ggaggcgagctgacgacacgcattctaccggc-3′	