The Assimilation of γ -Butyrolactone in *Agrobacterium tumefaciens* C58 Interferes with the Accumulation of the *N*-Acyl-Homoserine Lactone Signal

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Agrobacterium tumefaciens C58 communicates using *N*-acyl-homoserine lactones (acyl-HSL) and contains two lactonase-encoding genes, *attM* and *aiiB*, the products of which are capable of inactivating the acyl-HSL signal. In *A. tumefaciens* A6, the expression of the *attKLM* operon is controlled by the transcriptional repressor encoded by an adjacent gene, *attJ*. An *attJ::Tn5* mutant does not accumulate acyl-HSL because of the constitutive expression of the lactonase AttM, the activity of which inactivates acyl-HSL. In this work, the *attKLM* operon of *A. tumefaciens* C58 was shown to be involved in an assimilative pathway of γ -butyrolactone (GBL), γ -hydroxybutyrate (GHB), and succinate semialdehyde (SSA), in which AttM and AttL are key enzymes for GBL and GHB assimilation. The expression of the *attKLM* promoter was activated in the presence of GBL, GHB, and SSA. Under these conditions, *A. tumefaciens* C58 did not accumulate the acyl-HSL that it naturally synthesizes, and also became able to inactivate exogenous acyl-HSL signals. Therefore, in *A. tumefaciens* C58, the assimilative pathway of γ -butyrolactone interferes with the acyl-HSL signaling.

Additional keywords: At plasmid, *N*-3-oxo-octanoyl-L-homoserine lactone, quorum sensing, Ti plasmid.

N-acyl-homoserine lactones (acyl-HSLs) are diffusible pheromones that are produced by many gram-negative bacteria. Beyond a threshold concentration, these signal molecules trigger the expression of target genes via an interaction with specific transcriptional regulators (Fuqua and Greenberg 2002). Numerous bacterial functions are acyl-HSL dependent, including the expression and horizontal transfer of genes encoding virulence factors in plant and animal pathogens (Whitehead et al. 2001). This cell-to-cell communication system that links cell density to gene regulation is termed quorum sensing (QS) (Fuqua et al. 1994).

The search for QS quenchers (i.e., chemical, biochemical, and biological factors disrupting the acyl-HSL-mediated communication) constitutes an original approach to develop novel, antibiotic-free therapies and treatments against pathogenic bacteria in medicine and agronomy (Camara et al. 2002; Zhang 2002). Several QS quenchers have been identified.

They encompass chemical compounds targeting the acyl-HSL synthesis and the turnover of acyl-HSL receptors (Hentzer et al. 2003; Hoang and Schweizer 1999; Manefield et al. 2002), as well as enzymes cleaving acyl-HSL molecules, such as lactonases and acylases (Dong et al. 2000; Lin et al. 2003), and acyl-HSL-degrading bacteria (Dong et al. 2000; Leadbetter and Greenberg 2000; Lin et al. 2003; Uroz et al. 2003). Transgenic plants that express one of these bacterial lactonases were constructed and appeared to be more resistant to QS pathogens than their wild-type parents (Dong et al. 2001).

The above-mentioned lactonases are zinc-hydrolases belonging to the AiiA family, named according to the enzyme AiiA from *Bacillus* sp. strain 240B1 (Dong et al. 2000). AiiA analogs were discovered in several acyl-HSL-degrading bacterial species (Dong et al. 2002; Park et al. 2003), and in a single acyl-HSL-producing species, the phytopathogen *Agrobacterium tumefaciens* (Carlier et al. 2003; Zhang et al. 2002). In the octopine-type *A. tumefaciens* strain A6, the AttM lactonase is encoded by the At plasmid (Zhang et al. 2002). In *A. tumefaciens* C58, two AiiA lactonases, AttM and AiiB, were identified (Carlier et al. 2003). They are encoded by genes located on the At and nopaline-type Ti plasmids, respectively.

