Different Regulation and Roles of Lactonases AiiB and AttM in *Agrobacterium tumefaciens* C58

Elise Haudecoeur, Mélanie Tannières, Amélie Cirou, Aurélie Raffoux, Yves Dessaux, and Denis Faure

Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Gif-sur-Yvette 91198, France

Submitted 5 September 2008. Accepted 30 December 2008.

The phytopathogen *Agrobacterium tumefaciens* C58 expresses two lactonases, AttM and AiiB. We showed that expression of the *aiiB* gene was controlled by agrocinopines A and B and required the agrocinopine-ABC transporter Acc, but was not affected by the level of quorum-sensing (QS) signal 3-oxo-octanoylhomoserine lactone (OC8-HSL). In the presence of agrocinopines, a constructed *aiiB* mutant accumulated OC8-HSL at a level 10-fold higher than that of the wild-type strain, and showed an exacerbated expression of a key QS-regulated function, conjugation of Ti plasmid (in vitro and in planta), as well as an increase of the number of emerging tumors on the host plant. The expression and acyl-HSL-degrading activity of AttM were evident in the presence of wounded tissues; however, in unwounded plant tumors, the QS-regulated functions were weakly affected in an *attM* mutant. By contrast, we observed that *attM* conferred a selective advantage in the course of colonization of plant tumors. Finally, polymerase chain reaction survey of genes *attM* and *aiiB* showed that they were not strictly conserved in the genus *Agrobacterium*. This work proved that the lactonases AttM and AiiB are regulated by different plant signals and are implicated in different functions in the course of the *A. tumefaciens* C58–host interaction.

Agrobacterium tumefaciens can infect a broad range of dicotyledonous plants and transfer an oncogenic DNA fragment, the T-DNA, from its tumor-inducing (Ti) plasmid to the nuclear genome of the plant hosts. In transformed plant tissues, expression of T-DNA genes leads to uncontrolled synthesis of growth regulators, auxin, and cytokinins, resulting in formation of a tumor, a phenomenon known as crown gall disease (Escobar and Dandekar 2003). T-DNA also encodes for synthesis of opines (Schell et al. 1979), which are specific growth substrates and signals used by the virulent *Agrobacterium* spp. Some opines, called conjugative opines, are required for synthesis of a quorum-sensing (QS) signal, 3-oxo-octanoyl-homoserine lactone (OC8-HSL). The QS pathway controls amplification of copy number (Li and Farrand 2000) and conjugative transfer (Piper et al., 1993; Zhang et al. 1993) of Ti plasmid. A link between a high copy number of Ti plasmid and severity of tumor symptoms was established (Pappas and Winans 2003) and, consequently, antivirulence strategies targeting QS have been proposed to attenuate *Agrobacterium*-induced symptoms on plants (Chevrot et al. 2006; Molina et al. 2003).

In *A. tumefaciens* C58–induced tumors, conjugative agrocinopines A and B tightly control synthesis of the OC8-HSL sig-

nal at the transcriptional level. In the presence of these opines, the AccR-mediated transcriptional repression of *arc* operon (*orfA-orfB-splA-traR-mcpA*) is released (Beck von Bodman et al. 1992; Piper et al. 1999); subsequently, transcriptional regulator TraR binds OC8-HSL (Qin et al. 2007), allowing expression of the OC8-HSL-synthase TraI at a high level, as well as that of *tra* and *rep* operons which are required for conjugative transfer and control of Ti plasmid replication, respectively. However, the QS pathway is downregulated by a conserved antagonist, TraM, which directly interacts with TraR (Luo et al. 2000; Swiderska et al. 2001): TraM prevents the binding of TraR/OC8-HSL complexes to the target DNA sequences of QS-regulated promoters. In the presence of conjugative opines, the antagonist effect of TraM would be compensated by synthesis of TraR at a high rate.

Enzymatic inactivation of the OC8-HSL signal by lactonases AttM and AiiB is proposed to play a role in the fine regulation of the QS-controlled functions in *A. tumefaciens* C58 (Carlier et al. 2003; Zhang et al. 2002). The expression of AttM is stimulated at the transcriptional level by several plant signals, such as γ -aminobutyrate (GABA) and its byproducts, γ -hydroxybutyrate (GHB) and succinic semialdehyde (SSA) (Carlier et al. 2004; Chevrot et al. 2006), as well as by salicylic acid (Yuan et al. 2007). The lactonase-encoding gene *attM* is part of the *attKLM* operon, the expression of which is controlled by transcriptional factor AttJ (Chai et al. 2007; Wang et al. 2006; Zhang et al. 2002). In addition to implication of AttM in the GBL-ring cleavage of OC8-HSL, the *attKLM* operon encodes for assimilation of GBL and GHB as a carbon source (Carlier et al. 2004; Chai et al. 2007). On the other hand, the structure of the Ti plasmid-encoded AiiB lactonase is established (Liu et al. 2007) but the regulation of the *aiiB* gene remains unknown (Carlier et al. 2003).

In this work, the regulation of both AttM and AiiB lactonase-encoding genes was compared and their involvement in QS-regulated functions, such as the horizontal transfer of Ti plasmid and the modulation of the number of emerging tumors, was evaluated. Their implication in the colonization of plant tumors by *A. tumefaciens* C58 and their presence in reference strains and soil isolates of the genus *Agrobacterium* were also investigated by polymerase chain reaction (PCR) screening.

RESULTS

Agrocinopines induce expression of the *aiiB* gene.

An *aiiB::lacZ* fusion was constructed and recombined into *A. tumefaciens* C58. Several classes of compounds were tested for their ability to induce the *aiiB::lacZ* expression: i) acyl-HSL with short or long acyl chains and, optionally, 3-hydroxy and 3-oxo substituted, such as C4HSL, C6HSL, OC6HSL, OHC6HSL, C8HSL, OC8-HSL, OHC8HSL, C10HSL, and

C12HSL; ii) GBL and compounds exhibiting a GBL ring, such as γ -caprolactone, γ -valerolactone, γ -heptanolactone, α -acetylbutyrolactone, α -amino- γ -butyrolactone, tetrahydrofuran, tetrahydrofuryl alcohol, succinic anhydride, D-pantoyllactone, α -methyl- γ -butyrolactone, tetroneic acid, and D-erythronic acid; and iii) other plant compounds such as GABA and acetosyringone, vanillic acid, ferulic acid, and sinapic acid. None of these compounds (applied at up to 1 mM) affected expression of the *aiiB* gene (data not shown). The *aiiB::lacZ* expression assays were also performed in the presence of sections of tomato stems and tumors induced by *A. tumefaciens* strains C58 or R10. The R10 and C58 T-DNAs encode the synthesis of different opines in the plant tumor (Fuqua and Winans 1996; Kim and Farrand 1997), which are octopine, histopine, lysopine, and mannityl opines (R10) and nopaline and agrocinopines A and B (C58). The *traI::lacZ* and *orfA::lacZ* transcriptional fusions of the *tra* and *arc* operons, the expression of which is regulated by agrocinopines, were also constructed and used as a positive control in the course of the agrocinopine tests. Although expression of the *aiiB::lacZ* fusion was not affected by slices of stem or R10-tumor, a 10-fold increase was detected in the presence of slices of C58 tumor (Fig. 1A). These results strongly suggested an opine-dependent expression of the *aiiB::lacZ* fusion. Therefore, induction assays were performed in the presence of purified opines from *A. tumefaciens* C58 tumors. Although the expression of *aiiB* was not affected by

nopaline (data not shown), it was significantly induced by the purified agrocinopines A and B (Fig. 1B).

