

# Production of Acyl-Homoserine Lactone Quorum-Sensing Signals by Gram-Negative Plant-Associated Bacteria

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Many gram-negative bacteria regulate expression of specialized gene sets in response to population density. This regulatory mechanism, called autoinduction or quorum-sensing, is based on the production by the bacteria of a small, diffusible signal molecule called the autoinducer. In the most well-studied systems the autoinducers are N-acylated derivatives of L-homoserine lactone (acyl-HSL). Signal specificity is conferred by the length, and the nature of the substitution at C-3, of the acyl side-chain. We evaluated four acyl-HSL bioreporters, based on *tra* of *Agrobacterium tumefaciens*, *lux* of *Vibrio fischeri*, *las* of *Pseudomonas aeruginosa*, and pigment production by *Chromobacterium violaceum*, for their ability to detect sets of 3-oxo acyl-HSLs, 3-hydroxy acyl-HSLs, and alkanoyl-HSLs with chain lengths ranging from C<sub>4</sub> to C<sub>12</sub>. The *traG::lacZ* fusion reporter from the *A. tumefaciens* Ti plasmid was the single most sensitive and versatile detector of the four. Using this reporter, we screened 106 isolates representing seven genera of bacteria that associate with plants. Most of the *Agrobacterium*, *Rhizobium*, and *Pantoea* isolates and about half of the *Erwinia* and *Pseudomonas* isolates gave positive reactions. Only a few isolates of *Xanthomonas* produced a detectable signal. We characterized the acyl-HSLs produced by a subset of the isolates by thin-layer chromatography. Among the pseudomonads and erwinias, most produced a single dominant activity chromatographing with the properties of N-(3-oxo-hexanoyl)-L-HSL. However, a few of the erwinias, and the *P. fluorescens* and *Ralstonia solanacearum* isolates, produced quite different signals, including 3-hydroxy forms, as well as active compounds that chromatographed with properties unlike any of our standards. The few positive xanthomonads, and almost all of the agrobacteria, produced small amounts of a compound with the chromatographic properties of N-(3-oxo-octanoyl)-L-HSL. Members of the genus *Rhizobium* showed the greatest diversity, with some producing as few as one and others producing as many as seven detectable signals. Several isolates pro-

duced extremely nonpolar compounds indicative of very long acyl side-chains. Production of these compounds suggests that quorum-sensing is common as a gene regulatory mechanism among gram-negative plant-associated bacteria.

Quorum-sensing, also called autoinduction, is a mechanism by which bacteria regulate the expression of specialized gene sets in response to their cell density. The bacteria gauge the size of their population by sensing the concentration of small, diffusible, signal molecules called autoinducers that they themselves produce. These molecules pass out of and back into the cells, sometimes by simple diffusion, thereby accumulating in the environment as a function of the growth of the cells. At some threshold concentration, corresponding to a critical population density, these signals serve as co-inducers to regulate transcription of target genes, the products of which presumably are of benefit to the bacteria in the particular habitat.

Autoinduction first was described in the marine symbiotic bacterium *Vibrio fischeri* (Nealson et al. 1970). This organism, which colonizes the light organs of certain marine fish and cephalopods, regulates expression of genes encoding bioluminescence in response to population density. LuxR, a transcriptional activator, requires a threshold concentration of the *V. fischeri* autoinducer, VAI, in order to activate transcription of the *lux* operon. In turn, the bacteria produce this autoinducer during their growth in the light organ. According to the current models, once it has reached the necessary concentration, VAI interacts with LuxR, converting the protein into a functional transcriptional activator. LuxR then activates transcription of the *lux* operon, the first gene of which is *luxI*. LuxI, the protein coded for by this gene, catalyzes the synthesis of VAI (for reviews see Dunlap and Greenberg 1991; Fuqua et al. 1994; Fuqua et al. 1996). Thus, activation by autoinduction results in the production of increased amounts of the signal and concomitant amplification of the transcription of the target operon. Although regulation of production of the signal is not part of the initial definition of autoinduction it is common among known quorum-sensing systems. But control of signal synthesis by autoinduction is not a de rigueur element of this regulatory paradigm; some organisms express their *I* genes constitutively (Beck von Bodman and Farrand 1995).

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Over the past few years a number of gram-negative bacteria, representing a range of genera, have been shown to regulate various gene systems by quorum-sensing (see, for example, Throup et al. 1995; Eberl et al. 1996; McClean et al. 1997; Puskas et al. 1997; Swift et al. 1997). Among the better characterized of these, *Agrobacterium tumefaciens* controls conjugal transfer of several plasmid types by quorum-sensing involving the transcriptional activator TraR and the diffusible signal molecule AAI (Piper et al. 1993; Fuqua and Winans 1994). *Pseudomonas aeruginosa* regulates several gene systems, including those required for the production of rhamnolipids (Ochsner and Reiser 1995), and extracellular enzymes and toxins (Passador et al. 1993) by two hierarchical quorum-sensing systems, each with its own transcriptional regulator and diffusible signal molecules (Latifi et al. 1996; Pearson et al. 1997). Another pseudomonad, *P. aureofaciens* 30-84, regulates production of phenazine antibiotics by quorum-sensing (Pierson et al. 1994). These antibiotics have been implicated in the ability of this bacterium to control take-all disease of wheat in disease-suppressive soils (Thomashow and Weller 1988). Four plant pathogens, *Erwinia carotovora* (Jones et al. 1993; Pirhonen et al. 1993), *E. stewartii* (now *Pantoea stewartii*; Beck von Bodman and Farrand 1995), *Ralstonia solanacearum* (Clough et al. 1997), and *Xanthomonas campestris* pv. *campestris* (Barber et al. 1997), regulate the expression of traits involved in pathogenesis by quorum-sensing systems. Significantly, in each case, while the regulatory strategy is similar, the target genes controlled by quorum-sensing are very different.

Most but not all of the autoinducers characterized to date are N-acylated derivatives of L-homoserine lactone (acyl-HSLs). Such acyl-HSLs produced by various organisms can differ with respect to the length of, nature of the substitution at the 3-carbon position of, and presence or absence of one or more unsaturations within, the acyl side-chain. For example, VAI is *N*-(3-oxo-hexanoyl)-L-HSL (Eberhard et al. 1981), while the *Agrobacterium* spp. autoinducer, AAI, is *N*-(3-oxooctanoyl)-L-HSL (Zhang et al. 1993). The active signal molecule produced by *Chromobacterium violaceum* is *N*-hexanoyl-L-HSL (McClean et al. 1997), while the autoinducer synonymous with small bacteriocin produced by some isolates of *Rhizobium* spp. is *N*-(3-hydroxy-7-*cis*-tetradecanoyl)-L-HSL (Gray et al. 1996; Schripsema et al. 1996). Some bacteria produce only a single detectable species of acyl-HSL (McClean et al. 1997) while others, such as *P. aeruginosa* and *R. etli*, can produce as many as seven compounds with detectable activity (Pearson et al. 1994; Winson et al. 1995; Rosemeyer et al. 1998).

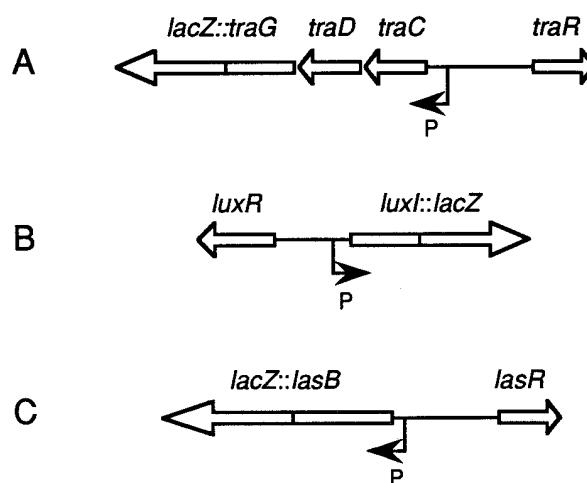
Although several bacteria that interact with plants produce compounds with acyl-HSL activity, there has been no systematic survey of this group of organisms for the production of these signal molecules. In this study we assessed the capacity of four reporter systems to detect a variety of acyl-HSL signal molecules differing with respect to the lengths of the side-chain and the nature of the substitution at the 3-carbon position. We used a qualitative, petri dish-based bioassay to screen 106 isolates of plant-associated, gram-negative bacteria representing seven genera for production of compounds with acyl-HSL signaling activity. We then assessed the complements of acyl-HSLs produced by a subset of these isolates by thin-layer chromatography (TLC).