In *A. tumefaciens* A6, the lactonase-encoding gene *attM* is part of the *attKLM* operon that is controlled by the transcriptional repressor AttJ. Upon Tn5 inactivation of *attJ*, the *attKLM* operon was constitutively expressed and the corresponding *A. tumefaciens* mutant did not accumulate *N*-3-oxo-octanoyl-L-homoserine lactone (oxo-C8HSL), suggesting that the lactonase AttM may be implicated in the modulation of the oxo-C8HSL signal (Zhang et al. 2002). In this work, *A. tumefaciens* strain C58 was chosen as a model to investigate additional functions of the *attKLM* operon. This operon encodes an assimilative pathway of γ -butyrolactone (GBL). Several chemicals that activate its transcription were identified, and novel physiological conditions, in which interference between the expression of *attKLM* and the accumulation of acyl-HSLs occurred, were defined.

RESULTS

The genes *attM* and *attL* encode key proteins involved in GBL assimilation.

The nucleotide sequence of the *attJKLM* genes in *A. tumefaciens* A6 is identical to that in *A. tumefaciens* C58. These *attKLM* genes are clustered in an operon (Fig. 1) that is, to date, unique among the sequenced bacterial genomes. The

attM gene encodes a lactonase that opens the γ -butyrolactone ring of acyl-HSL, and belongs to the zinc-hydrolase family (Carrier et al. 2003; Zhang et al. 2002). In silico analysis of the deduced amino acid sequences of the two other genes revealed high identities (up to 71%) between *AttK* and several NAD-succinate semialdehyde (SSA) dehydrogenases, and between *AttL* and several NAD alcohol dehydrogenases (up to 49%). The three enzymes (*AttK*, *AttL*, and *AttM*) encoded by this operon may participate in a degradative pathway of GBL, as suggested by the comparison with the catabolic pathway of GBL known in animals (Mason and Kerns 2003). This eukaryotic pathway involves three enzymatic activities: i) a lactonase that converts GBL into γ -hydroxybutyrate (GHB); ii) a NAD-alcohol dehydrogenase that oxidizes GHB into SSA; and iii) an NAD-SSA dehydrogenase, oxidizing SSA into succinic acid (SA), a compound of the triboxylic acid (TCA) cycle (Figure 2 shows a proposition of such a pathway in *A. tumefaciens* C58). The characterization of the two first enzymatic steps is crucial to identify a degradation pathway of GBL. To experimentally verify the involvement of *AttL* and *AttM* in such a pathway, these two proteins were individually, or simultaneously, expressed in *Escherichia coli*, a species that naturally assimilates SA and SSA. Although the expression of *attL* was sufficient to convert *E. coli* into an efficient GHB-assimilative bacterium, *attL* and *attM* were required simultaneously for the growth of *E. coli* on GBL (Table 1). The expression of *AttL*, however, had no effect on the acyl-HSL signal (Table 1).

The *attKLM* operon is required for the growth of *A. tumefaciens* on GBL.

Degradation assays revealed that *A. tumefaciens* C58 did not assimilate a large spectrum of substituted γ -butyrolactones: γ -valerolactone, homoserine lactone, and C6HSL cannot be used as sole carbon source. However, strain C58 was able to grow on GBL as sole carbon source, while its $\Delta(attJKLM)$ derivative C101 did not. This inability to grow on GBL also was observed when additional copies of the *attJ* gene encoding the *attKLM*-transcriptional repressor were introduced into *A. tumefaciens* C58 (Fig. 3A). The implication of these pAt-encoded genes in the assimilation of GBL was in agreement with the phenotypes of strains C58.C2 and C58.00, both lacking pAt, which also did not grow on GBL. In contrast, strain C58.C1, cured of pTi but still harboring pAt, grew on GBL. The mutant C101 also was affected in the assimilation of GHB and SSA, while no difference was observed between strains

C58 and C101 during their growth on SA (Fig. 3B). Based on all these results, the operon *attKLM* is proposed to be involved in an assimilative pathway of GBL.

GBL, GHB, and SSA stimulate the transcription of *attKLM*.

