Lehrstuhl für Mikrobiologie
der Technischen Universität München

Die Bedeutung bakterieller Zell-Zell Kommunikation für die Besiedelung von Pflanzenwurzeln

Anette Dorothea Charlotte Steidle

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. W. Höll
Prüfer der Dissertation:	1. Priv.-Doz. Dr. L. Eberl 2. Univ.-Prof. Dr. K.-H. Schleifer 3. apl. Prof. Dr. A. Hartmann, Ludwig-Maximilians-Universität München

Die Dissertation wurde am 20.06.2002 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 09.09.2002 angenommen.

INHALTSVERZEICHNIS

ABKÜRZUNGSVERZEICHNIS

AHL	*N*-Acyl-L-homoserinlacton
Ap^R	Ampicillinresistenz
bp	Basenpaare
C6-HSL	*N*-hexanoyl-L-homoserinlacton
C8-HSL	*N*-octanoyl-L-homoserinlacton
C10-HSL	*N*-decanoyl-L-homoserinlacton
C12-HSL	*N*-dodecanoyl-L-homoserinlacton
CFU	'colony forming units'
CLSM	konfokales Laser Scanning Mikroskop
Cm^R	Chloramphenicolresistenz
EPS	extrazelluläre polymere Substanzen
Gfp	grün fluoreszierendes Protein
Gm^R	Gentamicinresistenz
ISR	induzierte systemische Resistenz
Km^R	Kanamycinresistenz
MCS	'multiple cloning site'
OD	optische Dichte
Rfp	rot fluoreszierendes Protein
RT	Raumtemperatur
Sm^R	Streptomycinresistenz
Tet^R	Tetracyclinresistenz
ÜN	über Nacht
3-oxo-C6-HSL	*N*-(3-oxo-hexanoyl)-L-homoserinlacton
3-oxo-C8-HSL	*N*-(3-oxo-octanoyl)-L-homoserinlacton
3-oxo-C10-HSL	*N*-(3-oxo-decanoyl)-L-homoserinlacton
3-oxo-C12-HSL	*N*-(3-oxo-dodecanoyl)-L-homoserinlacton
3-oxo-C14-HSL	*N*-(3-oxo-tetradecanoyl)-L-homoserinlacton

A. EINLEITUNG

A.1 Das Ökosystem Rhizosphäre – ein lebenswichtiger Partner der Pflanze

Die Wurzeln der Pflanzen sind in erster Linie für die Aufnahme von Wasser und Nährstoffen aus dem Boden verantwortlich, geben aber gleichermaßen auch Biomasse (in Form von abgestorbenen Zellen) und Exsudate (organische Säuren, Aminosäuren, Zucker, Vitamine, Enzyme u. a.) an die Umgebung ab. Die Bodenmikroflora nutzt dieses organische Material als Nährstoffquelle. Daher ist es nicht verwunderlich, dass die höchste mikrobielle Aktivität im Boden in direkter Nachbarschaft zu den Pflanzen zu finden ist. Der Begriff Rhizosphäre, der 1904 von Lorenz Hiltner erstmals eingeführt wurde, bezeichnet die nur wenige Millimeter breite Zone des Bodens, in der das Wachstum von Mikroorganismen durch die Ausscheidungsprodukte der Pflanze stimuliert wird. Hier leben bis zu hundert Mal mehr Mikroorganismen als im unbewachsenen Boden (Weller und Thomashow, 1994). Die Rhizosphärengemeinschaft setzt sich aus Bakterien, Pilzen (z. B. Mykorrhiza), Protozoen, Algen, Nematoden und anderen Kleinstlebewesen (v. a. Arthropoden) zusammen. Die Oberfläche der Wurzel, auch Rhizoplane genannt, ist von einer mukoiden Schicht aus pflanzlichen und bakteriellen Polysacchariden – dem „Mucigel“ – bedeckt (Jenny und Grossenbacher, 1963). In diese Matrix sind die Bakterien eingebettet, die häufig Mikrokolonien an besonders nährstoffreichen Stellen bilden. Dies können z. B. Läsionen der Epidermis, Furchen zwischen Epidermiszellen oder Wurzelhaare sein. Wird der Wurzelcortex von Mikroorganismen besiedelt, so wird diese Zone als Endorhizosphäre bezeichnet.

Die Rhizosphäre ist durch eine hohe Dynamik gekennzeichnet, da sich die dort herrschenden Umweltbedingungen in Abhängigkeit vom Alter und Entwicklungszustand der Pflanze, aber auch von vielen abiotischen Faktoren (z. B. Nährstoff- und Wassergehalt des Bodens, Sauerstoffversorgung oder Temperatur) häufig ändern können. Daher zeichnen sich viele Rhizobakterien durch eine hohe physiologische Flexibilität aus (Sørensen, 1997).

Eine aktive, dynamische Rhizosphärenpopulation hat einen bedeutenden, wenn nicht gar essentiellen Einfluss auf den Ernährungs- und Gesundheitszustand der Pflanze (Bloemberg und Lugtenberg, 2001). Die Funktionen, die von der Bodenmikroflora in der Rhizosphäre übernommen werden, sollen daher im folgenden genauer beleuchtet werden.

Zunächst einmal sind die Mikroorganismen in der Lage, im Boden vorhandenes organisches Material zu mineralisieren, wodurch die Pflanze mit essentiellen Nährstoffen versorgt wird. Als Destruenten sind sie unentbehrlich für die Aufrechterhaltung der Stoffkreisläufe im

Boden. Aber nicht nur organische Nährstoffe, sondern auch viele Giftstoffe, die als Xenobiotika in den Boden gelangt sind, können von Rhizobakterien abgebaut werden. Für die biologische Bodensanierung kommen wegen ihres außergewöhnlichen metabolischen Potentials häufig Rhizobakterien der Gattung *Pseudomonas* zum Einsatz. Diese Bakterien können eine Reihe von toxischen Stoffen abbauen, darunter halogenierte Aromaten (z. B. polychlorierte Biphenyle aus Plastikwaren, Isolierungen, etc.), den Sprengstoff 2,4,6-Trinitrotoluol (TNT) und einige Pestizide (Hofer *et al*., 1996; Esteve-Núñez *et al*., 2001).

Über den Abbau von Stoffen hinaus sind die Mikroorganismen im Boden aber auch für die Synthese zahlreicher Substanzen verantwortlich, die der Pflanze von Nutzen sein können. So produzieren einige wachstumsfördernde Rhizobakterien - die sogenannten 'plant-growth-promoting rhizobacteria' - Vitamine und pflanzliche Wachstumshormone, z. B. Auxine oder Gibberelline, die vor allem die Wurzelbildung positiv beeinflussen (Bloemberg und Lugtenberg, 2001).

Die engste Assoziation mit der Wirtspflanze haben sicherlich symbiontische und parasitische (pathogene) Mikroorganismen. Die am weitesten verbreiteten Symbionten sind die Mykorrhizapilze, die in der Rhizosphäre vieler Pflanzen, vor allem Bäume, zu finden sind. Sie schützen die Wurzeln vor Austrocknung und versorgen ihren Wirt mit Nährstoffen (z. B. Phosphat). In diesem Zusammenhang sind auch die Stickstoff-fixierenden Bakterien von großer Bedeutung, die entweder endosymbiontisch mit der Pflanze assoziiert sind (z. B. *Rhizobium* spp.) oder frei im Boden vorkommen (z. B. *Azospirillum* spp.) und einen großen Beitrag zur Stickstoffversorgung der Pflanzen leisten. Die bakterielle Population der Rhizosphäre kann jedoch auch pathogene Mikroorganismen beherbergen, wie zum Beispiel Welke-Erreger (*Pantoea stewartii* und *Ralstonia solanacearum*), Fäule- und Nekrose-Erreger (*Erwinia* spp., *Xanthomonas* spp. und *Pseudomonas* spp.) oder *Agrobacterium tumefaciens*, das durch Übertragung von onkogener DNA bei der Pflanze Tumoren auslöst (Trevors und van Elsas, 1997). Daneben spielen vor allem Pilze eine bedeutende Rolle als Krankheitserreger im Boden (z. B. *Pythium* spp., *Rhizoctonia* spp., *Fusarium* spp., u. a.) (Thorn, 1997).

Eine besonders wichtige Aufgabe haben daher die antagonistischen Mikroorganismen (sog. 'biocontrol' Organismen) in der Rhizosphäre, welche die Pflanze vor Pathogenbefall schützen. Hierbei kommen unterschiedliche Mechanismen zum Einsatz, wie Konkurrenz um Nährstoffe und Substrat (Wurzeloberfläche), Stimulierung der induzierten systemischen Resistenz (ISR) bei der Pflanze, und vor allem die Synthese von Hemmstoffen, z. B. Fungiziden und Antibiotika (Bloemberg und Lugtenberg, 2001). Zu den am besten

charakterisierten antagonistischen Bakterien gehören Vertreter der Gattung *Pseudomonas*, die eine Reihe von antifungischen Metaboliten produzieren, wie Phenazine, Pyrrolnitrin, 2,4-Diacetylphloroglucinol oder Pyoluteorin. Zu ihrer antagonistischen Aktivität trägt aber auch die Synthese von eisenbindenden Siderophoren, Cyanid und einigen Exoenzymen (z. B. Chitinasen und Proteasen) bei (O'Sullivan und O'Gara, 1992).

In den letzten Jahren wurde angesichts der massiven Bodenkontamination durch Pestizide und der zunehmenden Resistenzentwicklung der Schadorganismen vermehrt der Einsatz von biologischen Schädlingsbekämpfungsmitteln in Betracht gezogen. Doch bisher haben es nur wenige dieser Produkte bis zur Marktreife gebracht, da häufig die Wirkung der eingesetzten 'biocontrol' Organismen zu gering oder inkonsistent ist. Schwachpunkte sind meist eine unzureichende Etablierung der eingeführten Organismen oder eine zu geringe antagonistische Aktivität im Feldversuch. Die Erforschung der molekularen Mechanismen der 'biocontrol' Aktivität und deren Regulation durch Umweltfaktoren ist daher eine wichtige Aufgabe (O'Sullivan und O'Gara, 1992; Cook *et al*., 1995). Desweiteren müssen die an der Kolonisierung beteiligten Vorgänge aufgeklärt werden, da die Besiedelung der Wurzel der erste entscheidende Schritt für die weitere Aktivität eines Bakteriums in der Rhizosphäre ist, sei es ein 'biocontrol' Organismus oder ein Krankheitserreger (Lugtenberg und Dekkers, 1999). Besondere Beachtung verdienen darüber hinaus die mikrobiellen Interaktionen in der Rhizosphäre, da die Wechselwirkung mit anderen Lebewesen sowohl die ökologische Fitness des eingesetzten Organismus als auch die gewünschte bakterielle Aktivität (z. B. die Synthese von Hemmstoffen) entscheidend beeinflussen kann.

Die Möglichkeiten für Interaktionen zwischen den Mitgliedern der mikrobiellen Rhizosphärengemeinschaft sind sicherlich mannigfaltig und bisher nur ansatzweise verstanden. Es hat sich jedoch gezeigt, dass die interzelluläre Kommunikation mittels Signalmolekülen eine bedeutende Rolle bei der Koordination einer Vielzahl bakterieller Aktivitäten spielt. Daher soll die Zell-Zell-Kommunikation von Bakterien in der Rhizosphäre und die zugrundeliegenden molekularen Mechanismen im nächsten Kapitel genauer beleuchtet werden.

A.2 *N*-Acyl-L-homoserinlactone als bakterielle Botenstoffe

In den letzten Jahren hat man erkannt, dass Bakterien nicht als isolierte Einzelzellen ihr Habitat besiedeln, sondern in komplexen, miteinander interagierenden Gemeinschaften existieren, die zu einer Reihe koordinierter Aktionen befähigt sind. Die Kommunikation der

Prokaryonten wird durch chemische Signalmoleküle vermittelt, die von den Zellen ausgeschieden werden und deren Wahrnehmung die unterschiedlichsten physiologischen Antworten auslöst (Miller und Bassler, 2001).

Die am besten untersuchten Botenstoffe Gram-negativer Bakterien sind die *N*-Acyl-L-homoserinlactone (AHL), mit deren Hilfe die Organismen ihre eigene Populationsdichte messen und in Abhängigkeit davon bestimmte Funktionen regulieren. Aus diesem Grund werden AHL-abhängige Kommunikationssysteme auch als 'Quorum sensing' Systeme bezeichnet, wobei das 'Quorum' die kleinste, zu koordinierten Aktionen befähigte Einheit darstellt (Fuqua *et al*., 1994).

Abbildung 1 zeigt die Struktur von AHL-Molekülen. Sie unterscheiden sich nur in der Länge und Substitution ihrer Acyl-Seitenkette. Bisher wurden Moleküle mit einer Kettenlänge von 4 bis 14 C-Atomen identifiziert, die Doppelbindungen enthalten können und teilweise an der C-3 Position eine Carbonyl- oder eine Hydroxylgruppe tragen.

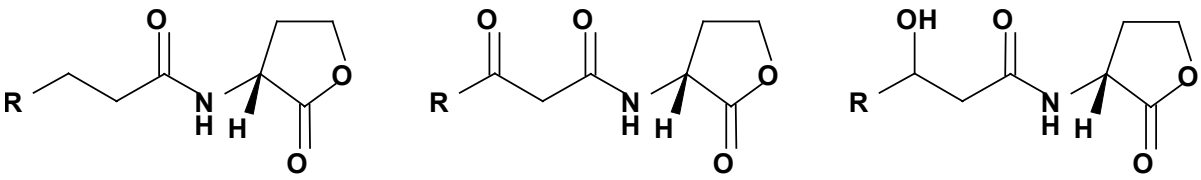


Abb. 1. Molekülstruktur von *N*-Acyl-L-homoserinlactonen

AHL-abhängige Kommunikationssysteme basieren auf zwei Proteinen: Einer AHL-Synthase, meist ein Mitglied der LuxI-Proteinfamilie, und einem AHL-Rezeptor, der zur LuxR-Familie transkriptioneller Regulatorproteine zählt (Übersichtsartikel Eberl, 1999 oder Whitehead *et al*., 2001). Bei geringen Populationsdichten produzieren die Zellen mittels der AHL-Synthase eine basale AHL-Konzentration. Sobald die Zelldichte ansteigt, reichern sich die Signalmoleküle im Habitat an. Da AHL-Moleküle größtenteils frei durch die bakterielle Zellmembran diffundieren können, steigt mit zunehmender Populationsdichte auch die intrazelluläre AHL-Konzentration an. Bei einer bestimmten kritischen Schwellen-konzentration bindet das Signalmolekül an das entsprechende LuxR-homologe Rezeptorprotein, welches nun seinerseits die Induktion oder Repression der Transkription von Zielgenen vermittelt. Da häufig auch die Transkription der AHL-Synthase auf diese Weise aktiviert wird, kommt es zu einer positiven Rückkopplung und die AHL-Produktion steigt sprunghaft an.

Zahlreiche auf diese Weise regulierte Funktionen spielen eine wichtige Rolle für die Interaktionen von Bakterien mit ihren eukaryontischen Wirten. So regulieren beispielsweise

einige phytopathogene Bakterien die Expression ihrer Virulenzfaktoren durch 'Quorum sensing'. *Erwinia carotovora* verursacht Weichfäule an einer Reihe von Kulturpflanzen, die durch die Aktivität von Zellwand-lytischen Exoenzymen wie Cellulasen und Pectinasen ausgelöst wird. Die Synthese dieser Virulenzfaktoren wird durch ein 'Quorum sensing' System kontrolliert, dem *N*-(3-Oxohexanoyl)-L-homoserinlacton (3-Oxo-C6-HSL) als Botenstoff dient (Jones *et al*., 1993; Pirhonen *et al*., 1993). Dieser Kontrolle unterliegt gleichzeitig die Produktion von β-Lactam Antibiotika, welche vermutlich Konkurrenten von der neu erschlossenen Nahrungsquelle fernhalten sollen (Bainton *et al*., 1992). Ebenfalls durch 3-Oxo-C6-HSL wird die Synthese von Exopolysacchariden in *Pantoea stewartii* induziert (von Bodman *et al*., 1998). Die Polysaccharidkapsel ist der Hauptvirulenzfaktor dieses Welke-Erregers, der durch Insekten übertragen wird. Im allgemeinen wird angenommen, dass die Produktion von Virulenzfaktoren bei hoher Zelldichte gewährleistet, dass die Erreger nicht frühzeitig vom pflanzlichen Abwehrsystem erkannt werden und erst dann einen koordinierten Angriff auf die Wirtspflanze starten, wenn die Zahl der Angreifer erfolgversprechend ist (Eberl, 1999). Bei *Agrobacterium tumefaciens*, das durch Übertragung der onkogenen T-DNA („Transfer-DNA") Tumoren in seiner Wirtspflanze verursacht, ist der konjugative Transfer des Ti („Tumor-induzierenden") Plasmids zwischen den Bakterien am Infektionsherd in Abhängigkeit von der Zelldichte reguliert. Interessanterweise unterliegt die Expression des *luxR*-homologen Gens *traR* einer zusätzlichen Kontrolle durch Signalstoffe der Pflanze (den Opinen, deren Synthese ebenfalls von der T-DNA gesteuert wird) (Piper *et al*., 1993; Zhang *et al*., 1993).

Die Verwendung von 'Quorum sensing' Systemen ist nicht nur auf pathogene Bakterien beschränkt. So sind in *Rhizobium leguminosarum*, einem Stickstoff-Fixierer, der spezifische Symbiosen mit Leguminosenpflanzen eingeht, bisher vier 'Quorum sensing' Systeme identifiziert worden, die ein regulatorisches Netzwerk ausbilden (Lithgow *et al*., 2000). An der Spitze der regulatorischen Hierarchie steht das Signalmolekül *N*-(3-Hydroxy-7-*cis*-tetradecenoyl)-L-homoserinlacton, welches ursprünglich aufgrund seiner wachstumshemmenden Aktivität als 'small bacteriocin' identifiziert worden war (Gray *et al*., 1996; Schripsema *et al*., 1996). Das komplexe 'Quorum sensing' Netzwerk kontrolliert eine Vielzahl von Funktionen, darunter physiologische Veränderungen beim Übergang in die stationäre Phase (Thorne und Williams, 1999), die Knöllchenbildung an der Wirtspflanze (Rodelas *et al*., 1999) und den konjugativen Plasmidtransfer (Lithgow *et al*., 2000).

Zur Erforschung der Rolle von AHL-Signalmolekülen bei der Zell-Zell-Kommunikation in der Rhizosphäre diente *Pseudomonas aureofaciens* als Modellorganismus. *P. aureofaciens*

30-84 produziert Phenazinantibiotika, die Weizenpflanzen vor Befall durch den Pilz *Gaeumannomyces graminis* var. *tritici*, dem Erreger der sog. 'take-all disease', schützen. Die Synthese dieser Phenazine wird in Abhängigkeit von der Zelldichte reguliert und steht unter Kontrolle des PhzI/PhzR 'Quorum sensing' Systems (Pierson *et al*., 1994; Wood und Pierson, 1996). Mit Hilfe eines AHL-Monitorstamms konnte gezeigt werden, dass die Phenazinsynthese in der Rhizosphäre von Weizen durch *N*-Hexanoyl-L-homoserinlacton induziert wird (Wood *et al*., 1997). Es wurde außerdem der erste Beweis dafür erbracht, dass AHL im natürlichen Habitat produziert werden. Eine weiterführende Arbeit demonstrierte, dass auch die Anwesenheit artfremder Bakterien, die zur Synthese von AHL befähigt sind, die Produktion von Phenazinen bei *P. aureofaciens* stimulieren kann (Pierson *et al*., 1998). Diese Resultate deuten an, dass AHL eine intergenerische Kommunikation ('cross-talk') zwischen unterschiedlichen Spezies der Rhizosphärenpopulation vermitteln können.

A.3 Ziele dieser Arbeit

Anknüpfend an die letztgenannte Studie sollte im Rahmen dieser Arbeit die AHL-vermittelte Zell-Zell-Kommunikation in der Rhizosphäre von Tomatenpflanzen *in situ* nachgewiesen werden. Im Vordergrund stand hierbei auch die Frage, ob AHL als Signalmoleküle zwischen verschiedenen Bakterienarten fungieren können. Der von Pierson *et al.* (1998) verwendete AHL-Biosensor *P. aureofaciens* 30-84Ice/I beruht auf einer Fusion des 'Quorum sensing'-regulierten *phzB* Promotors mit dem Reportergen *inaZ*. Durch Nachweis der InaZ- ('ice nucleation'-) Aktivität nach Reisolierung der Sensorzellen aus der Weizenrhizosphäre konnte die Produktion von AHL-Molekülen im natürlichen Habitat gezeigt werden. Über die räumliche Verteilung von AHL-Produzenten und Sensorzellen an der Wurzel ließ sich jedoch keine Aussage treffen. Deshalb sollten zunächst neue AHL-Biosensoren entwickelt werden, die eine Visualisierung der Zell-Zell-Kommunikation direkt an der Wurzel auf Einzelzellebene ermöglichen. Da die molekulare Kommunikation bisher nur in der Rhizosphäre von Weizen beobachtet worden war, wurde für unsere Studie die Tomate als Vertreter der dicotylen Kulturpflanzen ausgewählt.

Weiterhin sollte überprüft werden, inwieweit AHL-vermittelte Interaktionen bei der Kolonisierung der Wurzel und der Etablierung einer Population in der Rhizosphäre eine Rolle spielen. Diese Frage ist im Hinblick auf die Anwendung von Mikroorganismen in der biologischen Schädlingsbekämpfung von besonderem Interesse, da für den Erfolg einer solchen Maßnahme eine effiziente Besiedelung der Rhizosphäre durch die eingebrachten

Organismen unerlässlich ist. Die Bedeutung der intergenerischen Kommunikation in der Rhizosphäre für die Diversität und Dynamik der Mikroflora wurde in Kooperation mit der Arbeitsgruppe von Prof. A. Hartmann (Institut für Bodenökologie, GSF, Neuherberg) untersucht. Außerdem wurde in Zusammenarbeit mit der Arbeitsgruppe von Dr. C. Langebartels (Institut für biochemische Pflanzenpathologie, GSF, Neuherberg) der Effekt von AHL-Produzenten in der Rhizosphäre auf die induzierte systemische Resistenz der Tomatenpflanze getestet.

Um den Einfluss von AHL auf das Besiedelungsverhalten zu erforschen, wurde *Pseudomonas putida* als Modellorganismus ausgewählt. Diese Spezies ist für diese Untersuchungen aus mehreren Gründen besonders gut geeignet. Zum einen ist *P. putida* ein typisches Pflanzen-assoziiertes Bakterium und kann aus der Rhizosphäre zahlreicher Kulturpflanzen isoliert werden (Anhang 3; Elasri *et al*., 2001). Vertreter dieser Gattung zeigen häufig antagonistische Aktivität gegen phytopathogene Pilze oder sind in der Lage, das Wachstum von Pflanzen zu fördern, weshalb sie zur biologischen Schädlingsbekämpfung und Phytostimulation eingesetzt werden könnten. Für die Wahl eines *P. putida* Stammes sprach darüber hinaus vor allem die Tatsache, dass die Fähigkeit zur AHL-Produktion innerhalb dieser Spezies zwar weit verbreitet ist (Anhang 3; Sauer und Camper, 2001; Elasri *et al*., 2001; Kojic *et al*., 1999), jedoch bisher weder die in die AHL-Synthese involvierten Gene noch ein Zelldichte-abhängiger Phänotyp in *P. putida* beschrieben worden waren. Daher sollte im zweiten Teil dieser Arbeit das ‘Quorum sensing’ System von *P. putida* IsoF auf molekularer Ebene charakterisiert werden. Die Erzeugung AHL-negativer Mutanten sollte dann die Voraussetzung schaffen, um ‘Quorum sensing’-regulierte Phänotypen in *P. putida* zu identifizieren.

B. MATERIAL UND METHODEN

B.1 Organismen und Plasmide

In den folgenden Tabellen sind die in dieser Arbeit verwendeten Organismen (Tabelle B-1) und Plasmide (Tabelle B-2) aufgeführt.

Tabelle B-1. Verwendete Organismen

Stamm	Relevante Eigenschaften	Herkunft/Referenz
Serratia liquefaciens MG1	Wildtyp	Givskov *et al.*, 1992
S. liquefaciens MG44	AHL-negative Mutante von *S. liquefaciens* MG1, SwrI$^-$	Eberl *et al.*, 1996
Rahnella aquatilis T13	Wildtyp, Isolat aus der Tomatenrhizosphäre	Diese Arbeit
R. aquatilis TAA	Wildtyp, Isolat aus der Tomatenrhizosphäre	Diese Arbeit
Burkholderia cepacia H111	Wildtyp	Römling *et al*., 1994; Gotschlich *et al*., 2001
B. cepacia H111-I	Km^R; AHL-negative Mutante von *B. cepacia* H111, CepI$^-$	Gotschlich, 2001
B. cepacia H111-R	Km^R; AHL-negative Mutante von *B. cepacia* H111, CepR$^-$	Huber *et al*., 2001
Pseudomonas putida		
IsoF	Wildtyp, Isolat aus der Tomatenrhizosphäre	Diese Arbeit
F117	Km^R; *ppuI*::*npt* Mutante von IsoF	Diese Arbeit
IsoF-R	Km^R; *ppuR*::*npt* Mutante von IsoF	Diese Arbeit
IsoF-D4	*ppuA*::*luxAB* Mutante von IsoF	Diese Arbeit
F117-D1	Km^R; *ppuI*::*npt*, *ppuA*::*luxAB* Doppelmutante von IsoF	Diese Arbeit
KS35	F117 mit integrierter P_{lasB}-*gfp*(ASV)-P_{lac}-*lasR* Kassette	Diese Arbeit
Z2D	Wildtyp, Isolat aus der Tomatenrhizosphäre	Diese Arbeit
Escherichia coli		
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F*'proAB lacI*^{q} *ZΔM15* Tn*10* (Tet^R)]	Stratagene, La Jolla, USA
MT102	*araD139* (*ara-leu*)*7697* Δ*lac thi hsdR*	Stammsammlung Labor Eberl, TU München, Freising
HB101	*recA thi pro leu hsdR*$^-$*M*$^+$ Sm^R	Kessler *et al*., 1992
CC118 λ pir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ *pir* lysogen	Herrero *et al.*, 1990

Im Rahmen einer Kooperation mit G. Berg (Institut für Molekulare Physiologie und Biotechnologie, Mikrobiologie, Universität Rostock) wurden insgesamt 111 bakterielle

Isolate aus der Rhizosphäre von Kartoffel, Raps und Erdbeere und aus dem unbewachsenen Boden auf AHL-Produktion getestet. Die Herkunft dieser Stämme und ihre Identifizierung sind dem Anhang 3 (Tabellen 2 und 3) zu entnehmen.

Tabelle B-2. Verwendete Plasmide

Plasmide	Relevante Eigenschaften	Herkunft/Referenz
pAS-C8	pBBR1MCS-5 mit P_{cepI}-*gfp*(ASV)-P_{lac}-*cepR*; Gm^R	Diese Arbeit
pKR-C12	pBBR1MCS-5 mit P_{lasB}-*gfp*(ASV)-P_{lac}-*lasR*; Gm^R	Anhang 4
pJBA132	pME6031 mit *luxR*-P_{luxR}-P_{luxI} -*gfp*(ASV)-T_0-T_1; Tet^R	Andersen *et al.*, 2001
pSB403	Biolumineszentes AHL-Sensorplasmid; Tet^R	Winson *et al.*, 1998
pTn*5*-LAS	pTn*5*-Gm mit P_{lasB}-*gfp*(ASV)-P_{lac}-*lasR*; Gm^R, Ap^R	Hentzer *et al.*, 2002
pTn*5*-Red	pUTtc mit P_{lac}-*rfp*-T_0-T_1; Tc^R, Ap^R	Hentzer *et al.*, 2002
pUT-Kan-*dsred*	pUTkan mit P_{lac}-*rfp*-T_0-T_1; Km^R, Ap^R	Tolker-Nielsen *et al.*, 2000
pBK-miniTn*7*-gfp3	pUC19 mit miniTn*7*-$P_{A1/04/03}$-*gfp*mut3*- T_0-T_1, Ap^R, Km^R, Cm^R, Sm^R, *mob*$^+$	Koch *et al.*, 2001
pRK600	ColE1 RK2-Mob$^+$ RK2-Tra$^+$; Helferplasmid für konjugativen Transfer	Kessler *et al.*, 1992
pUX-BF13	Helferplasmid für Tn*7* Transposition	Bao *et al.*, 1991
pCR 2.1-TOPO	*lac*Zα, Klonierungsvektor, Ap^R, Km^R	Invitrogen, Carlsbad, USA
pGEM®-3Zf(+)	*lac*Zα, Klonierungsvektor; Ap^R	Promega, Madison, USA
pBBR1MCS-2	Klonierungsvektor mit breitem Wirtsbereich, *lac*Zα; Km^R	Kovach *et al.*, 1995
pBBR1MCS-5	Klonierungsvektor mit breitem Wirtsbereich, *lac*Zα; Gm^R	Kovach *et al.*, 1995
pEX18Gm	Gm^R, *oriT*$^+$ *sacB*$^+$, 'gene replacement vector' mit pUC18 MCS	Hoang *et al*., 1998
pGF1	chromosomales 2,9 kb *Sal*I Fragment aus IsoF in pGEM®-3Zf(+) kloniert, enthält *ppuI-rsaL-ppuR*	Diese Arbeit
pGF3	chromosomales 1,4 kb *Eco*RI Fragment aus IsoF in pBBR1MCS-5 kloniert, enthält *ppuI*	Diese Arbeit
pGF4	chromosomales 13 kb *Sac*I Fragment aus F117 in pGEM®-3Zf(+) kloniert, enthält das *ppu* Gencluster und flankierende Bereiche	Diese Arbeit
pEXF1	Gm^R, Km^R, pEX18Gm Derivat zur Inaktivierung von *ppuI*	Diese Arbeit
pEXF2	Gm^R, pEX18Gm Derivat zur Inaktivierung von *ppuA*	Diese Arbeit
pEXF3	Gm^R, Km^R, pEX18Gm Derivat zur Inaktivierung von *ppuR*	Diese Arbeit
pPPUR1	pGEM®-3Zf(+) mit *ppuR* aus IsoF	Diese Arbeit
pGA-L14	Promotor-Testvektor mit breitem Wirtsbereich	Geiselhöringer, 1999
pAS-L22	pGA-L14 mit T_0-T_1-P_{ppuI}-*luxAB*	Diese Arbeit
pBBR-*cepI*$_{H111}$	pBBR1MCS-5 mit *cepI* aus *B. cepacia* H111	Gotschlich, 2001
pBAH27	pBBR1MCS-5 mit *cepR* aus *B. cepacia* H111	Huber *et al*., 2001

B.2 Isolierung von Bakterien aus der Rhizosphäre

Wurzelproben verschiedener Pflanzen (z. B. Tomaten) wurden präpariert und mit PBS-Puffer (10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 130 mM NaCl, pH 7,2) gewaschen, um die daran haftende Erde zu entfernen. Ein Gramm Wurzelmaterial wurde in 9 ml Saccharose-Lösung (4 % wt/vol) zermörsert. Aus der Wurzelsuspension wurden Verdünnungsreihen erstellt, und 100-µl Aliquots auf LB-Agar oder ABC-Agar ausplattiert. Einzelkolonien wurden isoliert und durch weitere Verdünnungsausstriche zur Reinkultur gebracht. Die so erhaltenen Isolate wurden nachfolgend auf AHL-Produktion wie unter Punkt B.6.2 beschrieben getestet.

B.3 Nährmedien und Zusatzstoffe

Alle Medien wurden durch Autoklavieren in einem Wasserdampf-Hochdruckautoklaven bei 121 °C und 1,2 bar Überdruck für 20 min sterilisiert. Hitzeempfindliche Zusatzstoffe wurden sterilfiltriert und anschließend den autoklavierten Medien zugegeben. Festmedien wurde 16 g/l Agar zugegeben.

Zur Herstellung von Selektivmedien und zur Plasmidstabilisierung bei der Kultivierung plasmidhaltiger Stämme wurden die entsprechenden Antibiotika in folgenden Konzentrationen eingesetzt: Ampicillin (100 µg/ml), Tetracyclin (10 µg/ml), Chloramphenicol (10 µg/ml), Gentamicin (20 µg/ml für *E. coli* und 50 µg/ml für *P. putida*), Kanamycin (50 µg/ml für *E. coli* und 100 µg/ml für *P. putida*), Streptomycin (50 µg/ml).

LB-Medium: (modifiziert nach Andersen *et al.*, 1998)

Casein-Hydrolysat	10 g
Hefe-Extrakt	5 g
NaCl	4 g
ad 1000 ml H_2O_{dest}	

ABC-Minimalmedium: (Clark und Maaløe, 1967)

A-10:	$(NH_4)_2SO_4$	20 g
	Na_2HPO_4	60 g
	KH_2PO_4	30 g
	NaCl	30 g
	ad 1000 ml H_2O_{dest}	

B:	1 M $MgCl_2$ x 6 H_2O	2 ml	(Stammlösung: 20,33 g/100 ml)
	0,5 M $CaCl_2$ x 2 H_2O	0,2 ml	(Stammlösung: 7,35 g/100 ml)
	0,01 M $FeCl_3$ x 6 H_2O	0,3 ml	(Stammlösung: 0,27 g/100 ml)
	ad 900 ml H_2O_{dest}		
C:	1 M Citrat		

Alle drei Bestandteile wurden getrennt autoklaviert. Anschließend wurden zu 900 ml B-Medium 100 ml A-10 und 10 ml Citrat gegeben.

Pseudomonas Isolations Agar (PIA): (Becton Dickinson, Sparks, USA)

Verwendung als Fertigmedium nach Angaben des Herstellers.

Würze-Agar: (Merck, Darmstadt)

Herstellung der Würze-Boullion aus Fertigmedium nach Angaben des Herstellers. Würze-Agar wurde mit 16 g/l Agar versetzt.

B.4 Konjugativer Plasmidtransfer

Plasmide wurden mittels eines triparentalen Mating-Ansatzes (de Lorenzo und Timmis, 1994) in *P. putida* und *S. liquefaciens* Zellen eingebracht. Dabei wurden der Donor- und Rezipientenstamm sowie der Helferstamm *E. coli* HB101 (pRK600) über Nacht in 5 ml LB mit den entsprechenden Antibiotikazusätzen kultiviert. Nach einer Subkultivierung bis zu einer OD_{600} von ca. 0,9 wurden je 2 ml der Kulturen geerntet, einmal in LB-Medium gewaschen und in 200 µl LB resuspendiert. Je 100 µl der Donor- und Helferzellen wurden gemischt und für 20 min bei RT inkubiert. Danach wurden die Rezipientenzellen im Verhältnis 1:1 zugegeben. Die Mischung wurde auf die Oberfläche vorgewärmter Agarplatten aufgetropft. Nach Inkubation über Nacht bei 30 °C wurden die Zellen mit Hilfe einer sterilen Einwegimpföse von den Platten abgekratzt und in 0,9 % NaCl resuspendiert. Verdünnungen wurden auf LB- bzw. PIA-Agarplatten ausplattiert, denen Antibiotika zur Selektion gegen Donor-, Helfer-, und untransformierte Rezipientenzellen zugegeben waren.

B.5 Molekulargenetische Methoden

Klonierungen, Restriktionsverdaus und Transformationen von *E. coli* wurden grundsätzlich entsprechend den von Sambrook *et al*. (1989) beschriebenen Standardtechniken durchgeführt.

Für PCR-Amplifikationen wurde die TaKaRa rTaq Polymerase (TaKaRa Shuzo, Shiga, Japan) oder die Herculase (Stratagene, La Jolla, USA) verwendet. Southern Blot Hybridisierungen wurden mit Hilfe des DNA Labeling and Detection Kits (Roche, Mannheim) durchgeführt. Folgende Kits von Qiagen (Hilden) wurden entsprechend den Angaben des Herstellers eingesetzt: QIAprep Spin Miniprep Kit (Isolierung von Plasmid-DNA), DNeasy Tissue Kit (Isolierung chromosomaler DNA) und QIAquick Gel Extraction Kit (Aufreinigung von DNA-Fragmenten aus Agarosegelen und Aufreinigung von PCR-Produkten).

Zur Visualisierung von Wurzel-assoziierten Bakterienzellen unter dem konfokalen Laser Scanning Mikroskop wurden die verwendeten Stämme mit dem grün fluoreszierenden Protein (Gfp) bzw. rot fluoreszierenden Protein (Rfp) markiert. Um einen Verlust des Markers in Abwesenheit von Selektionsdruck zu verhindern, wurde eine $P_{A1/04/03}$-*gfp*-T_0-T_1 (Andersen *et al.*, 1998) oder $P_{A1/04/03}$-*rfp*-T_0-T_1 (Tolker-Nielsen *et al.*, 2000) Transposonkassette ins Chromosom der Stämme integriert. Als Vektoren für die Transposonkassetten wurden pUTkan bzw. pUTtc verwendet, die über konjugativen Transfer in die Stämme eingebracht wurden. Die für die Anzucht der Biofilme verwendeten Stämme *P. putida* IsoF, F117 und IsoF-D4 wurden durch Integration einer miniTn*7*-$P_{A1/04/03}$-*gfp* Transposonkassette (Koch *et al.*, 2001) markiert. Dieses Verfahren hat den Vorteil, dass die Markerkassette an einer genau definierten, neutralen Stelle ins Chromosom integriert wird und so keine unerwünschten Mutationen verursacht (Koch *et al.*, 2001).

Tabelle B-3. Verwendete PCR-Primer (durch Primer in die PCR-Produkte eingeführte Restriktionsschnittstellen sind unterstrichen)

Primer	**Sequenz (5' → 3')**
PcepI-V3	CGGGATCCGACATCGGCATGTTGC
PcepI-R2	ACATGCATGCATGTCCTCGGATCTGTGC
gfp(ASV)-fwd	ACATGCATGCGTAAAGGAGAAGAAC
gfp(ASV)-rev	CCCAAGCTTATTAAACTGATGCAGC
CepR-Rb	GGGGTACCAACCTGACAAGTATGACAGCG
CepR-0Vb	GGGGTACCGGATGAGCATGGAGAAAAGC
PPUR5-V	CGGAGCTCTGAGGGACGGCGGTAG
PPUR5-R	CGGAATTCAACCTGGGTCGCATCACC
PPUR3-V	AACTAAGCTTCACGCCACAGCCAATGGC
PPUR3-R	AACTAAGCTTCTGTCGTGCACGACCTC

Primer	Sequenz (5' → 3')
PPUR-V	CGTCTAGACCACCGTCAAACGGTGC
PPUR-R	CGGAGCTCAGCGGTCGCCTGGAGG
PPUAII-V	CGGAATTCCCAAGCCGGTCTAGCG
PPUAII-R	CGGAGCTCGACCAGTTGCGCGCC
PPUAR-V	ACATGTCGACGCTAGTAAATAGGCTGCG
PPUAR-R	AACTAAGCTTCGGCATCAGCCAGGCC
PPUI-out	CGGGATCCATGTCATTGATCAGCGG
RSAL-out	CGGGATCCGAACCTTGACCAGAATTCG

B.5.1 Konstruktion von Plasmiden

Konstruktion des AHL-Sensorplasmids pAS-C8 (Anhang 4). Das AHL-Sensorplasmid pAS-C8 wurde in drei Schritten konstruiert. Zunächst wurde die *cepI* Promotorregion mit Hilfe der Primer PcepI-V3 und PcepI-R2 aus *B. cepacia* H111 amplifiziert. Das PCR-Produkt wurde mit *Sph*I und *Bam*HI verdaut und in den Vektor pGEM-3Zf(+) ligiert, wodurch Plasmid pAS1 entstand. In einem zweiten Schritt wurde *gfp*(ASV) mit den Primern *gfp*(ASV)-fwd und *gfp*(ASV)-rev aus pKR-C12 amplifiziert, mit *Sph*I und *Hin*dIII verdaut und nachfolgend in pAS1 ligiert. Das daraus resultierende Plasmid pAS2 enthält eine translationale Fusion von *cepI* mit *gfp*(ASV). Diese Kassette wurde anschließend unter Verwendung der *Bam*HI und *Hin*dIII Schnittstellen in den Vektor pBBR1MCS-5 überführt. In dieses Plasmid wurde schließlich unter Verwendung der *Kpn*I Schnittstelle das mit den Primern CepR-Rb und CepR-0Vb aus *B. cepacia* H111 amplifizierte *cepR* ligiert. Es wurde ein Klon isoliert, welcher *cepR* unter Kontrolle des *lac* Promotors enthält. Das Plasmid pAS-C8 beinhaltet somit eine translationale Fusion von *cepI* mit *gfp*(ASV) und *cepR* unter der Kontrolle des *lac* Promotors.

Die Konstruktionsschritte aller weiteren im Rahmen dieser Arbeit konstruierten Plasmide (siehe Tabelle B-2) sind dem Anhang 2 zu entnehmen.

B.5.2 DNA Sequenzanalyse

Die Sequenzierung von DNA wurde von den Firmen SequiServe (Vaterstetten) und MWG Biotech (Ebersberg) übernommen. Sequenzen wurden mittels der Suchmaschine BLAST des 'National Center for Biotechnology Information' (http://www.ncbi.nlm.nih.gov) mit anderen Sequenzen in der Datenbank 'GenBank' verglichen.

B.5.3 Knock-out Mutagenese durch homologe Rekombination

Um definierte *ppuI*-, *ppuR*- und *ppuA*-Mutanten von *P. putida* IsoF zu erzeugen, wurde eine von Schweizer und Hoang (1995) beschriebene Methode eingesetzt. Mit dieser Methode kann ein bestimmtes Gen mittels zweier aufeinander folgender Rekombinationsereignisse durch eine inaktivierte Kopie ersetzt werden. Die Konstruktion der auf dem Vektor pEX18Gm (Hoang *et al*., 1998) basierenden Suizidvektoren pEXF1, pEXF2 und pEXF3, die zur Einbringung der inaktiven Kopien von *ppuI*, *ppuA* und *ppuR* in die Zellen genutzt wurden, ist dem Anhang 2 zu entnehmen. Die Suizidvektoren wurden durch triparentales Mating in *P. putida* IsoF eingebracht. In einem ersten Schritt wurden die Transkonjuganten auf Gentamicin selektioniert, da der Vektor pEX18Gm für eine Gm-Resistenz kodiert. Somit wurden merodiploide Klone erhalten, welche durch homologe Rekombination den gesamten Suizidvektor ins Chromosom integriert haben. In einem zweiten Schritt wurden diese Zellen auf LB-Platten mit 5 % Saccharose überführt, um so Klone zu selektionieren, die durch ein zweites Rekombinationsereignis den Suizidvektor (*sacB*$^{+}$) wieder aus dem Chromosom ausgeschnitten haben. Da in diesem Schritt mit etwa 50 %-iger Wahrscheinlichkeit eine Rückrekombination zum Wildtyp-Allel auftritt, wurden die Klone zusätzlich auf Kanamycinresistenz, welche für die Erzeugung der Knock-out Kassetten *ppuI*::*npt* und *ppuR*::*npt* verwendet wurde, getestet. Bei der Erzeugung der *ppuA* Mutanten IsoF-D4 und F117-D1 wurde die Insertion von *ppuA*::*luxAB* ins Chromosom der Saccharose-resistenten Klone durch die Überprüfung ihrer Biolumineszenz bestätigt. Die korrekte Insertion der Knock-out Kassetten in das Chromosom der erzeugten Mutanten wurde außerdem durch Southern Blot Hybridisierung überprüft.

B.6 Nachweis von AHL-Molekülen

B.6.1 Bakterielle Reportersysteme

Zum Nachweis von AHL-Molekülen dienten verschiedene AHL-Biosensoren. Die Anwesenheit der Signalmoleküle wird bei diesen Sensoren durch die Expression eines Reportergens, z. B. bakterieller Luziferase oder dem grün fluoreszierenden Protein Gfp, angezeigt.

pSB403: Das Sensorplasmid pSB403 enthält das *luxR* Gen aus *Vibrio fischeri* zusammen mit einer transkriptionellen Fusion der *luxI* Promotorregion mit den Biolumineszenzgenen *luxCDABE* aus *Photorhabdus luminescens*. Da das *V. fischeri* 'Quorum sensing' System 3-Oxo-C6-HSL als Signalmolekül nutzt, zeigt das Sensorplasmid die höchste

Sensitivität für dieses AHL-Molekül. Es können jedoch auch andere AHL detektiert werden, wenn auch mit etwas geringerer Sensitivität (Winson *et al.*, 1998; Geisenberger *et al.*, 2000).

pJBA132: Das Sensorplasmid pJBA132 verwendet ebenfalls LuxR als AHL-Rezeptor, enthält jedoch das Reportergen *gfp*(ASV), eine instabile Variante des *gfp*mut3*.

pAS-C8: Das Sensorplasmid pAS-C8 basiert auf Komponenten des *cep* 'Quorum sensing' Systems aus *B. cepacia* H111 (siehe Punkt B.5.1). Es ist daher am sensitivsten für *N*-Octanoyl-L-homoserinlacton.

pKR-C12: Das Sensorplasmid pKR-C12 wurde aus den Komponenten des *las* 'Quorum sensing' Systems aus *Pseudomonas aeruginosa* konstruiert. Es ist sehr spezifisch für langkettige AHL-Moleküle.

Die Sensorplasmide pJBA132, pAS-C8 und pKR-C12 wurden für die Detektion von AHL-Molekülen in der Tomatenrhizosphäre mittels konjugativem Transfer in die beiden Stämme *P. putida* F117 und *S. liquefaciens* MG44 eingebracht.

Die resultierenden Biosensoren wurden nachfolgend hinsichtlich ihrer Sensitivität und Spezifität charakterisiert. Dazu wurden logarithmische Kulturen der Sensorstämme in den Kavitäten einer Mikrotiterplatte mit unterschiedlichen Konzentrationen (10000, 5000, 2500, 1250, 625, 310, 160, 80, 40 und 20 nM) der Signalmoleküle 3-Oxo-C12-HSL [*N*-(3-Oxododecanoyl)-L-homoserinlacton], 3-Oxo-C10-HSL [*N*-(3-Oxodecanoyl)-L-homoserinlacton], 3-Oxo-C6-HSL [*N*-(3-Oxohexanoyl)-L-homoserinlacton], C12-HSL [*N*-Dodecanoyl-L-homoserinlacton], C10-HSL [*N*-Decanoyl-L-homoserinlacton], C8-HSL [*N*-Octanoyl-L-homoserinlacton], C6-HSL [*N*-Hexanoyl-L-homoserinlacton], und C4-HSL [*N*-Butanoyl-L-homoserinlacton] gemischt. Nach 6 h Inkubation bei 30 °C erfolgte die Detektion der grünen Fluoreszenz (Emission bei 515 nm, Anregung bei 474 nm) in einem Lambda Fluoro 320 Plus Mikrotiterplatten Lesegerät (Bio-Tek Instruments, Winooski, USA). Die Daten wurden mit Hilfe der KC4 Software (Bio-Tek Instruments, Winooski, USA) ausgewertet und nach Abzug der Autofluoreszenz als RFU ('relative fluorescence units') gegen die AHL-Konzentrationen aufgetragen. Die synthetischen AHL-Moleküle wurden von den Firmen Fluka Chemie AG (Buchs, CH), Aurora Biosciences Corp. (Coralville, IA, USA) oder von P. Williams (School of Pharmaceutical Sciences, University of Nottingham, Nottingham, UK) bezogen.

Für die Quantifizierung von AHL-Molekülen von *P. putida* IsoF wurde routinemäßig der Monitorstamm *P. putida* F117 (pKR-C12) eingesetzt. Die zu bestimmenden Proben (z. B. zellfreie Kulturüberstände) wurden dazu mit einer logarithmischen Kultur des Monitorstammes in Kavitäten einer Mikrotiterplatte für 6 h bei 30 °C inkubiert. Die Detektion

der grünen Fluoreszenz erfolgte in einem Lambda Fluoro 320 Plus Mikrotiterplatten Lesegerät (Bio-Tek Instruments, Winooski, USA).

B.6.2 Nachweis der AHL-Produktion durch Kreuzausstrich

Werden AHL-produzierende Zellen auf einer LB-Agarplatte räumlich eng benachbart zu einem Sensorstamm ausgestrichen, so diffundieren die von dem zu untersuchenden Stamm produzierten AHL-Moleküle durch das Medium zu den Sensorzellen und induzieren dort die Expression des jeweiligen Reportergens. Die Agarplatten wurden bei 30 °C über Nacht inkubiert. Zur Detektion von Biolumineszenzsignalen wurde die ultrasensitive Photonenkamera C2400-40 (Hamamatsu, Herrsching) verwendet. Gfp-Signale konnten nach Anregung mit blauem Licht der Wellenlänge λ = 480 nm (Halogenlampe Intralux® 5000-1, Volpi AG, Schlieren, CH, mit Blau-Anregungsfilter F44-001, AHF-Analysentechnik, Tübingen) in einer Dunkelkammer mit bloßem Auge detektiert werden.

B.6.3 Identifizierung von AHL-Molekülen mittels Dünnschicht-Chromatographie

Hierzu wurden die AHL-Moleküle mit Dichlormethan aus dem Kulturüberstand des zu testenden Stamms extrahiert und mittels Dünnschicht-Chromatographie (DC) aufgetrennt. Danach wurden die DC-Platten mit Softagar überschichtet, der mit dem Sensorstamm *E. coli* MT102 (pSB403) beimpft war (Shaw *et al.*, 1997; Geisenberger *et al.*, 2000). Durch AHL-Moleküle induzierte Biolumineszenzsignale wurden durch Belichtung eines Röntgenfilms dokumentiert. Ein Vergleich der R_f-Werte der erhaltenen Signale mit denen von synthetischen AHL-Standards ermöglichte eine Identifizierung der extrahierten AHL-Moleküle. Der R_f-Wert ('retention factor') ist definiert als das Verhältnis der Wanderungsgeschwindigkeit einer Substanz zur Wanderungsgeschwindigkeit des Laufmittels bzw. das ihrer Laufstrecke zu der des Laufmittels. Eine ausführliche Beschreibung der Extraktion von AHL-Molekülen und ihrer Auftrennung mittels DC befindet sich in Anhang 1 und 2.

B.7 Messung der Biolumineszenz von rekombinanten Bakterienstämmen

Die bakterielle Luciferase LuxAB wurde im Rahmen dieser Arbeit als Reporter für Genexpressionsstudien verwendet. Die LuxAB Aktivität wurde durch Messung der Biolumineszenz von Flüssigkulturen der jeweiligen rekombinanten Stämme bestimmt. Da die bakterielle Luciferase ein langkettiges Aldehyd als Substrat benötigt, wurde 1 ml der Probe (ein Aliquot der Bakterienkultur) mit 1 µl *N*-Decanal versetzt und nach kurzem Mischen

sofort in einem EG&G Berthold MiniLumat LB9506 (Berthold, Bundoora, Australien) vermessen.

B.8 Bestimmung der antagonistischen Aktivität von Bakterien gegen phytopathogene Pilze

Die Anzucht der phytopathogenen Pilze *Cladosporium herbarium, Alternaria alternata*, *Botrytis cinerea* und *Fusarium oxysporum*, die freundlicherweise von G. Bahnweg (Institut für biochemische Pflanzenpathologie, GSF, Neuherberg) zur Verfügung gestellt wurden, erfolgte auf Würze-Agarplatten bei RT. Zur Gewinnung von Sporen wurden die Pilze für ca. 4 Wochen auf Haferflocken-Agar (10 g Haferflocken/l Agar) inkubiert und die Sporen anschließend mit 0,9 % NaCl-Lösung von der Kultur abgespült. Für die Agarblock-Inhibitionsassays (gegen *A. alternata*, *B. cinerea* und *F. oxysporum*) wurde ein mit Mycel bewachsener Agarblock in die Mitte einer Würze-Platte gelegt. In ca. 2 cm Abstand dazu wurden 20 µl Aliquots einer ÜN-Kultur der zu testenden Rhizobakterien auf die Platte aufgetropft. Nach 7- bis 14-tägiger Inkubation bei RT (je nach Wachstumsgeschwindigkeit des Pilzes) wurden die Hemmhöfe um die Bakterienkolonien ausgemessen. Bei Inhibitionsassays gegen *C. herbarium* bewährte sich die Herstellung eines mit Konidien beimpften Overlays. Dazu wurde aufgeschmolzener Würze-Weichagar (Würze-Boullion mit 0,7 % Agar) mit einer Konidiensuspension von *C. herbarium* inokuliert und auf eine Würze-Agarplatte gegossen. Nach Verfestigung des Weichagars wurden 20 µl einer Flüssigkultur der zu testenden Bakterienstämme auf die Platte aufgetropft, welche anschließend ca. 5 Tage bei RT inkubiert wurde. Die Auswertung erfolgte durch Ausmessen der Hemmhöfe um die Bakterienkolonien.

B.9 Anzucht von Tomatenpflanzen im gnotobiotischen System

Tomatensamen (*Lycopersicon esculentum* Micro-Tom, Meissner *et al.*, 1997) wurden von B. Nebelung, Everswinkel bezogen. Die Samen wurden durch Waschen in 70 % Ethanol für 3 min und anschließende Behandlung mit 5 % NaOCl-Lösung (+ 0,6 % Tween 20) für 20 min Oberflächen-sterilisiert. Die so behandelten Samen wurden nach fünfmaligem Waschen mit sterilem Wasser in Phytatrays (Sigma, Taufkirchen) ausgesät. Als Substrat diente 250 g steriler Quarzsand, der mit 25 ml steriler Nährlösung (nach Simons *et al.*, 1996) getränkt war. Die Phytatrays wurden mit einem Deckel verschlossen, mit Parafilm abgedichtet und in einer Klimakammer bei 25/20 °C (Tag/Nacht), 70 % Luftfeuchte und einem 14/10 h Tag-Nachtrhythmus für 14 bis 21 Tage inkubiert. 10 Tage nach der Aussaat (4 bis 5 Tage nach der

Auskeimung) wurden die Tomatenpflanzen mit den Bakterienstämmen beimpft. Die Bakterien wurden dazu in 250 ml LB Medium mit den entsprechenden Antibiotika-Zusätzen über Nacht angezogen und durch Zentrifugation geerntet. Die Zellen wurden in 10 mM $MgSO_4$ gewaschen und in 10 mM $MgSO_4$ auf eine Endkonzentration von 10^9 CFU/ml verdünnt. Drei Milliliter dieser Bakteriensuspension wurden zur Inokulation eines Phytatrays mit ca. 5 Tomatenpflanzen verwendet. Wurden die Pflanzen mit zwei Bakterienstämmen gleichzeitig inokuliert, so wurde eine 1:1 Mischung der Bakteriensuspensionen hergestellt und 3 ml der Mischung für die Inokulation verwendet.

B.10 Anzucht von Tomatenpflanzen im unsterilen System

Die wie oben beschrieben behandelten Tomatensamen wurden in unsteriler Anzuchterde (Fruehstorfer Einheitserde, Typ T, Patzer, Simmtal-Jossa) ausgesät und wie oben beschrieben in Klimakammern angezogen. Die Inokulation mit den Bakterien erfolgte 5 Wochen nach der Aussaat mit 10 ml Bakteriensuspension pro Pflanze.

B.11 Konfokale Laser Scanning Mikroskopie der Tomatenwurzeln

Alle mikroskopischen Aufnahmen der Tomatenwurzeln wurden mit einem LSM510 konfokalen Laser Scanning Mikroskop (Zeiss, Oberkochen) gemacht. Zur Detektion der Gfp- und Rfp-markierten Bakterien wurden ein Ar Laser (Gfp: Anregung 488 nm; Emissionsfilter BP 505-550) und ein HeNe Laser (Rfp: Anregung 543 nm; Emissionsfilter LP 560) in Kombination mit der vom Hersteller gelieferten Software (Version 2.1) verwendet.
Die Wurzelproben wurden 1 bis 10 Tage nach der Inokulation der Bakterien genommen, kurz mit PBS-Puffer abgespült und direkt mikroskopiert.

B.12 Untersuchung von Biofilmen in Durchflusskammern

B.12.1 Anzucht der Biofilme

Die Biofilme wurden in künstlichen Durchflusskammern mit ABC-Medium, das 0,1 mM Citrat enthielt, bei RT angezogen. Das Durchflusskammersystem wurde wie von Christensen *et al.* (1999) beschrieben aufgebaut. Ein Deckglas (Knittel Gläser, Braunschweig) stellte das Substrat dar. Übernachtkulturen Gfp-markierter Zellen in LB-Medium wurden bis zu einer OD_{600} von 0,7 subkultiviert. Sodann wurden sie in 0,9 % NaCl auf eine OD_{600} von 0,1 verdünnt. Die Durchflusskammern wurden mit je 250 µl dieser Verdünnungen beimpft. Nach einer Stunde, in der sich die Bakterien am Substrat anheften konnten, wurde mittels einer

peristaltischen Watson-Marlow 205S Pumpe ein konstanter Medienfluss von 0,2 mm/s erzeugt.

B.12.2 Biofilmanalyse und Bildverarbeitung

Zur mikroskopischen Inspektion der Biofilme wurde ein konfokales Laser Scanning Mikroskop (Zeiss LSM510, Zeiss, Oberkochen), das mit einem 40x/1,3 Ölimersions Objektiv ausgestattet war, eingesetzt. Die Bilder wurden mit der 488 nm Laserlinie eines Ar/Kr Lasers aufgenommen. Die Bildverarbeitung erfolgte mit Hilfe des IMARIS Softwarepakets (Bitplane, Zürich, CH).

C. ERGEBNISSE UND DISKUSSION

C.1 Häufigkeit und Diversität AHL-produzierender Bakterien in der Rhizosphäre

In den letzten Jahren wurden große Fortschritte bei der Erforschung der molekularen Mechanismen der 'Quorum sensing' Systeme zahlreicher Bakterien gemacht. Die ökologische Bedeutung dieser bakteriellen Kommunikationssystme wurde bisher jedoch kaum untersucht. Ziel dieser Arbeit war es daher, die Bedeutung AHL-vermittelter Kommunikation zwischen den verschiedenen bakteriellen Populationen der Rhizosphäre für die Diversität und Dynamik der Mikroflora zu beurteilen.