## RESULTS

### Assessing reporter systems.

Acyl-HSL signal molecules are produced and are active at very low concentrations. This precludes the use of most specific and nonspecific chromogenic reagents for detecting these signals in culture supernatants. Moreover, these compounds lack chromophores that strongly absorb light in the visible and UV wavelengths. Thus, these compounds are usually detected by bioassays with, as indicators, constructs in which an exogenously supplied acyl-HSL is required to express an easily detectable trait. We tested four systems for their capacity to detect acyl-HSLs. Three of these, *tra*, *lux*, and *las*, use fusions of the regulated promoter to *lacZ* and can be assayed by any of the methods developed for detecting  $\beta$ -galactosidase activity. The schematic structures of these reporters are shown in Figure 1. The fourth, *Chromobacterium violaceum*, controls the production of violacein, a violet pigment, by quorum-sensing (McClean et al. 1997). Strain CV026blu is a mutant of the wild-type strain that is unable to produce its own acyl-HSL signal but responds to certain exogenous active signal molecules (McClean et al. 1997).

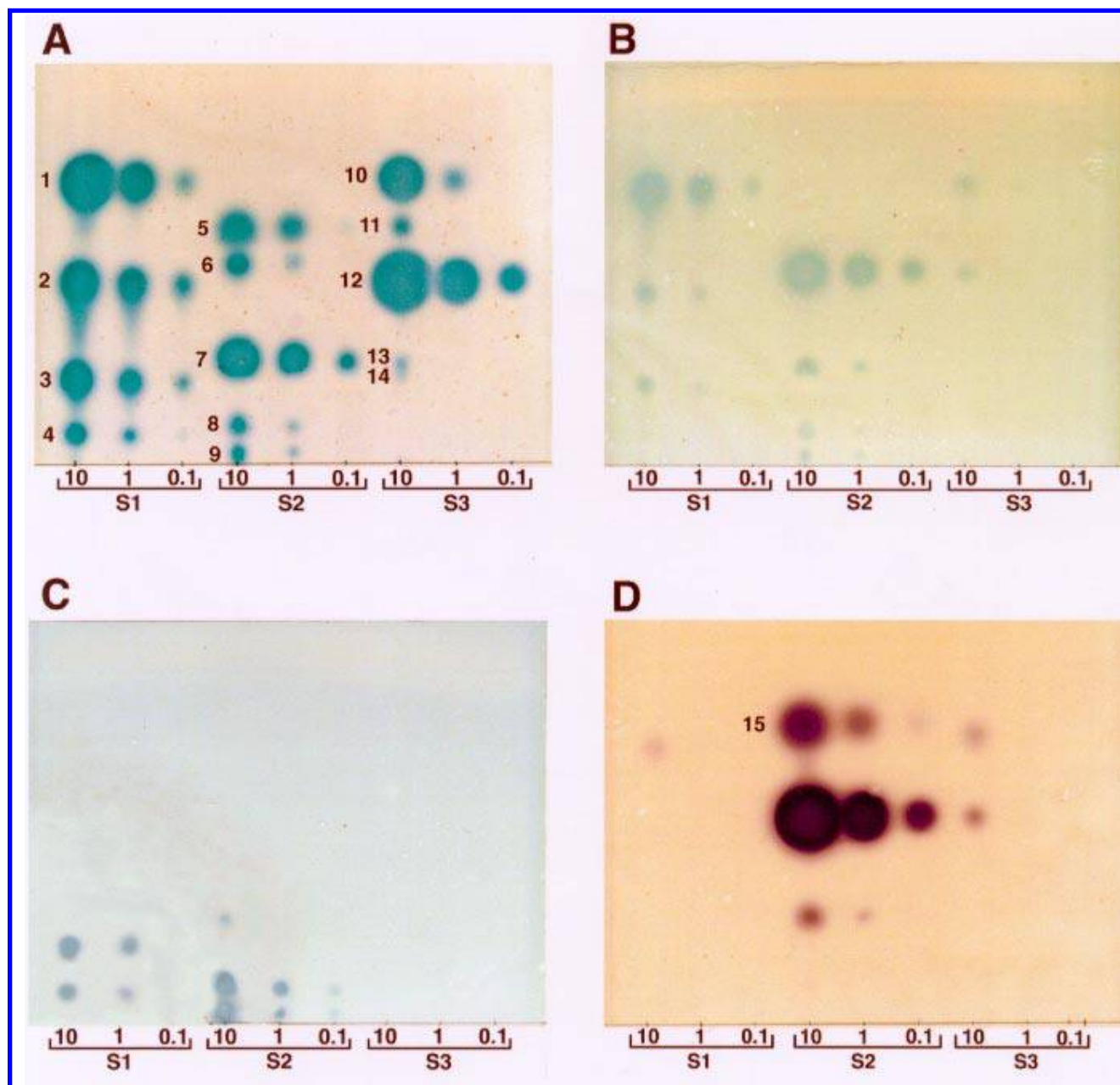
We assessed the four bioreporters for their ability to detect a range of acyl-HSL standards, and for their sensitivity to these compounds. Following chromatography of three sets of standards at three relative concentrations on four C<sub>18</sub> reversed-phase TLC plates, each plate was overlaid with a soft agar suspension of one of the four indicator strains as described in



**Fig. 1.** Structures of *lacZ* reporter fusions for the detection of compounds with *N*-acyl-L-homoserine lactone activity. **A**, *traG::lacZ* reporter. Plasmid pZLR4 contains the *traCDG* operon along with its promoter region from pTic58. The insert was derived from pTHB58*traG::Tn3lacI*, which contains a transcriptional fusion of *lacZ* to *traG* from Tn3HoHo1 to *traG* (Farrand et al. 1996). The clone also contains *traR* from pTic58 cloned as a 1.8-kb fragment into the *EcoRI* site of the vector. **B**, *luxI::lacZ* reporter. The insert, cloned in pSUP102, contains *luxR*, the intergenic *luxR-luxI* promoter region, and the 5' portion of *luxI* fused to *lacZ* to form pWH141 (Dunlap and Kuo 1992). **C**, *lasB::lacZ* reporter. Plasmid pKDT17 contains the *lasB* promoter region and the 5' portion of *lasB* fused to *lacZ* cloned into pUCP18 (Pearson et al. 1994). The plasmid also contains the *lasR* gene cloned as a separate fragment. Strains harboring these reporter plasmids lack the appropriate *I* gene and do not produce their own *N*-acyl-L-homoserine lactone signal molecules. However, they will activate transcription of the reporter fusion in response to an exogenous source of active autoinducer.

