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Acyl-Homoserine Lactone Production Is More Common among Plant-Associated *Pseudomonas* spp. than among Soilborne *Pseudomonas* spp.†

MIENA ELASRI,[1] SANDRINE DELORME,[2] PHILIPPE LEMANCEAU,[2] GORDON STEWART,[3] BRIDGET LAUE,[3] ERIC GLICKMANN,[1] PHIL M. OGER,[1] AND YVES DESSAUX[1]*

Institut des Sciences Végétales, CNRS UPR040, 91198 Gif-sur-Yvette Cedex,[1] and UMR INRA-Université de Bourgogne BBCE-IPM, CMSE-INRA, 21034 Dijon Cedex,[2] France, and University of Nottingham, Leicester, Sutton Bonington, United Kingdom[3]

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A total of 137 soilborne and plant-associated bacterial strains belonging to different *Pseudomonas* species were tested for their ability to synthesize *N*-acyl-homoserine lactones (NAHL). Fifty-four strains synthesized NAHL. Interestingly, NAHL production appears to be more common among plant-associated than among soilborne *Pseudomonas* spp. Indeed, 40% of the analyzed *Pseudomonas syringae* strains produced NAHL which were identified most often as the short-chain NAHL, *N*-hexanoyl-L-homoserine lactone, *N*-(3-oxo-hexanoyl)-homoserine lactone, and *N*-(3-oxo-octanoyl)-L-homoserine lactone (no absolute correlation between genomospecies of *P. syringae* and their ability to produce NAHL could be found). Six strains of fluorescent pseudomonads, belonging to the species *P. chlororaphis*, *P. fluorescens*, and *P. putida*, isolated from the plant rhizosphere produced different types of NAHL. In contrast, none of the strains isolated from soil samples were shown to produce NAHL. The gene encoding the NAHL synthase in *P. syringae* pv. maculicola was isolated by complementation of an NAHL-deficient *Chromobacterium* mutant. Sequence analysis revealed the existence of a *luxI* homologue that we named *psmI*. This gene is sufficient to confer NAHL synthesis upon its bacterial host and has strong homology to *psyI* and *ahlI*, two genes involved in NAHL production in *P. syringae* pv. tabaci and *P. syringae* pv. syringae, respectively. We identified another open reading frame that we termed *psmR*, transcribed convergently in relation to *psmI* and partly overlapping *psmI*; this gene encodes a putative LuxR regulatory protein. This gene organization, with *luxI* and *luxR* homologues facing each other and overlapping, has been found so far only in the enteric bacteria *Erwinia* and *Pantoea* and in the related species *P. syringae* pv. tabaci.

In most habitats, microbes compete to ensure their survival and multiplication. In the plant and soil environments, the mechanisms that allow a bacterium to outcompete other microbes are diverse (for reviews, see references 6, 8, and 47). Some of these traits are expressed constitutively, i.e., at any moment in the life of the microbial cell. Others are expressed only at times most favorable to allow an efficient biological effect. The regulation of these processes may depend on environmental parameters or alterations sensed by the microbes (see, for example, references 25 and 34; for a review, see reference 5), such as changes in microbial cell density. Indeed, microbes have evolved regulatory systems allowing gene expression only when the microbial cell density is appropriate. Such a phenomenon, which couples the microbial cell density to the expression of the relevant biological traits, is known as quorum sensing (QS) (for reviews, see references 20, 24, 41, and 46).

The mechanism underlying QS has been described for several microbes, including gram-negative bacteria, gram-positive bacteria, and streptomycetes (26). It involves the synthesis of low-molecular-weight molecules that diffuse in and out of the bacterial cell. As the bacterial population density increases, the amount of signal molecules synthesized and, consequently, their concentration in the environment increase. Once a critical concentration is reached, the signal molecules can be bound by a ligand protein, which acts as a transcription regulator in the microbial cell, allowing, upon binding, the expression of QS-regulated genes (for reviews, see references 20, 24, 41, and 46).

In gram-negative bacteria, the QS signal molecules are almost exclusively *N*-acyl-homoserine lactones (NAHL). Light emission by the fish symbiont *Photobacterium fischeri* (also known as *Vibrio fischeri*) was the first biological function known to be regulated in a QS-dependent fashion (13). In this bacterium, the NAHL-derived mediator was identified as *N*-(3-oxo-hexanoyl)-homoserine lactone (OHHL) (14), the synthesis of which relies on the activity of the NAHL synthase LuxI (encoded at the *luxI* locus). OHHL can be bound by LuxR (encoded by the *luxR* gene), which in turn is converted, upon binding, to a functional transcription regulator (for a review, see reference 46). This regulator attaches to a 20-nucleotide, inverted-repeated sequence located upstream of the operon encoding the proteins responsible for luminescence (16), al-

* Corresponding author. Mailing address: ISV, Bâtiment 23, CNRS, Ave. de la Terrasse, 91198 Gif-sur-Yvette Cedex, France. Phone: 33 1 6982 3690. Fax: 33 1 6982 3695. E-mail: yves.dessaux@isv.cnrs-gir.fr.

† This paper is dedicated to the memory of Gordon Stewart.

lowing its transcription. This palindrome sequence is known as the *lux* operator or the *lux* box. It has been found in the promoter regions of several QS-regulated genes (see, for example, references 1, 2, and 19). Interestingly, the first gene of the *lux* operon is *luxI*. Thus, the activation of the *lux* operon stimulates the production of the protein responsible for the production of OHHL (44).

Several bacterial traits are known to be regulated by LuxI- and LuxR-like proteins as a function of cell density (41). Indeed, *luxI* and *luxR* sequences have been detected in various gram-negative bacteria, and the production of NAHL signal molecules is widespread (4). These characteristics apply to plant-associated bacteria whether they are beneficial or deleterious for plant growth and health (37). Among the systems described so far, the conjugal transfer of the Ti plasmid of *Agrobacterium tumefaciens* depends on the presence of the relevant opine(s) in the environment as well as on a QS regulatory mechanism (18, 39). Similarly, the production of macerating exoenzymes (40) or that of the antibiotic carbapenem by *Erwinia carotovora* strains is regulated in a cell-density-dependent fashion (33; for a review, see reference 46). All the above functions involve the production of different NAHL, which differ from one species to the other or one strain to the other but whose structures are closely related.

