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Identifizierung von Genen in *Burkholderia cepacia*, die für die Formation von Biofilmen auf abiotischen Oberflächen von Bedeutung sind

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Abkürzungsverzeichnis

AHL	*N*-Acyl-L-homoserinlacton
Bcc.	*Burkholderia cepacia*-Komplex
bp	Basenpaare
C6-HSL	Hexanoyl-homoserinlacton
C8-HSL	Octanoyl-homoserinlacton
c-di-GMP	bis-(2´,5´)-zyklische Diguaninsäure
CF	zystische Fibrose (Syn. Mukoviszidose)
CFTR	cystic fibrosis transmembrane conductance regulator
CLSM	konfokales Laser-Scanning-Mikroskop
EPS	extrazelluläre polymere Substanzen
GFP	grün fluoreszierendes Protein
MCS	'multiple cloning site'
QS	Quorum sensing
SDS	Natriumdodecylsulfat
U	Uridin
3-Oxo-C6-HSL	*N*-(3-Oxohexanoyl)-homoserinlacton
3-Oxo-C8-HSL	*N*-(3-Oxooctanoyl)-homoserinlacton
3-Oxo-C12-HSL	*N*-(3-Oxododecanoyl)-homoserinlacton
Ψ	Pseudouridin

A. EINLEITUNG

A.1 *Burkholderia cepacia* – Freund oder Feind?

Burkholderia cepacia, ein in der Umwelt weit verbreitetes Gram-negatives Bakterium, wurde ursprünglich als Verursacher von Zwiebelfäule isoliert (Burkholder, 1950). Inzwischen ist jedoch bekannt, dass die Bedeutung dieses Organismus über seine pflanzenpathogenen Eigenschaften weit hinaus geht. Aufgrund eines äußerst vielseitigen Stoffwechsels ist *B. cepacia* in der Lage, eine Vielzahl von Substanzen wie industrielle Abfälle oder Herbizide abzubauen. Dies macht einen Einsatz dieses Bakteriums zur Dekontaminierung verunreinigter Böden denkbar (Krumme *et al*., 1993; Bhat *et al*., 1994). Daneben ist *B. cepacia* auch ein potentieller Biokontrollorganismus. Es produziert eine Reihe von antimikrobiellen Substanzen wie Pyrrolnitrine, Cepalycine, Siderophore und Bacteriocin-ähnliche Stoffe, die zur Hemmung von pflanzenpathogenen Bakterien und Pilzen und zur Förderung des Pflanzenwachstums genutzt werden können (Aoki *et al*., 1993; Kang *et al*., 1998; LiPuma und Mahenthiralingam, 1999). Da jedoch in den letzten Jahrzehnten zunehmend deutlich wurde, dass *B. cepacia* auch opportunistisch humanpathogen ist, stehen dem Interesse von Landwirtschaft und Industrie an einer kommerziellen Nutzung von *B. cepacia* massive Vorbehalte von medizinischer Seite entgegen (Govan und Vandamme, 1998; Jones *et al*., 2001).

Obwohl *B. cepacia* mit Infektionen (meist Lungenentzündungen) bei Patienten mit verschiedenen Primärerkrankungen und gelegentlich auch bei gesunden Personen assoziiert ist, stellt dieses Bakterium in erster Linie ein Problem für Mukoviszidosepatienten dar (Koch und Høiby, 1993; Govan und Deretic, 1996; Jones *et al*., 2001). Mukoviszidose oder zystische Fibrose (CF) ist eine häufige Erbkrankheit, bei der Mutationen im 'cystic fibrosis transmembrane conductance regulator' (CFTR) zu einem Defekt bei der Chloridsekretion von Epithelien führen (Tümmler und Kiewitz, 1999). Die daraus resultierende Produktion eines dehydrierten und zähen Schleims hat multiple klinische Auswirkungen. So werden z.B. in den Atemwegen die Zilien in ihrer Reinigungsfunktion behindert, wodurch die Kolonisierung der Lungenepithelien durch Bakterien erleichtert wird. Dies sind anfangs v.a. *Hämophilus influenzae* und *Staphylococcus aureus,* später im Krankheitsverlauf erfolgt eine meist dauerhafte Kolonisierung mit *Pseudomonas aeruginosa*. Mit einer Infektionsrate, die regional zwischen 5 und 70 % schwankt, wird ein Teil der Patienten zusätzlich mit *B. cepacia* infiziert (Johansen *et al*., 1998; Hutchison und Govan, 1999). Der Krankheitsverlauf nach einer Co-Infektion mit *B. cepacia* ist variabel, und neben asymptomatischen Infektionen treten auch schwerwiegende Komplikationen auf. Wegen der hohen intrinsischen Antibiotikaresistenz

von *B. cepacia* sind diese Infektionen schwer zu kontrollieren. Eine Kolonisierung mit *B. cepacia* verringert die Lebenserwartung eines CF-Patienten von durchschnittlich 30 Jahren auf 15 bis 20 Jahre, dabei erliegen ca. 20 % der Patienten dem sogenannten '*cepacia*-Syndrom', einer innerhalb kurzer Zeit zum Tode führenden nekrotisierenden Lungenentzündung (Isles *et al*., 1984; Hutchison und Govan, 1999; Jones *et al*., 2001). Zu dieser erheblichen Verschlechterung der Prognose kommen auch soziale Einschränkungen, da aufgrund der hohen Übertragungsrate von Patient zu Patient eine strikte Trennung von *B. cepacia*-positiven und *B. cepacia*-negativen Patienten praktiziert wird.

Taxonomische Studien machten deutlich, dass verschiedene ursprünglich als '*B. cepacia*' klassifizierte Isolate äußerst heterogen waren. Vandamme *et al*. (1997) unterteilten die Art deshalb in fünf Genomovare (der Begriff 'Genomovar' wurde als Bezeichnung für phänotypisch ähnliche genomische Arten eingeführt), die zusammen als '*B. cepacia*-Komplex' bezeichnet wurden. Heute gehören dem *B. cepacia*-Komplex acht Genomovare an: *B. cepacia* Bcc. I, Bcc. III, Bcc. VI, sowie die inzwischen als eigenständige Arten anerkannten *B. multivorans*, *B. stabilis*, *B. vietnamiensis*, *B. ambifaria* und *B. pyrrocinia*, wobei der Typstamm dem Genomovar I (Bcc. I) zugeordnet wird (Coenye *et al*., 2001b). Viele besonders pathogene und leicht übertragbare Stämme sind Vertreter des *B. cepacia* Genomovar III, dem auch das von uns untersuchte Isolat H111 angehört. Pathogene Stämme können jedoch in jedem der Genomovare gefunden werden, und es ist derzeit nicht möglich, innerhalb des *B. cepacia*-Komplexes eine klare Unterscheidung zwischen pathogenen Keimen und harmlosen Umweltisolaten vorzunehmen (Vandamme *et al*., 1997; Govan und Vandamme, 1998; Mahenthiralingam *et al*., 2000; Balandreau *et al*., 2001; Coenye *et al*., 2001a).

A.2 Biofilme – Bedeutung und Entstehung

In ihrer natürlichen Umgebung leben die meisten Bakterien als sogenannte Biofilme – Oberflächen-assoziierte vielzellige Gemeinschaften mit geordneter Struktur, die in eine Matrix aus extrazellulären polymeren Substanzen (EPS) eingebettet sind (Stickler, 1999; Tolker-Nielsen und Molin, 2000; Watnick und Kolter, 2000). Man geht heute davon aus, dass die im Labor zumeist untersuchte planktonische Lebensweise in der Natur lediglich dazu dient, von einer Oberfläche zu einer anderen zu gelangen. Diese Erkenntnis ist von enormer Bedeutung, da sich die Physiologie Oberflächen-assoziierter Bakterien grundlegend von der planktonischer Zellen unterscheidet. Im medizinischen Bereich besonders wichtig ist die drastisch erhöhte Resistenz von Biofilmen gegen Antibiotika und die Immunabwehr. Dafür kommen verschiedene Ursachen in Frage, die vermutlich alle, wenn auch in

unterschiedlichem Ausmaß, zu diesem Phänomen beitragen (Hoyle *et al*., 1990; von Eiff *et al*., 1999; Xu *et al*., 2000; Stewart und Costerton, 2001; Mah und O´Toole, 2001; Lewis, 2001). (i) Das Antibiotikum kann nicht in tiefere Schichten des Biofilms eindringen. Obwohl die EPS-Matrix keine undurchdringliche Barriere für antimikrobielle Substanzen darstellt, kann sie die Diffusion mancher Stoffe verlangsamen. Der dadurch effektivere Abbau der entsprechenden Substanzen in den oberen Schichten des Biofilms kann so weiter innen liegende Zellen schützen. Vor allem aber stellt die EPS-Matrix eine bedeutende physikalische Barriere für die Komponenten des Immunsystems dar. (ii) In Biofilmen herrschen Konzentrationsgradienten, die verschiedene Mikrohabitate schaffen. In tieferen Schichten eines Biofilms können niedrige Nährstoff- und Sauerstoffkonzentrationen und die Anhäufung von Stoffwechselendprodukten das Wachstum von Bakterien verlangsamen oder zum Stillstand bringen. Im allgemeinen steigt die Antibiotikaresistenz beim Übergang von der exponentiellen Wachstumsphase in die stationäre Phase. Niedrigere pH-Werte in tieferen Schichten des Biofilms können die Aktivität einiger Antibiotika hemmen. Zudem können die Wachstumsbedingungen in Biofilmen eine allgemeine Stressantwort induzieren, welche durch die dadurch hervorgerufenen physiologischen Veränderungen einen weiteren Schutz vor antimikrobiellen Substanzen bietet. (iii) Biofilmbildung wird zunehmend als Entwicklungsprozess gesehen, in dessen Verlauf die Zellen eine Differenzierung durchlaufen (O´Toole *et al*., 2000). So wird heute vermutet, dass eine kleine Subpopulation von Mikroorganismen eines Biofilms einen Biofilm-spezifischen hochresistenten Phänotyp ausbildet, vergleichbar mit der Zelldifferenzierung, die zur Sporenbildung führt. Diese Hypothese wird durch Experimente gestützt, in denen erhöhte Resistenz in neu gebildeten Biofilmen nachgewiesen wurde, die noch zu dünn sind, um die Diffusion von antimikrobiellen Substanzen oder Stoffwechselprodukten zu behindern. Die Bedeutung hochresistenter Zellen, die als ‘Persister’ bezeichnet wurden, wird auch durch eine kürzlich veröffentlichte Studie unterstrichen (Spoering und Lewis, 2001). Darin wird gezeigt, dass die Resistenz von *P. aeruginosa*-Biofilmen gegen bestimmte Antibiotika nicht höher ist als die von stationären Flüssigkulturen, was darauf schließen lässt, dass hauptsächlich eine hohe Zelldichte und die dadurch steigende Zahl von ‘Persistern’ für die erhöhte Antibiotikatoleranz verantwortlich ist.

Schätzungen gehen davon aus, dass etwa 65 % der Infektionen in der entwickelten Welt im Zusammenhang mit der Ausbildung von Biofilmen stehen. Darunter sind, neben CF-Lungenentzündungen, u.a. Karies und Paradontose, Mittelohrentzündung, Gallengangsinfektionen, Endokarditis und Osteomyelitis. Dazu kommen nosokomiale Infektionen wie Bakteriämie oder Harnwegsinfektionen, die meist von Biofilmen auf medizinischen Vorrichtungen wie Kathetern oder künstlichen Herzklappen verursacht werden (Costerton *et*

al., 1999; Costerton, 2001). Dies unterstreicht die Bedeutung, die einer Kontrolle der Biofilmbildung für die wirksame Bekämpfung chronischer Krankheiten zukommt.

P. aeruginosa bildet bei der chronischen Kolonisierung der Lungen von CF-Patienten dicke Biofilme, die in Alginat, ein Polysaccharid, das von *P. aeruginosa* während der Infektion in großen Mengen produziert wird, eingebettet sind (Lam *et al*., 1980; Costerton, 2001). Auch *B. cepacia* ist in der Lage, spezifisch an Schleim und Epithelzellen zu binden (Sajjan *et al*., 1992; Sajjan und Forstner, 1993). *B. cepacia* infiziert meist Patienten, die bereits mit *P. aeruginosa* kolonisiert sind. Es wird angenommen, dass die Modifizierung von Epithelzellen durch *P. aeruginosa*-Exoprodukte zur Exposition von Rezeptoren führt, was die Anheftung von *B. cepacia* erleichtert (Saiman *et al*., 1990; Sajjan *et al*., 2000). Diese Beobachtungen sind, zusätzlich zur chronischen Natur von Lungenentzündungen bei zystischer Fibrose, ein Hinweis darauf, dass *P. aeruginosa* und *B. cepacia* als gemischte Biofilme in der CF-Lunge wachsen.

Während der letzten Jahre wurden an einer Reihe von Bakterien Faktoren untersucht, die zur Entwicklung von Biofilmen beitragen. Dabei wurde deutlich, dass von verschiedenen Bakterienarten unterschiedliche Mechanismen zur Biofilmbildung genutzt werden, die zudem in Abhängigkeit von den gegebenen Umweltbedingungen variieren können (Pratt und Kolter, 1998; O´Toole und Kolter, 1998a; O´Toole und Kolter, 1998b; Watnick und Kolter, 1999; Danese *et al*., 2000). Auf der Basis dieser Untersuchungen wurde ein allgemeines Modell für die Biofilmbildung Gram-negativer Bakterien entwickelt. Danach ist für die anfängliche Anheftung an ein Substrat Beweglichkeit mittels Flagellen oder Typ I-Pili erforderlich, um die abstoßenden Kräfte, die von der Oberfläche ausgehen, zu überwinden. Sobald der Kontakt hergestellt ist, werden Typ IV-Pili oder Flagellen für ein Entlanggleiten an der Oberfläche genutzt, um Mikrokolonien zu bilden. Pili, Adhäsine und in der Membran verankerte Oberflächenproteine spielen auch eine entscheidende Rolle bei der spezifischen und unspezifischen Bindung der Zellen an verschiedene Substrate. Exopolysaccharide tragen zur Stabilisierung der dreidimensionalen Struktur von Biofilmen bei (Pratt und Kolter, 1999; O´Toole *et al*., 2000; Watnick und Kolter, 2000). Interessanterweise wurde festgestellt, dass bei *P. aeruginosa* die Reifung von Biofilmen durch Kommunikation zwischen Zellen, das sogenannte ‘Quorum sensing’ (QS), gesteuert wird. Dies unterstützt die Auffassung, dass Biofilme als multizelluläre Verbände anzusehen sind (Davies *et al*., 1998).

A.3 'Quorum sensing' – eine primitive Form der Vielzelligkeit

Neben der oben beschriebenen Existenz von Bakterien in Biofilmen hat vor allem die Entdeckung, dass Bakterien in der Lage sind, miteinander zu kommunizieren, zu einer Abkehr von der lange vorherrschenden Wahrnehmung bakterieller Populationen als Ansammlungen isolierter einzelzelliger Organismen geführt.

Prokaryoten interagieren miteinander mittels kleiner diffusionsfähiger Moleküle, die zur Koordination der Expression bestimmter Gene genutzt werden. Diese Kontrolle in Abhängigkeit von der Populationsdichte wird als 'Quorum sensing' (QS) bezeichnet, wobei das 'Quorum' als die kleinste Einheit definiert ist, die zu koordinierten Aktionen in der Lage ist (Fuqua *et al*., 1994).

Am weitesten verbreitet bei Gram-negativen Bakterien sind Kommunikationssysteme, die *N*-Acyl-L-homoserinlactone (AHL) als Botenstoffe einsetzen. Das älteste bekannte Beispiel für einen 'Quorum sensing'-regulierten Vorgang ist die Biolumineszenz bei *Photobacterium fischeri* (Nealson *et al.*, 1970). Dieses marine Bakterium kommt nicht nur in geringer Konzentration im Meerwasser vor, sondern auch in den Lichtorganen verschiedener Tiefseefische und Quallen, die es mit Zelldichten von bis zu 10^{10} Zellen/ml kolonisiert. Das QS-System von *P. fischeri* besteht aus einer AHL-Synthase, welche vom *luxI* Gen kodiert wird, und aus einem von *luxR* kodierten Regulator. LuxI, das die Bildung von *N*-(3-Oxohexanoyl)-homoserinlacton (3-Oxo-C6-HSL) katalysiert, wird konstitutiv in geringen Mengen exprimiert. Mit steigender Zelldichte akkumuliert das frei diffundierbare 3-Oxo-C6-HSL im Medium, bis eine kritische Grenzkonzentration erreicht wird, bei der es an LuxR bindet. Der LuxR/3-Oxo-C6-HSL Komplex wiederum bindet an ein 20 bp Palindrom in der *luxI* Promotorregion, die sogenannte *lux*-Box, und aktiviert die Transkription des *luxICDABE* Operons, das alle für die Biolumineszenz benötigten Gene enthält. Da auch *luxI* Teil dieses Operons ist, führt dies zu einer positiven Rückkopplung und einer sprunghaften Zunahme der Lichtemission (Engebrecht und Silverman, 1984; Kaplan und Greenberg, 1985). Durch diesen Mechanismus wird sicher gestellt, dass der bioluminezente Phänotyp nur in den Lichtorganen ausgebildet wird, wo er Teil einer Symbiose zwischen Tiefseefisch und Bakterium ist, nicht aber im Meerwasser, wo die Lichtemission sinnlos und energetisch unvorteilhaft wäre.