Materials and Methods. The *A. tumefaciens* reporter strain detected the broadest range of acyl-HSL derivatives of all three classes (Fig. 2A). Moreover, this reporter showed the greatest sensitivity, detecting most of the compounds even at one-tenth of the arbitrary standard concentration in the mixes. As we noted previously (Shaw et al. 1997) this strain did not

detect *N*-butanoyl-L-HSL, even at 10-fold higher amounts than that present in the standard mix (Figure 2A). The *C. violaceum* reporter, which recognizes *N*-hexanoyl-L-HSL as the cognate signal (McClellan et al. 1997), was extremely sensitive to the short- and medium-chain length alkanoyl-HSL standards, and easily detected the *N*-butanoyl-HSL (Fig. 2D,



**Fig. 2.** Specificities and sensitivities of the reporter systems to *N*-acylated derivatives of L-homoserine lactone (acyl-HSL). Mixtures of the acyl-HSL standards were spotted onto  $C_{18}$  reversed phase thin-layer plates and chromatographed with methanol/water (60:40, vol/vol). Following development, the plates were air dried and overlaid with suspensions of the indicator strains: **A**, *Agrobacterium tumefaciens* NT1(pZLR4); **B**, *Escherichia coli* XL1Blue(pWH141); **C**, *E. coli* MG4(pKDT17); and **D**, *Chromobacterium violaceum* CV026blu, all as described in Materials and Methods. Standard mixes are as follows: S1, 3-oxo derivatives with acyl chain lengths of (1)  $C_6$ , (2)  $C_8$ , (3)  $C_{10}$ , (4)  $C_{12}$ ; S2, alkanoyl derivatives with acyl chain lengths of (5) unknown contaminant, (15)  $C_4$ , (6)  $C_6$ , (7)  $C_8$ , (8)  $C_{10}$ , (9)  $C_{12}$ ; S3, 3-hydroxy derivatives with acyl chain lengths of (10)  $C_6$ , (11) unknown component, (12)  $C_8$ , (13) *N*-octanoyl-HSL, (14)  $C_{10}$ . S3 sample was prepared as an ethyl acetate extract of culture supernatants of *Pseudomonas fluorescens* 2-79 as described in Materials and Methods. Each set of standards was applied to the plate at three relative concentrations. 1 = a set of concentrations of each species that gives easily and reproducibly detectable spots with the *traG::lacZ* reporter as previously described (Shaw et al. 1997). 0.1 and 10 = one-tenth and 10-times the loading amounts of the standards present in concentration sets 1, respectively. In all cases, samples were loaded in a total volume of 1  $\mu$ l.

spot 15). However, as previously reported (McClellan et al. 1997), this strain lacks sensitivity to alkanoyl-HSLs with longer chain lengths and to most of the 3-oxo- derivatives. The strain also did not detect any of the 3-hydroxy- derivatives; the two spots in the  $\times 10$  S3 lane of Figure 2D correspond to the *N*-hexanoyl-L-HSL and *N*-octanoyl-L-HSL species present in the extract from *P. fluorescens* we use as our source of 3-hydroxy acyl-HSL standards (data not shown). The *lux* reporter detected most of the 3-oxo- and alkanoyl-standards but did not detect *N*-butanoyl-L-HSL or any of the 3-hydroxy forms at the concentrations tested (Fig. 2B). Like the *C. violaceum* reporter, the *lux* indicator detected the *N*-hexanoyl-L-HSL and *N*-octanoyl-L-HSL species present in the 3-hydroxy standard mix (Figure 2B,  $\times 10$  S3 lane). Moreover, the *lux* reporter responded with less intensity and was considerably less sensitive to the compounds, compared with the *tra* reporter. The *las* reporter responded strongly to *N*-decanoyl-L-HSL and *N*-dodecanoyl-L-HSL and less strongly to the *N*-(3-oxo-decanoyl)-L-HSL and *N*-(3-oxo-dodecanoyl)-L-HSL standards, and did not detect any of the 3-hydroxy compounds at the concentrations tested (Fig. 2C). Nor did the *las* reporter detect any of the shorter chain,  $C_4$ ,  $C_6$ , and  $C_8$  signals at the standard concentrations, although it weakly detected *N*-octanoyl-L-HSL at the  $\times 10$  concentration (Fig. 2D, lane 4). We should note that our alkanoyl- standard mix contains a contaminant detectable by the *tra* reporter as the fastest migrating activity (Shaw et al. 1997; see Figure 2A, spot 5). We do not know the nature of this compound, but it is not detected by any of the other reporters. Interestingly, this compound comigrates with an unknown active species present in the 3-hydroxy standard mix prepared from a culture of *P. fluorescens* 2-79 (Fig. 2A, spot 11). This unknown signal also is not detected by any of the other reporters.

Given the range and sensitivity of detection shown by the *A. tumefaciens tra* reporter we used this system for all of our assays unless otherwise noted.

### Surveying plant-associated bacteria.

Using our petri dish assay, we surveyed 106 isolates representing seven genera of plant-associated, gram-negative bacteria—*Agrobacterium*, *Erwinia*, *Pantoea*, *Pseudomonas*, *Ralstonia*, *Rhizobium*, and *Xanthomonas*—for the production of compounds with acyl-HSL activity. The tested strains represent pathogens and a few nonpathogenic and race mutants, commensals, and symbionts. The results, along with the source of the organism and the medium used for the tests, are presented in Table 1.

With the exception of the xanthomonads, production of compounds giving a positive signal was common among isolates from all genera tested. However, within each genus, production of these activities often was species dependent. For example, all three isolates of *E. carotovora* tested produced an easily detectable activity, but none of the three *E. amylovora* isolates tested gave a positive reaction. Moreover, among the isolates of *E. chrysanthemi* and *E. herbicola* tested, some gave a positive response while others failed to produce a detectable activity. Similar diversity was seen within and among biovars of *Pseudomonas syringae*. With the exception of the two phenazine producers, *P. aureofaciens* 30-84 and *P. fluorescens* 2-79, other pseudomonads, including *P. putida* and *P. fluorescens*, generally failed to give a positive reaction.

Most of the *Agrobacterium* spp. isolates produced a very weak reaction from the indicator. The remainder, including one strain of *A. rhizogenes*, one strain of *A. tumefaciens*, and two of the *A. vitis* isolates, failed to give a positive reaction. Similarly, all but one of the rhizobial isolates gave a distinctly positive reaction.

The xanthomonads proved particularly difficult to assess. Several isolates, including *X. campestris* pv. *campestris* 4546, *X. campestris* pv. *pelargoni* X-5, and *X. oryzae* pv. *oryzicola* BLS303, clearly produced an active species (see below). Other isolates often induced in the indicator overlay a very narrow zone of diffusing blue color around the colony. However, these reactions were not reproducible. Moreover, such isolates occasionally gave a comparable ring of activity even when the indicator strain was omitted from the overlay. These isolates evidently produce some extracellular factor that hydrolyzes X-Gal. Given this, we retested each positive isolate on a plate lacking the indicator strain to rule out such false-positive reactions. No other isolates gave such a reaction.

### Assessing acyl-HSL complements by TLC.

TLC, coupled with a bioreporter, provides a simple and rapid technique for assessing the minimum number of different acyl-HSL species produced by a given organism (Shaw et al. 1997). The technique also provides limited and preliminary information concerning the nature of these compounds present in extracts of the culture supernatants. However, one cannot assign structures to the active signals based on chromatographic properties alone.

We analyzed by TLC extracts from culture supernatants of selected isolates, representing each of the genera listed in Table 1, that had given positive reactions in the petri dish assay. In each case we detected the separated acyl-HSLs with two indicators: the *traG::lacZ* reporter from *A. tumefaciens*, and, to detect the short-chain alkanoyl-HSLs, the *C. violaceum* reporter (see above). The results, illustrated in Figure 3 for a representative group of the strains tested, can be summarized as follows. The isolates of the *Pseudomonas syringae* group tested all produced as the dominant activity a compound that is chromatographically indistinguishable from the *N*-(3-oxo-hexanoyl)-L-HSL standard (Fig. 3A, lanes 1–3). Many of these isolates also produced at barely detectable levels a compound that co-chromatographed with *N*-(3-oxo-octanoyl)-L-HSL. The *C. violaceum* indicator detected a weakly reactive component that could be *N*-hexanoyl-L-HSL in extracts prepared from culture supernatants of each of the three *P. syringae* strains (data not shown). Similarly, most of the *Erwinia* isolates we examined produced an active compound that co-chromatographed with *N*-(3-oxo-hexanoyl)-L-HSL as the dominant signal molecule (Fig. 3B, lanes 2 and 3). However, there were a few exceptions. *Erwinia carotovora* subsp. *atroseptica* produced at least four detectable species (Fig. 3B, lane 2). Two of these chromatographed with the characteristics of *N*-(3-oxo-hexanoyl)-L-HSL and *N*-(3-oxo-octanoyl)-L-HSL. The remaining activities chromatographed with characteristics unlike any of our standards. One of these has properties similar to the unknown contaminant in the alkanoyl-HSL standards mix while the second is more polar than even our  $C_4$  standards.