Zunächst sollte die Häufigkeit und Diversität der AHL-produzierenden Bakterien in der Rhizosphäre untersucht werden. In mehreren unabhängigen Studien wurde gezeigt, dass ein beachtlicher Anteil der Gram-negativen Rhizosphärenbakterien unterschiedlicher Kulturpflanzen zur AHL-Produktion befähigt ist. In Zusammenarbeit mit G. Berg (Institut für molekulare Physiologie und Biotechnologie, Mikrobiologie; Universität Rostock) wurden Isolate aus der Rhizosphäre von Erdbeere, Kartoffel, Raps und aus dem Boden auf ihre Fähigkeit zur AHL-Produktion getestet. Durch Cross-Streaks gegen den AHL-Monitorstamm *E. coli* MT102 (pSB403) (Winson *et al.*, 1998) wurde die Produktion von AHL bei ca. 41 % (45/111) der getesteten Stämme nachgewiesen. Die Stämme, welche alle aufgrund ihrer antagonistischen Aktivität gegen den phytopathogenen Pilz *Verticillium dahliae* isoliert worden waren, wurden mittels Analyse der Fettsäurezusammensetzung (FAME) und Sequenzanalyse der 16S rDNA größtenteils der Gattung *Pseudomonas* spp. zugeordnet (Tabelle 1, Anhang 3). Daneben wurden häufig AHL-produzierende Vertreter der *Enterobacteriaceae* identifiziert, vor allem *Serratia* spp., *Enterobacter* spp. und *Pantoea* spp. (Tabelle 2, Anhang 3).

Darüber hinaus wurden in unserem Labor bei der Isolierung von AHL-Produzenten aus der Rhizosphäre unterschiedlicher Kulturpflanzen (z. B. Tomate, Mais, Raps, Weizen) häufig Vertreter der Gattungen *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Rahnella* und *Aeromonas* identifiziert (Daten nicht gezeigt).

Diese Ergebnisse stehen in gutem Einklang mit vorausgehenden Studien, die ebenfalls zeigen, dass sehr viele Pflanzen-assoziierte *Proteobacteria* zur Synthese von AHL-Signalmolekülen befähigt sind. Cha *et al*. (1998) demonstrierten, dass die meisten der untersuchten *Agrobacterium*, *Rhizobium* und *Pantoea* Isolate und etwa die Hälfte der getesteten *Erwinia*

und *Pseudomonas* Isolate eine positive Reaktion mit einem AHL-Biosensor ergaben. Die 'Quorum sensing' Systeme einiger dieser Organismen sind bereits auf molekularer Ebene charakterisiert worden und regulieren größtenteils bakterielle Funktionen, die für die Interaktion mit der Pflanze von großer Bedeutung sind (siehe Einleitung). Diese Annahme wird durch eine umfassende Studie zur AHL-Produktion bei Pflanzen-assoziierten Bakterien untermauert. Hierin waren 40 % der aus der Rhizosphäre isolierten Pseudomonaden zur Synthese von meist mehreren AHL-Molekülen fähig, wohingegen die aus unbewachsenem Boden isolierten Stämme keinen der verwendeten AHL-Biosensoren aktivieren konnten (Elasri *et al*., 2001). Diese Beobachtung dient als Beweis für die Hypothese, dass die Wahrscheinlichkeit der AHL-Produktion eines Bakteriums umso höher ist, je enger dieses mit einer Pflanze assoziiert ist. Dies trifft insbesondere für phytopathogene Mikroorganismen zu, welche durch die Wahrnehmung der eigenen Populationsdichte die Expression ihrer Virulenzfaktoren so koordinieren können, dass die Wirtspflanze förmlich „überrannt" wird und nicht mehr rechtzeitig eine Abwehrreaktion aktivieren kann (Eberl, 1999).

An dieser Stelle soll noch angemerkt werden, dass keine der oben genannten Arbeiten den Anteil von AHL-produzierenden Bakterien an der Gesamtpopulation der Rhizosphäre erfasst hat. Sowohl bei der im Rahmen dieser Arbeit durchgeführten Durchmusterung von *Verticillium*-Antagonisten als auch in der oben erwähnten Studie von Elasri *et al*. (2001) wurden hauptsächlich Pseudomonaden untersucht und in beiden Fällen repräsentieren die getesteten Stämme keinen Querschnitt der Gesamtpopulation sondern eine vorher ausgewählte Teilpopulation der Mikroflora einer Pflanze. Eine sicherlich repräsentativere Zahl geht aus der Studie von Pierson *et al*. (1998) hervor, im Rahmen derer insgesamt 700 Rhizosphärenisolate unterschiedlicher geographischer Herkunft auf AHL-Produktion getestet wurden. Hierbei waren insgesamt 8 % der Isolate in der Lage, einen AHL-Biosensor zu aktivieren. In unserem Labor wurden bei der Isolierung von Wurzel-assoziierten Bakterien auf Standardmedien (LB-Medium oder ABC-Medium) ca. 10 bis 60 % aller Isolate (je nach untersuchter Wirtspflanze) positiv auf AHL-Produktion getestet (Daten nicht gezeigt). Wie bereits aus der Studie von Berg *et al*. (Anhang 3) hervorgeht, scheint auch die Pflanzenart einen Einfluss auf die Zusammensetzung der bakteriellen Population in der Rhizosphäre auszuüben, was möglicherweise eine artspezifische Anreicherung von AHL-Produzenten in der Rhizosphäre mancher Pflanzen zur Folge haben könnte. So beherbergt beispielsweise die Reisrhizosphäre eine große Anzahl AHL-produzierender *Burkholderia* spp. (V. Albrecht, 2002).

C.2 Charakterisierung der von Rhizobakterien produzierten AHL-Moleküle

Im Hinblick auf die Untersuchung der bakteriellen Zell-Zell-Kommunikation in der Rhizosphäre sollten als nächstes die von den Bakterien verwendeten Botenstoffe, die *N*-Acyl-L-homoserinlactone, genauer charakterisiert werden. Eine schnelle und einfache Methode zur Bestimmung der von einem Organismus produzierten AHL-Moleküle ist die Auftrennung der aus dem Kulturüberstand extrahierten AHL über Dünnschichtchromatographie und anschließende Detektion mit Hilfe eines AHL-Biosensors. Durch den Vergleich mit Referenzsubstanzen kann auf die Molekülstruktur der AHL geschlossen werden, da das chromatographische Laufverhalten der Substanzen durch die Länge und Substitution der Acylseitenkette bestimmt wird. Darüber hinaus erhält man Informationen über die Anzahl an unterschiedlichen AHL-Molekülen, die von dem jeweiligen Organismus produziert werden. Hierbei muss jedoch immer die Spezifität und Sensitivität des verwendeten AHL-Sensors berücksichtigt werden. So werden beispielsweise besonders lang- oder kurzkettige AHL-Moleküle nur eingeschränkt von dem in unserem Labor routinemäßig eingesetzten Monitorstamm *E. coli* MT102 (pSB403) (Winson *et al.*, 1998) erkannt.

Die Charakterisierung der AHL-Spektren zahlreicher Rhizosphärenisolate brachte zwei wichtige Erkenntnisse. Zum einen stellte sich heraus, dass verschiedene Stämme der selben Art durchaus unterschiedliche AHL-Muster aufweisen können. Die AHL-Profile von 12 *P. putida* Stämmen, die aus der Rhizosphäre von Raps und Kartoffel und aus dem Boden isoliert worden waren, wurden durch Dünnschichtchromatographie bestimmt, wobei sich zwei verschiedene Muster unterscheiden ließen (Abb. 2). Einige Stämme waren zur Synthese sowohl kurz- als auch langkettiger AHL-Moleküle fähig (Abb. 2: 8Kz4, 7Bp13/2, 9Rx4/2, 9Rx3/2, 2Rr14, 9Rx11, 7Kc1 und 9Rx6/2), während die anderen Isolate hauptsächlich C6-HSL und geringe Mengen an 3-Oxo-C6-HSL produzierten (Abb. 2: 2Bx4, 10Bp12, 8Rr22 und 6Kp8). Diese Stämme waren daher auch nicht in der Lage, den für langkettige AHL-Moleküle spezifischen AHL-Sensorstamm *P. putida* F117 (pKR-C12) im Cross-Streak zu aktivieren (siehe Anhang 3, Tabelle 3; die Stämme 8Rr22 und 6Kp8 wurden der *P. putida* Gruppe B zugeordnet und sind daher nicht in dieser Tabelle aufgeführt). Die Variabilität von AHL-Mustern innerhalb einer Art ist nicht nur auf *P. putida* beschränkt, sondern wurde auch im Rahmen anderer Studien bei Vertretern der Gattungen *Rahnella* (Anhang 1), *Erwinia* oder *Rhizobium* (Cha *et al*., 1998) beobachtet.

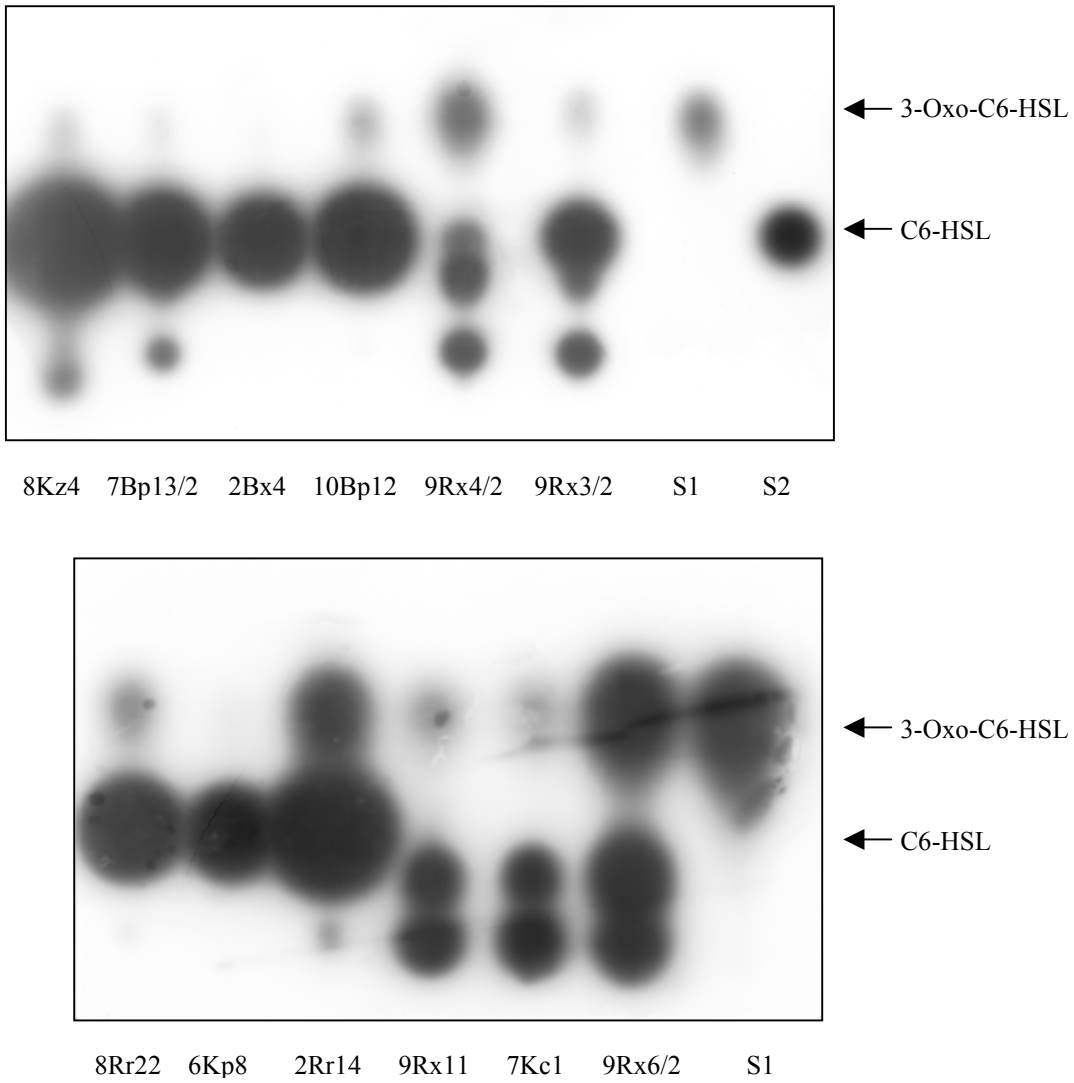


Abb. 2. AHL-Profile von *P. putida* Stämmen, die aus der Rhizosphäre von Raps und Kartoffel oder aus unbewachsenem Boden isoliert wurden (zur Herkunft der Stämme siehe Anhang 3). Als Referenzsubstanzen wurden 3-Oxo-C6-HSL (S1) und C6-HSL (S2) verwendet.

Wie hier am Beispiel von *P. putida* gezeigt (siehe auch Anhang 1 und 3), sind viele Rhizosphärenbakterien zur Synthese mehrerer verschiedener AHL-Moleküle fähig (Cha *et al*., 1998; Brelles-Mariño und Bedmar, 2001). Die Synthese mehrerer Signalmoleküle, welche abhängig von ihrer Struktur von unterschiedlichen Rezeptoren erkannt werden können, kann einerseits darauf hindeuten, dass innerhalb der Zelle eine AHL-abhängige Regulationskaskade existiert, die eine differentielle Genregulation ermöglicht. Oft wird die Synthese der verschiedenen AHL-Moleküle auch von unterschiedlichen AHL-Synthasen bewerkstelligt, zum Beispiel in *P. aeruginosa* duch LasI und RhlI (Winson *et al.*, 1995). Andererseits könnte ein breites Spektrum von Botenstoffen auch als eine Art universelle Sprache eingesetzt werden, die auch über die Artgrenze hinweg verstanden wird. Diese Möglichkeit erscheint besonders plausibel, wenn Vertreter unterschiedlicher Spezies, die ein Habitat besiedeln, die

gleichen AHL-Moleküle produzieren. Die Zell-Zell-Kommunikation zwischen verschiedenen Spezies einer Population, auch als ‘cross-talk’ bezeichnet, ermöglicht eine wesentlich komplexere Interaktion der Individuen innerhalb des Habitats, was weitreichende Folgen für die Dynamik und Struktur der Rhizosphärenpopulation haben könnte. Die Untersuchung und Visualisierung dieser intergenerischen Kommunikation mittels AHL-Signalmolekülen war eines der Hauptziele dieser Arbeit. Im folgenden sollen nun die wichtigsten Schritte dieser Studie aufgezeigt und diskutiert werden, die experimentellen Details der Arbeit sind dem Anhang 1 zu entnehmen. An dieser Stelle soll erwähnt werden, dass ein Teil der hier (in Punkt C.3 bis C.6) beschriebenen Experimente von Katja Sigl im Rahmen ihrer Diplomarbeit durchgeführt wurde.

C.3 Konstruktion von AHL-Biosensoren auf Gfp-Basis

Zum Nachweis der AHL-Produktion war es bislang notwendig, die aus der Rhizosphäre isolierten Bakterien im Labor zu kultivieren. Um jedoch die tatsächliche Bedeutung AHL-vermittelter Kommunikation in der Rhizosphäre beurteilen zu können, sollten im Rahmen dieser Arbeit Techniken und Werkzeuge entwickelt werden, die es erlauben, die Signalmoleküle im natürlichen Habitat auf Einzelzellebene nachzuweisen. Zu diesem Zweck wurden Biosensoren entwickelt, welche auf die Anwesenheit von AHL mit der Expression des grün fluoreszierenden Proteins (Gfp) (Chalfie *et al.*, 1994) antworten. So können die aktivierten AHL-Sensorzellen mittels Epifluoreszenzmikroskopie oder konfokaler Laser-Scanning-Mikroskopie (CLSM) direkt an der Pflanzenwurzel detektiert, und ihre Assoziation mit anderen Fluoreszenz-markierten Bakterienzellen (z. B. AHL-Produzenten) untersucht werden. Gegenüber anderen Reportern, wie der bakteriellen Luciferase oder β-Galaktosidase, weist Gfp den Vorteil auf, dass seine Detektion vollkommen unabhängig von exogenen Substraten oder Cofaktoren ist, da es nur einer Bestrahlung mit Licht im nahen UV-Bereich bedarf (Hauptabsorptionsmaximum bei 395 nm und lokales Maximum bei 475 nm). Durch Aminosäuresubstitutionen im Gfp-Chromophor konnten Gfp-Derivate mit verschobenen Anregungswellenlängen erzeugt werden (wie das hier verwendete Gfpmut3*, das zwischen 480 und 490 nm absorbiert). Diese sog. ‘red-shifted’ Gfp-Varianten weisen eine stärkere Signalintensität auf und können besser von dem im CLSM verwendeten Argon-Laser angeregt werden (Heim *et al.*, 1995; Cormack *et al.*, 1996). Die Nachteile der Gfp-Reportergentechnologie sind das sog. ‘photobleaching’, das Ausbleichen des Fluoreszenzsignals bei längerer Bestrahlung durch das Laserlicht des

Epifluoreszenzmikroskops, und die geringere Sensitivität im Vergleich zu den Reportern Luciferase oder β-Galaktosidase. Um Schwankungen der AHL-Konzentration im Verlauf des bakteriellen 'cross-talk' verfolgen zu können, wurde für die Konstruktion der Sensoren eine instabile Gfp-Variante, Gfp(ASV), verwendet. Dieses modifizierte Gfpmut3* weist eine C-terminale Aminosäuresequenz auf, die das Protein dem Abbau durch intrazelluläre spezifische Proteasen zugänglich macht. Dadurch wird die Halbwertszeit des Proteins in der Zelle von mehr als 24 Stunden (Gfpmut3*) auf ca. 190 Minuten reduziert (Andersen *et al.*, 1998).

Die Funktionsweise eines AHL-Biosensors und die zur Konstruktion der AHL-Sensorplasmide verwendeten Komponenten sollen hier nun am Beispiel des im Rahmen dieser Arbeit konstruierten Plasmids pAS-C8 erläutert werden (siehe Punkt B.5.1 und Anhang 4). Typischerweise besteht ein AHL-Sensorplasmid aus zwei funktionellen Bestandteilen. Erstens, einer transkriptionellen oder translationalen Fusion eines AHL-regulierten Gens mit einem Reportergen, hier *gfp*(ASV), und zweitens einem *luxR*-homologen Gen, das unter die Kontrolle eines konstitutiven Promotors gebracht wurde. Da weder das Plasmid noch der Wirtsstamm für eine AHL-Synthase kodieren, kommt es zur Expression von *gfp*(ASV), wenn AHL-Moleküle von außen zugegeben werden.

Für die Konstruktion von pAS-C8 wurde als AHL-Rezeptor CepR aus *Burkholderia cepacia* ausgewählt (Abb. 3). Das *cep* 'Quorum sensing' System verwendet *N*-Octanoyl-L-homoserinlacton (C8-HSL) als Signalmolekül (Lewenza *et al.*, 1999), weshalb pAS-C8 vor allem AHL-Moleküle mit mittlerer Kettenlänge gut erkennen sollte. Die Expression von *cepR* wird durch den *lac* Promotor kontrolliert, was in den meisten Organismen zu einer starken, konstitutiven Expression des Rezeptors führt. Die Expression von *gfp*(ASV) wird durch den *cepI* Promotor reguliert, der durch den CepR/AHL-Komplex aktiviert wird und in Abwesenheit von AHL-Molekülen praktisch keine detektierbare Hintergrundaktivität aufweist. Die P_{cepI}-*gfp*(ASV)-P_{lac}-*cepR* Sensorkassette wurde in das Plasmid pBBR1MCS-5 ligiert, welches einen breiten Wirtsbereich hat und durch konjugativen Transfer mobilisierbar ist. Das daraus resultierende Plasmid pAS-C8 stellt somit ein konjugatives AHL-Sensorplasmid auf Gfp-Basis mit breitem Wirtsbereich dar.

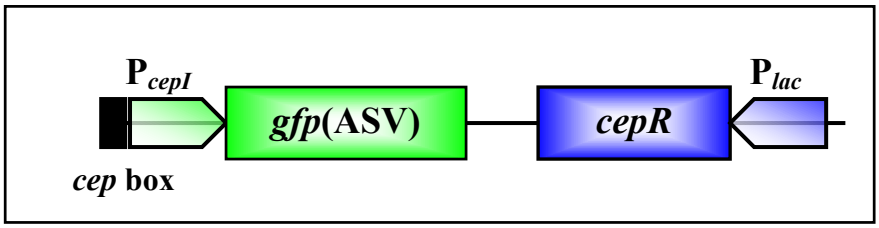


Abb. 3. Schematische Darstellung der AHL-Sensorkassette in pAS-C8. Die *cep*-Box ist ein 20 bp-Palindrom im Promotorbereich von *cepI*, das als Bindungsstelle für CepR fungiert.

Um ein möglichst breites Spektrum an AHL-Molekülen detektieren zu können, wurden zusätzlich noch die beiden Plasmide pKR-C12 (Anhang 4) und pJBA132 (Andersen *et al.*, 2001) zur Konstruktion der AHL-Biosensoren verwendet. Plasmid pJBA132 basiert auf den Komponenten des *lux* 'Quorum sensing' Systems von *V. fischeri* und kodiert für LuxR als AHL-Rezeptor und für eine transkriptionelle Fusion des *luxI* Promotors mit *gfp*(ASV). Da das *lux* 'Quorum sensing' System in *V. fischeri* durch 3-Oxo-C6-HSL aktiviert wird, zeigt der Sensor die höchste Sensitivität für dieses Molekül, erkennt jedoch auch verwandte Signalmoleküle, allerdings mit geringerer Sensitivität (Andersen *et al.*, 2001). Für die Konstruktion des Sensorplasmids pKR-C12 wurden *lasR* und eine translationale Fusion des *lasB* Elastase Gens mit *gfp*(ASV) verwendet. Diese Komponenten entstammen dem *las* 'Quorum sensing' System von *P. aeruginosa* und erkennen vor allem *N*-(3-Oxododecanoyl)-L-homoserinlacton (3-Oxo-C12-HSL) und andere langkettige AHL-Moleküle (Anhang 4). Auch die beiden Plasmide pJBA132 und pKR-C12 basieren auf Replikons mit breitem Wirtsbereich und sind durch konjugativen Transfer mobilisierbar. Um geeignete Biosensoren zur Detektion von AHL in der Rhizosphäre zu erhalten, wurden die drei genannten Plasmide in AHL-Nullmutanten von *P. putida* (*P. putida* F117) und *S. liquefaciens* (*S. liquefaciens* MG44) überführt. Diese beiden Organismen wurden ausgewählt, da sie sehr häufig in der Rhizosphäre von Pflanzen vorkommen und eine stabile Kolonisierung der Wurzel über einen längeren Zeitraum aufweisen.

Der Nachteil von Plasmid-kodierten AHL-Sensoren ist ihre verminderte Stabilität in Abwesenheit von Selektionsdruck. Obwohl Plasmid pKR-C12 im Laborversuch über längere Zeit stabil an die Tochterzellen weitergegeben wurde (nur ca. 20 % Plasmidverlust über 100 Generationen) (K. Sigl, 2001), sollte zusätzlich ein Biosensor konstruiert werden, der die Sensorkassette stabil ins Chromosom integriert trägt. Zu diesem Zweck wurde die P_{lasB}-*gfp*(ASV)-P_{lac}-*lasR* Sensorkassette ins Chromosom von *P. putida* F117 integriert, wodurch sie auch ohne Selektionsdruck stabil segregiert wird.

Die Bindungsaffinität eines AHL-Moleküls zu den unterschiedlichen LuxR-homologen Proteinen wird durch die Länge und Substitution seiner Acylseitenkette bestimmt. Hinzu kommt jedoch, dass sowohl die Spezifität als auch die Sensitivität der Sensorplasmide zu einem gewissen Maß durch den Wirtstamm beeinflusst wird (Anhang 4; Andersen *et al.*, 2001). Daher war es notwendig, die rekombinanten AHL-Monitorstämme hinsichtlich ihrer Sensitivität für unterschiedliche AHL-Moleküle zu charakterisieren. Aus Abb. 1 in Anhang 1 ist zu entnehmen, dass die beiden *las* Sensoren [*P. putida* F117 (pKR-C12) und *P. putida* F117::Tn*5*-LAS] sehr spezifisch für langkettige AHL-Moleküle sind, wohingegen *S. liquefaciens* MG44 (pJBA132) und *P. putida* F117 (pAS-C8) vorwiegend AHL mit kurzer und mittlerer Kettenlänge erkennen. Diese Beobachtungen entsprachen weitgehend den Erwartungen, da die am besten erkannten Moleküle die natürlichen Liganden der jeweiligen LuxR-homolgen Rezeptoren darstellen. Die beiden Monitorstämme *P. putida* F117::Tn*5*-LAS und *P. putida* F117 (pKR-C12) verwenden das gleiche AHL-Reportersystem und zeigen daher auch eine vergleichbare Spezifität. Da die ins Chromosom integrierte Sensorkassette jedoch nur in einer Kopie pro Zelle vorliegt, zeigt der Stamm F117::Tn*5*LAS eine deutlich verringerte Signalintensität im Vergleich zu F117 (pKR-C12). Bei der Charakterisierung der rekombinanten AHL-Monitorstämme zeigte sich weiterhin, dass die drei verwendeten Sensorplasmide nicht in beiden Bakterienstämmen gleich gut funktionierten. So konnten beispielsweise selbst in Anwesenheit von 10 µM 3-oxo-C12-HSL keine grün fluoreszierenden Zellen von *S. liquefaciens* MG44 (pKR-C12) detektiert werden, während schon geringste Mengen dieses Signalmoleküls zur Aktivierung des Sensorplasmids in *P. putida* F117 (pKR-C12) ausreichten (Daten nicht gezeigt). Die Ursache für die unterschiedliche Sensitivität der Biosensoren ist bislang nicht völlig klar. Man weiß jedoch, dass vor allem langkettige AHL-Moleküle nicht mehr frei durch die Zellhülle diffundieren können (Pearson *et al.*, 1999) und die Zelle daher vermutlich geeignete Transportsysteme zur Aufnahme der Signalstoffe besitzen muss.

Um ein möglichst breites Spektrum an AHL-Molekülen mit hoher Sensitivität detektieren zu können, wurden für die weiteren Experimente die Sensorstämme ausgewählt, mit denen im *in vitro* Versuch die höchste Signalintensität für die jeweils erkannten AHL-Moleküle erhalten wurde. Die hierbei ermittelten Detektionslimits der drei ausgewählten Sensorstämme *P. putida* F117 (pAS-C8), F117 (pKR-C12) und *S. liquefaciens* MG44 (pJBA132) lagen jeweils unter 10 nM für das am besten erkannte Signalmolekül. Auch auf Einzelzellebene waren geringste Mengen der AHL-Moleküle ausreichend (weniger als 5 nM), um die grün fluoreszierenden Sensorzellen mit Hilfe eines Epifluoreszenzmikroskops detektieren zu

können. Bei der Verwendung der Biosensoren für ‘Cross-Streaks’ gegen AHL-produzierende Rhizosphärenisolate konnte das Gfp-Signal bei Bestrahlung der Platten mit blauem Licht sogar mit bloßem Auge detektiert werden.
Diese ‘Cross-Streaks’ dienten im nächsten Schritt als Anhaltspunkt für die Auswahl der AHL-Produzenten zur Inokulation der Tomatenpflanzen in den nachfolgenden Experimenten. Es wurden insgesamt ca. 300 Rhizosphärenisolate gegen die Biosensoren ausgestrichen, wobei etwa 12 % eine Aktivierung eines oder mehrerer AHL-Monitorstämme auslösten. Es wurden schließlich vier aus der Tomatenrhizosphäre isolierte Stämme ausgewählt, welche im ‘Cross-Streak’ eine besonders starke Aktivierung des Sensors aufwiesen (Tabelle 3, Anhang 1). Aufgrund einer 16S rDNA Sequenzanalyse wurden die Stämme T13 und TAA als *Rahnella aquatilis*, die Stämme IsoF und Z2D als *P. putida* identifiziert (siehe Anhang 1).

C.4 Zell-Zell-Kommunikation in der Rhizosphäre von gnotobiotisch gezogenen Tomatenpflanzen

Um die AHL-vermittelte Zell-Zell-Kommunikation unter genau definierten und reproduzierbaren Bedingungen untersuchen zu können, wurde ein gnotobiotisches System zur Anzucht von Tomatenpflanzen entwickelt. Hierbei wird die Pflanze unter sterilen Bedingungen in Quarzsand angezogen und kann mit den gewünschten Bakterienstämmen beimpft werden. Die Verwendung einer speziellen „Zwerg-Tomate“ der Sorte Micro-Tom, die nur eine Höhe von ca. 20 cm erreicht (Meissner *et al.*, 1997), ermöglichte die Anzucht der Pflanzen in abgeschlossenen, sterilen Anzuchtgefäßen. Die Verwendung eines gnotobiotischen Systems hat drei entscheidende Vorteile: (a) die Biosensoren können gezielt, ohne störende Begleitflora mit AHL-Produzenten co-inokuliert werden, welche den jeweiligen Sensorstamm aufgrund ihres AHL-Profils optimal aktivieren, (b) die geringe Autofluoreszenz des verwendeten Quarzsands im Vergleich zu herkömmlicher Anzuchterde erleichtert die Detektion der fluoreszenz-markierten Zellen und (c) ein hohes Maß an Reproduzierbarkeit.
Fünf Tage nach der Auskeimung wurden die Tomatenkeimlinge unter sterilen Bedingungen mit einer Mischung aus AHL-Produzent und Monitorstamm beimpft. Nach 10-tägiger Inkubation in der Klimakammer wurden die Pflanzen geerntet, und die Wurzeln mit PBS abgespült, um den anhaftenden Sand zu entfernen. Dann wurden die Wurzeln sofort unter dem CLSM mikroskopiert.

In mehreren unabhängigen Experimenten, in denen die beschriebenen Biosensoren zusammen mit den ausgewählten AHL-Produzenten die Tomatenwurzel besiedelten, konnte eine deutliche Aktivierung der Sensoren beobachtet werden (siehe Anhang 1, Abb. 3). Die Experimente im gnotobiotischen System zeigen deutlich, dass biologisch relevante Konzentrationen der Signalmoleküle an der Wurzel gebildet werden, und diese nicht nur innerhalb einer Art sondern auch über die Artgrenzen hinweg die Genexpression regulieren können. Gleichzeitig wurde durch Kontrollexperimente bestätigt, dass die Sensoren hochspezifisch auf die Anwesenheit von AHL-Molekülen reagieren, da in Abwesenheit von AHL-produzierenden Bakterien keine Aktivierung der Sensoren an der Wurzel zu detektieren war. Die Visualisierung der nicht-aktivierten Sensorzellen wurde durch eine konstitutive Markierung der Stämme mit dem rot fluoreszierenden Protein (Rfp oder DsRed; Matz *et al.*, 1999) ermöglicht. Dieses Markerprotein wurde ebenfalls dazu verwendet, um einige der AHL-produzierenden Bakterien gleichzeitig mit den grün fluoreszierenden Sensorzellen detektieren zu können. Auf diese Weise war es möglich, die Besiedelungsmuster der beiden Bakterienpopulationen mit Hilfe des CLSM zu rekonstruieren. Es zeigte sich hierbei, dass Sensorzellen und AHL-Produzenten nicht immer in direkter räumlicher Nachbarschaft zu finden waren. Diese Beobachtung weist darauf hin, dass sich die Signalmoleküle innerhalb des Habitats über eine gewisse Distanz hinweg ausbreiten können. Diese Vermutung sollte nachfolgend durch einige gezielte Experimente bestätigt werden. In einem Vorversuch wurden Tomatenpflanzen im gnotobiotischen System mit dem Rfp-markierten Sensorstamm *P. putida* F117::Tn*5*LAS beimpft. Nach einer punktförmigen Inokulation einer definierten Menge an AHL-Molekülen am unteren Ende des Tomatenstamms wurde unter dem Epifluoreszenzmikroskop ausgemessen, in welchen Wurzelbereichen nach 24 h aktivierte AHL-Sensorzellen zu finden waren. Es konnte beobachtet werden, dass sich bei der Inokulation von 50 µl einer 100 µM 3-Oxo-C12-HSL Lösung am Übergang zwischen Wurzel und Stamm die AHL-Moleküle bis zur Wurzelspitze (bis zu ca. 5 cm Entfernung) hin ausbreiteten, um dort Sensorzellen zu aktivieren. Wurden nur 10 µl der gleichen AHL-Lösung appliziert, breiteten sich die AHL-Moleküle nur etwa 1 bis 2 cm weit an der Wurzel entlang aus. Da in diesem Experiment sehr hohe Konzentrationen der Signalstoffe eingebracht wurden, ist die gemessene Diffusionsstrecke sicherlich nicht mit den natürlichen Bedingungen vergleichbar. Daher wurde die Ausbreitung der AHL-Moleküle innerhalb der Rhizosphäre in einem zweiten Versuch auf Einzelzellebene untersucht. In Zusammenarbeit mit C. Dürr (Institut für Bodenökologie, GSF, Neuherberg) und F. Dazzo (Department of Microbiology & Molecular Genetics, Michigan State University, East Lansing, USA) wurde

ein mikroskopisches Bild einer Tomatenwurzel, die von dem AHL-Sensorstamm *P. putida* F117 (pKR-C12) und dem Rfp-markierten AHL-Produzenten *P. putida* IsoF besiedelt war, mit Hilfe des Computerprogramms CMEIAS ausgewertet (Liu *et al.*, 2001). Hierbei wurden anhand einer digitalisierten CLSM-Aufnahme der gesamten Wurzel die Abstände zwischen den einzelnen AHL-Produzenten und aktivierten Sensorzellen ausgemessen und statistisch ausgewertet. Die Entfernung zwischen einem AHL-Produzenten und einer Sensorzelle lag meist zwischen 4 und 10 µm, es wurden aber auch Werte bis zu 65 µm gemessen (C. Dürr, 2001).

Elektronenmikroskopische Untersuchungen haben gezeigt, dass die Epidermis von Pflanzenwurzeln von einer polymeren Matrix, dem sogenannten „Mucigel“ (Jenny und Grossenbacher, 1963), überzogen ist, in welche die Bakterien eingebettet sind (Chin-A-Woeng *et al.*, 1997). Diese mukoide Matrix würde ein geeignetes Medium für die Ausbreitung der Signalmoleküle entlang der Wurzeloberfläche darstellen, da sie möglicherweise die Diffusion der Stoffe in den umgebenden Boden vermindert und dadurch die lokale Konzentration der AHL erhalten bleibt.

C.5 Nachweis der AHL-vermittelten Kommunikation im natürlichen Habitat

Die Beobachtung, dass bakterieller ’cross-talk’ zwischen Einzelzellen im gnotobiotischen System stattfindet, legte nahe, die AHL-vermittelte Kommunikation auch in der natürlichen Population der Tomatenrhizosphäre zu untersuchen. Daher wurden die Tomatenpflanzen in unsteriler Erde über einen Zeitraum von fünf Wochen angezogen, so dass sich eine natürliche Mikroflora an der Wurzel ausbilden konnte. Dann wurde der Rfp-markierte AHL-Monitorstamm *P. putida* F117::Tn*5*LAS, der wegen der Stabilität der Sensorkassette auch unter dem erhöhten Konkurrenzdruck durch die bereits bestehende Population am besten für dieses Experiment geeignet war, in die Rhizosphäre inokuliert. Bei der anschließenden Untersuchung der Tomatenwurzeln unter dem CLSM war besonders interessant, dass auch dann aktivierte Sensorzellen an der Wurzel detektiert werden konnten, wenn kein zusätzlicher AHL-Produzent zusammen mit dem Sensorstamm eingebracht worden war (Abb. 3F, Anhang 1). Dieses Experiment zeigt, dass Mitglieder der natürlichen Rhizosphärenpopulation signifikante Mengen an AHL-Molekülen produzieren, und diese von anderen Zellen wahrgenommen werden können. Dies legt die Vermutung nahe, dass AHL-vermittelte Kommunikation im natürlichen Habitat eine Rolle bei der Koordination der unterschiedlichen

bakteriellen Funktionen spielt. Eine Untersuchung des Besiedelungsverhaltens von Gfp-markierten *Pseudomonas fluorescens* Zellen in der Rhizosphäre von Gerstenpflanzen konnte zeigen, dass *P. fluorescens* meist Mikrokolonien an der Wurzeloberfläche bildet, welche häufig mit Zellen der natürlichen Mikroflora (diese wurden durch DAPI angefärbt) eng assoziiert sind (Normander *et al.*, 1999). Solche gemischten Mikrokolonien, die in das Mucigel eingebettet sind, bieten ideale Voraussetzungen für bakteriellen 'cross-talk'.

Das Konzept der Zell-Zell-Kommunikation zwischen verschiedenen Spezies wurde erstmals 1979 aufgrund der Beobachtung aufgestellt, dass *Vibrio harveyi* durch die Zugabe von Kulturüberständen anderer, nicht lumineszenter Bakterien zur Biolumineszenz angeregt werden konnte (Greenberg *et al.*, 1979). Die Struktur des damals noch unbekannten „Alloinducers" konnte erst über 20 Jahre später aufgeklärt werden. Es handelt sich dabei um ein Furanosyl-Borat-Diester (AI-2) (Chen *et al.*, 2002), welches in *V. harveyi* von LuxS synthetisiert und von dem periplasmatischen Protein LuxP gebunden wird. Inzwischen wurde AI-2 Aktivität auch in Kulturüberständen anderer Bakterien, darunter *E. coli, Vibrio cholerae, Yersinia entercolitica* und *Salmonella enterica* Serovar Typhimurium, nachgewiesen (Surette *et al.*, 1999; Bassler *et al.*, 1997). Datenbankrecherchen brachten außerdem *luxS* homologe Gene in zahlreichen Gram-negativen und Gram-positiven Bakterien zu Tage, was zu der Vermutung führte, dass es sich bei AI-2 um ein universelles Signalmolekül handeln könnte, das Interaktionen in einer Reihe von natürlichen Habitaten vermittelt (Schauder *et al.*, 2001). Diese Hypothese wird allerdings noch kontrovers diskutiert, da LuxS homologe Proteine eine entscheidende Rolle bei der Rückgewinnung von S-Adenosyl-Methionin (SAM), einem zentralen Metaboliten bei Methylierungsreaktionen in der Zelle, spielen. Daher ist das Vorhandensein dieses Enzyms kein eindeutiger Beweis für die Verwendung von AI-2 als Kommunikationssignal, da ihm in erster Linie eine metabolische Rolle in der Zelle zukommt und AI-2 möglicherweise nur in einigen Organismen als echtes 'Quorum sensing' Signal verwendet wird (Winzer *et al.*, 2002).

Als gesichert gilt jedoch die Rolle von AHL als Signalmoleküle im bakteriellen 'cross-talk'. So wurde beispielsweise ebenfalls mit Hilfe von Gfp-basierenden AHL-Monitorstämmen die Zell-Zell-Kommunikation in gemischten Biofilmen von *B. cepacia* und *P. aeruginosa* nachgewiesen (Anhang 4). In Übereinstimmung mit der bereits genannten Studie von Pierson *et al.* (1998) (Punkt A.2 und A.3) konnte durch die Verwendung von AHL-Monitorstämmen eine intergenerische Kommunikation von Bakterien in der Rhizosphäre gezeigt werden. Aus diesen Erkenntnissen lässt sich ein Szenario entwickeln, in dem das 'Quorum', welches ein Individuum in seinem Habitat wahrnimmt, nicht nur aus Mitgliedern der eigenen Population

besteht, sondern gleichzeitig auch durch aktivierende und hemmende Signale von artfremden Organismen beeinflusst wird. Der „Pool“ an Signalmolekülen kann hierbei natürlich auch von AHL-abbauenden Organismen, die erst kürzlich beschrieben wurden, verändert werden (Dong *et al.*, 2000; Leadbetter und Greenberg, 2000). Somit kann die Produktion von universellen Signalmolekülen, bzw. deren Abbau, für die unterschiedlichen Organismen in einem komplexen Habitat von entscheidendem Vorteil im Wettbewerb gegenüber Konkurrenten sein. Für den biologischen Pflanzenschutz könnten sich aus dieser Erkenntnis neue Perspektiven ergeben.

C.6 Mögliche Implikationen der Zell-Zell-Kommunikation in der Rhizosphäre für den biologischen Pflanzenschutz

Entscheidend für den Erfolg einer Maßnahme im biologischen Pflanzenschutz ist zum einen die stabile Besiedelung der Rhizosphäre der Kulturpflanze durch die eingesetzten Organismen (Chin-A-Woeng *et al.*, 2000) und zum anderen ein hohes Maß der gewünschten bakteriellen Aktivität, also z. B. die Synthese von Hemmstoffen gegen die zu bekämpfenden Krankheitserreger. In einer Reihe von potentiellen ‘biocontrol’ Organismen wird die Synthese von Antibiotika in Abhängigkeit von der Zelldichte reguliert, was auf die Beteiligung eines ‘Quorum sensing’ Systems hindeutet. Ein prominentes Beispiel hierfür ist die Produktion von Phenazinantibiotika durch *P. aureofaciens* 30-84, welche das Wachstum von *Gaeumannomyces graminis* var. *tritici*, dem Erreger der ‘take-all disease’ von Weizen hemmen (siehe Einleitung). Da für die Synthese der Phenazine die Anwesenheit einer kritischen Konzentration von Hexanoyl-homoserinlacton erforderlich ist, ist ein Verständnis der Zell-Zell-Kommunikation in der Rhizosphäre für die Optimierung von Schutzmaßnahmen gegen die ‘take-all disease’ unerlässlich, zumal gezeigt werden konnte, dass die Produktion von Phenazinen auch zur ökologischen Fitness des Organismus, d. h. zu seiner Fähigkeit sich im Habitat zu etablieren, beiträgt (Mazzola *et al.*, 1992). Häufig ist gerade die mangelnde Etablierung der ‘biocontrol’ Organismen in der Rhizosphäre limitierend für den Erfolg einer biologischen Pflanzenschutzmaßnahme (Bloemberg und Lugtenberg, 2001). Erst kürzlich wurde ebenfalls an *P. aureofaciens* 30-84 gezeigt, dass bei diesem Vorgang AHL-vermittelte Kommunikationssysteme eine unmittelbare Rolle spielen könnten. Der Stamm verfügt neben PhzRI über ein zweites ‘Quorum sensing’ System, CsaRI, welches zwar nicht in die Regulation der Phenazinbiosynthese involviert ist, dafür aber die Oberflächeneigenschaften der bakteriellen Zelle kontrolliert. Mutanten, bei denen eines oder beide ‘Quorum sensing’

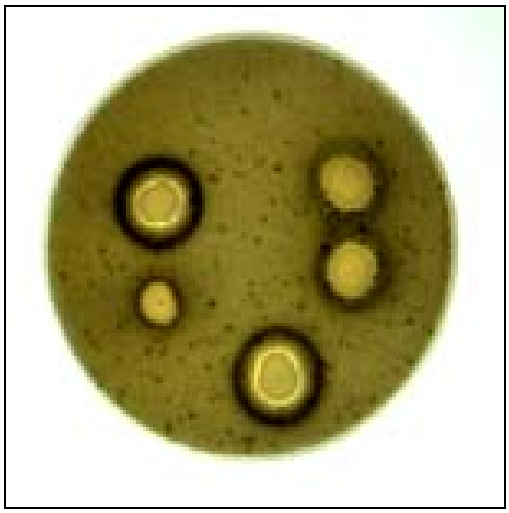
Systeme inaktiviert wurden, zeigten eine signifikant verminderte Fähigkeit zur Kolonisierung der Weizenrhizosphäre (Zhang und Pierson, 2001).

Im Rahmen dieser Arbeit konnte gezeigt werden, dass auch bei *B. cepacia* und *S. liquefaciens*, zwei Bakterienarten, die attraktive Kandidaten für den biologischen Pflanzenschutz darstellen, ein 'Quorum sensing' System an der Kontrolle der 'biocontrol' Aktivität beteiligt ist. Es wurde hierzu die antagonistische Aktivität der AHL-produzierenden Wildtypstämme *B. cepacia* H111 und *S. liquefaciens* MG1 gegen mehrere phytopathogene Pilze mit der Aktivität der entsprechenden AHL-Nullmutanten verglichen. Hierbei zeigten sich deutliche Unterschiede zwischen Wildtyp und Mutanten in der Fähigkeit das Wachstum der getesteten Pilze zu hemmen. Wie in Abb. 4 gezeigt, hemmt *B. cepacia* H111 das Wachstum des Pilzes *Cladosporium herbarium* in einem Inhibitionsassay, was durch die Ausbildung eines Hemmhofes um die Bakterienkolonie und eine lokale Aktivierung der Sporenbildung durch *C. herbarium* dokumentiert wird. Die AHL-Nullmutanten H111-I (*cepI*) und H111-R (*cepR*) sind dagegen nur eingeschränkt in der Lage, das Wachstum des Pilzes zu hemmen (keine Ausbildung von Hemmhöfen, nur geringe Sporulation des Pilzes). Die Komplementation dieses Defekts war im Fall von H111-R durch das für *cepR* kodierende Plasmid pBAH27 *in trans* bzw. durch die Zugabe von AHL zum Medium der *cepI* Mutante (nicht gezeigt) möglich. Es konnte keine Komplementation der antagonistischen Aktivität von H111-I durch das für *cepI* kodierende Plasmid pBBR-$cepI_{H111}$ *in trans* beobachtet werden, obwohl die AHL-Produktion in Stamm H111-I (pBBR-$cepI_{H111}$) wieder hergestellt ist (Gotschlich, 2001). Dieses Phänomen wurde ebenfalls bei der Untersuchung der Protease-Aktivität des Stammes beobachtet und scheint auf einem Kopiezahl-Effekt zu beruhen (Gotschlich, 2001). Durch die Einbringung des Plasmids pBBR-$cepI_{H111}$ in die Zelle wird die Anzahl der möglichen Bindungsstellen (*lux*-Boxen) für CepR drastisch erhöht (das *cepI* Gen wurde inklusive seiner Promotor-Region in pBBR-$cepI_{H111}$ kloniert). Dadurch kommt es vermutlich zu einer kompetitiven Hemmung der Expression von AHL-abhängigen Genen in diesem Stamm. Hierbei ist die Konzentration des CepR Proteins limitierend, da CepR als Komplex mit C8-HSL an die *lux*-Box im *cepI* Promotorbereich bindet und somit nicht mehr für die transkriptionelle Aktivierung anderer Zielgene zur Verfügung steht.

Auch im Inhibitionsassay gegen die Pilze *Alternaria alternata*, *Botrytis cinerea* und *Fusarium oxysporum* konnte bei der AHL-negativen Mutante H111-I im Gegensatz zum Wildtyp keine Hemmung des Pilzwachstums detektiert werden (Daten nicht gezeigt).

H111-R (pBAH27)

H111-I (pBBR-*cepI*$_{H111}$)



H111-R (*cepR::npt*)

H111-I (*cepI::npt*)

H111 (Wildtyp)

Abb. 4. Inhibitionsassay von *B. cepacia* H111 und den AHL-negativen Mutanten H111-I und H111-R gegen den phytopathogenen Pilz *C. herbarium*. Eine Würze-Platte wurde mit Konidien von *C. herbarium* in einem Softagar-Overlay überschichtet. Die Bakterienkulturen wurden punktförmig aufgetropft und bildeten Kolonien mit einem Durchmesser von ca. 10 mm aus. Die Hemmung des Pilzwachstums durch H111 und die komplementierte *cepR* Mutante resultierte in ca. 2 mm breiten Hemmhöfen um die Kolonien und in einer lokalen Aktivierung der Sporenbildung bei *C. herbarium* (dunkle Ringe).

Ganz ähnliche Beobachtungen wurden auch beim Vergleich der beiden Stämme *S. liquefaciens* MG1 (Wildtyp) und MG44 (AHL-negative Mutante) gemacht. Bei einem Inhibitionsassay gegen *A. alternata* zeigte MG1 eine starke Hemmung des Pilzwachstums (5 bis 10 mm Hemmhof um die Bakterienkolonie), wohingegen MG44 keine Aktivität aufwies und teilweise vom Pilzmycel überwachsen wurde (Abb. 5).

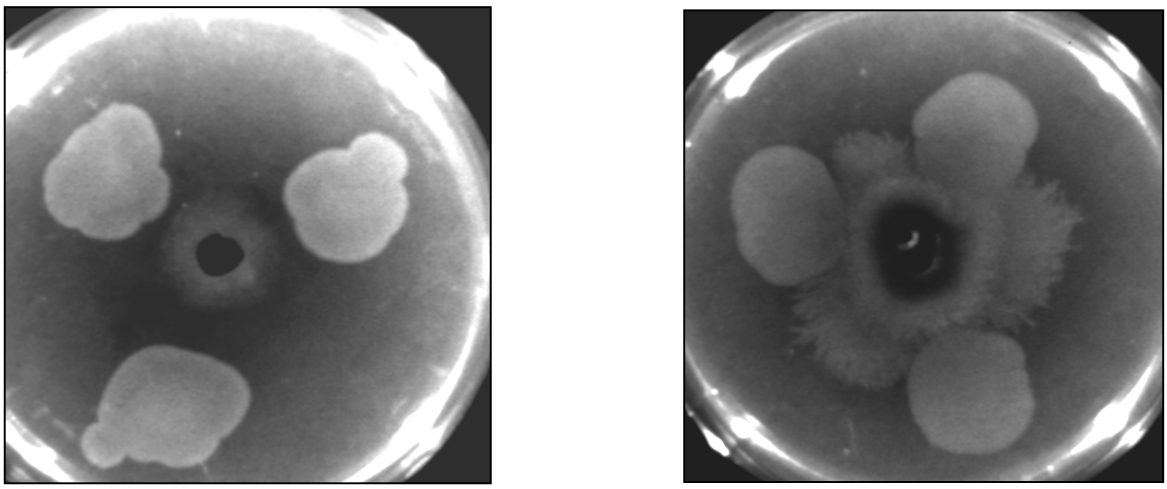


Abb. 5. Inhibitionsassay von *S. liquefaciens* MG1 (links) und der AHL-negativen Mutante MG44 (rechts) gegen den phytopathogenen Pilz *A. alternata*. In der Mitte einer Würze-Platte wurde ein mit Pilzmycel bewachsener Agarblock platziert und in ca. 2 cm Abstand dazu die Bakterienkulturen aufgetropft (je drei Tropfen pro Platte). Das Pilzmycel breitete sich innerhalb weniger Tage radial aus, wurde aber durch die antifungische Aktivität von MG1 am weiteren Wachstum gehindert (Hemmhöfe 5 – 10 mm). Die AHL-negative Mutante MG44 hemmte das Wachstum des Mycels kaum, wurde sogar teilweise vom Pilz überwachsen.

Die molekularen Mechanismen der antagonistischen Aktivität von *S. liquefaciens* MG1 und *B. cepacia* H111 konnten bisher noch nicht aufgeklärt werden. Es wurde jedoch gezeigt, dass die Synthese der extrazellulären Chitinase in beiden Arten durch das 'Quorum sensing' System reguliert ist (K. Riedel, persönl. Mitteilung; Gotschlich, 2001). Die Chitinase-Aktivität trägt sicherlich zur Hemmung des Pilzwachstums bei (Chitin ist ein Bestandteil der Pilzzellwand), ist aber vermutlich nicht allein für die Ausbildung der Hemmhöfe verantwortlich.
B. cepacia H111 produziert neben der Chitinase eine antifungisch wirksame Substanz, die mit den Zellen assoziert ist und nur in geringem Maß ins Medium abgegeben wird. Dafür spricht zum einen die geringere Hemmhofbildung, die in allen Pilz-Assays im Vergleich zu *S. liquefaciens* beobachtet werden konnte. Darüber hinaus war im sterilfiltrierten Kulturüberstand von H111 keine antifungische Aktivität detektierbar, es konnte jedoch ein aktives Extrakt aus ganzen Zellen von H111 gewonnen werden. Die mit Ethylacetat extrahierte Substanz zeigte starke Aktivität gegen alle Pilze, die auch durch lebende Zellen von H111 gehemmt wurden. Im Gegensatz dazu konnte aus Zellen von H111-I kein antifungisches Extrakt mit Ethylacetat gewonnen werden (Daten nicht gezeigt). Eine chemische Charakterisierung der Substanz konnte im Rahmen dieser Arbeit nicht mehr durchgeführt werden.

Neueste Studien berichten, dass AHL nicht nur als Signalmoleküle innerhalb der prokaryontischen Welt von Bedeutung sind. Es konnte gezeigt werden, dass eines der von *P. aeruginosa* produzierten AHL-Moleküle (3-Oxo-C12-HSL) für die Auslösung von Entzündungsreaktionen im Mausmodell verantwortlich ist und die Produktion von Interleukinen und anderen Entzündungsmediatoren anregt (Smith *et al.*, 2002). Interleukine sind ein wichtiger Botenstoff bei der Immunantwort des Menschen. Heute weiß man, dass auch Pflanzen eine Art von Immunantwort besitzen, die durch eine Reihe von Signaltransduktionswegen vermittelt wird. Um zu untersuchen, ob die Anwesenheit von AHL-Produzenten in der Rhizosphäre einen stimulierenden Effekt auf das Abwehrsystem der Pflanze hat, wurden in Zusammenarbeit mit R. Schuhegger (Institut für biochemische Pflanzenpathologie, GSF, Neuherberg) Infektionsversuche an Tomatenpflanzen mit dem Spross-pathogenen Pilz *A. alternata* durchgeführt. Eine Besiedelung der Tomatenrhizosphäre mit dem AHL-produzierenden Stamm *S. liquefaciens* MG1 führte zu einer signifikant verminderten Nekrosenbildung bei nachfolgender Infektion mit *A. alternata*. Durch real-time PCR konnte quantitativ nachgewiesen werden, dass die Blätter dieser Pflanzen durch deutlich

weniger Pilzzellen befallen waren, als die der Kontrollpflanzen ohne *S. liquefaciens*. Eine Inokulation der Pflanzenwurzeln mit der AHL-negativen Mutante MG44 führte nur zu einer geringfügigen Reduktion der Nekrosenbildung, d. h. die 'biocontrol' Aktivität von *S. liquefaciens* ist wenigstens zum Teil von der AHL-Produktion abhängig (R. Schuhegger, persönl. Mitteilung).

In Zusammenarbeit mit G. Berg (Universität Rostock) wurde eine Reihe von Isolaten aus der Rhizosphäre von unterschiedlichen Kulturpflanzen, die antagonistische Aktivität gegen den Pilz *V. dahliae* zeigen, genotypisch und phänotypisch charakterisiert. Aus diesen Arbeiten ging hervor, dass es sich bei einem Großteil der Isolate um Vertreter der Spezies *P. putida* handelt. Wie bereits beschrieben, war etwa die Hälfte der untersuchten Stämme zur AHL-Produktion fähig. Interessanterweise korrelierte die Produktion der Signalmoleküle auffällig mit der Fähigkeit zur Synthese von β-Indolylessigsäure (IES, engl. IAA). Diese von vielen Rhizobakterien produzierte Substanz gehört zur Gruppe der Auxine, das sind das Pflanzenwachstum fördernde Hormone. Aufgrund dieser auffälligen Korrelation stellte sich die Frage, ob in *P. putida* ein 'Quorum sensing' System existiert, welches bakterielle Aktivitäten kontrolliert, die für die Eigenschaften dieser Stämme als Wachstumsförderer oder als biologisches Schädlingsbekämpfungsmittel eine Rolle spielen.

C.7 Identifizierung eines 'Quorum sensing' Systems in *Pseudomonas putida* IsoF

C.7.1 Klonierung und Sequenzierung der in die AHL-Synthese involvierten Gene

Obwohl bereits wiederholt beschrieben wurde, dass Pflanzen-assoziierte *P. putida* Stämme zur AHL-Synthese befähigt sind (Kojic *et al.*, 1999; Elasri *et al.*, 2001; Sauer und Camper, 2001; Anhang 1 und 3), wurden bisher weder die zugrunde liegenden genetischen Determinanten noch ein AHL-regulierter Phänotyp identifiziert. Daher sollten im Rahmen dieser Arbeit die an der AHL-Synthese beteiligten Gene in *P. putida* IsoF charakterisiert und AHL-regulierte Funktionen identifiziert werden. An dieser Stelle sollen zusammenfassend die wichtigsten Fragestellungen und Ergebnisse der Charakterisierung des 'Quorum sensing' Systems in *P. putida* IsoF dargestellt werden. Die Einzelheiten dieser Arbeit sind dem Anhang 2 zu entnehmen.

P. putida IsoF wurde aus der Rhizosphäre einer Tomatenpflanze isoliert und aufgrund biochemischer Charakteristika der *P. putida* Gruppe A zugeordnet. Der Stamm hemmt das Wachstum mehrerer phytopathogener Pilze (*A. alternata, C. herbarium, Pythium ultimum* und

Rhizoctonia solani) und hat die Fähigkeit, das Wachstum von Pflanzen positiv zu beeinflussen. Darüber hinaus produziert er große Mengen an AHL-Molekülen (Konzentrationen bis zu 3 µM). Diese wurden durch Dünnschichtchromatographie als 3-Oxo-C6-, 3-Oxo-C8-, 3-Oxo-C10- und 3-Oxo-C12-HSL identifiziert (Abb. 2, Anhang 1 und Abb. 4, Anhang 2). Die an der AHL-Synthese beteiligten Gene konnten kloniert und sequenziert werden. Es wurden ein *luxI*-homologes AHL-Synthasegen, *ppuI,* und ein *luxR*-homologes Gen, *ppuR*, das für ein AHL-Rezeptorprotein kodiert, identifiziert. Die beiden Genprodukte haben die größte Ähnlichkeit zu LasI und LasR, welche zusammen ein 'Quorum sensing' System in *P. aeruginosa* bilden, das vorwiegend 3-oxo-C12-HSL als Signalmolekül verwendet (Passador *et al.*, 1993; Gambello und Iglewski, 1991). Eine weitere Homologie zum *las* System aus *P. aeruginosa* zeigte sich in der Anwesenheit eines *rsaL*-homologen Gens, welches zwischen *ppuI* und *ppuR* lokalisiert ist (siehe Abb. 6 und Punkt C.7.5). In *P. aeruginosa* befindet sich *rsaL* in der intergenerischen Region zwischen *lasI* und *lasR* und kodiert für einen negativen Regulator des *las* Systems (De Kievit *et al.*, 1999).