Inzwischen ist bekannt, dass 'Quorum sensing' mittels AHL-Molekülen kein auf wenige Organismen beschränktes kurioses Phänomen ist, sondern dass viele Gram-negative Bakterien Variationen dieses Systems nutzen, um verschiedenste Prozesse zu regulieren (s. auch aktuelle Übersichtsartikel von Fuqua *et al*., 2001 und Whitehead *et al*., 2001). Dazu zählen konjugativer Plasmidtransfer (Piper *et al*., 1993; Zhang *et al*., 1993) und verschiedene Formen

bakterieller Motilität (Eberl *et al*., 1996; Glessner *et al*., 1999) ebenso wie die Produktion von Antibiotika und Virulenzfaktoren (McGowan *et al*., 1995; Pearson *et al*., 1997) oder die Biofilmbildung (Davies *et al*., 1998).

Die durch QS regulierten Gene sind meist an symbiontischen oder pathogenen Interaktionen mit eukaryotischen Wirten beteiligt (Parsek und Greenberg, 2000). So dient die Regulation von Genen durch 'Quorum sensing' bei pathogenen Organismen im allgemeinen dazu, dass die Keime bis zum Erreichen einer kritischen Populationsdichte vor dem Immunsystem verborgen bleiben. Sobald das 'Quorum' erreicht ist, können die Bakterien durch die koordinierte Expression von Virulenzfaktoren die Immunabwehr überwältigen und eine Infektion etablieren.

Auch *B. cepacia* reguliert die Expression potentieller Virulenzfaktoren wie Protease, Lipase und des Siderophors Ornibactin durch ein 'Quorum sensing'-System, welches aus der AHL-Synthase CepI und dem Regulator CepR besteht (Lewenza *et al*., 1999; Lewenza und Sokol, 2001). Ähnlich wie beim *lux*-System ist auch die Expression von *cepI* durch CepR reguliert (Lewenza und Sokol, 2001). Das von *B. cepacia* produzierte Signalmolekül wurde als Octanoyl-homoserinlacton (C8-HSL) identifiziert (Lewenza *et al*., 1999).

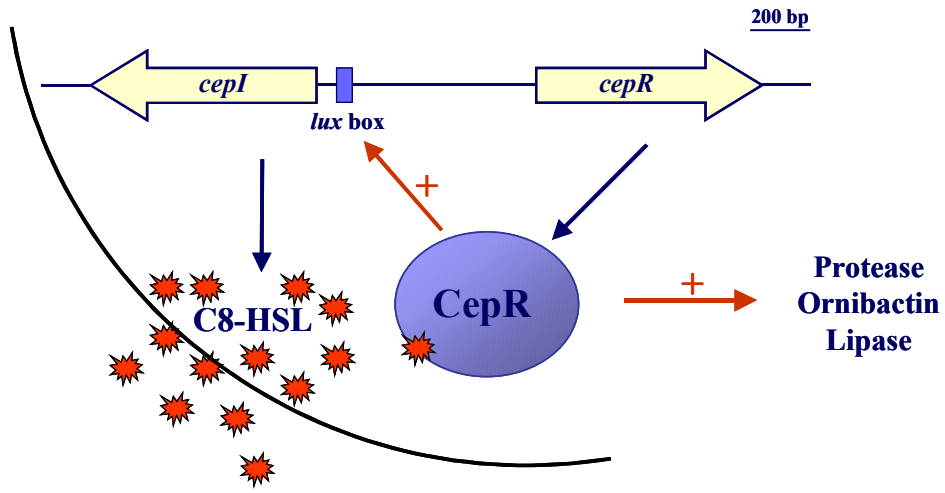

Abbildung 1. Schematische Darstellung des QS-Regulons von *B. cepacia*. Die AHL-Synthase CepI ist für die Produktion des Signalmoleküls C8-HSL, welches frei durch die bakterielle Membran diffundieren kann, verantwortlich. Erreicht die C8-HSL-Konzentration einen kritischen Schwellenwert, so bindet dieses Signalmolekül an den transkriptionellen Regulator CepR, der nun die Expression von Zielgenen aktiviert. Neben der Produktion extrazellulärer Virulenzfaktoren kontrolliert der CepR/C8-HSL Komplex auch die Expression von *cepI*.

A.4 Ziele der Arbeit

Ziel dieser Arbeit war eine Analyse der Faktoren, die zur Biofilmbildung von *B. cepacia* H111 auf abiotischen Oberflächen beitragen.

Zunächst mussten geeignete Methoden und Vektoren zur genetischen Manipulation von *B. cepacia* gefunden werden, die zum einen die Erzeugung von Mutanten und zum anderen das Einbringen von Genen in den Organismus ermöglichten.

Um an der Biofilmbildung beteiligte Gene zu finden, wurde eine Bibliothek von 5000 durch zufällige Transposoninsertionen entstandenen Mutanten erstellt. Diese Bibliothek sollte auf Mutanten durchsucht werden, die Defekte in der Biofilmbildung aufwiesen. Durch die Sequenzierung der das Transposon flankierenden DNA-Regionen dieser Mutanten und deren Abgleich mit Sequenzen in Datenbanken sollten die jeweils mutierten Gene identifiziert werden. Um die Rolle der Zell-Zell-Kommunikation bei der Biofilmbildung aufzuklären, sollten außerdem gerichtet konstruierte *cepI*- und *cepR*-Mutanten untersucht werden.

Zusätzlich sollte die Entwicklung von Biofilmen ausgesuchter Mutanten über mehrere Tage hinweg genauer untersucht werden. Dazu sollten künstliche Durchflusskammern eingesetzt werden, die in Kombination mit konfokaler Laser-Scanning-Mikroskopie eine direkte Beobachtung der Entstehung der dreidimensionalen Struktur von Biofilmen ermöglichen.

Auch die Bildung gemischter Biofilme von *P. aeruginosa* und *B. cepacia* in Durchfluss-kammern sollte untersucht werden. Von besonderem Interesse war dabei die Möglichkeit artübergreifender Kommunikation in diesen gemischten Zellverbänden.

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
E. coli		
XL1-Blue	*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*	T. Hansen, Novo Nordisk A/S
CC118 (λpir)	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA thi-1 rpsE rpoB argE*(Am) *recA* λpir lysogen	Herrero *et al*., 1990
HB101	*recA thi pro leu hsdR*$^-$*M*$^+$ Sm^r	Kessler *et al*., 1992
B. cepacia		
H111	klinisches Isolat, Genomovar III	Römling *et al*., 1994; Gotschlich *et al*., 2001
H111-I	Km^r; *cepI* ::Km Mutante von H111	Gotschlich, 2001
H111-R	Km^r; *cepR*::Km Mutante von H111	diese Arbeit
P. aeruginosa		
PAO1	Wildtyp	Holloway, 1955
PAO1-JP2	*lasI rhlI* Mutante von PAO1, Hg^r,Tc^r	Pearson *et al*., 1997
SH1	klinisches Isolat	Römling *et al.*, 1994
SH38	klinisches Isolat	Römling *et al.*, 1994

Tabelle B-2. Verwendete Plasmide

Plasmid	Relevante Eigenschaften	Herkunft/Referenz
pAG	Gm^r, Km^r; pEX18Gm Derivat zur Inaktivierung von *cepI*	Gotschlich, 2001
pAS-C8	Gm^r ; pBBR1MCS-5 mit P_{cepI}-*gfp*(ASV) P_{lac}-*cepR*	Riedel *et al*., 2001
pBAH8	pBBR1MCS-5 mit $P_{A1/04/03}$-*gfp mut3*-T_o-T_1	diese Arbeit
pBAH27	pBBR1MCS-5 mit dem *cepR* Gen von *B. cepacia* H111	diese Arbeit
pBAH33	Gm^r, Km^r; pEX19Gm Derivat zur Inaktivierung von *cepR*	diese Arbeit
pBBR1MCS-5	Gm^r; Klonierungsvektor	Kovach *et al*., 1995
pCR 2.1-TOPO	Ap^r, Km^r; Klonierungsvektor	Invitrogen, Carlsbad, USA
pEX18Gm	Gm^r; *oriT*$^+$ *sacB*$^+$; 'gene replacement vector' mit pUC18 MCS	Hoang *et al*., 1998
pEX19Gm	Gm^r; *oriT*$^+$ *sacB*$^+$; 'gene replacement vector' mit pUC19 MCS	Hoang *et al*., 1998
pGEM-3Zf(+)	Ap^r; Klonierungsvektor	Promega, Madison, USA
pKR-C12	Gm^r ; pBBR1MCS-5 mit P_{lasB}-*gfp*(ASV) P_{lac}-*lasR*	Riedel *et al*., 2001
pMH94	Ap^r, Km^r; Vektor für mini-Tn*5* Tel^r *kilAtelAB*-$P_{A1/04/03}$-*gfp mut3*-T_o-T_1	Hentzer, M. und Parsek, M.R., unveröffentlicht
pRK600	Cm^r; ColE1*oriV*; RK-2Mob$^+$ RK2-Tra$^+$; Helferplasmid für triparentale Matings	de Lorenzo und Timmis, 1994
pSB403	Tc^r; *luxRI´*::*luxCDABE* transkriptionelle Fusion; biolumineszentes AHL Sensorplasmid	Winson *et al*., 1998b
pUTmini-Tn*5* Km2-*luxCDABE*	Km^r; Vektor für mini-Tn*5* Km2-*luxCDABE*	Winson *et al*., 1998a

B.2 Nähr- und Indikatormedien

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. Gegebenenfalls wurde den Medien 16 g/l Agar zur Verfestigung zugesetzt.

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

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

Caseinhydrolysat	10 g
Hefeextrakt	5 g
NaCl	4 g
$H_2O_{dest.}$ ad 1 l	

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	
	$H_2O_{dest.}$ ad 1 l		
B:	1 M $MgCl_2$ x 6 H_2O	2 ml	(Stock: 20,33 g/100 ml)
	0,5 M $CaCl_2$ x 2 H_2O	0,2 ml	(Stock: 7,351 g/100 ml)
	0,01 M $FeCl_3$ x 6 H_2O	0,3 ml	(Stock: 0,2703 g/100 ml)
	$H_2O_{dest.}$ ad 900 ml		
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* Isolation Agar (PIA)** (Becton Dickinson, Sparks, USA)

Trypton	6,7 g
Hefeextrakt	3,3 g
NaCl	1,7 g
Agar	10 g
PIA	12,5 g
$H_2O_{dest.}$ ad 1 l	

Chromazurol S (CAS)-Agar (Schwyn und Neilands, 1987, modifiziert)

10 x LB-Medium	Caseinhydrolysat	10 g
	Hefeextrakt	5 g
	NaCl	5 g
	$H_2O_{dest.}$ ad 100 ml	
10 x MM 9	NH_4Cl	1 g
	KH_2PO_4	0,3 g
	NaCl	0,5 g
	H_20_{dest} ad 100 ml	
CAS-Stammlösung	Chomazurol S	60,5 mg
	$H_2O_{dest.}$ ad 50 ml	
	1 mM $FeCl_3$ x 6 H_2O	10 ml
	Hexadecyltrimethylammonium-bromid (HDTMA)	72,9 mg
	$H_2O_{dest.}$ ad 40 ml	

Herstellung der CAS-Stammlösung: Chromazurol S in $H_2O_{dest.}$ lösen, Eisenchloridlösung zugeben und mischen, in HDTMA-Lösung einrühren und autoklavieren. 10x LB und 10x MM9 ebenfalls getrennt autoklavieren.

PIPES-Agar	10x MM9	100 ml
	H_20_{dest}	500 ml
	Piperazindiethansulfonsäure (PIPES)	31,1g
	pH 6,8 mit 4 N NaOH	
	$H_2O_{dest.}$ ad 850 ml	
	Agar	16 g

Herstellung des PIPES-Agars: 10x MM9 mit $H_2O_{dest.}$ mischen, unter ständigem Rühren PIPES zugeben und pH mit NaOH auf 6,8 einstellen. Auf 850 ml auffüllen. Nach Zugabe von 16 g Agar autoklavieren.

Nach Abkühlung des PIPES-Agar auf 50 °C unter sterilen Bedingungen 30 ml 10x LB-Medium, 10 ml 20 % Glukose (sterilfiltriert), 2 ml 1 M $MgSO_4$-Lösung, 2 ml 1 M Na_2SO_4-

Lösung, 1 ml 0,1 M $CaCl_2$-Lösung, und 100 ml CAS-Stammlösung zugeben und sofort Platten gießen.

Chitinase-Agar (Connell *et al*., 1998, modifiziert)

Zugabe von wässrigen Lösungen von Ethylenglycolchitin (Endkonzentration 0,0375 %) und Trypanblau (0,01 %) (beide Zusätze von Sigma, Deisenhofen) zu autoklaviertem LB-Medium.

Magermilch-Agar

LB-Agar mit 2 % Magermilch

B.3 Konjugativer Plasmidtransfer

Plasmide wurden mittels eines triparentalen Mating-Ansatzes (de Lorenzo und Timmis, 1994) in *B. cepacia*-Zellen eingebracht. Dabei wurden der Donor- und der Rezipientenstamm sowie der Helferstamm *E. coli* HB101 (pRK600) über Nacht in 5 ml LB mit den nötigen 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 vermischt und für 30 min bei Raumtemperatur inkubiert. Danach wurden die Rezipientenzellen zugegeben. Die Mischung wurde auf die Oberfläche vorgewärmter Agarplatten aufgetropft. Nach Inkubation über Nacht bei 37 °C wurden die Zellen mit Hilfe einer sterilen Einwegimpföse von den Platten abgenommen und in 0,9 % NaCl resuspendiert. Verdünnungen wurden auf LB- bzw. PIA-Agarplatten, denen Antibiotika zur Selektion gegen Donor-, Helfer- und untransformierte Rezipientenzellen zugegeben waren, ausplattiert.

B.4 Molekulargenetische Methoden

Klonierungen, Restriktionsansätze 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) verwendet. Folgende Kits von Qiagen (Hilden) wurden entsprechend den Angaben des Herstellers eingesetzt: der QIAprep Spin Miniprep Kit zur Isolierung von Plasmid-DNA, der DNeasy Tissue Kit zur Gewinnung von chromosomaler DNA und der QIAquick Gel Extraction Kit zur Aufreinigung von DNA-Fragmenten aus Agarose-Gelen.

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

Primer	Sequenz (5´ → 3´)
PCR-Primer	
cepR-R	GGGGTACCAACCTGACAAGTATGACAGCG
cepR-OV	GGGGTACCGGATGAGCATGGAGAAAAGC
intercep-f-Eco	GGAATTCGAGATCCGCCGCGAGTTCG
intercep-r-Sac	GATCCGCTGGAAGAGCTCC
cepR-f-Sph	ACATGCATGCGCTCGGATTCGAATACTGC
cepR-r-Hind	CCCAAGCTTAGAAGCTCGAGCAGATCGC
igR-f-Eco	GGAATTCCCAGTATTCGAATCCGAGCCGC
igR-r-Sac	CGAGCTCGGGATGTCCTCGGATCTGTGC
cepI-f-Bam	CGGGATCCCGCCTTCGTTCACGAGGAAGGG
cepI-r-Hind	CCCAAGCTTGGGCGCGCGTTCCGGCTCAGG
ARB6	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
luxCext2	AGTCATTCAATATTGGCAGG
ARB2	GGCCACGCGTCGACTAGTAC
luxCint2	GGATTGCACTAAATCATCAC
Sequenzierprimer	
i-end	CAGATCTGATCAAGAGACAG
o-end	CACTTGTGTATAAGAGTCAG

B.4.1 Konstruktion von Plasmiden

Um Gene in *B. cepacia* einzubringen, wurde das Plasmid pBBR1MCS-5 (Kovach *et al*., 1995) genutzt, das anders als viele andere gebräuchliche Vektoren in der Lage ist, in *B. cepacia* zu replizieren.

Komplementierung der *cepR*-Mutante H111-R: zur Komplementierung von H111-R wurde das Plasmid pBAH27 (*cepR*$^+$) konstruiert (s. Anhang 1). Dazu wurde das *cepR*-Gen von *B. cepacia* H111 mit den Primern cepR-R und cepR-OV amplifiziert. Das Produkt wurde mit *Kpn*I verdaut, und mit dem Vektor pBBR1MCS-5 ligiert.

Markierung der Zellen mit GFP: zur Konstruktion von pBAH8 (Anhang 2) wurde die $P_{A1/04/03}$-RBSII-*gfp mut3*-T_o-T_1 Kassette von pJBA28 (Andersen *et al*., 1998) als 2 kb Fragment in den Vektor pBBR1MCS-5 ligiert.

B.4.2 Identifizierung der Transposon-Insertionsstellen

Um die DNA-Regionen, welche die Insertionsstellen des mini-Tn*5* Transposons in den *B. cepacia*-Mutanten flankierten, zu bestimmen, wurden zwei Methoden eingesetzt: Klonieren oder Amplifikation mittels Arbitrary PCR. Um die an das I-Ende des mini-Tn*5* Transposons anschließende DNA-Region zu identifizieren, wurde die chromosomale DNA mit *Sph*I verdaut und mit dem Vektor pGEM 3Zf(+) ligiert. Nach Transformation von *E. coli* XL1-Blue wurden Kanamycin-resistente Klone isoliert. Bei diesen hatte die Kanamycin-Resistenz des Transposons zusammen mit der flankierenden DNA-Region in den Vektor inseriert.