Strain 2-79, the single isolate of *P. fluorescens* that gave a positive reaction in the plate assay, produced five species detectable with the *tra* reporter. These correspond to *N*-(3-

**Table 1.** Strains, their characteristics and sources, and their production of compounds with *N*-acyl-L-homoserine lactone activity<sup>a</sup>

Genus, species, biovar or pathovar	Strain	Source	Medium <sup>b</sup>	R <sup>c</sup>	Genus, species, biovar or pathovar	Strain	Source	Medium <sup>b</sup>	R <sup>c</sup>
<i>Erwinia amylovora</i>	273	S. M. Ries	ABM/LB	–	<i>X. campestris</i> pv. <i>campestris</i>	NA5	J. E. Leach	LB	–
	FB01	D. L. Coplin	ABM/LB	– <sup>d</sup>		477	J. E. Leach	LB	+
	FB03	D. L. Coplin	ABM/LB	– <sup>d</sup>		4546	J. E. Leach	LB	+
<i>E. carotovora</i> bv. <i>atroseptica</i>	SR8	D. L. Coplin	ABM	+++		4547	J. E. Leach	LB	– <sup>d</sup>
<i>E. carotovora</i> bv. <i>carotovora</i>	SR29	D. L. Coplin	ABM	+++	<i>X. campestris</i> pv. <i>cerealis</i>	NCPPB1943	J. E. Leach	LB	– <sup>d</sup>
	DM2105	D. L. Coplin	ABM	+++	<i>X. campestris</i> pv. <i>glycines</i>	FI	I. Hwang	LB	+
<i>E. chrysanthemi</i>	FI	S. M. Ries	ABM	–		426	J. E. Leach	LB	– <sup>d</sup>
	873	A. Collmer	ABM	+++		479	J. E. Leach	LB	–
	D759	A. Collmer	ABM	+++	<i>X. campestris</i> pv. <i>holicola</i>	86	J. E. Leach	LB	– <sup>d</sup>
	16	A. Collmer	ABM	+++		429	J. E. Leach	LB	–
	SR120A	A. Collmer	ABM/LB	– <sup>d</sup>	<i>X. campestris</i> pv. <i>malva- cearum</i>	R-2	J. E. Leach	LB	– <sup>d</sup>
	3937	A. Collmer	ABM/LB	–	<i>X. campestris</i> pv. <i>pelargoni</i>	X-1	J. E. Leach	LB	– <sup>e</sup>
	D706	A. Collmer	ABM/LB	–		X-5	J. E. Leach	LB	+
<i>E. herbicola</i>	FI	S. M. Ries	ABM/LB	–	<i>X. campestris</i> pv. <i>vesicatoria</i>	FI	I. Hwang	LB	–
	DC531	D. L. Coplin	ABM	+++		82-8	B. Staskawicz	LB	– <sup>d</sup>
	DC535	D. L. Coplin	ABM/LB	– <sup>d</sup>		82-8avrBS2	B. Staskawicz	LB	–
<i>E. rhapontici</i>	DC541	D. L. Coplin	ABM/LB	– <sup>d</sup>	<i>X. oryzae</i> pv. <i>oryzae</i>	PX061	J. E. Leach	LB	– <sup>d</sup>
	DC542	D. L. Coplin	ABM/LB	–		PX099	J. E. Leach	LB	–
<i>Pantoea stewartii</i>	FI	S. M. Ries	ABM	+++	<i>X. oryzae</i> pv. <i>oryzicola</i>	BLS179	J. E. Leach	LB	+
	ESR5	D. L. Coplin	ABM	+++		BLS303	J. E. Leach	LB	+
	SS104	D. L. Coplin	ABM	+++	<i>Agrobacterium radiobacter</i>	K84	OC	ABMB	+
	SW2	D. L. Coplin	ABM	+++		K299	OC	ABMB	+
<i>Pseudomonas aureofaciens</i>	30-84	L. Thomashow	VB	+	<i>A. rhizogenes</i>	A4	OC	ABMB	+
<i>P. fluorescens</i>	2-79	L. Thomashow	VB/ABM	+++		8196	OC	ABM	– <sup>d</sup>
	1855.344	OC	VB/KB	– <sup>d</sup>		K599	OC	ABM	+
<i>P. putida</i>	B53	OC	VB/KB	– <sup>d</sup>	<i>A. tumefaciens</i>	C58	OC	ABM	+
	PRS2000	OC	VB/KB	–		C58ΔaccR	OC	ABM	+++
<i>P. syringae</i> pv. <i>angulata</i>	PA1	OC	VB	+++		A6	OC	ABM	+
<i>P. syringae</i> pv. <i>coronofaciens</i>	PC27	OC	VB	+++		B6	OC	ABM	+
<i>P. syringae</i> pv. <i>glycinea</i>	PG79	OC	VB	+++		15955	OC	ABM	+
<i>P. syringae</i> pv. <i>maculicola</i>	4326	D. N. Nunn	VB/KB	–		15955tra <sup>c</sup>	OC	ABM	+++
<i>P. syringae</i> pv. <i>melea</i>	PM1020	D. N. Nunn	VB/KB	–		Bo542	OC	ABM	+
<i>P. syringae</i> pv. <i>phaseolicola</i>	3121	N. Panopoulos	VB/KB	– <sup>d</sup>		Chry5	S. Pueppke	ABM	+
<i>P. syringae</i> pv. <i>savastanoi</i>	1020	Y. Dessaux	VB	++		Ant4	Y. Dessaux	ABM	+
(olive)	1670	Y. Dessaux	VB	++		AB2/73	OC	ABMB	+
<i>P. syringae</i> pv. <i>savastanoi</i>	2088	Y. Dessaux	VB/KB	–		K108	A. Kerr	ABM	– <sup>d</sup>
(oleander)	T12-1	Y. Dessaux	VB	++	<i>A. vitis</i>	K308	L. W. Moore	ABM	–
<i>P. syringae</i> pv. <i>savastanoi</i>	2093	Y. Dessaux	VB/KB	–		Hm1	L. Otten	ABM	– <sup>d</sup>
(ash)	2169	Y. Dessaux	VB	++		Ag57	E. W. Nester	ABM	+
	2174	Y. Dessaux	VB/KB	–	<i>Rhizobium etli</i>	CFN42	S. Brom	YEM	+++
<i>P. syringae</i> pv. <i>syringae</i>	FI	S. M. Ries	VB/KB	–		G12	T. Reitz	YEM	++
	Cit7	S. E. Lindow	VB/KB	– <sup>d</sup>	<i>R. fredii</i>	USDA191	G. Elkan	YEM	+
	B728a	D. K. Willis	VB	+++	<i>R. leguminosarum</i> bv. <i>phaseoli</i>	127K14	W. Lepps	YEM	++
						14482	E. P. Greenberg	YEM	++
<i>P. syringae</i> pv. <i>tabaci</i>	BR2.004	OC	VB	+++	<i>R. leguminosarum</i> bv. <i>trifolii</i>	162E8	W. Lepps	YEM	++
	BR2.024	OC	VB	+++		14480	E. P. Greenberg	YEM	++
	81	OC	VB	+++	<i>R. leguminosarum</i> bv. <i>viciae</i>	128C53	W. Lepps	YEM	– <sup>d</sup>
	11528	OC	VB	+++		175F3	W. Lepps	YEM	++
<i>P. syringae</i> pv. <i>tomato</i>	DC3000	D. K. Willis	VB/KB	–		10004	K. Gray	YEM	++
	2212	OC	VB/KB	–	<i>R. meliloti</i>	Rm41	A. Kondorosi	YEM	+++
<i>Ralstonia solanacearum</i>	AW1	T. P. Denny	CPG/VB	+++		AK631 <sup>c</sup>	A. Kondorosi	YEM	+++
	B1	L. Sequeira	CPG/VB	+++		Rm1021	K. Gray	YEM	+
	K60 <sup>e</sup>	L. Sequeira	CPG/VB	+++		L5-30	S. Long	YEM	++
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	FI	I. Hwang	LB	–					

<sup>a</sup> Abbreviations: OC, our collection; ABM, AB minimal mannitol medium; ABMB, ABM supplemented with biotin; KB, King's medium B; LB, Luria-Bertani medium (low salt); VB, Vogel-Bonner medium; YEM, yeast extract mannitol medium; FI, field isolate.