The expression of gene *aiiB* requires the agrocinopine-specific ABC transporter Acc.

The uptake of agrocinopines A and B and, consequently, the expression of agrocinopine-dependent functions, require the *acc*-encoded ABC-transporter. The expression of *aiiB::lacZ* in the presence of opines is affected in an *accA* mutant (Fig. 1C). This demonstrates that the import of agrocinopines is necessary to induce the expression of the *aiiB* gene. However, no synergistic effect of agrocinopines and OC8-HSL (or C6HSL) on the expression of the *aiiB::lacZ* fusion was observed (data not shown), suggesting that the expression of *aiiB* would be independent of the level of acyl-HSL. The first observation was confirmed in an *accR* mutant, in which the OC8-HSL concentration in culture supernatant was higher than in that of the wild-type strain (373 ± 50 nM versus 0.5 ± 0.2 nM). The expression of *aiiB::lacZ* fusion in the *accR* and wild-type backgrounds was similar (Fig. 1D), indicating that the pathways for the OC8-HSL biosynthesis (via *AccR*, *TraR*, and *TraI*) and degradation (via *AiiB*) are independent.

Both AiiB and AttM may modulate the accumulation of OC8-HSL.

The implication of AiiB in the modulation of OC8-HSL level was evaluated by comparing C58-110 (*attM⁻aiiB⁺*) and C58-111 (*attM⁻aiiB⁻*) mutants. In the absence of agrocinopines, *A. tumefaciens* derivatives C58-110 and C58-111 produced a similar, low level of OC8-HSL (0.5 nM). In the presence of these opines, OC8-HSL concentration in the culture supernatant of C58-110 and C58-111 reached 11 and 98 nM, respectively (Fig. 2A), suggesting that AiiB indeed modulates accumulation of the OC8-HSL signal. Remarkably, when a high concentration of OC8-HSL (200 nM) was added at the beginning of the culture, a similar level of OC8-HSL in culture medium of strains C58-110 and C58-111 was observed (Fig. 2B). The addition of OC8-HSL at such high concentration likely saturates the degradation system or strongly activates the synthesis system. In both cases, this experiment suggests that the impact of AiiB on the accumulation of OC8-HSL is mostly significant when concentration of the QS signal is limited (e.g., when synthesis begins).

The transcription of the *attKLM* operon is not affected in presence of opines (Carrier et al. 2004) but it is stimulated in the presence of GABA (Chevrot et al. 2006) and its byproducts GHB and SSA (Carrier et al. 2004, 2006; Chai et al. 2007; Zhang et al. 2004). Experiments revealed that the concentration of GABA was higher in tumor (0.57 μ mol/g FW) than stem tissues (0.16 μ mol/g FW) and increased rapidly after wounding to reach 0.68 and 2.69 μ mol/g FW in stem and tumor tissues, respectively (Fig. 2C). Moreover, in the presence of wounded stem and tumor, the *attKLM* operon was expressed (Fig. 2D) and the *A. tumefaciens* C58-102 (*aiiB⁻*) derivative was able to inactivate the added OC8-HSL (Fig. 2E). In contrast, the C58-111 derivative (*attM⁻aiiB⁻*) was not able to degrade OC8-HSL (Fig. 2E), suggesting that, in the presence of wounded plant tissues (stem and tumor), the AttM lactonase may efficiently cleave the OC8-HSL signal.

AiiB modulates the conjugation frequency of the Ti plasmid.

The OC8-HSL-degradation ability of AttM and AiiB suggests that these lactonases would be able to downregulate the QS-regulated horizontal transfer of Ti plasmid. To test this hypothesis, conjugation frequencies were measured using lactonase-defective derivatives as donor strains. *A. tumefaciens* C58.00,

Fig. 1. Expression of *aiiB::lacZ* in the presence of agrocinopines A and B. Expression of the *aiiB::lacZ*, *traI::lacZ*, and *orfA::lacZ* transcriptional fusions was measured in *Agrobacterium tumefaciens* strain C58 grown in AB medium supplemented or not (control) with **A**, crude extracts of *A. tumefaciens* C58- or R10-tumors or **B**, electrophoretically separated fractions (F1 to F6) from *A. tumefaciens* C58-tumors. Agrocinopines A and B (fractions F3 and F4) and fructose, used as a nonmigrating marker, were revealed by silver nitrate staining after migration on 3M Whatman paper (B). Expression of the transcriptional fusions was measured in the wild-type background of *A. tumefaciens* C58 as well as the mutants **C**, *accA* and **D**, *accR* in the absence (white bars) or presence (black bars) of crude extracts of C58-induced tumors. All those experiments were done at least in duplicate and generated comparable results. Under the same conditions, the *A. tumefaciens* strain C58, free of any transcriptional fusion *lacZ*, showed an undetectable β -galactosidase activity.

Fig. 2. Impact of AiiB and AttM on the accumulation of the 3-oxo-octanoylhomoserine lactone (OC8-HSL) signal. The level of OC8-HSL was measured in culture supernatants of *Agrobacterium tumefaciens* C58-110 (*attM*) and C58-111 (*attMaiB*) derivatives in the presence of agrocinopines and in the **A**, absence or **B**, presence of exogenous OC8-HSL (200 nM) that was added at the beginning of the culture. **C**, The level of γ -aminobutyrate (GABA) was measured in tomato stems at infection time and in *A. tumefaciens* C58-induced tumors harvested 5 weeks postinfection. **D**, The expression of the *attK::lacZ* fusion in *A. tumefaciens* C58 and **E**, the level of OC8-HSL (initially introduced at 200 nM) in cultures of *A. tumefaciens* C58-102 (*aaiB*⁻) and C58-111 (*attM*⁻*aaiB*⁻) were measured in the presence of wounded stem and tumor tissues. A culture of *A. tumefaciens* C58 harboring the *attK::lacZ* fusion in **D**, AB medium and **E**, an uninoculated medium served as controls. The experiments were done in triplicate and bars indicate the standard deviation.

which is lacking Ti and At plasmids, was used as the recipient strain. Ti plasmid conjugation was analyzed in the niche where it naturally occurs, the plant tumor (Table 1). A *traI* donor strain, defective for QS-signal biosynthesis, was used as negative control: no transconjugants were recovered from the conjugation assay. In contrast, transconjugants in tumors infected with C58-105 (*aaiB*⁻*attM*⁺) were much more abundant than in tumors infected with C58-107 (*aaiB*⁺*attM*⁺). The large standard deviation is explained by absence of detection of transconjugants in some tumors. Therefore, the downregulation of Ti plasmid conjugation by the OC8-HSL-cleaving lactonase AiiB is substantial under this experimental condition. Nevertheless, because agrocinopines A and B stimulate the synthesis of abundant OC8-HSL signals, plant tumor remains the most favorable niche for conjugation, as evidenced by the isolation of transconjugants with donor C58-107. However, when the C58-105 (*aaiB*⁻*attM*⁺) and C58-111 (*aaiB*⁻*attM*⁻) donors were compared, the inactivation of *attM* did not stimulate the accumulation of transconjugants (Table 1), suggesting that, in this experiment, the impact of the AttM lactonase on conjugation is limited.