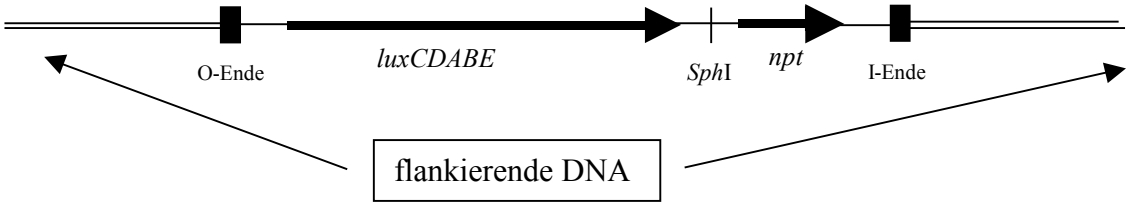

Abbildung 2. schematische Darstellung des Transposons mini-Tn*5*Km2-*luxCDABE (npt*: Neomycin Phosphotransferase, vermittelt Kanamycinresistenz)

Um die DNA am O-Ende des Transposons zu amplifizieren, wurde eine leicht abgeänderte Version eines Arbitrary PCR Protokolls verwendet (O´Toole und Kolter, 1998a; Pratt und Kolter, 1998).

Einer der beiden in der ersten Amplifikationsrunde eingesetzten Primer ist spezifisch für das Transposon (in diesem Fall *luxC*: luxCext2), während der andere unspezifisch im Genom bindet (ARB 6). In dieser ersten Runde entstand ein Gemisch von Produkten unterschiedlicher Länge. In einer zweiten PCR-Runde wurden diese Produkte mit spezifischen Primern weiter amplifiziert. Dies waren luxCint2 (bindet auch in *luxC*, aber nahe dem 3´-Ende, d.h. näher am Übergang Transposon-Chromosom) und ARB2 (entspricht dem 5´-Ende von ARB6). Die Produkte der zweiten Runde wurden aufgereinigt und entsprechend den Herstellerangaben in den Vektor pCR2.1 TOPO ligiert. Die Reaktionsbedingungen für die Arbitrary PCR sind in Anhang 2 ausführlich dargestellt.

B.4.3 DNA-Sequenzanalyse

Die Sequenzierung von DNA nach der Dideoxynukleotid Kettenabbruchmethode (Sanger *et al*., 1977) erfolgte mit Hilfe eines LI-COR 4200 DNA Sequenzanalysators (LI-COR, Lincoln, USA). Zur Sequenzierung der das I-Ende bzw. das O-Ende des mini-Tn*5* Transposons flankierenden DNA-Regionen wurden die Primer i-end und o-end eingesetzt. DNA

Sequenzen wurden mittels der Suchmaschine BLAST des 'National Center for Biotechnology Information' (http://www.ncbi.nlm.nih.gov/) mit anderen Sequenzen in GenBank verglichen.

B.4.4 Knock-out Mutagenese mit Hilfe von homologer Rekombination

Um definierte *cepI*- und *cepR*-Mutanten zu erzeugen, wurde eine von Schweizer und Hoang (1995) beschriebene Methode eingesetzt. Mit dieser Methode kann ein bestimmtes Gen mittels zweier aufeinanderfolgender Rekombinationsereignisse durch eine inaktivierte Kopie ersetzt werden. Die Konstruktion der auf pEX-Vektoren (Hoang *et al*., 1998) basierenden Suizid-Vektoren pAG und pBAH33, die zum Einbringen der inaktivierten *cepI*- bzw. *cepR*-Genkopien in die Zellen genutzt wurden, ist in Anhang 1 ausführlich beschrieben. Zur Konstruktion der *cepR*-Mutante wurden zwei DNA-Fragmente amplifiziert: eines enthielt die intergenerische Region und die ersten 75 bp von *cepR* (Primer: intercep-f-Eco und intercep-r-Sac), das andere enthielt 760 bp der 3´-Region von *cepR* (Primer: cepR-f-Sph und cepR-r-Hind). Mit Hilfe der durch die Primer eingeführten Restriktionsschnittstellen wurden beide PCR-Produkte nacheinander mit dem Vektor pEX19Gm ligiert. Am Ende wurde das *npt*-Gen aus dem Transposon Tn903 (Oka *et al*., 1981), dessen Produkt Resistenz gegen Kanamycin vermittelt, in den Vektor kloniert. Das resultierende Konstrukt, pBAH33, wurde mittels des oben beschriebenen triparentalen Matingansatzes in *B. cepacia* H111 eingebracht. Da das Gen für Gentamicinresistenz auf dem pEX19Gm Vektor im Falle eines doppelten Cross Over Ereignisses verloren geht, wurde auf Klone gescreent, die resistent gegen Kanamycin, aber sensitiv gegen Gentamicin waren. Eine Mutante, H111-R, wurde ausgewählt und ihre korrekte genetische Struktur durch Southern Blot Analyse bestätigt.
Die Konstruktion von H111-I erfolgte analog: die Primer igR-f-Eco und igR-r-Sac dienten zur Amplifikation der intergenerischen Region, während die 5´-Region von *cepI* mit Hilfe des Primerpaars cepI-f-Bam und cepI-r-Hind amplifiziert wurde.

B.5 Identifizierung Biofilm-negativer Mutanten

B.5.1 Konstruktion der *B. cepacia* H111 Mutantenbank

Die *B. cepacia* H111 Mutantenbank entstand durch zufällige Insertion des Transposons mini-Tn*5*Km2-*luxCDABE* mit Hilfe des oben beschriebenen triparentalen Matings. Transkonjuganten wurden auf LB-Agar mit Kanamycin und Tetracyclin selektiert. Diese zufälligen Insertionsmutanten wurden gepickt und in 150 µl LB in den Kavitäten von Polypropylen Mikrotiterplatten (Nunc, Roskilde, Dk) inkubiert. Zur Lagerung wurde 75 µl 50 % (v/v) Glycerin zugegeben und die Mikrotiterplatten bei –80 °C eingefroren.

B.5.2 Screening nach Mutanten mit Defekten in der Biofilmbildung

Die Biofilmbildung in Polystyrol-Mikrotiterplatten wurde nach einer von O´Toole und Kolter (1998a) und Pratt und Kolter (1998) beschriebenen Methode getestet. Die Zellen wurden in den Kavitäten der Mikrotiterplatten in 100 µl ABC Medium bei 30 °C inkubiert. Nach 48 h wurde das Medium abgesaugt und 100 µl einer 1 %igen wässrigen Kristallviolettlösung zugegeben. Nach 20 min Färben bei Raumtemperatur wurde das Kristallviolett entfernt und die Kavitäten sorgfältig gewaschen, so dass nur die Zellen, die an den Wänden der Kavitäten hafteten, gefärbt blieben. Um diese zu quantifizieren, wurde das Kristallviolett in 100 µl DMSO gelöst und die Absorption bei 570 nm gegen DMSO gemessen.

B.6 Nachweis von AHL-Molekülen

B.6.1 Bakterielle Reportersysteme

Zum Nachweis von AHL-Molekülen dienten verschiedene bakterielle Reportersysteme. Das Prinzip hierbei ist, dass die Anwesenheit von AHL-Molekülen durch die Induktion der Expression von Reportergenen, in diesem Fall den Bioluminenszenzgenen *luxCDABE* bzw. des Gens für das grün fluoreszierende Protein, *gfp*, angezeigt wird.

pSB403: Das biolumineszenten Sensorplasmid pSB403 enthält das *luxR* Gen von *Photobacterium fischeri* zusammen mit einer transkriptionellen Fusion der *luxI* Promotorregion mit den Biolumineszenzgenen *luxCDABE* von *Photorhabdus luminescens*. Da das *P. fischeri* QS-System 3-oxo-C6-HSL nutzt, zeigt das Sensorplasmid die höchste Sensitivität für dieses AHL-Molekül. Es können jedoch auch andere AHL-Moleküle detektiert werden, wenn auch mit leicht verringerter Sensitivität (Winson *et al*., 1998b; Geisenberger *et al*., 2000).

pAS-C8: Das Sensorplasmid pAS-C8 basiert auf Komponenten des *B. cepacia* H111 *cep*-Systems und weist eine translationelle *cepI-gfp*(ASV) Fusion sowie das *cepR* Gen unter der Kontrolle des P_{lac} Promotors auf (Riedel *et al*., 2001). Es ist höchst sensitiv für C8-HSL.

pKR-C12: Dieses Sensorplasmid wurde auf ähnliche Weise auf der Basis des *las*-Systems von *P. aeruginosa* konstruiert. Es ist daher am sensitivsten für 3-Oxo-C12-HSL (Riedel *et al*., 2001).

B.6.2 Nachweis der AHL-Produktion durch Kreuzausstrich

Werden AHL-produzierende Zellen auf einer LB-Agarplatte räumlich eng benachbart zum Sensorstamm ausgestrichen, so diffundieren die vom zu untersuchenden Stamm produzierten AHL-Moleküle durch das Medium zu den Sensorzellen und induzieren dort die Expression

der Reportergene. 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 Quantifizierung von AHL-Molekülen

Die zu testenden Kulturen wurden in LB-Medium bis zu einer OD_{600} von 3,0 angezogen. 100 µl des sterilfiltrierten Kulturüberstandes wurden zu 100 µl einer exponentiellen Kultur von *E. coli* MT102 (pSB403) gegeben, die in den Kavitäten einer FluoroNunc Polysorp Mikrotiterplatte (Nunc, Roskilde, Dk) vorgelegt waren. Nach einer Inkubation bei 30 °C für 6 h wurde die Biolumineszenz mit einem Lamda Fluoro 320 Plus Reader (Bio-Tek Instruments, Winooski, USA) gemessen.

B.6.4 Identifizierung von AHL-Molekülen mittels Dünnschicht-Chromatographie

Hierzu wurden die AHL-Moleküle aus dem Kulturüberstand des zu testenden Stamms extrahiert und mittels Dünnschicht-Chromatographie (DC) aufgetrennt. Danach wurden die DC-Platten mit Softagar, der mit dem Sensorstamm *E. coli* MT102 (pSB403) beimpft war, überschichtet (Shaw *et al*., 1997; Geisenberger *et al*., 2000). Durch AHL-Moleküle induzierte Biolumineszenz wurde durch das Belichten eines Röntgenfilms detektiert. Ein Vergleich der R_f-Werte der erhaltenen Signale mit denen von synthetischen AHL-Standards ermöglichte eine Identifizierung der aus den Kulturüberständen 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 findet sich in Anhang 1.

B.7 Extraktion von Zelloberflächenproteinen

Grundsätzlich wurden proteinbiochemische Standardmethoden (Harlow und Lane, 1998) angewendet. Membranproteine wurden nach einem leicht modifizierten Protokoll von Kawai *et al*. (1998) isoliert. Zellen von 2 ml einer in LB-Medium gewachsenen Kultur mit einer OD_{600} von 3 wurden geerntet, in PBS-Puffer (8 g NaCl; 0,2 g KCl; 1,44 g Na_2HPO_4 x 2 H_2O; 0,2 g KH_2PO_4; $H_2O_{dest.}$ ad 1 l; pH 7,2) gewaschen und in 400 µl einer 0,3 % SDS (Natriumdodecylsulfat)-Lösung aufgenommen. Nach 3 min Inkubation bei Raumtemperatur

und Zentrifugation bei 13.000 rpm für 15 min befanden sich die Zelloberflächenproteine im Überstand. Die Proteinkonzentrationen wurden mit Hilfe des Coomassie Plus Protein Assay Reagent (Pierce, Rockford, USA) entsprechend den Angaben des Herstellers kolorimetisch bestimmt. Die Proteine (je 1,3 µg) wurden auf 12 % SDS Polyacrylamidgelen (SDS-PAGE) (Laemmli, 1970) aufgetrennt, und die Proteinbanden wurden mittels Silberfärbung detektiert.

B.8 Untersuchungen der Exoenzym- und Siderophorproduktion

Die Produktion von Protease, Chitinase und Siderophoren wurde mit Hilfe der in Abschnitt B.2 beschriebenen Indikatormedien untersucht. Zur Untersuchung der Lipaseaktivität wurde das Tributyrin Agar Base-Fertigmedium mit 1 % Glycerintributyrat von Merck (Darmstadt) nach den Angaben des Herstellers zubereitet.
Die Aktivität der Exoenzyme Protease, Lipase und Chitinase wurde durch klare Höfe um die Kolonien nach Inkubation bei 37 °C über Nacht angezeigt. Siderophorproduktion resultierte in einem Farbumschlag von blau nach orange auf den CAS-Indikatorplatten, der durch den Entzug des Eisens aus dem CAS-Farbkomplex zustande kam.

B.9 Untersuchung der Motilität der Mutanten

Schwimmen: flagellengetriebene Schwimm-Motilität wurde auf ABC-Medium, dem zur Verfestigung 0,3 % Agar zugegeben waren, getestet. Die zu testende Kultur wurde mit einem sterilen Zahnstocher stichförmig in der Mitte der Agarplatte angeimpft, und über Nacht bei 30 °C inkubiert.
Schwärmen: Schwärmermotilität wurde getestet wie oben beschrieben. Jedoch war die Agarkonzentration 0,4 %, und dem Medium wurden Casaminosäuren in einer Endkonzentration von 0,1 % zugegeben.

In beiden Fällen wurden die Platten während der Inkubation mit Frischhaltefolie abgedeckt, um eine Austrocknung und die daraus resultierende Veränderung der Agarkonzentration zu vermeiden.

B.10 Bestimmung der Zelloberflächen-Hydrophobizität

Hierzu wurde eine von Rosenberg *et al*. (1980) beschriebene Methode mit einigen Modifikationen angewendet. Die Zellen wurden entweder bis zur frühen exponentiellen oder bis zur stationären Phase inkubiert. Nach der Zellernte und zweimaligem Waschen in PUM-

Puffer (22,2 g K_2HPO_4 x 3 H_2O; 7,26 g KH_2PO_4; 1,8 g Harnstoff; 0,2 g $MgSO_4$ x 7 H_2O ad 1 l; pH 7,1) wurden die Zellen in PUM auf eine OD_{600} von 1 gebracht. In Glasröhrchen wurden 160 µl *n*-Hexadecan (Merck, Darmstadt) zu 1,6 ml Zellsuspension gegeben. Nach einer Präinkubation von 10 min bei 30 °C wurden die Röhrchen 90 s lang geschüttelt. Anschließend wurden die Röhrchen für 15 min stehen gelassen, um eine Phasentrennung zwischen Hexadecan und wässriger Phase zu ermöglichen. Die OD_{600} der wässrigen Phase wurde bestimmt und die bakterielle Adhäsion an den Kohlenwasserstoff ('bacterial adhesion to hydrocarbon', BATH) wurde nach folgender Formel berechnet: $[1 - (OD_2/OD_1)] \times 100$; dabei war OD_1 = Zelldichte am Anfang des Experiments und OD_2 = Zelldichte nach der Inkubation der Zellen mit dem Kohlenwasserstoff.

B.11 Untersuchung der Biofilmbildung in Durchflusskammern

B.11.1 Anzucht der Biofilme

Die Biofilme wurden in künstlichen Durchflusskammern mit AB-Medium, das 1 mM Glukose enthielt (ABG), bei 30 °C 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 ABG-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 300 µl dieser Verdünnungen beimpft. Nach einer Stunde, in der sich die Bakterien am Substat anheften konnten, wurde mittels einer peristaltischen Watson-Marlow 205S Pumpe ein konstanter Medienfluss von 0,7 mm/s erzeugt.

B.11.2 Biofilmanalyse und Bildverarbeitung

Zur mikroskopischen Inspektion der Biofilme wurde ein konfokales Laser-Scanning-Mikroskop (TCS4D, Leica Lasertechnik, Heidelberg), das mit einem 63x/1,32-0,6 Ölimmersions Objektiv ausgestattet war, eingesetzt. Für die statistische Auswertung der Biofilmstrukturen wurde ein 40x/0,75 Luftobjektiv benutzt. Die Bilder wurden mit der 488 nm Laserlinie eines Ar/Kr Lasers aufgenommen. Die Bildverarbeitung erfolgte mit Hilfe des IMARIS Softwarepakets (Bitplane, Zürich, CH).

B.11.3 Statistische Auswertung der Biofilmdaten

Um eine statistische Aussage über die Biofilmstrukturen treffen zu können, wurden zwei unabhängige Runden von Biofilmversuchen durchgeführt. Zusätzlich wurden alle Stämme in jeder Runde in zwei separaten Kanälen angezogen. Von jedem Kanal wurden sieben 'image

stacks' aufgenommen und mit Hilfe des Computerprogramms COMSTAT (Heydorn *et al*., 2000) analysiert (s. Anhang 1 und 2).

C. ERGEBNISSE UND DISKUSSION

C.1 Biofilmbildung in Mikrotiterplatten als Modellsystem

In den letzten Jahren wurden viele Anstrengungen unternommen, um Faktoren, die bei der bakteriellen Biofilmbildung eine Rolle spielen, aufzuklären. Eine Möglichkeit dazu ist das Screening einer durch zufällige Transposoninsertionen erzeugten Mutantenbank nach Mutanten mit Defekten in der Biofilmbildung in den Kavitäten von Mikrotiterplatten (O´Toole und Kolter, 1998a und 1998b). Diese Methode hat den Vorteil, dass mit relativ geringem Aufwand eine große Zahl von Mutanten untersucht werden kann. Gene, die für die Ausbildung eines Biofilms benötigt werden, können dann durch die Sequenzierung der das Transposon umgebenden DNA-Region und den Abgleich der erhaltenen Sequenz mit Sequenzen in GenBank mithilfe der BLAST-Software (Altschul *et al*., 1990) identifiziert werden. Darüber hinaus ist es möglich, durch Veränderungen des Mediums, der Inkubationstemperatur und der Inkubationszeit den Einfluss von Umweltbedingungen auf die Biofilmbildung zu erforschen.