Durch Konstruktion der *ppuI* Knock-out Mutante *P. putida* F117 wurde gezeigt, dass PpuI nach derzeitigem Wissen die einzige AHL-Synthase in *P. putida* IsoF ist, da selbst in hochkonzentrierten Extrakten des Kulturüberstandes von F117 keine detektierbaren Mengen an AHL mehr zu finden waren. Dieser Defekt konnte durch die Einbringung eines Plasmid-kodierten *ppuI in trans* vollständig komplementiert werden. Darüber hinaus weist ein *E. coli* Stamm, der *ppuI* heterolog exprimiert, das gleiche AHL-Profil wie *P. putida* IsoF auf (Abb. 4, Anhang 2). Zusammenfassend kann man aus diesen Ergebnissen schließen, dass das Genprodukt von *ppuI* die Synthese von mindestens vier unterschiedlichen AHL-Molekülen aus Substraten katalysiert, die sowohl in *P. putida* als auch in *E. coli* vorhanden sind.

Im Brennpunkt des Interesses stand im folgenden die Identifizierung von Genen, deren Transkription durch das *ppu* System reguliert wird. Im Promotorbereich von 'Quorum sensing'-regulierten Genen befindet sich in der Regel eine palindromische 20 bp-Signatursequenz, die sog. *lux*-Box, die spezifisch von Proteinen der LuxR Familie erkannt wird. Die Bindung des Regulator/AHL-Komplexes an die *lux*-Box aktiviert in den meisten Fällen die Transkription des Gens bzw. Operons. In den flankierenden Bereichen von *ppuR* konnten zwei potentielle *lux*-Boxen identifiziert werden. Die beiden Sequenzmotive, die eine sehr hohe Ähnlichkeit zu *lux*-Boxen aus *P. aeruginosa* aufweisen (Whiteley und Greenberg, 2001), befinden sich im Promotorbereich von *ppuI* und strangaufwärts von *ppuR* vor einem offenen Leserahmen, der als *ppuA* bezeichnet wurde (siehe Abb. 2, Anhang 2). Dieses Resultat legte die Vermutung nahe, dass sowohl *ppuI* als auch *ppuA* einer Zelldichte-

abhängigen Genregulation unterliegen könnten. Diese Hypothese wurde nachfolgend genauer untersucht, wobei vor allem Methoden der Reportergentechnologie zum Einsatz kamen.

C.7.2 Die Expression der AHL-Synthase *ppuI* unterliegt einer positiven Rückkopplung

Um die Regulation der Expression der AHL-Synthase *ppuI* in *P. putida* IsoF zu untersuchen, wurden zwei verschiedene experimentelle Ansätze gewählt. In einem ersten Schritt wurde die Menge der gebildeten AHL-Moleküle in einer Kultur von IsoF über den Verlauf der Wachstumskurve bestimmt. Hierbei konnte ein ca. 20-facher Anstieg der AHL-Konzentration beim Übergang von der logarithmischen zur stationären Wachstumsphase der Kultur beobachtet werden, was auf eine Zelldichte-abhängige Regulation der AHL-Synthese hindeutet (Abb. 5A, Anhang 2). Um die Wachstumsphasen-abhängige Transkription von *ppuI* in *P. putida* IsoF verfolgen zu können, wurde die durch PCR amplifizierte *ppuI* Promotorregion in den Promotor-Testvektor pGA-L14 ligiert. Die Orientierung des Inserts wurde so gewählt, dass die Transkription des Reportergens *luxAB* durch den *ppuI* Promotor kontrolliert wird. Das daraus resultierende Plasmid pAS-L22 wurde in *P. putida* IsoF eingebracht und die Biolumineszenz des rekombinanten Stammes über die Wachstumskurve verfolgt. In Übereinstimmung mit den AHL-Messungen stieg die Biolumineszenz der Zellen im Laufe der spät-exponentiellen Wachstumsphase signifikant (ca. 10-fach) an, was die Vermutung bestätigt, dass die Expression von *ppuI* in Abhängigkeit von der Zelldichte reguliert wird (Abb. 5B, Anhang 2). Um die Rolle von PpuR bei der Kontrolle der Transkription von *ppuI* zu untersuchen, wurde ein rekombinanter *E. coli* Stamm erzeugt, der neben dem Promotor-Testvektor pAS-L22 noch das Plasmid pPPUR1 trägt, welches *ppuR* unter der Kontrolle des *lac* Promotors enthält. Nach Zugabe von 3-Oxo-C12-HSL zu einer Kultur des Stammes *E. coli* (pAS-L22, pPPUR1) stieg die Lichtemission der Zellen um das Zehnfache an. Dieses Experiment veranschaulicht, dass PpuR in Verbindung mit passenden AHL-Molekülen ausreicht, um die Expression von *ppuI* zu aktivieren. Dies wird auch durch die Tatsache untermauert, dass die *ppuR* Knock-out Mutante *P. putida* IsoF-R keine detektierbaren Mengen an AHL mehr produziert (Daten nicht gezeigt).

Die Autoregulation einer AHL-Synthase wurde erstmals an *luxI* gezeigt und ist Bestandteil des ‘Quorum sensing’ Paradigmas (Fuqua *et al.*, 1994; Salmond *et al.*, 1995). Sobald ein basaler Schwellenwert an Signalmolekülen die Bindung des Regulator/AHL-Komplexes an die *lux*-Box des AHL-Synthasegens ermöglicht, steigt durch den positiven

Rückkopplungseffekt die AHL-Konzentration in einer Kultur sprunghaft an, was eine extreme Verstärkung der Expression aller anderen Zelldichte-abhängigen Gene zur Folge hat.

C.7.3 Identifizierung von *ppuA* als Mitglied des *ppu* 'Quorum sensing' Regulons

Bei der Sequenzanalyse der flankierenden DNA-Region von *ppuR* wurde strangaufwärts ein offener Leserahmen identifiziert, der *ppuA* genannt wurde und der für ein Protein mit signifikanter Ähnlichkeit zu Fettsäure-CoA-Ligasen aus unterschiedlichen Organismen kodiert.

Das Vorhandensein einer *lux*-Box 31 bp strangaufwärts des *ppuA* Startcodons deutet darauf hin, dass die Transkription von *ppuA* ebenfalls unter Kontrolle von PpuR stehen könnte. Um diese Hypothese zu überprüfen, wurde eine transkriptionelle Fusion von *ppuA* mit einer promotorlosen *luxAB* Kassette auf dem Chromosom der *ppuI* Mutante F117 erzeugt (siehe Abb. 3, Anhang 2). Da der daraus resultierende Stamm *P. putida* F117-D1 aufgrund des Defekts in der AHL-Synthase keine Signalmoleküle synthetisiert, sollte die Transkription von AHL-regulierten Genen unterbunden sein. In der Tat konnte über die gesamte Wachstumsphase hinweg nur eine sehr geringe Lichtemission mit diesem Stamm gemessen werden, was auf eine sehr schwache Aktivität des *ppuA* Promotors in Abwesenheit von Signalmolekülen hindeutet. Wurde jedoch zu einer Kultur von F117-D1 Homoserinlacton zugegeben (1 µM 3-Oxo-C10-HSL), so stieg die Biolumineszenz der Zellen innerhalb von 90 Minuten um das 56-fache an (Abb. 6, Anhang 2). Dieses Ergebnis zeigt, dass die Aktivität des *ppuA* Promotors AHL-abhängig ist, und die identifizierte *lux*-Box vermutlich als Bindungsstelle für PpuR fungieren kann. Darüber hinaus wurde untersucht, welche AHL-Moleküle eine Aktivierung des *ppuA* Promotors auslösen. Es konnte gezeigt werden, dass die stärkste Induktion der *ppuA::luxAB* Expression durch die Zugabe von 3-Oxo-C10- oder 3-Oxo-C12-HSL erzielt wurde. Dieses Resultat verwundert nicht, da diese beiden Moleküle die Hauptprodukte der PpuI AHL-Synthase darstellen. Die minimale Konzentration dieser beiden Signalstoffe, die zur messbaren Aktivierung der Biolumineszenz ausreichte, lag unter 10 nM. Ein Anstieg der Lichtemission wurde auch nach der Zugabe von 1 µM 3-Oxo-C14-HSL gemessen, wohingegen die Zugabe von 3-Oxo-C6- oder 3-Oxo-C8-HSL keinen Effekt hatte. AHL-Moleküle ohne 3-Oxo-Gruppe waren ebenfalls nicht in der Lage, die Expression von *ppuA* zu stimulieren (Abb. 7, Anhang 2).

Seit einigen Jahren ist bekannt, dass 'Quorum sensing'-regulierte Prozesse, wie zum Beispiel die Produktion von Virulenzfaktoren bei *E. carotovora,* die Schwärmermotilität von *S.*

liquefaciens oder die Ausbildung von Biofilmen von *P. aeruginosa* durch natürliche Inhibitoren, welche von der marinen Rotalge *Delisea pulchra* produziert werden, gehemmt werden können (Givskov *et al.*, 1996; Rasmussen *et al.*, 2000; Manefield *et al.*, 2001; Hentzer *et al.*, 2002). Es handelt sich bei diesen Substanzen um halogenierte Furanone, welche vermutlich die Stabilität des R-Proteins beeinträchtigen und so spezifisch in die AHL-abhängige Genregulation eingreifen (Manefield *et al.*, 2002). Als weiterer Beweis für die Regulation der Transkription von *ppuA* durch PpuR kann daher die Tatsache angesehen werden, dass die Zugabe von halogenierten Furanonen die Aktivierung der *ppuA::luxAB* Expression durch 3-Oxo-C12-HSL um 50 % verminderte (Daten nicht gezeigt).

C.7.4 Das 'Quorum sensing' System von *P. putida* IsoF kontrolliert die Reifung von Biofilmen

In ihrer natürlichen Umgebung leben die meisten Bakterien als sessile, an eine Oberfläche angeheftete Zellen, die von einer extrazellulären polymeren Matrix (EPS) umgeben sind. Diese Form der Lebensweise wird auch Biofilm genannt und hat für die Bakterien entscheidende Vorteile (Davey und O'Toole, 2000; Watnick und Kolter, 2000). Sie leben in einer vielzelligen, strukturierten Gemeinschaft, die optimale Bedingungen für eine Vielzahl von Interaktionen bietet (Austausch von Signalmolekülen, DNA-Transfer, Transfer von Metaboliten) und die es ihnen ermöglicht, auch widrige Umweltbedingungen zu überstehen. Besonders kennzeichnend für Biofilme ist ihre erhöhte Resistenz gegenüber Antibiotika und Desinfektionsmitteln, was im medizinischen und industriellen Bereich sehr häufig zu Problemen führt (Costerton *et al.*, 1999; Stewart und Costerton, 2001). Durch die enge Assoziation der einzelnen Zellen, die meist Mikrokolonien innerhalb des Biofilms bilden, entstehen optimale Bedingungen für eine Zell-Zell-Kommunikation, die den Bakterien eine Koordination ihrer Aktivitäten ermöglicht. So ist es nicht verwunderlich, dass 'Quorum sensing' Systeme auch in die Ausbildung und Reifung der Biofilme involviert sind. Es wurde bereits gezeigt, dass AHL-regulierte Prozesse an der Biofilmbildung von *Aeromonas hydrophila*, *B. cepacia* und *P. aeruginosa* (Lynch *et al.*, 2002; Huber *et al.*, 2001; Davies *et al.*, 1998) beteiligt sind, und sogar ein AHL-vermittelter 'cross-talk' zwischen *B. cepacia* und *P. aeruginosa* in einem gemischten Biofilm stattfindet (Anhang 4).

In Anbetracht dieser Erkenntnisse sollte untersucht werden, ob die Biofilmbildung von *P. putida* IsoF durch das PpuR/PpuI 'Quorum sensing' System gesteuert wird. Die Gfp-markierten Zellen wurden dazu in künstlichen Durchflusskammern angezogen, welche direkt

mit einem CLSM mikroskopiert werden können, was eine Rekonstruktion der dreidimensionalen Strukturen der Biofilme erlaubt. Es wurde beobachtet, dass sich die Biofilme von *P. putida* IsoF und der AHL-negativen Mutante F117 während der ersten zwei Tage nach der Inokulation der Zellen weitgehend gleichen, danach aber signifikante Unterschiede in der dreidimensionalen Struktur auftreten. Während sich die Wildtyp-Zellen zu einem sehr homogenen, unstrukturierten Biofilm differenzieren, der das Substrat vollständig bedeckt, bilden die Zellen von F117 sehr charakteristische Mikrokolonien, die einen Durchmesser von bis zu 40 µm erreichen, jedoch nie das gesamte Substrat bedecken (Abb. 8, Anhang 2). Die Zugabe von AHL zum Medium von F117 resultiert in Biofilmen, deren Struktur der des Wildtyp-Biofilms gleicht. Aus diesen Ergebnissen kann man schließen, dass nur die Reifung des *P. putida* Biofilms Zelldichte-abhängig ist, nicht aber die Anheftung der Zellen an die Glasoberfläche. Diese Hypothese erscheint sehr einleuchtend, wenn man bedenkt, dass die anfängliche Besiedelung des Substrats meist durch planktonische Einzelzellen erfolgt, die vermutlich zunächst nur eine geringe Populationsdichte aufweisen und erst nach Bildung von Mikrokolonien ausreichende Mengen an Signalmolekülen für die Zell-Zell-Kommunikation produzieren können. Unsere Ergebnisse ergänzen sehr gut die Studie von Sauer und Camper (2001), die anhand von Proteomanalysen zeigte, dass die frühen Stadien der Biofilmbildung bei *P. putida* nicht durch AHL-abhängige Prozesse gesteuert werden.

Da *ppuA* bislang das einzige AHL-regulierte Gen mit unbekannter Funktion in IsoF ist und die AHL-negative Mutante F117 auch keine weiteren offensichtlichen phänotypischen Unterschiede zum Wildtyp aufweist (Daten nicht gezeigt), wurde angenommen, dass *ppuA* in die Reifung der Biofilme von *P. putida* involviert sein könnte. Eine mikroskopische Untersuchung von Biofilmen der *ppuA* Mutante IsoF-D4 zeigte, dass dieser Stamm Biofilme bildet, die ähnlich wie bei F117 charakteristische Mikrokolonien aufweisen. Im Gegensatz zu F117 zeigte jedoch die Zugabe von AHL zum Medium von IsoF-D4 keinen Effekt, weshalb eine direkte Beteiligung von PpuA an der Biofilmbildung anzunehmen ist. Die Unfähigkeit der *ppuI* Mutante Wildtyp-Biofilme zu bilden, könnte also auf den Defekt in der Expression von *ppuA* zurückzuführen sein. Die molekulare Funktion von PpuA in IsoF konnte im Rahmen dieser Arbeit nicht aufgeklärt werden. Es wäre jedoch aufgrund der Ähnlichkeit von PpuA zu Fettsäure-CoA-Ligasen denkbar, dass das Protein eine Rolle bei der Synthese von Zelloberflächenbestandteilen spielt und somit Einfluss auf die adhäsiven Eigenschaften der Zellen ausübt. Häufig sind Fettsäure-CoA-Ligasen an der Aufnahme von langkettigen Fettsäuren aus dem Medium verantwortlich, die teilweise direkt in die Zellmembran

eingebaut werden können. Erst kürzlich wurde ein zweites ‘Quorum sensing’ System in *P. aureofaciens* identifiziert, das aus den LuxI/R-Homologen CsaI/R besteht und die Biosynthese von Komponenten der bakteriellen Zelloberfläche kontrolliert. Es wurde festgestellt, dass die abweichende Koloniemorphologie der *csaR* Mutante auf eine Änderung der Fettsäurezusammensetzung der Zellmembran zurückzuführen ist (Zhang und Pierson, 2001). Die Analyse der Fettsäurezusammensetzung der Zellmembran von IsoF und F117 ergab jedoch keine auffälligen Unterschiede (Daten nicht gezeigt).

Ein Abgleich der *ppuA* Sequenz mit der BLASTX Datenbank ergab, dass das Genprodukt die höchste Ähnlichkeit zu AttG aus *Rhodococcus fascians* aufweist. Die AttG Fettsäure-CoA-Ligase ist an der Synthese eines autoregulatorischen Signalstoffes beteiligt, der essentiell für die Expression von Virulenzfaktoren des Organismus bei der Infektion von Tabakpflanzen ist (Maes *et al.*, 2001). Interessanterweise weist das *att* Regulationssystem in *R. fascians* Ähnlichkeiten zu ‘Quorum sensing’ Systemen auf, da seine Expression ebenfalls Zelldichte-abhängig ist und einer positiven Rückkopplung durch die gebildeten Signalmoleküle unterliegt. Es besteht daher auch die Möglichkeit, dass *ppuA* nicht direkt, sondern indirekt an der Biofilmdifferenzierung beteiligt ist und beispielsweise in die Synthese weiterer, bisher nicht identifizierter Signalstoffe involviert ist. Darüber hinaus kann nicht ausgeschlossen werden, dass noch andere, bisher unbekannte Faktoren an der Zelldichte-abhängigen Differenzierung der *P. putida* IsoF Biofilme beteiligt sind. Proteomanalysen des *P. putida* Stammes ATCC 39168 haben gezeigt, dass die Expression von 16 Proteinen durch die Zugabe von AHL-Molekülen zum Medium der Zellen beeinflusst wurde. Dies deutet darauf hin, dass das ‘Quorum sensing’ System als globaler Regulator in *P. putida* fungiert (Sauer und Camper, 2001).

Zusammenfasssend soll folgendes Modell des *ppu* ‘Quorum sensing’ Systems in *P. putida* IsoF den bisherigen Wissensstand veranschaulichen (Abb. 6).

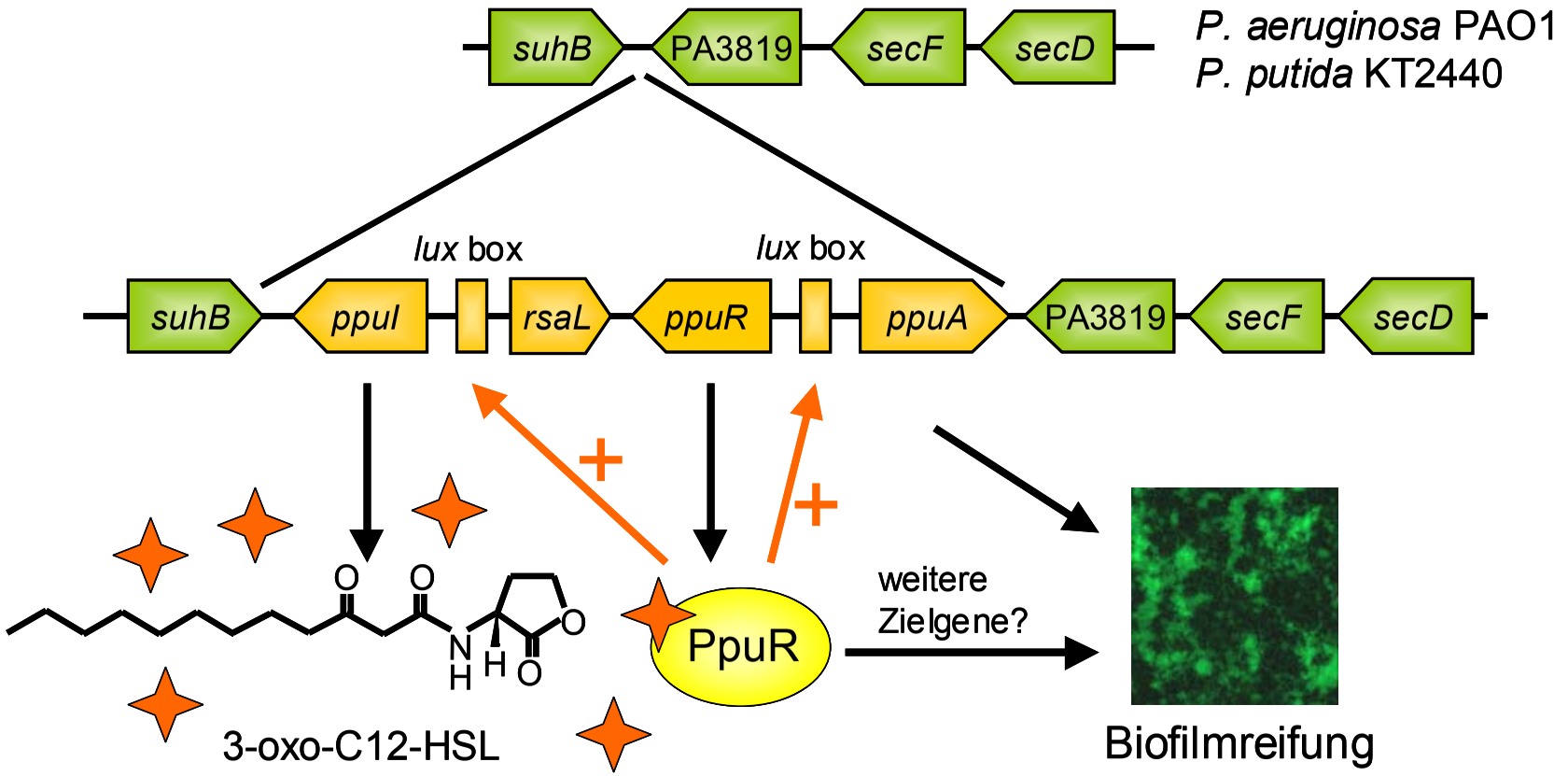


Abb. 6. Modell des PpuI/PpuR ‚Quorum sensing' Systems in *P. putida* IsoF. Es besteht aus dem Transkriptionsregulator PpuR und der AHL Synthase PpuI, welche die Synthese von vorwiegend 3-Oxo-C10- und 3-Oxo-C12-HSL katalysiert. Aufgrund der Anwesenheit einer *lux*-Box in ihrer Promotorregion wurden *ppuI* und *ppuA* als Zielgene der Zelldichte-abhängigen Genregulation identifiziert. Die Differenzierung von Biofilmen wird durch das *ppu* ‚Quorum sensing' System reguliert. Eine Beteiligung von PpuA an diesem Vorgang konnte gezeigt werden. Das *ppu* Gencluster wird von den Genen *suhB* und PA3819-*secD*-*secF* flankiert, die ein hohes Maß an Homologie zu chromosomalen Bereichen in *P. aeruginosa* PAO1 und *P. putida* KT2440 aufweisen.

C.7.5 Untersuchung der flankierenden Regionen des *ppu* Genclusters – Hinweise auf einen horizontalen Gentransfer?

Ein Vergleich der Sequenz des *ppuI-rsaL-ppuR-ppuA* Genclusters mit der Genomsequenz von *P. putida* KT2440 (www.tigr.org) und *P. aeruginosa* PAO1 (www.pseudomonas.com) ergab, dass keines der vier Gene in *P. putida* KT2440 existiert, der Bereich von *ppuI-rsaL-ppuR* allerdings in hohem Maße homolog zu *lasI-rsaL-lasR* aus *P. aeruginosa* PAO1 ist. Eine homologe Sequenz zu *ppuA* konnte nicht gefunden werden. Auffallend bei der Analyse der flankierenden Regionen des *ppu* Genclusters war das hohe Maß an Identität dieser Sequenzen zu hochkonservierten Bereichen des *P. aeruginosa* PAO1 und *P. putida* KT2440 Genoms (Abb. 6, *suhB*-PA3819-*secD*-*secF*). Aufgrund dieser Beobachtung wird vermutet, dass das *ppu* Gencluster eine „Insel“ in einer hochkonservierten chromosomalen Region bildet und möglicherweise durch horizontalen Gentransfer erworben wurde. Diese Vermutung wird durch das Vorhandensein mehrerer repetitiver Sequenzen (‘inverted repeats’ und ‘direct repeats’) in der intergenerischen Region von *ppuA* und PA3819 gestützt. Solche Sequenzmotive können auf DNA-Rekombinationsereignisse hindeuten. Es konnten jedoch keine Homologien zu Transposasen oder anderen IS-Elementen (‘insertion sequence’) gefunden werden.

Eine Southern Blot Analyse chromosomaler DNA anderer AHL-produzierender *P. putida* Stämme ergab keine positiven Hybridisierungssignale mit *ppuI* bzw. *ppuR* als Sonde, was darauf hindeutet, dass diese Stämme keine *ppuI* oder *ppuR* homologen Gene tragen. Mit Hilfe eines PCR-Assays konnte darüber hinaus bei keinem der untersuchten Stämme eine Insertion zwischen *suhB* und PA3819 nachgewiesen werden. Obwohl die Produktion von AHL-Molekülen innerhalb der *P. putida* Gruppe sehr weit verbreitet ist, scheint das *ppu* ‘Quorum sensing’ System von *P. putida* IsoF bisher einzigartig zu sein.

C.7.6 Untersuchung der Rolle des ‘Quorum sensing’ Systems hinsichtlich der ökologischen Fitness von *P. putida* IsoF

Im Hinblick auf die Bedeutung der AHL-vermittelten Kommunikation für die mikrobielle Ökologie der Rhizosphäre sollen abschließend noch drei Eigenschaften beleuchtet werden, die für die Aktivität von *P. putida* in der Rhizosphäre wichtig sind. Es wurde die Rolle der Zelldichte-abhängigen Genregulation in *P. putida* IsoF bei der Kolonisierung von

Tomatenwurzeln, der antagonistischen Aktivität gegenüber phytopathogenen Pilzen und der Förderung des Pflanzenwachstums untersucht.

Ein quantitativer Vergleich der Kolonisierung der Tomatenrhizosphäre durch *P. putida* IsoF und die AHL-negative Mutante F117 ergab keine signifikanten Unterschiede in der Fähigkeit der beiden Stämme die Tomatenwurzel zu besiedeln (C. Dürr, 2001). Im Gegensatz zu *P. aureofaciens* (Zhang und Pierson, 2001) scheint also 'Quorum sensing' in *P. putida* IsoF für die erfolgreiche Kolonisierung der Rhizosphäre nicht ausschlaggebend zu sein.

Inhibitionsassays gegen einige phytopathogene Pilze zeigten, dass *P. putida* IsoF antagonistische Aktivität gegen *A. alternata, C. herbarium, P. ultimum* und *R. solani* aufweist. Die Fähigkeit das Wachstum dieser Pilze zu hemmen war bei der *ppuI* Mutante F117 in gleichem Maße zu beobachten wie beim Wildtyp (Daten nicht gezeigt). Die Synthese der nicht näher charakterisierten antifungischen Substanz(en) ist daher vermutlich nicht durch das *ppu* 'Quorum sensing' System reguliert. Im Gegensatz dazu ist die Synthese von Antibiotika oder Fungiziden in vielen anderen *Pseudomonas* Arten Zelldichte-abhängig. So spielen 'Quorum sensing' Systeme bei der Synthese von Phenazinen in *P. aureofaciens* 30-84 (Pierson *et al.*, 1994; Wood und Pierson, 1996) und *Pseudomonas chlororaphis* PCL1391 (Chin-A-Woeng *et al.*, 2001), der Bildung des Polyketid-Antibiotikums Mupirocin in *P. fluorescens* NCIMB 10586 (El-Sayed *et al.*, 2001) und wahrscheinlich bei der Produktion des Fungizids 2,4-Diacetylphloroglucinol in *P. fluorescens* F113 (Laue *et al.*, 2000) eine Rolle.

Zur Untersuchung der Phytostimulation wurden axenische Erdbeerkeimlinge mit *P. putida* IsoF bzw. F117 inokuliert und nach fünf Wochen die Größe der Pflanze, Anzahl der Primärblätter, Wurzellänge, Anzahl der Seitenwurzeln und Entwicklungszustand der Wurzelhaare beurteilt. Die Inokulation mit *P. putida* hatte nur einen Effekt auf den Entwicklungszustand der Seitenwurzeln. Im Vergleich zur Kontrolle ohne Bakterien war die Anzahl der Seitenwurzeln ca. 4-fach erhöht, wenn die Keimlinge mit *P. putida* IsoF inokuliert worden waren. Eine Inokulation der Keimlinge mit *P. putida* F117 resultierte in einem etwas geringeren (3-fachen) Anstieg der Seitenwurzelzahl (G. Berg, persönl. Mitteilung). Dieses Ergebnis deutet an, dass *P. putida* einen positiven Effekt auf das Wachstum von Pflanzen ausübt und diese Fähigkeit zu einem gewissen Maß mit der Homoserinlacton-Produktion korreliert. Diese Vermutung wird durch die Beobachtung gestützt, dass fast alle im Rahmen dieser Arbeit untersuchten AHL-produzierenden *P. putida* A Isolate auch zur Synthese des Auxins β-Indolylessigsäure fähig waren, die AHL-negativen Stämme mit einer Ausnahme dagegen keine IES-Synthese zeigten (Anhang 3). Da die Phytostimulation von IsoF bisher nur an einer Wirtspflanze und über einen relativ kurzen Zeitraum untersucht worden ist, sollte der

Einfluss von AHL auf diese Fähigkeit jedoch noch durch weitere Experimente aufgeklärt werden.

C.8 Ausblick

Im Rahmen dieser Arbeit konnte gezeigt werden, dass eine AHL-vermittelte Kommunikation zwischen Bakterien in der Tomatenrhizosphäre stattfindet. Für das Verständnis der bakteriellen Interaktionen in der Rhizosphäre ist es daher in Zukunft von großer Bedeutung die Faktoren zu untersuchen, die den „Pool" an Signalmolekülen im Habitat beeinflussen. Erst kürzlich wurden zwei Bakterienarten beschrieben, die zum Abbau von AHL-Molekülen fähig sind (Dong *et al.*, 2000; Leadbetter und Greenberg, 2000). Ein möglicher Einsatz solcher AHL-Abbauer bzw. deren Enzyme gegen pflanzenpathogene Organismen, welche die Expression ihrer Virulenzfaktoren durch 'Quorum sensing' regulieren, scheint nun möglich. Dong *et al.* (2001) zeigten, dass transgene Pflanzen, welche die AHL-Lactonase AiiA exprimieren, tatsächlich gegen eine Infektion mit *E. carotovora* resistent waren. Der Vorteil einer solchen Maßnahme ist die gezielte Hemmung der Virulenz ohne dabei das Wachstum der Erreger zu beeinträchtigen, so dass keine Resistenzbildung wie beim Einsatz von Antibiotika eintreten kann. Ein anderer Lösungsansatz ist die Erzeugung transgener Pflanzen, die AHL-Moleküle synthetisieren (Fray *et al.*, 1999). Durch die Einbringung der YenI AHL-Synthase aus *Y. enterocolitica* in die Chloroplasten einer transgenen Tabakpflanze konnte eine AHL-vermittelte Kommunikation der Pflanze mit Bakterien (*E. carotovora* und *P. aureofaciens*) ermöglicht werden. Eine weiterführende Arbeit zeigte, dass transgene Tabakpflanzen, welche die AHL-Synthase ExpI aus *E. carotovora* exprimieren, eine erhöhte Resistenz gegen den Befall durch diesen Erreger aufweisen (Mae *et al.*, 2001). Wird in *E. carotovora* die Synthese der Virulenzfaktoren bereits bei niedriger Zelldichte durch pflanzliche AHL aktiviert, so können die Krankheitserreger die Pflanze zwar anfänglich befallen, machen sich dadurch aber frühzeitig bemerkbar. Die Pflanze kann daraufhin rechtzeitig eine lokale und systemische Abwehrreaktion aktivieren und sich so vor einer weiteren Schädigung schützen.

An dieser Stelle soll nochmals die Möglichkeit einer AHL-vermittelten Kommunikation von Bakterien mit Pflanzen diskutiert werden. Es konnte bereits gezeigt werden, dass die Besiedelung der Rhizosphäre durch AHL-produzierende Bakterien einen positiven Effekt auf die Resistenz der Pflanze gegenüber Pathogenbefall haben kann (siehe Punkt C.6). Dieser Effekt war durch einen Defekt in der AHL-Synthese des Bakteriums drastisch reduziert. Es ist

bekannt, dass nicht-pathogene Pseudomonaden eine sog. induzierte systemische Resistenz (ISR) in der Pflanze auslösen, was dazu führt, dass das „Immunsystem“ der Pflanze aktiviert wird (Van Loon *et al.*, 1998). In Zusammenarbeit mit R. Schuhegger (Institut für biochemische Pflanzenpathologie, GSF, Neuherberg) wurde untersucht, ob die Induktion der pflanzlichen Immunantwort durch AHL-Moleküle ausgelöst werden kann. Erste Ergebnisse zeigen, dass die Anwesenheit von Homoserinlactonen in der Rhizosphäre die Konzentration von Botenstoffen, die an der Induktion der systemischen Resistenz beteiligt sind, lokal deutlich ansteigen lässt und auch die Expression von pflanzlichen Abwehrgenen aktiviert wird. Im Mittelpunkt des Interesses stehen nun weiterhin die molekularen Mechanismen der Signaltransduktion zwischen Bakterium und Pflanze und die Effekte dieser Interaktion auf das pflanzliche Abwehrsystem.

D. ZUSAMMENFASSUNG

Viele Gram-negative Bakterien produzieren *N*-Acyl-L-homoserinlactone (AHL), die als Signalstoffe in die Umgebung ausgeschieden werden und den Organismen eine Koordination bestimmter Aktivitäten in Abhängigkeit von ihrer Populationsdichte ermöglichen. Die Erforschung dieser Zell-Zell-Kommunikationssysteme, die auch als 'Quorum sensing' Systeme bezeichnet werden, erfolgte bisher hauptsächlich an Reinkulturen unter Laborbedingungen, weshalb wenig über die tatsächliche Bedeutung der AHL-vermittelten Kommunikation in natürlichen Habitaten bekannt ist.

Um die Bedeutung dieser Form von Zell-Zell-Kommunikation für die Dynamik und Ökologie der Rhizosphäre von Tomatenpflanzen zu untersuchen, wurden neue AHL-Monitorstämme auf Gfp-Basis konstruiert, die eine Detektion der Signalmoleküle *in situ* und auf Einzelzellebene erlauben. Zu diesem Zweck wurden drei AHL-Sensorplasmide, die aufgrund ihrer unterschiedlichen Spezifitäten die Erfassung eines breiten Spektrums von AHL-Molekülen erlauben, in AHL-negative Mutanten von *P. putida* und *S. liquefaciens* überführt. Diese Biosensoren wurden dann zur Visualisierung der Zell-Zell-Kommunikation zwischen definierten bakteriellen Populationen in der Rhizosphäre von gnotobiotisch gezogenen Tomatenpflanzen verwendet. Außerdem wurde ein Biosensor konstruiert, der die AHL-Monitorkassette stabil ins Chromosom integriert hat und deshalb besonders geeignet für den Nachweis von AHL-Molekülen im natürlichen Habitat ist, wo der erhöhte Konkurrenzdruck auf die Zellen zu einem Verlust der Plasmid-kodierten Sensoren führen könnte. Dieser Monitorstamm wurde erfolgreich zur Detektion von AHL-Signalmolekülen eingesetzt, die von der natürlichen Mikroflora einer Tomatenwurzel in unsterilem Boden produziert wurden. Diese Ergebnisse führten zu dem Schluss, dass AHL als universelle Signalmoleküle zwischen unterschiedlichen Populationen in der Rhizosphäre dienen können und somit komplexe Interaktionen ermöglichen, die sich auf eine Reihe von bakteriellen Aktivitäten in diesem Habitat auswirken können.

Diese Annahme wurde durch eine Evaluierung der Häufigkeit und Diversität von AHL-Produzenten in der Rhizosphäre untermauert. Es wurde gezeigt, dass eine Reihe von Bakterienarten, die aus der Rhizosphäre unterschiedlicher Kulturpflanzen und aus dem Boden isoliert wurden, zur AHL-Produktion befähigt sind und darüber hinaus häufig ähnliche AHL-Profile aufweisen.

Um die Rolle von AHL-abhängiger Genregulation bei der Kolonisierung der Wurzel zu untersuchen, wurde *P. putida* als Modellorganismus ausgewählt. Vertreter dieser Spezies

lassen sich häufig aus der Rhizosphäre von Kulturpflanzen isolieren und kommen für den Einsatz in der biologischen Schädlingsbekämpfung, der Phytostimulation oder der biologischen Bodensanierung in Frage, wo eine effiziente Etablierung der eingesetzten Bakterien von großer Bedeutung für den Erfolg der Maßnahme ist.

Erstmals wurden im Rahmen dieser Arbeit die Gene charakterisiert, die in die Synthese von AHL-Molekülen in *P. putida* involviert sind. Es wurden in *P. putida* IsoF die Gene für einen Transkriptionsregulator der *luxR* Familie (*ppuR*) und eine AHL-Synthase (*ppuI*) identifiziert, die zusammen ein 'Quorum sensing' System bilden. Es konnte gezeigt werden, dass die Transkription von *ppuI* und einem weiteren Gen (*ppuA*), das für eine potentielle Fettsäure-CoA-Ligase kodiert, in Abhängigkeit von der Zelldichte reguliert wird. Darüber hinaus wird die Reifung von Biofilmen auf einer abiotischen Oberfläche in *P. putida* IsoF durch das 'Quorum sensing' System kontrolliert, nicht aber die Kolonisierung der Tomatenwurzel. Im Gegensatz zu anderen *Pseudomonas* spp. ist die antagonistische Aktivität gegen phytopathogene Pilze in *P. putida* IsoF nicht AHL-abhängig.

E. LITERATURVERZEICHNIS

1. **Albrecht, V.** 2002. Charakterisierung von *N*-Acyl-L-homoserinlacton-produzierenden Rhizosphärenbakterien verschiedener Pflanzen. Diplomarbeit am Lehrstuhl für Mikrobiologie, Technische Universität München.
2. **Andersen, J. B., C. Sternberg, L. K. Poulsen, S. P. Bjørn, M. Givskov, and S. Molin.** 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol. **64:**2240-2246.
3. **Andersen, J. B., A. Heydorn, M. Hentzer, L. Eberl, O. Geisenberger, B. B. Christensen, S. Molin, and M. Givskov.** 2001. *gfp*-based *N*-acyl-homoserine lactone sensor systems for detection of bacterial communication. Appl. Environ. Microbiol. **67:**575-585.
4. **Bainton, N. J., P. Stead, S. R. Chhabra, B. W. Bycroft, G. P. Salmond, and P. Williams.** 1992. *N*-(3-oxo-hexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochem. J. **288:**997-1004.
5. **Bao, Y., D. P Lies, H. Fu, and G.P. Roberts.** 1991. An improved Tn*7*-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene **109**:167-168.
6. **Bassler, B. L., E. P. Greenberg, and A. M. Stevens.** 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J. Bacteriol. **179:**4043-4045.
7. **Bloemberg, G. V., and B. J. J. Lugtenberg.** 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. **4**:343-350.
8. **Brelles-Mariño, G., and E. J. Bedmar.** 2001. Detection, purification and characterization of quorum-sensing signal molecules in plant-associated bacteria. J. Biotechnol. **91:**197-209.
9. **Cha, C., P. Gao, Y. Chen, P. D. Shaw, and S. K. Farrand.** 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact. **11:**1119-1129.
10. **Chalfie, M., Y. Tu, G. Euskirchen, W. Ward, and D. C. Prasher.** 1994. Green fluorescent protein as a marker for gene expression. Science **263:**802-805.
11. **Chen, X., S. Schauder, N. Potier, A. van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson.** 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature **415:**545-549.

12. **Chin-A-Woeng, T. F. C., W. de Priester, A. J. van der Bij, and B. J. J. Lugtenberg.** 1997. Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. Mol. Plant-Microbe Interact. **10:**79-86.
13. **Chin-A-Woeng, T. F., G. V. Bloemberg, I. H. Mulders, L. C. Dekkers, and B. J. Lugtenberg.** 2000. Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. Mol. Plant Microbe Interact. **13:**1340-1345.
14. **Chin-A-Woeng, T. F., D. van den Broek, G. de Voer, K. M. van der Drift, S. Tuinman, J. E. Thomas-Oates, B. J. Lugtenberg, and G. V. Bloemberg.** 2001. Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium. Mol. Plant Microbe Interact. 14:969-979.
15. **Christensen, B. B., C. Sternberg, J. B. Andersen, R. J. Palmer, Jr., A. T. Nielsen, M. Givskov, and S. Molin.** 1999. Molecular tools for the study of biofilm physiology. Methods Enzmol. **310:**20-42.
16. **Clark, J. D., and O. Maaløe.** 1967. DNA replication and the division cycle in *Escherichia coli*. J. Mol. Biol. **23:**99-112.
17. **Cook, R. J., L. S. Thomashow, D. M. Weller, D. Fujimoto, M. Mazzola, G. Bangera, and D. S. Kim.** 1995. Molecular mechanisms of defense by rhizobacteria against root disease. Proc. Natl. Acad. Sci., USA **92:**4197-4201.
18. **Cormack, B. P., R. Valdivia, and S. Falkow.** 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene **173:**33-38.
19. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318-1322.
20. **Davey, M. E., and G. A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64:**847-867.
21. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295-298.
22. **De Kievit, T., P. C. Seed, J. Nezezon, L. Passador, and B. H. Iglewski.** 1999. RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. J. Bacteriol. **181:**2175-2184.

23. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived mini-transposons. Methods Enzymol. **235:**386-405.
24. **Dong, Y. H., J. L. Xu, X. Z. Li, and L. H. Zhang.** 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc. Natl. Acad. Sci., USA **97:**3526-3531.
25. **Dong, Y. H., L. H. Wang, J. L. Xu, H. B. Zhang, X. F. Zhang, and L. H. Zhang.** 2001. Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. Nature **411:**813-817.
26. **Dürr, C.** 2001. Besiedelung der Rhizosphäre von Tomaten mit dem Autoinducer-produzierenden Bakterium *Pseudomonas putida* Isolat F. Diplomarbeit am Lehrstuhl für Mikrobiologie, Ludwig-Maximilians-Universität München.
27. **Eberl, L., M. K. Winson, C. Sternberg, G. S. A. B. Stewart, G. Christiansen, S. R. Chhabra, B. Bycroft, P. Williams, S. Molin, and M. Givskov.** 1996. Involvement of *N*-acyl-L-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. Mol. Microbiol. **20:**127-136.
28. **Eberl, L.** 1999. *N*-Acyl homoserinelactone-mediated gene regulation in gram-negative bacteria. System. Appl. Microbiol. **22:**493-506.
29. **Elasri, M., S. Delorme, P. Lemanceau, G. Stewart, B. Laue, E. Glickmann, P. M. Oger, and Y. Dessaux.** 2001. Acyl-homoserine lactone production is more common among plant-associated *Pseudomonas* spp. than among soilborne *Pseudomonas* spp. Appl. Environ. Microbiol. **67:**1198-1209.
30. **El-Sayed, A. K., J. Hothersall, and C. M. Thomas.** 2001. Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens* NCIMB 10586. Microbiology **147:**2127-2139.
31. **Esteve-Núñez, A., A. Caballerao, and J. L. Ramos.** 2001. Biological degradation of 2,4,6-trinitrotoluene. Microbiol. Mol. Biol. Rev. **65:**335-52.
32. **Fray, R. G., J. P. Throup, M. Daykin, A. Wallace, P. Williams, G. S. A. B. Stewart, and D. Grierson.** 1999. Plants genetically modified to produce *N*-acylhomoserine lactones communicate with bacteria. Nature Biotech. **17:**1017-1020.
33. **Fuqua, W. C., S. C. Winans and E. P. Greenberg. 1994.** Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176**:269-275.

34. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene: a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000-3009.
35. **Geiselhöringer, A.** 1999. Konstruktion von Vektoren mit einem breiten Wirtsbereich zur Analyse von Stickstoff-abhängigen Promotoren. Diplomarbeit am Lehrstuhl für Mikrobiologie, Technische Universität München.
36. **Geisenberger, O., M. Givskov, K. Riedel, N. Høiby, B. Tümmler, and L. Eberl.** 2000. Production of *N*-acyl-L-homoserine lactones by *P. aeruginosa* isolates from chronic lung infections associated with cystic fibrosis. FEMS Microbiol. Lett. **184:**273-278.
37. **Givskov, M., and S. Molin.** 1992. Expression of extracellular phospholipase from *Serratia liquefaciens* is growth-phase-dependent, catabolite-repressed and regulated by anaerobiosis. Mol. Microbiol. **6:**1363-1374.
38. **Givskov, M., R. de Nys, M. Manefield, L. Gram, R. Maximilien, L. Eberl, S. Molin, P. Steinberg, and S. Kjelleberg.** 1996. Eukaryotic interference with homoserine lactone-mediated procaryotic signalling. J. Bacteriol. **178:**6618-6622.
39. **Gotschlich, A., B. Huber, O. Geisenberger, A. Tögl, A. Steidle, K. Riedel, P. Hill, B. Tümmler, P. Vandamme, B. Middleton, M. Camara, P. Williams, A. Hardman, and L. Eberl.** 2001. Synthesis of multiple *N*-acyl-homoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex. System. Appl. Microbiol. **24:**1-14.
40. **Gotschlich, A.** 2001. Nachweis, Verbreitung und Bedeutung von *N*-Acyl-L-homoserinlactonen bei Vertretern des *Burkholderia cepacia* Komplexes. Dissertation am Lehrstuhl für Mikrobiologie, Technische Universität München.
41. **Gray, K. M., J. P. Pearson, J. A. Downie, B. E. Boboye, and E. P. Greenberg.** 1996. Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of stationary phase and rhizosphere-expressed genes. J. Bacteriol. **178:**372-376.
42. **Greenberg E. P., J. W. Hastings, and S. Ulitzur.** 1979. Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. Arch. Microbiol. **120:**87-91.
43. **Heim, R., A. B. Cubitt, and R. Y. Tsien.** 1995. Improved green fluorescence. Nature **373:**663-664.
44. **Hentzer, M., K. Riedel, T. B. Rasmussen, A. Heydorn, J. B. Andersen, M. R. Parsek, S. A. Rice, L. Eberl, S. Molin, N. Høiby, S. Kjelleberg, and M. Givskov.**

2002. Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiology **148:**87-102.

45. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557-6567.
46. **Hiltner, L.** 1904. Über neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache. Arb. Dtsch. Landwirt. Ges. **98:**59-78.
47. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene. **212:**77-86.
48. **Hofer, B., R. Blasco, M. Megharaj, M. Seeger, D. McKay, R. M. Wittich, D. H. Pieper, and K. N. Timmis.** 1996. Pseudomonads designed for bioremediation and circumvention of undesired routes. *In*: T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (eds.), Molecular biology of Pseudomonads. ASM Press, Washington, D.C., p. 121-131.
49. **Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl.** 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology **147:**2517-2528.
50. **Jenny, H., and K. Grossenbacher.** 1963. Root-soil boundary zones as seen in the electron microscope. Soil Sci. Soc. Am. Proc. **27:**273-277.
51. **Jones, S., B. Yu, N. J. Bainton, M. Birdsall, B. W. Bycroft, S. R. Chhabra, A. J. Cox, P. Golby, P. J. Reeves, S. Stephens, et al.** 1993. The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. Embo J. **12:**2477-2482.
52. **Kessler, B., V. De Lorenzo, and K. N. Timmis.** 1992. A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the *Pm* promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. **233:**293-301.

53. **Koch, B., L. E. Jensen, and O. Nybroe.** 2001. A panel of Tn*7*-based vectors for insertion of the *gfp* marker gene or for delivery of cloned DNA into gram-negative bacteria at a neutral chromosomal site. J. Microbiol. Methods **45**:187-195.
54. **Kojic, M., G. Degrassi, and V. Venturi.** 1999. Cloning and characterisation of the *rpoS* gene from plant-growth-promoting *Pseudomonas putida* WCS358: RpoS is not involved in siderophore and homoserine lactone production. Biochim. Biophys. Acta. **1489:**413-420.
55. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175-176.
56. **Laue, B. E., Y. Jiang, S. R. Chhabra, S. Jacob, G. S. A. B. Stewart, A. Hardman, J. A. Downie, F. O'Gara, and P. Williams.** 2000. The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium* small bacteriocin, *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone, via HtdS, a putative novel *N*-acylhomoserine lactone synthase. Microbiology **146:**2469-2480.
57. **Leadbetter, J. R. and E. P. Greenberg.** 2000. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. J. Bacteriol. **182:**6921-6926.
58. **Lewenza, S., B. Conway, E. P. Greenberg, and P. A. Sokol.** 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J. Bacteriol. **181**:748-756.
59. **Lithgow, J. K., A. Wilkinson, A. Hardman, B. Rodelas, F. Wisniewski-Dye, P. Williams, and J. A. Downie.** 2000. The regulatory locus *cinRI* in *Rhizobium leguminosarum* controls a network of quorum-sensing loci. Mol. Microbiol. **37:**81-97.
60. **Liu, J., F. B. Dazzo, O. Glagoleva, B. Yu, and A. K. Jain.** 2001. CMEIAS: A computer-aided system for the image analysis of bacterial morphotypes in microbial communities. Microb. Ecol. **41:**173-194.
61. **Lynch, M. J., S. Swift, D. F. Kirke, C. W. Keevil, C. E. R. Dodd, and P. Williams.** 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environ. Microbiol. **4:**18-28.
62. **Lugtenberg, B. J. J., and L. C. Dekkers.** 1999. What makes *Pseudomonas* bacteria rhizosphere competent? Environ. Microbiol. **1:**9-13.

63. **Mae, A., M. Montesano, V. Koiv, and E. T. Palva.** 2001. Transgenic plants producing the bacterial pheromone *N*-acyl homoserine lactone exhibit enhanced resistance to the bacterial phytopathogen *Erwinia carotovora*. Mol. Plant Microbe Interact. **14:**1035-1042.

64. **Maes, T., D. Vereecke, T. Ritsema, K. Cornelis, H. N. T. Thu, M. van Montagu, M. Holsters, and K. Goethals.** 2001. The *att* locus of *Rhodococcus fascians* strain D188 is essential for full virulence on tabacco through the production of an autoregulatory compound. Mol. Microbiol. **42:**13-28.

65. **Manefield, M., M. Welch, M. Givskov, G. P. C. Salmond, and S. Kjelleberg.** 2001. Halogenated furanones from the red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*. FEMS Microbiol. Lett. **205:**131-138.

66. **Manefield, M., T. B. Rasmussen, M. Hentzer, J. B. Andersen, P. Steinberg, S. Kjelleberg, and M. Givskov.** 2002. Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. Microbiology **148:**1119-1127.

67. **Matz, M. M. V., A. F. Fradkov, Y. A. Labas, A. P. Savitsky, A. G. Zaraisky, M. L. Markelov, and S. A. Lukyanov.** 1999. Fluorescent proteins from nonbioluminescent *Anthozoa* species. Nature Biotech. **17:**969-973.

68. **Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson III.** 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl. Environ. Microbiol. **58:**2616-2624.

69. **Meissner, R., Y. Jacobson, S. Melamed, S. Levyatuv, G. Shalev, A. Ashri, Y. Elkind, and A. Levy.** 1997. A new model system for tomato genetics. Plant J. **12:**1465-1472.

70. **Miller, M. B., and B. L. Bassler.** 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. **55:**165-199.

71. **Normander, B., N. B. Hendriksen, and O. Nybroe.** 1999. Green fluorescent protein-marked *Pseudomonas fluorescens*: localization, viability, and activity in the natural barley rhizosphere. Appl. Environ. Microbiol. **65:**4646-4651.

72. **O'Sullivan, D. J., and F. O'Gara.** 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol. Rev. **56:**662-676.

73. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127-1130.
74. **Pearson, J. P., C. Van Delden, and B. H. Iglewski.** 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J. Bacteriol. **181:**1203-1210.
75. **Pierson, III, L. S., V. D. Keppenne, and D. W. Wood.** 1994. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. J. Bacteriol. **176:**3966-3974.
76. **Pierson, E. A., D. W. Wood, J. A. Cannon, F. M. Blachere, and L. S. Pierson III.** 1998. Interpopulation signaling via *N*-acyl-homoserine lactones among bacteria in the wheat rhizosphere. Mol. Plant-Microbe Interact. **11:**1078-1084.
77. **Piper, K. R., S. B. von Bodman, and S. K. Farrand.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature. **362:**448-450.
78. **Pirhonen, M., D. Flego, R. Heikinheimo, and E. T. Palva.** 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. Embo J. **12:**2467-2476.
79. **Rasmussen, T. B., M. Manefield, J. B. Andersen, L. Eberl, U. Anthoni, C. Christophersen, P. Steinberg, S. Kjelleberg, and M. Givskov.** 2000. How *Delisea pulchra* furanones affect quorum sensing and swarming motility in *Serratia liquefaciens* MG1. Microbiology **146:**3237-3244.
80. **Rodelas, B., J. K. Lithgow, F. Wisniewski-Dye, A. Hardman, A. Wilkinson, A. Economou, P. Williams, and J. A. Downie.** 1999. Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. viciae. J. Bacteriol. **181:**3816-3823.
81. **Römling, U., B. Fiedler, J. Bosshammer, D. Grothues, J. Greipel, H. von der Hardt, and B. Tümmler.** 1994. Epidemiology of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis. J. Infect. Dis. **170:**1617-1621.
82. **Salmond, G. P. C., B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1995. The bacterial 'enigma': cracking the code of cell-cell communication. Mol. Microbiol. **16**:615-624.
83. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. 2nd Edition. New York. Cold Spring Harbor Laboratory.

84. **Sauer, K., and A. K. Camper.** 2001. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. J. Bacteriol. **183:**6579-6589.

85. **Schauder, S., K. Shokat, M. G. Surette, and B. L. Bassler.** 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum sensing signal molecule. Mol. Microbiol. **41:**463-476.

86. **Schripsema, J., K. E. de Rudder, T. B. van Vliet, P. P. Lankhorst, E. de Vroom, J. W. Kijne, and A. A. van Brussel.** 1996. Bacteriocin small of *Rhizobium leguminosarum* belongs to the class of *N*-acyl-L-homoserine lactone molecules, known as autoinducers and as quorum sensing co-transcription factors. J. Bacteriol. **178:**366-371.

87. **Schweizer, H. P., and T. T. Hoang**. 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15-22.

88. **Shaw, P. D., G. Ping, S. Daly, C. Cha, J. E. Cronan Jr., K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. USA **94:**6036-6041.

89. **Sigl, K.** 2001. In situ Visualisierung von Zell-Zell-Kommunikation mittels Homoserinlactonen in der Tomaten-Rhizosphäre. Diplomarbeit am Lehrstuhl für Mikrobiologie, Technische Universität München.

90. **Simons, M., A. J. van der Bij, I. Brand, L. A. de Weger, C. A. Wijffelman, and B. J. J. Lugtenberg.** 1996. Gnotobiotic system for studying rhizosphere colonization by plant growth promoting *Pseudomonas* bacteria. Mol. Plant-Microbe Interact. **9:**600-607.

91. **Smith, R. S., S. G. Harris, R. Phipps, and B. Iglewski.** 2002. The *Pseudomonas aeruginosa* quorum-sensing molecule *N*-(3-Oxododecanoyl)-homoserine lactone contributes to virulence and induces inflammation in vivo. J. Bacteriol. **184:**1132-1139.

92. **Sørensen, J.** 1997. The rhizosphere as a habitat for soil microorganisms. *In*: Van Elsas, J. D., J. T. Trevors, and E. M. H. Wellington (eds.) Modern Soil Microbiology, Marcel Dekker, Inc. New York, Basel, Hongkong, p. 21-45.

93. **Stewart, P. S., and J. W. Costerton.** 2001. Antibiotic resistance of bacteria in biofilms. Lancet **358:**135-138.

94. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: A new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. USA **96:**1639-1644.

95. **Thorn, G.** 1997. The fungi in soil. *In*: Van Elsas, J. D., J. T. Trevors, and E. M. H. Wellington (eds.) Modern Soil Microbiology, Marcel Dekker, Inc. New York, Basel, Hongkong, p. 63-127

96. **Thorne, S. H., and H. D. Williams.** 1999. Cell density-dependent starvation survival of *Rhizobium leguminosarum* bv. phaseoli: identification of the role of an *N*-acyl homoserine lactone in adaptation to stationary-phase survival. J. Bacteriol. **181:**981-990.

97. **Tolker-Nielsen, T., U. C. Brinch, P. C. Ragas, J. B. Andersen, C. S. Jacobsen, and S. Molin.** 2000. Development and dynamics of *Pseudomonas* sp. biofilms. J. Bacteriol. **182:**6482-6489.

98. **Trevors, J. T. and J. D. van Elsas.** 1997. Microbial interactions in soil. *In*: Van Elsas, J. D., J. T. Trevors, and E. M. H. Wellington (eds.), Modern Soil Microbiology, Marcel Dekker, Inc. New York, Basel, Hongkong, p. 215-243.

99. **Van Loon, L. C., P. A. H. M. Bakker, and C. M. J. Pieterse.** 1998. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. **36:**453-483.

100. **Von Bodman, S. B., D. R. Majerczak, and D. L. Coplin.** 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. stewartii. Proc. Natl. Acad. Sci. USA **95:**7687-7692.

101. **Watnick, P., and R. Kolter.** 2000. Biofilm, city of microbes. J. Bacteriol. **182:**2675-2679.

102. **Weller, D. M., and L. S. Tomashow.** 1994. Current challenges in introducing beneficial microorganisms into the rhizosphere. *In*: F. O'Gara, D. N. Dowling, and B. Boesten (eds.), Molecular ecology of rhizosphere microorganisms. VCH Weinheim, New York, Basel, Cambridge, Tokyo, p. 1-18.

103. **Whitehead, N. A., A. M. L. Barnard, H. Slater, N. J. L. Simpson, G. P. C. Salmond.** 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Rev. **25:**365-404.

104. **Whiteley, M., and E. P. Greenberg.** 2001. Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. J. Bacteriol. **183:**5529-5534.

105. **Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, *et al.*** 1995. Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci., USA **92:**9427-9431.

106. **Winson, M. K., S. Swift, L. Fish, J. P. Throup, F. Jørgensen, S. R. Chhabra, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart.** 1998. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl-homoserine lactone-mediated quorum sensing. FEMS Microbiol. Lett. **163:**185-192.

107. **Winzer, K., K. R. Hardie, and P. Williams.** 2002. Bacterial cell-to-cell communication: sorry, can't talk now – gone to lunch! Curr. Opinion Microbiol. **5:**216-222.

108. **Wood, D. W., and L. S. Pierson III.** 1996. The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. Gene **168:**49-53.

109. **Wood, D. W., F. Gong, M. M. Daykin, P. Williams, and L. S. Pierson III.** 1997. *N*-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. J. Bacteriol. **179:**7663-7670.

110. **Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate.** 1993. *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. Nature **362:**446-448.

111. **Zhang, Z., and L. S. Pierson III.** 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. Appl. Environ. Microbiol. **67:**4305-4315.