Fluorescent pseudomonads have also developed QS-regulated synthesis of secondary metabolites implicated in antagonistic activities against plant pathogens, such as phenazines and pyoverdines (36, 45, 51). Because of the role of QS in the regulation of important physiological processes in plant-bacterium associations and because NAHL production in plant-associated *Pseudomonas* species has been investigated only in a small number of studies (4, 12), we decided to conduct a broad survey of NAHL production among pseudomonads isolated from plant tissues, the plant rhizosphere, or the bulk soil. NAHL production was not uncommon among plant-associated pseudomonads but was not detected in soil isolates. Among the plant-associated bacteria, members of the pathogenic *Pseudomonas syringae* and related species often produced NAHL. We therefore decided to characterize the genetic organization of the NAHL-dependent regulatory system in a representative isolate, *P. syringae* pv. maculicola strain CFBP 10912–9. Genes involved in QS regulation were isolated and analyzed. They appear to be distantly related members of the *luxR-luxI* gene family, with an unusual organization characterized by *luxR* and *luxI* facing and overlapping each other.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *Pseudomonas* strains and their origins and characteristics are listed in Tables 1, 2 and 3. The cytochrome *c* oxidase-positive strains of fluorescent pseudomonads are a representative subset of the diversity of a larger collection of strains ($n = 340$) isolated from two bulk soils and the rhizosphere of two plant species cultivated in these two soils (28). All bacterial strains were different, even though they belong to the same genomospecies and the same pathovar. Three complementary NAHL biosensors were used: *A. tumefaciens* strain NT1(pDCI41E33) (43), *Chromobacterium violaceum* strain CVO26 (32), and *Escherichia coli* strain JM109(pSB401) (50). Plasmid pDCI41E33 from *A. tumefaciens* harbors *traR* but not *traI* and contains a *traG*::*lacZ* fusion. CVO26 is a mini-Tn*5*-generated mutant of *C. violaceum* strain ATCC 31532 with all genes involved in violacein production and mutated copies of two regulatory genes, making violacein production dependent upon exogenous NAHL. Plasmid pSB401 harbors the *luxR* gene and the *lux* operon of *P. fischeri* with a deleted *luxI* region, making light emission dependent on the presence of exogenous NAHL. Other *E. coli* strains used were DH5α (42) and DB82(pRK2013) (11).

Pseudomonas strains were grown at 25°C, *Chromobacterium* and *Agrobacterium* strains were grown at 28 to 29°C, and *E. coli* strains were grown at 37°C. The media used were modified Luria-Bertani medium (LBm) (49); modified King B medium (KBm) (23); and ABM or ABG medium, which consisted of AB minimal medium (7) supplemented with mannitol or glucose at 0.5% (wt/vol), respectively. When required, antibiotics were incorporated into the media at the following concentrations: ampicillin (100 μg/ml), carbenicillin (100 μg/ml), rifampin (150 μg/ml), tetracycline (10 μg/ml), and kanamycin (100 μg/ml); however, *E. coli* strain DB82(pRK2013) was grown in the presence of 50 μg of kanamycin/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 40 μg/ml when necessary.

Chemicals. QS systems involve the production of several NAHL with closely related structures. We designate these molecules as a function of the number of carbon atoms of the lipid moiety and as a function of the substitution at position 3 of the fatty acid chains. The molecules are designated as follows: HSL, *N*-acyl-L-homoserine lactone; BHL, *N*-butanoyl-L-homoserine lactone (or C4-HSL); HHL, *N*-hexanoyl-L-homoserine lactone (or C6-HSL); OHL, *N*-octanoyl-L-homoserine lactone (or C8-HSL); DHL, *N*-decanoyl-L-homoserine lactone (or C10-HSL); OBHL, *N*-(3-oxo-butanoyl)-L-homoserine lactone (or 3-oxo-C4-HSL); OHHL, *N*-(3-oxo-hexanoyl)-L-homoserine lactone (or 3-oxo-C6-HSL); OOHL, *N*-(3-oxo-octanoyl)-L-homoserine lactone (or 3-oxo-C8-HSL); ODHL, *N*-(3-oxo-decanoyl)-L-homoserine lactone (or 3-oxo-C10 HSL); and OdDHL, *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (or 3-oxo-C12-HSL). Authentic NAHL samples were kindly provided by Paul Williams (University of Nottingham).

NAHL extraction. For rapid determination of NAHL production in liquid medium, 1-ml samples of the strains were cultured in KBm. Strains were grown to stationary phase and centrifuged for 10 min at ca. 10,000 × *g* and 4°C. A 250-μl sample of the native culture supernatant was used to load wells in plate assays as described below. Strains identified as positive were further tested on ABG medium and retested on KBm. The spent culture supernatant was extracted as follows. Bacteria were grown in 5 ml of ABG medium or KBm to stationary phase. The media were centrifuged for 10 min at 7,500 × *g* and 4°C. The supernatant was extracted twice with 100% ethyl acetate to yield 10 ml of extract. The extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness in a rotary evaporator at room temperature. The dried extract was resuspended in 600 μl of 100% ethyl acetate, evaporated again in a rotary evaporator at room temperature, and resuspended in 50 μl of 100% ethyl acetate. The final volume of the extract was therefore 1/100 that of the original culture medium.

NAHL detection and characterization. Individual strains were screened for the production of NAHL by two different assays. First, strains were screened on solid medium in a streak plate assay as described by Piper et al. (38) using the NAHL biosensors *C. violaceum* CVO26 and *E. coli* JM109(pSB401) on LBm and *A. tumefaciens* NT1(pDCI41E33) on ABM supplemented with 40 μg of X-Gal/ml. This assay was used to survey NAHL production by growing bacteria. Second, the presence of NAHL in native culture supernatants was assayed by the plate assay of McClean et al. (32) using the same three biosensors. Assay plates were incubated for 24 h for *Chromobacterium* and *E. coli* and for up to 48 h for *Agrobacterium.* NAHL produced by strains positive in any of the above-mentioned screens were further characterized by thin-layer chromatography (TLC) essentially as described by Shaw et al. (43) using the *Agrobacterium* sensor or by McClean et al. (32) using the *Chromobacterium* sensor. The concentrated extract was spotted onto a C_{18} reverse-phase TLC plate developed with a methanol-water solvent mixture. After elution, the plate was overlaid with soft agar containing bacterial indicators; this procedure generated a chromogenic compound upon sensing of a trace amount of one or more NAHL.

Modifications to the previously published protocols were as follows. TLC plates were C_{18} reverse-phase TLC plates (Silicagel; 60 Å, 20 by 20 cm, 0.2-mm thick). Elution buffers were methanol-water (60/40, vol/vol) for general use and methanol-water (70/30, vol/vol) for improved resolution of NAHL with acyl chains longer than eight carbon atoms. Characterization of the NAHL was based on the evaluation of the R_fs and shapes of the spots and on the differential responses of the sensor strains (including "reverse detection" by *Chromobacterium* [32]). An NAHL multistandard was always spotted on the TLC plates before migration of and along with the analyzed samples. The distributions of NAHL producers in different classes, defined according to their origins and to their relationships with the plant (soilborne isolates, nonpathogenic isolates from

TABLE 1. Names, origins, and NAHL-producing capacities of strains of cytochrome *c* oxidase-negative species of fluorescent pseudomonads[a]