The low copy-number plasmids pMIR121 and pMIR122, harboring the transcriptional fusions *attJ::lacZ* and *attK::lacZ*, were introduced into *A. tumefaciens* C58 by electroporation. The addition of GBL to bacterial cultures strongly increased the expression of the fusion *attK::lacZ*, while an equivalent supplementation with mannitol had no effect. In contrast, the expression of the *attJ::lacZ* fusion was only slightly affected by GBL supplementation (Fig. 4A). The other lactones, such as γ -valerolactone and homoserine lactone, did not induce the expression of the *attKLM* operon (data not shown), but the two metabolic intermediates of the GBL assimilative pathway, GHB and SSA, were efficient inducers (Fig. 4B).

Interferences between the expression of *attKLM* and the acyl-HSL signals.

When the *attKLM* operon was not induced (e.g. in the presence of mannitol as sole carbon source), the expression of *attK::lacZ* transcriptional fusion barely was affected by the addition of C6HSL and oxo-C8HSL, and the concentrations of oxo-C8HSL are equivalent in the cultures of *A. tumefaciens* C58 and its derivative C101 (Fig. 5). These results suggested that, in the absence of *attKLM* inducers in AB-mannitol medium, the expression of the *attKLM* operon is not induced by and does not affect the acyl-HSL concentra-

Fig. 1. Schematic representation of the *attJKLM* region of *Agrobacterium tumefaciens* C58 and relevant plasmids used in this study. *A. tumefaciens* C101 is an *A. tumefaciens* C58 derivative in which the *attJKLM* genes were replaced by *aph* gene conferring kanamycin resistance. The double lines represent the DNA of cloning vectors, while single lines and open boxes represent pAt-DNA from *A. tumefaciens* C58. The genes are not drawn to scale relative to each other. Pk and Plac are strong bacterial promoters.

Fig. 2. Proposal for γ -butyrolactone (GBL)-assimilation pathway in *Agrobacterium tumefaciens* C58. The capacity of lactonase AttM to inactivate *N*-acyl-homoserine lactone (acyl-HSL), such as *N*-3-oxo-octanoyl-L-homoserine lactone ($R = NH-CO-CH_2-CO-(CH_2)_4-CH_3$) and C6HSL ($R = NH-CO-(CH_2)_4-CH_3$), was described previously (Carrier et al. 2003; Zhang et al. 2002). The role of lactonase AttM, γ -hydroxybutyrate (GHB)-dehydrogenase AttL, and succinate semialdehyde (SSA)-dehydrogenase AttK in GBL-assimilative pathway of *A. tumefaciens* C58 is discussed in this work.

tion in *A. tumefaciens* C58 cultures. In contrast, when this bacterium grew in AB-GBL medium (data not shown) and in AB-mannitol medium, which was supplemented with the *attK* inducers SSA, GHB, or GBL, it did not accumulate oxo-C8HSL, whereas it did when grown in AB-mannitol supplemented with SA (Fig. 6). Moreover, *A. tumefaciens* C58 cells, in which the *attKLM* operon was induced by GBL, became able to inactivate exogenous C6HSL and oxo-C8HSL signals (Table 2). This acyl-HSL inactivating activity also was observed when the *A. tumefaciens* C58 cells were induced by GHB and SSA, whereas incubation in the presence of SA had no effect (Table 2). In similar experiments, the mutant C101 appeared to be insensitive to the addition of inducers, confirming the genetic link between the disappearance of acyl-HSL in *A. tumefaciens* C58 cultures and the expression of the GBL-degradation pathway encoded by *attJKLM* genes.

Fig. 3. Growth of *Agrobacterium tumefaciens* on γ -butyrolactone (GBL), γ -hydroxybutyrate (GHB), succinic acid (SA), and succinate semialdehyde (SSA). **A**, Growth of *A. tumefaciens* C58 (closed squares), *A. tumefaciens* C101 (open squares), and *A. tumefaciens* C58 harboring p6000 (closed triangles) or pMIR113 (open triangles), was monitored in AB-GBL medium. **B**, Growth curves of *A. tumefaciens* C58 (closed squares) and C101 (open squares) in AB-GHB, AB-SSA, and AB-SA were compared. The experiments were done in triplicate; standard deviations were lower than symbol size.