F. ANHANG

Anhang 1

Visualization of *N*-Acylhomoserine Lactone-Mediated Cell-Cell Communication between Bacteria Colonizing the Tomato Rhizosphere

ANETTE STEIDLE, KATJA SIGL, REGINA SCHUHEGGER, ALEXANDRA IHRING, MARKUS SCHMID, STEPHAN GANTNER, MARION STOFFELS, KATHRIN RIEDEL, MICHAEL GIVSKOV, ANTON HARTMANN, CHRISTIAN LANGEBARTELS, AND LEO EBERL

Veröffentlicht in: *Applied and Environmental Microbiology*, 67:5761-5770 (2001)

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2001, p. 5761–5770 Vol. 67, No. 12
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.12.5761–5770.2001

Visualization of *N*-Acylhomoserine Lactone-Mediated Cell-Cell Communication between Bacteria Colonizing the Tomato Rhizosphere

ANETTE STEIDLE,[1] KATJA SIGL,[1] REGINA SCHUHEGGER,[2] ALEXANDRA IHRING,[1] MARKUS SCHMID,[1] STEPHAN GANTNER,[3] MARION STOFFELS,[3] KATHRIN RIEDEL,[1] MICHAEL GIVSKOV,[4] ANTON HARTMANN,[3] CHRISTIAN LANGEBARTELS,[2] AND LEO EBERL[1]*

Lehrstuhl für Mikrobiologie, Technische Universität München, D-85350 Freising,[1] and Institut für Biochemische Pflanzenpathologie[2] and Institut für Bodenökologie,[3] GSF-Forschungszentrum, D-85764 Neuherberg, Germany, and Department of Microbiology, DTU, DK-2800 Lyngby, Denmark[4]

Received 4 June 2001/Accepted 12 September 2001

Given that a large proportion of the bacteria colonizing the roots of plants is capable of producing *N*-acyl-L-homoserine lactone (AHL) molecules, it appears likely that these bacterial pheromones may serve as signals for communication between cells of different species. In this study, we have developed and characterized novel Gfp-based monitor strains that allow in situ visualization of AHL-mediated communication between individual cells in the plant rhizosphere. For this purpose, three Gfp-based AHL sensor plasmids that respond to different spectra of AHL molecules were transferred into AHL-negative derivatives of *Pseudomonas putida* IsoF and *Serratia liquefaciens* MG1, two strains that are capable of colonizing tomato roots. These AHL monitor strains were used to visualize communication between defined bacterial populations in the rhizosphere of axenically grown tomato plants. Furthermore, we integrated into the chromosome of AHL-negative *P. putida* strain F117 an AHL sensor cassette that responds to the presence of long-chain AHLs with the expression of Gfp. This monitor strain was used to demonstrate that the indigenous bacterial community colonizing the roots of tomato plants growing in nonsterile soil produces AHL molecules. The results strongly support the view that AHL signal molecules serve as a universal language for communication between the different bacterial populations of the rhizosphere consortium.

In recent years, it has become evident that bacteria not only exist as individual cells but also often coordinate their activities and act in a concerted manner similar to that of multicellular organisms. Such interactions require sophisticated cell-cell communication systems to adjust the various functions within a bacterial community. In fact, many gram-negative bacteria have been shown to produce *N*-acyl-L-homoserine lactone (AHL) signal molecules, which are utilized by the bacteria to monitor their own population densities in a process known as quorum sensing (for reviews, see references 11 and 15). These regulatory systems typically rely on two proteins, an AHL synthase, usually a member of the LuxI family of proteins, and an AHL receptor protein, belonging to the LuxR family of transcriptional regulators. At low population densities, cells produce a basal level of AHL via the activity of the AHL synthase. As the cell density increases, AHLs accumulate in the growth medium. On reaching a critical threshold concentration, the AHL molecule binds to its cognate receptor, which in turn activates or represses the expression of target genes.

AHL-based quorum-sensing systems have been identified in a number of plant-associated bacteria and have been shown to control the expression of various functions in response to the density of the population (for a recent review, see reference 35). In *Agrobacterium tumefaciens*, the causative agent of crown gall tumors, a quorum-sensing system relying on *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL) is involved in the regulation of Ti plasmid conjugative transfer (36, 53). In plant-pathogenic bacteria which belong to the genus *Erwinia* and which cause soft rot disease, the production of cell wall-degrading enzymes as well as the synthesis of the antibiotic carbapenem is subject to *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL)-mediated gene regulation (28). Similarly, 3-oxo-C6-HSL is produced by *Pantoea stuartii*, the causative agent of Stewart's wilt and leaf blight of corn, and was shown to control the production of an exopolysaccharide capsule which represents the major virulence determinant of the bacterium (47). It is generally believed that the expression of virulence determinants at high cell densities ensures a rapid and coordinated attack to overwhelm the plant before it is able to mount an efficient defense response.

Furthermore, quorum-sensing systems have also been identified in bacteria which are beneficial for the plant. A well-known example is *Pseudomonas aureofaciens* strain 30-84, which is used as a biocontrol agent to protect wheat from take-all disease, caused by *Gaeumannomyces graminis* var. *tritici*. When this strain is present in the rhizosphere of wheat, the severity of the disease is strongly reduced due to the production of phenazine antibiotics which are active against the ascomycete fungus. The synthesis of these antibiotic compounds has been demonstrated to be regulated by a quorum-sensing system that utilizes the AHL molecule *N*-hexanoyl-L-homoserine lactone (C6-HSL) (51). Recent work has shown that in *Rhizobium leguminosarum*, at least four different AHL

* Corresponding author. Mailing address: Department of Microbiology, Technical University of Munich, Am Hochanger 4, D-85350 Freising, Germany. Phone: 49 8161 715446. Fax: 49 8161 715475. E-mail: EBERL@mikro.biologie.tu-muenchen.de.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Bacterial strains		
Pseudomonas putida IsoF	Wild-type isolate from tomato roots	This work
Pseudomonas putida F117	AHL-negative derivative of IsoF; PpuI$^-$	This work
Pseudomonas putida KS35	F117 with integrated P_{lasB}-*gfp*(ASV) P_{lac}-*lasR* cassette	This work
Pseudomonas putida Z2D	Wild-type isolate from tomato roots	This work
Serratia liquefaciens MG44	AHL-negative mutant of *S. liquefaciens* MG1; SwrI$^-$	12
Rahnella aquatilis T13	Wild-type isolate from tomato roots	This work
Rahnella aquatilis TAA	Wild-type isolate from tomato roots	This work
Escherichia coli MT102	*araD139* (*ara-leu*)*7697* Δ*lac thi hsdR*	Laboratory collection
Escherichia coli HB101	*recA thi pro leu hsdR*$^-$*M*$^+$ Sm^r	24
Escherichia coli CC118 (λ*pir*)	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-I rpsE rpoB argE*(Am) *recA1* λ*pir* lysogen	20
Plasmids		
pAS-C8	pBBR1MCS-5 carrying P_{cepI}-*gfp*(ASV)-P_{lac}-*cepR*; Gm^r	38
pKR-C12	pBBR1MCS-5 carrying P_{lasB}-*gfp*(ASV)-P_{lac}-*lasR*; Gm^r	38
pTn*5*-LAS	pTn*5*-based delivery plasmid carrying P_{lasB}-*gfp*(ASV) P_{lac}-*lasR*; Gm^r Ap^r	Hentzer et al., submitted
pJBA132	pME6031 carrying *luxR*-P_{luxR}-P_{lux1}-*gfp*(ASV)-T_0-T_1; Tet^r	1
pSB403	Bioluminescent broad-host-range AHL sensor plasmid; Tet^r	50
pUT-Kan-*dsred*	pUT-Kan carrying P_{lac}-*rfp*-T_0-T_1; Kan^r Ap^r	45
pTn*5*-Red	Tn*5*-based delivery plasmid carrying P_{lac}-*dsred*-T_0-T_1; Tet^r Ap^r	Hentzer et al., submitted
pGEM-3Zf(+)	*lac*Zα cloning vector; Ap^r	Promega, Madison, Wis.
pBBR1MCS-5	Broad-host-range plasmid; *lac*Zα; Gm^r	25
pRK600	ColE1 RK2-Mob$^+$ RK2-Tra$^+$; helper plasmid; Cm^r	8

production loci direct the synthesis of multiple AHLs (27). Among these molecules is *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone (18, 40), a compound which was first referred to as small bacteriocin because it inhibits the growth of certain sensitive strains of *R. leguminosarum* (49). The highly sophisticated quorum-sensing network which operates in *R. leguminosarum* is involved in the control of various functions, including stationary-phase adaptation (44), the ability to nodulate peas (39), and the conjugal transfer of plasmid pRL1JI (27).

Despite rapidly increasing knowledge of the molecular mechanisms of the quorum-sensing cascades in various plant-associated bacteria, little information exists about whether AHLs are in fact produced under in situ conditions. In previous reports, circumstantial evidence was presented that *P. aureofaciens* produces C6-HSL when colonizing the rhizosphere of wheat (34, 51). Moreover, based on cross-stimulation of transcriptional fusions of reporter genes *lacZ* and *inaZ* to the phenazine biosynthetic operon, it was inferred that AHL molecules can serve as interpopulation signals in the wheat rhizosphere (34). However, no information is currently available on AHL-mediated communication in the rhizosphere of a dicot plant species.

The aim of this study was to develop novel tools that are suitable for in situ visualization of AHL-mediated communication between individual cells in the rhizosphere of tomato plants. This was accomplished by transferring three different Gfp-based AHL sensor plasmids into AHL-negative derivatives of two plant-associated bacteria, *Pseudomonas putida* IsoF and *Serratia liquefaciens* MG1. The resulting strains were rigorously characterized with respect to their sensitivities for various AHL molecules. These AHL monitor strains were then used to monitor AHL-based communication between cells of selected bacterial strains in the rhizosphere of axenically grown tomato plants in a nondestructive, online manner. Furthermore, using a *P. putida* monitor strain carrying a chromosomally integrated long-chain AHL sensor cassette, we demonstrated that the indigenous bacterial community of the tomato rhizosphere in fact produces AHLs.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown aerobically in modified Luria-Bertani (LB) medium (4) containing 4 g of NaCl/liter instead of 10 g of NaCl/liter at 30 or 37°C (*Escherichia coli*). Solid media contained 15 g of agar/liter. Antibiotics were added as required at final concentrations of 10 mg of tetracycline/liter, 25 mg of gentamicin/liter, 50 mg of kanamycin/liter, and 100 mg of ampicillin/liter. Root-associated bacteria were isolated as follows. Root samples were collected and rinsed in sterile phosphate-buffered saline (PBS) to remove adhering soil particles. One gram of root material was then macerated in 9 ml of sucrose solution (4% [wt/vol]) with a mortar and pestle. Serial 10-fold dilutions of the suspensions were prepared, and 100-μl aliquots were plated on LB agar. Single colonies were further purified and screened for AHL production by cross-streaking against *E. coli* MT102(pSB403) (50) as described below.

To identify strains of interest, their 16S ribosomal DNAs (rDNAs) were sequenced. Amplification of 16S rDNAs (*E. coli* positions 8 to 1526) was performed using primers 616V (5′-AGAGTTTGATYMTGGCTCAG-3′) and 630R (5′-CAKAAAGGAGGTGATCC-3′). The amplicons were then sequenced using primers 607V (5′-GGGCTACACACGTGC-3′), 609V (5′-GGATTAGATACC CBDGTA-3′), 610RII (5′-ACCGCKRCTGCTGGCAC-3′), and 612II (5′-GTA AGGTTYTNCGCGT-3′). For phylogenetic affiliation of the strains, the sequences were analyzed using the ARB software package (http://www.arb-home.de).

Tagging of bacterial strains with Gfp and DsRed. To monitor single bacterial cells in the tomato rhizosphere, strains were tagged with green fluorescent protein (Gfp) or red fluorescent protein (DsRed). To avoid loss of the marker in the absence of antibiotic pressure, a $P_{A1/04/03}$-*gfp*-T_0-T_1 (2) or a $P_{A1/04/03}$-*dsred*-T0-T1 (45) transposon cassette was randomly inserted into the chromosomes of respective strains using the delivery vector pUTkan or pUTtc. Plasmids were mobilized from *E. coli* CC118(λ*pir*) to the respective strains by triparental mating using *E. coli* HB101(pRK600) as a helper as described previously (7). Fluorescent exconjugants that grew indistinguishably from the parental strains on plates and on tomato roots (data not shown) were used in the experiments described here.

Characterization of AHL monitor strains. To determine the specificity and sensitivity of the different Gfp-based AHL monitor strains, respective overnight

cultures were diluted four times into fresh LB medium, incubated for 1 h at 30°C, and then distributed in 200-μl aliquots into wells of a microtiter plate. 3-oxo-C12-HSL [*N*-(3-oxo-dodecanoyl)-L-homoserine lactone], 3-oxo-C10-HSL [*N*-(3-oxo-decanoyl)-L-homoserine lactone], 3-oxo-C6-HSL, C12-HSL [*N*-dodecanoyl-L-homoserine lactone], C10-HSL [*N*-decanoyl-L-homoserine lactone], C8-HSL [*N*-octanoyl-L-homoserine lactone], C6-HSL, and C4-HSL [*N*-butanoyl-L-homoserine lactone] were added to the wells at final concentrations of 10,000, 5,000, 2,500, 1,250, 625, 310, 160, 80, 40, and 20 nM. Following 6 h of incubation at 30°C, green fluorescence was measured using the microtiter plate reader Lambda Fluoro 320 Plus (MWG Biotech, Ebersberg, Germany) with an excitation wavelength of 474 nm and emission detection at 515 nm. Data were processed with KC4 software (Bio-Tek Instruments). The fluorescence measurements were corrected for autofluorescence and plotted as a function of AHL concentrations. Synthetic AHL molecules either were purchased from Fluka Chemie AG, Buchs, Switzerland, or were kindly provided by P. Williams (School of Pharmaceutical Sciences, University of Nottingham, Nottingham, United Kingdom).

Characterization of AHLs produced by rhizosphere isolates. The production of AHLs by bacterial isolates was investigated with the aid of the bioluminescent sensor plasmid pSB403 (50). This sensor plasmid contains the *Photobacterium fischeri luxR* gene together with the *luxI* promoter region as a transcriptional fusion to bioluminescence genes *luxCDABE* of *Photorhabdus luminescens*. The quorum-sensing system of *P. fischeri* relies on 3-oxo-C6-HSL, and the sensor plasmid consequently exhibits the highest sensitivity for this AHL molecule. However, several other AHL molecules are detected by the sensor, albeit with somewhat reduced sensitivity (50). Bioluminescence was detected either with a highly sensitive photon-counting camera (C2400-40; Hamamatsu Photonics, Herrsching, Germany) or by exposure of an X-ray film (X-Ray-90; AGFA-Gevaert, Munich, Germany). We also used different Gfp-based biosensors for the detection of AHLs. For this, the production of AHLs was monitored as the expression of green fluorescence. This was accomplished by illuminating plates with blue light using an HQ 480/40 filter (F44-001; AHF-Analysentechnik, Tübingen, Germany) in combination with a halogen lamp (Intralux 5000-1; Volpi, Schlieren, Switzerland) as a light source. Illumination took place in a dark box equipped with the C2400-40 camera connected to a Pentax CCTV camera lens and an HQ 535/20 filter (F42-001; AHF-Analysentechnik).

For a more detailed analysis, the AHL molecules were extracted from spent culture supernatants of the strains and separated by thin-layer chromatography (TLC), and AHL spots were visualized by overlaying the TLC plates with soft agar seeded with the sensor strain *E. coli* MT102(pSB403) as described previously (17, 41). Routinely, AHLs were extracted twice with dichloromethane (250:100 supernatant/dichloromethane) from 250 ml of sterile filtered supernatants of cultures grown in LB medium at 30°C to an optical density at 600 nm of 1.0. The combined extracts were dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. Residues were dissolved in 250 μl of ethyl acetate. Ten-microliter samples were applied to C_{18} reversed-phase TLC plates (Merck no. 1.15389) and dried with a stream of cold air. Samples were separated by using methanol (60% [vol/vol]) in water as the mobile phase. For the detection of AHLs, each TLC plate was overlaid with a thin film of 0.8% (wt/vol) LB agar (143 ml) seeded with 7 ml of an exponentially grown AHL biosensor and then was incubated at 30°C for 24 h. The tentative identification of AHLs present in spent culture supernatant extracts was achieved by comparison of mobilities (R_f values) to those for the synthetic AHL standards.

Growth of tomato seedlings in a gnotobiotic test system. Tomato seeds (*Lycopersicon esculentum* Micro-Tom) (31) provided by B. Nebelung, Everswinkel, Germany, were surface sterilized by soaking first in 70% ethanol for 3 min and subsequently in a 5% sodium hypochlorite solution (with 0.6% Tween 20) for 20 min. Seeds were thoroughly rinsed five times in sterile water and sown in Phytatrays (Sigma, Taufkirchen, Germany) filled with 250 g of sterile quartz sand and 25 ml of sterile nutrient solution (42). The Phytatrays were covered with a lid, sealed with Parafilm, and incubated for 14 to 21 days in a growth chamber at 25°C (day) and 20°C (night) with 70% humidity and a 14 h-10 h day-night cycle. Plants were inoculated with bacteria 10 days after seeding (4 to 5 days after germination). Bacterial strains were grown overnight in 250 ml of LB medium with antibiotics when necessary and harvested by centrifugation. Cells were washed in 10 mM $MgSO_4$ and diluted in 10 mM $MgSO_4$ to a final concentration of 10^9 CFU/ml. Three milliliters of the bacterial suspension was used to inoculate a Phytatray with five tomato plants. For inoculation of the seedlings with two strains, AHL producer and monitor strains were mixed in a 1:1 ratio, and 3 ml of the mixture was used to inoculate one Phytatray.

Growth of tomato seedlings in potting soil. To examine molecular communication in the naturally occurring rhizosphere of tomato plants, tomato seedlings were grown in nonsterile substrate (46). Seeds were surface sterilized as described above, sown in potting substrate (Fruehstorfer Einheitserde, type T; Patzer, Simmtal-Jossa, Germany), and incubated as described above. Plants were inoculated five weeks after seeding with 10 ml of bacterial suspension per plant as described above.

Confocal laser scanning microscopy. All microscopic observations and image acquisitions for root-associated bacteria were performed using an LSM 510 scanning confocal microscope (Zeiss, Jena, Germany) equipped with an Ar ion laser (Gfp: excitation, 488 nm; emission filter BP 505-550) and an HeNe laser (DsRed: excitation, 543 nm; emission filter LP 560) together with the standard software package delivered with the instrument (version 2.1). Tomato roots were sampled for microscopy 1 to 10 days postinoculation. The roots were washed in PBS to remove sand particles, mounted in PBS on a microscope slide, and examined immediately.

RESULTS

Construction and characterization of Gfp-based AHL monitor strains suitable for in situ studies of cell-cell communication in the rhizosphere. In order to monitor a maximum of different AHL molecules, we used three Gfp-based AHL sensor plasmids in this study. Each of these sensor plasmids is capable of detecting a certain range of AHL molecules, depending on the components used for its construction.

The sensor plasmid pJBA132 (1) is based on components of the *lux* quorum-sensing system of *Vibrio fischeri* and contains a P*luxI-gfp*(ASV) transcriptional fusion together with the *luxR* gene on the broad-host-range plasmid pME6031. The *lux* quorum-sensing system utilizes 3-oxo-C6-HSL (10), and the sensor plasmid consequently exhibits the highest sensitivity for this signal molecule. However, this sensor plasmid is also responsive to various related AHL molecules, albeit with decreased sensitivity (1). Noteworthy is the fact that for the construction of pJBA132 (as well as for that of the AHL sensors described below), an unstable variant of Gfpmut3*, namely, Gfp(ASV), was used (2). This Gfp variant carries a C-terminal peptide tag which makes the protein prone to degradation by housekeeping-intracellular tail-specific proteases (Clp). Due to the high rate of turnover of the reporter protein, online monitoring of transient communication is possible.

Both plasmid pKR-C12 (38) and the hybrid transposon mini Tn*5*-LAS (19a) contain an AHL sensor cassette which is based on components of the *las* quorum-sensing system of *Pseudomonas aeruginosa*. Specifically, this cassette consists of a P*lasB-gfp*(ASV) translational fusion together with the *lasR* gene placed under the control of P*lac*. As expected, because the cognate AHL of the *las* system is 3-oxo-C12-HSL (33), this system is most sensitive for 3-oxo-C12-HSL and other long-chain AHL molecules.

Finally, for the detection of AHL molecules with medium-length acyl side chains, we used plasmid pAS-C8 (38). This sensor plasmid was constructed from components of the *cep* system of *Burkholderia cepacia*, which utilizes the signal molecule C8-HSL (17, 26), and contains a P*cepI-gfp*(ASV) translational fusion together with the *cepR* gene transcribed from the P*lac* promoter of the broad-host-range plasmid pBBR1MCS-5. This sensor plasmid responds most efficiently to C8-HSL and, with a lower efficiency, to related AHL molecules.

As we intended to use these sensor plasmids for the analysis of AHL-mediated communication between bacterial cells in the rhizosphere, we transferred them to two strains capable of colonizing tomato roots. *P. putida* IsoF was originally isolated from the tomato rhizosphere and was subsequently shown to

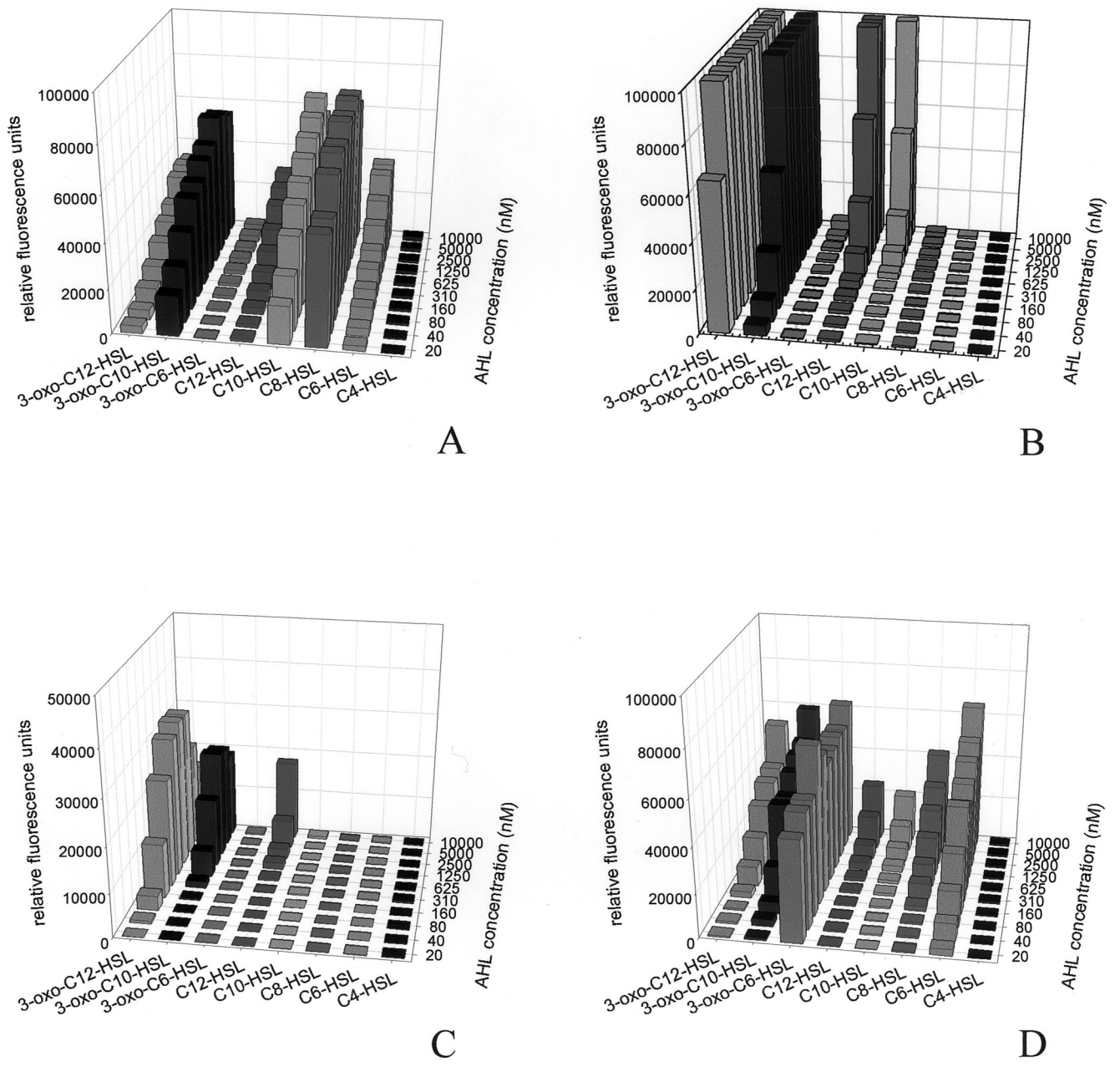


FIG. 1. Characterization of the AHL monitor strains used in this study. The monitor strains *P. putida* F117(pAS-C8) (A), *P. putida* F117(pKR-C12) (B), *P. putida* KS35 (F117::Tn*5*-LAS) (C), and *S. liquefaciens* MG44(pJBA132) (D) were grown in the presence of different AHL compounds in a concentration range from 0 to 10 μM. The green fluorescence of the cultures was recorded with a microtiter plate reader at 515 nm. The measured values were corrected for autofluorescence and plotted as a function of AHL concentration. The AHL compounds assayed are shown at the bottom of each panel.

be an excellent root colonizer (data not shown). Since this strain produces various AHL molecules due to the presence of the *ppuI* AHL synthase gene, a defined *ppuI* knockout mutant, F117, which no longer produces AHLs (data not shown), was used as the host. Likewise, *S. liquefaciens* MG44 (12), a defined AHL-negative derivative of wild-type MG1, was used as a host for the AHL sensor plasmids. *S. liquefaciens* MG1, which was isolated from the surface of a cucumber (16), can persist for extended periods of time in the rhizosphere of tomatoes (data not shown). Both *P. putida* and *S. liquefaciens* are typical inhabitants of the rhizosphere, and the presence of members of these genera often promotes the growth of the host plant (22, 29, 48).

The presence of the AHL sensor cassettes on mobilizable broad-host-range vectors enabled us to transfer them to the AHL-negative strains *P. putida* F117 and *S. liquefaciens* MG44, giving rise to six different monitor strains. Recent work has shown that both the specificity and the sensitivity of the AHL sensor plasmids described above are dependent to some degree on the strain background (1, 38; Hentzer et al., submit-

TABLE 2. Activation of the AHL monitor strains in cross-streak experiments[a]

Rhizosphere isolate	Result obtained with:			
	MG44 (pJBA132)	KS35	F117 (pKR-C12)	F117 (pAS-C8)
P. putida IsoF	+++	+++	+++	+++
P. putida Z2D	+++	+++	+++	+
R. aquatilis T13	+++	−	−	++
R. aquatilis TAA	+++	+	+++	+++

[a] The four monitor strains, MG44(pJBA132), F117(pKR-C12), F117(pAS-C8), and KS35 (F117::Tn*5*-LAS), were cross-streaked against various rhizosphere isolates on LB agar plates. Following 24 h of incubation at 30°C, the production of Gfp(ASV) by the monitor strains was visualized by epifluorescence microscopy. Levels of activation are indicated as follows: +++, strong activation, diffusion of AHL of >1 cm; ++, activation, diffusion of AHL of 0.5 to 1 cm; +, weak activation, diffusion of AHL of <0.5 cm; −, no detectable activation.

ted). We therefore characterized the various monitor strains with respect to their sensitivity for different AHL molecules. This was accomplished by measuring the Gfp fluorescence of cultures exposed to various AHL concentrations in a microtiter dish assay as described in Materials and Methods.

To our great surprise, some of the AHL sensor plasmids worked well in one of the strains but not in the other. For example, in the presence of 10 nM 3-oxo-C6-HSL, pJBA132 gave rise to strong fluorescence in the *S. liquefaciens* MG44 background; however, it was only weakly stimulated at concentrations of up to 1 μM 3-oxo-C6-HSL in the *P. putida* F117 background (Fig. 1 and data not shown). In contrast, MG44 harboring plasmid pKR-C12 was very insensitive for 3-oxo-C12-HSL, while the presence of this plasmid in the F117 background allowed the detection of less than a 20 nM concentration of this AHL molecule. Likewise, the sensor plasmid pAS-C8 did not work in the MG44 background, while in the F117 background, it allowed the detection of less than 10 nM C8-HSL. At present, the reasons for the failure of proper functioning of the AHL sensor plasmids in certain strain backgrounds are unclear and will require further investigation. For this study, we used the three sensor plasmids in the strain background that allowed for the most sensitive detection of the respective AHL molecules, i.e., plasmids pKR-C12 and pAS-C8 in *P. putida* F117 and plasmid pJBA132 in *S. liquefaciens* MG44.

The three monitor strains responded to different spectra of AHL molecules. In good agreement with other studies (38; Hentzer et al., submitted), F117 harboring plasmid pKR-C12 responded not only to 3-oxo-C12-HSL (detection limit of less than 10 nM) but also to 3-oxo-C10-HSL, C10-HSL, and C12-HSL, albeit with reduced sensitivity (Fig. 1). As expected, monitor strain F117(pAS-C8) was most sensitive for C8-HSL (detection limit of 10 nM) but was also, with lower efficiency, stimulated by C10-HSL, 3-oxo-C10-HSL, C6-HSL, C12-HSL, and 3-oxo-C12-HSL. MG44 harboring the 3-oxo-C6-HSL sensor plasmid pJBA132 was activated by a broad spectrum of AHL molecules. This sensor exhibited the highest sensitivity for 3-oxo-C6-HSL (detection limit of 5 nM), followed by C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, and C8-HSL.

We also determined the detection limits of the three monitor strains for single-cell analysis by inspection of samples by epifluorescence microscopy. For the most efficient AHL molecules, concentrations of less than 5 nM were sufficient to stimulate the sensors to a level allowing the unequivocal detection of green fluorescent cells.

Assessment of interspecies communication in cross-streaking experiments. To test the monitor strains for their suitability for detecting AHL production in situ, they were cross-streaked against various bacterial strains isolated from tomato roots. Of

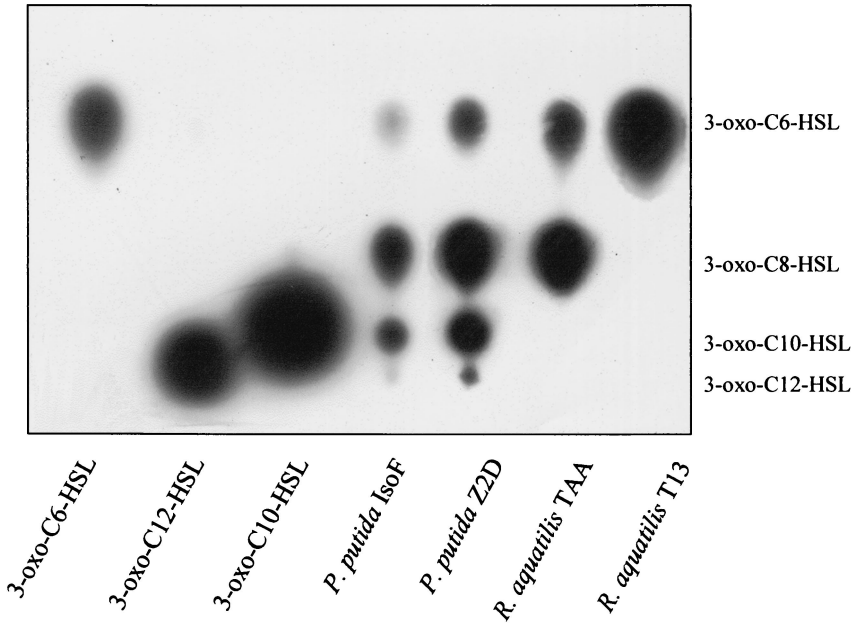


FIG. 2. TLC analysis of AHLs produced by bacterial strains isolated from the tomato rhizosphere. AHLs extracted from cell-free culture supernatants of *P. putida* IsoF, *P. putida* Z2D, *R. aquatilis* TAA, and *R. aquatilis* T13 were separated by TLC, and spots were detected with the aid of the AHL biosensor *E. coli* MT102(pSB403). Synthetic AHLs were included as reference compounds, as indicated.

300 strains tested, approximately 12% provoked a positive signal with one or more of the monitor strains (data not shown). Three strains gave rise to particularly strong signals with at least one of the monitor strains, and these were chosen for further investigations (Table 2). Sequence analysis of the 16S rDNAs identified strains T13 and TAA as a *Rahnella* sp. (more than 99% similarity to *Rahnella aquatilis*) and strain Z2D as a *Pseudomonas* sp. (more than 98% similarity to *P. putida*).

AHL profiles of the rhizosphere isolates used in this study. TLC in combination with AHL biosensors provides a simple and rapid technique for characterizing and quantifying the AHL species produced by a given organism (17, 41). Dichloromethane extracts of spent culture supernatants of *P. putida* IsoF, *P. putida* Z2D, *R. aquatilis* TAA, and *R. aquatilis* T13 were analyzed as described in Materials and Methods. The two *P. putida* strains, Z2D and IsoF, exhibited very similar AHL patterns (Fig. 2). Using the bioluminescent monitor strain *E. coli* MT102(pSB403), four different AHL species were detected. Based on their mobilities (R_f values) and by including appropriate reference compounds, these molecules were tentatively identified as 3-oxo-C12-HSL, 3-oxo-C10-HSL, 3-oxo-C8-HSL, and 3-oxo-C6-HSL. The fact that these strains produced several long-chain AHL molecules provides a rationale for the strong stimulation of the F117(pKR-C12) monitor strain (Table 2).

The two *R. aquatilis* strains differed in their AHL patterns. While only a single AHL species, which was found to comigrate with 3-oxo-C6-HSL, was detected in the supernatant of strain T13, two AHL species were produced by strain TAA, one comigrating with 3-oxo-C6-HSL and the other comigrating with 3-oxo-C8-HSL. The production of different AHLs by the two strains also explains the observed differences in their abilities to activate the three AHL monitor strains in cross-streaking experiments. While strain T13 strongly stimulated the MG44(pJBA132) sensor, it did not activate F117(pKR-C12). In contrast, both sensors responded equally well to the presence of strain TAA, indicating the production of both long- and short-chain AHL molecules.

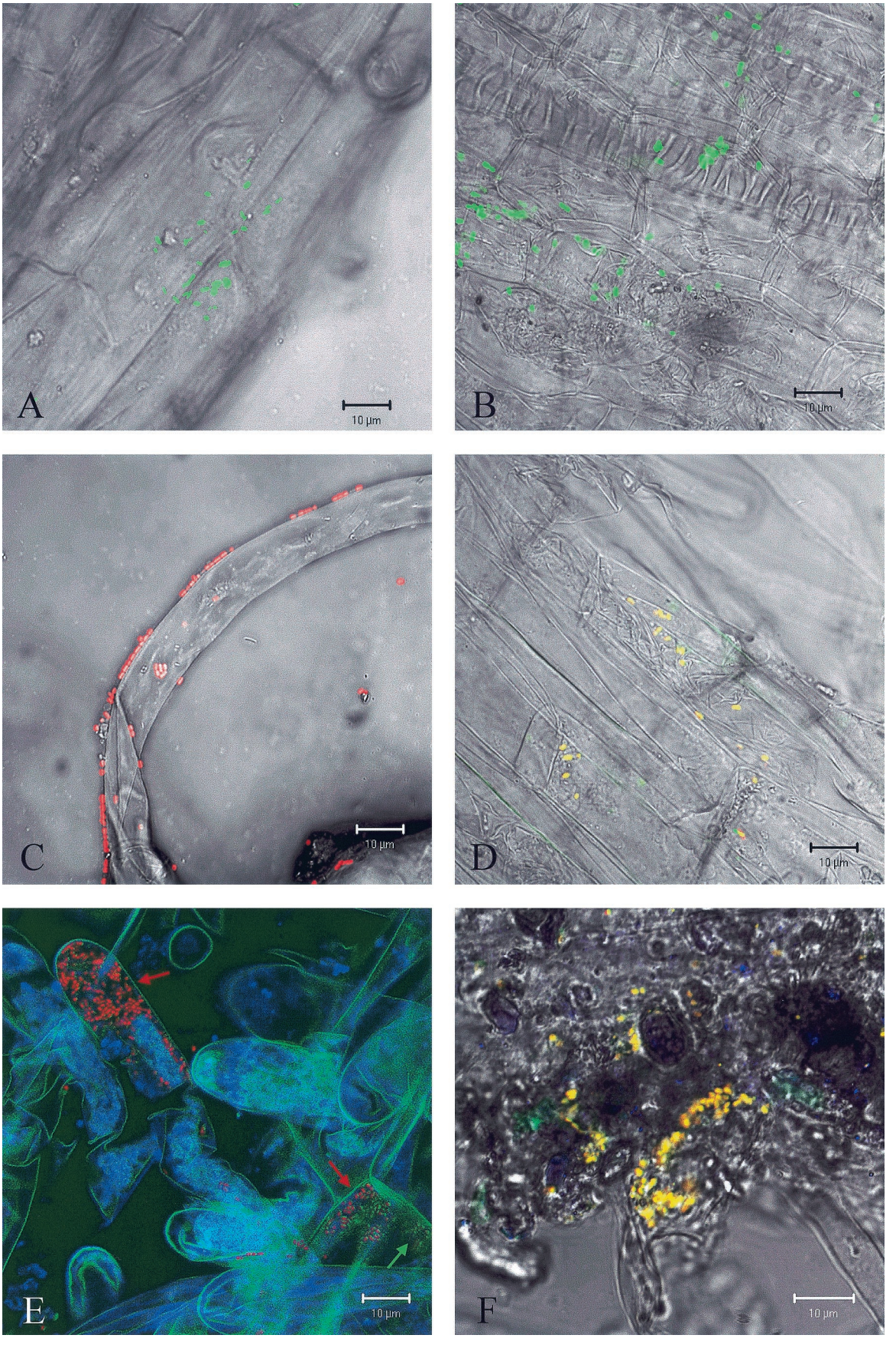
Cell-cell communication in the rhizosphere of axenic tomato plants. To investigate whether bacteria colonizing the rhizosphere do produce AHL molecules in situ, we inoculated axenically cultured tomato plants with an AHL monitor strain together with an AHL-producing rhizosphere isolate. The use of a gnotobiotic system has three major advantages: (i) the sensor and AHL-producing strains can be freely chosen, and thus pairs that show good results in the cross-streaking experiments can be analyzed under in situ conditions; (ii) there is a low autofluorescence background due to the use of sterile quartz sand instead of soil for the growth of tomatoes; and (iii) there is a high level of reproducibility. Four to 5 days after germination of the tomato seeds, the plants were inoculated with a mixture of a sensor strain and an AHL-producing strain. Ten days after inoculation, the plants were harvested and the roots were rinsed with PBS to remove adherent quartz particles. For detection of fluorescent cells, root samples were inspected with a confocal laser scanning microscope.

In all instances in which the sensor strains were coinoculated with an AHL-producing strain capable of activating the sensor strains in cross-streaking experiments, green fluorescent cells were detected, as shown in Fig. 3A and B for the pairs MG44(pJBA132)-T13 and F117(pAS-C8)-Z2D, respectively. As expected, no signals were detected when the sensor strains were inoculated alone (data not shown). In order to visualize the presence of the sensor strains in the rhizosphere, they were chromosomally tagged with DsRed. When these tagged monitor strains were used for inoculation of axenic tomato plants, red fluorescent cells were clearly visible on the surface of the roots, but none of the cells exhibited green fluorescence (Fig. 3C). However, in the presence of an AHL-producing strain, most cells were found to exhibit red as well as green fluorescence. As a consequence of the coexpression of Gfp and DsRed, the cells appeared yellow in the red-green double-exposure photomicrograph shown in Fig. 3D. In these experiments, we observed that activated cells of the monitor strain were not necessarily associated with microcolonies, suggesting that the AHLs may spread within the rhizosphere. To address this issue in better detail, we inoculated a tomato plant with the monitor strain MG44(pJBA132) together with *R. aquatilis* T13 tagged with DsRed. In support of our hypothesis, we observed that red fluorescent cells, which indicate the location of T13 cells, were often separated from the green fluorescent cells indicating the activated monitor cells (Fig. 3E).

In conclusion, these experiments provide strong evidence that AHLs are produced by bacteria colonizing the rhizosphere of axenically grown tomato plants and that these signals can be perceived by other bacteria. Moreover, our data suggest that AHLs are capable of spreading over relatively long distances in the rhizosphere.

Cell-cell communication in the rhizosphere of tomato plants grown in soil. The results obtained with the gnotobiotic tomato system encouraged us to investigate the possibility of AHL-

FIG. 3. AHL-mediated cell-cell communication in the tomato rhizosphere. Axenically grown tomato plants were inoculated with a mixture of a Gfp-based AHL monitor strain and an AHL-producing tomato isolate. (A) *S. liquefaciens* MG44(pJBA132) together with *R. aquatilis* T13. (B) *P. putida* F117(pAS-C8) together with *P. putida* Z2D. Root samples taken 10 days after inoculation were inspected by confocal laser scanning microscopy. Perception of AHL signal molecules is indicated by the appearance of green fluorescent cells. To visualize the distribution of the monitor strain on the tomato root, the monitor strain F117(pKR-C12) was tagged with DsRed. (D) In the presence of *P. putida* Z2D, most cells of the red fluorescent monitor strain were found also to be green fluorescent, as indicated by the appearance of yellow cells in the red-green double-exposure photograph. (C) When the monitor strain was inoculated alone, only red fluorescent cells were observed, excluding the possibility that the monitor strain is stimulated by the plant. Green fluorescent cells of the monitor strain were often found to be separated from the AHL-producing strains. This finding was particularly apparent when the monitor strain was coinoculated with a DsRed-tagged AHL-producing strain. (E) Results of a colonization experiment using a mixture of the monitor strain MG44(pJBA132) and a DsRed-tagged derivative of *R. aquatilis* T13 (red arrow). The green arrow indicates the position of activated sensor cells. (F) The DsRed-tagged monitor strain KS35 (F117::Tn*5*-LAS) was also stimulated when it was used for the inoculation of tomato plants grown in nonsterile soil, indicating the production of AHL molecules by the indigenous rhizosphere community.


A
10 µm
B
10 µm
C
10 µm
D
10 µm
E
10 µm
F
10 µm

mediated communication in the rhizosphere of soil-grown tomato plants. Despite the fact that the sensor plasmids were relatively stable in test tube experiments, e.g., less that 20% plasmid loss after 100 generations (data not shown), we were concerned that, due to increased competition pressure, plasmid loss might be a problem in the nonsterile system. Hence, we integrated the *las*-based monitor cassette into the chromosome of the DsRed-tagged strain *P. putida* F117. As shown in Fig. 1C, this monitor strain exhibited virtually the same specificity as the plasmid-based monitor strain, albeit with a slightly reduced sensitivity due to the reduced copy number of the sensor cassette. Half-maximal activation was observed in the presence of approximately 160 nM 3-oxo-C12-HSL in the microtiter assay, and a detection limit of about 40 nM was obtained for single-cell inspection. The specificity remained highest for 3-oxo-C12-HSL, followed by 3-oxo-C10-HSL and C12-HSL.

In a first step, this monitor strain was used to inoculate tomato plants grown in nonsterile soil together with the untagged wild-type strain IsoF. As for the axenically grown plants, we observed that most red fluorescent cells also exhibited green fluorescence, indicating that IsoF also produces AHL in the natural root habitat. However, in contrast to the gnotobiotic system, we also detected green fluorescent cells when the monitor strain was inoculated alone (Fig. 3F). These results clearly demonstrate that the indigenous rhizosphere community produces AHLs at concentrations high enough to activate the monitor strain and thus provide strong evidence that these signal molecules are utilized by the consortium for communication between different populations.

DISCUSSION

Evidence that has accumulated over the past few years has firmly established that a large number of *Proteobacteria* synthesize AHL signal molecules (for reviews, see references 11 and 15). This appears to be particularly true for bacteria which are associated with plants. In an extensive survey, Cha et al. (5) demonstrated that the majority of plant-associated bacteria produce AHL signal molecules. Specifically, they showed that almost all tested isolates of the genera *Agrobacterium*, *Pantoea*, and *Rhizobium* and about half of the erwinias and pseudomonads tested synthesized detectable levels of AHLs. In contrast, only a few AHL producers could be identified among *Xanthomonas* isolates. More recently, Elasri et al. (13) screened 137 soilborne and plant-associated strains belonging to different *Pseudomonas* species for their ability to synthesize AHLs. Of all the strains tested, 54 were positive for AHL production. Most interestingly, however, was the observation that plant-associated and plant-pathogenic bacteria produced AHLs more frequently than did soilborne strains. On the basis of these results, the authors suggested that the closer the relationship of the bacteria with the host plant, the higher the probability that it produces AHLs. We screened over 300 bacterial strains isolated from the rhizosphere of tomato plants on standard laboratory media for their ability to synthesize AHLs. Approximately 12% of the strains provoked a clear response in one or more of the AHL monitor strains described in this report. This result is in very good agreement with the results of Pierson et al. (34), who screened 700 strains of wheat root-associated bacteria with the aid of different AHL biosensors and found that about 8% of the strains were able to activate at least one of the sensors.

Given that many gram-negative bacteria that colonize the roots of tomato plants utilize the same chemical language, it appears likely that AHL signal molecules are used not only as population density sensors in one species but also for communication between cells of different species. The concept of interspecies communication was already introduced in 1979, when it was observed that *Vibrio harveyi* was stimulated to produce light following the addition of cell-free culture fluid from several species of nonluminous bacteria (19). This stimulation was attributed to the presence of a so-called autoinducer, the structure of which remained unidentified at that time. It was not until recently that it was shown that *V. harveyi* in fact utilizes two interlinked quorum-sensing systems to control the bioluminescence phenotype (14). One of these systems relies on the AHL molecule 3-hydroxy-L-butanoyl-homoserine lactone (3-OH-C4-HSL), while the cognate signal molecule (HAI-2) of the second system has not yet been identified. However, while HAI-2 has been demonstrated to be produced by a large number of bacteria (43), including *E. coli* and *Salmonella enterica* serovar Typhimurium, 3-OH-C4-HSL has so far been detected only in culture supernatants of *Vibrio parahaemolyticus* and *Xenorhabdus nematophilus* as well as *V. harveyi* (3, 9).

Evidence for interpopulation signaling via AHL molecules has been presented in previous studies. McKenney et al. (30) demonstrated that spent culture supernatants of *P. aeruginosa* enhance virulence factor production in *B. cepacia* and that this effect was attributable to the presence of AHLs in the *P. aeruginosa* supernatants. More important in the context of this study is the recent demonstration of AHL-mediated cross-communication in the rhizosphere of wheat (34). Using an AHL-specific reporter strain, *P. aureofaciens* 30-84Ice/I, which expresses ice nucleation activity from the AHL-controlled phenazine promoter of the organism, the authors demonstrated that AHLs produced by coinoculated bacterial populations are perceived by the reporter strain (34). These results provided strong evidence that AHLs may serve as interpopulation signals in the wheat rhizosphere. However, despite the fact that nucleation activity can be measured with a very high sensitivity even in complex samples, this reporter strain does not allow the detection of gene expression at the single-cell level. Moreover, since the measurement of nucleation activity requires processing of the sample, information about the spatial localization of the cells is lost.

To obtain direct visual evidence for AHL-mediated cross-communication between bacteria, we used three Gfp-based AHL sensor cassettes which respond to different spectra of AHL molecules, depending on the components used for their construction. In previous studies, Gfp-based AHL sensor plasmids were used for the detection of AHL molecules in the lung tissues of mice infected with *P. aeruginosa* (52), for visualization of interspecies communication between *P. aeruginosa* and *B. cepacia* in mixed biofilms (38), and for the analysis of quorum-sensing inhibition by halogenated furanone compounds in swarming colonies of *S. liquefaciens* MG1 (37) and in *P. aeruginosa* biofilms (Hentzer et al., submitted). In order to visualize cell-cell communication in the tomato rhizosphere, different

AHL sensor plasmids were transferred into *P. putida* F117 and *S. liquefaciens* MG44, both of which are good root colonizers. To our surprise, we observed that the functionality of the sensor plasmids was highly dependent on the background strain. While plasmids pKR-C12 and pAS-C8 worked well in *P. putida* F117, they did not work in *S. liquefaciens* MG44. Conversely, pJBA132 functioned only in the latter strain. The reasons for these effects are at present unclear. However, given that cells are freely permeable for short-chain AHLs but that long-chain AHLs are actively transported (32), it is tempting to speculate that differences in the presence and/or specificity of long-chain AHL transporters in the two strains may account for the observed differences.

To monitor bacterial cells in the rhizosphere of tomato plants, we integrated a cassette expressing DsRed from *Discosoma* sp. into the chromosomes of various strains. The tagging of strains served two purposes: (i) to ensure the presence of the AHL monitor strains in the rhizosphere when no activation was observed and (ii) to visualize the spatial arrangement of AHL monitor strains and coinoculated AHL producer strains. These experiments also revealed important information about differences in the colonization patterns of the strains used in this study. In agreement with previous studies of the colonization behavior of pseudomonads (6), we observed that *P. putida* IsoF cells tended to form microcolonies on the root surface. Similar colonization patterns were also observed with the two *Rahnella* strains used in this study. In contrast, *S. liquefaciens* cells were preliminarily found as single cells and did not form aggregates on the surface of the root.

Using axenically grown tomato plants that were inoculated with a mixture of an AHL monitor and an AHL producer, we were able to visualize in situ cell-cell communication between defined bacterial populations. Our experiments revealed that activated cells of the monitor strains were not necessarily associated with microcolonies, indicating that AHLs are capable of spreading over relatively long distances along the root surface. In fact, we observed that microcolonies of the two *P. putida* F117 monitor strains were usually well separated from those of the cocolonizing AHL producer strains. Previous work has shown that the root epidermis is often covered by a distinct matrix, the so-called mucigel (21). Recent electron microscopy studies provided evidence that microcolonies of a *Pseudomonas fluorescens* biocontrol strain are embedded in this mucoid matrix (6). Hence, one possible explanation of our observations could be that AHL signal molecules spread within the mucigel layer. This hypothesis is further corroborated by the finding that inoculation of 50 μl of 3-oxo-C12-HSL (100 μM) at a single location on the top part of a tomato root colonized by *P. putida* KS35 leads to the activation of sensor cells on the entire root surface after 12 h of incubation (data not shown). However, further work will be needed to assess the full extent of the mobility of the different AHL molecules within the rhizosphere.

Despite that fact that only a minority of bacterial isolates from the tomato rhizosphere were capable of synthesizing AHL molecules, we were able to detect the production of AHLs by the indigenous rhizosphere community of plants grown in nonsterile soil. Given that the AHL monitor F117::Tn*5*-LAS used in these experiments is activated only in the presence of at least 40 nM 3-oxo-C12-HSL, for which the sensor is most sensitive, it is reasonable to assume that the concentrations of AHLs in the rhizosphere are biologically meaningful. For *V. fischeri* it has been demonstrated that a 3-oxo-C6-HSL concentration as low as 10 nM is sufficient to trigger the induction of bioluminescence (23), representing the archetypic AHL-controlled phenotype.

In conclusion, our data provide strong evidence that AHL molecules are produced by the bacterial consortium naturally colonizing the roots of tomato plants and strongly support the view that these molecules may act as signals for coordinating the functions of the different populations within the community. Work is currently under way to investigate the role of AHL-mediated cell-cell communication in the composition and architecture of the rhizosphere community.

ACKNOWLEDGMENTS

We thank H. P. Schweitzer and M. E. Kovach for providing bacterial strains and plasmids and P. Williams for the generous gift of synthetic AHLs.

This work was supported by the BMBF.

REFERENCES

1. **Andersen, J. B., A. Heydorn, M. Hentzer, L. Eberl, O. Geisenberger, B. B. Christensen, S. Molin, and M. Givskov.** 2001. *gfp*-based *N*-acyl-homoserine lactone sensor systems for detection of bacterial communication. Appl. Environ. Microbiol. **67:**575–585.
2. **Andersen, J. B., C. Sternberg, L. K. Poulsen, S. P. Bjørn, M. Givskov, and S. Molin.** 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol. **64:**2240–2246.
3. **Bassler, B. L., E. P. Greenberg, and A. M. Stevens.** 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J. Bacteriol. **179:**4043–4045.
4. **Bertani, G.** 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62:**293–300.
5. **Cha, C., P. Gao, Y. Chen, P. D. Shaw, and S. K. Farrand.** 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact. **11:**1119–1129.
6. **Chin-A-Woeng, T. F. C., W. de Priester, A. J. van der Bij, and B. J. J. Lugtenberg.** 1997. Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. Mol. Plant-Microbe Interact. **10:**79–86.
7. **Christensen, B. B., C. Sternberg, J. B. Andersen, R. J. Palmer, Jr., A. T. Nielsen, M. Givskov, and S. Molin.** 1999. Molecular tools for study of biofilm physiology. Methods Enzymol. **310:**20–42.
8. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived mini-transposons. Methods Enzymol. **235:**386–405.
9. **Dunphy, G., C. Miyamoto, and E. Meighen.** 1997. A homoserine lactone autoinducer regulates virulence of an insect-pathogenic bacterium, *Xenorhabdus nematophilus* (*Enterobacteriaceae*). J. Bacteriol. **179:**5288–5291.
10. **Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry **20:**2444–2449.
11. **Eberl, L.** 1999. *N*-Acyl homoserinelactone-mediated gene regulation in gram-negative bacteria. Syst. Appl. Microbiol. **22:**493–506.
12. **Eberl, L., M. K. Winson, C. Sternberg, G. S. A. B. Stewart, G. Christiansen, S. R. Chhabra, B. Bycroft, P. Williams, S. Molin, and M. Givskov.** 1996. Involvement of *N*-acyl-L-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. Mol. Microbiol. **20:**127–136.
13. **Elasri, M., S. Delorme, P. Lemanceau, G. Stewart, B. Laue, E. Glickmann, P. M. Oger, and Y. Dessaux.** 2001. Acyl-homoserine lactone production is more common among plant-associated *Pseudomonas* spp. than among soilborne *Pseudomonas* spp. Appl. Environ. Microbiol. **67:**1198–1209.
14. **Freeman, J. A., and B. L. Bassler.** 1999. Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. J. Bacteriol. **181:**899–906.
15. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727–751.
16. **Givskov, M., and S. Molin.** 1992. Expression of extracellular phospholipase from *Serratia liquefaciens* is growth-phase-dependent, catabolite-repressed and regulated by anaerobiosis. Mol. Microbiol. **6:**1363–1374.
17. **Gotschlich, A., B. Huber, O. Geisenberger, A. Tögl, A. Steidle, K. Riedel, P. Hill, B. Tümmler, P. Vandamme, B. Middleton, M. Camara, P. Williams, A.**

Hardman, and L. Eberl. 2001. Synthesis of multiple *N*-acyl-homoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex. Syst. Appl. Microbiol. **24:**1–14.

18. **Gray, K. M., J. P. Pearson, J. A. Downie, B. E. Boboye, and E. P. Greenberg.** 1996. Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of stationary phase and rhizosphere-expressed genes. J. Bacteriol. **178:**372–376.
19. **Greenberg, E. P., J. W. Hastings, and S. Ulitzur.** 1979. Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. Arch. Microbiol. **120:**87–91.

19a. **Hentzer, M., K. Riedel, T. B. Rasmussen, A. Heydron, J. B. Anderson, M. R. Parsek, S. Rice, L. Eberl, S. Molin, S. Kjelleberg, and Giuskov.** Interference with *Oseydinibas aerygubisa* quorum-sensing by a halogenated furanone compound. Microbiology, in press.

20. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
21. **Jenny, H., and K. Grossenbacher.** 1963. Root-soil boundary zones as seen in the electron microscope. Soil Sci. Soc. Am. Proc. **27:**273–277.
22. **Kalbe, C., P. Marten, and G. Berg.** 1996. Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. Microbiol. Res. **151:**433–439.
23. **Kaplan, H. B., and E. P. Greenberg.** 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. J. Bacteriol. **163:**1210–1214.
24. **Kessler, B., V. de Lorenzo, and K. N. Timmis.** 1992. A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the *Pm* promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. **233:**293–301.
25. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, I. I., and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
26. **Lewenza, S., B. Conway, E. P. Greenberg, and P. A. Sokol.** 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J. Bacteriol. **181:**748–756.
27. **Lithgow, J. K., A. Wilkinson, A. Hardman, B. Rodelas, F. Wisniewski-Dye, P. Williams, and J. A. Downie.** 2000. The regulatory locus *cinRI* in *Rhizobium leguminosarum* controls a network of quorum-sensing loci. Mol. Microbiol. **37:**81–97.
28. **Liu, Y., Y. Cui, A. Mukherjee, and A. K. Chatterjee.** 1998. Characterization of a novel RNA regulator of *Erwinia carotovora ssp. carotovora* that controls production of extracellular enzymes and secondary metabolites. Mol. Microbiol. **29:**219–234.
29. **McCullagh, M., R. Utkhede, J. G. Menzies, Z. K. Punja, and T. C. Paulitz.** 1996. Evaluation of plant growth-promoting rhizobacteria for biological control of *Pythium* root rot of cucumbers grown in rockwool and effects on yield. Eur. J. Plant Pathol. **102:**747–755.
30. **McKenney, D., K. E. Brown, and D. G. Allison.** 1995. Influence of *Pseudomonas aeruginosa* exoproducts on virulence factor production in *Burkholderia cepacia*: evidence of interspecies communication. J. Bacteriol. **177:**6989–6992.
31. **Meissner, R., Y. Jacobson, S. Melamed, S. Levyatuv, G. Shalev, A. Ashri, Y. Elkind, and A. Levy.** 1997. A new model system for tomato genetics. Plant J. **12:**1465–1472.
32. **Pearson, J. P., C. Van Delden, and B. H. Iglewski.** 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J. Bacteriol. **181:**1203–1210.
33. **Pearson, J. P., E. C. Pesci, and B. H. Iglewski.** 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179:**5756–5767.
34. **Pierson, E. A., D. W. Wood, J. A. Cannon, F. M. Blachere, and L. S. Pierson III.** 1998. Interpopulation signaling via *N*-acyl-homoserine lactones among bacteria in the wheat rhizosphere. Mol. Plant-Microbe Interact. **11:**1078–1084.
35. **Pierson, L. S., III., D. W. Wood, and E. A. Pierson.** 1998. Homoserine lactone-mediated regulation in plant-associated bacteria. Annu. Rev. Phytopathol. **36:**207–225.
36. **Piper, K. R., S. B. von Bodman, and S. K. Farrand.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature **362:**448–450.
37. **Rasmussen, T. B., M. Manefield, J. B. Andersen, L. Eberl, U. Anthoni, C. Christophersen, P. Steinberg, S. Kjelleberg, and M. Givskov.** 2000. How *Delisea pulchra* furanones affect quorum-sensing and swarming motility in *Serratia liquefaciens* MG1. Microbiology **146:**3237–3244.
38. **Riedel, K., M. Hentzer, O. Geisenberger, B. Huber, A. Steidle, A. Gotschlich, H. Wu, M. Givskov, S. Molin, and L. Eberl.** AHL-mediated intergeneric communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology, in press.
39. **Rodelas, B., J. K. Lithgow, F. Wisniewski-Dye, A. Hardman, A. Wilkinson, A. Economou, P. Williams, and J. A. Downie.** 1999. Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. viciae. J. Bacteriol. **181:**3816–3823.
40. **Schripsema, J., K. E. de Rudder, T. B. van Vliet, P. P. Lankhorst, E. de Vroom, J. W. Kijne, and A. A. van Brussel.** 1996. Bacteriocin small of *Rhizobium leguminosarum* belongs to the class of *N*-acyl-L-homoserine lactone molecules known as autoinducers and as quorum sensing co-transcription factors. J. Bacteriol. **178:**366–371.
41. **Shaw, P. D., G. Ping, S. Daly, C. Cha, J. E. Cronan, Jr., K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. USA **94:**6036–6041.
42. **Simons, M., A. J. van der Bij, I. Brand, L. A. de Weger, C. A. Wijffelman, and B. J. J. Lugtenberg.** 1996. Gnotobiotic system for studying rhizosphere colonization by plant growth promoting *Pseudomonas* bacteria. Mol. Plant-Microbe Interact. **9:**600–607.
43. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. USA **96:**1639–1644.
44. **Thorne, S. H., and H. D. Williams.** 1999. Cell density-dependent starvation survival of *Rhizobium leguminosarum* bv. phaseoli: identification or the role of an *N*-acyl-homoserine lactone in adaptation to stationary-phase survival. J. Bacteriol. **181:**981–990.
45. **Tolker-Nielsen, T., U. C. Brinch, P. C. Ragas, J. B. Andersen, C. S. Jacobsen, and S. Molin.** 2000. Development and dynamics of *Pseudomonas* sp. biofilms. J. Bacteriol. **182:**6482–6489.
46. **Tuomainen, J., C. Betz, J. Kangasjärvi, D. Ernst, Z. H. Yin, C. Langebartels, and H. Sandermann.** 1997. Ozone induction of ethylene emission in tomato plants: regulation by differential accumulation of transcripts for the biosynthetic enzymes. Plant J. **12:**1151–1162.
47. **Von Bodman, S. B., D. R. Majerczak, and D. L. Coplin.** 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii subsp. stewartii*. Proc. Natl. Acad. Sci. USA **95:**7687–7692.
48. **Weller, D. M.** 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26:**379–407.
49. **Wijffelman, C. A., E. Pees, A. A. N. van Brussel, and P. J. J. Hooykaas.** 1983. Repression of small bacteriocin excretion in *Rhizobium leguminosarum* and *Rhizobium trifolii* by transmissible plasmids. Mol. Gen. Genet. **192:**171–176.
50. **Winson, M. K., S. Swift, L. Fish, J. P. Throup, F. Jørgensen, S. R. Chhabra, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart.** 1998. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl-homoserine lactone-mediated quorum sensing. FEMS Microbiol. Lett. **163:**185–192.
51. **Wood, D. W., F. Gong, M. M. Daykin, P. Williams, and L. S. Pierson III.** 1997. *N*-Acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30–84 in the wheat rhizosphere. J. Bacteriol. **179:**7663–7670.
52. **Wu, H., Z. Song, M. Hentzer, J. B. Andersen, A. Heydorn, K. Mathee, C. Moser, L. Eberl, S. Molin, N. Høiby, and M. Givskov.** 2000. Detection of *N*-acyl-homoserine lactones in lung tissues infected with *Pseudomonas aeruginosa*. Microbiology **146:**2481–2493.
53. **Zhang, L., P. J. Murphy, A. Kerr, and E. M. Tate.** 1993. *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. Nature **362:**446–448.

Anhang 2

Identification and characterization of a *N*-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* IsoF

ANETTE STEIDLE, MARIE ALLESEN-HOLM, KATHRIN RIEDEL, GABRIELE BERG, MICHAEL GIVSKOV, SØREN MOLIN, AND LEO EBERL

APPLIED AND ENVIRONMENTAL MICROBIOLOGY

Eingereicht am 03.06.2002

Identification and characterization of a *N*-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* IsoF

Anette Steidle[1], Marie Allesen-Holm[2], Kathrin Riedel[1], Gabriele Berg[3], Michael Givskov[2], Søren Molin[2] and Leo Eberl[1*]

[1]Department of Microbiology, TUM, Am Hochanger 4, D-85350 Freising, Germany
[2]Department of Microbiology, DTU, Building 301, DK-2800 Lyngby, Denmark
[3]Department of Microbiology, University of Rostock, D-18051 Rostock, Germany

Corresponding author: Leo Eberl. Tel: +49 8161 715446, Fax: +49 8161 715475
e-mail: EBERL@mikro.biologie.tu-muenchen.de

Running title: Quorum sensing in *Pseudomonas putida.*

Keywords: Quorum sensing, biofilm, *Pseudomonas putida*

Subject category: Genetics and Molecular Biology

Abstract

Recent reports have shown that several strains of *Pseudomonas putida* produce *N*-acylhomoserine lactones (AHLs). These signal molecules enable bacteria to co-ordinately express certain phenotypic traits in a density-dependent manner in a process referred to as quorum sensing. In this study we have cloned a genomic region of the plant growth promoting *P. putida* strain IsoF that, when present in *trans*, provoked induction of a bioluminescent AHL reporter plasmid. Sequence analysis identified a gene cluster consisting of four genes: *ppuI* and *ppuR*, whose predicted amino acid sequences are highly similar to proteins of the LuxI-LuxR family, an open reading frame (ORF) located in the intergenic region between *ppuI* and *ppuR* with significant homology to *rsaL* from *Pseudomonas aeruginosa*, and a gene, designated *ppuA,* present upstream of *ppuR*, the deduced amino acid sequence of which shows similarity to long chain fatty acid CoA ligases from various organisms. Using a transcriptional *ppuA*::*luxAB* fusion we demonstrate that expression of *ppuA* is AHL-dependent. Furthermore, transcription of the AHL synthase *ppuI* is shown to be subject to quorum sensing regulation, creating a positive feedback loop. Sequencing of the DNA regions flanking the *ppu* gene cluster indicated that the four genes form an island in the *suhB*-PA3819 intergenic region of the currently sequenced *P. putida* strain KT2440. While the wild type strain formed very homogenous biofilms, both a *ppuI* and a *ppuA* mutant formed structured biofilms with characteristic microcolonies and water-filled channels. These results suggest that the quorum-sensing system is influencing biofilm structural development.

Introduction

Evidence that accumulated over the past few years established that numerous bacteria employ cell-cell communication systems that rely on small signal molecules to express certain phenotypic traits in a density-dependent manner. This regulatory principle is now generally referred to as quorum sensing (for reviews see 13, 17, 49). Most quorum-sensing systems thus far identified in gram-negative bacteria utilize *N*-acyl-homoserine lactone (AHL) signal molecules, which are usually synthesized by a member of the LuxI protein family. As the density of the population increases, AHL molecules accumulate in the growth medium and upon reaching a critical threshold concentration bind to their cognate receptors (a member of the LuxR protein family). The receptor/AHL complex then activates or represses target gene expression.

AHL-dependent quorum-sensing systems have been identified in more than 50 species, which inhabit various ecosystems. Many of the AHL-producing bacteria, however, are associated with plants. In fact, Cha *et al*. (6) demonstrated that the majority of plant-associated bacteria produce AHL signal molecules. Specifically, they showed that almost all tested isolates of the genera *Agrobacterium*, *Pantoea*, and *Rhizobium*, and about half of the erwinias and pseudomonads tested synthesized detectable levels of AHLs. By contrast, only a few AHL producers could be identified among *Xanthomonas* isolates. More recently, Elasri *et al*. (14) screened 137 soilborne and plant-associated strains belonging to different *Pseudomonas* species. Of all the strains tested 54 (39 %) were positive for AHL production. In the same vein Pierson *et al*. (37), who screened 700 isolates of wheat root-associated bacteria by the aid of different AHL biosensors, showed that about 8 % of the strains were able to activate at least one of the sensors. Given that a large proportion of the bacteria colonizing the roots of plants produces AHL signals it is not too surprising that these pheromones not only serve as population density sensors of one species but also for communication between cells of different species (37, 45). These interactions appear to be important for coordinating the functions of the different populations within the rhizosphere community.

Many root-colonizing fluorescent pseudomonads can promote plant growth and exhibit biocontrol potential against certain pathogens (48, 38). A well-known example is *Pseudomonas aureofaciens* strain 30-84, which is used as a biocontrol agent to protect wheat from take-all disease caused by *Gaeumannomyces graminis* var. *tritici.* When this strain is present in the rhizosphere of wheat the severity of the disease is strongly reduced due to the

production of phenazine antibiotics, which are active against the ascomycete fungus. Synthesis of these antibiotic compounds is regulated by the PhzR-PhzI quorum-sensing system that utilizes the AHL molecule *N*-hexanoyl-L-homoserine lactone (C6-HSL) (54). Recently, a second quorum-sensing system, which was designated CsaR-CsaI, was identified in the strain (55). This system is not involved in the regulation of phenazine production but controls biosynthesis of cell surface components and plays an important role in the rhizosphere competence of the strain. *Pseudomonas fluorescens* F113 is capable of protecting sugar beet seedling from damping-off disease caused by *Pythium ultimum* (16, 43). The biocontrol capability of the strain resides, at least in part, in the production of the antifungal agent 2,4-diacetylphloroglucinol. Evidence has been provided that *P. fluorescens* F113 produces at least three different AHLs with the aid of a novel AHL synthase, HdtS, which is not related to members of the LuxI protein family (29). Although no definite phenotype has yet been linked to the quorum-sensing system operating in this strain, it has been speculated that AHLs may be involved in regulating the biocontrol properties of the organism.

Some *Pseudomonas putida* strains not only synthesize secondary metabolites, which exhibit antagonistic activities against plant pathogens (48), but they also have the ability to promote plant growth and to degrade toxic organic compounds. Thus, *P. putida* is a highly attractive candidate for agricultural and environmental uses. Recent work has shown that some *P. putida* strains produce AHL signal molecules (26, 14, 42, 3), suggesting that they are capable of triggering expression of certain functions in a cell density-dependent manner. However, neither have the genes encoding the components of the *P. putida* quorum-sensing system been identified nor has a definite AHL-regulated gene or phenotype been described. In this study, we report the cloning and molecular characterization of the *ppu* quorum-sensing locus of *P. putida* IsoF.

Materials and Methods

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Unless otherwise stated, all strains were grown aerobically in modified Luria-Bertani medium (4) containing 4 g of NaCl/l instead of 10 g of NaCl/l at 30 °C (*Pseudomonas putida*) or 37 °C (*Escherichia coli*). Solid media contained 15 g/l agar. For selection of pseudomonads, cells were grown on *Pseudomonas* Isolation Agar (PIA) (Difco). Antibiotics were added as required at final concentrations of 10 mg/l tetracycline, 25 mg/l gentamicin for *E. coli* and 50 mg/l for *P. putida*, 50 mg/l kanamycin for *E. coli* and 100 mg/l for *P. putida*, 10 mg/l chloramphenicol, 50 mg/l streptomycin, and 100 mg/l ampicillin. Growth of liquid cultures was monitored spectrophotometrically by a Ultraspec Plus spectrophotometer (Pharmacia) by measurement of optical density at 600 nm (OD_{600}).

Conjugative plasmid transfer. Plasmids were delivered to *P. putida* by tri-parental mating as described previously (7). Briefly, donor and recipient strains as well as helper strain *E. coli* HB101(pRK600) were grown overnight in 5 ml LB supplied with the appropriate antibiotics. Following subculturing to an OD_{600} of 0.9, the cells from 2 ml culture were harvested by centrifugation, washed, and resuspended in 200 µl LB. Donor and helper cells (100 µl each) were carefully mixed and incubated for 20 min. at room temperature. Then, 200 µl of the recipient cells were added and the mixture was spot-inoculated onto the surface of prewarmed LB plates. After overnight incubation at 30 °C, the cells were scraped off and resuspended in 1 ml of 0.9 % NaCl. Serial dilutions were plated on PIA containing the appropriate antibiotic for selection of transconjugants.

DNA manipulation and nucleotide sequencing. Cloning, restriction enzyme analysis, and transformation of *E. coli* were performed essentially as described previously (41). Polymerase chain reaction (PCR) was performed using Herculase (Stratagene, La Jolla, CA, USA). Plasmid DNA was isolated with the QIAprep Spin Miniprep kit and chromosomal DNA from *P. putida* was purified with the DNeasy Tissue kit. DNA fragments were purified from agarose gels using the QIAquick Gel Extraction kit (all kits are from QIAGEN, Hilden, Germany). DNA probes were labelled with Digoxygenin and detected using the DNA labelling and detection kit (Roche, Mannheim, Germany).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Bacterial strains		
Escherichia coli		
CC118 λ *pir*	Δ*(ara-leu) araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ *pir* lysogen	20
HB101	*recA thi pro leu hsd*$^{-}$*M*$^{+}$ Sm^{R}	24
MT102	*araD139* (*ara-leu*)7697 Δ*lac thi hsdR*	Laboratory collection
XL1Blue	*recA1 endA1 gyrA*96 *thi-1 hsdR17 supE44 relA1 lac* [*F'proAB lacI*^{q} ZΔM15 Tn*10* (Tet^{R})]	Stratagene
Pseudomonas putida		
IsoF	wild type isolate from tomato roots	45
F117	Km^{R}, *ppuI*::*npt* mutant of IsoF	This study
IsoF-D4	*ppuA*::*luxAB* mutant of IsoF	This study
F117-D1	Km^{R}, *ppuI*::*npt*, *ppuA*::*luxAB* double mutant of IsoF	This study
Plasmids		
pAS-L22	pGA-L14 containing a P_{ppuI}-*luxAB* transcriptional fusion	This study
pBBR1MCS-2	broad host range plasmid, *lacZ*α; Kan^{R}	27
pBBR1MCS-5	broad host range plasmid, *lacZ*α; Gm^{R}	27
pBK-miniTn*7*-gfp3	pUC19-based delivery plasmid for miniTn*7*-gfp3; Km^{R}, Ap^{R}, Cm^{R}, Sm^{R}, *mob*$^{+}$	25
pEX18Gm	Gm^{R}, *oriT*$^{+}$ *sacB*$^{+}$, gene replacement vector with multiple cloning site from pUC18	21
pEXF1	Gm^{R}, Km^{R}, pEX18Gm derivative for inactivation of *ppuI*	This study
pEXF2	Gm^{R}, pEX18Gm derivative for inactivation of *ppuA*	This study
pGA-L14	broad host range *luxAB*-based promoter probe vector	Laboratory collection
pGEM®-3Zf(+)	*lacZ*α, cloning vector; Ap^{R}	Promega, Madison, USA
pGF1	2.9 kb *Sal*I chromosomal fragment containing *ppuI-rsaL-ppuR* of IsoF cloned into pGEM®-3Zf(+)	This study
pGF3	1.4 kb *Eco*RI chromosomal fragment containing *ppuI* of IsoF cloned into pBBR1MCS-5	This study
pGF4	Km^{R}, 13 kb *Sac*I chromosomal fragment containing the *ppu* locus of F117 plus flanking regions cloned into pGEM®-3Zf(+)	This study
pKR-C12	AHL sensor plasmid; pBBR1MCS-5 carrying P_{lasB}-*gfp*(ASV)-P_{lac}-*lasR*; Gm^{R}	39
pPPUR1	pGEM®-3Zf(+) containing the *ppuR* gene of IsoF	This study
pRK600	ColE1 RK2-Mob^{+}, RK2-Tra^{+}; helper plasmid; Cm^{R}	12
pSB403	bioluminescent AHL sensor plasmid; Tet^{R}	53
pUX-BF13	R6K replicon-based helper plasmid, providing the Tn*7* transposition function in *trans*, Ap^{R}, *mob*$^{+}$	2

For flow-chamber experiments, the strains were tagged with the green fluorescent protein (GFP). This was accomplished by the insertion of a miniTn*7*-$P_{A1/04/03}$-*gfp*-T_0T_1 transposon cassette into the chromosomes of target strains using the suicide construct pBK-miniTn*7*-gfp3 (25). Plasmid pBK-miniTn*7*-gfp3 was delivered to target strains by four-parental mating using *E. coli* HB101(pRK600) and *E. coli* XL1Blue(pUX-BF13) as helper strains.

Cloning of *ppuI* and *ppuR*. The AHL synthase of *P. putida* IsoF was cloned by complementation of an AHL biosensor strain as described previously (47). Briefly, a chromosomal bank of *P. putida* IsoF was constructed in plasmid pGEM-3Zf(+) using the restriction enzyme *Sal*I. The resulting plasmids were electroporated into *E. coli* MT102 harboring the AHL sensor plasmid pSB403 (53). This sensor plasmid contains the *Photobacterium fischeri luxR* gene together with a transcriptional fusion of the *luxI* promoter to the promoterless bioluminescence gene cluster *luxCDABE* of *Photorhabdus luminescens.* In the presence of a recombinant plasmid containing a *luxI* homologous gene light emission of plasmid pSB403 is induced due to the synthesis of AHLs. Bioluminescent clones were detected using a highly sensitive photon-counting camera (C2400-40; Hamamatsu Photonics, Herrsching, Germany). A recombinant plasmid, designated pGF1, conferring a bioluminescent phenotype when present in *E. coli* MT102(pSB403), was isolated. DNA sequencing of the plasmid, which contained a 2.9 kb chromosomal *Sal*I DNA fragment, was performed using the M13 universal sequencing primers and specifically designed primers as sequence information became available. DNA sequences were compared to other sequences in GenBank using the on-line BLAST search engine (1) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Construction of a defined *ppuI* mutant. A defined *ppuI* mutant was constructed by the gene replacement method described by Hoang *et al.* (21). The mutant allele was constructed from pGF1 by blunt end ligation of the *npt* gene from Transposon Tn*903* (32) into a unique *Apa*I restriction site at position 225 bp of *ppuI*. Next, he *ppuI*::*npt* cassette was cloned into the *Sal*I site of pEX18Gm. The resulting plasmid, pEXF1, was mobilized into IsoF by triparental mating and gene replacement mutants were selected on PIA medium containing 100 mg/l kanamycin and 5 % sucrose. Insertion of the kanamycin cassette into the *ppuI* gene was confirmed by Southern Blot hybridisation using an 1.4 kb *Eco*RI fragment of pGF1 containing *ppuI* as a probe. This 1.4 kb fragment was also cloned into the *Eco*RI site of pBBR1MCS-5 and the resulting plasmid pGF3 was used for complementation purposes.

Cloning and chromosomal inactivation of *ppuA*. A gene library of *P. putida* F117 was constructed by ligating *Sac*I digested chromosomal DNA into the compatible sites of pGEM-3Zf(+). Following electroporation of *E. coli* XL1Blue kanamycin-resistant clones were selected. Restriction analysis of one transformant, named pGF4, revealed the presence of a 13 kb chromosomal fragment containing *ppuI::npt*. Using sequence information from pGF1, the

flanking regions of *ppuI::npt* and *ppuR* were sequenced by primer walking. A defined *ppuA* mutant was constructed as follows. Two DNA fragments were amplified: an 1.1 kb *Eco*RI-*Sac*I fragment containing part of the *ppuA* gene using primers PPUAII-V (5'-CGGAATTCCCAAGCCGGTCTAGCG-3') and PPUAII-R (5'-CGGAGCTCGACCAGTTGCGCGCC-3') and an 1.3 kb *Sal*I-*Hin*dIII fragment containing the *ppuA* start codon and the complete *ppuR* gene using primers PPUAR-V (5'-ACATGTCGACGCTAGTAAATAGGCTGCG-3') and PPUAR-R (5'-AACTAAGCTTCGGCATCAGCCAGGCC-3'). Using the restriction sites introduced by the PCR primers (respective sites are underlined) these two DNA fragments were successively inserted into the compatible sites of pEX18Gm. Next, the promoterless *luxAB* cassette from pJMS10 (Kristensen et al., 1995) was cloned as a 2.5 kb *Xba*I-*Sal*I fragment into the vector cut with the same enzymes. The resulting construct was transferred to both IsoF and F117 by triparental mating and integrants were selected on PIA medium containing gentamicin. Merodiploids were resolved by plating on LB medium containing 5 % sucrose. The genetic structures of the resulting strains, which were designated IsoF-D4 (*ppuA*::*luxAB*) and F117-D1 (*ppuI*::*npt, ppuA*::*luxAB*), were confirmed by Southern Blot analysis.

Analysis of *ppuA* promoter activity. As strain F117-D1 bears a promoterless *luxAB* cassette within the *ppuA* gene, transcriptional regulation of *ppuA* can be followed by light measurements. Cells of 1 ml of overnight culture were harvested by centrifugation, washed in LB and inoculated into 100 ml of fresh LB medium to a final OD_{600} of 0.05. The cultures were incubated at 30 °C under constant agitation and samples were taken at regular time intervals. Following addition of 1 µl of *N*-decanal (Sigma, Deisenhofen, Germany) to 10 µl of sample, bioluminescence was measured using the EG&G Berthold MiniLumat LB9506 (Berthold, Bundoora, Australia). Specific luminescence units (SLU) were defined as relative bioluminescence per unit of optical density at 600 nm. Stimulation of *ppuA* promoter activity by different AHL molecules was analysed by measuring bioluminescence of F117-D1 after addition of synthetic AHL standards, which were either purchased from Fluka Chemie AG (Buchs, Switzerland) or were kindly provided by P. Williams (School of Pharmaceutical Sciences, University of Nottingham, UK). To this end, cells of an overnight culture of F117-D1 were diluted 5 times in fresh LB medium, incubated 1h at 30 °C, and then 1.5 ml aliquots were added to 1.5 ml of LB medium containing 2 µM of 3-oxo-C14-HSL [*N*-(3-oxo-tetradecanoyl)-L-homoserine lactone], 3-oxo-C12-HSL [*N*-(3-oxo-dodecanoyl)-L-homoserine lactone], 3-oxo-C10-HSL [*N*-(3-oxo-decanoyl)-L-homoserine lactone], 3-oxo-C8-HSL [*N*-(3-

oxo-octanoyl)-L-homoserine lactone], 3-oxo-C6-HSL [*N*-(3-oxo-hexanoyl)-L-homoserine lactone], C12-HSL [*N*-dodecanoyl-L-homoserine lactone], C10-HSL [*N*-decanoyl-L-homoserine lactone], C8-HSL [*N*-octanoyl-L-homoserine lactone], C6-HSL [*N*-hexanoyl-L-homoserine lactone], and C4-HSL [*N*-butanoyl-L-homoserine lactone].

Anlaysis of *ppuI* promoter activity. The intergenic region between *rsaL* and *ppuI* was PCR amplified using the primers PPUI_out (5'-CGGGATCCATGTCATTGATCAGCGG-3') and RSAL_out (5'-CGGGATCCGAACCTTGACCAGAATTCG-3'). The 360 bp amplicon was digested with *Bam*HI (restriction sites are underlined) and cloned into the promoter probe vector pGA-L14 cut with the same enzyme. The resulting plasmid, pAS-L22, containing the insert in the orientation that P_{ppuI} drives transcription of *luxAB* was transferred to *P. putida* IsoF. Bioluminescence of the resulting strain IsoF (pAS-L22) was monitored along the growth curve.

Detection and characterization of AHLs. AHLs were extracted twice with dichloromethane (250:100 supernatant/dichloromethane) from 250 ml sterile-filtered supernatants of cultures grown in LB medium at 30 °C to an OD_{600} of 1.0. The combined extracts were dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. Residues were dissolved in 250 µl ethyl acetate. 10 µl samples were then applied to C_{18} reversed-phase TLC plates (Merck No. 1.15389., Merck, Darmstadt, Germany) and dried with a stream of cold air. Samples were separated by using methanol (60 % vol/vol) in water as the mobile phase. For detection of AHLs the TLC plate was overlaid with a thin film of 0.8 % (w/v) LB agar (143 ml) seeded with 7 ml of an exponentially grown AHL biosensor and was then incubated at 30 °C for 24 hours. The tentative identification of AHLs present in spent culture supernatant extracts was achieved by comparison of mobilities (R_f-values) relative to those for the synthetic AHL standards.

For quantification of AHL molecules 100 µl of filter sterilized culture supernatants were incubated with 100 µl logarithmic culture of the AHL monitor strain F117(pKR-C12) in a microtitre plate (all samples were measured in triplicate). The AHL monitor plasmid pKR-C12 contains a P_{lasB}-*gfp*(ASV) translational fusion together with the *lasR* gene placed under control of P_{lac}. This sensor strain is most sensitive for 3-oxo-C12- and 3-oxo-C10-HSL. Following 6 h of incubation at 30 °C, the green fluorescence was measured using the microtitre plate reader Lambda Fluoro 320 Plus (MWG Biotech, Ebersberg, Germany) with an excitation wavelength of 474 nm and emmission detection at 515 nm. Data were processed

with the KC4 software (Bio-Tek Instruments, Winooski, USA). Background fluorescence of the AHL monitor strains was determined by incubation of the sensors with LB medium as a negative control. Specific fluorescence units (SFU) are defined as relative fluorescence units (RFU) corrected for background fluorescence per unit of optical density at 600 nm.

Monitoring biofilm formation by confocal laser scanning microscopy (CLSM) and image analysis. Biofilms were grown in artificial flow cells supplied with AB medium (8) containing 0.1 mM citrate. The flow system was assembled and prepared as described previously (7). The substratum consisted of a microscope glass coverslip (Knittel Gläser, Braunschweig, Germany). Overnight cultures in LB medium were subcultured to an OD_{600} of 0.7 before dilution in 0.9 % NaCl to an OD_{600} of 0.1. Aliquots (250 µl) of these dilutions were used to inoculate the flow channels. Medium flow was kept at a constant rate of 0.2 mm s^{-1} by a Watson-Marlow 205S peristaltic pump. The flow channels were incubated at room temperature.

Microscopic inspection and image acquisition were performed on a confocal laser scanning microscope (Zeiss LSM510, Zeiss, Oberkochen, Germany) equipped with a 40 x /1.3 oil objective. Image scanning was carried out with the 488 nm laser line of an Ar/Kr laser. Captured images were visualized using the IMARIS software package (Bitplane AG, Zürich, Switzerland) running on an Indigo 2 workstation (Silicon Graphics, Mountain View, CA., USA).

Nucleotide sequence accession number. The nucleotide sequence of the *ppu* locus and flanking region has been deposited in Genbank under accession number AY115588.

Results

Cloning and sequencing of the *P. putida luxI* and *luxR* homologs *ppuI* and *ppuR*. The plant-associated *P. putida* strain IsoF produces a wide spectrum of AHL signal molecules, including 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL, and 3-oxo-C12-HSL (45). In order to clone the gene(s) directing synthesis of these signal molecules we employed a previously described strategy for the isolation of *luxI* homologs which is based on complementation *in trans* of an AHL biosensor (47). For this purpose *E. coli* MT102 harboring the *lux*-based AHL sensor plasmid pSB403 (53) was transformed with a plasmid library of the *P. putida* IsoF genome. Bioluminescent colonies were picked and were found to contain a 2.9 kb *Sal*I chromosomal fragment. DNA sequencing revealed the presence of two open reading frames (ORFs) with homology to *luxI* and *luxR* genes. One ORF, designated *ppuI*, spans 591 bases and is predicted to encode a 22.0 kDa protein that is homologous to members of the LuxI family of AHL synthases (Fig. 1a). PpuI has 56 % identity (74 % similarity) with *Pseudomonas aeruginosa* LasI (34, 35) and 51 % identity (69 % similarity) with *Pseudomonas fluorescens* NCIMB 10586 MupI (15). PpuI contains each of the ten amino acids that are completely conserved among members of the LuxI family (33). The *ppuI* promoter region contains putative –10 and –35 *E. coli* consensus sequences. In addition, adjacent to the –35 site is a 20-bp imperfect palindrome with high similarity to *lux* box-like elements, in particular with operator sequences present upstream of quorum sensing-regulated genes in *P. aeruginosa* (50; Fig.2). These inverted repeats are thought to represent binding sites of LuxR homologs.

The second ORF, designated *ppuR*, is located upstream of *ppuI* and is transcribed in the same direction (Fig. 3). The *ppuR* gene spans 714 bases and encodes a protein with a predicted molecular mass of 27.0 kDa. Over the entire protein, PpuR has 40 % identity (62 % similarity) with *P. fluorescens* NCIMB 10586 MupR (15) and 42 % identity (62 % similarity) with *P. aeruginosa* LasR (18) (Fig. 1B). PpuR has all seven invariant amino acids typical of the LuxR family (17).

A further ORF, tentatively designated *rsaL*, which potentially encodes a 76-amino-acid protein of 8.6 kDa, was identified in the intergenic region between *ppuI* and *ppuR*; it is divergently oriented to *ppuI* and separated from it by 138 nucleotides (Fig. 3). When translated, this ORF was 36 % identical (66 % similar) to the regulatory protein RsaL of *P. aeruginosa*, which functions as a negative regulator of the *P. aeruginosa* quorum-sensing cascade (10).

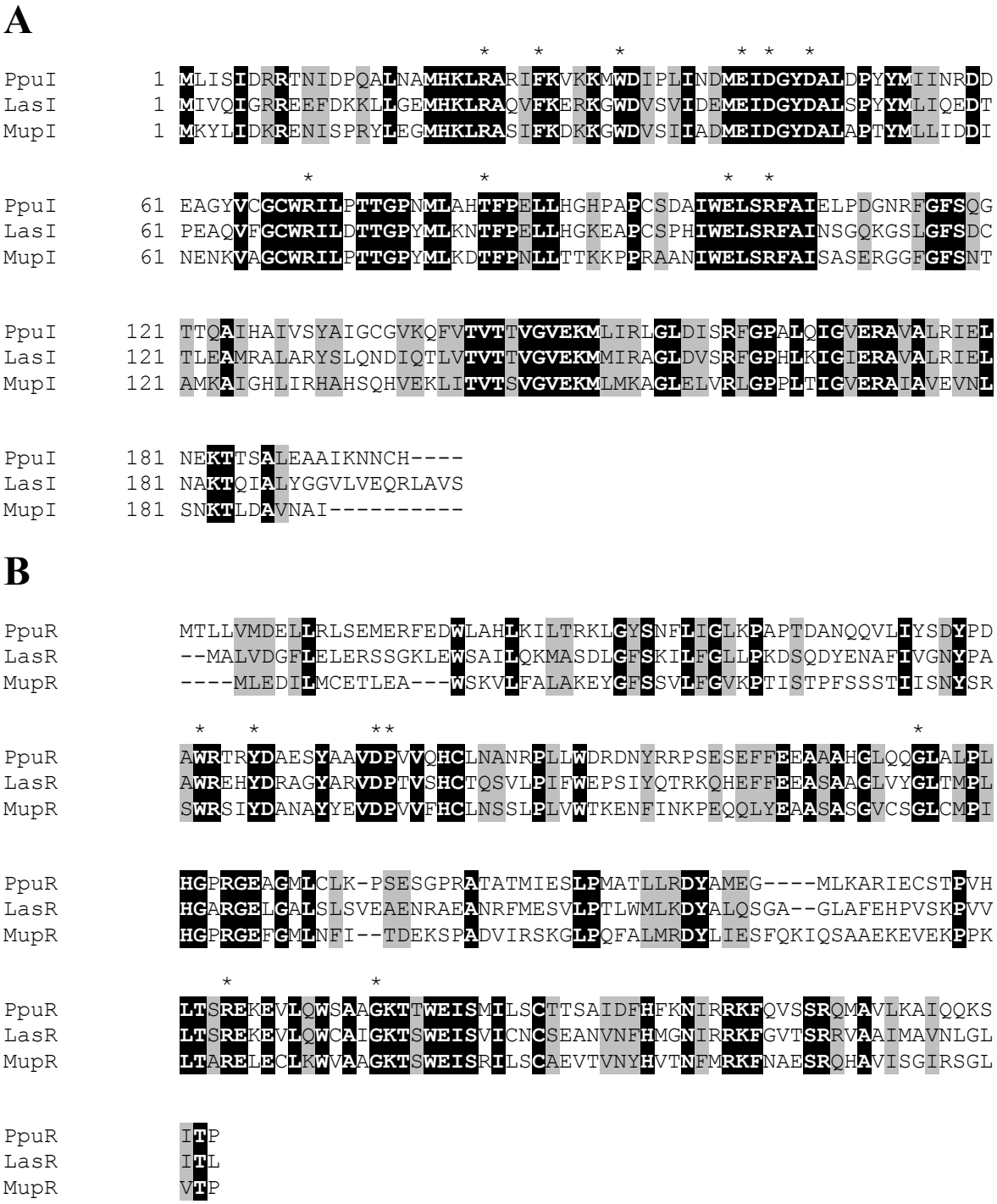
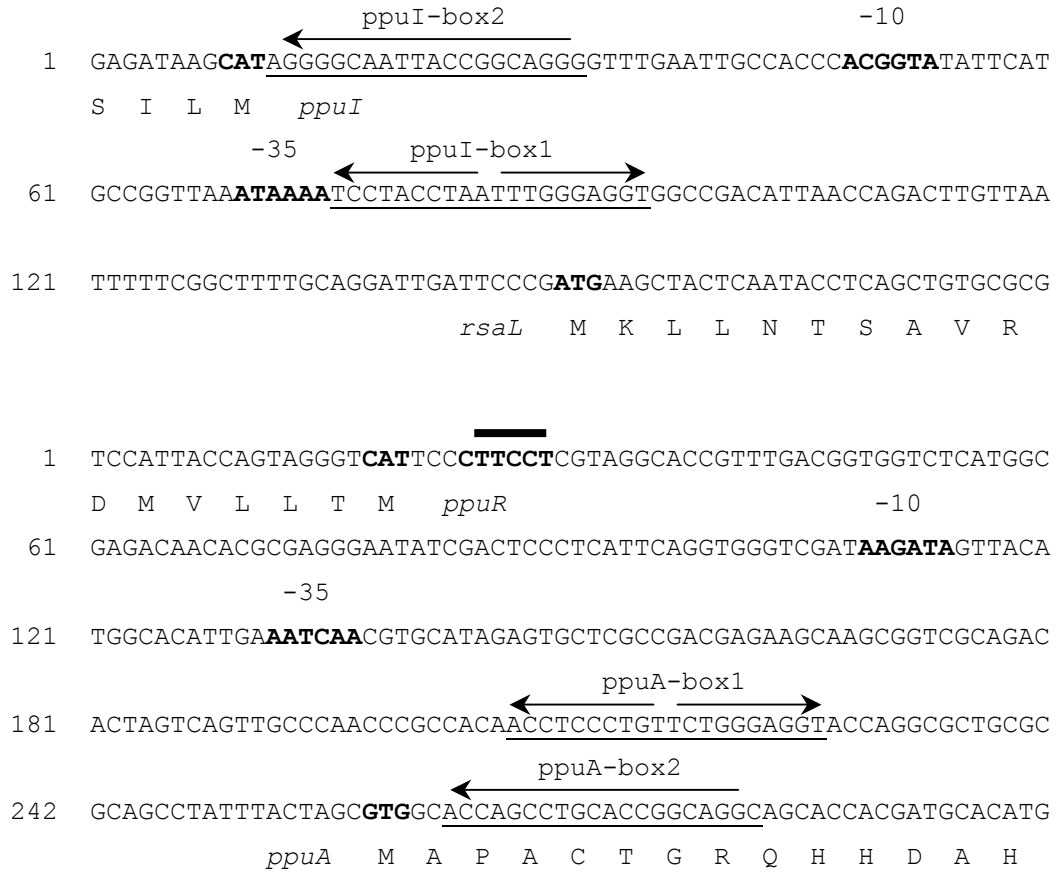


Fig. 1. (A) Protein sequence alignments of PpuI (*P. putida* IsoF), LasI (*P. aeruginosa* PAO1), and MupI (*P. fluorescens* NCIMB 10586). Shaded in black are identical amino acids, shaded in grey are similar amino acids, the asterisks indicate the ten amino acids that are highly conserved among all members of the LuxI family. (B) Protein sequence alignments of PpuR (*P. putida* IsoF), LasR (*P. aeruginosa* PAO1), and MupR (*P. fluorescens* NCIMB 10586). Asterisks indicate the seven amino acids that are identical in all members of the LuxR family.

A



B

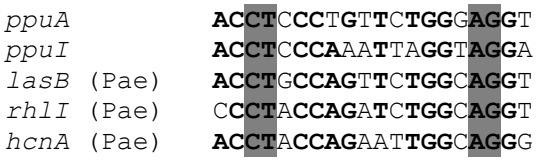


Fig. 2. (A) Nucleotide sequence analysis of the intergenic regions of *ppuI-rsaL* and *ppuR-ppuA*, respectively. Putative –35 and –10 promoter sequences are shown in bold type. A putative ribosome binding site upstream of *ppuR* is indicated by a line above the sequence. Two inverted repeats exhibiting similarity to *lux* boxes were identified: One of the repeats, denoted as ppuI-box1, is located in the *ppuI-rsaL* intergenic region, the second repeat, denoted as ppuA-box1, is present in the *ppuR-ppuA* intergenic region. Two additional non-palindromic sequences, denoted as ppuI-box2 and ppuA-box2, were identified in the 5'-regions of *ppuI* and *ppuA*, respectively. (B) Nucleotide sequence comparison of *lux* box-like sequences of the *ppu* locus and previously identified sequences involved in cell density-dependent regulation of *lasB*, *rhlI*, and *hcnA* in *P. aeruginosa*. Nucleotides in bold type are identical in at least five of the six sequences. The nucleotides shaded in grey represent the minimal consensus sequence defined for operators of quorum sensing-controlled (*qsc*) genes in *P. aeruginosa* (50).

Furthermore, the 2.9 kb *Sal*I fragment contains a partial ORF downstream of *ppuI* and oriented in the opposite direction with high homology to *suhB* genes from various bacteria (see below).

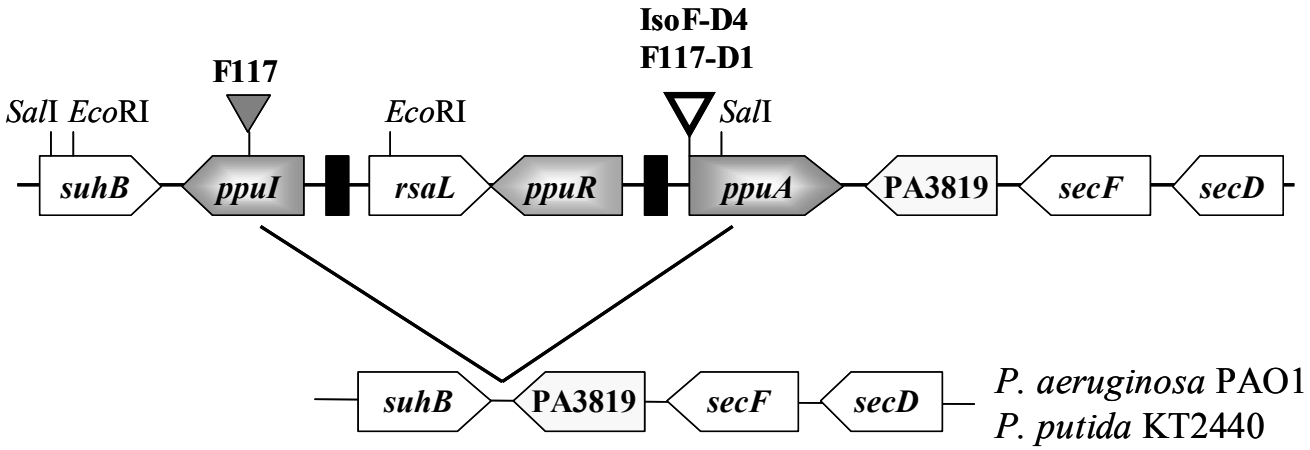


Fig. 3. Genetic organization of the *ppu* locus in *P. putida* IsoF. The identified ORFs are represented by arrows indicating the direction of transcription. Two *lux* box-like sequences in the intergenic regions of *ppuI-rsaL* and *ppuR-ppuA* are indicated as black boxes. Relevant restriction sites are shown. The position of the *npt* cassettes in the *ppuI* mutant F117 is indicated by a grey triangle. The position of the *luxAB* cassette in the *ppuA* mutant IsoF-D4 and the *ppuI ppuA* double mutant F117-D1 is indicated by an open triangle. The panel below the *ppu* locus shows the chromosomal regions of *P. aeruginosa* PAO1 and *P. putida* KT2440 containing *suhB*, PA3819, *secF*, and *secD*. The *ppu* gene cluster of IsoF appears to form an island in a region that is highly conserved in *P. putida* KT2440 and *P. aeruginosa* PAO1.

PpuI directs the synthesis of multiple AHLs. Thin layer chromatography (TLC) in combination with AHL biosensors provides a simple and rapid technique to characterize and quantify the AHL species produced by a given organism (44). Consistent with previous studies (14, 45), we identified four AHL species with mobility properties being similar to those of 3-oxo-C12-, 3-oxo-C10-, 3-oxo-C8-, and 3-oxo-C6-HSL in spent culture supernatants of *P. putida* IsoF (Fig. 4). The AHL profiles of *E. coli* strains harboring a plasmid with an intact *ppuI* gene, e.g. pGF1 or pGF3, were indistinguishable from the one of the *P. putida* wild type (Fig. 4). Inactivation of *ppuI* by insertion of an antibiotic marker gene into the *Apa*I site of pGF1 abolished AHL production in *E. coli* (data not shown).

Using a gene replacement method, we constructed a *P. putida* IsoF mutant strain, designated F117, in which the chromosomal *ppuI* gene is interrupted by the insertion of a kanamycin-resistance cassette (see Materials and Methods; Fig. 3). Even in highly concentrated dichloromethane extracts of spent culture supernatants from *P. putida* F117 no AHL molecules were detected. However, production of all four AHL species was restored (to wild type levels) when F117 was complemented with plasmid pGF3, which contains the *ppuI* gene as a 1.4 kb *Eco*RI fragment inserted into the broad host-range vector pBBR1MCS-5 (Fig. 4). In conclusion, these data demonstrate that a single polypeptide encoded by the *ppuI* gene is sufficient for the production of AHL signal molecules from precursors present in both *P. putida* and *E. coli*.

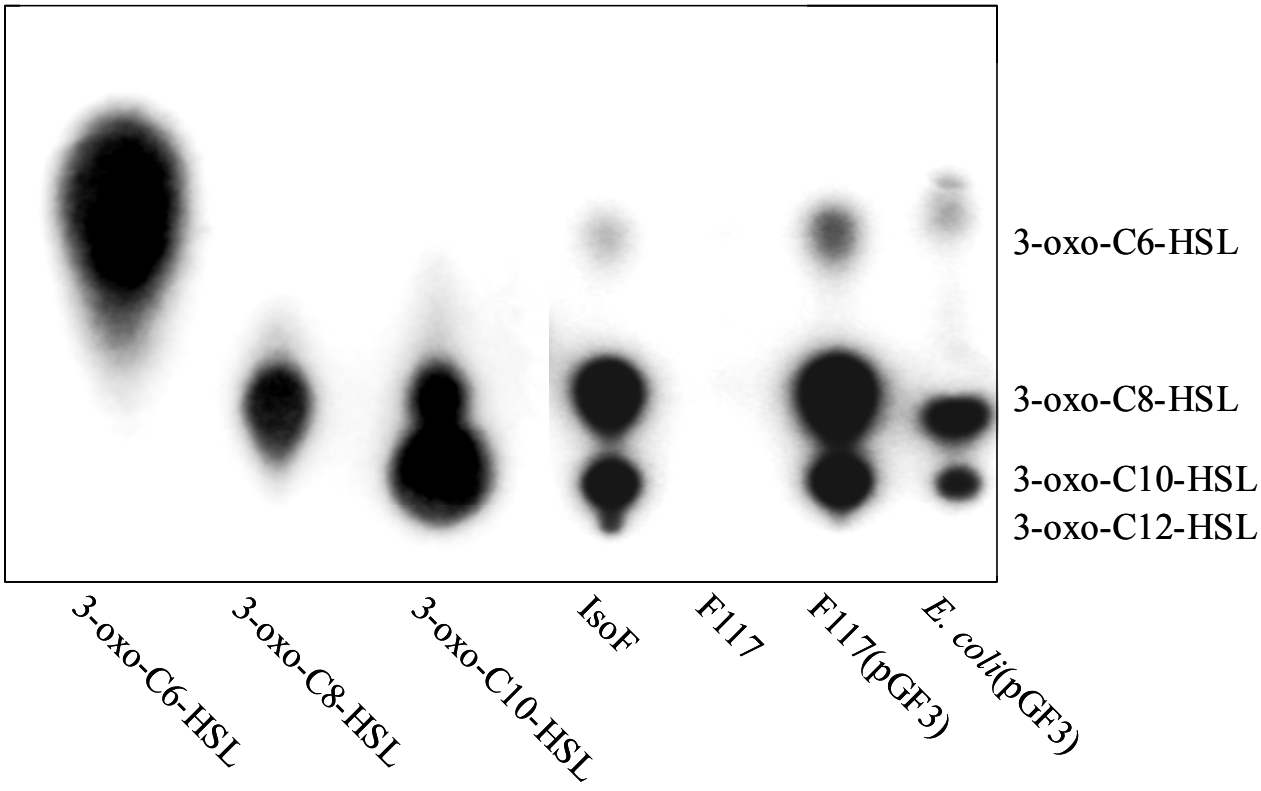


Fig. 4. TLC analysis of AHLs produced by the wild type IsoF, the *ppuI* mutant F117, F117 harboring plasmid pGF3 (*ppuI*$^+$), and *E. coli* XL1Blue harboring pGF3 (*ppuI*$^+$). Synthetic AHLs were included as reference compounds, as indicated.

Expression of *ppuI* is subject to positive feedback regulation. The finding that a *lux* box-like element is present in the promoter region of *ppuI* (Fig. 2) prompted us to investigate whether AHL synthesis in *P. putida* IsoF is autoregulated. In a first step, we determined the AHL concentrations of spent culture supernatants prepared at various time points during growth of a culture of the wild type strain. In support of the view that expression of *ppuI* is induced in a density-dependent fashion we observed that the AHL concentrations increased dramatically (almost 20-fold) between OD_{600} of 1.0 and 2.0 (Fig. 5A). We next constructed a transcriptional fusion of the *ppuI* promoter region to the promoterless *luxAB* genes on vector pGA-L14. The resulting plasmid, pAS-L22, was transferred to *P. putida* IsoF and the bioluminescence of the recombinant strain was determined along the growth curve. In agreement with our AHL measurements, transcription of *ppuI* was specifically induced (approximately 9-fold) at the transition between exponential growth and stationary phase (Fig. 5B). The approach taken to investigate the role of PpuR for transcriptional activation of *ppuI* was to complement the P_{ppuI}-*luxAB* promoter fusion on pAS-L22 present in *E. coli* MT102 with plasmid pPPUR1, which contains the *ppuR* gene under control of the P_{lac}

promoter of pGEM-3Zf(+). We observed that bioluminescence increased 10-fold upon addition of 1 µM 3-oxo-C12-HSL to the medium, indicating that transcription of *ppuI* is positively regulated by the PpuR/AHL complex.

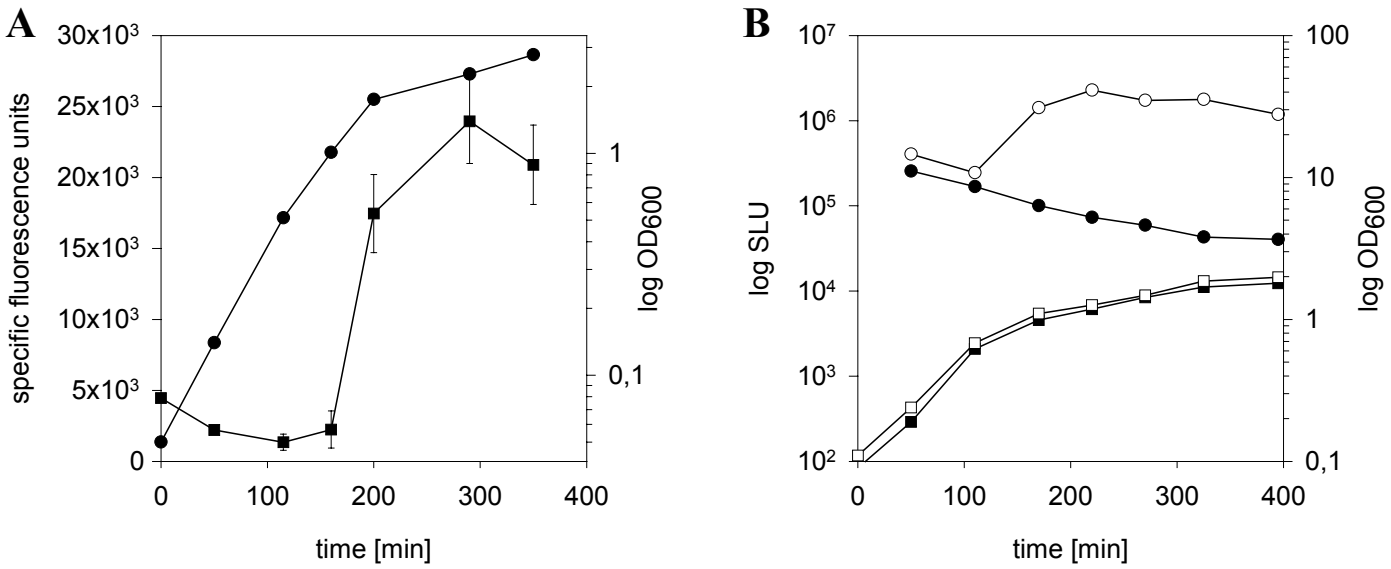


Fig. 5. AHL production of *P. putida* IsoF is growth phase-dependent. (A) Samples of spent culture supernatants were taken at regular time intervals throughout the growth curve and AHLs were quantified by the aid of the GFP-based AHL-monitor strain *P. putida* F117(pKR-C12). Specific fluorescence units (■) were defined as relative fluorescence units per unit of optical density at 600 nm. Mean values of three replicates are shown with standard deviations. Growth (•) was measured spectrophotometrically at 600 nm. (B) Transcription of *ppuI* is induced in the late exponential phase. IsoF harboring plasmid pAS-L22 (open symbols), which contains a transcriptional *ppuI*::*luxAB* fusion, and IsoF harboring the promoter probe vector pGA-L14 (closed symbols) were grown in LB medium and bioluminescence (○, •) and optical density (□, ■) were measured at regular time intervals.

Sequence analysis of the DNA regions flanking *ppuI* and *ppuR*. We noticed the presence of an additional *lux*-box like element upstream of *ppuR* close to the end of the 2.9 kb chromosomal *Sal*I fragment. This sequence is strikingly similar to the inverted repeat identified in the promoter region of *ppuI* (Fig. 2). This observation led us to speculate that a quorum sensing-regulated gene might be present upstream of *ppuR*. To address this issue we decided to determine the DNA sequences flanking *ppuR* and *ppuI*. For this purpose we digested chromosomal DNA of F117 with *Sac*I and cloned the resulting fragments into pGEM-3Zf(+). Following transformation of *E. coli* XL1Blue, a Km^r clone, designated pGF4, containing a 13 kb chromosomal DNA fragment was isolated. Sequence analysis of the *ppuR* upstream region revealed an ORF, denoted *ppuA*, the predicted product of which has 509 amino acids and a molecular mass of 55 kDa. PpuA showed significant similarity to various

long chain fatty acid CoA ligases and to non-ribosomal peptide synthetases. The best identity was 24 % to the AttG CoA ligase of *Rhodococcus fascians* (41 % similarity). This protein is predicted to be involved in the synthesis of an autoregulatory compound, which is essential for full virulence on tabacco (31). PpuA contains a putative AMP-binding domain (amino acids 176 to 187) and a sequence motif exhibiting similarity to the fatty acyl-CoA synthetase (FACS) signature motif. This 25-amino acid consensus sequence DGWLHTGDIGXWXPXGXLKIIDRKK is thought to compose part of the fatty acid binding site and was shown to be essential for catalytic activity of the enzyme (5). However, PpuA contains only 5 of 9 of the amino acid residues described to be invariant or highly conserved.

The DNA sequence downstream of *ppuA* shows striking similarity to chromosomal regions of *P. putida* KT2440 (89 % identity over a DNA stretch of 1.8 kb) and *P. aeruginosa* PAO1. In *P. aeruginosa*, this region encodes for the conserved hypothetical protein PA3819, SecF, and SecD. SecF and SecD are highly conserved proteins involved in protein export, PA3819 is a putative membrane protein of unknown function. The *ppuI* flanking region was highly homologous to the regions present downstream of PA3819 in *P. putida* KT2440 (93 % identity over a stretch of 5.5 kb) and *P. aeruginosa* PAO1. The genetic organization of the corresponding chromosomal regions in *P. aeruginosa* PAO1, *P. putida* KT2440 and *P. putida* IsoF is depicted in Fig. 3. The ORF downstream of *ppuI* showed 82 % identity (91 % similarity) to SuhB of *P. aeruginosa* and 96 % identity (98 % similarity) to SuhB of *P. putida* KT2440. SuhB of *E. coli* posses inositol monophosphatase activity and was suggested to participate in the post-transcriptional control of gene expression by modulating mRNA decay (23).

On the basis of these data we suggest that the *ppu* locus of *P. putida* IsoF comprises four genes, *ppuI*, *rsaL*, *ppuR*, and *ppuA*, which form an island in a chromosomal region that is highly conserved between *P. putida* KT2440 and *P. aeruginosa* PAO1.

Expression of *ppuA* is AHL-dependent. To analyse regulation of *ppuA* expression we constructed a *ppuA*::*luxAB* transcriptional fusion in the chromosome of the *ppuI* mutant F117. This was accomplished by allelic exchange of the wild type *ppuA* gene with the recombinant *ppuA*::*luxAB* allele present on the suicide plasmid pEXF2 (see Materials and Methods for details). When the resulting mutant, which was denoted F117-D1, was grown in LB medium light levels were low throughout the growth curve (Fig. 6). However, upon addition of 1 µM 3-oxo-C10-HSL *luxAB* activity increased dramatically (56-fold) within 90 minutes.

We also determined the response of F117-D1 to different AHL molecules. To this end, early log phase cells of F117-D1 were supplemented with 1 µM of AHL (Fig. 7) and bioluminescence was measured after 4 h incubation at 30 °C. As expected, *ppuA* promoter activity was highest when 3-oxo-C10-HSL or 3-oxo-C12-HSL, the two most abundant AHL molecules produced by the organism, were added to the cultures. A response was also observed with 3-oxo-C14-HSL, while 3-oxo-C8-HSL or 3-oxo-C6-HSL had no effect. Molecules without a 3-oxo group were also unable to stimulate *ppuA* expression at a concentration of 1 µM. The minimal concentration of 3-oxo-C10-HSL and 3-oxo-C12-HSL required for activation of bioluminescence in F117-D1 was below 10 nM (data not shown). These results demonstrate that expression of *ppuA* is quorum sensing-regulated.

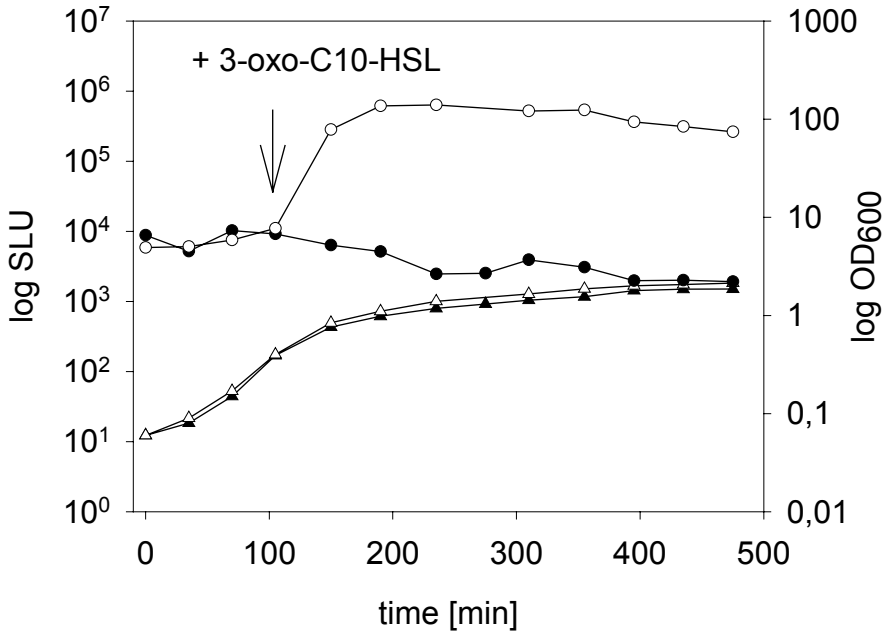


Fig. 6. Transcription of *ppuA* is quorum sensing-regulated. Bioluminescence of strain F117-D1 (*ppuA*::*luxAB*, *ppuI*), in which the promoterless *luxAB* genes are transcriptionally fused with the chromosomal *ppuA* gene was monitored in the presence (open symbols) or absence (closed symbols) of 1 µM 3-oxo-C10-HSL. Parallel cultures of F117-D1 were grown in LB medium and bioluminescence (•, ○) and OD_{600} (▲, Δ) was followed along the growth curve. At the timepoint indicated by the arrow, one culture was supplemented with 1 µM 3-oxo-C10-HSL.