Implication of AiiB in conjugation of Ti plasmid was confirmed by in vitro experiments in the presence of purified agrocinopines A and B. *A. tumefaciens* C58.00 was used as a recipient and strains C58-107 (*aaiB*⁺*attM*⁺) and C58-105 (*aaiB*⁻*attM*⁺) as alternative donors (Fig. 3). The Ti plasmid transconjugants that emerged earlier were those obtained with the lactonase AiiB-defective strain C58-105 as the donor. The higher efficiency for conjugation of this strain was correlated both with an early appearance (at day 2) and a higher level of OC8-HSL in culture medium (Fig. 3).

AiiB modulates the emergence of tumors.

The implication of QS in the modulation of virulence (number of emerging tumors) was initially reported in *A. tumefaciens* R10 overexpressing *traR* (Pappas and Winans 2003) and then observed in other *A. tumefaciens* isolates (Chevrot et al. 2006; Molina et al. 2003). To evaluate the contribution of the OC8-HSL-cleaving activity in virulence on whole plants, aggressiveness of wild-type strain C58 was compared with that of its *attM* (C58-110), *aaiB* (C58-102), and *attMaiB* (C58-111) derivatives. Virulence assays were performed on tomato and tobacco plants under greenhouse culture conditions (Fig. 4). The aggressiveness was measured by counting the number of emerging tumors within each infected gash. All the mutants were more virulent than wild-type strain C58. Remarkably, the *aaiB* mutant was more virulent than the *attM* and *attMaiB* double mutants; this feature would be explained by the loss of nutritive advantage conferred by AttM in the tumor environment (discussed below). Because the *aaiB* mutant was the most aggressive strain, we performed virulence assays with a complemented *aaiB* mutant that harbored plasmid pMIR103 expressing the AiiB lactonase (Carlier et al. 2003). The complemented mutant *aaiB* was less virulent than the same mutant harboring the empty vector pME6010 (Fig. 4C).

Table 1. Conjugation frequencies of tumor-inducing plasmid with C58.00 as a recipient^a

Donor strains		Conjugation frequency in plant tumors	
Name	Characteristics	Transconjugant/donor	Transconjugant/recipient
C58-107	<i>traI</i> ⁺ <i>aaiB</i> ⁺ <i>attM</i> ⁺	$2 \times 10^{-7} \pm 1 \times 10^{-7}$	$3 \times 10^{-7} \pm 1 \times 10^{-7}$
C58-105	<i>traI</i> ⁺ <i>aaiB</i> ⁻ <i>attM</i> ⁺	$2 \times 10^{-5} \pm 4 \times 10^{-5}$	$2 \times 10^{-5} \pm 8 \times 10^{-5}$
C58-111	<i>traI</i> ⁺ <i>aaiB</i> ⁻ <i>attM</i> ⁻	$3 \times 10^{-6} \pm 7 \times 10^{-6}$	$4 \times 10^{-6} \pm 5 \times 10^{-6}$
C58-115	<i>traI</i> ⁻ <i>aaiB</i> ⁺ <i>attM</i> ⁺	$<3 \times 10^{-8}$	$<3 \times 10^{-8}$

^a Values are average values obtained from nine replicates; when no transconjugants were observed, a frequency below detection limit was calculated. Number of recipients includes that of transconjugants.

AttM contributes to fitness of *A. tumefaciens* C58 in plant tumor.

Competitions between C58-107 (*attM*⁺*aiiB*⁺) and C58-110 (*attM*⁺*aiiB*⁺), and between C58-107 (*attM*⁺*aiiB*⁺) and C58-105 (*attM*⁺*aiiB*⁻) were performed by co-inoculating two strains (at an initial ratio of 1/1) in wounded stems of tomato plants. The percentage of each mutant was measured among agrobacteria collected from emerging tumors. In a first assay performed on six 6-week-old plant tumors, C58-107 represented more than 95% of the tested isolates, therefore dominating C58-110. In contrast, the proportion of C58-107 (*attM*⁺*aiiB*⁺) and C58-105 (*attM*⁺*aiiB*⁻) remained equal in six 6-week old plant tumors (data not shown). To confirm these results, mutants C58-107 (*attM*⁺) and C58-110 (*attM*⁻) were reconstructed to generate mutant strains independent from those used in the first series of experiments. Competition experiments performed with the second series of mutants (Fig. 5B and D) confirmed that C58-107 (*attM*⁺) was a better fit in the tumor environment than was C58-110 (*attM*⁻). Additionally, a competition assay was performed in tomato rhizosphere, revealing that mutation *attM* had no effect on the fitness of *A. tumefaciens* C58 in this plant environment (Fig. 5A and C).

PCR screening of *attM* and *aiiB* genes in *Agrobacterium* strains.

The presence (+) or absence (-) of strong homologs of *attM* and *aiiB* genes was investigated by PCR amplification in several strains, including type strains (T) belonging to *Agrobacterium* spp., as well as in the genomic draft of *A. vitis* S4 and *A. radiobacter* K84 (also referred as *Rhizobium rhizogenes* K84). At the genus level, the analyses yielded the following results: *A. tumefaciens* B6^T (*attM*⁻*aiiB*⁻), *A. larrymoorei* AF3.10^T (*attM*⁻*aiiB*⁻), *A. vitis* K309^T (*attM*⁻*aiiB*⁻) and S4 (*attM*⁻*aiiB*⁻), and *R. rhizogenes* CFBP2408^T (*attM*⁻*aiiB*⁻) and K84 (*attM*⁻*aiiB*⁺). However, the *Agrobacterium* biovar1 (formally *A. tumefaciens* species) is organized in nine genomic species (G1 to G9) (Popoff et al. 1984), in which *A. tumefaciens* B6^T and C58^T

are type strains for G4 and G8, respectively. The *aiiB* or *attM* genes were detected in strains CIP497-74^T (G2) (*attM*⁺*aiiB*⁻) and Zutra3/1^T (G7) (*attM*⁻*aiiB*⁺) but seemed to be absent in strains TT111^T (G1), CIP107443^T (G3), CIP107444^T (G5), NCPPB925^T (G6), and HaywardO363^T (G9). The amplified *attM* and *aiiB* genes were cloned and expressed in *Escherichia coli* host, to which they conferred the capacity to inactivate NAHL (C6-HSL and OC8-HSL) (data not shown).

The above differences among reference strains prompted investigation of the presence of *attM* and *aiiB* genes in natural agrobacterial populations. *Agrobacterium* biovar1 isolates were recovered from tomato and tobacco rhizospheres (soil from Gif-sur-Yvette, north of France) in which they reached approximately 7×10^5 and 3×10^5 CFU/g of soil (i.e., 0.5 and 1% of the total cultivable bacterial community), respectively. The 628 isolates were collected from three independent replicates for each of the two host plants. Although none of the isolates yielded a PCR amplification signal with the *aiiB* primers, 93% (tomato host) to 98% (tobacco host) of them positively responded to *attM* amplification assays and inactivated C6-HSL (introduced at 25 μ M), as strain C58 and A6, after 48 h of incubation (Wang et al. 2006).

Aside from this PCR survey, one cannot exclude that low stringency hybridization would permit the detection of additional *aiiB* and *attM* homologs, of which the encoded protein would exhibit lactonase activity against NAHL.