Genomospecies[b]	Pathovar	Strain[c]	Host plant	NAHL synthesized
P. syringae	syringae	CFBP 1392	*Syringa vulgaris*	
	syringae	Meyer	*Phaseolus vulgaris*	HHL, OHHL
	aptata	CFBP 1617	*Beta vulgaris*	
	lapsa	CFBP 1731	*Triticum aestivum*	
	papulans	CFBP 1754	*Malus sylvestris*	
	pisi	CFBP 2105	*Pisum sativum*	
		CFBP 10267	*Pisum sativum*	
	atrofaciens	CFBP 2213	*Triticum aestivum*	
	aceris	CFBP 2339	*Acer* sp.	OHHL, OOHL
	panici	CFBP 2345	*Panicum* sp.	
	dysoxylis	CFBP 2356	*Dysoxylum spectabile*	
	japonica	CFBP 2896	*Hordeum vulgare*	
P. savastanoi	phaseolicola	CFBP 1390	*Phaseolus vulgaris*	
	ulmi	CFBP 1407	*Ulmus* sp.	HHL, OHHL, OOHL
	mori	CFBP 1642	*Morus alba*	
	lachrymans	CFBP 1644t	*Cucumis sativus*	HHL, OHHL, OOHL
	savastanoi	CFBP 1670	*Olea europaea*	HHL, OHHL
		CFBP 62	*Olea europaea*	HHL, OHHL
		CFBP 1020	*Olea europaea*	HHL, OHHL
		CFBP 2088	*Nerium oleander*	HHL, OHHL
		CFBP T12-1	*Nerium oleander*	HHL, OHHL
		CFBP 2093	*Fraxinus excelsior*	HHL, OHHL
		CFBP 2167	*Fraxinus excelsior*	HHL, OHHL
		CFBP 2169	*Fraxinus excelsior*	HHL, OHHL
		CFBP 2172	*Fraxinus excelsior*	HHL, OHHL
		CFBP 2174	*Fraxinus excelsior*	HHL, OHHL
		CFBP T36-10	*Fraxinus excelsior*	HHL, OHHL
		CFBP 2094	*Fraxinus excelsior*	HHL, OHHL
		CFBP 2176	*Fraxinus excelsior*	HHL, OHHL
	sesami	CFBP 1671	*Sesamum indicum*	
	tabaci	CFBP 2106	*Sesamum indicum*	HHL, OHHL
	morsprunorum	CFBP 2116t	*Prunus cerasus*	
	glycinea	CFBP 2214	*Glycinea max*	
		CFBP 1645	*Glycinea javanica*	
	ciccaronei	CFBP 2342	*Ceratonia siliqua*	HHL, OHHL
	eriobotryae	CFBP 2343	*Eriobotrya japonica*	HHL, OHHL
	mellea	CFBP 2344	*Nicotiana tabacum*	HHL, OHHL, OOHL
	aesculi	CFBP 2894	*Aesculus indica*	HHL, OHHL, OOHL
	hibisci	CFBP 2895	*Hibiscus japonica*	HHL, OHHL, OOHL
	myricae	CFBP 2897	*Myrica rubra*	HHL, OHHL
		CFBP 11005	*Myrica rubra*	HHL, OHHL
	photiniae	CFBP 2899	*Photinia glabra*	HHL, OHHL
		CFBP 11033	*Photinia glabra*	HHL, OHHL, OOHL
	dencropanacis	CFBP 3226	*Dendropanax trifidus*	OHHL
	amygdali	CFBP 3340	*Prunus amydalus*	
		CFBP 10788	*Prunus amydalus*	
	ficuserectae	CFBP 3224	*Ficus erecta*	HHL, OHHL
	meliae	CFBP 3225	*Melia azedarach*	HHL, OHHL
P. tomato	persicae	CFBP 1573	*Prunus persicae*	
		CFBP 1067	*Prunus persicae*	
	anthirrini	CFBP 1620	*Antirrhinum majus*	OHHL
	maculicola	CFBP 1657	*Brassica oleracea*	OHHL, OOHL
		CFBP 11056	*Brassica oleracea*	HHL, OHHL, OOHL
		CFBP 1637	*Raphanus sativus*	
		CFBP 11079-5	*Raphanus sativus*	OHHL, OOHL
		CFBP 10912-9	*Raphanus sativus*	OHHL, OOHL
	viburni	CFBP 1702	*Viburnum* sp.	OHHL, OOHL
	berberidis	CFBP 1727	*Berberidis* sp.	
		CFBP 3208	*Berberidis gagnepainii*	
	apii	CFBP 2103	*Apium graveolens*	OHHL, OOHL
	tomato	CFBP 2212	*Lycopersicon esculentum*	OHHL
		CFBP 8486	*Lycopersicon esculentum*	
		CFBP 10199	*Lycopersicon esculentum*	
		CFBP 10208	*Lycopersicon esculentum*	
	delphinii	CFBP 2215	*Delphinium* sp.	HHL, OHHL
	passiflorae	CFBP 2346	*Passiflora edulis*	OHHL, OOHL
	morsprunorum	CFBP 2351	*Prunus domestica*	

Continued on following page

TABLE 1—*Continued*

Genomospecies[b]	Pathovar	Strain[c]	Host plant	NAHL synthesized
	lachrymans	CFBP 2440	*Cucumis sativus*	
	philadelphi	CFBP 2898	*Philadelphus coronarius*	
	ribicola	CFBP 10971t	*Ribes aureum*	HHL, OHHL
	primulae	CFBP 11007t	*Primula* sp.	BHL, HHL
P. porri	porri	CFBP 1908	*Allium porrum*	
		CFBP 2395	*Allium porrum*	
	garcae	CFBP 1634	*Coffea arabica*	
	striafaciens	CFBP 1674	*Avena sativa*	
		CFBP 1686t	*Avena sativa*	
	coronofaciens	CFBP 2216	*Avena sativa*	
	atropurpurea	CFBP 2340	*Lolium multiforum*	
	oryzae	CFBP 3228	*Oryza sativa*	
	zizaniae	CFBP 11040	*Zizania aquatica*	
P. avellanae	avellanae	CFBP 10963	*Corylus avellana*	
	theae	CFBP 2353	*Thea sinensis*	
P. helianthi	helianthi	CFBP 2067	*Helianthus annuus*	OHHL, HHL
	tagetis	CFBP 1694	*Tagetes erecta*	OHHL, HHL
P. tremae	tremae	CFBP 3229	*Trema orientalis*	
P. cannabina	cannabina	CFBP 2341	*Cannabis sativa*	
		CFBP 1619	*Cannabis sativa*	
P. viridiflava	viridiflava	CFBP 2107	*Phaseolus* sp.	
	primulae	CFBP 1660	*Primula* sp.	
	ribicola	CFBP 2348	*Ribes aureum*	

[a] Strains were grown in both ABG medium and KBm.
[b] As defined by Gardan et al. (21) and Gardan et al. (22).
[c] CFBP, Collection Française des Bactéries Phytopathogènes (French Collection of Plant-Pathogenic Bacteria), Institut National de la Recherche Agromomique, Angers, France. The bacteria assayed were all unique strains.

roots or leaves, and pathogenic isolates), were compared pairwise to a theoretical even distribution by a chi-square test.

DNA manipulations and sequencing. All molecular techniques, such as DNA extraction and restriction analysis, were performed using standard protocols (42). Routine cloning at various sites of the ColE1-derived vector pUC19 (or related vectors) was done with *E. coli* strain DH5α as a recipient strain. A genomic DNA bank was obtained from 10912–9 by cloning the dephosphorylated restriction products of a partial *Sau*3AI digestion of genomic DNA from this strain at the *Bam*HI site of the broad-host-range cosmid vector pCP13/B (10) according to standard protocols (42). When necessary, cosmid clones were transferred to gram-negative recipients by triparental mating with DB82(pRK2013) (11).

Sequencing was performed in part at the Institut des Sciences Végétales with an Applied Biosystems 370 sequencer and in part at Eurogentec (Herstal, Belgium). Sequences were assembled with Sequencer software (GeneCodes, Ann Harbor, Mich.). Nucleotide and amino acid sequence comparisons were made using the BLAST protocol available online at the National Center for Biotechnology Information website. Multiple alignments were performed using the Pileup subroutine of the GCG package (version 10; GCG Inc., Madison, Wis.).