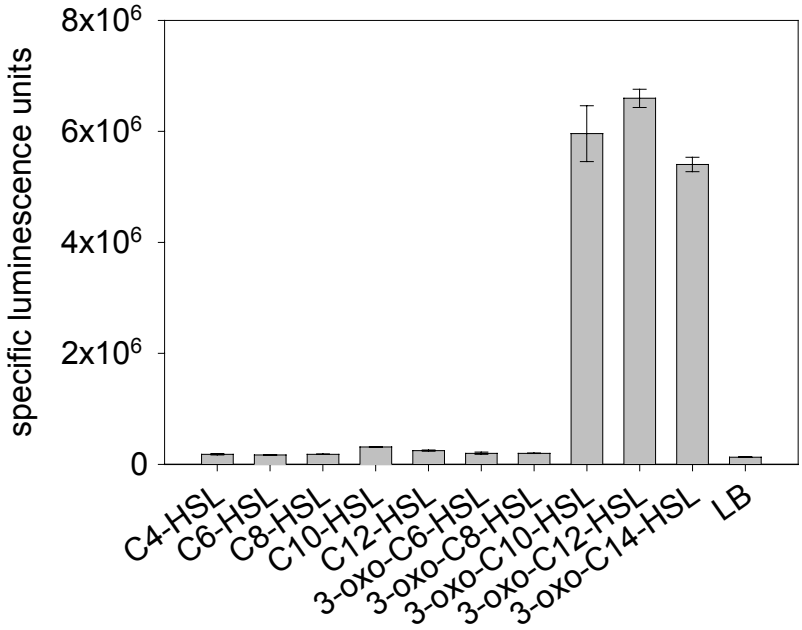


Fig. 7. Response of *P. putida* F117-D1 (*ppuA*::*luxAB*, *ppuI*) to different AHL molecules. F117-D1 was grown in the presence of various synthetic AHL compounds and bioluminescence of the cultures was determined at an OD_{600} of 1.0. Mean values of the specific luminescence units obtained from three independent experiments are shown with standard deviations.

The *ppu* quorum-sensing system influences biofilm structural development. Previous work has provided evidence that AHL-dependent quorum-sensing systems are involved in the regulation of biofilm formation (9, 22, 30). These findings prompted us to investigate the role of quorum sensing in biofilm formation of *P. putida* IsoF. Biofilms were grown in artificial flow cells, which allow biofilm development to be followed on a glass surface under highly defined conditions in real time (7). Moreover, by the use of a confocal laser scanning microscope (CLSM) the three-dimensional structures of the biofilm can be reconstructed. For this analysis it was necessary to tag *P. putida* IsoF and the two mutants F117 and IsoF-D4 with GFP. This was accomplished by inserting a $P_{A1/04/03}$-*gfp*-T_0T_1 transposon cassette into the chromosome of each of the three strains using the suicide plasmid pBK-miniTn*7*-gfp3. The tagged strains used for further investigations were carefully tested with respect to growth rates and were found to be indistinguishable from the parental strains (data not shown).

Parallel flow chambers were inoculated with each of the three GFP-tagged strains and biofilm development was monitored for 8 days. Visual inspection of CLSM images revealed that the structures of the biofilms formed during the first two days showed only minor differences. Thereafter, however, the *ppuI* mutant developed into a highly structured biofilm with characteristic microcolonies, while cells of the wild type uniformly covered the surface,

giving rise to unstructured biofilms (Fig. 8). Addition of 3-oxo-C12-HSL to the growth medium of F117 gave rise to an unstructured biofilm which was virtually indistinguishable from the one formed by the wild type. We also analysed the biofilm formed by the *ppuA* mutant IsoF-D4. This biofilm was strikingly similar to the one formed by F117, suggesting that the inability of the *ppuI* mutant to form biofilms with a wild type structure may be due to a defect in *ppuA* expression.

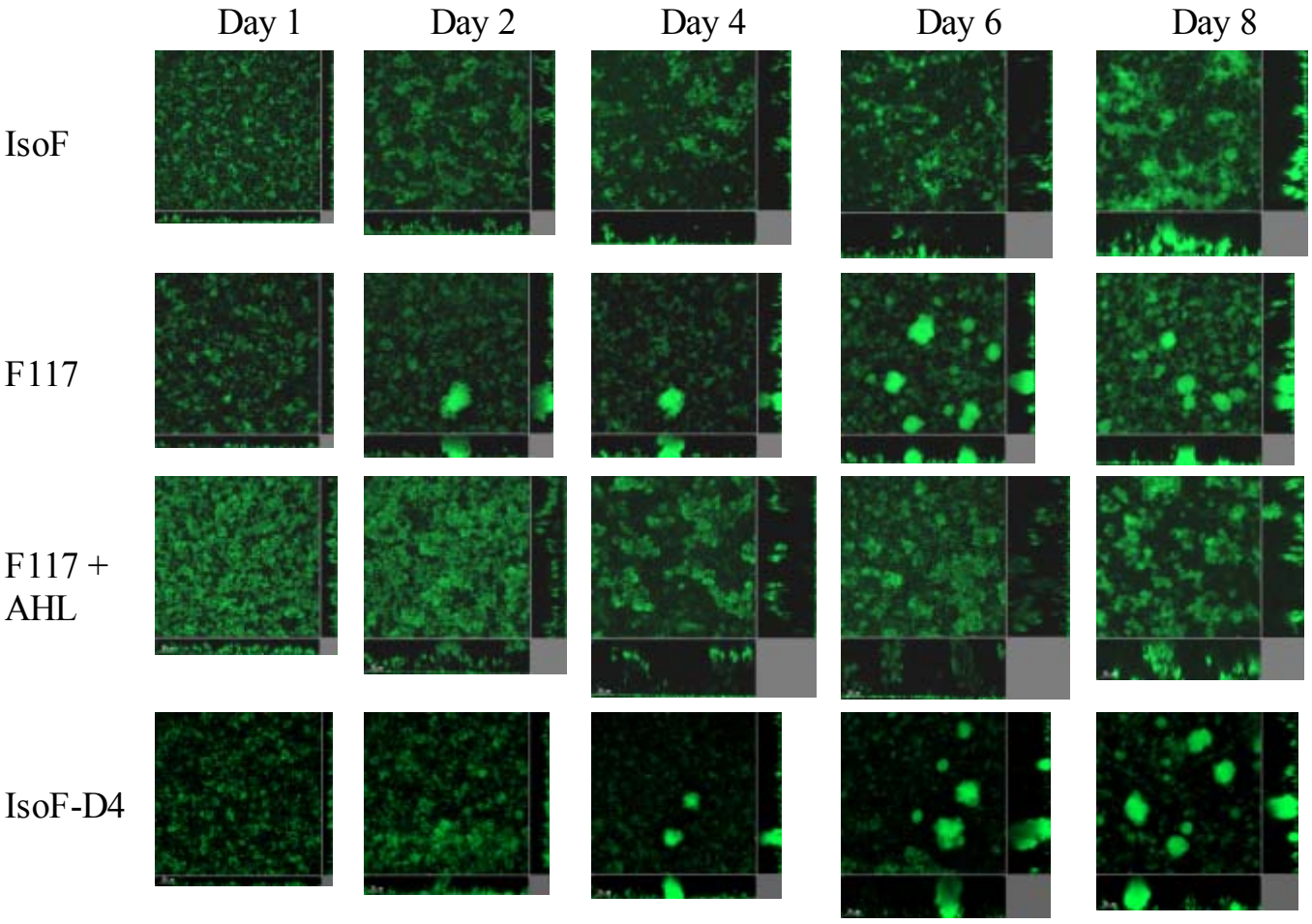


Fig. 8. Biofilm formation of *P. putida* IsoF is quorum sensing-regulated. Flow chambers were inoculated with *gfp*-tagged derivatives of IsoF, the *ppuI* mutant F117 in the presence or absence of 2 µM 3-oxo-C12-HSL, and the *ppuA* mutant IsoF-D4. CLSM images were taken on day 1, 2, 4, 6, and 8 post inoculation. The larger central plots are simulated fluorescence projections. Shown in the right and lower frames are vertical sections through the biofilms.

Discussion

Several reports have shown that some *P. putida* strains produce AHL signal molecules, indicating that they employ quorum-sensing systems to coordinate expression of certain phenotypic traits in a density-dependent manner (26, 14, 42). In this study, we cloned and sequenced the quorum-sensing locus of *P. putida* IsoF, a strain, which has been isolated from the tomato rhizosphere (45). The IsoF quorum-sensing system, termed *ppu*, is most similar to the *las* system of *P. aeruginosa*. PpuI and PpuR share the greatest similarities with LasI and LasR and both systems employ 3-oxo-C12-HSL as signal molecule. In fact, when *ppuI* is expressed in a *P. aeruginosa lasI* mutant, which due to the genetic lesion no longer produces extracellular proteolytic activity, the protease synthesis defect is fully restored (data not shown), indicating that LasI and PpuI are functionally interchangeable. Furthermore, an ORF with significant similarity to *rsaL*, which is present in the intergenic region between *lasI* and *lasR*, was identified in the region between *ppuI* and *ppuR*. In *P. aeruginosa* RsaL acts as a repressor of the *las* system, preventing premature activation of the quorum-sensing cascade at low cell densities. Further work will be needed to analyse the function of the *rsaL* homolog in *P. putida* IsoF.

The *las* system of *P. aeruginosa* is on top of a hierarchically arranged signalling cascade (36). The lower level quorum-sensing circuit, the *rhl* system, consists of the transcriptional activator RhlR and the AHL synthase RhlI, which directs the synthesis of *N*-butanoyl-L-homoserine lactone (C4-HSL). This quorum-sensing cascade regulates expression of a battery of extracellular virulence factors such as exoenzymes (elastase, alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, pyoverdin), and toxines (exotoxin A) (for reviews see 11, 51).

We have no indication that a second quorum-sensing system is present in *P. putida* IsoF. No AHL molecules could be detected in spent culture supernatants of the *ppuI* mutant F117. Furthermore, when supernatants of *E. coli* cultures expressing *ppuI* were analysed by TLC the AHL pattern was indistinguishable from the one of the IsoF wild type. Thus, PpuI directs the synthesis of four AHL molecules, which were tentatively identified as 3-oxo-C12-, 3-oxo-C10-, 3-oxo-C8-, and 3-oxo-C6-HSL (Fig.4; 14, 45). This result is reminiscent of the situation found with LasI from *P. aeruginosa*, which catalyses the synthesis of primarily 3-oxo-C12-HSL together with small amounts of 3-oxo-C10, 3-oxo-C8-, and 3-oxo-C6-HSL (35, 52, 19).

Two *lux*-box like sequences with striking similarity to operator sequences present in the promoter regions of quorum sensing-regulated genes in *P. aeruginosa* (50) were identified within the *ppu* locus. One of these palindromic sequences is present upstream of *ppuA* and the second one is located in the promoter region of *ppuI*. As both genes are quorum sensing-regulated it appears likely that these sequences are essential for AHL-dependent gene expression. Interestingly, two additional sequences, which show limited homology to the *lux*-box like elements but are not inverted repeats, are present in the 5' regions of *ppuA* and *ppuI* (Fig. 2). While the function of these sequences remains to be elucidated, it is interesting to note that similar arrangements of *lux*-box like sequences are also found in the promoter region of *lasB* in *P. aeruginosa* (40) and *mupA* in *P. fluorescens* NCIMB 10586 (15). In the case of *P. aeruginosa* both operator sequences are involved in AHL-dependent *lasB* expression (40).

Previous work has shown that the *las* quorum-sensing system of *P. aeruginosa* is involved in the regulation of biofilm formation (9). When compared with the wild type a *lasI* mutant of *P. aeruginosa* only formed flat and undifferentiated biofilms, suggesting that the *las* system is required for the development of a typical biofilm structure. Importantly, the *lasI* mutant biofilm exhibited greater sensitivity to the biocide sodium dodecyl sulfate in comparison to the wild type biofilm. More recently, it was shown that AHL-dependent quorum sensing plays a role in biofilm development of *Burkholderia cepacia* and *Aeromonas hydrophila* (22, 30). In both cases AHL-negative mutants showed defects in the late stages of biofilm development and thus were unable to form biofilms with a typical wild type structure.

P. putida IsoF formed very homogenous rather unstructured biofilms, while the *ppuI* mutant F117 formed structured biofilms with characteristic microcolonies and water-filled channels. Most interestingly, when AHL signal molecules were added to the medium the mutant biofilm lost its structure and converted into an unstructured biofilm that was similar to the one formed by the wild type.

Upstream of *ppuR* we identified a gene, designated *ppuA,* which is expressed in an AHL-dependent manner. Our data indicate an involvement of *ppuA* in biofilm maturation as the biofilm formed by IsoF-D4 (*ppuA*) was virtually indistinguishable from the one formed by F117 (*ppuI*). In a recent study, Zhang and Pierson (55) identified a second quorum-sensing system in *P. aureofaciens*, which consists of the LuxI/LuxR homologs CsaI/CsaR. This regulatory circuit does not interact with the *phz* quorum-sensing system for phenazine production but does interact with it for protease production. More important in the context of this study, however, was the finding that the *csa* system is involved in regulating biosynthesis of cell surface components. It was suggested that the *csaR* mutant had alterations in fatty acid

composition or relative percentages of some fatty acids compared to the wild type. Given that the *ppuA* gene in *P. putida* IsoF is similar to long chain fatty acid CoA ligases, we speculated that AHL-dependent expression of PpuA may in some way alter the fatty acid composition of the cell membrane. This could change the surface properties of the cells, which, in turn, would affect biofilm structures. However, a comparison of the whole cell fatty acid profiles of the wild type and the *ppuI* mutant F117 did not reveal any significant differences (data not shown).

At present we cannot exclude the possibility that quorum sensing-regulated genes outside of the *ppu* locus exist in *P. putida* IsoF, which encode for factors that affect biofilm structure. By employing proteomics Sauer and Camper (42) showed that 16 proteins are differentially expressed in the *P. putida* strain ATCC 39168 when the medium was supplemented with AHL signal molecules, indicating that the quorum-sensing system is a global regulator in this strain. As only one of the identified proteins, PotF, was found to be regulated by both adhesion to an abiotic surface and AHL addition, it was concluded that quorum sensing does not play a role in the initial attachment process, but may play a role in the late stages of biofilm development. Work is currently under way to define the quorum-sensing regulon of *P. putida* IsoF and to identify target genes outside of the *ppu* gene cluster.

Comparison of the sequence retrieved from pGF4 with the unfinished *P. putida* KT2440 genome database (http://www.tigr.org) revealed that none of the four genes contained in the *ppu* locus is present in the KT2440 genome. A comparison with the *P. aeruginosa* PAO1 genome (46) showed that the *ppuR-rsaL-ppuI* region is highly homologous to the *lasR-rsaL-lasI* region of PAO1. However, no *ppuA* homolog was found in the PAO1 genome. Most interestingly, the regions flanking the *ppu* gene cluster of *P. putida* IsoF are virtually identical with the *suhB*-PA3819-*secD*-*secF* chromosomal regions of both *P. aeruginosa* and *P. putida* (Fig. 3). The gene order of this region is even conserved in *E. coli*. Hence, the *ppuR-rsaL-ppuI-ppuA* gene cluster of *P. putida* IsoF forms an island within a highly conserved genomic region. These data may indicate that the *ppu* locus was acquired via horizontal gene transfer and was subsequently integrated into the *suhB*-PA3819 intergenic region. We searched the sequence for the presence of IS-elements using the IS-database of the Centre National de la Recherche Scientifique (http://www-appli.biotoul.fr) but could not detect any significant homologies to IS elements available in this database. However, we noticed the presence of five copies of a short DNA sequence (5'-CCAAGCCGGDC-3') within an approximately 200 bp DNA fragment spanning the region between the C-terminal ends of *ppuA* and PA3819 (data not shown). Two of these sequences form an inverted repeat

while the remaining three sequences are present as direct repeats. It is tempting to speculate that these repeats were involved in the acquisition of the *ppu* gene cluster.

In a recent survey, we screened over 80 *P. putida* strains for their ability to produce AHLs. Of all the strains tested, 44 % provoked a positive signal with at least one of the AHL biosensors used (3). Southern blot analyses of 9 AHL-producing isolates using DNA fragments containing part of the *ppuI* or *ppuR* gene as probes failed to give positive hybridisation signals, indicating that the investigated strains to not carry homologous genes. Moreover, using a PCR assay we were able to show that none of the AHL positive strains contain an insertion in the *suhB*-PA3819 intergenic region. These results show that, although production of AHL signal molecules is widespread among different *P. putida* isolates, the *ppu* gene cluster is so far unique for *P. putida* IsoF.

ACKNOWLEDGMENTS

We thank H. P. Schweitzer and M. E. Kovach for providing bacterial strains and plasmids, P. Williams for the generous gift of synthetic AHLs, and P. Kämpfer for analysing whole-cell cellular fatty acids. Preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org.

This work was supported by the BMBF.

References

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.
2. **Bao, Y., D. P. Lies, H. Fu, and G.P. Roberts.** 1991. An improved Tn*7*-based system for the single-copy insertion of cloned genes into chromosones of Gram-negative bacteria. Gene **109**:167-168.
3. **Berg, G., N. Roskot, A. Steidle, L. Eberl, A. Zock, and K. Smalla.** 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Appl. Environ. Microbiol. **68**, in press.
4. **Bertani, G.** 1951. Studies on lysogenesis I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62:**293-300.
5. **Black, P. N., Q. Zhang, J. D. Weimar, and C. DiRusso.** 1997. Mutational analysis of a fatty acyl-coenzyme A synthetase signature motif identifies seven amino acid residues that modulate fatty acid substrate specificity. J. Biol. Chem. **272:**4896-4903.
6. **Cha, C., P. Gao, Y. Chen, P. D. Shaw, and S. K. Farrand.** 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact. **11:**1119-1129.
7. **Christensen, B. B., C. Sternberg, J. B. Andersen, R. J. Palmer, A. T. Nielsen, M. Givskov, and S. Molin.** 1999. Molecular tools for the study of biofilm physiology. Methods Enzymol. **310:**20-42.
8. **Clark, J. D., and O. Maaløe.** 1967. DNA replication and the division cycle in *Escherichia coli*. J. Mol. Biol. **23:**99-112.
9. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295-298.
10. **De Kievit, T., P. C. Seed, J. Nezezon, L. Passador, and B. H. Iglewski.** 1999. RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. J. Bacteriol. **181:**2175-2184.
11. **De Kievit, T., and B. H. Iglewski.** 2000. Bacterial quorum sensing in pathogenic relationships. Infect. Immun. **68:**4839-4849.
12. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived mini-transposons. Methods Enzymol. **235:**386-405.
13. **Eberl, L.** 1999. *N*-Acyl homoserinelactone-mediated gene regulation in gram-negative bacteria. System. Appl. Microbiol. **22:**493-506.
14. **Elasri, M., S. Delorme, P. Lemanceau, G. Stewart, B. Laue, E. Glickmann, P. M. Oger, and Y. Dessaux.** 2001. Acyl-homoserine lactone production is more common among plant-associated *Pseudomonas* spp. than among soilborne *Pseudomonas* spp. Appl. Environ. Microbiol. **67:**1198-1209.
15. **El-Sayed, K. A., J. Hothersall, and C. M. Thomas.** 2001. Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens* NCIMB 10586. Microbiology **147:**2127-2139.
16. **Fenton, A. M., P. M. Stephens, J. Crowley, M. O'Callaghan, and F. O'Gara.** 1992. Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl. Eviron. Microbiol. **58:**3873-3878.

17. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727-751.
18. **Gambello M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000-3009.
19. **Geisenberger, O., M. Givskov, K. Riedel, N. Hoiby, B. Tümmler, and L. Eberl.** 2000. Production of *N*-acyl-L-homoserine lactones by *P. aeruginosa* isolates from chronic lung infections associated with cystic fibrosis. FEMS Microbiol. Lett. **184:**273-278.
20. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557-6567.
21. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.
22. **Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl.** 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology **147:**2517-2528.
23. **Inada, T. and Y. Nakamura.** 1995. Lethal double-stranded RNA processing activity of ribonuclease III in the absence of *suhB* protein of *Escherichia coli*. Biochimie **77:**294-302.
24. **Kessler, B., V. de Lorenzo, and K. N. Timmis.** 1992. A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the *Pm* promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. **233:**293-301.
25. **Koch, B., L. E. Jensen, and O. Nybroe.** 2001. A panel of Tn*7*-based vectors for insertion of the *gfp* marker gene or for delivery of cloned DNA into gram-negative bacteria at a neutral chromosomal site. J. Microbiol. Methods **45**:187-195.
26. **Kojic, M., G. Gegrassi, and V. Venturi.** 1999. Cloning and characterization of the *rpoS* gene from plant growth-promoting *Pseudomonas putida* WCS358: RpoS is not involved in siderophore and homoserine lactone production. Biochim. Biophys. Acta **1489:**413-420.
27. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175-176.
28. **Kristensen, C. S., L. Eberl, J. M. Sanches-Romero, M. Givskov, S. Molin, and V. de Lorenzo.** 1995. Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. J. Bacteriol. **177:**52-58.
29. **Laue, B. E., Y. Jiang, S. R. Chhabra, S. Jacob, G. S. A. B. Stewart, A. Hardman, J. A. Downie, F. O'Gara, and P. Williams.** 2000. The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium* small bacteriocin, *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone, via HtdS, a putative novel *N*-acylhomoserine lactone synthase. Microbiology **146:**2469-2480.

30. **Lynch, M. J., S. Swift, D. F. Kirke, C. W. Keevil, C. E. R. Dodd, and P. Williams.** 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environ. Microbiol. **4:**18-28.
31. **Maes, T., D. Vereecke, T. Ritsema, K. Cornelis, H. N. T. Thu, M. Van Montagu, M. Holsters, and K. Goethals.** 2001. The *att* locus of *Rhodococcus fascians* strain D188 is essential for full virulence on tobacco through the production of an autoregulatory compound. Mol. Microbiol. **42:**13-28.
32. **Oka, A., H. Sugisaki, and M. Takanami.** 1981. Nucleotide sequence of the kanamycin resistance transposon Tn*903*. J. Mol. Biol. **147**:217-226.
33. **Parsek, M. R., A. L. Schaefer, and E. P. Greenberg.** 1997. Analysis of random and site-directed mutations in *rhlI*, a *Pseudomonas aeruginosa* gene encoding an acylhomoserine lactone synthase. Mol. Microbiol. **26:**301-310.
34. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127-1130.
35. **Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA **91:**197-201.
36. **Pesci, E. C., J. P. Pearson, P. C. Seed, and B. H. Iglewski.** 1997. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **181:**2175-2184.
37. **Pierson, E. A., D. W. Wood, J. A. Cannon, F. M. Blachere, and L. S. Pierson III.** 1998. Interpopulation signaling via *N*-acyl-homoserine lactones among bacteria in the wheat rhizosphere. Mol. Plant-Microbe Interact. **11:**1078-1084.
38. **Rainey, P. B.** 1999. Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. Environ. Microbiol. **1:**143-257.
39. **Riedel, K., M. Hentzer, O. Geisenberger, B. Huber, A. Steidle, H. Wu, N. Høiby, M. Givskov, S. Molin, and L. Eberl.** 2001. *N*-Acylhomoserine-lactone-mediated intergeneric communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology **147:**3249-3262.
40. **Rust, L., E. C. Pesci, and B. H. Iglewski.** 1996. Analysis of the *Pseudomonas aeruginosa* elastase (*lasB*) regulatory region. J. Bacteriol. **178:**1134-1140.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
42. **Sauer, K., and A. K. Camper.** 2001. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. J. Bacteriol. **183:**6579-6589.
43. **Shanahan, P., D. J. O'Sullivan, P. Simpson, G. Glennon, and F. O'Gara.** 1992. Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters affecting its production. Appl. Eviron. Microbiol. **58:**353-358.
44. **Shaw, P. D., G. Ping, S. Daly, C. Cha, J. E. Cronan Jr., K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. USA **94:**6036-6041.
45. **Steidle, A., K. Sigl, R. Schuhegger, A. Ihring, M. Schmid, S. Gantner, M. Stoffels, K. Riedel, M. Givskov, A. Hartmann, C. Langebartels, and L. Eberl.** 2001. Visualization of *N*-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. Appl. Environ. Microbiol. **67:**5761-5770.

46. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. L. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K.-S. Wong, Z. Wu, I. Paulsen, J. Reizer, M. H. Saier, R. E. W. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1: an opportunistic pathogen. Nature **406:**959-964.
47. **Swift, S., M. K. Winson, P. F. Chan, N. J. Bainton, M. Birdsall, P. J. Reeves, C. E. D. Rees, S. R. Chhabra, P. J. Hill, J. P. Throup, B. W. Bycroft, G. P. C. Salmond, P. Williams, and G. S. A. B. Stewart.** 1993. A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. Mol. Microbiol. **10:**511-520.
48. **Weller, D. M.** 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26:**379-407.
49. **Whitehead, N. A., A. M. L. Barnard, H. Slater, N. J. L. Simpson, and G. P. C. Salmond.** 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Rev. **25:**365-404.
50. **Whiteley, M., and E. P. Greenberg.** 2001. Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. J. Bacteriol. **183:**5529-5534.
51. **Williams, P., M. Camara, A. Hardman, S. Swift, D. Milton, V. J. Hope, K. Winzer, B. Middleton, D. I. Pritchard, and B. W. Bycroft.** 2000. Quorum sensing and the population-dependent control of virulence. Phil. Trans. R. Soc. Lond. B. **355:**667-680.
52. **Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. C. Salmond, B. W. Bycroft, A. Lazdunski, G. S. A. B. Stewart, and P. Williams.** 1995. Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA. **92:**9427-9431.
53. **Winson, M. K., S. Swift, L. Fish, J. P. Throup, F. Jørgensen, S. R. Chhabra, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart.** 1998. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl-homoserine lactone-mediated quorum sensing. FEMS Microbiol. Lett. **163:**185-192.
54. **Wood, D. W., F. Gong, M. M. Daykin, P. Williams, and L. S. Pierson III.** 1997. *N*-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. J. Bacteriol. **179:**7663-7670.
55. **Zhang, Z., and L. S. Pierson III.** 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. Appl. Environ. Microbiol. **67:**4305-4315.

Anhang 3

Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants

GABRIELE BERG, NICOLLE ROSKOT, ANETTE STEIDLE, LEO EBERL, ANGELA ZOCK, and KORNELIA SMALLA

Veröffentlicht in: ***Applied and Environmental Microbiology*****, 68:3328-3338 (2002)**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2002, p. 3328–3338 Vol. 68, No. 7
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.7.3328–3338.2002

Plant-Dependent Genotypic and Phenotypic Diversity of Antagonistic Rhizobacteria Isolated from Different *Verticillium* Host Plants

Gabriele Berg,[1]* Nicolle Roskot,[1] Anette Steidle,[2] Leo Eberl,[2] Angela Zock,[3] and Kornelia Smalla[3]

Department of Microbiology, University of Rostock, D-18055 Rostock,[1] Department of Microbiology, Technical University of Munich, D-85350 Freising,[2] and Federal Biological Research Center for Agriculture and Forestry, D-38104 Braunschweig,[3] Germany

Received 10 December 2001/Accepted 11 April 2002

To study the effect of plant species on the abundance and diversity of bacterial antagonists, the abundance, the phenotypic diversity, and the genotypic diversity of rhizobacteria isolated from potato, oilseed rape, and strawberry and from bulk soil which showed antagonistic activity towards the soilborne pathogen *Verticillium dahliae* Kleb. were analyzed. Rhizosphere and soil samples were taken five times over two growing seasons in 1998 and 1999 from a randomized field trial. Bacterial isolates were obtained after plating on R2A (Difco, Detroit, Mich.) or enrichment in microtiter plates containing high-molecular-weight substrates followed by plating on R2A. A total of 5,854 bacteria isolated from the rhizosphere of strawberry, potato, or oilseed rape or bulk soil from fallow were screened by dual testing for in vitro antagonism towards *Verticillium*. The proportion of isolates with antagonistic activity was highest for strawberry rhizosphere (9.5%), followed by oilseed rape (6.3%), potato (3.7%), and soil (3.3%). The 331 *Verticillium* antagonists were identified by their fatty acid methyl ester profiles. They were characterized by testing their in vitro antagonism against other pathogenic fungi; their glucanolytic, chitinolytic, and proteolytic activities; and their BOX-PCR fingerprints. The abundance and composition of *Verticillium* antagonists was plant species dependent. A rather high proportion of antagonists from the strawberry rhizosphere was identified as *Pseudomonas putida* B (69%), while antagonists belonging to the *Enterobacteriaceae* (*Serratia* spp., *Pantoea agglomerans*) were mainly isolated from the rhizosphere of oilseed rape. For *P. putida* A and B plant-specific genotypes were observed, suggesting that these bacteria were specifically enriched in each rhizosphere.

The study of root-associated bacteria and their antagonistic potential is important not only for understanding their ecological role in the rhizosphere and the interaction with plants but also for any biotechnological application, e.g., biological control of soilborne plant pathogens. *Verticillium* wilt caused by the soilborne fungus *Verticillium dahliae* Kleb. is an important disease responsible for dramatic yield losses in many crops (46). Since microsclerotia of *V. dahliae* that develop in the senescing tissues of the dead plant may persist in soil for several years in the absence of a susceptible host, chemical control is nearly impossible (25). In the coming years, the phasing out of methyl bromide as a control measure for *Verticillium* wilt will have a great impact on the accumulation of microsclerotia in soil (46). The pathogen has a broad host range which includes many important crops, such as strawberry, potato, and oilseed rape. Efficacious control methods for *Verticillium* wilt are urgently needed for commercial crop production.

An environmentally friendly alternative to protect roots against fungal pathogens is rhizobacterium-mediated biological control (6, 47). Numerous studies have demonstrated the ability of several rhizobacteria to suppress diseases caused by fungal plant pathogens (12, 22, 48). One of the difficulties in developing rhizobacteria as a viable alternative is that many biological control agents are found to be too variable in their performance. According to Raaijmakers and Weller (35), variable expression of genes involved in disease suppression and poor root colonization are the major factors contributing to this inconsistency. Mechanisms of bacterial antagonism toward plant-pathogenic fungi include the competition for nutrients and space, the production of antibiotics, and the production of fungal cell wall-degrading enzymes (8, 13, 24). The production of antifungal metabolites is subject to complex regulation, allowing the bacteria to sense their own population density and to respond to different environmental factors (6, 9). Successful biological control requires not only a better understanding of the complex regulation of antifungal metabolite production by antagonists in response to environmental factors but also a better picture of what triggers root colonization and of the dynamics and composition of bacterial rhizosphere communities. Thus, little is known about plant specificity of antagonistic root-associated bacteria, which are an important functional group of beneficial bacteria in the rhizosphere (44). A few studies have indicated a plant-dependent composition of culturable bacteria (5, 15, 16, 21, 23, 28). Recently, denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S ribosomal DNA (rDNA) genes from community DNA were used to study dominant bacterial populations in the rhizosphere of the three *V. dahliae* Kleb. host plants—strawberry, potato, and oilseed rape—over two growing seasons (43). Using this cultivation-independent approach, a plant-dependent abundance of dominant bacterial populations could be shown for most of the sampling times. To examine the rhizosphere effect and the impact of the plant species on the

* Corresponding author. Mailing address: Universität Rostock, Fachbereich Biowissenschaften, Institut für Molekulare Physiologie und Biotechnologie, Mikrobiologie, D-18051 Rostock, Germany. Phone: 49 381 4986154. Fax: 49 381 4986152. E-mail: gabriele.berg@biologie.uni-rostock.de.

abundance and diversity of *Verticillium* antagonists, in the same study fallow soil and rhizosphere samples of strawberry (*Fragaria* × *ananassa* [Duchense] Decaisne and Naudin [family, *Rosaceae*]), oilseed rape (*Brassica napus* L. [family, *Brassicaceae*]), and potato (*Solanum tuberosum* L. [family, *Solanaceae*]) were analyzed by a cultivation-dependent approach. Bacterial isolates obtained after plating on R2A and enrichment in microtiter plates containing high-molecular-weight substrates were screened by dual culture for antagonistic activity against *V. dahliae*. A comprehensive phenotypic and genotypic characterization of the antagonists provided new data on plant-dependent diversity of *Verticillium* antagonists.

This field study performed over two growth periods showed that different bacterial populations which are potentially antagonistic towards *V. dahliae* were enriched in the rhizosphere of different *Verticillium* host plants. The enormous phenotypic and genotypic diversity revealed at the subspecies level and an improved understanding of the plant-dependent bacterial diversity will contribute to the development of improved biological control strains.

MATERIALS AND METHODS

Experimental design. Three different crop plants, potatoes (cultivar Element), oilseed rape (cultivar Express), and strawberries (cultivar Elsanta), were grown in a randomized block design with six replicates per crop plant and six unplanted plots. Field design and sampling were carried out according to the method of Smalla et al. (43).

Isolation of bacteria. Roots with adhering soil from five plants from one plot were sampled into sterile petri dishes and then transported to the laboratory. Three grams of each sample was transferred into a sterile stomacher bag. To extract the rhizosphere microorganisms from the roots, 27 ml of demineralized water was added and samples were homogenized in a stomacher laboratory blender for 60 s (BagMixer; Interscience, St. Nom, France). This procedure was repeated three times for each sample. Samples were serially diluted with sterile 0.85% NaCl and plated onto R2A (Difco, Detroit, Mich.). Plates were incubated for 5 days at 20°C, and CFU were counted after 5 days to calculate the means of colonies ($\log_{10}$ CFU) based on fresh weight (FW). Per treatment (strawberry, potato, oilseed rape, and fallow) and sampling time, 48 colonies with different colony morphologies were picked from dilution plates with 20 to 100 colonies. To enrich bacteria with hydrolytic activities, microtiter plates with high-molecular-weight substrates were used (AZO-CM-cellulose and AZO-xylan [Megazym, Sydney, Australia], casein [Gibco, Paisley, United Kingdom], and chitin [Sigma, Deisenhofen, Germany]). The plates were filled with the following contents: 1.5 g of peptone of casein (Gibco); 0.5 g of peptone of soy (Gibco); 0.5 g of NaCl liter^{-1} (pH 7.3); and azurine-dyed, cross-linked (AZCL) substrates, chitin, and casein (0.5 g liter^{-1}). The combined supernatants of six replicates per treatment were inoculated in serial dilution. The contents of wells of the last completely grown row were combined after 5 days' incubation, and 100 μl of this solution was plated after serial dilution on R2A. Between 15 and 20 colonies were isolated per high-molecular-weight substrate, purified, and stored at −70°C in broth containing 15% glycerol. Isolates obtained by plating were purified and stored at −70°C in broth containing 15% glycerol.

Screening of antagonistic bacteria. Bacterial isolates were screened for their activity towards *V. dahliae* Kleb. by a dual-culture in vitro assay on Waksman agar containing 5 g of proteose peptone (Merck, Darmstadt, Germany), 10 g of glucose (Merck), 3 g of meat extract (Chemex, Munich, Germany), 5 g of NaCl (Merck), 20 g of agar (Difco), and distilled water (to 1 liter), pH 6.8. Zones of inhibition were measured after 5 days of incubation at 20°C according to the method of Berg (3). All strains were tested in three independent replicates with (i) *V. dahliae* Kleb. V16 (isolated from *S. tuberosum* L.), (ii) *V. dahliae* V25 (isolated from *Brassica napus* L.), (3), and *V. dahliae* V35 (isolated from *Fragaria* × *ananassa* [Duchense] Decaisne and Naudin). Only bacterial isolates which showed antagonistic activity towards *V. dahliae* were tested for their antagonism towards *Rhizoctonia solani* Kühn and *Sclerotinia sclerotiorum* Lib. (culture collection of the University of Rostock, Department of Microbiology) and *Phytophthora cactorum* (Lebert and Cohn) J. Schröt. PF8 (from the culture collection of the Federal Biological Research Center for Agriculture and Forestry, Darmstadt, Germany). These fungi were routinely grown on Sabouraud medium (Gibco) and stored at −70°C in broth containing 15% glycerol.

Identification of bacterial antagonists. All antagonists were identified based on whole-cell cellular fatty acids; derivatized to methyl esters, i.e., fatty acid methyl esters (FAMEs); and analyzed by gas chromatography using the MIDI system (Microbial Identification System, Inc., Newark, N.J.). In addition, some strains were identified by 16S rDNA sequencing and aligned with the reference 16S rRNA gene sequence using the BLAST algorithm according to the method of Altschul et al. (1). Species richness, expressed as the number of species as a function (ratio) of the total number of individuals, was determined by the index proposed by Menhinick (27).

Screening for strains with endo-digesting hydrolytic activity. Chitinase activity (β-1,4-glucosamine polymer degradation) was tested in chitin minimal medium, according to the method of Chernin et al. (7). Clearing zones were detected 5 days after incubation at 30°C. β-Glucanase activity was tested using chromogenic AZCL substrates (Megazym). Formation of blue haloes was recorded until 5 days after incubation. Protease activity (casein degradation) was determined from clearing zones in skim milk agar (50 ml of sterilized skim milk mixed at 55°C with 50 ml of one-fifth volume of tryptic soy agar and 4% agar) after 5 days of incubation at 30°C.

Production of secondary metabolites. The ability of bacterial isolates to produce indole-3-acetic acid (IAA) was checked using the microplate method developed by Sawar and Kremer (41). The direct proof of cyanide production was made with an Aquaquant-14417-Testsystem (Merck) with culture broth (48 h) of the isolates. The ability of isolates to produce fluorescent siderophores was tested by plating bacteria on King's medium B (20) and incubating for 2 days at 25°C. Plates were inspected under 366-nm UV light.

BOX-PCR genomic fingerprints. Bacterial DNA was prepared following the protocol of Andersen and McKay (2) modified for genomic DNA. BOX-PCR was done as described by Rademaker and De Bruijn (37) using the BOXA1R primer 5′-CTA CGG CAA GGC GAC GCT GAC G-3′. PCR amplification was performed with a Peltier thermal cycler (PTC-200; Biozym Diagnostic, Hessisch Oldendorf, Germany) using an initial denaturation step at 95°C for 6 min and subsequently 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 65°C for 8 min followed by final extension at 65°C for 16 min. A 10-μl aliquot of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5× Tris-borate-EDTA buffer for 6 h, stained with ethidium bromide, and photographed under UV transillumination. The reproducibility of the results was verified in three independent experiments.

PCR detection of *phlD* and *chiA* genes. Amplifications with gene-specific primer Phl2 (36) were performed in the following mix: 1 μl of target DNA, a 10 pM concentration of each primer, and 17 μl of PCR SuperMix High Fidelity (Gibco). PCR was performed under the following conditions: 3 min at 95°C followed by 29 cycles consisting of 1 min at 94°C, 45 s at 48°C, and 45 s at 72°C for 45 s. PCR was finished by a primer extension step at 72°C for 5 min. For *chiA* gene detection the following primers were aligned from *Serratia marcescens* (accession no. A25090) for amplification: ChiA1 (5′-ATG CGC AAA TTT AAT AACC-3′) and ChiA2 (3′-CCG ATT GAA CGCG-5′). PCR was performed in the same mix listed above using a 0.1 pM concentration of each primer. PCR was performed with 29 cycles consisting of 1 min at 95°C, 1 min at 55°C, and 55 s at 48°C subsequently followed by a 10-min final extension step at 72°C. PCR-amplified DNAs were detected by using a 1.5% agarose gel. The gels were stained with ethidium bromide for 30 min, and the PCR products were visualized with a UV transilluminator. The reproducibility of the results was verified in two independent experiments, and *S. marcescens* was used as a positive control.

Characterization of AHLs produced by rhizosphere isolates. Production of *N*-acylhomoserine lactones (AHLs) by bacterial isolates was investigated with the aid of the bioluminescent sensor plasmid pSB403 (49). This sensor plasmid contains the *Photobacterium fischeri luxR* gene together with the *luxI* promoter region as a transcriptional fusion to the bioluminescence genes *luxCDABE* of *Photorhabdus luminescens.* The quorum-sensing system of *P. fischeri* relies on *N*-(3-oxo-hexanoyl)-homoserine lactone (3-oxo-C_6-HSL), and the sensor plasmid consequently exhibits the highest sensitivity for this AHL molecule. However, several other AHL molecules are detected by the sensor, albeit with somewhat-reduced sensitivity (49). We also employed the green fluorescent protein-based biosensor *Pseudomonas putida* F117(pKR-C12) (45) for the detection of long-chain AHLs. Plasmid pKR-C12 (40) contains an AHL sensor cassette which is based on components of the *las* quorum-sensing system of *Pseudomonas aeruginosa*. Specifically, this cassette consists of a P*lasB-gfp*(ASV) translational fusion together with the *lasR* gene placed under control of P*lac.* Expectedly, as the cognate AHL of the *las* system is 3-oxo-C_{12}-HSL (31), this system is most sensitive for 3-oxo-C_{12}-HSL and other long-chain AHL molecules. In this case, production of AHLs was monitored by the expression of green fluorescence. This

was accomplished by illuminating plates with blue light using an HQ 480/40 filter (F44-001; AHF-Analysentechnik, Tübingen, Germany) in combination with a halogen lamp (Intralux 5000-1; Volpi, Schlieren, Switzerland) as a light source. Illumination took place in a dark box equipped with the C2400-40 camera connected to a Pentax CCTV camera lens and an HQ 535/20 filter (F42-001; AHF-Analysentechnik).

Computer-assisted cluster analysis. Computer-assisted evaluation of BOX-PCR-generated fingerprints was made using the GelCompare program (version 4.1; Applied Math, Kortrijk, Belgium). The cluster analysis was performed with Ward's algorithm and the unweighted pair-group method using arithmetic averages (UPGMA) algorithm. The physiological analysis data were converted to a binary code, and interisolate relationships were measured by the Euclidian metric algorithm and UPGMA in the program STATISTICA (StatSoft, Hamburg, Germany).

RESULTS

Isolation of bacteria from the rhizospheres and from soil. At all sampling times the CFU counts were approximately 1 order of magnitude higher for rhizosphere samples than for soil from fallow plots. The CFU numbers determined for rhizosphere samples were rather similar for the different plants (strawberry and potato, $\log_{10}$ 8.3 ± 2.5 (strawberry) or 3.1 (potato) CFU g^{-1} root FW; oilseed rape, $\log_{10}$ 8.1 ± 1.7 CFU g^{-1} root FW) and showed no significant seasonal changes. A total of approximately 120 isolates per treatment (strawberry, potato, oilseed rape, and bulk soil) and per sampling time was used in the initial screening of antagonistic activity towards *V. dahliae* Kleb.

Screening for isolates antagonistic to *V. dahliae*. A total of 5,854 bacterial isolates were screened for their ability to suppress *V. dahliae* in an in vitro dual-culture assay. Initially 334 isolates were found which were active against *V. dahliae*, of which 67 were strongly active, with inhibition zones larger than 10 mm. Although similar numbers of isolates from each of the treatments were tested, the proportion of isolates with antagonistic activity was different. The proportion of isolates with antifungal activity was highest for strawberry rhizosphere (9.5%), followed by oilseed rape rhizosphere (6.3%), potato rhizosphere (3.7%), and fallow soil (3.3%). The proportion of *Verticillium* antagonists isolated from R2A plates after previous enrichment in high-molecular-weight substrate plates (7 to 8%) was significantly higher than that for isolates obtained after direct plating on R2A, of which only 3% showed antagonistic activity.

Diversity of *Verticillium* antagonists. The majority of the in vitro antagonists ($n = 286$) were identified by fatty acid analysis (Table 1). Based on their fatty acid profiles, 46 different bacterial species were identified. The richness of antagonistic species was plant species dependent. The highest number of different species with antagonistic activity was isolated from oilseed rape rhizosphere ($n = 30$), while only 18 or 12 different species were found in the rhizosphere of strawberry and potato, respectively. Interestingly, antagonists isolated after previous enrichment in high-molecular-weight substrates belonged mainly to the fast-growing γ-subdivision of the proteobacteria. The species composition did not depend on the kind of high-molecular-weight substrate. The diversity of antagonists obtained after direct plating onto R2A was higher, and gram-positive bacteria such as *Bacillus* spp. and *Streptomyces* spp. were only obtained by the direct plating approach. Gram-positive antagonistic isolates accounted only for a rather-small proportion of the *Verticillium* antagonists (11 of 286), and with one exception these isolates originated from bulk soil.

TABLE 1. List of bacterial species with antagonistic properties isolated from the rhizosphere of strawberry, potato, and oilseed rape, and uncultivated soil in the vegetation period 1998 to 1999

Bacterial species[a]	No. of isolates[b] obtained from:			
	Rhizosphere			Soil
	Strawberry	Potato	Oilseed rape	
Acidovorax avenea			1	
Acinetobacter baumannii	1			
Acinetobacter calcoaceticus	1 (1)			
Agrobacterium rhizogenes			1	
Agrobacterium tumefaciens	1			
Bacillus circulans				1
Bacillus laterosporus				1
Bacillus megaterium	1 (1)			
Bacillus mycoides				1
Bacillus pumilus				1
Burkholderia cepacia	1		1	
Brevibacterium acetylicum				2 (2)
Chryseobacterium balustinum			1	
Chryseobacterium indologenes			1	
Comamonas acidovorans	2 (2)	1 (1)		
Cytophaga johnsonae		1 (1)		
Enterobacter agglomerans			1	
Enterobacter intermedius			3	
Janthinobacterium lividum		1		
Kluyvera cryorescens		1 (1)		
Kocuria kristinae			1	
Pantoea agglomerans			3	1
Pasteurella anatipestifer			1 (1)	
Proteus vulgaris	1		2	
Pseudomonas chlororaphis		4 (1)	8	
Pseudomonas corrugata	3	3	1	1
Pseudomonas fluorescens	7 (3)	2	9 (4)	4
Pseudomonas marginalis	1	1	3	2
Pseudomonas putida A	6	6	13	10
Pseudomonas putida B	86 (2)	24 (1)	9 (1)	
Pseudomonas syringae	7	3 (2)	1	
Pseudomonas tolaasii	1 (1)		3 (1)	
Pseudomonas viridiflava			1	
Salmonella enterica Typhimurium	2 (2)		1 (1)	
Serratia fonticola			1	
Serratia grimesii	2		3	1
Serratia odorifera			1	
Serratia plymuthica			3	
Serratia proteamaculans			2	1
Stenotrophomonas maltophilia	1	2	2	
Streptomyces albidoflavus				2 (1)
Streptomyces hygroscopicus				1 (1)
Streptoverticillium reticulum				2
Weeksella zoohelcum			2 (1)	
Xenorhabdus nematophilia	1 (1)		1 (1)	
Xenorhabdus luminescens			1 (1)	
Total no. of species	18	12	30	15
Total no. of isolates	125	49	81	31
Richness (*d*)	1.61	1.71	3.33	2.69

[a] Identification by FAME analysis of isolates identified with a similarity index of <0.5.

[b] Numbers in parentheses indicate how many strains were isolated.

Only four species were obtained from the rhizospheres of all three plants and from soil—*Pseudomonas corrugata*, *Pseudomonas fluorescens*, *Pseudomonas marginalis*, and *P. putida* A—while *P. putida* B, *Pseudomonas syringae*, and *Stenotrophomonas maltophilia* were isolated from the rhizosphere of all three plants but not from soil. The highest number by far of

antagonistic isolates from the rhizosphere and from soil belonged to the *P. fluorescens* intrageneric cluster (strawberry, 111 of 125 antagonists; potato, 43 of 49 antagonists; oilseed rape, 48 of 81 antagonists; soil, 17 of 31 antagonists), with a high proportion of isolates from the *P. putida* lineage. The proportion of antagonists belonging to this *P. fluorescens* cluster was particularly high for strawberry and potato plants (approximately 90%), while their proportion in the rhizosphere of oilseed rape and soil accounted only for 59 and 55%, respectively. The frequent isolation of *P. putida* B from the strawberry rhizosphere (86 of 125 antagonists) was striking, leading to a rather low evenness of antagonistic isolates from strawberry. The diversity indices calculated for species richness were 3.3 for oilseed rape, 2.7 for soil, 1.7 for potato, and 1.6 for strawberry. The highest number of antagonists belonging to different species was observed for antagonists from the rhizosphere of oilseed rape. Different species of enterobacterial genera (*Serratia*, *Enterobacter*, *Pantoea*, and *Weeksella*) with antagonistic activity were isolated only from the rhizosphere of oilseed rape. Thus, enterobacterial species seem to be in particular enriched in the rhizosphere of oilseed rape. However, a large proportion of species (21 of 66 antagonists) was isolated from one plant species, often being isolated only once.

Antifungal activity and production of antifungal metabolites of *Verticillium* antagonists. All 286 *Verticillium* antagonists were tested in vitro for their activity against the plant pathogens *R. solani* (basidiomycete with a chitin-glucan-containing cell wall), *S. sclerotiorum* (ascomycete with a chitin-glucan-containing cell wall), and *P. cactorum* (oomycete with a cellulose-containing cell wall) and for the production of hydrolytic enzymes. Generally, the fungi grew as well as the bacterial isolates on Waksman agar. Inhibition was clearly discerned by limited growth or the complete absence of fungal mycelium in the inhibition zone surrounding a bacterial colony. *Verticillium* antagonists assigned to the same species often showed different patterns of antagonistic activity. While the majority of *P. putida* B isolates (mainly isolates from the strawberry rhizosphere) showed activity only against *V. dahliae*, a few *P. putida* B isolates also antagonized *R. solani*, *S. sclerotiorum*, and/or *P. cactorum. Verticillium* antagonists which showed a broad range activity and also suppressed *R. solani*, *S. sclerotiorum*, and *P. cactorum* originated from the rhizosphere of oilseed rape (*Serratia* spp. [$n = 7$]; *P. fluorescens* [$n = 1$]) and bulk soil (*Bacillus circulans* [$n = 1$]; *P. marginalis* [$n = 2$]). Altogether, more isolates with antagonistic activity against *R. solani* than against *S. sclerotiorum* and *P. cactorum* were found.

Strawberry. A large proportion of the antagonists isolated from the rhizosphere of strawberry showed antifungal activity only against *V. dahliae* (81 of 125 antagonists; 65%), most of them identified as *P. putida* B. About 30% of isolates also suppressed *R. solani* (37 of 125 antagonists), while activity against *S. sclerotiorum* and *P. cactorum* was found only for 9 of 125 antagonists (7%) and 8 of 125 antagonists (6.4%), respectively. For most of the *Verticillium* antagonists (121 of 125) proteolytic activity was detected, while only three of the isolates had chitinolytic activity and none showed glucanolytic activity. Isolates with chitinolytic activity were identified by FAME analysis as *Serratia* ($n = 2$) and *P. fluorescens* ($n = 1$).

Potato. Approximately 60% of the *Verticillium* antagonists isolated from the potato rhizosphere also antagonized *R. solani*, 20% had antagonistic activity towards *S. sclerotiorum*, and 18% had antagonistic activity towards *P. cactorum*. Fifteen isolates were suppressive towards three of the pathogens tested. Similarly to the strawberry isolates almost all isolates had proteolytic activity, while only two isolates (*S. maltophilia* and *Serratia proteamaculans*) showed chitinolytic activity.

Oilseed rape. Antagonistic activity against *R. solani* was observed for 53% of the *Verticillium* antagonists, while approximately 47% antagonized *S. sclerotiorum* and 23% were active against *P. cactorum*. Isolates which showed activity against *R. solani* and *S. sclerotiorum* most often belonged to the *Enterobacteriaceae*. Seven *Serratia* isolates were active against all pathogens tested here. Almost all *Verticillium* antagonists from oilseed rape showed clearing zones on skim milk agar plates, suggesting proteolytic activity. The proportion of antagonists with chitinolytic activity (19 of 81 antagonists; 23%) was higher than that for the strains from the rhizosphere of strawberry and potato plants. All strains with chitinolytic activity belonged to the *Enterobacteriaceae*. β-1,3-Glucanolytic activity was observed for five isolates belonging to taxonomically different groups.

Fallow soil. In contrast to the rhizosphere isolates, a higher proportion of *Verticillium* antagonists also suppressed *R. solani* (24 of 31; 77%) and *S. sclerotiorum* (19 of 31; 61%). Isolates displaying antagonistic activity towards these pathogens were most often identified as *P. putida* A. Two *P. marginalis* strains and one *B. circulans* strain were able to antagonize all pathogens tested here.

Characterization of *Verticillium* antagonists belonging to the *Enterobacteriaceae*. *Verticillium* antagonists assigned by FAME analysis to 13 different species belonging to the *Enterobacteriaceae* were mainly isolated from the rhizosphere of oilseed rape. BOX-PCR performed with genomic DNA yielded fingerprints with 12 to 35 amplification products, ranging from 100 to 3,000 bp. Isolates identified by FAME analysis as different species often displayed very similar BOX patterns (Fig. 1). This observation was confirmed when GelCompare was used for comparison of BOX patterns. Intraspecies diversity of BOX patterns analyzed in three independent replicates of isolate 9Ec15 (replicates I to III) was shown to be 89% similarity. Analysis of BOX patterns with more than 89% similarity resulted in three different cluster or genotype groups. Group 1 contained isolates identified with rather high similarity (of >0.8) as *Enterobacter*, *Salmonella*, *Serratia*, *Proteus*, and *Xenorhabdus*. The BOX patterns of the isolates which belong to this group are very similar or nearly identical (e.g., *Serratia odorifera* 4Rx13, *Xenorhabdus luminescens* 3Rp5, *Serratia plymuthica* 3Rr8). Cluster group 2 included isolates from *Serratia* and *Proteus* with highly homogeneous BOX patterns. Group 3 showed a more heterogeneous pattern than the other groups and contained 11 isolates belonging to four genera or seven species. To clarify these ambiguous results of the FAME analysis, partial 16S rDNA sequencing was done for 12 of the enteric isolates, each representing a BOX cluster. 16S rDNA sequencing revealed that 7 of the 12 isolates were most similar to *S. proteamaculans*, 4 were most similar to *S. plymuthica*, and 1 was most similar to *Pantoea agglomerans* (Table 2). Thus, the number of different species identified by FAME analysis, and consequently the diversity index calculated for richness of oilseed rape (Table 1), is an overestimate, and the corrected

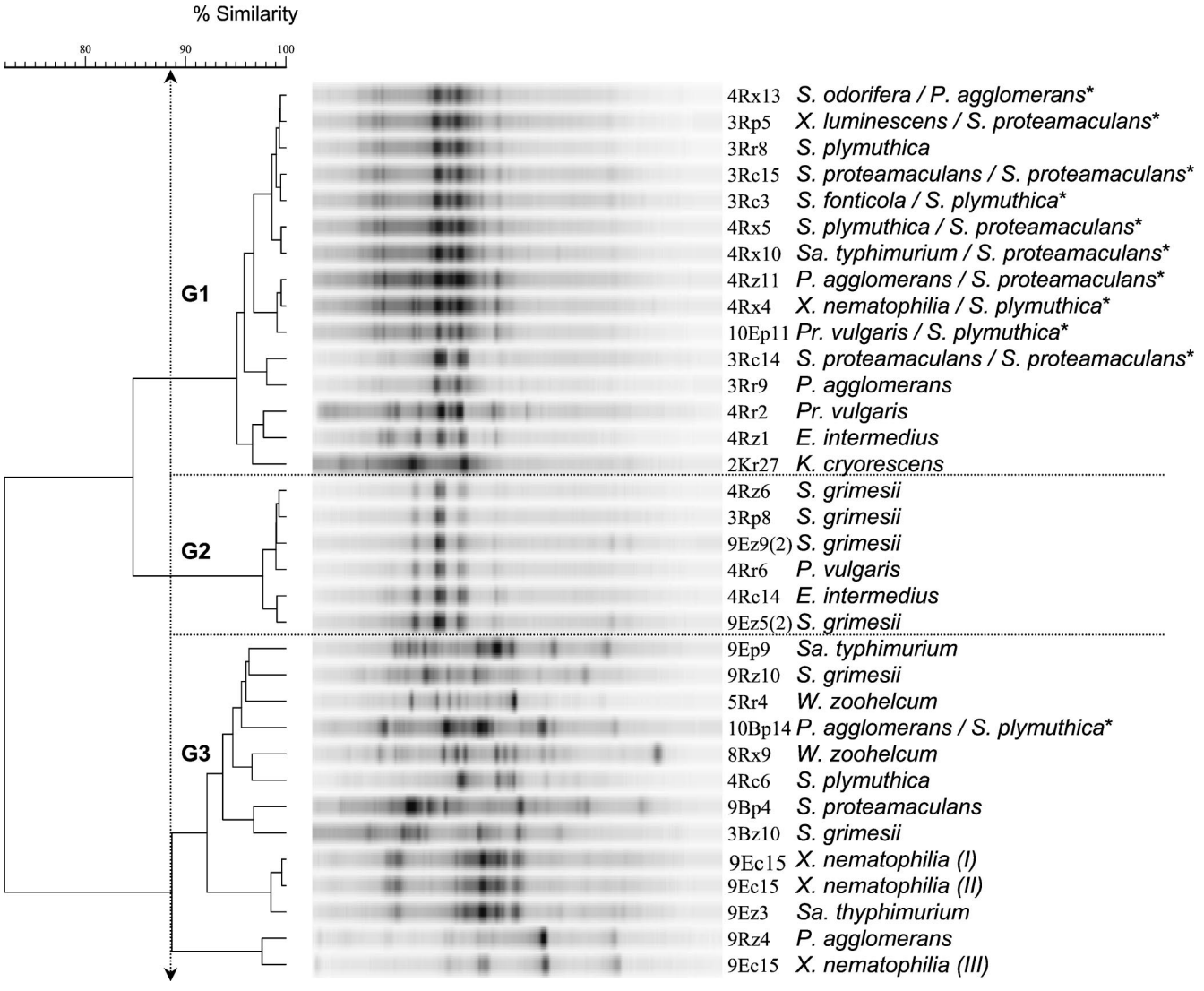


FIG. 1. Dendrogram showing the relationship of 32 *Enterobacteriaceae* isolates identified by FAME analysis from strawberry, potato, and oilseed rape rhizospheres and uncultivated soil based on BOX-PCR fingerprints using cluster analysis. An asterisk indicates a second identification by 16S rDNA sequencing. *K.*, *Kluyvera*; *P.*, *Pantoea*; *S.*, *Serratia*; *Pr.*, *Proteus*; *Sa.*, *Salmonella*; *W.*, *Weeksella*; *X.*, *Xenorhabdus*.

richness index would be 2.2. Although *Serratia* isolates which were active against all pathogens tested belonged to different BOX clusters, they all showed in vitro lytic activity. The majority of isolates were able to degrade chitin in plate assays. Additionally, when the molecular approach was used, the *chiA* gene was found in most of the chitinolytic strains. With the exception of four strains, enterics were proteolytic, while only three strains showed glucanolytic activity. Production of short-chain AHL signal molecules was detected for 15 out of 32 strains tested in cross-streaks against the sensor strain *Escherichia coli* MT102(pSB403). We also investigated the synthesis of long-chain AHLs with the aid of the biosensor *P. putida* F117(pKR-C12). However, only one strain (9Ep9) of all the *Enterobacteriaceae* tested gave a positive result, indicating that synthesis of long-chain AHL molecules is rare among members of this family. No obvious correlation between the production of AHL signal molecules and chitinolytic activity, proteolytic activity, or antagonism against fungi was observed.