<sup>b</sup> When two media are shown, the strain was tested in both.

<sup>c</sup> Reaction: Assessed by petri dish assay and *traG::lacZ* reporter as described in Materials and Methods. +++, wide (3 cm dia.); ++, somewhat narrower (1.5–3 cm dia.); +, very narrow (1.5 cm dia.); –, no detectable zones of diffusing blue color in the overlay surrounding colony or culture supernatant being tested. In most cases the colony or spot was approximately 0.5 to 1 cm in diameter.

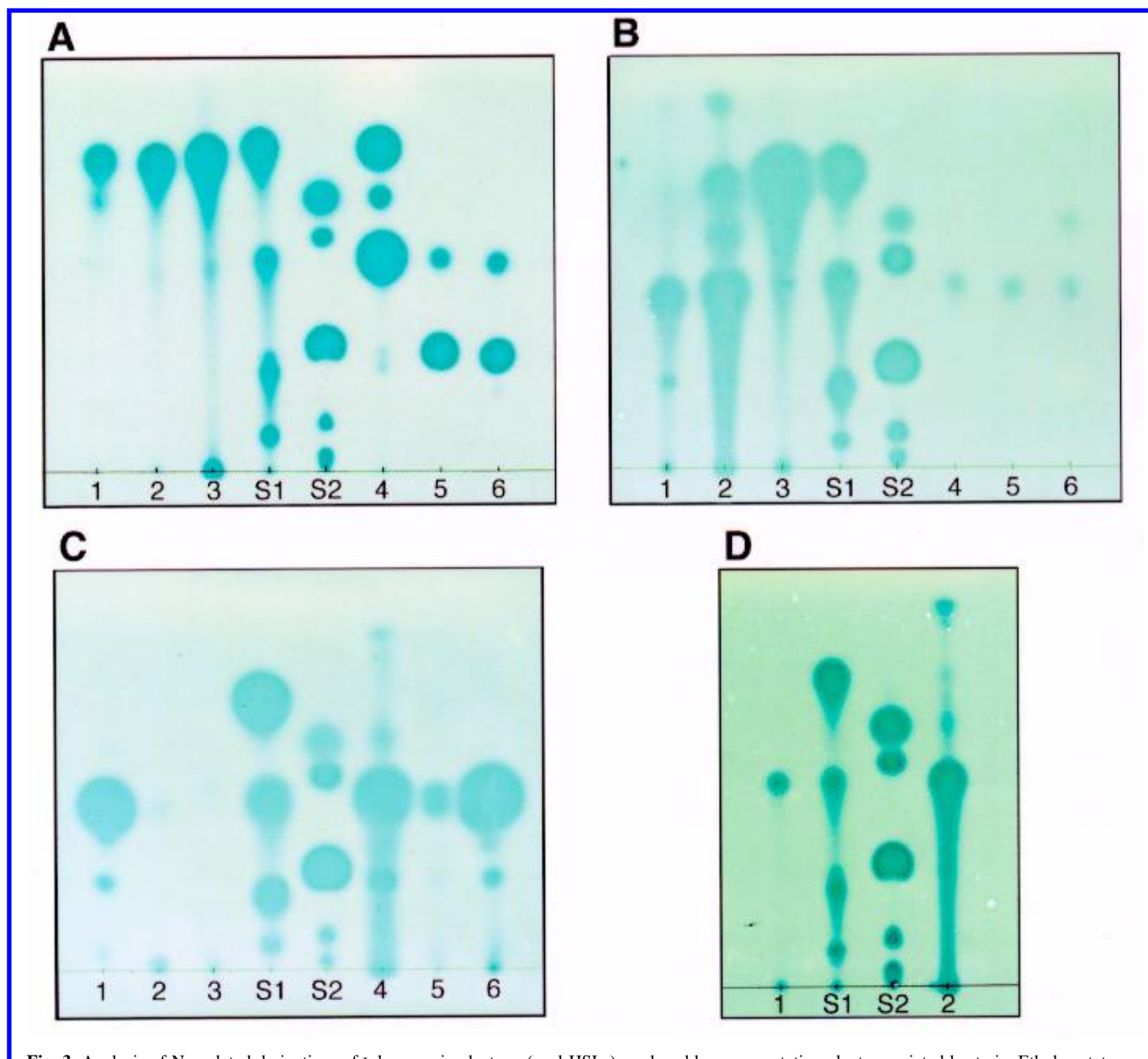
<sup>d</sup> Strains giving a negative response on petri dish assay that were tested by thin-layer chromatography. No compounds with *N*-acyl-L-homoserine lactone activity were detected in extracts of culture supernatants from any of these strains.

<sup>e</sup> Strain K60 is a spontaneous, nonpathogenic mutant of *R. solanacearum* B1; AK631 is a spontaneous compact-colony morphology mutant of *R. meliloti* Rm41 (Forrai et al. 1983).



hydroxy-hexanoyl)-L-HSL, *N*-(3-hydroxy-octanoyl)-L-HSL, *N*-(3-hydroxy-decanoyl)-L-HSL, and *N*-octanoyl-L-HSL we previously identified in extracts of culture supernatants from this bacterium (Shaw et al. 1997). The *C. violaceum* reporter detected an additional weak activity corresponding to *N*-hexanoyl-L-HSL in extracts of this strain (data not shown). We also examined three strains of *Ralstonia solanacearum*, the two pathogenic isolates, AW1 and B1, and the spontaneous nonpathogenic mutant of B1, strain K60. As we previously

reported, strain B1 produces two species detectable with the *traG::lacZ* reporter. One corresponds to *N*-octanoyl-L-HSL and the second chromatographed with the characteristics of *N*-(3-hydroxy-octanoyl)-L-HSL (data not shown). The independent pathogenic isolate AW1 produced an identical pattern (Fig. 3A, lane 5). Strain K60, the nonpathogenic variant of B1, produced the same two active species at indistinguishable levels (Fig. 3A, lane 6). The *C. violaceum* reporter detected only a single active component co-migrating with *N*-octanoyl-



**Fig. 3.** Analysis of *N*-acylated derivatives of L-homoserine lactone (acyl HSLs) produced by representative plant associated bacteria. Ethyl acetate extracts were prepared from culture supernatants of a subset of isolates giving a positive reaction in the plate assays as described in the text. Samples of each extract were chromatographed on two  $C_{18}$  thin-layer chromatography (TLC) plates. Following development, one plate was overlaid with the *traG::lacZ* reporter, the other with the *Chromobacterium violaceum* reporter. Only the plates overlaid with the *traG* reporter are shown. **A**, Lane 1, *Pseudomonas syringae* pv. *syringae* B728a; lane 2, *P. syringae* pv. *savastanoi* Olive 1670; lane 3, *P. syringae* pv. *coronofaciens* PC27; lane 4, *P. fluorescens* 2-79; lane 5, *Ralstonia solanacearum* K60; lane 6, *R. solanacearum* AW1. **B**, Lane 1, *Agrobacterium tumefaciens* Bo542; lane 2, *Erwinia carotovora* pv. *atroseptica* SR8; lane 3, *E. carotovora* pv. *carotovora* DM2105; lane 4, *Xanthomonas campestris* pv. *pelargoni* X-5; lane 5, *X. campestris* pv. *campestris* 4546; lane 6, *X. oryzae* pv. *oryzicola* BLS303. **C**, Lane 1, *Rhizobium leguminosarum* bv. *trifolii* 162E8; lane 2, *R. fredii* USDA191; lane 3, *R. meliloti* Rm1021; lane 4, *R. meliloti* Rm41; lane 5, *R. leguminosarum* bv. *trifolii* 14480; lane 6, *R. leguminosarum* bv. *phaseoli* 14482. **D**, Lane 1, *A. tumefaciens* C58; lane 2, *A. tumefaciens* NT1(pTiC58 $\Delta$ accR). S1 and S2 are the 3-oxo-acyl HSL and alkanoyl HSL standards as described in Figure 2.