DISCUSSION

The regulation and function of the lactonases AttM and AiiB, encoded by the At and Ti plasmids, respectively, of *A. tumefaciens* C58 (Carlier et al. 2003; Zhang et al. 2002), were analyzed. Results revealed that i) the *aiiB* gene is regulated at a transcriptional level by agrocinopines A and B (Fig. 1A and B), and such expression requires the agrocinopine ABC transporter Acc (Fig. 1C); ii) expression of *aiiB* is independent of the level of OC8-HSL and is not repressed by AccR (Fig. 1D); therefore, AiiB does not exert a retrocontrol on QS signal synthesis; iii) in the presence of agrocinopines, the accumulation of OC8-HSL signals is limited by AiiB, but this effect may be bypassed by the addition of an excess of OC8-HSL (Fig. 2); and iv) in an *aiiB* mutant, the Ti plasmid conjugation is increased in vitro (Fig. 3) and in planta (Table 1), as well as the number of emerging tumors (Fig. 4). From these observations, it can be proposed that the TiC58 plasmid-encoded AiiB lactonase contributes to the modulation of QS signaling in *A. tumefaciens* C58. The function of this lactonase cannot be described as a simple on-off regulator but as a fine modulator that essentially delays the release of OC8-HSL in *A. tumefaciens* C58 (Fig. 2) and the horizontal transfer of Ti plasmid (Fig. 3). Noticeably, PCR screening suggests that occurrence of the *aiiB* gene would be rare among representative strains of the *Agrobacterium* genus as well as among soil populations. It was amplified only in a couple of strains, including the biocontrol strain K84. However, one cannot exclude that low-stringency hybridization would permit the detection of other genes encoding AiiB homologs, of which the capacity to inactivate the QS signal should be experimentally tested. Therefore, the AiiB-mediated regulation of QS appears to be a strain-specific characteristic. In the particular case of *A. tumefaciens* C58, the lactonase AiiB is not a unique pathway for downregulation of QS because the crucial implication of the Ti plasmid-encoded regulator TraM is well documented (Luo et al. 2000; Swiderska et al. 2001). Unlike lactonase AiiB that directly acts on OC8-HSL, TraM (via protein-protein interaction with TraR) indirectly limits the production of the OC8-HSL by preventing or inhibiting positive autoregulation on TraI. Questions arise

Fig. 3. In vitro conjugation. Emergence of tumor-inducing (Ti) plasmid transconjugants (upper graph) and concentration of 3-oxo-octanoylhomoserine lactone (OC8-HSL) in culture medium (bottom graph) were monitored in two parallel conjugation assays with recipient strain C58-00 and donor strain C58-105 (*attM*⁺*aiiB*⁻) or C58-107 (*attM*⁺*aiiB*⁺). Four replicates were done.

about the biological role of such QS antagonisms in *Agrobacterium*. Both TraM and AiiB would avoid an excessive and inappropriate accumulation of OC8-HSL in the plant environment and, consequently, an activation of the acyl-HSL-induced plant defenses (Mathesius et al. 2003; Schuëgger et al. 2006). Consistent with this hypothesis, the receptor TraR shows a very high affinity (1 to 2 nM) to OC8-HSL signal (Luo and Farrand 2003), while agrocinopines promote an accumulation of OC8-HSL up to 100 nM in an *A. tumefaciens* C58-derivative defective for AiiB (Fig. 2A).

Regulation and functions of the AttM lactonase are more complex than those of AiiB. This work establishes that AttM

Fig. 4. Virulence assays on tobacco and tomato plants. Photos and graphs show greenhouse virulence assays ($n = 16$) on **A**, tomato and **B**, tobacco plants that were inoculated with *Agrobacterium tumefaciens* C58 wild type (wt) and its derivatives C58-110 (*attM*), C58-102 (*aiiB*), and C58-111 (*attMaiiB*). **C**, Graphs show greenhouse virulence assays ($n = 8$) on tomato plants that were inoculated with *A. tumefaciens* C58 wt and C58-102 derivative (both harboring the pME6010 vector) and with C58-102 complemented with pMIR103 expressing lactonase AiiB. Bacterial genotypes showing statistical differences (Kruskal-Wallis test, $P < 0.01$) in their aggressiveness categories are noted by different letters (a, b, and c). All data are collected from two independent replicates.

mediates the degradation of OC8-HSL in wounded plant tissues (Fig. 2E), suggesting that the AttM lactonase may temporarily inactivate the QS signal after wounding of stem and tumor tissues. However, GABA, which participates in plant response to abiotic and biotic stresses (Shelp et al. 2006), is also accumulated at a high level in unwounded *A. tumefaciens*-induced tumors of tomato plants (Fig. 2C) and *Arabidopsis thaliana* (Deeken et al. 2006); however, the impact of the AttM lactonase on the expression of QS functions is weak (Table 1; Fig. 4). This observation was also reported by Khan and Farrand (2009). Three nonexclusive hypotheses may be proposed: i) the AttM-mediated inactivation of OC8-HSL would be balanced by a strongest synthesis of this signal in plant tumors; ii) the decrease of the competitiveness of *attM* defective mutant in plant tumors (Fig. 5) would contribute to underestimate the conjugation competence of this bacterial genotype; and iii) the GABA-controlled expression of *attKLM* in planta would be attenuated by another plant signal. The hypothesis of a mechanism that downregulates the GABA-mediated expression of *attKLM* operon in plant tumors is supported by the following observation: expression of *attKLM* was lower in the presence of tumor than stem tissues (Fig. 2D) while the level of GABA was higher in tumor than stem tissues (Fig. 2C).

In addition to its involvement in the inactivation of the QS signal, the lactonase AttM, together with AttK and AttL, contribute to assimilation of GBL and GHB as carbon sources (Carlier et al. 2004). Competition assays demonstrated that the *attM* gene was advantageous for the agrobacterial host in the tumor environment but neutral in the rhizosphere (Fig. 5). This feature reveals a selective advantage conferred by the *At* plasmid to agrobacteria in plant tumors and may participate in maintenance of the *attM* gene among *A. tumefaciens* populations. Remarkably, the *attKLM* operon is also present in the

Fig. 5. Competition assay in rhizosphere and tumor of tomato plants. Colonization of **A**, tomato rhizosphere and **B**, tumors by total cultivable bacteria (open squares) and agrobacteria (full squares) was expressed as CFU/g fresh weight of **A**, root or **B**, tumor. At each time, three independent samples were examined and standard deviation was lower than 10% of mean values. In the **C**, rhizosphere and **D**, tumors, the percentage of both competitors C58-107 (*attM⁺aiiB⁺*) (gray bars) and C58-110 (*attM⁻aiiB⁺*) (white bars) was calculated on at least 48 isolates per sample.