DISCUSSION

A. tumefaciens C58 communicates using oxo-C8HSL and contains two lactonase-encoding genes, *attM* and *attB* (Carlier et al. 2003). In *A. tumefaciens* A6, the *attKLM* operon seems to be implied in the turnover of oxo-C8HSL (Zhang et al. 2002). In this article, a novel function of the *attKLM* operon is defined in *A. tumefaciens* C58.

Genetic analysis of the *attJKLM* genes supports their implication in an assimilative pathway for GBL and GHB. Aside from the demonstration that GBL assimilation interferes with QS regulation, this work constitutes the first description of a complete catabolic pathway of GBL in a bacterium (Fig. 2). Comparison between this bacterial pathway and that known in animals (Mason and Kerns 2003) evidences three common enzymatic steps: the opening of the GBL ring to yield GHB, the oxidation of GHB into SSA, and the oxidation of SSA into SA, a compound of the TCA cycle.

Fig. 4. Expression of the *attJ::lacZ* and *attK::lacZ* transcriptional fusions in *Agrobacterium tumefaciens* C58. The β -galactosidase activity of the bacteria was measured from four independent cultures and expressed in Miller units. Prior to β -galactosidase assays, the cells were incubated for 2 h with **A**, increasing concentrations of mannitol (open squares) and γ -butyrolactone (black squares) and **B**, mannitol (open squares), γ -hydroxybutyrate (black squares), and succinate semialdehyde (gray squares). Open circles represent β -galactosidase activity after addition of water as control.

Table 1. Expression of *attL* and *attM* in *Escherichia coli*

Plasmids harbored by <i>E. coli</i> DH5 α		Growth on different compounds as sole carbon source ^a				
Name	Expressed genes	SA	SSA	GHB	GBL	Inactivation ^b
None	None	1.07 $\pm$ 0.02	0.19 $\pm$ 0.01	...	...	No
pGEM	None	0.85 $\pm$ 0.01	0.24 $\pm$ 0.01	...	...	No
pMIR117	<i>attL</i>	0.90 $\pm$ 0.02	0.33 $\pm$ 0.01	0.88 $\pm$ 0.01	...	No
pMIR102	<i>attM</i>	0.84 $\pm$ 0.02	0.27 $\pm$ 0.01	...	...	Yes
pMIR117 and p6010	<i>attL</i>	0.81 $\pm$ 0.04	0.30 $\pm$ 0.05	0.54 $\pm$ 0.02	...	No
pMIR117 and pMIR102	<i>attL</i> , <i>attM</i>	0.83 $\pm$ 0.02	0.23 $\pm$ 0.01	0.46 $\pm$ 0.01	0.82 $\pm$ 0.14	Yes

^a Optical density at 600 nm was measured daily during 3 days, only the final mean values were shown. SA = succinic acid, SSA = succinate semialdehyde, GHB = γ -hydroxybutyrate, GBL = γ -butyrolactone.

^b Inactivation of *N*-acyl-homoserine lactone (acyl-HSL) signal measured after 24 h of incubation in presence of C6HSL at 25 μ M; ... = no growth.

In *A. tumefaciens*, these enzymatic reactions are likely to be catalyzed by AttM (γ -butyrolactonase), AttL (homolog to alcohol dehydrogenases IV of COG1454), and AttK (homolog to NAD-dependent dehydrogenases of COG1012), respectively. The COG families were defined by Tatusov and associates (2001). Unlike SA and SSA, which are common intermediates in several bacterial and eukaryotic pathways, the occurrence of GHB and GBL in nature is quite limited. GHB essentially is described as the immediate catabolic intermediate of GBL, and as a psychoactive substance (Mason and Kerns 2003). On the other hand, GBL is produced for industrial purposes, and is found in plants (Lee and Shibamoto 2000) and in fermented beverages such as wines (Vose et al. 2000). The fact that *A. tumefaciens* evolved a GBL-catabolic operon strongly suggests that it may be confronted by this compound during its life in soils and on plant surface. In agreement with this hypothesis, proteome analysis showed that the proteins AttM and AttK were expressed by *A. tumefaciens* C58 when it was exposed to tomato roots (Rosen et al. 2003). Indeed, the catabolic property conferred by the *attKLM* genes could confer a selective advantage upon *A. tumefaciens* populations in such plant environments. However, this assertion needs to be investigated further.