Erkenntnisse über die Biofilmbildung in Mikrotiterplatten können zwar nicht ungeprüft auf die Biofilmbildung in anderen Systemen, etwa bei chronischen Infektionen, übertragen werden. Allerdings spielen zentrale Faktoren, deren Bedeutung für die Biofilmbildung in Mikrotiterplatten-Versuchen gezeigt wurde, auch bei der Kolonisierung von Geweben eine wesentliche Rolle. Beispiele hierfür sind die Beweglichkeit der Zellen (Tans-Kersten *et al*., 2001), Pili (Sauer *et al*., 2000), die Matrix aus extrazellulären polymeren Substanzen (EPS) (Pier, 2000; Watnick *et al*., 2001) und die Zell-Zell-Kommunikation (Singh *et al*., 2000). Für die Übertragbarkeit der mithilfe des Mikrotiterplattentests gewonnenen Ergebnisse spricht auch unsere Beobachtung, dass alle in der Biofilmbildung in Mikrotiterplatten auffälligen *B. cepacia*-Mutanten, die zusätzlich in Durchflusskammern getestet wurden, auch in diesem grundlegend anderem System schwächere und anders strukturierte Biofilme bildeten als der Wildtyp. Darüber hinaus wurde deutlich, dass einige der bei *B. cepacia* identifizierten Gene auch für die Biofilmbildung anderer Arten sowohl auf abiotischen als auch auf biotischen Oberflächen von Bedeutung sind.

C.2 Beweglichkeit und Biofilmbildung

Genetische Untersuchungen haben gezeigt, dass der Vorgang der Biofilmbildung nicht zufällig abläuft, sondern aus einer geordneten Abfolge von Ereignissen besteht (Pratt und Kolter, 1999; Watnick und Kolter, 2000; O´Toole *et al*., 2000). Dabei werden drei Entwicklungsschritte unterschieden: (i) Anheftung von Zellen an eine Oberfläche, (ii) Bildung von Mikrokolonien durch diese Zellen und (iii) Differenzierung von Mikrokolonien zu ausgereiften Biofilmen.

In diesem Modell spielt die Beweglichkeit der Zellen eine zentrale Rolle. Insbesondere müssen Abstoßungskräfte an Oberflächen durch aktives Schwimmen der Bakterien überwunden werden, um den ersten Schritt, die Anheftung der Zellen, zu ermöglichen. Unerwarteterweise zeigte jedoch nur eine der bei den hier beschriebenen Versuchen isolierten Biofilm-defizienten Mutanten, m16, einen unbeweglichen Phänotyp (Anhang 2). Dies deutet darauf hin, dass die relativ lange Inkubationszeit der Biofilme von 48 Stunden dazu führte, dass bevorzugt Mutanten mit Defekten in späteren Entwicklungsstadien der Biofilmbildung isoliert wurden und nicht solche mit Defekten in der anfänglichen Anheftung. Um diese Hypothese zu testen, wurde der zeitliche Verlauf der Biofilmbildung von *B. cepacia* H111 mit dem einer isogenen, unbeweglichen *motA*-Mutante verglichen. Diese Mutante ist zwar in der Lage, Flagellen zu synthetisieren, da diese jedoch nicht rotieren können, sind die Zellen sozusagen paralysiert (Blair und Berg, 1990; Macnab, 1996). Es konnte gezeigt werden, dass die Biofilmbildung der *motA*-Mutante nur in den ersten acht Stunden signifikant von der des Wildtyps abweicht. Bei einer darüber hinaus gehenden Inkubationsdauer ist der durch die *motA*-Mutante gebildete Biofilm nicht mehr von dem des Wildtyps zu unterscheiden (Anhang 2). Offenbar beschleunigt also Schwimmen eine erste Anheftung an die Oberfläche, spielt aber keine wesentliche Rolle für die spätere Biofilmentwicklung. Dies erklärt, warum außer der Mutante m16, bei der ein Defekt in der DNA-Rekombination offenbar multiple phänotypische Auswirkungen hat, keine unbeweglichen Mutanten gefunden wurden. Gleichzeitig wird deutlich, dass die hier beschriebene Screeningmethode in erster Linie die Identifizierung von Faktoren ermöglicht, die für spätere Stadien der Biofilmbildung, d.h. für die Ausbildung einer typischen dreidimensionalen Struktur, von Bedeutung sind. Dies zeigt sich auch durch den Vergleich der Biofilmstrukturen: alle von uns getesteten Mutanten bildeten in Durchflusskammern Biofilme, deren Architektur stark von der des Wildtyps abwich (Anhang 1 und 2).

C.3 Gene, welche die Biofilmbildung bei *B. cepacia* beeinflussen

Das Screening der *B. cepacia*-Mutantenbank erbrachte insgesamt 18 Mutanten mit einer im Vergleich zum Wildtyp schwächeren Biofilmbildung in den Kavitäten von Mikrotiterplatten. Da eine verminderte Biofilmbildung auch durch unspezifische Wachstumsdefekte verursacht werden kann, wurden fünf Mutanten aufgrund ihrer im Vergleich zum Wildtyp längeren Verdopplungszeit von der Studie ausgeschlossen. Die restlichen 13 Mutanten wurden näher untersucht.

Bei elf dieser Mutanten konnten die inaktivierten Gene aufgrund von Ähnlichkeiten mit bereits bekannten Genen identifiziert werden, wodurch Rückschlüsse auf ihre Funktion möglich waren. Die mutierten Gene konnten folgenden Kategorien zugeordnet werden: (i) Gene, welche am Aufbau oder der Zusammensetzung der Zelloberfläche (Zelloberflächenproteine oder Zellmembran) beteiligt sind, (ii) Gene, welche die Zellmorphologie beeinflussen, (iii) Gene, die für regulatorische Faktoren kodieren. Bei den Regulationsfaktoren scheint das *cep* QS-System eine entscheidende Rolle einzunehmen: bei fünf der isolierten Mutanten war die AHL-Produktion stark reduziert oder nicht mehr nachweisbar.

Zelloberfläche. Vier Mutationen betrafen Gene, deren Produkte entweder Teil der Zelloberfläche sind oder zu ihrem Aufbau beitragen. Mutante m7 trägt eine Insertion in *tolA*. TolA ist Teil eines Multiproteinkomplexes, der am Aufbau einer intakten äußeren Membran bei verschiedenen Gram-negativen Bakterien beteiligt ist (Dennis *et al*., 1996; Lazzaroni *et al*., 1999; Llamas *et al*., 2000). Typisch für *tolA*-Mutanten ist eine uneinheitliche Zellmorphologie, wie sie auch bei m7 beobachtet werden konnte (Anhang 2). Bei *E. coli* gibt es Hinweise darauf, dass TolA die Prozessierung der Untereinheiten des O-Antigens beeinflusst (Gaspar *et al*., 2000). Diese Veränderung der Zelloberfläche führt im Fall von Mutante m7 zu einer drastischen Änderung der Biofilmstruktur: es sind keine der für *B. cepacia* H111 charakteristischen Mikrokolonien zu finden, vielmehr wachsen die Zellen völlig unstrukturiert (Anhang 2).

Die Mutanten m13 und m15 sind im gleichen Gen mutiert. Dieses Gen hat hohe Ähnlichkeit mit *bap* von *Staphylococcus aureus* (Cucarella *et al*., 2001), *esp* von *Enterococcus faecalis* (Toledo-Arana *et al*., 2001) und *mus-20* von *Pseudomonas putida* (Espinosa-Urgel *et al*., 2000). Diese drei Gene sind an der Biofilmbildung auf Kunststoff (*bap* und *esp*) bzw. der Kolonisierung von Maiskörnern (*mus-20*) beteiligt. Es wird angenommen, dass die von ihnen kodierten Proteine an der Zelloberfläche lokalisiert sind. Wie die Mutationen in *esp* oder *bap* (Toledo-Arana *et al*., 2001) hat auch die Mutation im entsprechenden *B. cepacia*-Gen eine reduzierte Hydrophobizität der Zellen zur Folge. Außerdem haben die Mutanten m13 und m15 im Gegensatz zum Wildtyp, der glatte Kolonien bildet, eine rauhe Koloniemorphologie.

Beide Beobachtungen sprechen dafür, dass auch bei diesen beiden Mutanten die Zelloberfläche verändert ist, obwohl beim Vergleich der Zelloberflächenproteine von *B. cepacia* H111 und Mutante m15 nur kleinere Unterschiede zu erkennen waren. Wie bei der Mutation in *tolA* resultiert auch diese Veränderung der Zelloberfläche in einer stark vom Wildtyp abweichenden Biofilmstruktur. Mutante m15 wächst in einzelnen großen Zellaggregaten, ohne die Oberfläche vollständig zu kolonisieren (Anhang 2).

Mutante MA2 ist in *gspE* mutiert, welches für eine Komponente des Typ II-Sekretionssystems kodiert. Dieses System ist Teil des 'General secretory pathway' oder 'Typ II-Sekretionswegs' Gram-negativer Bakterien (Pugsley, 1993) und ist verantwortlich für die Translokation von Proteinen vom Periplasma über die äußere Membran in den Extrazellularraum. GspE von *B. cepacia* H111 hat eine hohe Ähnlichkeit zu GspE von *Burkholderia pseudomallei*, das den Typ II-Sekretionsweg für den Export von Protease, Lipase und Phospholipase nutzt (DeShazer *et al*., 1999). Diese Daten erklären unsere Beobachtung, dass die Mutante MA2 keine extrazelluläre proteolytische und lipolytische Aktivität aufweist. Es gibt mehrere Möglichkeiten, wie das Typ II-Sekretionssystem die Biofilmbildung von *B. cepacia* beeinflussen könnte. Ähnlich wie es für ein Oberflächenprotein beschrieben wurde, das Teil eines ABC-Transportsystems ist und gleichzeitig als Adhäsin fungiert (Kolenbrander *et al*., 1998), könnten Komponenten des Typ II-Sekretionssystems direkt an der Biofilmbildung beteiligt sein. Es ist aber auch möglich, dass das Typ II-Sekretionssystem für den Export eines für die Biofilmbildung wichtigen Proteins verantwortlich ist. In diesem Zusammenhang ist erwähnenswert, dass Bestandteile des Typ II-Sekretionssystems Ähnlichkeiten zu Proteinen aufweisen, die am Aufbau von Typ IV-Pili beteiligt sind, was einen gemeinsamen evolutionären Ursprung der beiden Systeme wahrscheinlich macht (Hobbs und Mattick, 1993; Lory, 1998; Nunn, 1999). Typ IV-Pili spielen eine wesentliche Rolle für die Biofilmbildung von *P. aeruginosa* (O´Toole und Kolter, 1998b).

Zellform. Bei Mutante MA19 ist das Transposon in *rodA* (Stoker *et al*., 1983) inseriert. RodA ist, zusammen mit dem Penicillin-Bindeprotein II (PBP2) (Spratt, 1975) für die Aufrechterhaltung der Stäbchenform während der Zellteilung verantwortlich, da beide Proteine für die Mureinbiosynthese zur Verlängerung der Zellwand benötigt werden. Eine Mutation in einem der beiden Gene führt zur Bildung runder Zellen (Begg and Donachie, 1985). Dem entsprechend sind MA19-Zellen nicht stäbchenförmig wie der Wildtyp, sondern kokkoid (Anhang 2). Eine Erklärung für die veränderte Biofilmstruktur von MA19 könnte die aufgrund der kokkoiden Zellen höhere Packungsdichte in den Mikrokolonien sein. Da *rodA* am Aufbau der Zellhülle beteiligt ist, besteht aber auch in diesem Fall die Möglichkeit, dass veränderte Eigenschaften der Zelloberfläche verantwortlich für den Biofilmdefekt sind.

In einer kürzlich veröffentlichten Studie zeigten Whiteley *et al*. (2001), dass bei sessilen *P. aeruginosa*-Zellen die Expression von etwa 0,5 % der Gene im Vergleich zu planktonischen Zellen aktiviert ist, während die Expression von weiteren 0,5 % der Gene reprimiert ist. Unter den hoch regulierten Genen war nicht nur *tolA*, sondern auch *mreC*, dessen Produkt wie RodA für die Aufrechterhaltung der Stäbchenform benötigt wird. Diese Ergebnisse unterstreichen die Bedeutung von Zellform und Zelloberfläche für die Biofilmbildung.

Rekombination – Phasenvariation? Die Mutante m16 ist in *recR* mutiert. RecR ist zusammen mit RecO und RecF an rekombinatorischen DNA-Reparaturvorgängen und der Einleitung der SOS-Antwort beteiligt (Kuzminov, 1999). Man nimmt an, dass RecR und RecO dabei einen Komplex bilden, welcher die Bindung des RecA Proteins an einzelsträngige DNA erleichtert, die mit SSB ('single stranded DNA binding protein') bedeckt ist. Dementsprechend sind Mutanten mit Defekten im *recFOR*-Weg empfindlicher gegen UV-Strahlung und DNA-schädigende Chemikalien (Kuzminov, 1999; Keller *et al*., 2001), was auch bei Mutante m16 beobachtet werden konnte. Der Zusammenhang von DNA-Reparatur und Biofilmbildung scheint auf den ersten Blick nicht offensichtlich. Es konnte aber gezeigt werden, dass RecA auch bei der Genregulation eine wichtige Rolle spielen kann. So ist die Expression extrazellulärer Proteine bei *Serratia marcescens* und *Erwinia carotovora* RecA-abhängig (Zink *et al*., 1985; Ball *et al*., 1990). Darüberhinaus ist RecA auch für die Amplifikation des Choleratoxin-Gens bei *Vibrio cholerae* (Goldberg und Mekalanos, 1986) und die Antigenvariation der Pili bei *Neisseria gonorrhoeae* (Koomey *et al*., 1987) verantwortlich. Höchst interessant ist die Beobachtung, dass *V. cholerae recA*-Mutanten Defekte bei der Kolonisierung des Darms von Versuchstieren aufwiesen. Dies wurde darauf zurückgeführt, dass DNA-Rekombination auch für die Expression von Kolonisierungsfaktoren nötig sein könnte (Kumar *et al*., 1994).

Zwei der Mutanten mit Defekten in der Biofilmbildung, m14 und m19, tragen eine Transposoninsertion in DNA-Abschnitten, die keine signifikante Ähnlichkeit mit bereits charakterisierten Genen haben. Dies zeigt, dass auch neuartige, bisher unbekannte Faktoren an der Biofilmbildung von *B. cepacia* beteiligt sind.

C.4 Die zentrale Bedeutung des 'Quorum sensing'-Systems von *B. cepacia* für die Biofilmbildung

Da Bakterien in Biofilmen wesentlich dichter wachsen als in den meisten natürlich vorkommenden planktonischen Kulturen, scheint diese Lebensform ideale Voraussetzungen für Zell-Zell-Kommunikation zu bieten. Tatsächlich konnten aus Biofilmen, die auf so unterschiedlichen Habitaten wie Flusskieseln, Blasenkathetern oder Lungenepithelien gewachsen waren, AHL-Moleküle isoliert werden (McLean *et al*., 1997; Stickler *et al*., 1998; Singh *et al*., 2000). Darüber hinaus wurde gezeigt, dass das *las* QS-System von *P. aeruginosa* direkt an der Regulation der Biofilmbildung beteiligt ist. Eine *lasI*-Mutante bildete nur dünne und undifferenzierte Biofilme, die darüber hinaus sensitiver gegen das Detergens SDS waren (Davies *et al*., 1998). Unsere Ergebnisse machen deutlich, dass Zell-Zell-Kommunikation auch bei *B. cepacia* von zentraler Bedeutung für die Biofilmbildung ist.

Eine der Biofilm-defizienten Mutanten, m64, ist in *cepR* mutiert. Da der QS-Regulator CepR an der Expression der AHL-Synthase CepI beteiligt ist (Lewenza und Sokol, 2001), produziert die Mutante m64 keine nachweisbaren Mengen an AHL-Molekülen. Um die Auswirkungen von Mutationen in den *cep*-Genen auf die Biofilmbildung genauer zu untersuchen, wurden zwei definierte *cep*-Mutanten, H111-I (*cepI*-) und H111-R (*cepR*-) konstruiert. Biofilme beider Mutanten sind nicht nur deutlich schwächer als die von *B. cepacia* H111, sie weichen zudem auch in ihrer Struktur stark von ihnen ab. Neben einer geringeren Dicke und Biomasse ist auch ihre Fähigkeit, die Oberfläche zu kolonisieren, stark vermindert. Darüber hinaus ist die Heterogenität der von beiden Mutanten gebildeten Biofilme im Vergleich zum Wildtyp deutlich erhöht (Anhang 1).

Es wurden noch vier weitere Biofilm-defiziente Mutanten mit eingeschränkter bzw. fehlender AHL-Produktion identifiziert, nämlich m17, m20, MA18 und MA24 (Anhang 2). Die beiden Mutanten m17 und m20 tragen Transposoninsertionen in einem Gen mit hoher Ähnlichkeit zu *yciR* von *E. coli*. Das von diesem Gen kodierte Protein YciR weist zwei konservierte Motive, EAL und GGDEF auf, die bei Gram-negativen wie bei Gram-positiven Bakterien weit verbreitet sind. Beide Domänen können auch in der abgeleiteten Aminosäuresequenz der das Transposon flankierenden DNA-Sequenz beider Mutanten gefunden werden. Da diese Motive oft in Kombination mit an Signaltransduktions-Systemen beteiligten Domänen gefunden wurden, wurde schon früher angenommen, dass sie eine regulatorische Funktion ausüben (Parkinson und Kofoid, 1992; Alridge und Jenal, 1999). Verschiedene Studien zeigen, dass Proteine mit GGDEF- und EAL-Domänen an der Regulation der c-di-GMP-Konzentration in der Zelle beteiligt sind, wobei c-di-GMP [bis-(2´,5´)-zyklische Diguaninsäure] als allosterischer Aktivator bestimmter Proteine wirkt (Tal *et al*., 1998; Ausmees *et al*., 2001).