Fig. 6. Model for regulation of the synthesis and degradation of 3-oxo-octanoylhomoserine lactone (OC8-HSL) by plant signals. γ -Aminobutyrate (GABA) and agrocinoipines penetrate into *Agrobacterium tumefaciens* cells via ABC-transporters (cylinders), while pathways for the transportation of GBL, γ -hydroxybutyrate (GHB), and succinic semialdehyde (SSA) are still not known. The enzyme allowing conversion of GABA into SSA is also unknown in *A. tumefaciens*. Although agrocinoipines are required for synthesis of OC8-HSL signals, they also contribute to trigger the expression of lactonase AiiB; the *attKLM* operon converts GBL, GHB, and SSA into succinate (Suc) that can enter into TCA cycle. The repressing activity of AttJ is modulated by SSA and GHB. Both of the lactonases AttM and AiiB would act as modulator of the level of OC8-HSL. The dotted and double arrows indicate regulatory pathways and enzyme reactions, respectively. OC8HS, 3-oxo-octanoyl-homoserine; SAM, S-adenosyl methionine.

nodule inducers *R. leguminosarum* biovar *viciae* 3841 (Young et al. 2006) and *Azorhizobium caulinodans* ORS571 (Lee et al. 2008). In the case of *R. leguminosarum* 3841, the lactonase activity toward NAHL was experimentally confirmed when AttM-homolog was expressed in *E. coli* (Riaz et al. 2008). Both *Agrobacterium tumefaciens*-induced plant tumors (Deeken et al. 2006) (Fig. 3A) and N-fixing nodules (Miller et al. 1991; Prell et al. 2002; Vance and Heichel 1991) are GABA-rich plant environments. Therefore, byproducts of GABA metabolism, such as SSA, GHB, and GBL (Bouché and Fromm 2004) would accumulate in both nodules and tumors and would be assimilated by agrobacteria and rhizobia via *attKLM* operon; the precise level of GHB and GBL remains to be experimentally determined in both nodule and tumor tissues.

In conclusion, the biological roles of the *A. tumefaciens* lactonases AttM and AiiB are diverse. They appear to be fine modulators of QS-regulated functions and, thus, should be integrated in a regulation model of QS in *A. tumefaciens* C58 (Fig. 6). Whereas the TraI-mediated synthesis of OC8-HSL is controlled by a regulatory cascade (implicating AccR, TraR, and TraM) that is initiated by the T-DNA-encoded agrocinoipines A and B, the inactivation of OC8-HSL is controlled by diverse signals, such as agrocinoipines in the case of AiiB and stress-induced plant compounds, such as GABA, GHB, and salicylic acid, in the case of AttM. The combination of all these signals likely contributes to an optimization of the expression of virulence functions in *A. tumefaciens* C58 (Yuan et al. 2008). Additionally, AttM confers a selective advantage to *A. tumefaciens* in the tumor environment, a feature that is probably related to the assimilative function of the *attKLM* operon.

MATERIALS AND METHODS

Strains, media, and chemicals.

Strains are listed in Table 2. *E. coli* cells were grown at 37°C in Luria-Bertani modified (LBm) medium, in which the NaCl concentration was 5 g/liter. *A. tumefaciens* strains were

Table 2. Bacterial strains and plasmids

Strains ^a	Relevant characteristics ^b	Source or reference
<i>E. coli</i>	supE44 Δ lacU169(Φ 80lacZ Δ M15) hsdR17deoR recA1 endA1 gyrA96 thi-1 relA1	Hanahan et al. 1983
<i>A. tumefaciens</i>		
C58	Wild type, containing Ti (nopaline/agrocinoipines) and At plasmids, producing OC8-HSL	Laboratory collection
C58-C	C58 derivative, free of Ti plasmid, Rf ^r	This study
C58-00	C58 derivative, free of Ti and At plasmids, Rf ^r and Sm ^r	Laboratory collection
C58-102	C58 derivative, <i>aaiB::aphA</i> , Km ^r	This study
C58-105	C58 derivative, <i>aaiB::acc1</i> , Gm ^r	This study
C58-107	C58 derivative, <i>atu6148::acc1</i> , Gm ^r	This study
C58-110	C58 derivative, <i>attM::acc1</i> , Gm ^r	Chevrot et al. 2006
C58-111	C58-102 derivative, <i>attM::acc1 aaiB::aphA</i> , Km ^r Gm ^r	This study
C58-115	C58 derivative, <i>traI::acc1</i> , Gm ^r	This study
C58-121	C58 derivative, <i>accR::acc1</i> , Gm ^r	This study
C58-127	C58 derivative, <i>aaiB::lacZ</i> fusion on Ti plasmid, Km ^r	This study
C58-132	C58-121 derivative, <i>aaiB::lacZ</i> fusion on Ti plasmid, Km ^r Gm ^r	This study
K476	C58 derivative, <i>accA::Tn3HoHo1</i> , Cb ^r	Kim and Farrand 1997
NT1(pZLR4)	Harboring fusion <i>traG::lacZ</i> , OC8-HSL biosensor, Gm ^r	Piper et al. 1993
Plasmids		
pKm34S	Source of Km-cassette	Dennis and Zylstra 1998
pGm34S	Source of Gm-cassette	Dennis and Zylstra 1998
pKOK5	Source of Km- <i>lacZ</i> cassette, Km ^r Ap ^r	Kokotek et al. 1989
pME6010	Broad host-range vector, pVS1 derivative, low copy number, Tc ^r	Heeb et al. 2000
pME6031	Broad host-range vector, pVS1 derivative, low copy number, Tc ^r	Heeb et al. 2000
pMIR103	pME6010 expressing <i>aaiB</i>	Carlier et al. 2003
pMIR124	pME6031 harboring <i>traI::lacZ</i> fusion	This study
pMIR163	pME6031 harboring <i>orfA::lacZ</i> fusion	This study
pMIR122	pME6031 harboring <i>attK::lacZ</i> fusion	Carlier et al. 2004

^a *E. coli* = *Escherichia coli* DH5 α and *A. tumefaciens* = *Agrobacterium tumefaciens* C58 derivatives.

^b Ti = tumor inducing; OC8-HSL = 3-oxo-octanoyl-homoserine lactone; Rf^r, Sm^r, Km^r, Gm^r, Cb^r, Ap^r, and Tc^r = resistant to rifampicin, kanamycin, gentamycin, carbenicillin, ampicillin, and tetracycline, respectively.

routinely grown at 28°C in AB minimal medium (Chilton et al. 1974) supplemented with mannitol (0.2%), or in LBm or TY rich medium (Beringer 1974). When appropriate, antibiotics were used at the following concentrations: ampicillin, 50 mg/liter; carbenicillin, 100 mg/liter; gentamycin, 20 mg/liter; kanamycin, 50 mg/liter; rifampicin, 100 mg/liter; and tetracycline, 10 mg/liter. All chemicals were from commercial sources, except opines that were synthesized in our laboratory or purified as described below. Agrocinosins A and B were extracted in H₂O (3 ml/g of tissue) from 2 g (fresh weight) of *A. tumefaciens* C58-induced tumors on tomato stems. After centrifugation, the supernatant was lyophilized and dissolved in 1/10 the original volume of H₂O. Crude extract was acidified by addition of 2 M acetic acid (20 mM final concentration) and submitted to high voltage paper electrophoresis (Whatman 3MM paper) in a formic-acetic acid buffer (Ellis and Murphy 1981) for 20 min at 65 V/cm. Silver nitrate staining (Tun-Garrido et al. 2003) was performed on external sections of the paper to visualize the agrocinosins. Electrophoretic mobilities (Em) were evaluated relative to Orange G (Em = 1.0) and xylene cyanol with fructose as the nonmigrating marker. Agrocinosins present in unstained strips of the paper were eluted with water, vacuum-concentrated, and dissolved in a final volume of 500 µl of water.

Construction of mutants and plasmids.