Characterization of *P. putida* A strains. Approximately 12.2% of *Verticillium* antagonists were identified by FAME analysis as *P. putida* A. *P. putida* A isolates which antagonized *V. dahliae* originated from soil ($n = 10$), oilseed rape ($n = 13$), strawberry ($n = 6$), and potato ($n = 6$), indicating the absence of a plant-specific enrichment. *P. putida* A strains were mainly isolated in the second year (31 of 35 antagonists) and were obtained after enrichment or after direct plating onto R2A. Therefore, we analyzed for this subset of *Verticillium* antagonists a range of phenotypic and genotypic traits to find out whether a plant-specific enrichment of particular phenotypes and genotypes was detectable at a subspecies level (Table 3). The phenotypic characterization comprised the analysis of antifungal activity; the production of lytic enzymes (glucanases,

TABLE 2. Taxonomic classification and characterization of bacterial isolates with antagonistic properties belonging to *Enterobacteriaceae*

Genotype group[a]	Strain	FAME analysis		16S rDNA sequencing		Activity against[b]:				Lytic enzyme production[c]				AHL production	
		Identification	ID	Identification	% Similarity (strain)	*V. dahliae*	*R. solani*	*S. sclerotiorum*	*P. cactorum*	Glucanase	Chitinase	*chiA* product	Protease	MT 102[d]	FI17[e]
G1	4Rx13	*Serratia odorifera*	0.735	*Pantoea agglomerans*	99 (100–860)	+++	++	+++	−	−	−	−	+	+	−
		Pantoea agglomerans	0.701												
	3Rp5	*Xenorhabdus luminescens*	0.014	*S. proteamaculans*	99 (DSM4543)	+++	+	++++	+	−	+	+	+	−	−
	3Rr8	*Serratia plymuthica*	0.890			++	+++	+	+	−	+	+	+	−	−
	3Rc15	*Serratia proteamaculans*	0.839	*S. proteamaculans*	99 (DSM4543)	+++	++	++++	−	−	+	+	+	−	−
	3Rc3	*Serratia fonticola*	0.640	*S. plymuthica*	99 (DSM4540)	+++	++	++++	+	−	+	+	+	−	−
	4Rx5	*Serratia plymuthica*	0.715	*S. proteamaculans*	99 (DSM4543)	+++	+	+	−	−	+	+	+	+	−
	4Rx10	*Salmonella enterica* serovar Typhimurium	0.405	*S. proteamaculans*	99 (DSM4543)	++	+++	+	−		−	+	+	+	−
	4Rz11	*Pantoea agglomerans*	0.723	*S. proteamaculans*	99 (DSM4543)	++	+++	+	+	−	+	+	+	+	−
	4Rx4	*Xenorhabdus nematophilia*	0.146	*S. plymuthica*	99 (DSM4540)	+++	+	+	+	−	+	−	+	+	−
	10Ep11	*Proteus vulgaris*	0.834	*S. plymuthica*	98 (124–822)	++	+++	++	−	−	−	−	+	+	−
	3Rc14	*Pantoea agglomerans*	0.871												
		Klebsiella pneumoniae	0.829			+++	++	++++	−	+	+	+	−	−	−
	3Rr9	*Serratia proteamaculans*	0.793	*S. proteamaculans*	99 (DSM4543)	++	++	++++	+	−	+	+	+	−	−
	4Rr2	*Proteus vulgaris*	0.758			+	+++	++++	+	−	+	+	+	+	−
		Serratia grimesii	0.706												
	4Rz1	*Enterobacter intermedius*	0.591			++	+++	+	−		+	+	+	+	−
	2Kr27	*Kluyvera cryorescens*	0.383	*S. proteamaculans*	99 (DSM4543)	+	−	−	−	−	+	+	+	−	−
G2	4Rz6	*Serratia grimesii*	0.718			++	+++	+	−	−	+	+	+	+	−
		Pantoea agglomerans	0.714												
	3Rp8	*Serratia grimesii*	0.614			+++	+++	++++	−	−	+	−	+	+	−
		Pantoea agglomerans	0.589												
	9Ez29	*Serratia grimesii*	0.768			++	++++	+	−	−	+	+	+	+	−
		Pantoea agglomerans	0.765												
	4Rr6	*Proteus vulgaris*	0.721			++	+++	+	+	−	+	+	+	+	−
		Pantoea agglomerans	0.677												
	4Rc14	*Enterobacter intermedius*	0.736												
			0.675			+	+++	+	−	−	+	+	+	+	−
		Serratia grimesii													
	9Ez25	*Serratia grimesii*	0.777			++	−	+	−	−	+	+	+	+	−
G3	9Ep9	*Salmonella enterica* serovar Typhimurium	0.496			+	−	−	−	−	−	−	+	+	+
	9Rz10	*Serratia grimesii*	0.928			++++	++++	+	−	−	−	−	−	−	−
	5Rr4	*Weeksella zoohelcum*	0.560			++	+++	+++	−		+	−	−	−	−
	10Bp14	*Pantoea agglomerans*	0.884	*S. plymuthica*	99 (DSM4540)	+	+	+	−	−	−	+	−	−	−
	8Rx9	*Weeksella zoohelcum*	0.478			+++	−	−	−	+	−	−	+	−	−
	4Rc6	*Serratia plymuthica*	0.790			++	++	++++	−	+	+	+	+	−	−
	9Bp4	*Serratia proteamaculans*	0.740			+	−	−	−	−	−	+	+	−	−
	3Bz10	*Serratia grimesii*	0.928			+	−	−	−	−	−	+	+	−	−
	9Ec15	*Xenorhabdus nematophilia*	0.096			++	+	−	−	−	−	−	+	−	−
	9Ez3	*Salmonella enterica* serovar Typhimurium	0.398			++	−	−	−	−	−	−	+	−	−
	9Rz4	*Pantoea agglomerans*	0.89			++++	+++	++++	−	−	−	−	+	−	−
		Serratia grimesii	0.872			++++	+++	++++	−	−	−	−	+	−	−

[a] Grouping at 89% similarity.
[b] Antagonism toward these four species was determined by dual-culture assay. Results indicate the width of the zone of inhibition as follows: +, 0 to 5 mm; ++, 5 to 10 mm; +++, 10 to 15 mm; ++++, >15 mm.
[c] β-1,3-Glucanase, protease, and chitinase activities were demonstrated by plate assay (+, hydrolysis; −, no hydrolysis). The *chiA* gene was detected by a PCR approach.
[d] *E. coli* MT 102(pSB403) for detection of short-chain AHLs.
[e] *P. putida* F117(pKR-C12) for detection of long chain AHLs.

TABLE 3. Phenotypic characterization of *P. putida* A isolates belonging to the genotypic clusters defined in Fig. 2

Genotype group[a]	Strain	Origin		Activity against[b]:				Lytic enzyme production[c]			Secondary metabolite production				AHL production		Phenotype group
				V. dahliae	*R. solani*	*S. sclerotiorum*	*P. cactorum*	Glucanase	Chitinase	Protease	King B[d]	DAPG[e]	HCN[f]	IAA[g]	MT 102[h]	F117[i]	
G1	8Kz4	Potato	Cellulose	++	++	++	−	−	−	+	−	−	+	+	+	−	2
	8Kz8	Potato	Cellulose	++	++	+	−	−	−	+	−	−	+	+	+	−	2
	4Kc15	Potato	Chitin	+	+++	+	−	−	−	+	−	−	+	+	+	−	1
G2	9Rp10/2	Rape	Casein	+	+++	+	−	−	−	+	−	−	−	+	+	−	5
G3	2Rr14	Rape	R2A	+	+++	+	−	−	−	+	−	−	−	+	+	−	5
	8Rr24	Rape	R2A	+++	++	+	−	−	−	−	−	−	+	+	+	−	3
G4	9Rz12/2	Rape	Cellulose	+	−	−	−	−	−	+	+	−	−	−	−	−	5
	9Rz10/2	Rape	Cellulose	+	−	−	−	−	−	+	+	−	−	−	−	−	5
	9Rc9	Rape	Chitin	+	+++	+++	−	−	−	+	+	+	−	−	−	−	7
	9Rp2	Rape	Casein	+	−	−	+	−	−	+	+	−	−	−	−	−	7
	9Rp10	Rape	Casein	+	−	−	+	−	−	+	+	−	−	−	−	−	5
G5	8Kr32	Potato	R2A	+	++	−	−	−	−	+	+	−	−	−	−	ND	7
G6	7Ec5	Strawberry	Chitin	++	+	−	−	−	−	+	−	+	+	−	−	ND	6
G7	7Bp13/2	Soil	Casein	++	+	−	+	−	−	+	+	+	−	−	+	+	5
G8	7Ez9/2	Strawberry	Cellulose	++	−	−	−	−	−	+		−			−	−	
	10Er28	Strawberry	R2A	++	++	+	−	−	−	+	−	−	+	+	+	−	1
G9	2Bx14	Soil	Xylan	+	+++	+	−	−	−	+	−	−	+	+	+	−	1
	9Bc2	Soil	Chitin	++	+++	++	−	−	−	−	−	−	+	+	+	−	3
G10	4Kc13	Potato	Chitin	+	+++	++	−	−	−	+	−	−	+	+	+	−	1
	10Kx10/2	Potato	Xylan	+	+++	+	−	−	−	+	−	−	+	+	+	−	1
G11	9Bp8	Soil	Casein	+	+++	+	−	−	−	+	−	−	+	+	+	−	2
	10Bp12	Soil	Casein	++	+++	+	−	−	−	+	−	−	+	+	+	−	1
G12	9Bc8	Soil	Chitin	++	+++	++	−	−	−	+	−	+	+	+	+	−	1
	9Bc10	Soil	Chitin	+	++	+	−	−	−	+	−	+	+	+	+	+	1
	9Bc3	Soil	Chitin	++	+++	+	−	−	−	+	−	−	+	+	+	−	1
G13	2Bx4	Soil	Xylan	+	+++	+	−	−	−	+	−	+	+	+	+	−	1
	2 Bx13	Soil	Xylan	+	+++	+	−	−	−	+	−	−	+	+	+	−	1
G14	9Rx5	Rape	Xylan	+	−	−	+	−	−	+	+	+	−	+	+	+	5
G15	9Rx7	Rape	Xylan	+	−	−	−	−	−	+	+	+	−	+	+	+	5
G16	9Rx24	Rape	Xylan	+	−	−	+	−	−	+	+	−	−	+	+	+	5
	9Rx29	Rape	Xylan	++	−	+++	+	−	−	+	+	−	−	+	+	+	5
G17	9Rx11	Rape	Xylan	++	−	−	−	−	−	+	+	−	−	+	+	+	5
G18	9Ex1/2	Strawberry	Xylan	+	−	−	−	−	−	+	+	+	+	+	−	ND	4
	7Ez14/2	Strawberry	Cellulose	+++	−	−	−	−	−	+	−	+	+	−	−	−	6
	7Ex1	Strawberry	Xylan	+++	−	−	−	−	−	+	−	+	+	−	−	ND	6

[a] Grouping at 89% similarity
[b] Antagonism toward these four species was determined by dual-culture assay. Results indicate the width of the zone of inhibition as follows: +, 0 to 5 mm; ++, 5 to 10 mm; +++, 10 to 15 mm; ++++, >15 mm.
[c] β-1,3-Glucanase, protease, and chitinase activities were determined by plate assay (+, hydrolysis; −, no hydrolysis).
[d] Fluorescence on King's B agar.
[e] DAPG, 2,4-diacetylphloroglucinol; PCR approach.
[f] Production of cyanide as shown by the Merck Schnelltest: (+, > 0.001 mg liter^{-1}).
[g] Microplate method of Sawar and Kremer (41) (+, > 0.1 μg ml^{-1}).
[h] *E. coli* MT 102(pSB403) for detection of short-chain AHLs.
[i] *P. putida* F117(pKR-C12) for detection of long-chain AHLs.
[j] Grouping at 75% similarity.

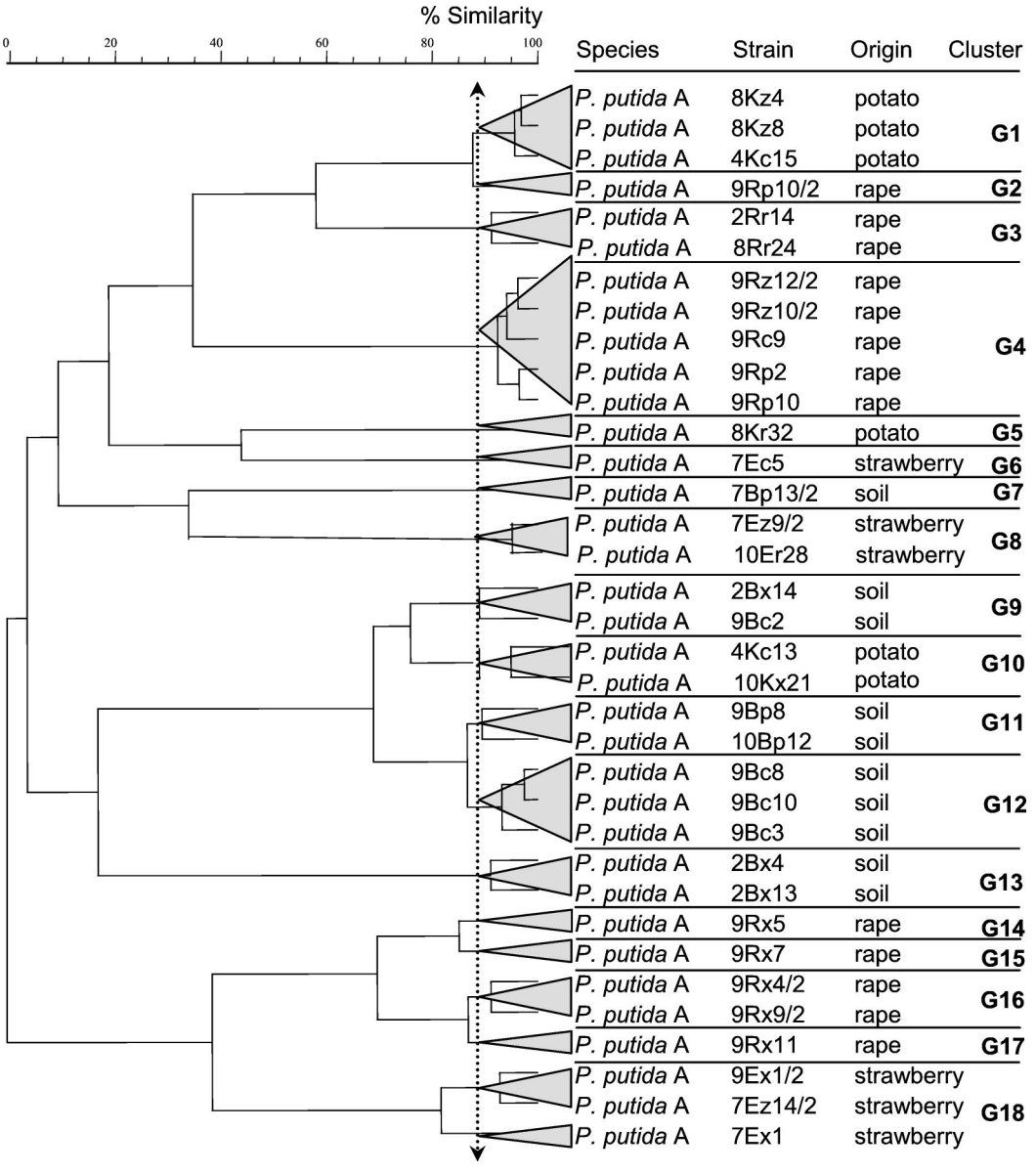


FIG. 2. Dendrogram showing the relationship of 35 *P. putida* A isolates from strawberry, potato, and oilseed rape rhizospheres and uncultivated soil based on BOX-PCR fingerprints and using cluster analysis.

chitinases, and proteases); fluorescence on King's B medium under UV light; and the production of cyanide, IAA, and AHL. BOX-PCR fingerprints were used to analyze the genomic relatedness between *P. putida* A isolates (Fig. 2). Furthermore, PCR was used to detect the presence of the *phlD* gene. While almost all *P. putida* A isolates showed in vitro proteolytic activity (33 of 35 antagonists; 94%), glucanolytic or chitinolytic activity was detected for none of them. Twenty *P. putida* A isolates were able to produce cyanide (HCN), which acts as an inducer of plant resistance. The production of the plant growth hormone IAA was detected for 71% of the *P. putida* A isolates. AHLs were detected for approximately 71% of the *P. putida* A isolates when the sensor strain *E. coli* MT102(pSB403) was used. With two exceptions a correlation of IAA and AHL production was detected. *P. putida* isolates which did not produce AHL and IAA fell into distinct genotype clusters (clusters G4, G5, G6, and G18). By employing the biosensor *P. putida* F117(pKR-C12), production of long-chain AHL molecules could be demonstrated for isolates of the clusters G14, G15, G16, and G17. Interestingly, all these strains were isolated from oilseed rape and grouped into one phenotype cluster (cluster 5). Only one soil isolate (7Bp13/2) was found to produce long-chain AHLs. This strain also belongs to phenotype cluster 5. The *phlD* gene was detected by PCR in isolates belonging to different genomic clusters originating from soil (4), oilseed rape (2), and strawberry (4).

Characterization of *Verticillium* antagonists identified as *P. putida* B. More than one-third of all *Verticillium* antagonists isolated were identified by FAME analysis as *P. putida* B. *P. putida* B strains antagonistic towards *V. dahliae* were isolated

from the rhizosphere of strawberries at all sampling times. Thus, *P. putida* B strains clearly resembled the most-abundant group of *Verticillium* antagonists which were isolated from all plant rhizospheres. To explore the diversity of *P. putida* B isolates, a total of 98 isolates from the rhizospheres of strawberry, oilseed rape, and potato was characterized by BOX-PCR fingerprints. BOX-PCR fingerprints revealed an enormous diversity of *P. putida* B at the subspecies level (data not shown). Analysis of the BOX fingerprints by UPGMA using GelCompare resulted in a cutoff level of 85% in 38 groups, of which 19 groups contained only one isolate. While 14 of the 19 groups with more than one isolate consisted only of isolates from one plant species, five groups had isolates from potato and strawberry rhizosphere ($n = 3$) or strawberry and oilseed rape rhizosphere ($n = 2$). Eight groups contained isolates which were isolated from only one substrate. Six of the 19 groups with more than one isolate contained strains isolated at different sampling points in both years, six groups consisted of isolates from different sampling times but all isolated in the same year, and seven groups contained only isolates from the same sampling time. In a subset of *P. putida* B (44) isolates analyzed for AHL production, 11 isolates were found to be positive. Interestingly, only 3 of 27 tested strains from strawberry rhizosphere produced AHLs. These three strains activated both AHL sensor strains used in this study. In contrast, three out of seven oilseed rape rhizosphere strains and 5 out of 10 potato rhizosphere strains tested were AHL positive in cross-streaks against *E. coli* MT102(pSB403), and most of these strains (two of the three oilseed rape rhizosphere strains and four of the five potato rhizosphere strains) also activated the long-chain AHL sensor *P. putida* F117(pKR-C12). The *phlD* gene was detected by PCR in a considerable proportion of the isolates from the strawberry (64 of 86 antagonists) and the potato (15 of 24 antagonists) rhizospheres, while no PCR product was obtained from the nine *P. putida* B isolates from the oilseed rape rhizosphere.

DISCUSSION

Root exudates such as amino acids, sugars, and organic acids are an important nutritional source for bacteria colonizing the roots. The composition of root exudates was shown to vary depending on the plant species and the stage of plant development (17). Thus, the plant is supposed to profoundly influence the relative abundance of indigenous rhizobacteria as well as the population dynamics of introduced biological control strains. Recently Rainey (39) showed that *P. fluorescens* genes involved in nutrient acquisition, stress response, or secretion had elevated levels of expression during rhizosphere colonization. To come to an improved understanding of factors affecting the ability of bacteria to colonize the rhizosphere, the plant should be taken into account. To explore the rhizosphere effect of different plant species on abundance and diversity of antagonistic bacteria, isolates originating from the rhizosphere of field-grown host plants of *V. dahliae*—strawberry, potato, and oilseed rape—and from fallow soil were analyzed for their antagonistic properties. *Verticillium* antagonists selected by dual-culture tests were identified by FAME analysis and characterized for their phenotypic and genotypic properties. To enrich bacteria with hydrolytic enzyme activities, the bacterial cells recovered from rhizosphere or bulk soil were incubated in microtiter plates with high-molecular-weight substrates. The previous incubation in high-molecular-weight-substrate microtiter plates resulted in an enrichment of fast-growing γ-proteobacteria. Thus, gram-positive bacteria were only isolated after direct plating onto R2A. DGGE analysis of DNA extracted from the cells recovered from the highest dilution row, which completely scored positive for growth, showed similar profiles for all substrates (data not shown). Obviously, a specific substrate-dependent enrichment of bacteria did not occur due to the presence of more easily degradable substrate (1/10 volume of tryptic soy agar). A reduction of the number of dominant DGGE bands was observed during incubation. Similar observations were made when rhizosphere communities were incubated in BIOLOG plates (42). Since *Verticillium* antagonists were isolated from the highest dilutions, they represent a considerable proportion of the culturable bacterial fraction. While no differences in the bacterial plate counts (CFU on R2A) were found between the different rhizospheres, the abundance, taxonomic composition, and diversity of *Verticillium* antagonists differed for the different treatments. Although we isolated the highest number of *Verticillium* antagonists from the rhizosphere of strawberry, their diversity in terms of richness was surprisingly low. A lower number of dominant bands was recently also found for the DGGE patterns of eubacterial populations from the strawberry rhizosphere compared to the more complex DGGE patterns of oilseed rape and potato rhizospheres (43). However, while the cultivation-independent approach indicated that the patterns were more similar between potato and oilseed rape compared to strawberry, this finding could not be confirmed for isolates with antagonistic activity towards *V. dahliae*. The proportion and taxonomic composition of the isolates were found to be specific for each of the plant species and soil. The most-remarkable findings were the high proportion of *P. putida* B isolates from the rhizosphere of strawberry and a high number of *Verticillium* antagonists belonging to the *Enterobacteriaceae* from the rhizosphere of oilseed rape. The widespread occurrence of *Serratia* species with in vitro antagonistic activity towards *V. dahliae* in the rhizosphere of oilseed rape was already reported by Kalbe et al. (18). The majority of *Verticillium* antagonists from all treatments belonged to the *P. fluorescens* intrageneric cluster (29), with a substantially lower number of *Pseudomonas* isolates from soil. Strains belonging to the genus *Pseudomonas* are the biological control agents which are best characterized at the molecular level (6, 30). Although *P. putida* A strains were isolated from all rhizospheres and soil, a clustering dependent on the origin of the isolates was observed when BOX-PCR profiles were compared. *P. putida* B isolates, which represented 42% of the collection of *Verticillium* antagonists, were exclusively isolated from the rhizosphere and not from bulk soil. Obviously, *P. putid*a B isolates are enriched from soil by root exudates, in particular those from strawberry plants. Again BOX-PCR fingerprints revealed a great diversity, and several of the genomic clusters contained only isolates isolated from one plant species. Several recently published studies used repetitive extragenic palindromic (REP)-PCR fingerprints (38), such as those obtained by BOX-, REP-, or enterobacterial repetitive intergenic consensus (ERIC)-PCR, to explore the diversity of pseudomonads originating from rhizospheres

and soils. Based on REP-PCR fingerprints all studies found an enormous genomic diversity of *Pseudomonas* spp. at the subspecies level (14, 26). Fromin et al. (14) reported that the genotypic structure of *Pseudomonas brassicacearum* populations analyzed by REP-PCR fingerprints are significantly influenced by the *Arabidopsis thaliana* genotype.

The proportion of *Verticillium* antagonists which were also suppressive to other pathogens tested here to some extent reflected the species composition of the collections obtained from each treatment. The proportion of *Verticillium* antagonists which were active also against *R. solani*, *S. sclerotiorum*, and *P. cactorum* was particularly high for isolates from oilseed rape rhizosphere and soil. Although significant differences were found in the production of hydrolytic enzymes, which is known to be an important mode of action in antagonism (8), no correlation was observed between the production of lytic enzymes and the range of fungal pathogens antagonized in vitro. Since antibiotics such as 2,4-diacetyl-phloroglucinol (encoded by *phl*) are major determinants of biological control of fungal pathogens and the *phlD* gene was shown to be conserved among Phl producers of worldwide origin (36), we have used a PCR screening approach to analyze the presence of the *phlD* gene in the *P. putida* A and B isolates. The *phlD* gene was detected in a surprisingly high number of *P. putida* B isolates from the rhizospheres of strawberry (74.4%) and potato (62.5%) but not in isolates from oilseed rape rhizosphere. The proportion of potential Phl producers among our *P. putida* B collection is considerably higher than previously reported frequencies of fluorescent *Pseudomonas* isolates from rhizospheres grown in disease-suppressive soils (19). In *P. putida* A the *phlD* gene was found in isolates from soil and strawberry, oilseed rape, and potato rhizospheres but was much less frequently detected. For many antagonists it was shown that the expression of genes involved in disease suppression (antifungal metabolites such as antibiotics or extracellular enzymes) is regulated in response to their own population densities, a phenomenon termed quorum sensing (10, 34). One prominent example is *Pseudomonas aureofaciens*, which is capable of protecting wheat from take-all disease, caused by the ascomycete fungus *Gaeumannomyces graminis* var. *tritici*. Disease suppression is due to the production of phenazine antibiotics, the synthesis of which is regulated by a quorum-sensing circuit (33). Evidence that accumulated over the past few years showed that AHL-mediated cell-cell communication is a widespread phenomenon among plant-associated bacteria (11, 34, 50). In this study all *P. putida* A strains, a subset of the *P. putida* B strains, and a subset of the *Serratia* and *Pantoea* strains were analyzed for the production of AHL. In contrast to the findings of Elasri et al. (11), who suggested that AHL production is more common among plant-associated bacteria than among pseudomonads originating from soil, we observed AHL production for *P. putida* A strains isolated from the rhizosphere and from soil. Recent work has shown that AHL signal molecules serve not only as population density sensors but also for communication between cells of different species colonizing the plant rhizosphere (32, 45). It has been speculated that AHL molecules may be important for coordinating the various functions of the different populations within the rhizosphere. Although AHLs were detected in a considerable proportion of the *P. putida* A and *P. putida* B strains tested, the functions regulated by AHL remain to be elucidated. About 50% of the *Serratia* and *Pantoea* strains were shown to produce AHLs, but neither exoenzyme production nor antifungal activity seemed to be associated with the production of signal molecules.

Sixty randomly selected isolates from this study were further characterized with regard to their plant growth-promoting activity in a strawberry seedling assay, and three selected isolates from each plant were characterized in greenhouse experiments (4). In this in vitro study, isolates from all plants were able to enhance plant growth in strawberries. However, the success of biological approaches to control plant diseases and enhance growth must be judged by their performance under field conditions. Raaijmakers and Weller (35) suggested that by matching rhizobacterium genotypes with crops for which they have colonization preference, root colonization could be increased. This study supports the notion that the rhizosphere of different plants might provide conditions (e.g., nutritional sources) differently supportive for biological control strains. The phenotypic and genotypic diversity found in natural populations and which was observed in the collection of *Verticillium* antagonists isolated in this study offers a tremendous resource for the improvement of biological control strains.

ACKNOWLEDGMENTS

We thank Hella Goschke (Rostock, Germany) for valuable technical assistance and Jessica Parzy (Braunschweig, Germany) for performing FAME analysis. Jens Frankowski (Rostock, Germany) was helpful in *chiA* gene detection.

This study was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46:**549–559.
3. **Berg, G.** 1996. Rhizobacteria of oil seed rape antagonistic to *Verticillium dahliae*. J. Plant Dis. Protect. **103:**20–30.
4. **Berg, G., A. Fritze, N. Roskot, and K. Smalla.** 2001. Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb. J. Appl. Microbiol. **156:**75–82.
5. **Berg, G., A. Buchner, E. H. M. Wellington, and K. Smalla.** 2000. Successful strategy for the selection of new strawberry-associated rhizobacteria antagonistic to Verticillium wilt. Can. J. Microbiol. **46:**1128–1137.
6. **Bloemberg, G. V., and B. J. J. Lugtenberg.** 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. **4:**343–350.
7. **Chernin, L., Z. Ismailov, S. Haran, and I. Chet.** 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Appl. Environ. Microbiol. **61:**1720–1726.
8. **Chet, I., A. Ordentlich, R. Shapira, and A. Oppenheim.** 1990. Mechanisms of biocontrol of soil-borne plant pathogens by rhizobacteria. Plant Soil **129:**85–92.
9. **Duffy, B. K., and G. Défago.** 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. **65:**2429–2438.
10. **Eberl, L.** 1999. N-acyl homoserinelactone-mediated gene regulation in gram-negative bacteria. Syst. Appl. Microbiol. **22:**493–506.
11. **Elasri, M., S. Delorme, P. Lemanceau, G. Stewart, B. Laue, E. Glickmann, P. M. Oger, and Y. Dessaux.** 2001. Acyl-homoserine lactone production is more common among plant-associated *Pseudomonas* spp. than among soilborne *Pseudomonas* spp. Appl. Environ. Microbiol. **67:**1198–1209.
12. **Emmert, E. A. B., and J. Handelsman.** 1999. Biocontrol of plant disease: a ($Gram^+$) positive perspective. FEMS Microbiol. Lett. **171:**1–9.
13. **Fravel, D. R.** 1988. Role of antibiosis in the biocontrol of plant diseases. Annu. Rev. Phytopathol. **26:**75–91.
14. **Fromin, N., W. Achouak, J. M. Thiéry, and T. Heulin.** 2001. The genotypic diversity of *Pseudomonas brassicacearum* populations isolated from roots of *Arabidopsis thaliana*: influence of plant genotype. FEMS Microbiol. Ecol. **37:**21–29.

15. **Germida, J. J., S. D. Siciliano, J. Renato de Freitas, and A. M. Seib.** 1998. Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). FEMS Microbiol. Ecol. **26:**43–50.
16. **Grayston, S. J., S. Wang, C. D. Campbell, and A. C. Edwards.** 1998. Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol. Biochem. **30:**369–378.
17. **Jaeger, C. H., III, S. E. Lindow, W. Miller, E. Clark, and M. K. Firestone.** 1999. Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. Appl. Environ. Microbiol. **65:** 2685–2690.
18. **Kalbe, C., P. Marten, and G. Berg.** 1996. Members of the genus *Serratia* as beneficial rhizobacteria of oilseed rape. Microbiol. Res. **151:**4433–4440.
19. **Keel, C., D. M. Weller, A. Natsch, G. Défago, R. J. Cook, and L. S. Thomashow.** 1996. Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Appl. Environ. Microbiol. **62:**552–563.
20. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44:**301–307.
21. **Kremer, R. J., M. F. T. Begonia, L. Stanlay, and E. T. Lanham.** 1990. Characterization of rhizobacteria associated with weed seedlings. Appl. Environ. Microbiol. **56:**1649–1655.
22. **Kurze, S., R. Dahl, H. Bahl, and G. Berg.** 2000. Biological control of soilborne pathogens in strawberry by *Serratia plymuthica* HRO-C48. Plant Dis. **85:**529–534.
23. **Lemanceau, P., T. Corberand, L. Gardan, X. Latour, G. Laguerre, J. M. Boeufgras, and C. Alabouvette.** 1995. Effect of two plant species, flax (*Linum usitatissinum* [sic] L.) and tomato (*Lycopersicum esculentum* Mill.), on the diversity of soilborne populations of fluorescent pseudomonads. Appl. Environ. Microbiol. **61:**1004–1012.
24. **Lugtenberg, B. J. J., L. Dekkers, and G. V. Bloemberg.** 2001. Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu. Rev. Phytopathol. **39:**461–490.
25. **Maas, J. L.** 1998. Compendium of strawberry diseases. APS Press, St. Paul, Minn.
26. **McSpadden Gardener, B. B., K. L. Schroeder, S. E. Kalloger, J. M. Raaijmakers, L. S. Thomashow, and D. M. Weller.** 2000. Genotypic and phenotypic diversity of *phlD*-containing *Pseudomonas* strains isolated from the rhizosphere of wheat. Appl. Environ. Microbiol. **66:**1939–1946.
27. **Menhinick, E. F.** 1964. A comparison of some species-individuals diversity indices applied to samples of field insects. Ecology **45:**859–861.
28. **Miller, H. J., G. Henken, and J. A. van Veen.** 1989. Variation and composition of bacterial populations in the rhizospheres of maize, wheat, and grass cultivars. Can. J. Microbiol. **35:**656–660.
29. **Moore, E. R. B., M. Mau, A. Arnscheidt, E. C. Böttger, R. A. Hutson, M. D. Collins, Y. van der Peer, R. de Wachter, and K. N. Timmis.** 1996. The determination and comparison of 16S rRNA gene sequences of species of the genus *Pseudomonas* (sensu stricto) and estimation of natural intrageneric relationships. Syst. Appl. Microbiol. **19:**478–492.
30. **O'Sullivan, D. J., and F. O'Gara.** 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol. Rev. **56:** 662–676.
31. **Pearson, J. P., E. C. Pesci, and B. H. Iglewski.** 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179:**5756–5767.
32. **Pierson, E. A., D. W. Wood, J. A. Cannon, F. M. Blachere, and L. S. Pierson III.** 1998. Interpopulation signaling via *N*-acyl-homoserine lactones among bacteria in the wheat rhizosphere. Mol. Plant-Microbe Interact. **11:**1078–1084.
33. **Pierson, L. S., III, V. D. Keppenne, and D. W. Wood.** 1994. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30–84 is regulated by PhzR in response to cell density. J. Bacteriol. **176:**3966–3974.
34. **Pierson, L. S., III, D. W. Wood, and E. A. Pierson.** 1998. Homoserine lactone-mediated gene regulation in plant-associated bacteria. Annu. Rev. Phytopathol. **36:**207–225.
35. **Raaijmakers, J. M., and D. M. Weller.** 2001. Exploiting genotypic diversity of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.: characterization of superior root-colonizing *P. fluorescens* strain Q8r1–96. Appl. Environ. Microbiol. **67:**2545–2554.
36. **Raaijmakers, J. M., D. M. Weller, and L. S. Thomashow.** 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl. Environ. Microbiol. **63:**881–887.
37. **Rademaker, J. L. W., and F. J. De Bruijn.** 1997. Characterization and classification of microbes by REP-PCR genomic fingerprinting and computer-assisted pattern analysis, p. 151–171. *In* G. Caetano-Anollés and P. M. Gresshoff (ed.), DNA markers: protocols, applications and overviews. J. Wiley & Sons, Inc., New York, N.Y.
38. **Rademaker, J. L. W., F. J. Louws, U. Rossbach, and F. J. de Bruijn.** 1999. Computer assisted pattern analysis of molecular fingerprints and data base construction, p. 1–33. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. DeBruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers. Dordrecht, The Netherlands.
39. **Rainey, P. B.** 1999. Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. Environ. Microbiol. **1:**243–257.
40. **Riedel, K., M. Hentzer, O. Geisenberger, B. Huber, A. Steidle, H. Wu, N. Hoiby, M. Givskov, S. Molin, and L. Eberl.** 2001. *N*-Acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology **147:**3249–3262.
41. **Sawar, M., and R. J. Kremer.** 1995. Determination of bacterially derived auxins using a microplate method. Lett. Appl. Microbiol. **20:**282–285.
42. **Smalla, K., U. Wachtendorf, H. Heuer, W.-T. Liu, and L. Forney.** 1998. Analysis of BIOLOG GN substrate utilization patterns by microbial communities. Appl. Environ. Microbiol. **64:**1220–1225.
43. **Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, N. Roskot, H. Heuer, and G. Berg.** 2001. Bacterial bulk and rhizosphere communities studied by denaturing gradient gel electrophoresis of PCR-amplified fragments of 16S rRNA genes: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. **67:**4742–4751
44. **Sørensen, J.** 1997. The rhizosphere as a habitat for soil microorganisms, p. 21–45. *In* J. D. Van Elsas, J. T. Trevors, and E. M. H. Wellington (ed.), Modern soil microbiology. Marcel Dekker, Inc., New York, N.Y.
45. **Steidle, A., K. Sigl, R. Schuhegger, A. Ihring, M. Schmid, S. Gantner, M. Stoffels, K. Riedel, M. Givskov, A. Hartmann, C. Langebartels, and L. Eberl.** 2001. Visualization of *N*-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. Appl. Environ. Microbiol. **67:**5761–5770.
46. **Tjamos, E. C., R. C. Rowe, J. B. Heale, and D. R. Fravel.** 2000. Advances in *Verticillium* research and disease management. APS Press, St. Paul, Minn.
47. **Weller, D. M.** 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26:**379–407.
48. **Whipps, J. M.** 1997. Ecological considerations involved in commercial development of biological control agents for soil-borne diseases, p. 525–545. *In* J. D. Van Elsas, J. T. Trevors, and E. M. H. Wellington (ed.), Modern soil microbiology. Marcel Dekker, Inc., New York, N.Y.
49. **Winson, M. K., S. Swift, L. Fish, J. P. Throup, F. Jørgensen, S. R. Chhabra, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart.** 1998. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl-homoserine lactone-mediated quorum sensing. FEMS Microbiol. Lett. **163:**185–192.
50. **Zhang, Z., and L. S. Pierson III.** 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. Appl. Environ. Microbiol. **67:**4305–4315.

Anhang 4

N-Acylhomoserine-lactone-mediated intergeneric communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms

KATHRIN RIEDEL, MORTEN HENTZER, OTTO GEISENBERGER, BIRGIT HUBER, ANETTE STEIDLE, HONG WU, NIELS HØIBY, MICHAEL GIVSKOV, SØREN MOLIN, AND LEO EBERL

Veröffentlicht in: *Microbiology*, 147:3249-3262 (2001)

Microbiology (2001), **147**, 3249–3262 Printed in Great Britain

N-Acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms

Kathrin Riedel,[1] Morten Hentzer,[2] Otto Geisenberger,[1] Birgit Huber,[1] Anette Steidle,[1] Hong Wu,[3] Niels Høiby,[3] Michael Givskov,[2] Søren Molin[2] and Leo Eberl[1]

Author for correspondence: Leo Eberl. Tel: +49 8161 715446. Fax: +49 8161 715475. e-mail: eberl@mikro.biologie.tu-muenchen.de

[1] Department of Microbiology, TUM, Am Hochanger 4, D-85350 Freising, Germany

[2] Department of Microbiology, DTU, Building 301, DK-2800 Lyngby, Denmark

[3] Department of Clinical Microbiology, University Hospital Copenhagen Ø, DK-2100, Denmark

***Pseudomonas aeruginosa* and *Burkholderia cepacia* are capable of forming mixed biofilms in the lungs of cystic fibrosis patients. Both bacteria employ quorum-sensing systems, which rely on *N*-acylhomoserine lactone (AHL) signal molecules, to co-ordinate expression of virulence factors with the formation of biofilms. As both bacteria utilize the same class of signal molecules the authors investigated whether communication between the species occurs. To address this issue, novel Gfp-based biosensors for non-destructive, *in situ* detection of AHLs were constructed and characterized. These sensors were used to visualize AHL-mediated communication in mixed biofilms, which were cultivated either in artificial flow chambers or in alginate beads in mouse lung tissue. In both model systems *B. cepacia* was capable of perceiving the AHL signals produced by *P. aeruginosa*, while the latter strain did not respond to the molecules produced by *B. cepacia*. Measurements of extracellular proteolytic activities of defined quorum-sensing mutants grown in media complemented with AHL extracts prepared from culture supernatants of various wild-type and mutant strains supported the view of unidirectional signalling between the two strains.**

Keywords: quorum sensing, cross-talk, intergeneric communication, cystic fibrosis

INTRODUCTION

Cystic fibrosis (CF) is the most common inherited lethal disease among Caucasians. The genetic lesion in CF leads to impaired epithelial chloride ion transport. This, in turn, leads to the production of a sticky dehydrated mucus in the ducts of exocrine glands, e.g. in the airways of the lungs. As a consequence, mucociliary and alveolar clearing are impaired and colonization of the lung epithelium by opportunistic bacterial pathogens leading to airway infections is facilitated. Early in life, CF patients are usually colonized by *Staphylococcus aureus* and non-capsulated *Haemophilus influenzae*, followed, later on, by mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*, which in most cases will establish chronic infections (Govan & Deretic, 1996; Tümmler & Kiewitz, 1999).

P. aeruginosa produces a wide variety of extracellular products, many of which contribute to its virulence. Expression of the majority of these virulence factors is not constitutive but is regulated in a cell-density-dependent manner. This form of gene regulation ensures that *P. aeruginosa* remains invisible to the immune system of the host until the opportunistic pathogen has reached a critical population density sufficient to overwhelm host defences and to establish the infection. Like many other Gram-negative bacteria, *P. aeruginosa* utilizes cell–cell communication systems that rely on

Abbreviations: AHL, *N*-acylhomoserine lactone; C4-, C6-, C8-, C10- and C-12-HSL, *N*-butanoyl, *N*-hexanoyl-, *N*-octanoyl, *N*-decanoyl- and *N*-dodecanoyl-L-homoserine lactone; 3-oxo-C10, 3-oxo-C12 and 3-oxo-C14-HSL, *N*-(3-oxodecanoyl)-, *N*-(3-oxododecanoyl)- and *N*-(3-oxotetradecanoyl)-L-homoserine lactone; CF, cystic fibrosis; CSLM, confocal scanning laser microscopy; DsRed, red fluorescent protein; Gfp, green fluorescent protein; QS, quorum sensing.

diffusible *N*-acylhomoserine lactone (AHL) signal molecules to monitor the size of the population in a process known as quorum sensing (for recent reviews see Van Delden & Iglewski, 1998; Williams *et al*., 2000; de Kievit & Iglewski, 2000). Typically, these communication systems depend on two proteins: an AHL synthase, usually a member of the LuxI family of proteins, and an AHL receptor protein, which belongs to the LuxR family of transcriptional regulators. At low population densities cells produce a basal level of AHL via the activity of the AHL synthase. As the cell density increases, the diffusible AHL signal molecule accumulates in the growth medium. On reaching a critical threshold concentration, the AHL binds to the cognate LuxR-type receptor protein, which in turn leads to the induction/repression of target genes. Two quorum-sensing systems have been identified in *P. aeruginosa*: the *las* system, consisting of the transcriptional activator LasR and the AHL synthase LasI, which directs the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), and the *rhl* system, consisting of RhlR and RhlI, which directs the synthesis of *N*-butanoyl-L-homoserine lactone (C4-HSL). The two systems do not operate independently as the *las* system positively regulates expression of both *rhlR* and *rhlI*. Thus, the two quorum-sensing systems of *P. aeruginosa* are hierarchically arranged, with the *las* system being on top of the signalling cascade. In complex interplays with additional regulators, including Vfr, GacA, RsaL and RpoS, the quorum-sensing cascade regulates expression of a battery of extracellular virulence factors such as exoenzymes (elastase, alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, pyoverdin) and toxins (exotoxin A). The importance of quorum sensing in the pathogenicity of *P. aeruginosa* has been demonstrated in a number of animal models including a *Caenorhabditis elegans* nematode model (Tan *et al*., 1999), the neonatal mouse model of pneumonia (Tang *et al*., 1996) and a burned mouse model (Rumbaugh *et al*., 1999). In all these animal models mutants defective in quorum sensing were substantially less virulent than the parent strains.

In recent years *B. cepacia* has emerged as another important pathogen in patients with CF (Govan & Deretic, 1996; Govan *et al*., 1996). In most cases infection with *B. cepacia* occurs in patients who are already colonized with *P. aeruginosa*. It has been suggested that *P. aeruginosa* produces an extracellular factor which modifies the epithelial cell surface of the lung in a way that facilitates attachment of *B. cepacia* (Saiman *et al*., 1990). Co-colonization can result in three clinical outcomes: asymptomatic carriage, slow and continuous decline in lung function, or, for approximately 20% of the patients, fulminant and fatal pneumonia, the so-called 'cepacia syndrome' (Isles *et al*., 1984).

Like *P. aeruginosa*, *B. cepacia* controls expression of various extracellular factors by an AHL-dependent quorum-sensing system, which consists of the AHL synthase CepI and the transcriptional regulator CepR (Lewenza *et al*., 1999; Gotschlich *et al*., 2001). The major signal molecule produced via CepI is *N*-octanoyl-L-homoserine lactone (C8-HSL). The *cep* system was shown to positively regulate production of extracellular proteolytic and chitinolytic activity and to repress synthesis of the siderophore ornibactin (Lewenza *et al*., 1999; Huber *et al*., 2001).

Recent work has presented strong evidence that *P. aeruginosa* cells exist as a biofilm in the CF lung (Singh *et al*., 2000). In the biofilm mode of growth the cells are embedded in a thick matrix of extracellular polymeric substances, can withstand host immune responses, and exhibit a dramatically increased resistance to antibiotics and biocides when compared to cells grown in liquid culture (Schierholz *et al*., 1999; Xu *et al*., 2000). Most interestingly, the formation of biofilms is a quorum-sensing-regulated process in *P. aeruginosa* as well as in *B. cepacia* (Davies *et al*., 1998; Huber *et al*., 2001). Both a *P. aeruginosa lasI* mutant and a *B. cepacia cepI* mutant form only flat and undifferentiated biofilms, suggesting that the respective quorum-sensing systems are in some way required for biofilm maturation. Importantly, the *P. aeruginosa lasI* mutant biofilm was also shown to be much more sensitive than the wild-type biofilm to the biocide sodium dodecyl sulfate.

During chronic co-infection *P. aeruginosa* and *B. cepacia* form mixed biofilms in the lungs of CF patients. Given that both bacteria utilize the same chemical language to control biofilm formation and expression of virulence factors it appears likely that not only are the two organisms capable of communicating with each other but that these interactions may also synergistically enhance the virulence of the consortium. In fact, McKenney *et al*. (1995) have shown that addition of spent culture supernatants of *P. aeruginosa* to the medium used for cultivation of *B. cepacia* enhances production of siderophores, lipase and protease of the latter species. The authors suggested that this stimulation of virulence factor production is caused by AHL molecules present in the *P. aeruginosa* supernatants.

In a recent study we analysed the AHL profiles, i.e. the types and amounts of AHL molecules, of sequential *P. aeruginosa* isolates from several chronically infected CF patients by TLC (Geisenberger *et al*., 2000). In one case the patient became transiently co-infected with an AHL-producing *B. cepacia* strain. During the co-infection period a dramatic reduction in the amounts of AHLs produced by the co-residing *P. aeruginosa* isolates was observed. However, 18 months after the last *B. cepacia*-positive sputum the initial *P. aeruginosa* AHL profile was regained. This observation led to the speculation that AHL-mediated cross-talk between the two pathogens may affect the virulence of the mixed consortium and that this change in pathogenic potential may in turn select for *P. aeruginosa* mutants producing lowered amounts of AHLs.

This study was initiated to investigate whether *P. aeruginosa* and *B. cepacia* are in fact capable of

communicating with each other using AHL signal molecules. Novel Gfp-based AHL sensor plasmids were constructed and these were used for *in situ* studies of cell–cell communication between the two organisms. Evidence is presented that in mixed biofilms intergeneric signalling only occurs in one direction, namely from *P. aeruginosa* to *B. cepacia*.

METHODS

Organisms and culture conditions. *Escherichia coli*, *Burkholderia cepacia* and *Pseudomonas aeruginosa* strains used in this study are listed in Table 1. Strains were grown in modified Luria–Bertani medium (Bertani, 1951) containing 4 g NaCl l^{-1} instead of 10 g NaCl l^{-1} or ABt minimal medium [AB minimal medium (Clark & Maaløe, 1967) supplemented with 2·5 mg thiamin l^{-1} and 1 mM glucose].

AHL extraction and TLC. AHL molecules were extracted with dichloromethane from culture supernatants (grown in minimal medium to an OD_{600} of 1·0) and separated by TLC as described by Geisenberger *et al.* (2000). The AHL molecules were visualized by overlaying the TLC plates with soft agar seeded with the sensor strain *E. coli* MT102(pSB403) (Winson *et al.*, 1998). After overnight incubation at 30 °C bioluminescent spots were detected by exposure of an X-ray film. By comparing the obtained R_F values of the spots with those of synthetic AHL standards a tentative identification of the AHLs was possible. Synthetic AHLs were either purchased from Fluka or were a generous gift from P. Williams, University of Nottingham, UK.

Measurement of proteolytic activity. Strains were grown overnight in LB medium in the presence or absence of 5 μM AHLs or dichloromethane extracts of spent culture supernatants from different strains. Proteolytic activity was measured as described by Ayora & Götz (1994). Briefly, 250 μl 2 % (w/v) azocasein (Sigma) in 50 mM Tris/HCl and 150 μl of the sterile filtered supernatants were incubated for 4 h at

Table 1. Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Relevant genotype and characteristics	Source or reference
E. coli		
MT102	F$^-$ *thi araD139 ara–leu*Δ*7679* Δ(*lacIOPZY*) *galU gal'K* r$^-$ m$^+$ Sm^R	T. Hansen, Novo Nordisk A/S
CC118 λpir	Δ(*ara–leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rps-1 rpoB argE*(Amp) *recA thi pro hsdRM*$^+$ RP4-2-Tc::Mu-Km::Tn*7* λ*pir*	Herrero *et al.* (1990)
P. aeruginosa		
PAO1	Wild-type *P. aeruginosa*	Holloway (1955)
PAO1-JP2	*lasI rhlI* derivative of PAO1; Hg^R Tc^R	Pearson *et al.* (1997)
SH1	Clinical isolate	Römling *et al.* (1994)
SH38	Clinical isolate	Römling *et al.* (1994)
B. cepacia		
H111	Clinical isolate	Römling *et al.* (1994)
H111-I	*cepI* derivative of H111	Huber *et al.* (2001)
Plasmids		
pSB403	Tc^R; broad-host-range AHL monitor plasmid	Winson *et al.* (1998)
pGEM-3Zf(+)	Ap^R; *lacZ*α, cloning vector	Promega
pBBR1MCS-5	Gm^R; broad-host-range vector	Kovach *et al.* (1995)
pMHLAS	Ap^R Gm^R; *Pseudomonas* shuttle vector carrying P_{lasB}–*gfp*(ASV) P_{lac}–*lasR*	Hentzer *et al.* (2002)
pKR-C12	Gm^R; pBBR1MCS-5 carrying P_{lasB}–*gfp*(ASV) P_{lac}–*lasR*	This study
pAS-C8	Gm^R; pBBR1MCS-5 carrying P_{cepI}–*gfp*(ASV) P_{lac}–*cepR*	This study
pUT-Tc-*dsred*	Ap^R Tc^R; Tn5-based delivery plasmid, carrying P_{lac}-*dsred*-T_0-T_1	Hentzer *et al.* (2002)
pUT-Tel-*dsred*	Ap^R Tel^R; Tn5-based delivery plasmid, carrying P_{lac}-*dsred*-T_0-T_1	M. Hentzer, unpublished
pUT-Gm-*dsred*	Ap^R Gm^R; Tn5-based delivery plasmid, carrying P_{lac}-*dsred*-T_0-T_1	M. Hentzer, unpublished
pRK600	Cm^R; oriColE1 RK2-Mob$^+$ RK2-Tra$^+$; helper plasmid in triparental conjugations	Kessler *et al.* (1992)
Primers		
cepI-fwd	5′-CGGGATCCGACATCGGCATGTTGC-3′	This study
cepI-rev	5′-ACATGCATGCATGTCCTCGGATCTGTGC-3′	This study
gfp(ASV)-fwd	5′-ACATGCATGCGTAAAGGAGAAGAAC-3′	This study
gfp(ASV)-rev	5′-CCCAAGCTTATTAAACTGATGCAGC-3′	This study
cepR-fwd	5′-GGGGTACCGGATGAGCATGGAGAAAAGC-3′	This study
cepR-rev	5′-GGGGTACCAACCTGACAAGTATGACAGCG-3′	This study

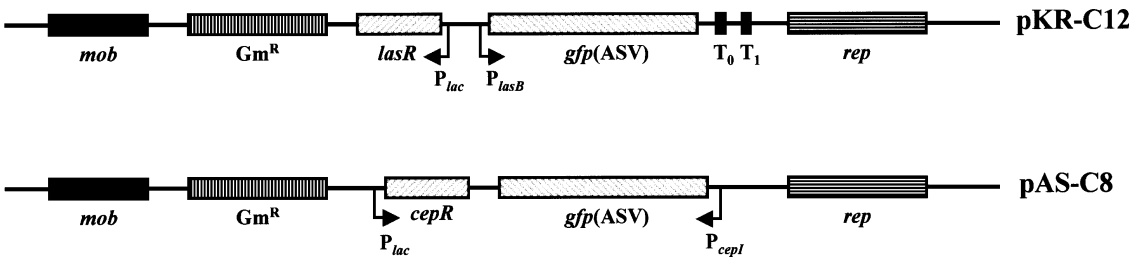


Fig. 1. Schematic drawings (not to scale) of the *las*- and *cep*-based AHL sensor plasmids used in this study. Plasmid pKR-C12 contains a *lasB–gfp*(ASV) translational fusion together with the *lasR* gene placed under control of P_{lac} on the broad-host-range plasmid pBBR1MCS-5. Plasmid pAS-C8 contains a *cepI–gfp*(ASV) translational fusion together with the *cepR* gene transcribed from the P_{lac} promoter of the broad-host-range plasmid pBBR1MCS-5. The genetic components are: P_{lasB}, elastase promoter fragment of PAO1; *gfp*(ASV), gene encoding an unstable Gfp; P_{cepI}, promoter fragment of the C8-HSL synthase of H111; *lasR* and *cepR*, genes encoding the transcriptional activators LasR and CepR, respectively; T_0, transcriptional terminator from phage lambda; T_1, transcriptional terminator from *rrnB* operon of *E. coli*; Gm^R, gentamicin-resistance marker; *rep*, replication gene; *mob*, mobilization gene.

37 °C. After precipitation of undigested substrate with 1·2 ml 10 % (w/v) trichloroacetic acid for 15 min at room temperature, followed by 10 min centrifugation at 15 000 r.p.m., 1·4 ml 1 M NaOH was added to the supernatant. The absorbance (A_{440}) of the supernatant was measured and relative protease activities were calculated as A_{440}/OD_{600}.

Plasmid and DNA manipulations. Purification, cloning, electrophoresis, and other manipulations of DNA were performed using standard techniques (Sambrook *et al*., 1989). *P. aeruginosa* strains were tagged with the red fluorescent protein DsRed by inserting the hybrid transposon mini-Tn*5 dsred* into the chromosome of the strains using a three-factor mating procedure (Christensen *et al*., 1999). The plasmids used in this study are listed in Table 1.

The broad-host-range 3-oxo-C12-HSL-sensor plasmid pKR-C12 (Fig. 1) was constructed as follows. A *Not*I cassette, containing divergently transcribed P_{lac}–*lasR* and P_{lasB}–*gfp*(ASV) translational fusions, was excised from plasmid pMHLAS (Hentzer *et al*., 2002), blunt-ended with Klenow fragment, and inserted into the unique *Sma*I site of the broad-host-range vector pBBR1MCS-5. The *cep*-based sensor pAS-C8 was constructed in a three-step cloning procedure. A 344 bp DNA fragment containing the start codon and upstream region of *cepI* was PCR amplified using the primers cepI-fwd and cepI-rev and chromosomal DNA of *B. cepacia* H111 (GenBank accession no. AF330025) as template. Following digestion with *Sph*I and *Bam*HI the PCR fragment was ligated into the corresponding sites of pGEM, giving rise to pAS1. The *gfp*(ASV) gene was amplified using the primer pair gfp(ASV)-1 and gfp(ASV)-2, and plasmid pMHLAS as template. The resulting 759 bp DNA fragment was digested with *Sph*I and *Hin*dIII and ligated into pAS1 cut with the same enzymes, yielding pAS2. In this construct the *cepI* promoter is translationally fused to *gfp*(ASV) at the start codon. This cassette was inserted into the broad-host-range plasmid pBBR1MCS-5 via the restriction sites *Bam*HI and *Hin*dIII. Next, the *cepR* gene of *B. cepacia* H111 (GenBank accession no. AF330020), which encodes the cognate C8-HSL receptor protein, was amplified using the primers cepR-fwd and cepR-rev. The resulting PCR product was digested with *Kpn*I and cloned into the corresponding site of pAS2. One clone, in which the *cepR* gene was placed downstream of the *lac* promoter of the plasmid, was chosen. This plasmid, which was designated pAS-C8, contains a P_{lac}–*cepR* transcriptional fusion together with a P_{cepI}–*gfp*(ASV) translational fusion transcribed in the opposite direction.

Both sensor plasmids, pKR-C12 and pAS-C8, were transferred to *P. aeruginosa* and *B. cepacia* strains by triparental mating (Christensen *et al*., 1999).

Characterization of AHL monitor strains. To determine the specificity and sensitivity of the different AHL monitor strains respective overnight cultures were diluted fourfold into fresh LB medium, incubated 1 h at 30 °C and then distributed in 200 µl aliquots into wells of a microtitre plate. C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, C10-HSL, 3-oxo-C10-HSL, C12-HSL and 3-oxo-C12-HSL were added to the wells at final concentrations of 5000, 2500, 1250, 600, 400, 100 and 25 nM. Following 6 h of incubation at 30 °C, green fluorescence of the monitor strains was measured using the microtitre plate reader Lambda fluoro 320 Plus (MWG Biotech) with an excitation wave length of 474 nm and emission detection at 515 nm. Data were processed with the KC4 software (Bio-Tek Instruments). Detection limits are defined as minimal AHL concentrations giving rise to at least 30 % of the activity of fully induced cultures.

Cross-streaking experiments. The monitor strain and the respective test strains were streaked close to each other to form a T. Following 24 h incubation at 30 °C, the plates were illuminated with blue light using an HQ 480/40 filter (F44–001; AHF-Analysentechnik) in combination with a halogen lamp (Intralux 5000-1; Volpi) as a light source. Illumination took place in a darkbox that was equipped with a light-sensitive camera (C2400-40; Hamamatsu) with a Pentax CCTV camera lens and an HQ 535/20 filter (F42-001; AHF-Analysentechnik). The Argus 20 image analysis system (Hamamatsu) was used for detection and documentation of green fluorescent areas within the monitor strain streak.