Die Cellulosesynthase von *Acetobacter xylinum* war das erste Enzym, für das eine Regulation der Aktivität durch c-di-GMP gezeigt werden konnte (Ross *et al*., 1987). Auch bei anderen Bakterien werden c-di-GMP-abhängige Regulationsmechanismen vermutet (Ausmees *et al*., 2001). Interessanterweise ist auch das GGDEF-Protein AdrA von *E. coli* und *Salmonella typhimurium* an der Regulation der Cellulosebiosynthese beteiligt. Cellulose ist in beiden Organismen für die Biofilmbildung und die Aggregation von Zellen nötig (Römling *et al*., 2000; Zogaj *et al*., 2001). Die Tatsache, dass m17 dünne Biofilme ohne Mikrokolonien bildet (Anhang 2), könnte darauf hinweisen, dass bei dieser Mutante ein Faktor fehlt, der die Autoaggregation von Zellen fördert.

MA18 ist in *suhB* mutiert. In *E. coli* führen Mutationen in *suhB* zur Unterdrückung verschiedener temperatursensitiver Mutationen (Shiba *et al*., 1984; Yano *et al*., 1990; Chang *et al*., 1991). Obwohl das *suhB*-Genprodukt Inositolmonophosphatase-Aktivität aufweist, ist seine Funktion als Suppressor offensichtlich davon unabhängig (Chen und Roberts, 2000). Es wird angenommen, dass SuhB an der posttranskriptionellen Kontrolle der Genexpression mitwirkt, indem es die Stabilität der mRNA moduliert (Inada und Nakamura, 1995).

Das bei der Mutante MA24 defekte Gen zeigt Homologien zu Pseudouridinsynthasen (ψ-Synthasen) verschiedener Bakterien (Charette und Gray, 2000). ψ-Synthasen katalysieren die gerichtete Isomerisierung von Uridin (U) in tRNA und rRNA zu Pseudouridin (ψ). Es ist unklar, inwiefern ψ-Synthasen die Zell-Zell-Kommunikation und die Biofilmbildung beeinflussen. Erschwerend kommt hinzu, dass ψ-Synthasen neben der Isomerisierung von U zusätzliche Funktionen haben können. So konnten Wachstumsdefekte von *E. coli*-Mutanten mit Defekten in Genen für verschiedene ψ-Synthasen durch Plasmide behoben werden, auf denen durch Punktmutationen zur Pseudouridylierung unfähige Derivate der entsprechenden ψ-Synthasen kodiert waren (Gutgsell *et al*., 2000; Gutgsell *et al*., 2001). Es ist jedoch in diesem Zusammenhang von Interesse, dass eine *P. aeruginosa*-Mutante mit einem Defekt in der ψ-Synthase *truB* verschiedene Defekte in der osmotischen Stressantwort aufweist und bei einer Temperatur von 43 °C in flüssigen, nicht aber auf festen Medien wachsen kann (Sage *et al*., 1997).

Bei keiner der drei Mutationen konnte die Biofilmbildung oder die Produktion von Protease, ein strikt QS-regulierter Phänotyp, durch die Zugabe von AHL-Molekülen zum Medium wieder hergestellt werden. Dagegen wurden beide Phänotypen vollkommen komplementiert, wenn die *cepI*-Mutante H111-I als Kontrolle verwendet wurde. Es kann daher ausgeschlossen werden, dass ein Defekt in der AHL-Synthese für den Biofilm-defizienten Phänotyp verantwortlich ist. Da CepR neben anderen QS-regulierten Genen auch die Expression von *cepI*, und damit die AHL-Synthese, kontrolliert (Lewenza und Sokol, 2001), könnten die Mutanten auch Defekte in der Expression von *cepR* aufweisen. Um diese Möglichkeit zu

untersuchen, wurde Plasmid pBAH27, welches ein vom *lac*-Promotor des Vektors abgelesenes *cepR*-Allel enthält, in die Mutanten m17 und MA18 eingebracht. Weder die AHL-Produktion noch die Biofilmbildung konnten durch pBAH27 wieder hergestellt werden, während diese Komplementierung bei der *cepR*-Mutante H111-R möglich war (Anhang 1). Bemerkenswerterweise wurde die extrazelluläre Proteaseaktivität bei Mutante m17 durch pBAH27 verstärkt, nicht aber bei MA18, was die Komplexität der *cep* QS-Regulationskaskade verdeutlicht.

Diese Ergebnisse zeigen, dass die Produkte von *yciR*, *suhB* und *yciL* dem *cep* QS-System übergeordnete Regulatoren darstellen. Darüber hinaus ist es wahrscheinlich, dass YciR und SuhB entweder die Konzentration oder die Aktivität von CepR in der Zelle beeinflussen. Noch ist unklar, ob diese Regulatoren auch die Expression von Genen außerhalb des *cep*-Regulons kontrollieren. Um dies näher zu untersuchen, wurde das Proteom von *B. cepacia* H111 mittels zweidimensionaler Gelelektrophorese mit dem der Mutanten m17, MA18 und MA24 sowie der *cepR*-Mutante m64 verglichen (unveröffentlichte Daten). Alle bei m64 im Vergleich zum Wildtyp stärker oder schwächer exprimierten Proteine waren auch bei m17, MA18 und MA24 in gleicher Weise differenziell reguliert. Darüber hinaus war bei jeder der drei Mutanten die Expression zusätzlicher Proteine aktiviert oder reprimiert. Diese Beobachtung bestätigt zum einen, dass die in m17, MA18 und MA24 mutierten Gene für Regulatoren kodieren, die dem *cep*-System übergeordnet sind; zum anderen ist sie ein Hinweis auf die Beteiligung dieser Regulatoren an der Kontrolle von Genen außerhalb des *cep*-Regulons.

In diesem Zusammenhang stellt sich auch die Frage, ob die Defekte in der Biofilmbildung bei m17, MA18 und MA24 lediglich durch deren QS-Defekt bedingt sind, oder ob die bei den drei Mutanten inaktivierten Gene darüber hinaus andere Faktoren regulieren, die an der Biofilmbildung beteiligt sind. Die Strukturen der von m17 und MA18 in Durchflusskammern gebildeten Biofilme sprechen für einen direkten Einfluss von YciR und SuhB auf die Biofilmbildung. Die Biofilme weisen nicht die typische Architektur der von H111-I oder H111-R gebildeten Biofilme auf, die sich durch überwiegendes Wachstum in charakteristischen Mikrokolonien und eine stark reduzierte Kolonisierung der Oberfläche auszeichnen (Anhang 1). Vielmehr sind die von m17 und MA18 gebildeten Biofilme eher strukturlos, d.h. es sind kaum Mikrokolonien vorhanden (Anhang 2, unveröffentlichte Daten).

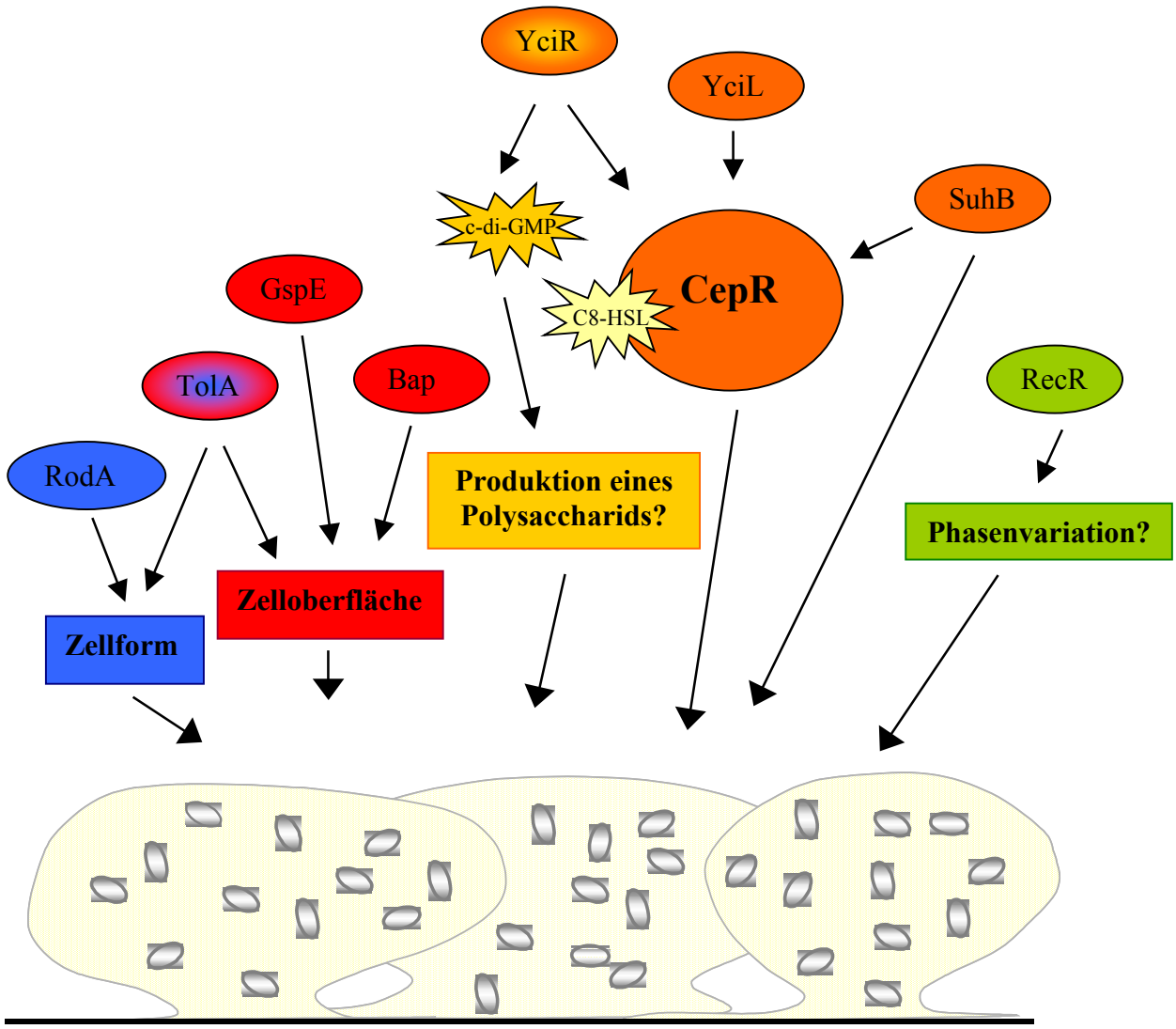

Abbildung 3. Zusammenfassende Darstellung der Genprodukte, die zur Entstehung eines reifen Biofilms bei *B. cepacia* H111 beitragen, sowie ihrer möglichen Funktionen. Die Zelloberfläche wird durch drei Proteine beeinflusst. Bap ist selbst ein Zelloberflächenprotein, GspE wird zum Aufbau eines Sekretionsystems benötigt, das entweder zur Sekretion von Adhäsinen beiträgt oder Komponenten enthält, die selbst als Adhäsine wirken, und Tol A ist Teil eines Proteinkomplexes, der am Aufbau der äußeren Membran beteiligt ist. Ebenso wie RodA, das zum Erhalt der Stäbchenform einer Zelle nötig ist, beeinflusst TolA darüber hinaus die Zellform. Fünf der Gene kodieren für regulatorische Faktoren. RecR ist an der DNA-Rekombination beteiligt, die zur phasenabhängigen Expression von Kolonisierungsfaktoren beitragen könnte. CepR ist Teil des QS-Systems von *B. cepacia*, SuhB, YciR, YciL sind dem QS-System übergeordnete Faktoren. Die Zell-Zell-Kommunikation beeinflusst auf unbekannte Weise die Biofilmreifung. SuhB und YciR haben wahrscheinlich zusätzlich einen direkten Einfluss auf die Biofilmbildung. YciR gehört zu einer Proteinfamilie, die in anderen Bakterien die Konzentration des Regulatormoleküls c-di-GMP kontrolliert. c-di-GMP ist u.a. an der Regulation der Produktion von Cellulose, welche bei der Biofilmbildung die Autoaggregation von Zellen fördern kann, beteiligt.

C.5 Intergenerische Kommunikation in gemischten Biofilmen

In den letzten Jahren wurde deutlich, dass 'Quorum sensing' mittels AHL-Molekülen in Gram-negativen Bakterien außerordentlich weit verbreitet ist (Eberl, 1999; Whitehead *et al*., 2001). In der Natur sind Reinkulturen von Mikroorganismen die große Ausnahme, vielmehr liegen nahezu immer Mischpopulationen vor. In vielen Fällen besiedeln AHL-produzierende Bakterien in enger räumlicher Nähe die gleichen ökologischen Nischen. Es ist daher naheliegend, dass QS nicht nur der Kommunikation innerhalb einer Art, sondern auch der zwischen Zellen verschiedener Arten dienen kann. So wurde beispielsweise vor kurzem mit Hilfe von sensitiven GFP-basierenden AHL-Sensoren die Zell-Zell-Kommunikation von Bakterien, welche die Tomatenrhizosphäre besiedeln, in situ gezeigt (Steidle *et al*., 2001).
B. cepacia und *P. aeruginosa* bilden bei einer chronischen Co-Infektion von CF-Patienten gemischte Biofilme in deren Lungen. Da bei beiden Organismen sowohl die Biofilmbildung als auch die Expression von Pathogenitätsfaktoren durch QS gesteuert wird (Winson *et al*., 1995; Davies *et al*., 1998; Lewenza *et al*., 1999; Anhang 1), könnte intergenerische Kommunikation einen wesentlichen Einfluss auf die Virulenz dieses Konsortiums haben. Diese AHL-vermittelten Wechselwirkungen könnten daher für die Verschlechterung der Lungenfunktion mancher mit *B. cepacia* und *P. aeruginosa* co-kolonisierter CF-Patienten (Isles *et al*., 1984; Jacques *et al*., 1998) verantwortlich sein. In der Tat konnten McKenney *et al*. (1995) zeigen, dass die Produktion verschiedener Virulenzfaktoren einer *B. cepacia*-Kultur durch die Zugabe von *P. aeruginosa*-Kulturüberstand zum Medium gesteigert wurde. Dies führten die Autoren auf das Vorhandensein von AHL-Molekülen in dem *P. aeruginosa*-Kulturüberstand zurück. Ein indirekter Hinweis auf Kommunikation zwischen *P. aeruginosa* und *B. cepacia* in Lungen von CF-Patienten ergab sich aus einer Studie, in der AHL-Profile von *P. aeruginosa*-Stämmen, die zu verschiedenen Zeitpunkten aus den Lungen infizierter CF-Patienten isoliert worden waren, verglichen wurden. Dabei war auffällig, dass während einer Co-Infektion mit *B. cepacia* H111 die Menge der von *P. aeruginosa* produzierten AHL-Moleküle drastisch zurückging (Geisenberger *et al*., 2000).
Mit Hilfe spezifischer AHL-Sensoren war es möglich, die Kommunikation von *B. cepacia* und *P. aeruginosa* sowohl in gemischten Biofilmen in Durchflusskammern als auch während der Co-Infektion von Mäuselungen nachzuweisen (Anhang 3). Dazu wurden zwei AHL-Sensoren eingesetzt, die am sensitivsten auf C8-HSL bzw. 3-oxo-C12-HSL [*N*-(3-Oxododecanoyl)-homoserinlacton] reagieren. C8-HSL ist das von *B. cepacia* H111 hauptsächlich produzierte AHL-Molekül (Lewenza *et al*., 1999; Anhang 4), während 3-oxo-C12-HSL das Produkt der AHL-Synthase LasI darstellt, welche Bestandteil eines der beiden QS-Systeme von *P. aeruginosa*, des *las*-Systems, ist (Pearson *et al*., 1994; Latifi *et al*., 1995).

Mit Hilfe dieser Sensorplasmide konnte gezeigt werden, dass tatsächlich AHL-vermittelte Kommunikation in gemischten Biofilmen aus *P. aeruginosa* und *B. cepacia* stattfindet, wenn auch nur in einer Richtung: während von *P. aeruginosa* produzierte AHL-Moleküle das *B. cepacia* QS-System aktivierten, konnte keine Aktivierung des *P. aeruginosa las*-Systems durch *B. cepacia* beobachtet werden. Diese Ergebnisse wurden durch Versuche zur Komplementierung der Proteaseaktivität, ein bei beiden Organismen streng QS-regulierter Phänotyp, bestätigt. Durch Zugabe von aus *P. aeruginosa*-Kulturüberstand extrahierten AHL-Molekülen zum Medium konnte der Proteasedefekt der *B. cepacia cepI*-Mutante H111-I aufgehoben werden. Demgegenüber war es nicht möglich, durch Zusatz von *B. cepacia* AHL-Extrakten die Proteaseaktivität einer *P. aeruginosa lasI*-Mutante signifikant zu erhöhen (Anhang 3).