The Km-cassette from p34S-Km (Dennis and Zylstra 1998) was cloned into pBS-SK harboring the 3' and 5' regions of the *aiiB* gene. The resulting plasmid, pMIR112, was electroporated into *A. tumefaciens* C58. Double crossovers were identified by carbenicillin sensitivity assays and verified by diagnostic PCR with appropriate primers. The resulting *aiiB::aphA* mutant was named C58-102. The double mutant *aiiB-attM* (C58-111) was obtained by electroporating the plasmid pMIR112 into the *attM::accI* (C58-110) mutant (Chevrot et al. 2006). Following a procedure similar to that described above, the Gm-cassette from the p34S-Gm plasmid (Dennis and Zylstra 1998) was used to obtain the *aiiB::accI* (C58-105), *atu6148::accI* (C58-107), *traI::accI* (C58-115), and *accR::accI* (C58-121) mutants in *A. tumefaciens* C58.

To construct the *aiiB::lacZ* transcriptional fusion, the DNA fragment containing the 5' region of the *aiiB* gene was amplified (primers 5'-TCTTCCCTCTCATCGCGACC and 5'-GGAAA GAACGGAGATCGGCAA), then cloned into the pGEM-T easy vector, in which the promoterless *aph-lacZ* cassette of pKOK5 (Kokotek and Lotz 1989) was thereafter introduced. The resulting construction was directly electroporated into the *A. tumefaciens* C58 strain and its *accR* and *accA* derivatives to create strains with single crossover Campbell-type insertions. The pMIR124 and pMIR163 plasmids harboring the *traI::lacZ* and *orfA::lacZ* reporter fusions, respectively, were created by amplifying DNA fragments containing the predicted region of each promoter, ligating it into pGEM-Teasy, and then subcloning it into the pME6031 plasmid (Heeb et al. 2000). The promoterless *aphA-lacZ* cassette of pKOK5 vector (Kokotek et al. 1989) was fused downstream of each promoter. The plasmids were finally transferred into *A. tumefaciens* C58 strains by electroporation.

β-Galactosidase assays, acyl-HSL detection, and quantification of GABA.

A. tumefaciens C58 derivatives carrying the *aiiB::lacZ* fusion were grown in AB medium for 20 h. Then, 250 µl of the different cultures were mixed with 500 µl of fresh AB medium, 250 µl of the different compounds to assay (final concentration 1 mM) in a 1-ml final volume, and incubated for 20 h until β-galactosidase activity was measured as described previously

(Carlier et al. 2004). Plant tissues were added at 1 g of fresh tissues per milliliter. Acyl-HSL detection was performed on TLC silica plates (Whatman, C18-reverse phase) as previously described (Shaw et al. 1997) using pure acyl-HSL as standards and the biosensor *A. tumefaciens* NT1 (pZLR4) (Cha et al. 1998). Acyl-HSL-degradation assays were performed by adding OC8-HSL (up to 200 nM) at the beginning of the culture, as previously described (Carlier et al. 2004). To quantify GABA, unwounded and wounded stems and tumors of tomato plants were immediately frozen in liquid nitrogen and extracted in aqueous methanol as previously described (Zhang and Bown 1997). Wounded tissues were generated by cutting 0.1-cm sections from tomato stems and then stored for 10 min at 25°C before being frozen. The composition of individual amino acids (including GABA) was determined by ion-exchange chromatography using the AminoTac JLC-500_V amino acid analyzer (Diaz et al. 2005).

Virulence assays.

In the greenhouse, virulence assays were conducted on stems of 8-week-old tobacco plants (*Nicotiana tabacum* cv. Samson) and 4-week-old tomato plants (*Lycopersicon esculentum* cv. Dona). Each stem was incised (4 cm) with a scalpel and infected by approximately 10⁶ CFU of *A. tumefaciens* C58 or its derivatives that were picked up from 48-h cultures on LBm agar plates. The number of tumors per incision was counted 5 weeks (tobacco) or 3 weeks (tomato) postinoculation. The aggressiveness of the strains was categorized into five classes: less than 5, from 5 to 14, 15 to 29, 30 to 50, or more than 50 tumors per infection site. Each virulence assay was performed in at least two independent experiments.

Conjugation assays.

In vitro tomato plants were infected by various donor *A. tumefaciens* strains harboring an antibiotic resistance gene on their Ti plasmid. The *A. tumefaciens* C58-107 mutant in which a Gm-cassette was inserted into the transposase-encoding gene *atu6148* on Ti plasmid was used as a control. Tumors (2 weeks old) that appeared were infected by the C58-00 recipient strain. The plants were immediately transferred onto fresh MS/2 medium and incubated at 24°C for 2 weeks. Tumors ($n \geq 9$) were crushed into sterile 0.8% NaCl solution and suspension dilutions were spotted onto LBm agar with the appropriate antibiotics for enumerating donors, recipients, and transconjugants. All transconjugants were verified by amplification of selected genetic markers.

For in vitro conjugation assay, donor (C58-107 and C58-105) and recipient (C58-00) strains were precultivated in AB medium in the absence of opines, then mixed at 1/1 ratio in AB medium (200 µl) supplemented with purified agrocinosins and incubated at 24°C. Four replicates were done. Suspension dilutions were spotted onto LBm agar with the appropriate antibiotics for enumerating donors, recipients, and transconjugants. Transconjugants were verified by amplification of selected genetic markers.

Competition assays.

Competitions were performed in tumor and rhizosphere compartments of tomato plants (*L. esculentum* cv. Dona). In the case of competition in plant tumors, bacterial suspension densities of each competitor were adjusted in 0.8% NaCl, the suspensions mixed in equal ratio, and then inoculated onto LBm plates that were incubated at 28°C for 48 h. Those mixed cultures were used to infect 5-week-old plants as described above in virulence assay.

In the case of competition in the rhizosphere, the two competitors were mixed in equal ratio and a unique cell suspension

was used to inoculate a nonsterile soil mixture (Loire River sand and compost, 1:1, vol/vol) to obtain a cell number of 10^4 CFU/g of soil. Tomato seed that were preliminarily germinated on sterilized soil were transplanted into pots containing the inoculated soil.

At appropriate times, three independent samples (tumor or rhizosphere) were harvested, crushed, suspended, and diluted in 0.8% NaCl for spreading on appropriate medium for counting. Total bacteria were enumerated on TY medium (Beringer 1974), while the inoculated agrobacteria were enumerated on tellurite medium (Mougel et al. 2001) supplemented with gentamycin because all competitors harbor the same Gm^r-cassette (*accI*). The percentage of each competitor was estimated on at least 48 randomly chosen isolates that were individually identified by PCR amplification of specific markers.

Isolation of rhizospheric *A. tumefaciens* (biovar1).

Disinfected seeds of tobacco and tomato lines were germinated in a soil mixture (1/1, vol/vol) consisting of sterile Loire River sand and nonsterile soil from the CNRS experimental field (Mérantaise, Gif-sur-Yvette), which was the source of soil bacteria. The pots (20 cm in diameter) were placed in the greenhouse under long-daylight conditions (16 h) at 17°C (night) and 24°C (day) and watered with tap water twice a day. Each pot contained three tobacco or tomato plants. Fifteen weeks after planting, three pots per condition (bare soil, tomato rhizosphere, and tobacco rhizosphere) were chosen for analysis of soil and rhizospheric samples. From each pot, 1 g of soil sample or root with adhering soil was resuspended in 10 ml of 0.8% sterile NaCl by very vigorous shaking for 3 min, and the resulting suspension was serially diluted. Appropriate dilutions were spread onto plates containing either the nonselective TY medium or the selective medium for agrobacteria amended with K₂TeO₃ (Mougel et al. 2001). After an incubation of 96 h at 24°C, total cultivable heterotrophic bacteria were counted as CFU.