L-HSL in the extracts from each of these three bacteria (data not shown).

Of the three xanthomonads examined, all produced a weakly detectable activity that chromatographed with the characteristics of *N*-(3-oxo-octanoyl)-L-HSL (Fig. 3B, lanes 4–6). One isolate, *X. oryzae* pv. *oryzicola* BLS303, produced, in addition, a compound that co-chromatographed with the unknown contaminant in our alkanoyl-HSL standard mix (Fig. 3B, lane 6). The *C. violaceum* reporter did not detect any active compounds in extracts from these strains (data not shown).

Among the agrobacteria tested, most produced barely detectable amounts of a single active compound that chromatographed with the properties of *N*-(3-oxo-octanoyl)-L-HSL (for example, see Figure 3D, lane 1). However, strain Bo542 produced easily detectable amounts of this signal and weakly detectable amounts of a species chromatographing with the characteristics of *N*-(3-oxo-decanoyl)-L-HSL (Fig. 3B, lane 1). No other activities were detected with the *C. violaceum* reporter (data not shown).

Unlike the agrobacteria, among the *Rhizobium* isolates tested we observed considerable diversity. Some, such as *R. meliloti* Rm1021, produced only a single, highly nonpolar activity (Fig. 3C, lane 3). *Rhizobium fredii* 191 produced barely detectable amounts of a species that co-chromatographed with *N*-(3-oxo-octanoyl)-L-HSL (Fig. 3C, lane 2) in addition to the strongly nonpolar compound. *R. leguminosarum* bv. *trifolii* 14480 produced a similar complement, but the *N*-(3-oxo-octanoyl)-L-HSL component was present in easily detectable amounts (Fig. 3C, lane 5). Other rhizobia produced several active compounds at easily detectable levels. For example, *R. leguminosarum* bv. *trifolii* 162E8 (Fig. 3C, lane 1) and *R. leguminosarum* bv. *phaseoli* 14482 (Fig. 3C, lane 6) each produced four detectable compounds. The most active of these chromatographed with the characteristics of *N*-(3-hydroxy-octanoyl)-L-HSL. In addition, each produced an active species that chromatographed with the properties of *N*-octanoyl-L-HSL. The two strains also produced a strongly nonpolar activity indistinguishable from that seen in extracts of *R. fredii* 191. *R. leguminosarum* bv. *trifolii* 162E8, but not *R. leguminosarum* bv. *phaseoli* 14482, also produced an activity, easily detectable by the *C. violaceum* reporter, that chromatographed with the characteristics of *N*-hexanoyl-L-HSL (data not shown). *R. meliloti* Rm41 produced a minimum of seven detectable active compounds (Fig. 3C, lane 4). The two most active species chromatographed with characteristics indistinguishable from *N*-(3-oxo-octanoyl)-L-HSL and *N*-octanoyl-L-HSL. The strain also produced a highly nonpolar species that barely moved from the origin, as well as two strongly polar activities that migrated faster than any of our standards. Finally, like *E. carotovora* SR8, the strain produced an activity that co-chromatographed with the contaminant present in our alkanoyl-HSL standard mix. Despite this complexity, extracts from this strain did not contain compounds detectable by the *C. violaceum* indicator (data not shown).

We also tested extracts from culture supernatants of a number of isolates that failed to give a positive reaction in the petri dish test. None of these strains produced compounds detectable on the TLC plates by either of the bioreporters (Table 1).

The IncRh1 Ti plasmids pTiC58 and pTiR10 confer production of *N*-(3-oxo-octanoyl)-L-HSL, the *Agrobacterium*

autoinducer (Zhang et al. 1993; Hwang et al. 1994; Fuqua and Winans 1994). However, production of this signal, synthesis of which is coded for by the *trai* gene (Hwang et al. 1994; Fuqua and Winans 1994; Moré et al. 1996), is repressed in the absence of the conjugal opine (Piper et al. 1993; Hwang et al. 1994; Fuqua and Winans 1994). We examined the complement of acyl-HSL signals produced by wild-type strain C58 and by the transfer-constitutive mutant, NT1(pTiC58 $\Delta$ accR). This latter strain constitutively expresses *trai* (Zhang et al. 1993; Hwang et al. 1994). Strain C58 produced only weakly detectable levels of *N*-(3-oxo-octanoyl)-L-HSL (Fig. 3D, lane 1), while the transfer-constitutive mutant produced this signal molecule in greatly elevated amounts (Fig. 3D, lane 2). Moreover, extracts from the transfer-constitutive mutant contained a minimum of six additional species, some chromatographing with characteristics unlike any of our standards. In fact, the pattern of activities produced by NT1(pTiC58 $\Delta$ accR) resembles that produced by *R. meliloti* Rm41 (compare Figure 3C, lane 4, with Figure 3D, lane 2).

## DISCUSSION

Our surveys with the petri dish assay indicate that production of compounds with acyl-HSL signaling activity is relatively common among gram-negative bacteria that interact with plants. One or more isolates from all seven genera surveyed produced such compounds (Table 1). Among the agrobacteria, pantoea, and rhizobia tested virtually all produced active signals, while about half of the erwinias and pseudomonads tested gave a positive reaction (Table 1). Production of detectable levels of acyl-HSLs was rarer among the xanthomonads tested. However, some isolates that tested positive in the petri dish assay clearly produce active compounds detectable by TLC (Fig. 3B, lanes 4–6). With the exception of the agrobacteria and rhizobia, production of a detectable acyl-HSL signal largely was species- or biovar-specific. Thus, most isolates of *E. carotovora* and *P. stewartii* produced detectable levels of these signals. Similarly, all three isolates of *P. syringae* pv. *tabaci* tested produced detectable acyl-HSLs. However, we noted considerable diversity within some species and pathovars. For example, only a subset of the *E. chrysanthemi* and *P. syringae* pv. *savastanoi* isolates tested produced active compounds. And, although the number of isolates examined is small, certain species and pathovars, most notably *E. amylovora* and *P. syringae* pvs. *tomato* and *phaseolicola*, characteristically did not produce detectable acyl-HSL signals.

Such failures to detect activity must be viewed with caution. First, the organisms may contain acyl-HSL-mediated signaling systems but produce their messenger compounds at levels below that detectable by the reporter. For example, the agrobacteria that have been examined in detail all super-regulate their quorum-sensing systems and produce their cognate acyl-HSL signals at very low levels when grown in standard laboratory medium (Hwang et al. 1994; Fuqua and Winans 1994). Alternatively, the organisms may produce signals, even in large amounts, that are not detectable by the reporter used in the assay. Use of several reporters with complementary specificities may minimize this problem. In this regard we have retested most of the isolates that failed to elicit a response with the *traG::lacZ* reporter against the *lux* and *C. violaceum* indicator strains. None produced signals that acti-

vated expression of either of these alternative reporters (data not shown).

In many instances, of those that produce detectable acyl-HSLs, all isolates of a given taxonomic group produce the same signal or set of signals. For example, as assessed by TLC, all acyl-HSL-positive isolates of *Erwinia* spp. as well as the various pathovars of *P. syringae* examined produced an activity that is chromatographically indistinguishable from *N*-(3-oxo-hexanoyl)-L-HSL as their major detectable signal molecule. Similarly, all isolates of *Agrobacterium* spp. produced *N*-(3-oxo-octanoyl)-L-HSL (Fig. 3). However, some but not all *erwinia* and a few *P. syringae* isolates produced additional active species. These additional signals may represent multiple quorum-sensing systems. A growing number of organisms, including *V. fischeri* (Kuo et al. 1994), *P. aeruginosa* (Pearson et al. 1994; Winson et al. 1995), and *R. etli* (Rosemeyer et al. 1998), express several, sometimes interrelated, quorum-sensing systems. Alternatively, they may be the products of a single acyl-HSL synthase; a number of these enzymes, including VsmI, LuxI, and RaiI, can produce several acyl-HSL derivatives (Kuo et al. 1994; Winson et al. 1995; Rosemeyer et al. 1998). Moreover, while TraI, the AI synthase of *A. tumefaciens* Ti plasmids, confers production of *N*-(3-oxo-octanoyl)-L-HSL as its major product, when expressed at high levels, this synthase produces additional acyl-HSLs at levels now detectable by the reporter strain (Fig. 3D).