In relation with the above problem, costs and benefits of the expression of *attKLM* should be examined, especially because this operon encodes the lactonase AttM that inactivates the acyl-HSL signals (Fig. 2). When the *attKLM* operon was not induced (i.e., in the absence of GBL, GHB, or SSA), the concentrations of oxo-C8HSL were identical in the culture supernatants of the wild-type strain C58 and its *attJKLM* mutant. Moreover, the acyl-HSLs, even added in excess to the growth media, do not induce the expression of the *attKLM* operon. All these features indicate that this operon would not participate, under these experimental conditions (in the absence of *attKLM*-inducers and *traR*-inducers), in the modulation of acyl-HSL level in *A. tumefaciens* C58. In *A. tumefaciens* A6, it was reported that the same operon may be implied in the turnover of oxo-C8HSL because its expression was induced when cells reached stationary phase (Zhang et al. 2002). In the case of *A. tumefaciens* C58, such growth-phase-dependent regulation of the *attKLM* operon remains to be carefully analyzed before any premature comparison with *A. tumefaciens* A6. However, it should be underlined that *A. tumefaciens* C58 contains a second lactonase-encoding gene, *aiiB* (Carlier et al. 2003), that also might contribute to the modulation of the oxo-C8HSL signal.

Fig. 5. *N*-acyl-homoserine lactone (acyl-HSL) accumulation in the absence of *attKLM*-inducers. In all these experiments, the *Agrobacterium tumefaciens* strains were cultivated in AB-mannitol. **A**, Concentration of *N*-3-oxo-octanoyl-L-homoserine lactone (oxo-C8HSL) in culture medium was compared during the growth of *A. tumefaciens* C58 (closed squares) and C101 (open squares). **B**, Expression of the *attK::lacZ* fusion was measured in *A. tumefaciens* C58, in the presence of increasing concentrations of C6HSL (open squares) and oxo-C8HSL (closed squares). Open circle represents β -galactosidase activity after addition of water as control.

In the presence of GBL, GHB, or SSA, *A. tumefaciens* C58 clearly inactivates endogenous and exogenous acyl-HSL signals, because the expression of the *attKLM* operon and, more precisely, the expression of the lactonase AttM is induced. The large spectrum of acyl-HSL cleaved by this enzyme (Carlier et al. 2003; Zhang et al. 2002) suggests that *A. tumefaciens* C58, under *attKLM* induction conditions, is able to

Fig. 6. *N*-acyl-homoserine lactone (acyl-HSL) level in the presence of *attKLM*-inducers. In all these experiments, the *Agrobacterium tumefaciens* strains were grown in AB-mannitol, supplemented with succinic acid (SA), succinate semialdehyde (SSA), γ -hydroxybutyrate (GHB), and γ -butyrolactone (GBL) at 0.2 g/liter. When cells reached stationary phase, after 15, 24, and 39 h of culture, **A**, the expression of the *attK::lacZ* fusion was measured in *A. tumefaciens* C58 harboring pMIR122, whereas **B**, the concentration of *N*-3-oxo-octanoyl-L-homoserine lactone (oxo-C8HSL) was evaluated in *A. tumefaciens* C58 culture medium. In the presence of SSA, GHB, and GBL, the *attK::lacZ* fusion was highly expressed, whereas a low concentration of oxo-C8HSL was detected in culture supernatants of *A. tumefaciens* C58.

disrupt any acyl-HSL signal; therefore, it becomes an efficient acyl-HSL-degrading bacterium. The *attM*-paralogous gene *aiiB*, that also encodes lactonase activity directed toward acyl-HSLs (Carlier et al. 2003), likely does not participate in this inducible acyl-HSL-degrading activity, because the *attJKLM* mutant that still harbors the *aiiB* gene on its pTi was unable to degrade acyl-HSL even in the presence of *attKLM* inducers. However, the physiological conditions in which the *aiiB* gene is expressed remain to be clarified before any role can be attributed to this gene and to the encoded peptide.