Mutagenesis of the *psmI* gene was performed by introducing a gentamicin resistance cassette obtained from pmGm (35) as an *Sma*I fragment at the *Ssp*I site of *psmI*, interrupting the open reading frame (ORF) at position 85. To evaluate homoserine lactone production, the wild-type *psmI* or *psmR* locus and the *psmI*::Gm construct were cloned into the broad-host-range vector pBBR1MCS-3 (27), which was further transferred to various gram-negative bacterial hosts by triparental mating using DB82(pRK2013) (11) as a helper.

Nucleotide sequence accession number. Sequences determined here have been deposited in GenBank under accession number AF234628.

RESULTS

NAHL production is more common among plant-associated than among soilborne *Pseudomonas* spp. Using the protocols described in Materials and Methods, strains which induced the production of violacein (or which inhibited it) in the *C. violaceum* sensor, strains which activated the biosynthesis of beta-galactosidase in *A. tumefaciens* NT1(pDCI41E33), and strains which induced light emission in *E. coli* JM109(pSB401) remained NAHL producers. The others were regarded as nonproducing strains under our experimental conditions. The results of this analysis and the identification of the synthesized NAHL molecules are shown in Tables 1, 2 and 3 and in Fig. 1. As the characterization of the NAHL molecules is based on the examination of the shapes and R_fs of the spots and on the differential responses of the sensors (with respect to standard reference samples), the identification results must be interpreted with care.

Among the 90 pathogenic strains of oxidase-negative fluorescent pseudomonads, 44 were identified as NAHL producers. Within this group, plant-pathogenic bacteria belonging to *P. syringae* and related species are listed by genomospecies (Table 1). These are taxonomic subdivisions which can be regarded de facto as species; indeed, strains belonging to the same genomospecies show a DNA-DNA relatedness of at least 66% (21, 22), whereas relatedness between strains belonging to different genospecies is lower than 59%. The ratio of NAHL producers varies from one genomospecies to another. Thus, among the strains of the genomospecies *P. syringae*, only 2 out of 12 produced OHHL and HHL; none of the strains of the genomospecies *P. porri*, *P. avellanae*, *P. tremae*, *P. cannabina*, and *P. viridiflava* synthesized NAHL (under our experimental

TABLE 2. Names, origins, and NAHL-producing capacities of strains of cytochrome *c* oxidase-positive species of fluorescent pseudomonads[a]

Species	Biovar	Strain[b]	Origin	Host plant[c]	NAHL synthesized
P. chlororaphis		DTR133	Rhizosphere	*Lycopersicon esculentum*	HHL, OHHL
P. fluorescens	II	DLRp214	Rhizoplane	*Linum usitatissimum*	
		CLRp812	Rhizoplane	*Linum usitatissimum*	
		CLE513	Root tissue	*Linum usitatissimum*	
		CS611	Bulk soil	NA	
		CTR1015	Rhizosphere	*Lycopersicon esculentum*	
		CTR212	Rhizosphere	*Lycopersicon esculentum*	
		C7R12	Rhizosphere	*Linum usitatissimum*	
	III	CS613	Bulk soil		
	IV	DLR426	Rhizosphere	*Linum usitatissimum*	
		DTR335	Rhizosphere	*Lycopersicon esculentum*	Unidentified
		DLE411J	Root tissue	*Linum usitatissimum*	
	VI	CLR711	Rhizosphere	*Linum usitatissimum*	HHL
		CTRp112	Rhizoplane	*Lycopersicon esculentum*	
	Undetermined	CFBP 2129	Water	NA	
		GRA-3	Rhizosphere	ND	
		CFBP 11393	Rhizosphere	*Lycopersicon esculentum*	OOHL, OHL, DHL
		CFBP 11363	Rhizosphere	*Zea mays*	OOHL, OHL, DHL
		CFBP 11350	Rhizosphere	*Zea mays*	
		CFBP 11388	Rhizosphere	*Lycopersicon esculentum*	
		CFBP 11345	Rhizosphere	*Zea mays*	
		CFBP 11273	Rhizosphere	*Glycine max*	
		CFBP 11346	Rhizosphere	*Zea mays*	
		CFBP 11369	Rhizosphere	*Zea mays*	
		CFBP 11387	Rhizosphere	*Lycopersicon esculentum*	
		CFBP 11385	Rhizosphere	*Lycopersicon esculentum*	
		CFBP 11366	Rhizosphere	*Zea mays*	
		CFBP 11394	Rhizosphere	*Lycopersicon esculentum*	
Intermediate species (*P. fluorescens* and *P. putida*)	Undetermined	DS824	Bulk soil	NA	
		DS821	Bulk soil	NA	
P. putida	A	DTRp621	Rhizoplane	*Lycopersicon esculentum*	
		DLR223	Rhizosphere	*Linum usitatissimum*	
		DLR228	Rhizosphere	*Linum usitatissimum*	
		DS1026	Bulk soil	NA	
		DLE3216	Root tissue	*Linum usitatissimum*	
		DS131	Bulk soil	NA	
		CS111	Bulk soil	NA	
		CS413	Bulk soil	NA	
		CS714	Bulk soil	NA	
	Undetermined	CFBP 2066	Bulk soil	NA	
		N5F5	Rhizosphere	*Beta vulgaris*	OHHL, OOHL, ODHL, one unidentified
P. cichorii		CFBP 2101	Plant	*Cichorium endivia*	

[a] Strains were grown in both ABG medium and KBm.
[b] See Table 1, footnote *c*. Strains other than CFBP strains were from our laboratories. The bacteria assayed were all unique strains.
[c] NA, not available; ND, not determined.

conditions). In contrast, NAHL-producing strains were predominant in three genomospecies. In the *P. savastanoi* genomospecies, 28 of 35 strains and, most notably, all 13 strains of *P. savastanoi* pv. savastanoi produced NAHL, while 12 of 23 strains of the genomospecies *P. tomato* produced NAHL. Finally, the two strains defining the *P. helianthi* genomospecies also produced NAHL.

Among the 42 oxidase-positive *Pseudomonas* strains, 27 were isolated from the rhizosphere and rhizoplane, 10 were from bulk soil, 4 were from plant tissues, and 1 was from water (Table 2). Within this group, only 6 of 42 strains produced NAHL. These six strains were isolated from the rhizosphere of four different plants. In contrast to their oxidase-negative counterparts, from which they are taxonomically distinct, oxidase-positive pseudomonads synthesized a variety of NAHL molecules, such as HHL, OHHL, OOHL, DHL, and ODHL. *P. putida* strain P5F5 also produced an unidentified NAHL, the migration of which was related to that of OdDHL but was not strictly identical (data not shown).