Members of some taxa, best exemplified by the rhizobia, showed considerable diversity with respect to the number and nature of acyl-HSL signals produced. Some isolates, such as *R. fredii* 191, produced only a single detectable compound while others, such as *R. meliloti* Rm41, produced a minimum of seven detectable species. The pattern produced by this latter isolate is reminiscent of that produced by *R. etli* CNPAF512 (Rosemeyer et al. 1998). Intriguingly, *R. leguminosarum* bv. *phaseoli* 14482 apparently produces a subset of the signals produced by *R. meliloti* Rm41 (Fig. 3C, lanes 4 and 6).

Most of the rhizobia tested produced as the dominant detectable activity a compound that chromatographs with the characteristics of *N*-(3-hydroxy-octanoyl)-L-HSL. Many of the *Rhizobium* isolates also produced a strongly nonpolar species that barely migrates in the 60:40 methanol-water solvent system (Fig. 3 and data not shown). Several biovars of *Rhizobium* produce a long-chain acyl-HSL, called RLAI, involved in regulating rhizosphere-expressed genes as well as a growth-inhibitory phenotype (Gray et al. 1996; Schripsema et al. 1996). This signal molecule, identified as *N*-(3-hydroxy-7-*cis*-tetradecanoyl)-L-HSL (Gray et al. 1996; Schripsema et al. 1996) is detectable by the *tra* reporter in its pure form (data not shown) and also in ethyl acetate extracts of culture supernatants from *R. leguminosarum* bv. *phaseoli* 14482, an isolate known to produce this signal (Fig. 3C, lane 6). Compounds with indistinguishable chromatographic characteristics were detected in extracts from culture supernatants of *R. leguminosarum* bv. *trifolii* strains 162E8 (Fig. 3C, lane 1) and 14480 (Fig. 3C, lane 5), and *R. leguminosarum* bv. *viciae* 10004 (data not shown). Whether these correspond to RLAI remains to be determined. Intriguingly, several rhizobial isolates, including *R. fredii* USDA191, *R. leguminosarum* bv. *phaseoli* 14482, and *R. meliloti* Rm41, produced activities even more nonpolar than RLAI (Fig. 3C, lanes 2, 4, and 6). The nature of these compounds is not known, but Gray et al. (1996) reported

that *R. meliloti* Rm1021 produces a signal molecule that is more nonpolar than RLAI. This could correspond to the activity, which remains at or near the base line, we detected in extracts from *R. meliloti* Rm41 (Fig. 3C, lane 4).

Using the *tra* reporter, we consistently detected two acyl-HSLs in culture supernatants of *Ralstonia solanacearum* (Fig. 3A, lanes 5 and 6; and Shaw et al. 1997). One chromatographs with the properties of *N*-octanoyl-L-HSL, while the more polar compound could be *N*-(3-hydroxy-octanoyl)-L-HSL. Flavier et al. (1997b) recently reported that, based on high-performance liquid chromatography (HPLC) elution profiles, *R. solanacearum* AW1 produces *N*-octanoyl-L-HSL and *N*-hexanoyl-L-HSL. We did not detect this latter compound on chromatograms overlaid with the *C. violaceum* reporter (data not shown). This is surprising, considering that *N*-hexanoyl-L-HSL is the cognate autoinducer for the quorum-sensing system that regulates production of violacein pigment in this organism (McClean et al. 1997). We have no explanation for this discrepancy. Interestingly, B1, a second wild-type isolate of *R. solanacearum*, as well as its spontaneous nonpathogenic mutant, K60, produced a pattern of acyl-HSLs indistinguishable in type and amount from that produced by AW1 (Fig. 3A and data not shown).

Surveys for acyl-HSL signal molecules and their subsequent analysis require sensitive bioreporters. These compounds generally are produced at levels so low as to escape detection by standard chemical methods. Our comparative analysis indicates that the *lacZ*-based *tra* reporter from the nopaline-type Ti plasmid pTiC58 is superior in both specificity and sensitivity, compared with any one of the three other reporter systems tested (Fig. 2). However, the *tra* reporter is not without limitations; it is not particularly sensitive to the short chain alkanoyl-HSLs including *N*-hexanoyl-L-HSL and does not respond at all to *N*-butanoyl- and *N*-(3-hydroxy-butanoyl)-L-HSL (Fig. 2A and data not shown). In this regard, the *C. violaceum* reporter serves as a useful complement; this strain responds with reasonable sensitivity to *N*-hexanoyl- and *N*-butanoyl-L-HSL (Fig. 2D). Thus, in our TLC analyses, we tested all of our samples with both indicators. This proved enlightening; several isolates, including all of the otherwise-positive *P. syringae* pathovars, *P. fluorescens* 2-79, and especially *R. leguminosarum* bv. *trifolii* 162E8, produced a compound with the chromatographic characteristics of *N*-hexanoyl-L-HSL that was detectable only with the *C. violaceum* reporter (data not shown). The *tra* reporter also detected activities with chromatographic properties unlike those of any of our standards. Some of these, for example spot 11 in the extracts from *P. fluorescens* 2-79 (Fig. 2A), could represent acyl-HSLs with atypical side chains having an odd number of carbons, branches, or one or more unsaturations. Others, such as the fast-migrating species present in extracts from cultures of *E. carotovora* pv. *atroseptica* SR8 (Fig. 3B, lane 2), *R. meliloti* Rm41 (Fig. 3C, lane 4), and *A. tumefaciens* NT1(pTiC58 $\Delta$ accR) (Fig. 3D, lane 2), could represent compounds with completely novel structures. However, the strongly polar compound in extracts from the *A. tumefaciens* strain is dependent on TraI; we detect it only in extracts from strains overexpressing the acyl-HSL synthase.

While acyl-HSLs constitute the most well-studied family of signal molecules associated with quorum-sensing systems, other types of compounds also are used. For example, few



xanthomonads produce detectable amounts of compounds with acyl-HSL activity. However, two isolates of *X. campestris* pv. *campestris* regulate several traits in a quorum-dependent fashion. Strain B-24 uses a diffusible, nonnitrogenous substituted lactone as a signal molecule to regulate production of xanthomonadin pigments as well as production of extracellular polysaccharides (EPS; Poplawsky and Chun 1997; Chun et al. 1997). Similarly, strain 8004 regulates production of extracellular enzymes involved in pathogenicity, as well as EPS, via an uncharacterized diffusible signal molecule (Barber et al. 1997). Based on an analysis of cross-signaling, these two xanthomonads apparently produce more than one somewhat interchangeable signals in a manner analogous to the production of different forms of acyl-HSLs (Poplawsky et al. 1998). These signals are not detectable by the *traG::lacZ* reporter (Poplawsky and Chun 1997; Barber et al. 1997). Organisms also may express more than one quorum-sensing system, each responding to a different class of signal molecules. For example, *R. solanacearum* utilizes a volatile fatty acid, 3-hydroxy palmitic acid methyl-ester (3-OH-PAME) to regulate traits involved in pathogenicity in a density-dependent manner (Clough et al. 1997; Flavier et al. 1997a). As described above, this organism also produces a minimum of two acyl-HSLs (Fig. 3A, lanes 5 and 6; Flavier et al. 1997b). The gene systems in *R. solanacearum* regulated by the acyl-HSLs remain unknown. However, work from Denny's group indicates that production of the acyl-HSL signals is controlled by the regulatory system responsive to 3-OH PAME (Flavier et al. 1997b).