A few functions only are known to be determined by At plasmid genes (Mathysse and McMahan 2001; Nair et al. 2003; Vaudequin-Dransart et al. 1998), whereas the pTi harbors most of the genes required for plant tumorigenesis (Zhu et al. 2000), as well as those involved in the synthesis (*traI*) and sensing (*traR*) of the QS signal, oxo-C8HSL. At this stage of knowledge about QS regulation in *A. tumefaciens* C58, the Ti plasmid may be regarded as an acyl-HSL emitter, and the At plasmid as an inducible acyl-HSL disrupter. Such a distribution of functions on separate replicons, but within the same bacterial cell, should be considered carefully with respect to further ecological interpretation and integration in the complex QS network of *A. tumefaciens* (Piper et al. 1993; Qin et al. 2000; Swiderska et al. 2001; Zhang et al. 1993; Zhu and Winans 2001). Similarly, novel acyl-HSL hydrolases (i.e., the *N*-acylases belonging to the family AiiD) have been described in different bacteria, such as *Ralstonia* sp. strain XJ12B and *Pseudomonas aeruginosa* PAO1 (Huang et al. 2003; Lin et al. 2003). The determination of the physiological conditions in which these acyl-HSL-degrading enzymes are expressed will constitute valuable and necessary data to assign biological functions to these enzymes, as in the case of the AiiA-lactonases related to AttM (Dong et al. 2002; Park et al. 2003).

MATERIALS AND METHODS

Bacterial strains.

A. tumefaciens C58, as well as its derivatives C58.C1, C58.C2, and C58.00 (Vaudequin-Dransart et al. 1998), which are cured for pTi, pAt, and pTi and pAt, respectively, were used in this study. The $\Delta(attJKLM)::aph$ mutant (Fig. 1), named *A. tumefaciens* C101, was obtained from strain C58 by a standard procedure for gene replacement via the introduction of a suicide plasmid by electroporation (Ugalde et al. 1998). Briefly, the *aph* gene purified from the plasmid p34S-Km (Dennis and Zylstra 1998) was used to replace the *attJKLM* region between nucleotides 143,303 and 146,511 (gi:17938588), according to the pAtC58 sequence (Wood et al. 2001). *A. tumefaciens* NTLR4 (Cha et al. 1998) and *Chromobacterium violaceum* CV026 (McClean et al. 1997) were used as acyl-HSL biosensors for oxo-C8HSL and C6HSL, respectively. *E. coli* DH5 α was the routine host for cloning and gene expression experiments.

Plasmids.

The maps of plasmids used in this study are given in Figure 1. The plasmid pMIR102, that confers the constitutive expression of the lactonase-encoding gene *attM* upon its host, was described previously (Carlier et al. 2003). The *attL* DNA region was amplified using primers 5'-ATACCTGTGCTCGGCCATC and 5'-TGCTGTCAGAAATGGGTCAG, and cloned into the pGEM-T easy cloning vector (Promega Corp., Madison, WI, U.S.A.) to yield pMIR117. Plasmid pMIR113 is a derivative of the broad host-range plasmid pME6000 (Maurhofer et al. 1998), in which an amplified fragment overlapping the *attJ* gene (primers 5'-CAAACCATCGACGCAATATG and 5'-GCGGGA-TCCCTGGGGTATTGG) was cloned at the *Bam*HI and *Pst*I sites. The divergent promoters of *attJ* and *attKLM* were amplified as a single DNA fragment (primers 5'-TCAGCCATGCA-CTATCCTTGA and 5'-GTCTAGCCATCCGCGGCTT) and fused with the promoterless *lacZ-aph* cassette of pKOK5 (Kokotek and Lotz 1989). Then, the *attJ::lacZ* and *attK::lacZ* transcriptional fusions were subcloned as *Sph*I-*Sac*I fragments into the broad host-range vector pME6031 (Heeb et al. 2000) to yield plasmids pMIR121 and pMIR122, respectively.