Overall, the data revealed that out of 137 different strains analyzed, 54 (40%) produced NAHL. Remarkably, NAHL-producing strains were found only among bacteria isolated from plants; none of the soilborne strains tested was able to synthesize these molecules. More precisely, the NAHL-producing strains were more abundant among the pathogenic

TABLE 3. Names, origins, and NAHL-producing capacities of strains of nonfluorescent species of *Pseudomonas*[a]

Species	Strain[b]	Origin[c]	Host plant	NAHL synthesized
P. stutzeri	CFBP 2443	ND	ND	
P. corrugata	8-1	Plant	*Lycopersicon esculentum*	HHL, OHHL, OHL
	82.23.6	Plant	*Lycopersicon esculentum*	HHL, OHHL, OHL
	632.2	Plant	*Lycopersicon esculentum*	HHL, OHHL, OHL
	83.83.4	Plant	*Lycopersicon esculentum*	HHL, OHHL, OHL

[a] Strains were grown in both ABG medium and KBm.
[b] See Table 1, footnote *c*. Strains other than the CFBP strain were from our laboratories. The bacteria assayed were all unique strains.
[c] ND, not determined.

strains (49%) than among the rhizosphere strains (28%) and the soilborne strains (0%). Statistical analysis of these data indicated that the frequencies of NAHL-producing strains differed significantly according to their origins and to their relationships with the plant ($\chi^2 = 11.53$, $P < 0.05$).

Cloning of the genes responsible for NAHL production in *P. syringae* pv. maculicola. The above results indicate that NAHL production is not uncommon among plant-associated *Pseudomonas* species and especially among strains of *P. syringae.* However, little is known about the biological traits regulated via QS in *P. syringae.* To identify the NAHL biosynthetic pathway and the relevant regulated biological functions, we attempted to identify the genes involved in the synthesis of these compounds by a *P. syringae* strain. The strain chosen for this study was *P. syringae* pv. maculicola strain CFBP 10912–9 (*P. tomato* genomospecies) (Table 1), essentially because it produces large amounts of OHHL and OOHL.

To isolate the genes responsible for NAHL production, a genomic cosmid library of 10912–9 DNA was conjugated en masse by triparental mating into a rifampin-resistant derivative of the biosensor CVO26. Screening of ca. 5,000 transconjugants selected as being resistant to tetracycline and rifampin was used to identify the genes encoding the production of NAHL; this procedure yielded 20 clones that restored violacein production in the biosensor. The recombinant plasmids in the clones, all with inserts ranging from 20 to 35 kb, contained one or more similarly sized *Eco*RI and *Xho*I fragments and could be organized in two classes according to their restriction patterns. A representative of each class, termed pMES-A and pMES-B, was retained for further studies. The two cosmids were transferred into the non-NAHL-producing *P. syringae* pv. persicae strain CFBP 1573 (Table 1). Both conferred to that strain the ability to produce NAHL. Furthermore, TLC profiles of concentrated culture supernatants from CFBP 1573(pMES-A) and 10912–9 were indistinguishable (Fig. 2). The region of overlap between the two cosmid classes was mapped, subcloned into the ColE1-based vector pUC19, and introduced into *E. coli* DH5α, which does not produce any detectable NAHL. Two pUC19-derived clones, containing a ca. 2-kb *Xho*I fragment (pMEX-A) and a 4.5-kb *Pst*I fragment (pMEP-A), conferred to that strain the ability to produce the same NAHL signal molecules as the parent strain, *P. syringae* pv. maculicola strain CFBP 10912–9 (Fig. 2), even in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG). Inter-

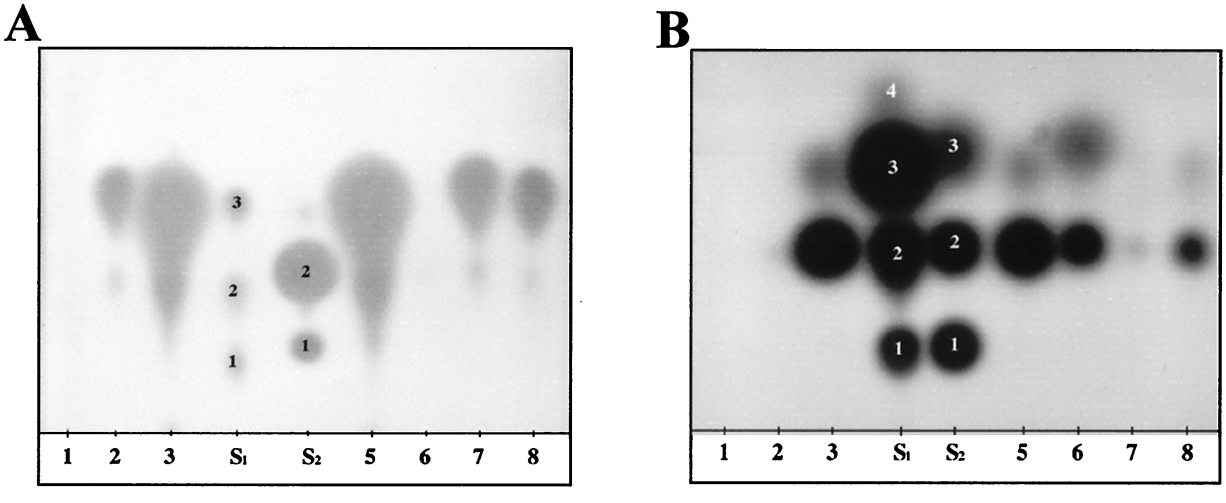


FIG. 1. Production of NAHL by various isolates of *Pseudomonas*. Supernatant extracts were obtained as indicated in Materials and Methods from strains of the *P. tomato* genomospecies: 1, CFBP 1573; 2, CFBP 1620; 3, CFBP 1702; 5, CFBP 2215; 6, CFBP 11007t; 7, CFBP 10912–9; and 8, CFBP 2212. S_1 was a mixture of keto-NAHL (OBHL, 4; OHHL, 3; OOHL, 2; and ODHL, 1) used as a migration standard; S_2, another migration standard, was a mixture of reduced NAHL (BHL, 3; HHL, 2; and OOHL, 1). (A) The sensor strain was *Agrobacterium* NT1(pDCI41E33) (43), which exhibits a high level of sensitivity toward reduced NAHL (concentration of standards according to Shaw et al. [43]). (B) The sensor strain was *Chromobacterium* CVO26 (32), which senses preferentially very-short-chain (C_6 and C_8) keto-NAHL (concentration of standards according to McClean et al. [32]).

FIG. 2. Production of NAHL by the cloned *psmI* gene. Concentrated extracts of bacterial spent supernatants were obtained and analyzed as indicated in Materials and Methods using the *Agrobacterium* sensor strain NT1(pDCI41E33) (43). Extracts were obtained from *P. tomato* pv. persicae CFBP 1573 (lane 1) and CFBP 1573(pMES-A) (lane 2). A result identical to that shown in lane 2 was obtained using wild-type *P. syringae* pv. maculicola strain CFBP 10912–9 (not shown). S, migration standard consisting of OHHL (top) and OOHL (middle).

estingly, the original cosmid clones, pMES-A and pMES-B, did not confer to the *E. coli* strain the ability to produce NAHL. Similarly, two pUC19-derived clones containing the 2-kb *Xho*I fragment and the 4.5-kb *Pst*I fragment in the orientations opposite those in pMEX-A and pMEP-A, respectively, did not induce the production of NAHL in DH5α.