Was sind die Gründe für diese einseitige Kommunikation? Offenbar weisen die AHL-bindenden R-Proteine in beiden Arten unterschiedliche Spezifitäten auf. *B. cepacia* H111 produziert C8-HSL und C6-HSL (Hexanoyl-homoserinlacton) im Verhältnis von ca. 10:1 (Anhang 4). Beide AHL-Moleküle sind nicht in der Lage, die QS-Systeme von *P. aeruginosa* zu aktivieren, der in erster Linie C4-HSL bzw. 3-Oxo-C12-HSL nutzt. *P. aeruginosa* wiederum produziert auch geringere Mengen an C6-HSL und 3-Oxo-C8-HSL [*N*-(3-Oxooctanoyl)-homoserinlacton], die beide schon in niedrigen Konzentrationen das *cep*-QS-System von *B. cepacia* aktivieren können (Anhang 3). Während die meisten AHL-positiven Stämme des *B. cepacia*-Komplex C8-HSL und C6-HSL produzieren, konnte gezeigt werden, dass einige Stämme, die zum Genomovar V (*B. vietnamiensis*) zählen, zusätzliche AHL-Moleküle mit Acyl-Seitenketten von C10 bis C14 synthetisieren (Anhang 4). Vorläufige Experimente haben ergeben, dass diese Stämme in der Lage sind, das QS-System von *P. aeruginosa* zu aktivieren, d.h. dass in diesen Fällen intergenerische Kommunikation in beiden Richtungen möglich ist.

C.6 'Quorum sensing' und Pathogenität

In dieser Arbeit wurde gezeigt, dass QS einen entscheidenden Einfluss auf die Biofilmbildung von *B. cepacia* H111 hat. Darüber hinaus wird wie bei anderen Mikroorganismen auch bei *B. cepacia* die Expression weiterer Proteine in Abhängigkeit von der Zelldichte reguliert. Darunter sind potentielle Pathogenitätsfaktoren wie Protease, Lipase und das Siderophor Ornibactin (Lewenza *et al*., 1999; Lewenza und Sokol, 2001). Im Zuge der Charakterisierung der QS-negativen Mutanten H111-I und H111-R wurde deutlich, dass auch bei *B. cepacia* H111 die Produktion von Protease und Siderophoren, nicht aber die von Lipase, QS-reguliert

ist. Darüber hinaus war die Chitinaseaktivität bei beiden Mutanten leicht reduziert (Anhang 1).

Bei der Untersuchung der Beweglichkeit von *B. cepacia* H111 QS-Mutanten zeigte sich, dass diese nicht mehr in der Lage sind zu schwärmen (Anhang 1). Schwärmen ist eine koordinierte Form der Beweglichkeit von Bakterien auf Oberflächen (Allison und Hughes, 1991; Harshey, 1994). Auch bei *P. aeruginosa* und *Serratia liquefaciens* ist das Schwärmen QS-reguliert (Eberl *et al*., 1996; Köhler *et al*., 2000). Bei beiden Bakterien kontrolliert das QS-System die Produktion oberflächenaktiver Substanzen. Im Fall von *P. aeruginosa* handelt es sich hierbei um Rhamnolipide, bei *S. liquefaciens* um das Lipopeptid Serrawettin W2 (Ochsner und Reiser, 1995; Lindum *et al*., 1998; Köhler *et al*., 2000). Die Verringerung der Oberflächenspannung durch diese Substanzen ist die Voraussetzung für die Schwärmbewegung, weshalb *S. liquefaciens* AHL-Mutanten nach Zugabe einer oberflächenaktiven Substanz zum Medium ihre Fähigkeit zu Schwärmen wiedererlangen (Lindum *et al*., 1998; Eberl *et al*., 1999). Bei *B. cepacia* H111 ist eine ähnliche Art der Regulation wahrscheinlich. Während beim Wildtyp rund um eine schwärmende Kolonie eine aus einer schleimigen Substanz bestehende Zone gebildet wird, fehlt diese bei den AHL-negativen Mutanten. Darüber hinaus zeigen die *cep*-Mutanten nach Zugabe von geringen Mengen an SDS, Surfactin oder Serrawettin zum Medium wieder normales Schwärmverhalten. Interessanterweise wurde bei einem anderen zum Genomovar III gehörenden *B. cepacia*-Stamm, J2315, ein Lipopeptid mit stark oberflächenaktiven Eigenschaften beschrieben, das Erythrozyten und Phagozyten von Säugetieren zerstört (Hutchison *et al*., 1998). Es ist daher naheliegend, dass auch die von *B. cepacia* H111 produzierte oberflächenaktive Substanz ein Virulenzfaktor ist.

Der Einfluss von ‘Quorum sensing’ auf die Biofilmbildung und die Produktion von Virulenzfaktoren, sowie die Möglichkeit der Beeinflussung der Pathogenität bakterieller Konsortien durch artübergreifende Kommunikation unterstreichen die Bedeutung der Erforschung von AHL-Blockern für die Behandlung chronischer Erkrankungen. Dieser Ansatz hat mehrere Vorteile: (i) Verringerung der Virulenz eines Organismus durch Hemmung der Expression von Pathogenitätsfaktoren, (ii) Verhinderung der Bildung ausgereifter Biofilme, dadurch fehlender Schutz der Bakterien vor Antibiotika und dem Immunsystem, (iii) geringere Gefahr der Selektion resistenter Keime, da die Bakterien durch QS-Hemmer nicht abgetötet werden.

In der Natur sind zwei Wege zur Hemmung der bakteriellen Kommunikation bekannt: Hemmung der AHL-Rezeptorproteine und Abbau der AHL-Signalmoleküle. Die erste Möglichkeit wird von der Rotalge *Delisea pulchra* genutzt, die dadurch ihre Blätter vor einer Kolonisierung durch Bakterien und der daraus resultierenden Fäulnis schützt (Givskov *et al*.,

1996; Kjelleberg *et al*., 1997). Diese Alge produziert sogenannte Furanone, deren Molekülstrukturen jenen von AHL-Molekülen ähneln. Da Furanone in der Lage sind, radioaktiv markierte AHL-Moleküle von ihrem entsprechenden Rezeptorprotein zu verdrängen, wurde vorgeschlagen, dass diese Substanzen kompetitiv die AHL-Bindungsstelle von Proteinen der LuxR-Familie besetzen (Manefield *et al*., 1999). Neuerdings wird dagegen vermutet, dass Furanone den raschen Abbau des Rezeptorproteins bewirken (Manefield *et al*., 2002). Wenn auch der genaue Wirkmechanismus der Furanone noch diskutiert wird, konnte doch gezeigt werden, dass Furanone in Konzentrationen, die das Wachstum von Bakterien nicht hemmen, spezifisch deren Kommunikation unterbinden, was wiederum Phänotypen wie die Produktion von Virulenzfaktoren oder die Ausbildung von Biofilmen unterdrückt (Rasmussen *et al*., 2000; Manefield *et al*., 2001; Hentzer *et al*., 2002).

Der zweite Weg, der Abbau von AHL-Molekülen, konnte für *Bacillus subtilis* und *Variovorax paradoxus* gezeigt werden (Dong *et al*., 2000; Leadbetter und Greenberg, 2000). Es wird angenommen, dass diese Bakterien durch die Störung der Kommunikation möglicher Nährstoffkonkurrenten einen Selektionsvorteil haben. Das *aiiA* Gen von *B. subtilis* kodiert für eine AHL-Lactonase, die verschiedene AHL-Moleküle inaktivieren kann. Um Möglichkeiten für die Anwendung dieses Enzyms zu untersuchen, wurde das *aiiA*-Gen in verschiedene Pflanzen eingebracht. Diese zeigten tatsächlich eine deutlich erhöhte Resistenz gegen das pflanzenpathogene Bakterium *Erwinia carotovora*, bei dem die Produktion von Virulenzfaktoren, ähnlich wie z.B. bei *P. aeruginosa*, unter der Kontrolle eines AHL-abhängigen QS-Systems steht (Dong *et al*., 2001).

C.7 Ausblick

Zweidimensionale Protein-Gelelektrophorese. Als Ergänzung zu dem in dieser Arbeit beschriebenen genetischen Ansatz zur Untersuchung der Biofilmbildung von *B. cepacia* sind Proteomanalysen geplant. Um Proteine zu identifizieren, deren Synthese durch das Wachstum auf Oberflächen induziert oder reprimiert wird, soll das Proteinprofil von in Flüssigmedium kultivierten *B. cepacia*-Zellen mit jenem sessiler Zellen verglichen werden. Proteine mit veränderter Expression in Biofilmen können nach Sequenzierung des N-Terminus durch einen Datenbankabgleich identifiziert werden. Da in der vorliegenden Arbeit gezeigt wurde, dass einige der für die Ausbildung von Biofilmen essentiellen Proteine mit der Zellmembran assoziiert sind, soll besonderes Augenmerk auf die Analyse von Membranfraktionen gelegt werden. Entsprechende Protokolle zur Zellfraktionierung und Probenvorbereitung werden zur Zeit innerhalb der Arbeitsgruppe entwickelt.

Das Oberflächenprotein AidA. Zwar wurde durch unsere bisherigen Arbeiten deutlich, dass die Biofilmdifferenzierung unter der Kontrolle des QS-Systems steht, doch noch sind die dafür verantwortlichen Strukturgene nicht bekannt. Bei der Analyse der Oberflächenproteine von *B. cepacia* H111 und der AHL-negativen Mutanten H111-I und H111-R mittels eindimensionaler Gelelektrophorese fiel ein 19 kDa-Protein auf, dessen Expression in beiden Mutanten stark reduziert ist. Da es möglich war, die Produktion dieses Proteins durch Zugabe von AHL-Molekülen zum Medium von H111-I wieder auf das Niveau des Wildtyps zu bringen, handelt es sich hierbei offenbar um ein QS-reguliertes Protein. Der Abgleich seiner N-terminalen Aminosäuresequenz mit verschiedenen Datenbanken ergab eine Ähnlichkeit von 55 % mit dem Protein AidA ('autoinducer dependent protein A') von *Ralstonia solanacearum*, einem mit *B. cepacia* nah verwandten pflanzenpathogenen Bakterium (Flavier *et al*., 1997). Diesem Protein konnte bisher noch keine Funktion zugeordnet werden, es ist jedoch bekannt, dass seine Expression vom QS-System von *R. solanacearum* kontrolliert wird.
Der relative hohe Anteil von AidA an den Zelloberflächenproteinen von *B. cepacia* von mehr als 15 % lässt vermuten, dass es sich hierbei um ein Strukturprotein, möglicherweise um ein Adhäsin, handelt. Um die Funktion dieses Proteins genauer zu charakterisieren, wurde es bereits in größerem Maßstab aufgereinigt und polyklonale Antikörper dagegen hergestellt. Diese Antikörper sollen dazu genutzt werden, um durch Immunfluoreszenzmarkierung bzw. Immunogoldmarkierung und anschließende Elektronenmikroskopie die genaue Lokalisation von AidA festzustellen. Auch funktionelle Analysen sind geplant. So soll untersucht werden, ob eine Inkubation von *B. cepacia* mit den anti-AidA Antikörpern die Fähigkeit der Bakterien zur Ausbildung von Biofilmen beeinflusst. Ein zweiter Ansatz zur funktionellen Charakterisierung ist die Konstruktion einer AidA-negativen Mutante. Das *B. cepacia*-Genom wird zur Zeit vom Sanger-Institut (http://www.sanger.ac.uk/) sequenziert. Da die Sequenz des Genoms mittlerweile fast vollständig veröffentlicht wurde, ist es nunmehr möglich, das *aidA*-Gen zu amplifizieren. Dies stellt die Basis für die Konstruktion einer definierten *aidA*-Mutante dar, die bezüglich ihrer Fähigkeit, Biofilme zu bilden, untersucht werden soll.

D. ZUSAMMENFASSUNG

Die Bildung von Biofilmen durch Bakterien ist die Ursache vieler chronischer Infektionen. Die beiden opportunistisch pathogenen Bakterien *Burkholderia cepacia* und *Pseudomonas aeruginosa* bilden oft gemischte Biofilme in den Lungen von Personen, die an zystischer Fibrose (CF) leiden.

Um Gene zu identifizieren, die an der Biofilmbildung von *B. cepacia* beteiligt sind, wurde eine Transposon-Mutantenbank in einem klinischen Isolat angelegt. 13 Mutanten wiesen Defekte in der Bildung von Biofilmen auf einer Polystyrol-Oberfläche auf. Die Sequenzanalyse der die Transposons flankierenden DNA erlaubte im Fall von elf der Mutanten eine Identifizierung der inaktivierten Gene. Es zeigte sich, dass neben der Zelloberfläche (Zelloberflächenproteine und Zellmembran) und der Zellform auch regulatorische Faktoren eine Rolle bei der Biofilmbildung spielen. Da die verwendete Screeningmethode die Isolierung von Mutanten mit Defekten in späten Stadien der Biofilmbildung begünstigte, sind die identifizierten Gene vorwiegend an der Reifung von Biofilmen beteiligt. Dies wurde auch durch die Untersuchung der dreidimensionalen Strukturen von Mutantenbiofilmen bestätigt: sie alle zeigten deutliche Abweichungen von der Architektur des vom Wildtyp gebildeten Biofilms.

Viele Bakterien nutzen *N*-Acyl-homoserinlacton-Signalmoleküle zur Zell-Zell-Kommunikation, um die Expression von Genen in Abhängigkeit von der Zelldichte zu koordinieren. Dieser Vorgang wird als ‘Quorum sensing’ (QS) bezeichnet. Die Beobachtung, dass fünf der regulatorischen Mutanten stark reduzierte Mengen von AHL-Molekülen produzierten, weist auf die zentrale Bedeutung von QS für die Bildung ausgereifter Biofilme durch *B. cepacia* hin. Eine der C8-HSL-negativen Mutanten trug eine Transposoninsertion im QS-Regulator *cepR*. Die anderen vier waren in offenbar dem *cep*-System übergeordneten Genen mutiert, was einen Einblick in die Komplexität der QS-Regulationskaskade bei *B. cepacia* erlaubte.

Darüber hinaus konnte gezeigt werden, dass in gemischten Biofilmen aus *B. cepacia* und *P. aeruginosa* auch artübergreifende Kommunikation stattfindet. Da ‘Quorum sensing’ von beiden Arten auch zur Regulation der Expression von Virulenzfaktoren genutzt wird, kann dieser Vorgang die Pathogenität des gemischten Zellverbundes entscheidend beeinflussen.

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F. ANHANG

Anhang 1

The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility

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The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility

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***Burkholderia cepacia* and *Pseudomonas aeruginosa* often co-exist as mixed biofilms in the lungs of patients suffering from cystic fibrosis (CF). Here, the isolation of random mini-Tn*5* insertion mutants of *B. cepacia* H111 defective in biofilm formation on an abiotic surface is reported. It is demonstrated that one of these mutants no longer produces *N*-acylhomoserine lactones (AHLs) due to an inactivation of the *cepR* gene. *cepR* and the *cepI* AHL synthase gene together constitute the *cep* quorum-sensing system of *B. cepacia*. By using a gene replacement method, two defined mutants, H111-I and H111-R, were constructed in which *cepI* and *cepR*, respectively, had been inactivated. These mutants were used to demonstrate that biofilm formation by *B. cepacia* H111 requires a functional *cep* quorum-sensing system. A detailed quantitative analysis of the biofilm structures formed by wild-type and mutant strains suggested that the quorum-sensing system is not involved in the regulation of initial cell attachment, but rather controls the maturation of the biofilm. Furthermore, it is shown that *B. cepacia* is capable of swarming motility, a form of surface translocation utilized by various bacteria to rapidly colonize appropriate substrata. Evidence is provided that swarming motility of *B. cepacia* is quorum-sensing-regulated, possibly through the control of biosurfactant production. Complementation of the *cepR* mutant H111-R with different biosurfactants restored swarming motility while biofilm formation was not significantly increased. This result suggests that swarming motility *per se* is not essential for biofilm formation on abiotic surfaces.**

Keywords: cystic fibrosis, *N*-acylhomoserine lactone, biosurfactant

INTRODUCTION

To date, most research in molecular microbiology has focused on the analysis of planktonic cells in pure cultures and it was not until recently that it was fully appreciated that in nature bacteria predominantly exist as sessile, surface-associated communities, referred to as biofilms (for recent reviews see O'Toole *et al*., 2000; Tolker-Nielsen & Molin, 2000; Watnick & Kolter, 2000). Many persistent and chronic bacterial infections, including periodontitis, otitis media, biliary tract infection and endocarditis, are intrinsically linked to the formation of biofilms. Moreover, various medical implants are prone to colonization by pathogenic bacteria (Costerton *et al*., 1999). Bacterial biofilm infections are particularly problematic as sessile bacteria can withstand host immune responses and are markedly more resistant to antibiotics (up to 1000-fold) and biocides than cells grown in suspension (Schierholz *et al*., 1999; Xu *et al*., 2000).

The opportunistic pathogenic bacterium *Pseudomonas aeruginosa* is capable of chronically colonizing the lungs of patients suffering from cystic fibrosis (CF), the most common lethal inherited disease among the Caucasian population (Koch & Høiby, 1993; Govan & Deretic, 1996; Tümmler & Kiewitz, 1999). During chronic infection, *P. aeruginosa* produces copious amounts of alginate, which forms a matrix completely embedding

Abbreviations: AHL, *N*-acylhomoserine lactone; CF, cystic fibrosis; CLSM, confocal laser scanning microscopy; GFP, green fluorescent protein; C8-HSL, *N*-octanoylhomoserine lactone; C6-HSL, *N*-hexanoylhomoserine lactone; 3-oxo-C6-HSL, *N*-(3-oxohexanoyl)homoserine lactone.

the cells, and becomes highly resistant to antibiotic treatment. These observations led to the suggestion that *P. aeruginosa* may exist as a biofilm in the CF lung (Lam *et al.*, 1980; Costerton *et al.*, 1999). This hypothesis was recently corroborated through profiling of *N*-acylhomoserine lactone (AHL) signal molecules (Singh *et al.*, 2000). *Burkholderia cepacia* has been recognized as another important pathogen in patients with CF. Infection with *B. cepacia* often occurs in patients who are already colonized with *P. aeruginosa*. In fact, it has been suggested that *P. aeruginosa* exoproducts may modify the epithelial cell surface of the lung such that attachment of *B. cepacia* is facilitated (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).