Natural occurrence of *attM* and *aiiB* genes.

The presence (+) or absence (–) of *attM* and *aiiB* genes was investigated by PCR amplification in several strains, including type strains belonging to *Agrobacterium* spp. as well as in the genomic draft of *A. vitis* S4 and *A. radiobacter* K84 (also referred as *R. rhizogenes* K84). Pairs of primers 5'-GCAACAGATCCGCTGGCCATTGG and 5'-TAATAATCGTCCTGCGCATCGTTG and 5'-GGCAGATTGCATGCCGACGC and 5'-TGAACCAGATCGCGTGACTT were used to amplify *attM* and *aiiB* genes, respectively, under standard PCR conditions as described by D'Angelo-Picard and associates (2005).

ACKNOWLEDGMENTS

We thank X. Nesme (University of Lyon, France) for providing the *Agrobacterium* reference strains from all the genomic groups and to S. K. Farrand (University of Illinois, U.S.A.) for mutant *accA*, as well as for fruitful discussions. This work is supported by Centre national de la Recherche Scientifique (France).

LITERATURE CITED

Beck von Bodman, S., Hayman, G. T., and Farrand, S. K. 1992. Opine catabolism and conjugal transfer of the nopaline Ti plasmid pTiC58 are co-ordinately regulated by a single repressor. *Biochemistry* 89:643-647.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* 84:188-198.

Bouché N, and Fromm, H. 2004. GABA in plants: Just a metabolite? *Trends Plant Sci.* 9:110-115.

Carlier, A., Uroz, S., Smadja, B., Latour, X., Fray, R., Dessaux, Y., and Faure, D. 2003. The Ti plasmid of *Agrobacterium tumefaciens* harbors

an *attM*-paralogous gene, *aiiB*, also encoding N-acyl homoserine lactonase activity. *Appl. Environ. Microbiol.* 69:4989-4993.

Carlier, A., Chevrot, R., Dessaux, Y., and Faure, D. 2004. The assimilation of γ -butyrolactone in *Agrobacterium tumefaciens* C58 interferes with the accumulation of the N-acyl-homoserine lactone signal. *Mol. Plant-Microbe Interact.* 17:951-957.

Cha, C., Gao, P., Chen, Y. C., Shaw, P. D., and Farrand, S. K. 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol. Plant-Microbe Interact.* 11:1119-1129.

Chai, Y., Tsai, C. C., Cho, H., and Winans, S. C. 2007. Reconstitution of the biochemical activities of the AttJ repressor and the AttK, AttL and AttM catalytic enzymes of *Agrobacterium tumefaciens*. *J. Bacteriol.* 189:3674-3679.

Chevrot, R., Rosen, R., Haudecoeur, E., Cirou, A., Shelp, B. J., Ron, E., and Faure, D. 2006. GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. U.S.A.* 103:7460-7464.

Chilton, M. D., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P., and Nester, E. W. 1974. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc. Natl. Acad. Sci. U.S.A.* 71:3672-3676.

D'Angelo-Picard, C., Faure, D., Penot, I., and Dessaux, Y. 2005. Diversity of N-acylhomoserine lactone-producing and -degrading bacteria in soil and tobacco rhizosphere. *Environ. Microbiol.* 7:1796-1808.

Deeken, R., Engelmann, J. C., Efetova, M., Czirjak, T., Müller, T., Kaiser, W. M., Tietz, O., Kriskhe, M., Mueller, M. J., Palme, K., Dandekar, T., and Hedrich, R. 2006. An integrated view of gene expression and solute profiles of *Arabidopsis* tumors: A genome-wide approach. *Plant Cell* 18:3617-3634.

Dennis, J. J., and Zylstra, G. J. 1998. Plasmids: Modular self-cloning minitransposon derivatives for rapid genetic analysis of gram negative bacterial genomes. *Appl. Environ. Microbiol.* 64:2710-2715.

Diaz, C., Purdy, S., Christ, A., Morot-Gaudry, J. F., Wingler, A., and Masclaux-Daubresse, C. 2005. Characterization of markers to determine the extent and variability of leaf senescence in *Arabidopsis*. A metabolic profiling approach. *Plant Physiol.* 138:898-908.

Ellis, J. G., and Murphy, P. J. 1981. Four new opines from crown gall tumours-their detection and properties. *Mol. Gen. Genet.* 181:36-43.

Escobar, M. A., and Dandekar, A. M. 2003. *Agrobacterium tumefaciens* as an agent of disease. *Trends Plant Sci.* 8:380-386.

Fuqua, C., and Winans, S. C. 1996. Localization of OccR-activated and TraR-activated promoters that express two ABC-type permeases and the traR gene of Ti plasmid pTiR10. *Mol. Microbiol.* 20:1199-1210.

Heeb, S., Itoh, Y., Nishijyo, T., Schneider, U., Kell, C., Wade, J., Walsh, U., O'Gara, F., and Haas, D. 2000. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. *Mol. Plant-Microbe Interact.* 13:232-237.

Khan, S. R., and Farrand, S. K. 2009. The BlecC (AttM) lactonase of *Agrobacterium tumefaciens* does not quench the quorum-sensing system that regulates Ti plasmid conjugative Transfer. *J. Bacteriol.* 191:1320-1329.

Kim, H., and Farrand, S. K. 1997. Characterization of the acc operon from the nopaline-type Ti plasmid pTiC58, which encodes utilization of agrocinopines A and B and susceptibility to agrocin 84. *J. Bacteriol.* 179:7559-7572.

Kokotek, W., and Lotz, W. 1989. Construction of a *lacZ*-kanamycin-resistance cassette, useful for site-directed mutagenesis and as a promoter probe. *Gene* 84:467-471.

Lee, K. B., De Backer, P., Aono, T., Liu, C. T., Suzuki, S., Suzuki, T., Kaneko, T., Yamada, M., Tabata, S., Kupfer, D. M., Najar, F. Z., Wiley, G. B., Roe, B., Binnewies, T. T., Ussery, D. W., D'Haese, W., Herder, J. D., Gevers, D., Vereecke, D., Holsters, M., and Oyaizu, H. 2008. The genome of the versatile nitrogen fixer *Azorhizobium caulinodans* ORS571. *BMC Genomics* 9:271.

Li, P. L., and Farrand, S. K. 2000. The replicator of the nopaline-type Ti plasmid is a member of the *repABC* family and is influenced by the TraR-dependent quorum-sensing regulatory system. *J. Bacteriol.* 182:179-188.

Liu, D., Thomas, P. W., Momb, J., Hoang, Q. Q., Petsko, G. A., Ringe, D., and Fast, W. 2007. Structure and specificity of a quorum-quenching lactonase (AiiB) from *Agrobacterium tumefaciens*. *Biochemistry* 46:11789-11799.