Sequence analysis and identification of *psmI* and *psmR*. The DNA sequence of the 2-kb *Xho*I/*Xho*I fragment and that of its adjacent regions were determined. DNA sequence analysis revealed the presence of three ORFs organized as shown in Fig. 3. One of the three ORFs, which we named *psmI*, could encode a 244-amino-acid protein with an estimated mass of 27.16 kDa. This sequence is closely related to those of two *luxI* gene homologues, *ahlI* (12) and *psyI* (P. M. Oger et al., unpublished data [GenBank accession number AF266600]), found in other members of the *P. syringae* genomospecies (Fig. 4). The product of this ORF is only distantly related to the other LuxI proteins. The *psmI* ORF is preceded by sequences with reasonable matches to consensus ribosome binding site (RBS) sequences and −10 and −35 promoter elements (Fig. 3). In addition, we were able to identify a sequence closely related to the *lux* box consensus sequence at positions −76 to −56 upstream of the *psmI* start codon, indicating that this ORF could be regulated by a QS-dependent regulator. Interestingly, this regulatory box lies between the best matches to −35 and −10 sequences and overlaps the putative −10 sequence (Fig. 3). Since *psmI* is the only ORF on the 2-kb *Xho*I fragment of pMEX-A whose product shows homology to NAHL synthases, its expression must be responsible for NAHL synthesis in DH5α(pMEX-A). Interestingly, the *Xho*I site used for the cloning lies within the *psmI* ORF. Therefore, the truncated *psmI* ORF of pMEX-A still encodes a functional or partially functional NAHL synthase.

The second complete ORF, which we called *psmR*, was found downstream of *psmI* (Fig. 3). It could encode a protein of 247 amino acids with a mass of 28.34 kDa. The deduced protein sequence exhibits a helix-turn-helix motif, suggesting that it can bind nucleic acid. In agreement with this information, the *psmR* gene product exhibits homology with the LuxR family of regulatory proteins (Fig. 5), especially with PsyR from *P. syringae* pv. tabaci (Oger et al., unpublished data; AF266600). As shown in Fig. 3, the *psmI* and *psmR* genes are transcribed convergently, and the 3′ ends of their coding regions overlap. Determination of the GC contents of both *psmI* and *psmR* yielded values (ca. 55 and 52%, respectively) slightly below those reported for other *P. tomato* genes (58%).

The third ORF, which we termed *orfA*, is located ca. 218 bp upstream of the putative start codon of *psmI* and is most probably transcribed divergently from it. It is closely related (85% identity) to the *orf1* gene, identified upstream of the *luxI* homologue *psyI* in *P. syringae* pv. tabaci. The putative products of *orfA* and *orf1* have weak homologies (32 and 54%, respectively) to the C-terminal ends of members of the histidinol-phosphate aminotransferase family and therefore could have a similar enzymatic function. However, the N-terminal ends of the characterized sequences of the putative proteins OrfA and Orf1 do not share homology with aminotransferases. No *lux* regulatory sequences could be identified upstream of *orfA* or *psmR*, suggesting that these genes may not be regulated by QS.

Analysis of *psmI* by complementation and mutational analysis. To confirm the involvement of *psmI* in NAHL synthesis, we recloned the 4.5-kb *Pst*I fragment in the broad-host-range vector pBBR1MCS-3 and transferred it by triparental mating to *P. fluorescens* recipient strain 1855.344, which does not produce any NAHL (4; unpublished results). Strain 1855.344 harboring the cloned 4.5-kb *Pst*I fragment now produced the same NAHL as wild-type strain 10912–9. A cassette encoding gentamycin resistance was cloned at the *Ssp*I site of the 4.5-kb *Pst*I fragment to disrupt *psmI* (Fig. 3). The resulting construction was also transferred to 1855.344. The resulting transconjugants did not produce any NAHL (data not shown).

DISCUSSION

The strain survey presented in the first part of this study indicates that NAHL production is not uncommon among plant-associated *Pseudomonas* strains. Several conclusions can be drawn. For instance, among oxidase-negative *Pseudomonas* strains, no clear correlation between taxonomic position and NAHL production is evident, with the exception of the 8 strains of the *P. porri* genomospecies (which did not appear to produce NAHL) and the 13 *P. savastanoi* strains (which all produced HHL and OHHL). During the course of this work, two surveys of NAHL production by gram-negative strains were published (4, 12). A few strains of the *P. syringae* group were examined for NAHL production. Some discrepancies between those studies and ours can be observed, e.g., for two *P. savastanoi* strains (CFBP 2088 and CFBP 2093). While Cha et al. (4) did not observe NAHL production for these strains, we report here that they do produce the NAHL signal molecules HHL and OHHL. This difference most likely results from the different growth conditions used in the two studies. However, this difference emphasizes that NAHL production among the strains analyzed by us and by others might have an even wider distribution. Strains which did not produce any NAHL under our experimental conditions might indeed produce such signal molecules under other growth conditions, e.g., in a more fa-