In both *P. aeruginosa* (for reviews see de Kievit & Iglewski, 2000; Parsek & Greenberg, 2000; Williams *et al.*, 2000) and *B. cepacia* (Lewenza *et al.*, 1999), expression of various virulence factors is controlled by AHL-dependent quorum-sensing systems. These regulatory systems ensure that pathogenic traits are only expressed when the bacterial population density is high enough to overwhelm the host before it is able to mount an efficient response. Interestingly, for *P. aeruginosa* it has been demonstrated that the architecture of biofilms formed on an abiotic surface is also quorum-sensing-controlled (Davies *et al.*, 1998). These results argue in favour of functional overlaps between factors necessary for biofilm formation and pathogenicity. The quorum-sensing system of *B. cepacia* K56-2 (genomovar III) has been recently identified (Lewenza *et al.*, 1999). This density-dependent regulatory system relies on two proteins: the AHL synthase CepI, which directs the synthesis of *N*-octanoylhomoserine lactone (C8-HSL) and, as a minor product, *N*-hexanoylhomoserine lactone (C6-HSL) (Gotschlich *et al.*, 2001), and CepR, which after binding of C8-HSL is thought to activate or repress transcription of target genes. The *cep* system was demonstrated to positively regulate protease production and to repress synthesis of the siderophore ornibactin (Lewenza *et al.*, 1999). Since the two bacteria not only form mixed biofilms in CF lungs but also utilize the same chemical language, it appears likely that the two species synergistically enhance the others' virulence (McKenney *et al.*, 1995).

The recent development of a simple biofilm assay has greatly facilitated the analysis of the genetic mechanisms underlying biofilm formation. In this assay, bacteria are grown in the wells of microtitre dishes in which the cells attach to the abiotic surface. Following removal of planktonic cells, the established biofilm is quantified after staining with crystal violet. Over the past few years, this assay has been extensively used to identify genes involved in biofilm formation in a number of bacteria, including *Escherichia coli* (Pratt & Kolter, 1998), *Pseudomonas fluorescens* (O'Toole & Kolter, 1998a), *P. aeruginosa* (O'Toole & Kolter, 1998b), *Vibrio cholerae* (Watnick & Kolter, 1999) and *Streptococcus gordonii* (Loo *et al.*, 2000). In the present study we have employed this assay to isolate random transposon insertion mutants in *B. cepacia* H111 that are defective in biofilm formation on a polystyrene surface. One of these mutants is demonstrated to bear the transposon within the *cepR* gene. This finding prompted us to investigate the role of the *cep* quorum-sensing system in the strain's ability to form biofilms. It is shown that both biofilm formation and swarming motility are *cep*-regulated phenotypes.

METHODS

Strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, the strains were grown at 37 °C in modified Luria–Bertani (LB) broth (Andersen *et al.*, 1998) or AB minimal medium (Clark & Maaløe, 1967) supplemented with 10 mM citrate. Solid media were routinely solidified with 1·5% (w/v) agar. Growth media for examination of swimming and swarming motility contained 0·3% and 0·4% agar, respectively (Eberl *et al.*, 1996). Antibiotics were added as required at final concentrations of 100 μg ampicillin ml^{-1}, 10 μg tetracycline ml^{-1}, 20 μg gentamicin ml^{-1} and 10 μg chloramphenicol ml^{-1}. Kanamycin was used at 50 μg ml^{-1} for *E. coli* and 100 μg ml^{-1} for *B. cepacia*. Tellurite concentration was 100 μg ml^{-1}. For complementation of H111-I, 200 nM C8-HSL and/or 200 nM C6-HSL were added. Growth of liquid cultures was monitored spectrophotometrically by an Ultrospec Plus spectrophotometer (Pharmacia) by measurement of optical density at 600 nm.

Conjugative plasmid transfer. Plasmids were delivered to *B. cepacia* by triparental mating as described by de Lorenzo & Timmis (1994). Briefly, donor and recipient strains and also the helper strain *E. coli* HB101(pRK600) were grown at 37 °C overnight in 5 ml LB supplied with the appropriate antibiotics. Following subculturing to an OD_{600} of 0·9, the cells from 2 ml of culture were harvested, washed and resuspended in 200 μl LB. Donor and helper cells (100 μl each) were mixed and incubated for 30 min at room temperature. Recipient cells (200 μl) were added and the mixture was spot-inoculated onto the surface of prewarmed LB agar plates. After overnight incubation at 37 °C, the cells were scraped off and were resuspended in 1 ml 0·9% NaCl. Serial dilutions were plated on LB medium containing antibiotics for counter-selection of donor, helper and untransformed recipient cells.

DNA manipulations and nucleotide sequencing. Cloning, restriction enzyme analysis and transformation of *E. coli* were performed essentially as described by Sambrook *et al.* (1989). PCR was performed using the TaKaRa rTaq DNA polymerase (TaKaRa Shuzo). Plasmid DNA was isolated with the QIAprep Spin Miniprep kit and chromosomal DNA from *B. cepacia* was purified with the DNeasy Tissue kit. DNA fragments were purified from agarose gels using the QIAquick Gel Extraction kit (all kits were from Qiagen).

For complementation of *B. cepacia* H111-R, we constructed plasmid pBAH27 (*cepR*$^+$) as follows. The *cepR* gene was PCR-amplified using primers cepR-R (5′-GGGGTACCAACCTG-ACAAGTATGACAGCG-3′) and cepR-OV (5′-GGGGTACC GGATGAGCATGGAGAAAAGC-3′) (*Kpn*I restriction sites are underlined). Following digestion with *Kpn*I, the PCR fragments were inserted into the broad-host-range vector pBBR1MCS-5 cut with the same enzyme. The plasmid

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q} *Z*ΔM15 Tn*10* (Tet^{R})]	Stratagene
MT102	*araD139* (*ara–leu*)*7697* Δ*lac thi hsdR*	Laboratory collection
CC118 (λpir)	Δ(*ara–leu*) *araD* Δ*lacX74 galE galK phoA thi-1 rpsE rpoB argE*(Am) *recA*, λpir lysogen	Herrero *et al.* (1990)
B. cepacia		
H111	CF isolate from a patient at the Medizinische Hochschule Hannover (1993), genomovar III	Römling *et al.* (1994); Gotschlich *et al.* (2001)
m64	CepR::Tn*5*Km2-*luxCDABE* derivative of H111	This study
H111-I	Km^{r}, *cepI*::Km mutant of H111	This study
H111-R	Km^{r}, *cepR*::Km mutant of H111	This study
Plasmids		
pAG	Gm^{r} Km^{r}, pEX18Gm derivative for inactivation of *cepI*	This study
pBAH27	pBBR1MCS-5 containing the *cepR* gene of *B. cepacia* H111	This study
pBAH33	Gm^{r} Km^{r}, pEX19Gm derivative for inactivation of *cepR*	This study
pBBR1MCS-5	Gm^{r}, broad-host-range cloning vector	Kovach *et al.* (1995)
pEX18Gm	Gm^{r}, *oriT*$^{+}$ *sacB*$^{+}$, gene replacement vector with multiple cloning site from pUC18	Hoang *et al.* (1998)
pEX19Gm	Gm^{r}, *oriT*$^{+}$ *sacB*$^{+}$, gene replacement vector with multiple cloning site from pUC19	Hoang *et al.* (1998)
pGEM-3Zf(+)	Ap^{r}, multicopy cloning vector	Promega
pMH94	Ap^{r} Km^{r}, delivery vector for mini-Tn*5* Tel^{r} *kilAtelAB*-$P_{A1/04/03}$-*gfp mut3*-T_0-T_1	M. Hentzer & M. R. Parsek (unpublished)
pRK600	Cm^{r}, ColE1*oriV*, RK-2Mob^{+} RK2-Tra^{+}, helper plasmid in triparental matings	de Lorenzo & Timmis (1994)
pSB403	Tc^{r}, *luxRI*′::*luxCDABE* transcriptional fusion, bioluminescent AHL sensor plasmid	Winson *et al.* (1998b)
pUTmini-Tn*5*Km2-*luxCDABE*	Km^{r}, delivery vector for miniTn*5*Km2-*luxCDABE*	Winson *et al.* (1998a)

containing the insert in the orientation placing the *cepR* gene downstream of the P_{lac} promoter of the cloning vector was chosen and this construct was designated pBAH27. For flow-chamber experiments, the strains were tagged with green fluorescent protein (GFP). This was accomplished by the insertion of a $P_{A1/04/03}$-*gfp*-T0-T1 transposon cassette (Andersen *et al.*, 1998) into the chromosomes of target strains using the suicide construct pMH94 (M. Hentzer & M. R. Parsek, unpublished results). Plasmid pMH94 was delivered to target strains by conjugative transfer and integrants were selected on PIA medium (Becton Dickinson Biosciences) containing tellurite.

Sequencing was performed by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) in a LI-COR 4200 DNA sequencer. The primer 5′-CAGATCTGATCAAGAG-ACAG-3′, which binds to the I-end of the Tn*5* transposon, was used for determination of the transposon insertion point in *B. cepacia* m64. DNA sequences were compared to other sequences in GenBank using the on-line BLAST search engine at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Construction of a *B. cepacia* H111 mutant bank. The hybrid transposon mini-Tn*5*Km2-*luxCDABE* was randomly inserted into the chromosome of *B. cepacia* H111 by the triparental mating procedure described above. Transconjugants were selected on LB medium containing kanamycin and tetracycline. These random insertion mutants were picked and grown in 150 µl LB medium in the wells of polypropylene MicroWell dishes (Nunc). For storage, 75 µl 50% (v/v) glycerol was added and the dishes were frozen at −80 °C.

Screen for mutants defective in biofilm formation. Biofilm formation in polystyrene microtitre dishes was assayed essentially as described by O'Toole & Kolter (1998a) and Pratt & Kolter (1998) with a few modifications. Cells were grown in the wells of the microtitre dishes in 100 µl AB medium supplemented with 10 mM citrate for 48 h at 30 °C. The medium was then removed and 100 µl of a 1% (w/v) aqueous solution of crystal violet was added. Following staining at room temperature for 20 min, the dye was removed and the wells were washed thoroughly. For quantification of attached cells, the crystal violet was solubilized in a 80:20 (v/v) mixture of ethanol and acetone and the absorbance was determined at 570 nm.

Detection and characterization of AHLs. Production of AHLs was investigated with the aid of the bioluminescent plasmid sensor pSB403 (Winson *et al.*, 1998b). 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 *Photobacterium fischeri* relies on *N*-(3-oxohexanoyl)homoserine lactone (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 (Winson *et al.*, 1998b; Geisenberger *et al.*, 2000). Bioluminescence was detected either with the highly sensitive photon-counting camera C2400-40 (Hamamatsu Photonics) or by exposure to an X-ray film. For more detailed analysis, the AHL molecules were extracted from spent culture supernatants of the strains, separated by 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 (Shaw *et al.*, 1997; Geisenberger *et al.*, 2000). Routinely, AHLs were extracted twice with dichloromethane (250:100 supernatant/dichloromethane) from 250 ml sterile-filtered supernatants of *B. cepacia* cultures grown in AB minimal medium containing 10 mM citrate 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. Samples (10 µl) were then 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 in water (60%, v/v) as the mobile phase. For detection of AHLs, the TLC plate was overlaid with a thin film of LB agar (143 ml) seeded with 7 ml of an exponentially grown AHL biosensor and was then 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) relative to those for the synthetic AHL standards.

For quantification of AHL signal molecules, 100 µl of filter-

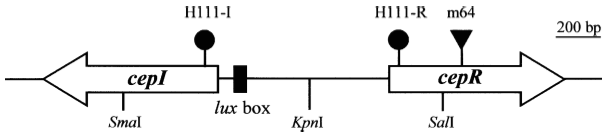

Fig. 1. Physical and genetic map of the *cep* quorum-sensing region of *B. cepacia* H111. Genes are marked by open arrows, with the direction of the arrowheads indicating the direction of transcription. A palindrome sequence in the *cepI* promoter region that is highly homologous to the *lux* box consensus sequence is indicated. Relevant restriction sites are shown. The location of the mini-Tn*5*Km2-*luxCDABE* element in *B. cepacia* m64 is indicated by a triangle. The positions of the *npt* cassettes in the *cepI* mutant H111-I and the *cepR* mutant H111-R are indicated by circles.

sterilized supernatants of cultures grown in LB medium to an OD_{600} of 3·0 was added to 100 µl of an exponential culture of *E. coli* MT102(pSB403) in the wells of a FluoroNunc Polysorp microtitre dish. Following incubation at 30 °C for 6 h, bioluminescence was measured with a Lamda Fluoro 320 Plus reader (Bio-Tek Instruments).

Exoenzyme and siderophore production. Exoenzyme and siderophore production was tested by streaking strains on appropriate indicator plates. Proteolytic activity was determined on LB medium supplemented with 2% skim milk, chitinolytic activity on ethylene glycol chitin agar (Connell *et al*., 1998) and lipolytic activity on tributyrin agar base containing 1% glycerol tributyrate (both Merck). Clear haloes around the colonies after incubation at 37 °C overnight indicated exoenzyme activity. Siderophore production was tested by growing strains on CAS agar (Schwyn & Neilands, 1987) for 24 h. Siderophores remove the iron from the CAS dye complex, resulting in a colour change around the colonies from blue to orange.

Construction of *B. cepacia* H111 *cepI* and *cepR* mutant strains. Defined *cep* mutants (Fig. 1) were constructed by the gene replacement method described by Hoang *et al*. (1998). For the construction of a *cepR* mutant, two DNA fragments were PCR-amplified: an 850 bp *Eco*RI–*Sac*I fragment spanning the intergeneric region plus the first 75 bp of *cepR* using the primer pair intercep-f-Eco (5′-GGAATTCGAGATCCGCCGCGAGTTCG-3′) and intercep-r-Sac (5′-GATCCGCTGGAAGAGCTCC-3′), and a 760 bp *Sph*I–*Hin*dIII fragment containing the 3′ region of the *cepR* gene using the primer pair cepR-f-Sph (5′-ACATGCATGCGCTCGGATTCGAATACTGC-3′) and cepR-r-Hind (5′-CCCAAGCTTAGAAGCTCGAGCAGATCGC-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 the gene replacement vector pEX19Gm (Hoang *et al*., 1998). Next, the *npt* gene from transposon Tn*903* (Oka *et al*., 1981), which confers resistance to kanamycin, was cloned as a 1·7 kb *Bam*HI fragment into the vector cut with the same enzyme. The final construct, which was designated pBAH33, was transferred to *B. cepacia* H111 and integrants were selected on LB medium containing kanamycin and tetracycline. To screen for gene replacement mutants, Kan^r clones were tested for gentamicin sensitivity as the gentamicin resistance gene is lost in the case of a double crossover event. One mutant, which was designated *B. cepacia* H111-R, was chosen and the correct genetic structure of the strain was confirmed by Southern Blot analysis. Construction of a defined *cepI* mutant was performed as described for the *cepR* mutant, except that the gene replacement vector pEX18Gm and two different PCR fragments were used. An 800 bp *Eco*RI–*Sac*I fragment spanning the intergeneric region plus 100 bp of *cepR* was amplified with the primer pair igR-f-Eco (5′-GGAATTCCCAGTATTCGAATCCGAGCCGC-3′) and igR-r-Sac (5′-CGAGCTCGGGATGTCCTCGGATCTGTGC3′), and a 650 bp *Bam*HI–*Hin*dIII fragment containing the 5′ region of *cepI* was amplified using the primer pair cepI-f-Bam (5′-CGGGATCCCGCCTTCGTTCACGAGGAAGGG-3′) and cepI-r-Hind (5′-CCCAAGCTTGGGCGCGCGTTCCGGCTCAGG-3′). The final gene replacement construct was designated pAG and the respective *B. cepacia* H111 *cepI* mutant was named H111-I.

Monitoring of biofilm formation by confocal laser scanning microscopy (CLSM) and image analysis. Biofilms were grown in artificial flow cells supplied with AB medium and containing 1 mM glucose (ABG). The flow system was assembled and prepared as described previously (Christensen *et al*., 1999). The substratum consisted of a microscope glass coverslip (Knittel Gläser). Overnight cultures in ABG medium were subcultured to an OD_{600} of 0·7 before dilution in 0·9% NaCl to an OD_{600} of 0·1. Aliquots (300 µl) of these dilutions were used to inoculate the flow channels. Medium flow was kept at a constant rate of 0·7 mm s^{-1} by a Watson-Marlow 205S peristaltic pump. Incubation temperature was 30 °C.

Microscopic inspection and image acquisition were performed on a confocal laser scanning microscope (TCS4D; Leica Lasertechnik) equipped with a 63 × /1.32-0.6 oil objective. For statistical evaluation of biofilm structures, a 40 × /0·75 air objective was used. 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) running on a Silicon Graphics Indigo 2 workstation.