Flow-chamber experiments. Surface-attached mixed-species biofilms were cultivated in artificial flow chambers (Møller *et al*., 1998) with channel dimensions of $1 \times 4 \times 40$ mm. The substratum consisted of a microscope coverslip (Knittel, 24×50 mm; Knittel Gläser) and the flow chambers were supplied with a flow of ABt minimal medium containing 1 mM glucose. The flow system was assembled and prepared as described previously (Møller *et al*., 1998). To cultivate mixed biofilms of *B. cepacia* and *P. aeruginosa*, flow chambers

were inoculated with 350 µl of a 1:1 mixture of exponentially growing cultures diluted to an OD_{600} of 0·1 in 0·9% NaCl. After inoculation, the medium flow was arrested for 1 h to allow efficient colonization of the glass surface. Medium flow was then started and the substrate was pumped through the flow chamber at a constant rate of 0·2 mm s^{-1} using a peristaltic pump (Watson Marlow 205S). After 24–72 h of cultivation at 30 °C, the mixed-species biofilms were inspected by confocal scanning laser microscopy (CSLM).

***In situ* hybridization of biofilms.** Embedding of mixed biofilms and *in situ* hybridization were performed as previously described (Christensen *et al*., 1999). Specific rRNA probes were used to visualize cells of *B. cepacia* and *P. aeruginosa*: Bcv13b (5′-GCTCATCCCATTTCGCTC-3′ – 23S rRNA) labelled with CY3 for H111, and Paa1448 (5′-GTAACCGT-CCCCCTTGCG-3′ – 16S rRNA) labelled with CY5 for SH1 and SH38.

Microscopy and image analysis. All microscopic observations and image acquisitions of biofilms were performed on a confocal scanning laser microsope (TCS4D; Leica Lasertechnik) equipped with a detector and a filterset for monitoring green fluorescent and red fluorescent protein. In addition, a reflection detector for bright-field images was installed. Images were obtained with a 63×/1·32 oil objective and image scanning was carried out with the 488 nm laser line from an Ar/Kr laser. Simulated fluorescence projections, and sections through the biofilms, were generated using the IMARIS software package (Bitplane) running on a Silicon Graphics Indigo 2 workstation. Images were further processed with the Photoshop software (Adobe).

Animal experiments. The mouse strain NMRI was obtained from the Panum Institute, Copenhagen University, Denmark. All animal experiments were performed after authorization from the National Animal Ethics Committee. Immobilization of *P. aeruginosa* and *B. cepacia* strains in seaweed alginate beads was performed as previously described (Wu *et al*., 2000). Cultures of *P. aeruginosa* and *B. cepacia* were adjusted to a concentration of $1{\cdot}0 \times 10^8$ c.f.u. ml^{-1} and a 2:3 mixture of these suspensions was used for immobilization. Intratracheal challenge with 0·04 ml of alginate beads was performed as described by Moser *et al*. (1997). Mice were anaesthetized by subcutaneous injection of a 1:1 mixture of etomidat (Janssen) and midazolam (Roche) at a dose of 10 ml per kg body weight and tracheotomized (Johansen *et al*., 1993). The animals were killed 1, 3 and 7 d after challenge by administering 20% pentobarbital (DAK) at 2 ml per kg body weight. Freeze microtomy of the lung tissue and CSLM of the 40–50 µm thick lung sections were performed as described previously (Wu *et al*., 2000).

RESULTS

AHL profiles of strains used in this study

In a first step to assess the possibility of AHL-mediated intergeneric signalling between *P. aeruginosa* and *B. cepacia* we determined the AHL profiles of bacterial isolates from one CF patient who became co-infected with *B. cepacia* (Geisenberger *et al*., 2000). *P. aeruginosa* SH1, a strain that was isolated from the patient prior to co-infection with *B. cepacia*, produced large amounts of AHL molecules. Using the bioluminescent plasmid sensor pSB403 in combination with TLC six different AHL molecules could be detected. On the basis of their

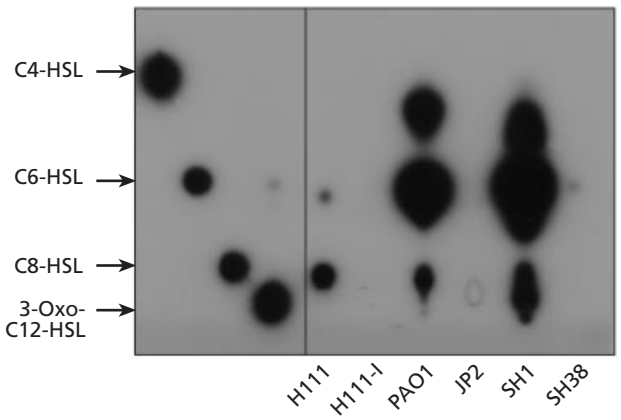


Fig. 2. TLC analysis of AHLs produced by strains of *P. aeruginosa* and *B. cepacia* used in this study. Samples were chromatographed on C_{18} reversed-phase thin-layer plates, developed with methanol/water (60:40, v/v) and spots were visualized by overlaying the TLC plates with *E. coli* MT102 harbouring the bioluminescent sensor plasmid pSB403. AHL standards were included as indicated.

mobilities (R_F values) and by including appropriate reference compounds it was concluded that these molecules represent 3-oxo-C12-HSL, 3-oxo-C8-HSL, 3-oxo-C14-HSL, C6-HSL and C8-HSL (Fig. 2; Geisenberger *et al*., 2000). As a control we also included the well-characterized *P. aeruginosa* strain PAO1 in this analysis. For this strain a very similar AHL profile was determined, except that we were unable to detect 3-oxo-C14-HSL (Fig. 2) and that the levels of 3-oxo-C8-HSL and 3-oxo-C12-HSL were significantly lower than those of SH1. This result is in good agreement with previous studies that demonstrated that in PAO1 LasI directs the synthesis of primarily 3-oxo-C12-HSL together with small amounts of 3-oxo-C8-HSL and 3-oxo-C6-HSL (Pearson *et al*., 1994) and that RhlI directs the synthesis of C4-HSL and C6-HSL in a molar ratio of 15:1 (Winson *et al*., 1995). However, C4-HSL could not be detected by the aid of plasmid pSB403 since this AHL sensor is very insensitive to this signal molecule (Winson *et al*., 1998). The production of both C4-HSL and C6-HSL was easily visualized by the use of *Chromobacterium violaceum* CV026 as sensor (Geisenberger *et al*., 2000). This sensor is highly sensitive to unsubstituted short-chain AHL molecules but is quite insensitive to other AHLs (McClean *et al*., 1997).

In contrast to SH1, strain SH38, which was isolated during the co-infection period, only produced trace amounts of C6-HSL. This situation is similar to that found with the *lasI rhlI* double mutant PAO1-JP2 (Pesci *et al*., 1997), which, as expected, does not produce any AHL molecules.

The genomovar III *B. cepacia* strain H111 originates from the same patient from whom *P. aeruginosa* strains SH1 and SH38 were isolated. Strain H111 produces C8-

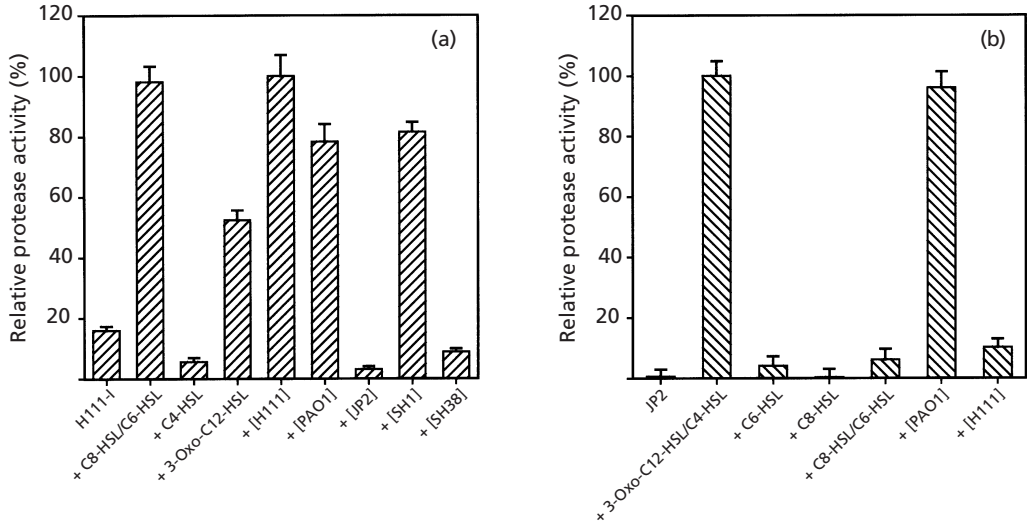


Fig. 3. Proteolytic activities of culture supernatants of (a) *B. cepacia* H111-I and (b) *P. aeruginosa* PAO1-JP2. The strains were grown in the absence or presence of different AHL molecules (at concentrations of 1 μM) or of dichloromethane extracts of supernatants prepared from cultures of the *P. aeruginosa* strains PAO1, PAO1-JP2 and SH1, and the *B. cepacia* strains SH38 and H111 (addition of extracts of the different strains is indicated by the strain name in brackets). Sterile filtered supernatants were used for measurements of proteolytic activities. The data represent mean values of three independent experiments. Error bars represent the standard errors of the means.

HSL and C6-HSL in a molar ratio of approximately 10:1 (Fig. 2; Gotschlich *et al*., 2001). The AHL profile of this *B. cepacia* clone was found to remain unchanged during the entire co-infection period (data not shown). No AHLs were detected in the supernatants of a recently constructed *cepI* mutant of this strain, which was designated H111-I (Fig. 2; Huber *et al*., 2001).

These results show that *P. aeruginosa* SH1 and *B. cepacia* H111, which were isolated from the same patient at the onset of co-colonization, produce C6-HSL, although in both cases in relatively small amounts. However, given that LuxR-type AHL receptor proteins exhibit a considerable degree of flexibility, i.e. molecules that are similar but not identical to the natural AHL ligand are capable of activating the receptor, these data support the idea that in the case of the formation of mixed biofilms the quorum-sensing systems operating in the two bacteria may cross-react.

P. aeruginosa supernatants restore protease production by *B. cepacia* H111-I

McKenney *et al*. (1995) showed that production of siderophores, lipase and protease of *B. cepacia* 10661 was stimulated when the growth medium was supplemented with concentrated spent culture supernatants of *P. aeruginosa* PAO1. They suggested that AHL molecules produced by PAO1 induce expression of extracellular products in *B. cepacia* 10661. However, the reported induction of exoproduct synthesis was relatively weak, being at best sevenfold for siderophore production. This only mild induction is, at least in part, due to the use of the *B. cepacia* wild-type strain, which itself produces AHLs, and thus complicates the analysis.

For a more detailed analysis we tested extracts of *P. aeruginosa* and *B. cepacia* wild-type strains for cross-stimulation of extracellular proteolytic activities of respective quorum-sensing-defective mutants. We chose to investigate effects on protease production as this phenotype is strictly regulated by quorum sensing in both bacteria (Passador *et al*., 1993; Lewenza *et al*., 1999). Accordingly, virtually no proteolytic activities were observed with the *cepI* mutant of *B. cepacia* or with the *lasI rhlI* double mutant of *P. aeruginosa* when compared with the wild-type strains (Fig. 3). Addition of a mixture of C8-HSL and C6-HSL (1 μM each) or extracts of H111 culture supernatants to the growth medium completely restored protease production of H111-I. The presence of 1 μM C4-HSL showed no effect, and the presence of 1 μM 3-oxo-C12-HSL partially complemented the defect. More importantly, extracts of the AHL-producing *P. aeruginosa* strains PAO1 and SH1 also restored protease production, while extracts of the AHL-negative strains PAO1-JP2 and SH38 did not. Conversely, extracts of H111 did not stimulate protease production of the *lasI rhlI* double mutant PAO1-JP2. Neither did the presence of C4-HSL, C8-HSL, C6-HSL, or a mixture of C8-HSL and C6-HSL, affect the results. Restoration of the defect was only observed when the medium was supplemented with 1 μM 3-oxo-C12-HSL, a mixture of 3-oxo-C12-HSL and C4-HSL (1 μM each), or an extract of the spent culture supernatant of PAO1. These data suggest that in principle *P. aeruginosa* and *B. cepacia* are capable of communicating with each other,

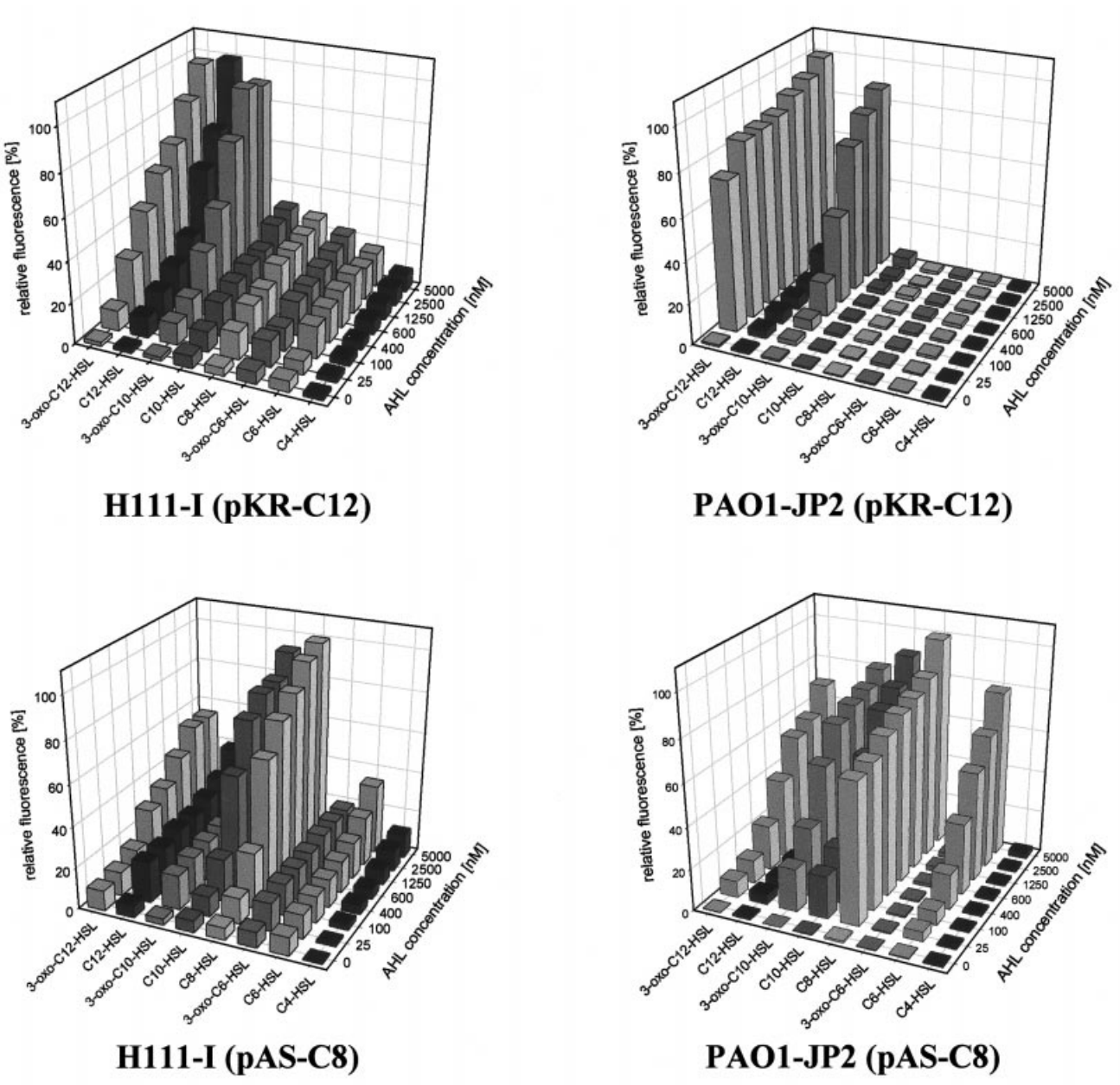


Fig. 4. Characterization of the AHL monitor strains. The monitor strains were grown in the presence of different AHL compounds at concentrations of 0–5000 nM. Green fluorescence of the cultures was recorded by a microtitre plate reader at a wavelength of 515 nm. Maximal induction levels of the monitor strains were set to 100 %.

albeit in an unidirectional manner, i.e. while *B. cepacia* is capable of perceiving AHL molecules from *P. aeruginosa*, the latter organism cannot utilize the signals produced by *B. cepacia* for triggering quorum sensing.

Construction and characterization of Gfp-based sensors for 3-oxo-C12-HSL and C8-HSL

To generate more direct evidence for communication between cells of *P. aeruginosa* and *B. cepacia* we constructed two Gfp-based AHL sensor plasmids as described in Methods (see also Fig. 1). The sensor plasmid pKR-C12 contains a translational fusion of the *lasB* elastase gene of *P. aeruginosa* to *gfp*(ASV), encoding an unstable version of the Gfpmut3* protein (Andersen *et al.*, 1998). Furthermore, the sensor contains the *lasR* gene, which encodes the cognate 3-oxo-C12-HSL receptor protein under control of a *lac*-type promoter. Since expression of *lasB* is controlled by the *las* quorum-sensing system, this sensor is expected to be most sensitive for 3-oxo-C12-HSL and related long-chain AHLs. The second sensor plasmid, pAS-C8, is based on the *cep* genes of *B. cepacia* and contains a translational *cepI–gfp*(ASV) fusion together with the *cepR* regulator

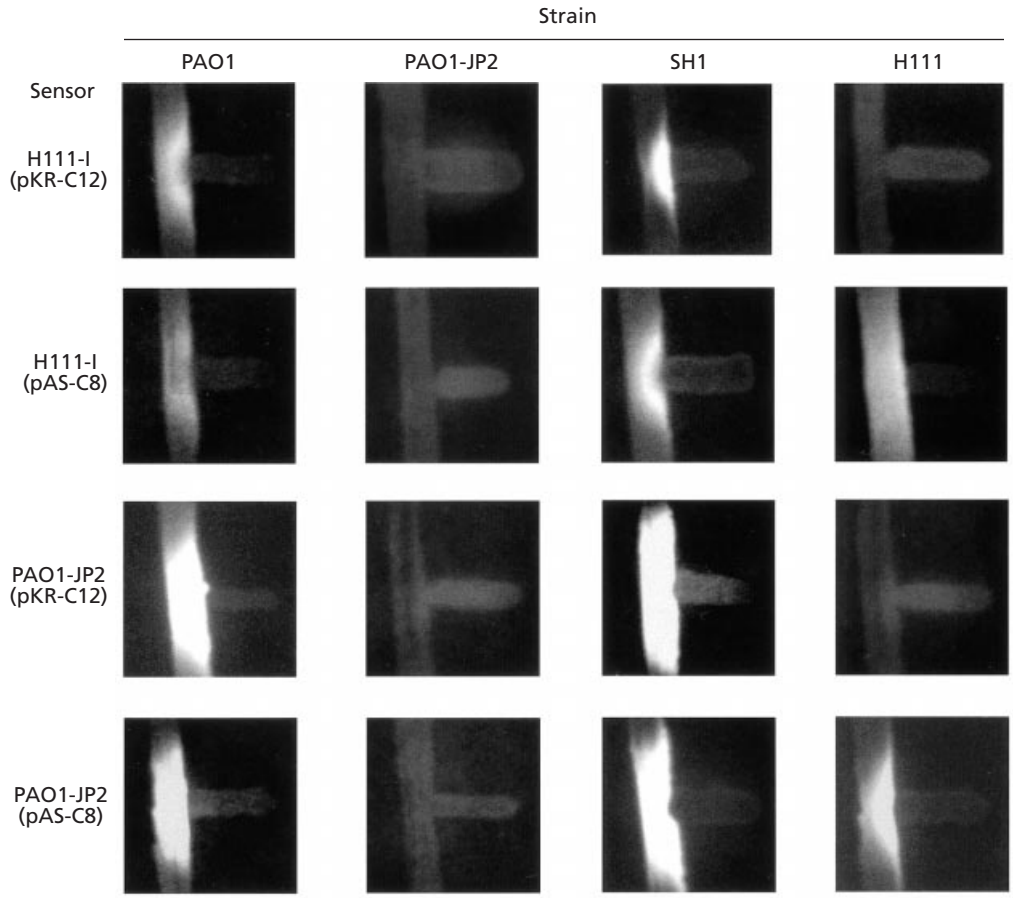


Fig. 5. Activation of the AHL monitor strains in cross-streak experiments. The four monitor strains H111-I(pKR-C12), H111-I(pAS-C8), PAO1-JP2(pKR-C12), PAO1-JP2(pAS-C8) and the test strains PAO1, PAO1-JP2, SH1 and H111 were cross-streaked on LB agar plates as described in Methods. Following 24 h of incubation at 30 °C production of Gfp(ASV) by the monitor strains was visualized by exciting the plates with blue light.

gene placed under control of P_{lac}. Since expression of *cepI* is auto-regulated (Lewenza *et al*., 1999; Huber *et al*., 2001), this sensor plasmid should be most sensitive for C8-HSL.

The presence of the two sensor cassettes on the mobilizable broad-host-range vector pBBR1MCS-5 (Kovach *et al*., 1995) enabled us to transfer the constructs to the AHL-negative strains PAO1-JP2 and H111-I, giving rise to the four monitor strains PAO1-JP2(pKR-C12), PAO1-JP2(pAS-C8), H111-I(pKR-C12) and H111-I(pAS-C8). We next tested the performance of these monitor strains with respect to their sensitivity for different AHL molecules. This was accomplished by measuring Gfp fluorescence of cultures exposed to various AHL concentrations. As expected, H111-I(pKR-C12) and PAO1-JP2(pKR-C12) exhibited the highest sensitivity to 3-oxo-C12-HSL and both sensors responded well to 3-oxo-C10-HSL (Fig. 4). Interestingly, while H111-I(pKR-C12) also responded to C12-HSL, PAO1-JP2(pKR-C12) was very insensitive to this molecule. The two monitor strains based on plasmid pAS-C8 were highly sensitive to C8-HSL and C10-HSL. However, both sensors were also responsive to a variety of related molecules, albeit with reduced sensitivity (Fig. 4).

Assessment of interspecies communication in 'cross-streaking' experiments

To test the different monitor strains for their applicability to visualize cell–cell communication *in situ*, the monitor strains were cross-streaked against the wild-type and mutant strains of *P. aeruginosa* and *B. cepacia*. In this simple assay AHL-mediated signalling can be monitored by the production of Gfp in the monitor strain. The results of these experiments are shown in Fig. 5. Strong green fluorescence was observed when SH1 or PAO1 was cross-streaked against the monitor strains H111-I(pKR-C12) and PAO1-JP2(pKR-C12). As expected, green fluorescence was somewhat weaker when PAO1 was cross-streaked against the monitor strains H111-I(pAS-C8) and PAO1-JP2(pAS-C8). On the other hand, when the *P. aeruginosa*-based monitor strains PAO1-JP2(pKR-C12) and PAO1-JP2(pAS-C8) were used to detect production of AHLs by H111, only the latter sensor gave rise to weak signals. Given that *lasB* is a quorum-sensing-regulated target gene, these

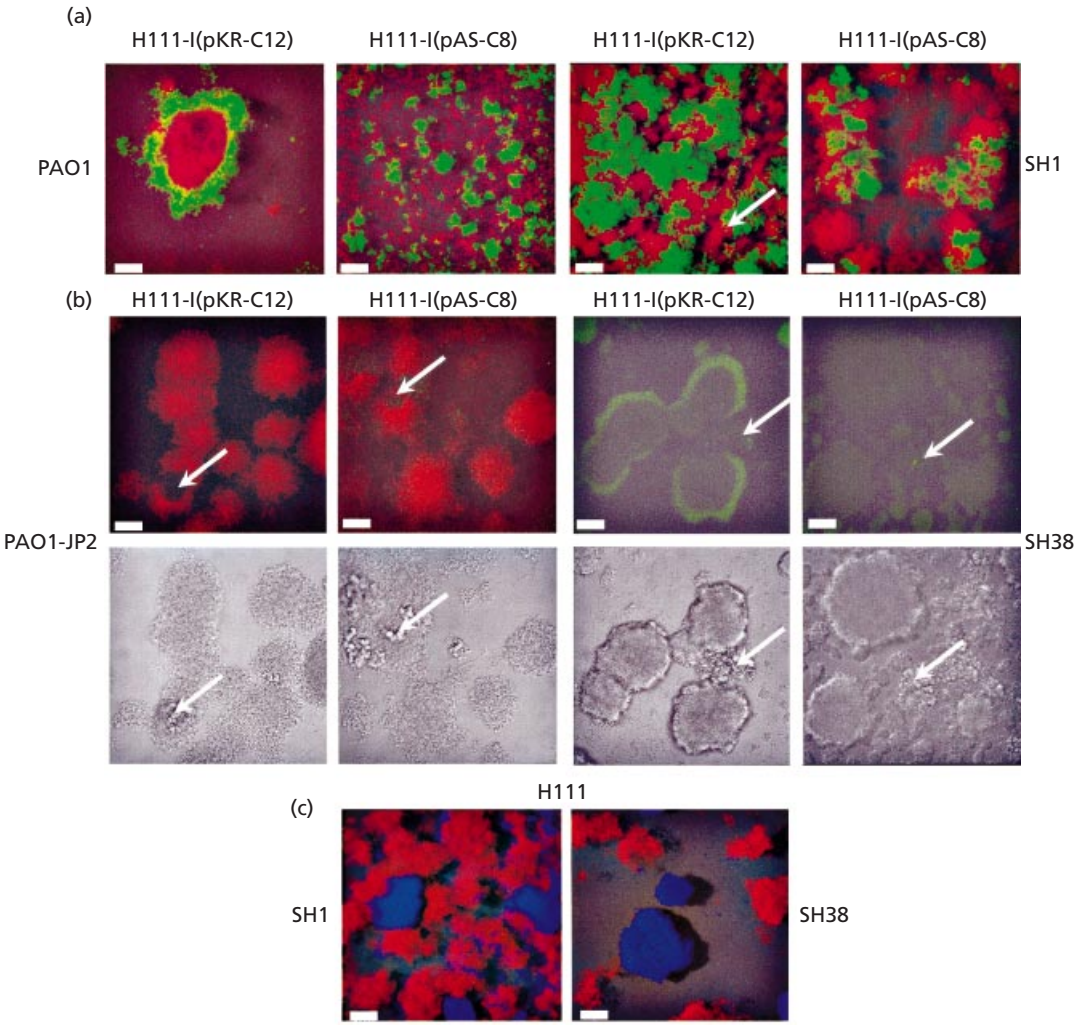


Fig. 6. Intergeneric communication between *P. aeruginosa* and *B. cepacia* in biofilms cultured in flow chambers. (a) Mixed biofilms of H111-I harbouring the sensor plasmids pKR-C12 (left panel) or pAS-C8 (right panel), and *dsred*-tagged derivatives of PAO1 and SH1. Perception of AHL molecules by the *B. cepacia* monitor strain is indicated by the appearance of green fluorescent cells; the distribution of *P. aeruginosa* cells is visualized by their red fluorescence. Simulated fluorescence projections were generated by CSLM 72 h post-inoculation. (b) Mixed biofilms of H111-I harbouring the sensor plasmids pKR-C12 (left panel) or pAS-C8 (right panel), and *dsred*-tagged derivatives of PAO1-JP2 and the untagged but auto-fluorescent strain SH38. White arrows indicate microcolonies of the *B. cepacia* monitor strain. Upper panels show epifluorescence images; lower panels are reflection images of the same microscopic field. (c) CSLM photograph of mixed biofilms formed by *B. cepacia* H111 and *P. aeruginosa* SH1 (left panel) or SH38 (right panel). *B. cepacia* was hybridized with the CY3-labelled (red) probe bcv13b, and *P. aeruginosa* was hybridized with probe paa1448 labelled with CY5 (blue). Bars, 20 μm.

results indicate that PAO1 may be unable to respond to the AHL signal molecules produced by H111. This result is consistent with our observation that extracts of *B. cepacia* H111 do not affect protease production of PAO1-JP2, while extracts of PAO1 did restore protease production by H111-I.

Visualization of intergeneric communication in biofilms grown in flow chambers

To investigate whether cell–cell communication would occur in mixed biofilms of *P. aeruginosa* and *B. cepacia* we used artificial flow chambers for culturing biofilms consisting of a monitor strain and an appropriate partner strain. Since our results suggest that *B. cepacia* is capable of responding to the AHLs produced by *P. aeruginosa* but not vice versa, we used the monitor strain H111-I(pKR-C12) in combination with different *P. aeruginosa* strains. To be able to easily monitor the *P. aeruginosa* strains in these experiments they were tagged with the red fluorescent protein DsRed. When mixed biofilms of H111-I(pKR-C12) and one of the AHL-producing strains, SH1 or PAO1, were inspected by CSLM bright green fluorescent cells were detected (Fig. 6a). By contrast, no green fluorescent *B. cepacia* cells were observed in mixed biofilms containing the AHL-

Table 2. Intergeneric communication between *P. aeruginosa* and *B. cepacia* in mouse lung tissue

In each of experiments I–IV, NMRI female mice were challenged with mixtures of *B. cepacia* H111-I harbouring an AHL sensor plasmid and different *dsred*-tagged *P. aeruginosa* strains. Three mice were killed on each of days 1, 3 and 7 after infection, and CLSM was employed to inspect tissue samples for green fluorescent cells. ++, Strong induction; +, induction; −, no induction of the monitor strain.

Expt	Bacterial strains inoculated (ratio 2:3 c.f.u. ml^{-1})	Day	Gfp signal
I	SH1 (DsRed) + H111-I(pKR-C12)	1	+
		3	++
		7	++
II	PAO1-JP2 (DsRed) + H111-I(pKR-C12)	1	−
		3	−
		7	−
III	SH1 (DsRed) + H111-I(pAS-C8)	1	+
		3	++
		7	+
IV	PAO1-JP2 (DsRed) + H111-I(pAS-C8)	1	−
		3	−
		7	−

negative strains PAO1-JP2 and SH38 (Fig. 6b). Similar results were obtained when the monitor strain H111-I(pAS-C8) was used instead of H111-I(pKR-C12) (Fig. 6a). These results show that in mixed biofilms *B. cepacia* is capable of perceiving the AHL signals produced by most, but not all, *P. aeruginosa* strains.

During the course of these experiments we further noticed that the various mixed biofilms investigated exhibited significant structural differences. In biofilms consisting of *B. cepacia* H111-I and AHL-producing *P. aeruginosa* strains the microcolonies of the two species were often closely associated (Fig. 6a) while in mixed consortia of H111-I and AHL-negative *P. aeruginosa* strains the microcolonies were more separated (Fig. 6b).

In a previous study it was shown that the *las* quorum-sensing system is directly involved in the regulation of biofilm formation (Davies *et al*., 1998). When compared with the wild-type, a *lasI* mutant of *P. aeruginosa* formed only flat and undifferentiated biofilms, suggesting that the *las* system is in some way required for the maturation of biofilms. However, we were unable to detect notable differences in the structures of single-species biofilms formed by PAO1 and PAO1-JP2 (data not shown). This apparent discrepancy may be attributable to the different media used in the two studies and/or to different experimental settings for growing biofilms.

To investigate the role of AHL production by *P. aeruginosa* in the structure of mixed biofilms in more detail we analysed the structures of biofilms formed by *B. cepacia* H111 and either the AHL producing *P. aeruginosa* strain SH1 or the AHL-negative strain SH38 (Fig. 6c). The spatial distribution of the bacteria in the consortium was investigated by CSLM after visualizing cells by fluorescent *in situ* hybridization. In biofilms formed by H111 and SH38 the two strains tended to grow in well-separated microcolonies. By contrast, in biofilms formed by H111 and SH1 the association of microcolonies was much tighter, and mixed microcolonies were observed, which were never observed in H111/SH38 biofilms. These results lend further support to the hypothesis that AHL production by *P. aeruginosa* plays an important role in determining the structure of the mixed consortium.

Evidence for intergeneric cell–cell communication in the lung tissue of infected mice

By the use of alginate-entrapped *P. aeruginosa* cells chronic lung infections can be established in mice (Moser *et al*., 1997). This animal model has recently been used in combination with a Gfp-based AHL monitor strain to show that *P. aeruginosa* produces AHL signal molecules when colonizing the lung tissue (Wu *et al*., 2000). To investigate whether AHL-mediated communication between *P. aeruginosa* and *B. cepacia* occurs during the course of a co-infection, mice were challenged with alginate beads containing a *B. cepacia* monitor strain together with different *dsred*-tagged *P. aeruginosa* strains. The mice were killed on day 1, 3 and 7 post-intratracheal challenge and the lung tissue was inspected by CSLM. The results of these investigations are summarized in Table 2. When the monitor strains H111-I(pKR-C12) or H111-I(pAS-C8) were used for co-infection together with the AHL-producing *P. aeruginosa* strain SH1, bright green fluorescent cells were

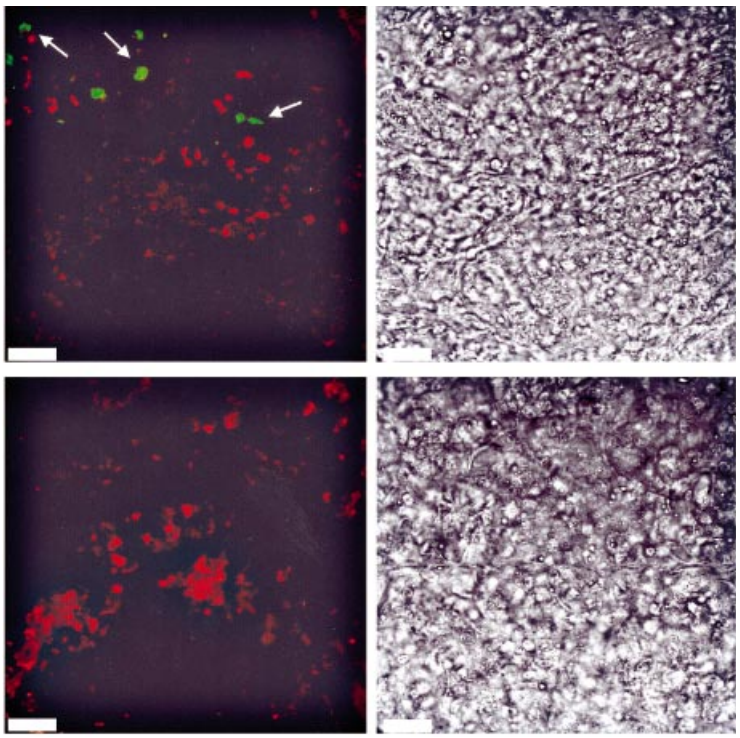


Fig. 7. Intergeneric communication between *P. aeruginosa* and *B. cepacia* in mouse lung tissue. Simulated fluorescence projections generated by CSLM of mouse tissue infected with alginate beads containing a mixture of H111-I(pAS-C8) and *dsred*-tagged derivatives of SH1 (top panel) and PAO1-JP2 (bottom panel) 72 h post-inoculation. Reflection images of the same microscopic views are shown on the right. Perception of AHL molecules by the *B. cepacia* monitor strain is indicated by the appearance of green fluorescent cells (as indicated by white arrows); the distribution of *P. aeruginosa* cells is visualized by their red fluorescence. Bars, 20 μm.

detected in the lung tissues (Fig. 7). As expected, no green fluorescent cells were detected in cases where the *B. cepacia* monitor strains were used together with the AHL-negative strain PAO1-JP2. These data provide strong evidence that unidirectional signalling between *P. aeruginosa* and *B. cepacia* does in fact occur during co-infection of mouse lung tissue.

DISCUSSION

Over the past few years evidence has emerged that quorum sensing is a common phenomenon in bacteria. To date, a large number of highly diverse signal molecules have been identified that are utilized by bacteria to sense their own population densities. Among Gram-negative bacteria, the most intensively investigated and probably the most widespread signal molecules are AHLs (for reviews see Swift *et al*., 1994; Fuqua *et al*., 1996; Salmond *et al*., 1995; Hardman *et al*., 1998; Eberl, 1999). Given that the vast majority of Gram-negative bacteria are capable of producing AHLs and that they often colonize the same environmental niche it is very tempting to assume that AHL signal molecules are used not only as cell-density sensors of one species but also for communication between cells of different species. A clinically highly relevant example of such a scenario is represented by mixed biofilms of *P. aeruginosa* and *B. cepacia* in the lungs of some CF patients. In most of these cases, the persons are already chronically colonized by *P. aeruginosa* before they become co-infected with *B. cepacia* (Govan & Deretic, 1996; Tümmler & Kiewitz, 1999). As both species employ AHL-dependent quorum-sensing systems to link biofilm formation with the expression of pathogenic traits (Davies *et al*., 1998; Parsek & Greenberg, 1999; Huber *et al*., 2001), intergeneric communication by the aid of AHLs may be of profound importance for the virulence of the mixed consortium.

To investigate whether AHL-mediated communication between *P. aeruginosa* and *B. cepacia* in mixed biofilms does occur we constructed two novel Gfp-based sensor plasmids, which are suitable for visualizing intergeneric communication at the single-cell level. The two plasmids respond to different spectra of AHL molecules, depending on the components used for their construction. The sensor plasmid pKR-C12 is based on components of the *P. aeruginosa* PAO1 *las* system and contains a *lasB–gfp*(ASV) translational fusion together with the *lasR* gene placed under control of P_{lac} on the broad-host-range plasmid pBBR1MCS-5 (Fig. 1). In good agreement with previous studies (Passador *et al*., 1993; Hentzer *et al*., 2002) this plasmid sensor exhibited the highest sensitivity for 3-oxo-C12-HSL irrespective of whether *P. aeruginosa* PAO1-JP2 or *B. cepacia* H111-I was used as host. However, the strain background had a significant effect on the detection limits. In the *P. aeruginosa* background we determined a detection limit for 3-oxo-C12-HSL of less than 10 nM for single cells (assessed by epifluorescence microscopy) and of less than 25 nM for measurements in microtitre assays, while the respective detection limits in the *B. cepacia* background were approximately 50 nM and 100 nM, respectively. Noteworthy in this context is the fact that an unstable variant of Gfp, Gfp(ASV) (Andersen *et al*., 1998), was used for the construction of the sensor to enable detection of transient bacterial communication. This Gfp variant carries a C-terminal peptide tag, which

makes the protein prone to degradation by housekeeping/intracellular tail-specific proteases (Clp). As constitutive expression of Gfp(ASV) from a P_{lac}-type promoter also results in significantly lower fluorescence levels in *B. cepacia* than in *P. aeruginosa* (unpublished result) we speculate that the Gfp variant may have a shorter half-life in *B. cepacia*.

With slightly reduced sensitivities both monitor strains also responded to 3-oxo-C10-HSL, but only in the *B. cepacia* background was the sensor stimulated by C12-HSL. At present the reason for this strain difference is unclear. Recent investigations concerning the uptake and efflux of 3-oxo-C12- and C4-HSL in *P. aeruginosa* revealed that cells are only freely permeable for the short-chain AHL. The long-chain AHL 3-oxo-C12-HSL was shown to be actively transported out of the cells by the *P. aeruginosa* MexAB–OprM multidrug efflux system (Evans *et al*., 1998; Pearson *et al*., 1999). Thus, variations in the presence and/or specificity of long-chain AHL transporters in the two strains may account for the observed strain-dependent difference in sensitivity for C10-HSL. Alternatively, variations in the copy number of the sensor plasmid in the two strains may be responsible for the different sensitivities for C10-HSL, as it has been demonstrated previously that the amount of TraR protein present in the cell determines the range of AHL molecules that are bound by the receptor (Zhu *et al*., 1998).

For the sensitive detection of C8-HSL we constructed a novel AHL sensor plasmid, designated pAS-C8, which is based on components of the *cep* quorum-sensing system of *B. cepacia* H111. This plasmid contains a *cepI–gfp*(ASV) translational fusion together with the *cepR* gene, which is transcribed from the P_{lac} promoter of the broad-host-range plasmid pBBR1MCS-5. As expected, this sensor plasmid responded most efficiently to C8-HSL, with a lower detection limit of less than 5 nM for single-cell analysis and of less than 25 nM in microtitre plate assays when the sensor plasmid was present in the *P. aeruginosa* PAO1-JP2 background. As for pKR-C12, the detection limits were higher in the *B. cepacia* H111-I background, namely 50 nM and 150 nM, respectively. The sensor was, with a lower efficiency, also stimulated by related molecules including C6- and C10-HSL. In contrast to pKR-C12, the strain background did not significantly influence the spectrum of AHL molecules detected by the sensor plasmid.

The AHL sensor plasmid pAS-C8, which is most sensitive for AHL molecules with a C_8 acyl side chain, nicely extends the range of AHLs that can currently be detected with the aid of Gfp-based AHL sensors. Beside *las*-based sensors, which are particularly suitable for detection of long-chain AHLs (Hentzer *et al*., 2002; this study), sensors for the detection of short-chain AHLs have been described. These sensors are based on components of the *lux* quorum-sensing system of *Vibrio fischeri* and were demonstrated to be highly sensitive for 3-oxo-C6-HSL (with a detection limit of 5 nM in an *E. coli* background) and other short-chain AHLs (Andersen *et al*., 2001). This series of Gfp-based sensors may prove to be highly valuable molecular tools for *in situ* visualization of AHL-mediated communication between individual bacterial cells in various natural habitats. Previously, we have used these sensors for detection of AHL signal molecules in the lung tissues of mice infected with *P. aeruginosa* (Wu *et al*., 2000), for visualization of interspecies communication in swarming colonies of *Serratia liquefaciens* (Eberl *et al*., 1999; Andersen *et al*., 2001), and for the analysis of quorum-sensing inhibition by halogenated furanone compounds in *P. aeruginosa* biofilms (Hentzer *et al*., 2002).

In this study we employed the C8- and 3-oxo-C12-HSL specific sensors to investigate the possibility of inter-generic communication between clinical isolates of *P. aeruginosa* and *B. cepacia*. Mixed consortia were either cultured in artificial flow chambers, which represent an artificial but highly controllable aquatic model system, or in the lungs of mice using alginate-entrapped bacteria. The detection of green fluorescent cells in both model systems as well as in cross-streak experiments provided evidence that the two bacteria utilize AHL molecules to interact with each other. Moreover, our data clearly show that communication between the two bacteria only occurs in one direction, namely from *P. aeruginosa* to *B. cepacia* and not vice versa.

It could be argued that the sensor plasmids used are artificial constructs that are valuable for detection of AHLs but do not necessarily indicate whether these AHLs are in fact perceived by the bacteria. In this respect it is important to bear in mind that both sensor plasmids used in these experiments are based on the promoter sequences of the genes *lasB* and *cepI*, which are controlled by the quorum-sensing systems of *P. aeruginosa* and *B. cepacia*, respectively. Hence, stimulation of these AHL sensor plasmids indeed indicates activation of target gene expression in the respective host bacterium. To further substantiate that production of AHLs by *P. aeruginosa* stimulates expression of target genes in *B. cepacia* we determined the effects of extracts of *P. aeruginosa* supernatants as well as of pure AHL compounds on restoration of extracellular proteolytic activity of the *cepI* mutant *B. cepacia* H111-I. Previous work has shown that production of an extracellular protease is tightly controlled by the *cep* quorum-sensing system of *B. cepacia* (Lewenza *et al*., 1999; Huber *et al*., 2001). Extracts of *P. aeruginosa* PAO1 and, more importantly, of SH1, the strain that colonized the lungs of the CF patient at the onset of co-infection with *B. cepacia* H111, restored protease production. By contrast, extracts of *B. cepacia* H111 supernatants did not stimulate protease production of the AHL-negative *P. aeruginosa* derivative PAO1-JP2, strongly supporting the view of unidirectional signalling between the two bacteria. Conceivably, this one-sided communication is a consequence of differences in the specificities of the AHL-binding R-homologues present in the two bacteria. The two AHLs produced by *B. cepacia* H111, C8- and C6-HSL, are very poor activators of the quorum-sensing systems of *P. aeruginosa* (Fig. 4), which

primarily utilize C4-HSL and 3-oxo-C12-HSL, respectively. On the other hand, *P. aeruginosa* produces C6- and 3-oxo-C8-HSL (Pearson *et al.*, 1994; Winson *et al.*, 1995; Geisenberger *et al.*, 2000; Fig. 2), two AHL molecules that are capable of activating the *cep* quorum-sensing system of *B. cepacia* at low concentrations (Fig. 4). Recent work has shown that most strains of the *B. cepacia* complex, which currently comprises six genomic species, produce C8- and C6-HSL (Gotschlich *et al.*, 2001). In this study it was further demonstrated that some strains belonging to the genomovar V (*Burkholderia vietnamiensis*) produce additional AHL molecules with acyl side chains ranging from C_{10} to C_{14}. These *B. vietnamiensis* strains are capable of stimulating the *P. aeruginosa* monitor strain PAO1-JP2(pKR-C12) in cross-streaking experiments (data not shown), indicating that in these cases intergeneric communication may occur in both directions. Work is currently under way to determine the role of AHL-mediated communication between *P. aeruginosa* and *B. cepacia* for the pathogenicity of the mixed consortium.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (EB 2051/1-2 and RI 969/2-1). We gratefully acknowledge the supply of clinical isolates of *P. aeruginosa* and *B. cepacia* by B. Tümmler, and P. Williams is thanked for the generous gift of synthetic AHLs.

REFERENCES

Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjorn, S. P., Givskov, M. & Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**, 2240–2246.

Andersen, J. B., Heydorn, A., Hentzer, M., Eberl, L., Geisenberger, O., Christensen, B. B., Molin, S. & Givskov, M. (2001). *gfp* based *N*-acyl-homoserine-lactone monitors for detection of bacterial communication. *Appl Environ Microbiol* **67**, 575–585.

Ayora, S. & Götz, F. (1994). Genetic and biochemical properties of an extracellular neutral metalloprotease from *Staphylococcus hyicus* subsp. *hyicus*. *Mol Gen Genet* **242**, 421–430.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**, 293–300.

Christensen, B. B., Sternberg, C., Andersen, J. B., Palmer, R. J., Jr, Nielsen, A. T., Givskov, M. & Molin, S. (1999). Molecular tools for study of biofilm physiology. *Methods Enzymol* **310**, 20–42.

Clark, D. J. & Maaløe, O. (1967). DNA replication and the division cycle in *Escherichia coli*. *J Mol Biol* **23**, 99–112.

Davies, G. D., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costeron, J. W. & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**, 295–298.

Eberl, L. (1999). *N*-Acyl homoserinelactone-mediated gene regulation in gram-negative bacteria. *Syst Appl Microbiol* **22**, 493–506.

Eberl, L., Molin, S. & Givskov, M. (1999). Surface motility of *Serratia liquefaciens* MG1. *J Bacteriol* **181**, 1703–1712.

Evans, K., Passador, L., Srikumar, R., Tsang, E., Nezezon, J. & Poole, K. (1998). Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **180**, 5443–5447.

Fuqua, C., Winans, S. C. & Greenberg, E. P. (1996). Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol* **50**, 727–751.

Geisenberger, O., Givskov, M., Riedel, K., Høiby, N., Tümmler, B. & Eberl, L. (2000). Production of *N*-acyl-L-homoserine lactones by *P. aeruginosa* isolates from chronic lung infections associated with cystic fibrosis. *FEMS Microbiol Lett* **184**, 273–278.

Gotschlich, A., Huber, B., Geisenberger, O. & 11 other authors (2001). Synthesis of multiple *N*-acyl-homoserine lactones is widespread among members of the *Burkholderia cepacia* complex. *Syst Appl Microbiol* **24**, 1–14.

Govan, J. R. W. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60**, 539–574.

Govan, J. R. W., Hughes, J. E. & Vandamme, P. (1996). *Burkholderia cepacia*: medical, taxonomic and ecological issues. *J Med Microbiol* **45**, 395–407.

Hardman, A. M., Stewart, G. S. & Williams, P. (1998). Quorum sensing and the cell-cell communication dependent regulation of gene expression in pathogenic and non-pathogenic bacteria. *Antonie Leeuwenhoek* **74**, 199–210.

Hentzer, M., Riedel, K., Rasmussen, T. B. & 9 other authors (2002). Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* **148** (in press).

Herrero, M., de Lorenzo, V. & Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* **172**, 6557–6567.

Holloway, B. W. (1955). Genetic recombination in *Pseudomonas aeruginosa*. *J Gen Microbiol* **13**, 572–581.

Huber, B., Riedel, K., Hentzer, M., Heydorn, A., Gotschlich, A., Givskov, M., Molin, S. & Eberl, L. (2001). The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. *Microbiology* **147**, 2517–2528.

Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P. & Levison, H. (1984). *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *J Pediatr* **104**, 206–210.

Johansen, H. K., Espersen, F., Pedersen, S. S., Hougen, H. P., Rygaard, J. & Høiby, N. (1993). Chronic *Pseudomonas aeruginosa* lung infection in normal and athymic rats. *APMIS* **101**, 207–225.

Kessler, B., de Lorenzo, V. & Timmis, K. N. (1992). A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. *Mol Gen Genet* **233**, 293–301.

de Kievit, T. R. & Iglewski, B. H. (2000). Bacterial quorum sensing in pathogenic relationships. *Infect Immun* **68**, 4839–4849.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.

Lewenza, S., Conway, B., Greenberg, E. P. & Sokol, P. A. (1999). Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepIR. *J Bacteriol* **181**, 748–756.

McClean, K. H., Winson, M. K., Fish, L. & 9 other authors (1997). Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **143**, 3703–3711.

McKenney, D., Brown, K. E. & Allison, D. G. (1995). Influence of *Pseudomonas aeruginosa* exoproducts on virulence factor production in *Burkholderia cepacia*: evidence of interspecies communication. *J Bacteriol* **177**, 6989–6992.

Møller, S., Sternberg, C., Andersen, J. B., Christensen, B. B., Ramos, J. L., Givskov, M. & Molin, S. (1998). In situ gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. *Appl Environ Microbiol* **64**, 721–732.

Moser, C., Johansen, H. K., Song, Z. J., Hougen, H. P., Rygaard, J. & Høiby, N. (1997). Chronic *Pseudomonas aeruginosa* lung infection is more severe in Th2 responding BALB/c mice compared to Th1 responding C3H/HeN mice. *APMIS* **105**, 838–842.

Parsek, M. R. & Greenberg, E. P. (1999). Quorum sensing signals in development of *Pseudomonas aeruginosa* biofilms. *Methods Enzymol* **310**, 43–55.

Passador, L., Cook, J. M., Gambello, M. J., Rust, L. & Iglewski, B. H. (1993). Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**, 1127–1130.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. & Greenberg, E. P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci U S A* **91**, 197–201.

Pearson, J. P., Van Delden, C. & Iglewski, B. H. (1999). Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol* **181**, 1203–1210.

Pesci, E. C., Pearson, J. P., Seed, P. C. & Iglewski, B. H. (1997). Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**, 3127–3132.

Römling, U., Fiedler, B., Bosshammer, J., Grothues, D., Greipel, J., von der Hardt, H. & Tümmler, B. (1994). Epidemiology of chronic *Pseudomonas aeruginosa* infections in cystc fibrosis. *J Infect Dis* **170**, 1617–1621.

Rumbaugh, K. P., Griswold, J. A., Iglewski, B. H. & Hamood, A. N. (1999). Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. *Infect Immun* **67**, 5854–5862.

Saiman, L., Cacalano, G. & Prince, A. (1990). *Pseudomonas cepacia* adherence to respiratory epithelial cells is enhanced by *Pseudomonas aeruginosa*. *Infect Immun* **58**, 2578–2584.

Salmond, G. P., Bycroft, B. W., Stewart, G. S. & Williams, P. (1995). The bacterial 'enigma': cracking the code of cell-cell communication. *Mol Microbiol* **16**, 615–624.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schierholz, J. M., Beuth, J., König, D., Nürnberger, A. & Pulverer, G. (1999). Antimicrobial substances and effects on sessile bacteria. *Zentbl Bakteriol* **289**, 165–177.

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J. & Greenberg, E. P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**, 762–764.

Swift, S., Throup, J. P., Williams, P., Salmond, G. P. & Stewart, G. S. (1994). Quorum sensing: a population-density component in the determination of bacterial phenotype. *Trends Biochem Sci* **21**, 214–219.

Tan, M. W., Mahajan-Miklos, S. & Ausubel, F. M. (1999). Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *Proc Natl Acad Sci U S A* **96**, 715–70.

Tang, H. B., DiMango, E., Bryan, R., Gambello, M., Iglewski, B. H., Goldberg, J. B. & Prince, A. (1996). Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. *Infect Immun* **64**, 37–43.

Tümmler, B. & Kiewitz, C. (1999). Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections. *Mol Med Today* **5**, 351–358.

Van Delden, C. & Iglewski, B. (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* **4**, 551–560.

Williams, P., Camara, M., Hardman, A. & 7 other authors (2000). Quorum sensing and the population-dependent control of virulence. *Philos Trans R Soc Lond B Biol Sci* **355**, 667–680.

Winson, M. K., Camara, M., Lafiti, A. & 10 other authors (1995). Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**, 9427–9431.

Winson, M. K., Swift, S., Fish, L., Throup, J. P., Jorgensen, F., Chhabra, S. R., Bycroft, B. W., Williams, P. & Stewart, G. S. A. B. (1998). Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl-homoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett* **163**, 185–192.

Wu, H., Song, Z., Hentzer, M. & 8 other authors (2000). Detection of *N*-acylhomoserine lactones in lung tissues of mice infected with *Pseudomonas aeruginosa*. *Microbiology* **146**, 2481–2493.

Xu, K. D., McFeter, G. A. & Stewart, P. S. (2000). Biofilm resistance to antimicrobial agents. *Microbiology* **146**, 547–549.

Zhu, J., Beaber, J. W., Moré, M. I., Fuqua, C., Eberhard, A. & Winans, S. C. (1998). Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. *J Bacteriol* **180**, 5398–5405.

Received 12 April 2001; revised 9 August 2001; accepted 13 August 2001.

PUBLIKATIONSLISTE

(in Klammern angegeben ist der von Anette Steidle geleistete Beitrag zu der jeweiligen Publikation)

1. Steidle, A., K. Sigl, R. Schuhegger, A. Ihring, M. Schmid, S. Gantner, M. Stoffels, K. Riedel, M. Givskov, A. Hartmann, C. Langebartels, and L. Eberl. 2001. Visualization of *N*-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. Appl. Environ. Microbiol. **67**:5761-5770.
(Durchführung der experimentellen Arbeit zusammen mit K. Sigl sowie das Verfassen des Manuskripts zusammen mit L. E.)

2. Steidle, A., M. Allesen-Holm, K. Riedel, G. Berg, M. Givskov, S. Molin, and L. Eberl. 2002. Identification and characterization of a *N*-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* IsoF. *Eingereicht.*
(der überwiegende Anteil an der experimentellen Arbeit sowie das Verfassen des Manuskripts zusammen mit L. E.)

3. Berg, G., N. Roskot, A. Steidle, L. Eberl, A. Zock, and K. Smalla. 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Appl. Environ. Microbiol. **68:**3328-3338.
(Untersuchung der Isolate auf AHL-Produktion)

4. Riedel, K., M. Hentzer, O. Geisenberger, B. Huber, A. Steidle, H. Wu, N. Høiby, M. Givskov, S. Molin, and L. Eberl. 2001. *N*-Acylhomoserine-lactone-mediated intergeneric communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology **147:**3249-3262.
(Konstruktion des AHL-Sensorplasmids pAS-C8)

5. Gotschlich, A., B. Huber, O. Geisenberger, A. Tögl, A. Steidle, K. Riedel, P. Hill, B. Tümmler, P. Vandamme, B. Middleton, M. Camara, P. Williams, A. Hardman, and L. Eberl. 2001. Synthesis of multiple *N*-acyl-homoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex. System. Appl. Microbiol. **24:**1-14.
(Klonierung der AHL-Synthase aus *B. cepacia* H193)

Die vorliegende Arbeit wurde am Lehrstuhl für Mikrobiologie der Technischen Universität München unter Leitung von Herrn Prof. Dr. K.-H. Schleifer im Zeitraum von Juli 1999 bis September 2002 angefertigt.

Mein herzlicher Dank gilt an dieser Stelle:

Herrn Prof. Dr. K.-H. Schleifer für die Möglichkeit, meine Arbeit an seinem Lehrstuhl durchzuführen und seine vielseitige Unterstützung.

Herrn PD Dr. Leo Eberl für seine sehr engagierte Betreuung, für viele gute Ideen und Anregungen und für sein Interesse an meiner Arbeit.

Dr. Kathrin Riedel für ihre außerordentliche Hilfsbereitschaft bei allen fachlichen Problemen und für ihren unermüdlichen Einsatz auf dem Gebiet „Aufbau und Pflege zwischenmenschlicher Beziehungen bei gutem Essen und Wein“.

Frau Beate Schumacher für ihre freundliche und ruhige Art und die Übernahme von Katja’s pflegeintensiver Stammsammlung.

Frau Sibylle Schadhauser und Jutta Elgner für die vielen Sequenzreaktionen, für die sie immer noch einen Platz auf einem Gel gefunden haben.

Frau Anna Leeb für ihre Unterstützung bei der Bewältigung bürokratischer Hürden, wie Reisekostenabrechnungen und Dienstreiseanträge.

Den „Eberl-Mädels“ Birgit Huber, Astrid Gotschlich, Manuela Köthe und Catalina Arevalo Ferro für die schöne Zeit in einem ganz selten hühnermäßigen Hühnerhaufen und die zahlreichen privaten Unternehmungen.

Allen Diplomandinnen und Diplomanden des Eberl-Labors für die erfrischende Abwechslung im Laboralltag. Vor allem meiner Ex-Diplomandin Katja Sigl für die erfolgreiche Zusammenarbeit im Kampf mit den „roten“ und „grünen“ Bakterien.

Den „Isos“ für ihre freundschaftliche Art, die abwechslungsreichen Kaffeepausen und die Mitwirkung bei einigen unvergesslichen Weihnachtsspielen.

Den „Wagners“ für die gute Zusammenarbeit und die Unterstützung am CLSM, LI-COR und bei so manchem Computerproblem.

Meinen Kollegen am IBÖ und BIOP der GSF, für ihre motivierte Mitarbeit am Tomatenprojekt.

Allen Kollaborationspartnern, die mit mir zusammen die Rätsel des Quorum sensing zu entschlüsseln suchten.

Meinen Freunden und Bekannten, die mich stets aufgemuntert und motiviert haben, für ihr Verständnis und ihre Geduld, wenn ich mal wieder keine Zeit hatte.

Und nicht zuletzt meinen Eltern, die mich in jeder Hinsicht unterstützt und mir meinen Weg geebnet haben.