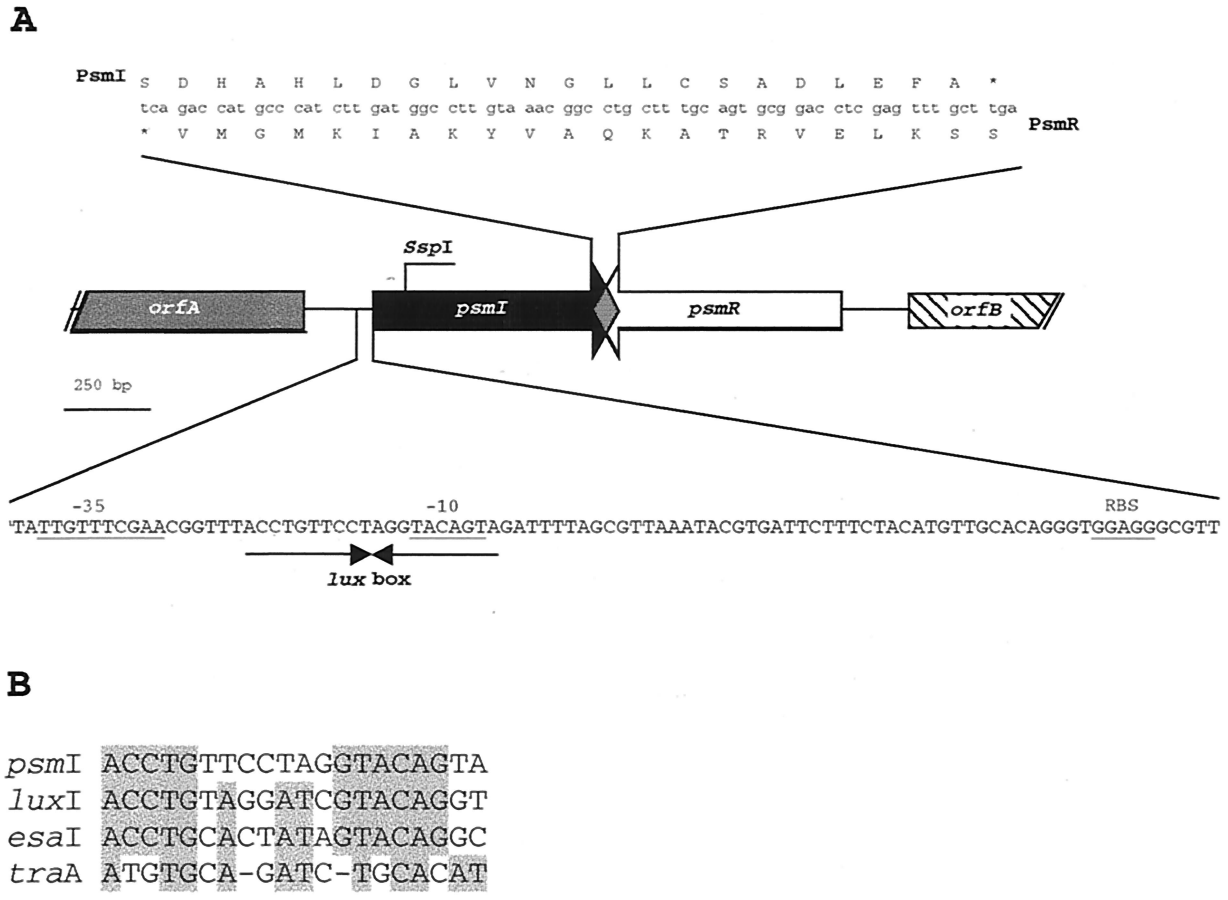


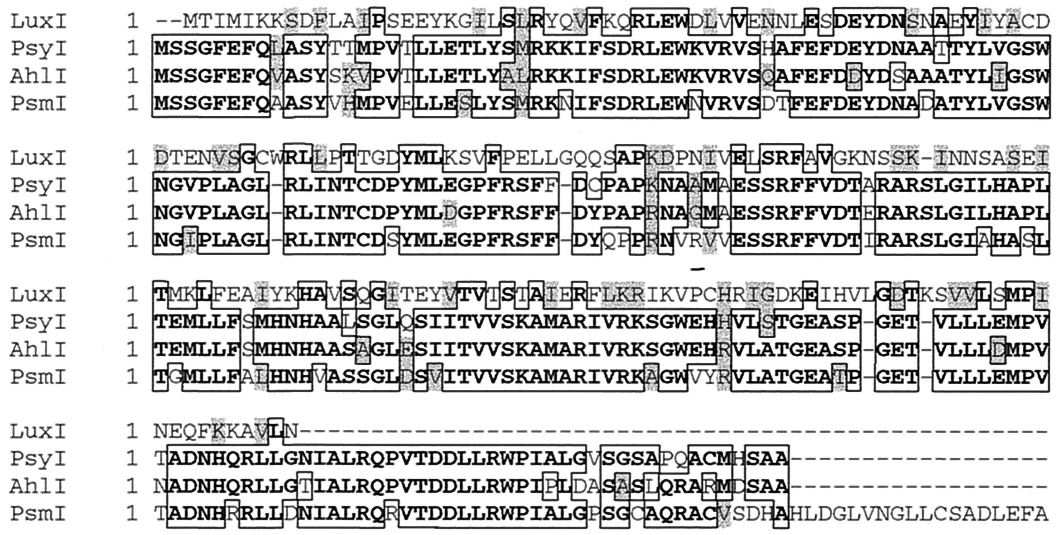
FIG. 3. (A) Genetic organization of the *psmI-psmR* locus from *P. syringae* pv. maculicola strain CFBP 10912–9. In the diagram in the center of panel A, the putative identified ORFs are represented by arrows indicating the direction of transcription. The upper diagram shows a detail of the overlap between the *psmI* and *psmR* genes. The lower diagram shows a detail of the promoter region of the *psmI* gene. The sequences of the putative regulatory elements (−35, −10, and RBS) are underlined. The position of the putative *lux* box is indicated by the convergent arrows. The gentamicin cassette used to disrupt the *psmI* gene was inserted at the *Ssp*I site shown above the *psmI* ORF. (B) Sequence alignment of *lux* boxes from the *psmI* gene of *P. syringae* pv. maculicola, the *luxI* gene of *P. fischeri*, the *esaI* gene of *P. stewartii*, and the *traA* gene of *A. tumefaciens*.

vorable or in an "inducing" environment, as reported for the opine-dependent NAHL synthesis involved in the regulation of Ti plasmid transfer in *Agrobacterium* (for a review, see reference 18).

As indicated above, most of the analyzed *Pseudomonas* strains produced short-chain NAHL (eight or fewer carbon atoms, e.g., HHL, OHHL, and OOHL). This feature is not due to a technical bias, since two of the biosensors are able to detect long-chain NAHL (*Chromobacterium* [reverse staining] and *Agrobacterium*) (32, 43). Indeed, one of the soil *P. putida* strains was found to produce ODHL and possibly OdDHL, two "long-chain" NAHL. The generalized production of HHL and OHHL by *P. syringae* strains is interesting and might reflect a common origin for the different QS systems in these strains.

Interestingly, our results go beyond those previously published (4, 12), as they indicate that NAHL production seems more common among pseudomonads closely associated with plants than among their soilborne counterparts. Indeed, the percentage of NAHL producers decreased from 49% among plant-pathogenic bacteria to 28 and 0% among nonpathogenic bacteria associated with the plant and soilborne bacteria, respectively. This finding suggests that the closer the relationship of the bacteria with the host plant, the higher the probability that it produces NAHL. That NAHL production appears to be more common among isolates closely associated with plant tissues may be related to the wealth of available carbon sources (31) in this highly competitive environment. This fact is consistent with the detection of QS-regulated functions in other plant-associated, plant-symbiont, or plant-pathogenic bacteria (for reviews, see references 20, 37, 41, and 46) or in other microbial hosts of rich ecotopes (e.g., *Photobacterium*, *Shigella*, and so forth). Alternatively, our results could reveal the limited ability of our experimental conditions to detect NAHL production in soilborne pseudomonads. If this is true, then the observed variation does not correlate with the mere presence of *luxI* and *luxR* loci but reflects differences in terms of regulation of these loci (e.g., additional regulatory levels, requirements for unknown inducers, and so forth). However, the statistical analysis demonstrates that these putative differences discriminating soilborne, plant-associated, and plant-pathogenic pseudomonads are statistically and biologically significant, as they correlate with the ecology of the bacteria.

A



B

Proteins	Organisms	Accession nb.	Identities	Similarities
PsyI	*Pseudomonas syringae* pv. *tabaci*	AF110468	78%	84%
AhlI	*Pseudomonas syringae* pv. *syringae*	AF072537	76%	85%
YtbI	*Yersinia pseudotuberculosis*	AF079136	37%	54%
CarI	*Erwinia carotovora*	X74299	34%	53%
EsaI	*Erwinia stewartii*	L32183	31%	48%
LuxI	*Vibrio fischeri*	M19039	30%	53%
TraI	*Agrobacterium tumefaciens*	AF057718	32%	49%

FIG. 4. Relatedness of the PsmI protein to other NAHL synthases. (A) Protein sequence alignment of PsmI with PsyI, AhlI, and LuxI, NAHL synthases from *P. syringae* pv. tabaci, *P. syringae* pv. syringae, and *P. fischeri*, respectively. Bold letters and boxes indicate identity; grey shading indicates similarity. (B) Similarity and identity with other LuxI-like proteins.