Although many of the tested organisms produce quorum-sensing signal molecules, the traits controlled by this regulatory system are known only for a few. Thus, *A. tumefaciens* regulates conjugal transfer of the Ti plasmids by quorum-sensing (Piper et al. 1993; Fuqua and Winans 1994), while *E. carotovora* regulates production and secretion of extracellular enzymes associated with pathogenicity (Jones et al. 1993; Pirhonen et al. 1993). Similarly, *P. stewartii* regulates another pathogenicity determinant, production of capsular polysaccharide by acyl-HSL-mediated autoinduction (Beck von Bodman and Farrand 1995). On the other hand, *P. syringae* pv. *tabaci*, although producing a quorum-sensing signal, does not use this signal to regulate expression of genes overtly required for pathogenesis (Y.-C. Chen, unpublished observations). Among the saprophytes, *P. fluorescens* 2-79 most probably regulates production of phenazine antibiotics by a quorum-sensing mechanism (Mavrodi et al. 1998) in a manner similar to that of *P. aureofaciens* 30-84 (Pierson et al. 1994). However, *P. fluorescens* 1855.344, which does not produce phenazines or other known antibiotics, also does not produce detectable amounts of an acyl-HSL signal. Thus, this regulatory strategy is used by these plant-associated bacteria to control a broad range of specialty traits. The challenge for the future will be to determine what traits are regulated by quorum-sensing in these organisms, as well as the roles of these traits in the biology of these bacteria, especially as they interact with their plant hosts.

## MATERIALS AND METHODS

### Bacterial strains and plasmids.

*Agrobacterium tumefaciens* NT1 is C58 cured of its Ti plasmid (Watson et al. 1975). The strain is prototrophic and

susceptible to most commonly used antibiotics. *Chromobacterium violaceum* CV026blu is a derivative of the autoinducer reporter strain CV026 (McClellan et al. 1997), and was obtained from Simon Swift. Plasmid pZLR4 contains *traR* and a *traG::lacZ* reporter fusion from pTic58 independently cloned into the broad-host-range vector pBBR1MCS5 (Kovach et al. 1995). This construct codes for resistance to ampicillin/carbenicillin and gentamicin. Plasmid pWH141, harbored by *Escherichia coli* XL1-Blue, contains *luxR* and a *luxI::lacZ* reporter fusion from *Vibrio fischeri* MJ1 cloned in pSUP102 (Dunlap and Kuo 1992) while pKDT17, present in *E. coli* MG4, contains *lasR* and a *lasB::lacZ* reporter fusion from *Pseudomonas aeruginosa* PAO1 cloned into pUCP18 (Pearson et al. 1994).

### Media and growth conditions.

Reporter strains were maintained on nutrient agar (Difco, Detroit, MI) or L agar (Sambrook et al. 1989) plates supplemented with the appropriate antibiotics. Antibiotics were used at the following concentrations when necessary. *A. tumefaciens*: carbenicillin, 100 µg/ml; gentamicin, 30 µg/ml. *E. coli*: ampicillin, 60 µg/ml. Cultures to be assayed for production of compounds with acyl-HSL activity were grown in the following: AB minimal medium supplemented with 0.2% mannitol (ABM; Chilton et al. 1974) and 2 µg of biotin per ml (ABMB) when necessary; King's B (KB) medium (King et al. 1954); Vogel-Bonner (VB) medium (Vogel and Bonner 1956); yeast extract mannitol (YEM) medium (Somasegaran and Hoben 1985); or CPG medium (Shaw et al. 1997), as noted in Table 1. Cultures of *E. coli* were grown at 37°C. Cultures of all other organisms were grown at 28°C. Liquid cultures were grown with shaking to ensure aeration.

### Chemicals.

Authentic alkanoyl- and *N*-3-oxo-acyl-homoserine lactones were synthesized as previously described (Shaw et al. 1997) or were the gifts of David Lynn (University of Chicago) or Anatol Eberhard (Ithaca College). The *Rhizobium* autoinducer, RLAI [*N*-(3-hydroxy-7-*cis*-tetradecanoyl)-L-HSL] was the generous gift of E. P. Greenberg (University of Iowa). A mix of 3-hydroxy acyl-HSLs was prepared from a culture supernatant of *Pseudomonas fluorescens* 2-79 (Shaw et al. 1997). Samples and standards were stored at -20°C in HPLC-grade ethyl acetate.

### Autoinducer bioassays.

Petri dish assays were performed as follows. A 3-ml volume of sterile 0.7% agar in distilled water was cooled to 45°C and inoculated with about 0.5 ml of a culture of the *A. tumefaciens* indicator strain grown overnight in ABM supplemented with carbenicillin. The suspension was mixed and layered over the surface of a petri dish (100 mm in diameter) containing 25 ml of ABM agar medium supplemented with 40 µg of 5-bromo-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. Generally, antibiotics were included in the media for overnight cultures, but not in the medium used for the overlays. The overlays were allowed to solidify, and colonies of organisms to be tested were inoculated as small patches onto the surface of the medium. When the organism to be tested did not grow on ABM, the isolate was grown to late exponential or early stationary phase in the appropriate medium (Table 1), the cells were re-

moved by centrifugation, and a 5- $\mu$ l volume of the supernatant was spotted onto the surface of the soft agar overlay. The plates were incubated for 1 to 2 days at 28°C.

TLC analysis was performed as described by Shaw et al. (1997). Strains to be tested were grown to late exponential or early stationary phase in 5-ml volumes of the appropriate liquid medium (Table 1). Cells were removed by centrifugation and the culture fluid was extracted twice with equal volumes of ethyl acetate. The organic phases were pooled, water was removed by addition of anhydrous Na<sub>2</sub>SO<sub>4</sub>, and the organic phases were taken to dryness under a stream of dry argon or nitrogen gas. Residues were redissolved in 50- $\mu$ l volumes of ethyl acetate and stored at -20°C. Components in the ethyl-acetate extracts were separated by chromatography on C<sub>18</sub> reversed-phase plates with a solvent system of methanol-water (60:40, vol/vol). The active acyl-HSLs were visualized by bioassay with the appropriate indicator strain. Preparations containing 0.7% agar were prepared in the medium appropriate to the indicator strain: ABM for *A. tumefaciens*, A medium (Sambrook et al. 1989) supplemented with glucose and 0.05% yeast extract for *E. coli* XL1-Blue, and L agar for *E. coli* MG4 and *C. violaceum* CV026blu. Medium used in assays involving the *tra*, *lux*, and *las* reporters was supplemented with X-Gal. The developed thin-layer plates, held in a Plexiglas jig, were overlaid with the soft agar suspensions, the agar was allowed to solidify, and the plates were incubated at 28°C overnight, all as described previously (Shaw et al. 1997).

#### Data preservation.

After development, the thin-layer plates were set out at room temperature in a fume hood until the overlay had completely dried down into a thin film. The dried plates were stored in flexible plastic sleeves. Images of the plates were archived with a digital color scanner and processed with Photoshop version 3.0 for the Macintosh (Adobe Systems, Mountain View, CA).

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## Erratum, Volume 11 no. 11, 1998

In the paper by Chung Cha, Ping Gao, Yu-Ching Chen, Paul D. Shaw, and Stephen K. Farrand (11:1119-1129) entitled "Production of Acyl-Homoserine Lactone Quorum-Sensing Signals by Gram-Negative Plant-Associated Bacteria" the *Ralstonia solanacearum* strains B1 and K60 were incorrectly identified as being the wild-type pathogenic strain and a spontaneous, nonpathogenic mutant of B1, respectively. This is reversed: strain K60 is the wild-type pathogen and strain B1 is the spontaneous, nonpathogenic mutant. Appropriate corrections, in which B1 should read as K60 and K60 should read as B1, should be made to footnote 'e' of Table 1, to the text of the Results section on page 1124, and to the text of the Discussion section on page 1126. Finally, lane 5 of Figure 3A shows an extract of a culture supernatant from *R. solanacearum* B1, the mutant, rather than from K60 as noted in the caption. This error does not affect any of the conclusions regarding production of acyl-HSL signal molecules by these strains of *R. solanacearum*.