Growth conditions.

E. coli was cultivated at 37°C in Luria Bertani rich medium and in M9 minimal medium (Sambrook et al. 1989), supplemented with NAD (0.66 g/liter) and the required carbon source (2 g/liter). *A. tumefaciens* strains were cultivated at 30°C in tryptone yeast medium and in AB minimal medium (Chilton et al. 1974), supplemented with NH₄Cl (1g/liter) and one of the following carbon sources (2 g/liter): mannitol, GBL, γ -valerolactone, homoserine lactone, GHB, or SA. In growth experiments, SSA was used at 0.3 g/liter. Mannitol was used as carbon source in all *Agrobacterium* precultures. The growth curves of *A. tumefaciens* and *E. coli* were computed by monitoring the optical density at 600 nm. When appropriate, antibiotics were used at the following concentrations: ampicillin at 50 mg/liter, kanamycin at 50 mg/liter, and tetracycline at 10 mg/liter.

Expression of transcriptional fusion.

The β -galactosidase activity was measured with *o*-nitrophenyl- β -D-galactopyranoside as a substrate, and expressed in Miller units (Sambrook et al. 1989). Four independent cultures were used for each experimental condition. In induction experiments, the cells containing the transcriptional fusions were cultivated in AB-mannitol medium for 24 h. Then, 360 μ l of these cultures were mixed with 40 μ l of the solutions of the compound to assay and incubated for 2 h at 24°C until the β -galactosidase assay was performed.

Acyl-HSL quantification.

The acyl-HSLs were quantified on KC18 reversed thin-layer chromatography (TLC) plates (Whatman, Maidstone, England) by comparison with an appropriate range of standards, as previously described (Cha et al. 1998; McClean et al. 1997). The

Table 2. Inactivation of exogenous *N*-acyl-homoserine lactones (acyl-HSLs) by *Agrobacterium tumefaciens* C58^a

Bacterial strains	Inactivation of C6HSL (25 μ M)					Inactivation of oxo-octanoyl-L-homoserine lactone (10 μ M)	
	Mannitol	GBL	GHB	SSA	SA	Mannitol	GBL
<i>A. tumefaciens</i> C58	No	Yes	Yes	Yes	No	No	Yes
<i>A. tumefaciens</i> C101	No	No	No	No	No	No	No

^a Degradation assay were performed with washed cells of *A. tumefaciens* C58 and C101. Before degradation assay, cells were incubated in the presence of mannitol, γ -butyrolactone (GBL), γ -hydroxybutyrate (GHB), succinate semialdehyde (SSA), and succinic acid (SA). "No" indicates more than 80% of the introduced acyl-HSLs remained after a 6-h incubation time, and "Yes" indicates less than 5% of the introduced acyl-HSLs remained after a 1-h incubation time.

oxo-C8HSL produced by *A. tumefaciens* were extracted with ethyl acetate from 1-ml or 8-ml samples of *Agrobacterium*–culture supernatants and concentrated 100 times before quantification. An acyl-HSL-disappearance assay was performed in *E. coli* after C6HSL supplementation (25 μ M) to the medium, as previously described (Carlier et al. 2003), and in *A. tumefaciens* after supplementation by C6HSL (25 μ M) and oxo-C8HSL (10 μ M). In these experiments, stationary phase cultures of *A. tumefaciens* were centrifuged and resuspended to a cell density of 5×10^7 CFU/ml into fresh AB medium containing mannitol, GBL, GHB, SSA, or SA as sole carbon source. After a 15-h incubation under shaking, the induced cells were washed with NaCl (0.8%) and inoculated at 10^9 CFU/ml into fresh AB medium supplemented with mannitol (0.2 g/liter) and NH_4Cl (0.1 g/liter), and with C6HSL (25 μ M) or oxo-C8HSL (10 μ M). After 1, 2, 3, and 6 h of incubation, the presence of residual acyl-HSLs in the culture media was assayed as described above. In all degradation assays, the complete disappearance of acyl-HSL was verified by separating samples in a methanol/water (60:40) solvent system used to develop KC18 reversed TLC plates.

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