The wide occurrence of NAHL producers among pseudomonads, especially among strains of *P. syringae* and related species, stands in contrast with the lack of information on the function(s) regulated in a QS-dependent way in these bacteria. A first step toward the elucidation and understanding of the QS-regulated functions in *P. syringae* strains involves the isolation of the relevant genes. Cloning and sequencing of the region responsible for NAHL production in *P. syringae* pv. maculicola led to the identification of two genes, *psmI* and *psmR*. To our knowledge, this is the first report of the presence of a *luxR* homologue in *P. syringae*. Sequence analysis of these genes revealed their high degree of homology to members of the *luxI* and *luxR* gene families, especially with the *psyI* and *psyR* genes from *P. syringae* pv. tabaci (Oger et al., unpublished results) and with the *ahlI* gene from *P. syringae* pv. syringae (12), respectively.

The *psmI* gene is likely to code for an NAHL synthase. Several lines of evidence support this hypothesis. First, it has strong homology to other genes encoding such enzymatic activities (Fig. 4). Second, the cloned gene conferred NAHL production upon nonproducing hosts (e.g., *P. syringae* pv. persicae, *P. fluorescens*, *E. coli*, and the two NAHL reporter strains tested). Third, bacteria hosting *psmI* exhibited a production pattern analogous to that of the *P. syringae* pv. maculicola strain. Finally, a mutated *psmI* gene does not confer NAHL production upon the bacterial host anymore. These features clearly indicate that the *psmI* gene is the necessary and sufficient genetic determinant accounting for the production of all NAHL signal molecules in *P. syringae* pv. maculicola CFBP 10912–9.

NAHL production in DH5α was observed only with clones harboring the *luxI* gene inserted into a pUC19 plasmid (e.g., pMEX-A) and under the control of the *lac* promoter and not with the full-size cosmid clones harboring both the *psmI* and the *psmR* genes, although the *psmI* gene was expressed in the *P. syringae* pv. persicae background. This result may have been due to the organization or sequence of the promoter regions of *P. syringae* pv. maculicola genes that are not recognized by the *E. coli* transcription machinery, although the *psmI* gene is preceded by reasonable matches to consensus −35 and −10 sequences. This observation has also been reported for several nonenteric bacterial species, such as *P. fluorescens*, *P. syringae* pv. tabaci, and *A. tumefaciens*. For example, the expression of *Agrobacterium* virulence genes in *E. coli* requires the presence of the alpha subunit of the RNA polymerase from *Agrobacterium* (29). Our data also suggest that PsmR could act as a

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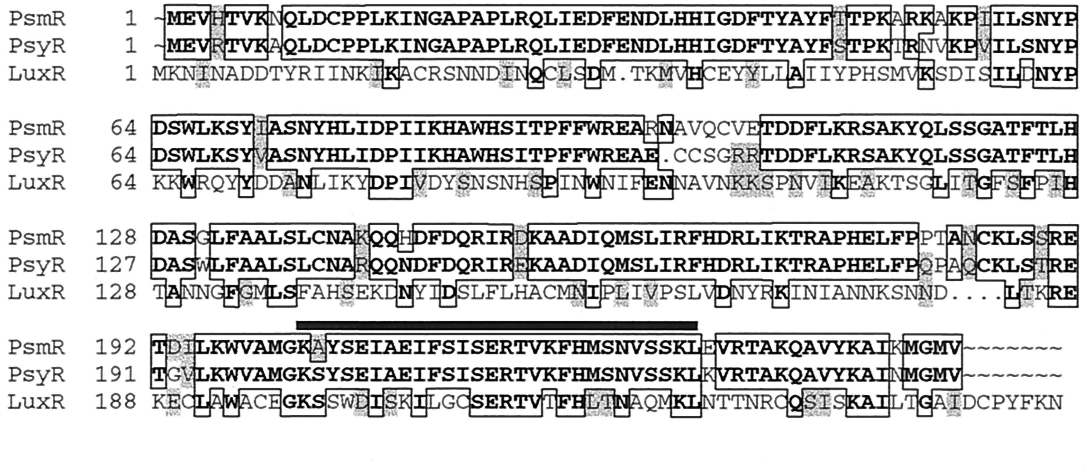

B

Proteins	Organisms	Accession nb.	Identities	Similarities
PsyR	*Pseudomonas syringae* pv. *tabaci*	AF110468	88%	92%
CarR	*Erwinia carotovora*	U17224	35%	57%
YtbR	*Yersinia pseudotuberculosis*	AF079136	34%	50%
EsaR	*Erwinia stewartii*	L32184	32%	50%
LuxR	*Vibrio fischeri*	M19039	26%	46%
TraR	*Agrobacterium tumefaciens*	AF010180	24%	42%

FIG. 5. Relatedness of the PsmR protein to other LuxR-type regulators. (A) Protein sequence alignment of PsmR with PsyR and LuxR from *P. syringae* pv. tabaci and *P. fischeri*, respectively. Bold letters and boxes indicate identity; grey shading indicates similarity. The black bar indicates the putative DNA-binding domain. (B) Similarity and identity with other LuxR-like proteins.

repressor which prevents the expression of *psmI*. In agreement with this hypothesis, a palindromic, *lux* box-like sequence has been detected within the promoter region of *psmI*, just upstream of the putative −10 sequence and overlapping it. Interestingly, this *lux* box is located at a position similar to that found in *P. stewartii* for a system involving the LuxR-like repressor protein EsaR (3). This finding is also consistent with the observation that in other systems which involve activator proteins, the regulatory *lux* sequences are located upstream of the proposed −35 elements (1, 16, 52). Recent results obtained by Luo and Farrand (30) confirmed that the activity of the LuxR-type regulator TraR, although an intrinsic property of the molecule, is also strongly affected by the positioning of the *lux* box (17).

Sequence data revealed a gene organization with two genes (*psmI* and *psmR*) facing each other and slightly overlapping. This organization is only the third example reported so far for bacteria. The first two were described for *Yersinia* (48) and *Erwinia* (*Pantoea*) (2). Although not demonstrated for *P. syringae* pv. maculicola or for any of the systems with convergently transcribed *luxR* and *luxI* genes, this organization may play an additional role in the regulation of the expression of the two genes, as the elevated transcription of one of the two may impair the expression of the other.

In some organisms, e.g., *A. tumefaciens* and *P. fischeri*, the genes regulated in a QS-dependent fashion are located downstream of the *traI* and *luxI* genes, respectively, and are coordinately regulated with these genes. In *P. syringae* pv. maculicola, this appears not to be the case. In this respect, the gene organization of *psmI* and *psmR* is similar to that in the enteric bacterium *Erwinia* (*Pantoea*), in which QS-regulated genes are not linked to the regulatory loci. The major difference between the organization of QS systems is of interest. In the systems in which a *luxI* homologue is the first gene of the LuxR-regulated operon, QS regulates only a single function, i.e., conjugal transfer for *A. tumefaciens* and bioluminescence for *P. fischeri*. In the systems in which a *luxI* homologue is not associated with a QS-regulated function, QS is most often involved in a complex regulatory scheme that controls the expression of more than one operon or function. This information suggests that QS may also regulate more than one trait in *P. syringae* pv. maculicola. Whether the functions regulated in a QS-dependent fashion in *P. syringae* pathovars are important for plant-microbe associations remains to be determined. However, with respect to previously published data, a possible correlation between NAHL synthesis and pathogenicity (2, 40), siderophore biogenesis (45), swarming (15), or biofilm formation (9) may be proposed.

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