Luo, Z. Q., and Farrand, S. K. 2003. In situ activation of the quorum-sensing transcription factor TraR by cognate and noncognate acyl-homoserine lactone ligands: Kinetics and consequences. *J. Bacteriol.* 185:5665-5672.

Luo, Z. Q., Qin, Y., and Farrand, S. K. 2000. The inactivator TraM interferes with the autoinducer-dependent binding of TraR to DNA by interacting with the C-terminal region of the quorum-sensing activator. *J. Biol. Chem.* 275:7713-7722.

- Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Caetano-Anolles, G., Rolfe, B. G., and Bauer, W. D. 2003. Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proc. Natl. Acad. Sci. U.S.A.* 100:1444-1449.
- Miller, R. W., McRae, D. G., and Joy, K. 1991. Glutamate and gamma-aminobutyrate metabolism in isolated *Rhizobium meliloti* bacteroids. *Mol. Plant-Microbe Interact.* 4:37-45.
- Molina, L., Constantinescu, F., Michel, L., Reimann, C., Duffy, B., and Dfago, G. 2003. Degradation of pathogen quorum-sensing molecules by soil bacteria: A preventive and curative biological control mechanism. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol.* 45:71-81.
- Mougel, C., Cournoyer, B., and Nesme, X. 2001. Novel tellurite-amended media and specific chromosomal and Ti plasmid probes for direct analysis of soil populations of *Agrobacterium* biovars 1 and 2. *Appl. Environ. Microbiol.* 67:65-74.
- Pappas, K. M., and Winans, S. C. 2003. A LuxR-type regulator from *Agrobacterium tumefaciens* elevates Ti plasmid copy number by activating transcription of plasmid replication genes. *Mol. Microbiol.* 48:1059-1073.
- Piper, K. R., Beck von Bodman, S., and Farrand, S. K. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* 362:448-450.
- Piper, K. R., Beck von Bodman, S., Hwang, I., and Farrand, S. K. 1999. Hierarchical gene regulatory systems arising from fortuitous gene associations: Controlling quorum-sensing by the opine regulon in *Agrobacterium*. *Mol. Microbiol.* 32:1077-1089.
- Popoff, M. Y., Kersters, K., Kiredjn, M., Miras, I., and Coynault, C. 1984. Taxonomic positions of *Agrobacterium* strains of hospital origin. *Ann. Microbiol. (Paris)* 135:427-442.
- Prell, J., Boesten, B., Poole, P., and Priefer, U. B. 2002. The *Rhizobium leguminosarum* bv. *viciae* VF39 γ -aminobutyrate (GABA) aminotransferase gene (*gabT*) is induced by GABA and highly expressed in bacteroids. *Microbiology* 148:615-623.
- Qin, Y., Su, S., and Farrand, S. K. 2007. Molecular basis of transcriptional antiactivation. TraM disrupts the TraR-DNA complex through stepwise interactions. *J. Biol. Chem.* 282:19979-19991.
- Riaz, K., Elmerich, C., Moreira, D., Raffoux, A., Dessaux, Y., and Faure, D. 2008. A metagenomic analysis of rhizospheric bacteria extends the diversity of quorum-quenching lactonases. *Environ. Microbiol.* 10:560-570.
- Schell, J., Van Montagu, M., De Beuckeleer, M., De Block, M., Depicker, A., De Wilde, M., Engler, G., Genetello, C., Hernalsteens, J. P., Holsters, M., Seurinck, J., Silva, B., Van Vliet, F., and Villarroel, R. 1979. Interactions and DNA transfer between *Agrobacterium tumefaciens*, the Ti-plasmid and the plant host. *Proc. R. Soc. Lond. Biol. Sci.* 204:251-266.
- Schuegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., Hutzler, P., Schmid, M., Van Breusegem, F., Eberl, L., Hartmann, A., and Langebartels, C. 2006. Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. *Plant Cell Environ.* 29:909-918.
- Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Rinchart, K. L., and Farrand, S. K. 1997. Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin layer chromatography. *Proc. Natl. Acad. Sci. U.S.A.* 94:6036-6041.
- Shelp, B. J., Bown, A. W., and Faure, D. 2006. Extracellular gamma-aminobutyrate mediates communication between plants and other organisms. *Plant Physiol.* 142:1350-1352.
- Swiderska, A., Berndtson, A. K., Cha, M. R., Li, L., Beaudoin, G. M. J., III, Zhu, J., and Fuqua, C. 2001. Inhibition of the *Agrobacterium tumefaciens* TraR quorum-sensing regulator. *J. Biol. Chem.* 276:49449-49458.
- Tun-Garrido, C., Bustos, P., Gonzalez, V., and Brom, S. 2003. Conjugative transfer of p42a from *Rhizobium etli* CFN42, which is required for mobilization of the symbiotic plasmid, is regulated by quorum-sensing. *J. Bacteriol.* 185:1681-1692.
- Vance, C. P., and Heichel, G. H. 1991. Carbon in N₂ fixation: Limitation or exquisite adaptation. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* 42:373-392.
- Wang, C., Zhang, H. B., Wang, L. H., and Zhang, L. H. 2006. Succinic semialdehyde couples stress responses to quorum-sensing signal decay in *Agrobacterium tumefaciens*. *Mol. Microbiol.* 62:45-56.
- Young, J. P., Crossman, L. C., Johnston, A. W., Thomson, N. R., Ghazoul, Z. F., Hull, K. H., Wexler, M., Curson, A. R., Todd, J. D., Poole, P. S., Mauchline, T. H., East, A. K., Quail, M. A., Churcher, C., Arrowsmith, C., Cherevach, I., Chillingworth, T., Clarke, K., Cronin, A., Davis, P., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Mungall, K., Norbertczak, H., Rabinowitsch, E., Sanders, M., Simmonds, M., Whitehead, S., and Parkhill, J. 2006. The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biol.* 7:R34.
- Yuan, Z. C., Edlind, M. P., Liu, P., Saenkham, P., Banta, L. M., Wise, A. A., Ronzone, E., Binns, A. N., Kerr, K., and Nester, E. W. 2007. The plant signal salicylic acid shuts down expression of the *vir* regulon and activates quorum-quenching genes in *Agrobacterium*. *Proc. Natl. Acad. Sci. U.S.A.* 104:11790-11795.
- Yuan, Z. C., Haudecoeur, E., Faure, D., Kerr, K. F., and Nester, E. W. 2008. Comparative transcriptome analysis of *Agrobacterium tumefaciens* in response to plant signals salicylic acid, indole-3-acetic acid, and gamma-amino butyric acid reveals signaling crosstalk and *Agrobacterium*-plant co-evolution. *Cell Microbiol.* 10:2339-2354.
- Zhang, G., and Bown, A. W. 1997. The rapid determination of gamma-aminobutyric acid. *Phytochemistry* 44:1007-1009.
- Zhang, L., Murphy, P. J., Kerr, A., and Tate, M. E. 1993. *Agrobacterium* conjugation and gene regulation by N-acyl homoserine lactones. *Nature* 362:446-448.
- Zhang, H. B., Wang, L. H., and Zhang L. H. 2002. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. U.S.A.* 99:4638-4643.
- Zhang, H. B., Wang, C., and Zhang, L. H. 2004. The quorumone degradation system of *Agrobacterium tumefaciens* is regulated by starvation signal and stress alarmone (p)ppGpp. *Mol. Microbiol.* 52:1389-1401.