For statistical evaluation of biofilm structures, three independent rounds of biofilm experiments were performed, and in each round, each strain was grown in two separate channels. Seven image stacks were taken of each channel every 24 h for 7 d after inoculation. These images were analysed by the computer program COMSTAT, which comprises various features for quantifying three-dimensional biofilm image stacks (Heydorn *et al*., 2000). The parameters used for characterization of biofilm architecture included biomass, substratum coverage, mean thickness and roughness coefficient.

RESULTS

Screening for biofilm-defective mutants

To determine the optimum experimental conditions for attachment of *B. cepacia* H111 to abiotic surfaces, biofilm assays were carried out under various conditions. While the strain formed thick biofilms in polystyrene microtitre dishes, no attachment was observed in dishes made of polypropylene. In contrast to other bacteria (O'Toole & Kolter, 1998a; Danese *et al*., 2000; Loo *et al*., 2000), biofilm formation by *B. cepacia* H111 is virtually independent of medium composition. AB minimal medium supplemented with glucose or citrate supported surface colonization as well as rich media such as LB. Furthermore, incubation temperatures ranging from 30 to 37 °C did not significantly affect biofilm yields (data not shown). For routine biofilm assays we used AB minimal medium containing 10 mM citrate and incubated the microtitre dishes for 48 h at 30 °C.

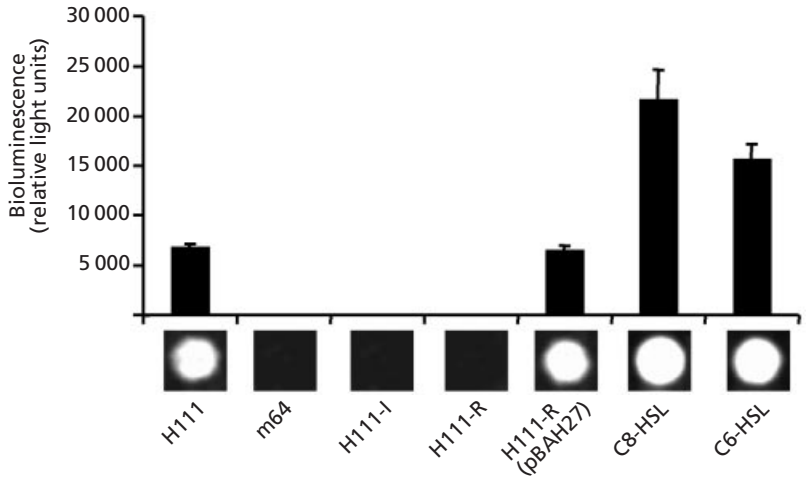

Fig. 2. AHL production by *B. cepacia* H111 is subject to positive feedback regulation. Aliquots (100 μl) of filter-sterilized culture supernatants of wild-type H111, the *cepI* mutant H111-I, the *cepR* mutants m64 and H111-R and the complemented *cepR* mutant H111-R(pBAH27) were mixed with an exponential culture of the AHL sensor MT102(pSB403) in a microtitre dish. Following incubation for 6 h, bioluminescence was quantified with a luminometer. Error bars represent the standard deviation of the mean for four independent wells. Bioluminescence was also visualized with the aid of a photon camera (pictures shown below the *x* axis). C8-HSL (10 μM) and C6-HSL (1 μM) standards were included as controls.

Table 2. Phenotypic characterization of *B. cepacia* H111 and *cep*-defective mutants

Production of AHLs was determined by testing culture supernatants for stimulation of the bioluminescent sensor plasmid pSB403. Synthesis of extracellular hydrolytic enzymes and siderophore activities were assessed by streaking strains on appropriate indicator plates as described in Methods. Swimming and swarming motility was tested on media solidified with 0·3% and 0·4% agar, respectively. Wild-type H111, the *cepR* mutant H111-R, the complemented *cepR* mutant H111-R(pBAH27) and the *cepI* mutant H111-I in the presence or absence of C8-HSL (200 nM) were investigated. +, Activity exhibited by the wild-type; (+) significantly reduced activity; –, no detectable activity; NA, not applicable.

Phenotype	H111	H111-R	H111-R (pBAH27)	H111-I	H111-I + AHL
AHL	+	–	+	–	NA
Protease	+	–	+	–	+
Chitinase	+	(+)	+	(+)	+
Lipase	+	+	+	+	+
Siderophore	+	(+)	+	(+)	+
Swarming	+	–	+	–	+
Swimming	+	+	+	+	+

The suicide vector pUT (de Lorenzo & Timmis, 1994) was used to deliver the hybrid transposon mini-Tn*5*Km2-*luxCDABE* (Winson *et al*., 1998a) into the *B. cepacia* H111 chromosome. A collection of 5000 random insertion mutants was screened for ability to form biofilms. A total of eighteen mutants which were to different degrees defective in biofilm formation was obtained (data not shown). During the course of a detailed phenotypical characterization of these mutants we noticed that one, m64, was deficient in the production of AHL signal molecules. As shown in Fig. 2, the wild-type strain H111 strongly activated the bioluminescent AHL sensor plasmid pSB403 while no activation was observed with m64. This result is reminiscent of the situation found with *P. aeruginosa*, for which it has been shown that development of a mature biofilm is quorum-sensing-regulated (Davies *et al*., 1998). We therefore focused our further investigations on the analysis of this mutant.

Since *B. cepacia* H111 produces two AHL molecules, C8-HSL and C6-HSL, in a ratio of 10:1 (Gotschlich *et al*., 2001), we next extracted the signal molecules from spent culture supernatants of the wild-type and the mutant and analysed them by TLC. This analysis showed that the mutant is impaired in the production of both molecules (data not shown). Addition of 200 nM C8-HSL to the growth medium did not restore biofilm formation (data not shown). To further characterize the nature of the transposon insertion in strain m64, the mutated locus was cloned into the cloning vector pGEM-3Zf(+) as a *Sph*I fragment, selecting for transposon-encoded kanamycin resistance. The resulting plasmid contains part of the transposon and approximately 3 kb of chromosomal DNA upstream of the transposon insertion point. Nucleotide sequence analysis of the flanking DNA revealed that the transposon had inserted into the *cepR* gene of *B. cepacia* H111 at position 356 (Fig. 1; GenBank accession no. AF330020). These data are fully consistent with previous results that showed that expression of *cepI* and thus AHL signal production is regulated by CepR (Lewenza *et al*., 1999). More importantly, these results suggest that the *cep* quorum-sensing system is involved in the regulation of biofilm formation in *B. cepacia* H111.

Construction and characterization of defined *B. cepacia* H111 *cep* mutants

To investigate the role of the *cepIR* genes in biofilm formation in greater detail, we constructed site-directed insertion mutations in the two genes by using a gene replacement method (see Methods for details and Fig. 1). The genetic structure of the two mutants, which were designated H111-I and H111-R, respectively, was confirmed by Southern blot analysis (data not shown).

As expected, neither the *cepI* mutant H111-I nor the *cepR* mutant H111-R produced detectable amounts of

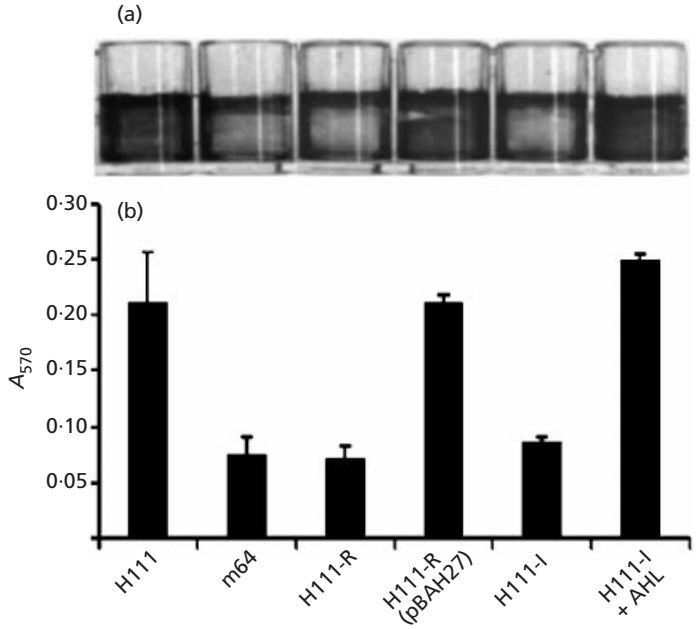

Fig. 3. The *cep* quorum-sensing system controls biofilm formation by *B. cepacia* H111. Strains were grown in AB minimal medium supplemented with 10 mM citrate in the wells of polypropylene microtitre dishes. After incubation for 48 h at 30 °C, planktonic cells were removed and attached cells were stained with crystal violet. Biofilms formed by wild-type H111, the *cepR* mutants m64 and H111-R, the complemented *cepR* mutant H111-R(pBAH27) and the *cepI* mutant H111-I in the presence or absence of C8-HSL (200 nM) are shown in (a); quantification of the biofilm-associated dye is shown in (b). Error bars represent the standard deviation of the mean for six independent wells.

AHLs (Fig. 2). However, production of AHLs was restored to wild-type levels when H111-R was complemented with plasmid pBAH27, which contains the *cepR* gene inserted into the broad-host-range vector pBBR-1MCS-5.

B. cepacia produces different siderophores and a number of exoenzymes that are thought to be pathogenesis factors in humans as well as in plants (Lonon *et al*., 1988; McKevitt *et al*., 1989; Gessner & Mortensen, 1990; Yohalem & Lorbeer, 1994; Darling *et al*., 1998). In a recent study it was shown that the *cep* system of *B. cepacia* K56-2 is involved in the regulation of the synthesis of extracellular enzymes and siderophores (Lewenza *et al*., 1999). We therefore tested the *B. cepacia* H111 wild-type and the two mutants H111-I and H111-R for the production of extracellular protease, lipase, chitinase and siderophores on appropriate indicator plates. The results of these investigations are summarized in Table 2. Consistent with the results reported by Lewenza *et al*. (1999), both mutants showed a clear reduction in protease activity. Furthermore, proteolytic activities of the mutants were completely restored when mutant H111-I was grown in the presence of 200 nM C8-HSL or when plasmid pBAH27 (*cepR*$^+$) was transferred to mutant H111-R. Both mutants were found to produce significantly lowered amounts of siderophores as assessed on CAS indicator plates. As for proteolytic activity, these defects were restored to wild-type levels by the external addition of 200 nM C8-HSL to H111-I or by complementation of H111-R with plasmid pBAH27 (*cepR*$^+$). These data are in contrast to the results of the above-mentioned study, which showed that inactivation of either *cepI* or *cepR* results in an up-regulation of siderophore production in *B. cepacia* K56-2. Most likely, this apparent discrepancy can be attributed to the different strains used in the studies. Likewise, while Lewenza *et al*. (1999) observed reduced lipase activity with the *cepR* but not with the *cepI* mutant of K56-2, we were unable to detect any difference in the lipase activities of H111, H111-I and H111-R. Chitinase activity was slightly reduced in the two mutants when compared with the wild-type, and since complementation (as described above) restored the defects we suggest that chitinase production in *B. cepacia* H111 is, at least in part, regulated by quorum sensing.

The *cep* system of *B. cepacia* H111 controls biofilm maturation

We next tested the two mutants H111-I and H111-R for their abilities to form biofilms in microtitre dishes as described above. In agreement with our initial observation with strain m64, we found that both mutants were defective in biofilm formation (Fig. 3). However, addition of 200 nM C8-HSL to the medium completely restored biofilm formation by mutant H111-I. Likewise, H111-R harbouring plasmid pBAH27 formed wild-type biofilms. As one reason for lowered biofilm formation capability is reduced growth, we determined the growth rates of H111, H111-I and H111-R in AB minimal medium supplemented with 10 mM citrate. Wild-type and mutant strains were found to grow equally well in liquid culture (data not shown).

To further analyse the role of the *cep* system in biofilm formation, we employed artificial flow cells, which allow biofilm development to be followed on a glass surface under highly defined conditions in real time (Christensen *et al*., 1999). Moreover, by the use of a confocal laser scanning microscope the three-dimensional structure of the biofilm can be reconstructed. For this analysis, it was necessary to tag *B. cepacia* H111 and the two mutants H111-I and H111-R with GFP. This was accomplished by inserting a $P_{A1/04/03}$-*gfp*-T0-T1 transposon cassette (Andersen *et al*., 1998) randomly

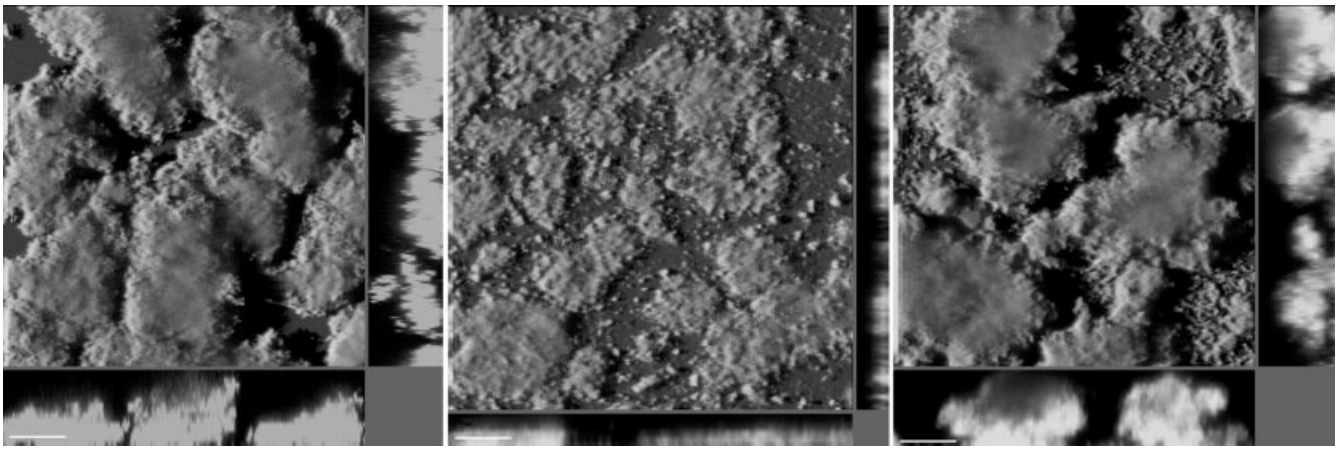

Fig. 4. Biofilm formation by *B. cepacia* H111 is quorum-sensing-regulated. Flow chambers were inoculated with *gfp*-tagged derivatives of wild-type H111 and of the *cepI* mutant H111-I in the presence or absence of 200 nM C8-HSL. The CLSM pictures shown were taken 4 d after inoculation. The larger central plots are simulated fluorescence projections. Shown in the right and lower frames are vertical sections through the biofilms. Bars, 20 μm.

into the chromosome of each of the three strains using the suicide plasmid pJMT6 (Sanchez-Romero *et al*., 1998). The tagged strains used for further investigations were carefully tested with respect to growth rates in liquid medium and biofilm formation in microtitre dishes 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 on a daily basis for 7 d. Visual inspection of CLSM images revealed that the biofilms formed by the two mutants not only differed in their substratum coverage and thickness, as had been anticipated from the microtitre plate assays, but also exhibited strikingly different structures (Fig. 4 and data not shown). Both wild-type and mutant strains formed characteristic microcolonies after initial surface attachment. However, while wild-type biofilms rapidly matured and covered most of the available surface space within 24 h, mutant biofilms were arrested in the microcolony stage and never colonized the entire surface during the course of the experiment.

To more accurately describe the differences in the biofilms formed by the wild-type and the *cep* mutants, we employed the computer program COMSTAT, which was recently developed for the quantitative characterization of biofilm structures (Heydorn *et al*., 2000). Out of ten image analysis features which the program provides for quantifying three-dimensional image stacks acquired by CLSM, we chose the following: biomass, mean thickness, substratum coverage and roughness coefficient. To generate data of statistical value, three independent rounds of biofilm experiments were performed. In each round, two flow chamber channels were inoculated with each strain tested. Seven image stacks per channel were taken every 24 h for 7 days and these were analysed with COMSTAT (Heydorn *et al*., 2000). In full agreement with the visual impression, the coefficients for mean thickness and for biomass were greatly reduced for the *cep* mutants when compared with the wild-type (Fig. 5). The two mutants colonize the surface less efficiently than the wild-type, a fact that is reflected by a higher value for substratum coverage for the latter strain. The roughness coefficient is a measure of the variance of biofilm thickness and the higher values of this coefficient for mutant biofilms indicate that they are more heterogeneous than wild-type biofilms. Importantly, in the presence of 200 nM C8-HSL, strain H111-I forms a biofilm that is completely indistinguishable from the one of the wild-type strain (Fig. 4 and Fig. 5a). Likewise, strain H111-R harbouring plasmid pBAH27 (*cepR*$^+$) forms a biofilm with a typical wild-type structure (Fig. 5b).

In conclusion, these results clearly show that the *cep* quorum-sensing system of *B. cepacia* H111 is involved in the control of biofilm formation. More specifically, our data suggest that the *cep* system may not be important for the initial attachment of cells to the surface but is essential for the differentiation of microcolonies, a process that is required for the development of a mature biofilm.

Swarming motility of *B. cepacia* H111 is regulated by the *cep* system

Genetic studies have shown that the formation of a mature biofilm proceeds through an ordered series of steps (for recent reviews see Pratt & Kolter, 1999; Watnick & Kolter, 2000; O'Toole *et al*., 2000). In this model, motility plays a major role in biofilm formation. Flagella-mediated motility is believed to be required to overcome repulsive forces at the surface of the substratum. Furthermore, once the initial contact to the surface is established, cells are thought to move on top

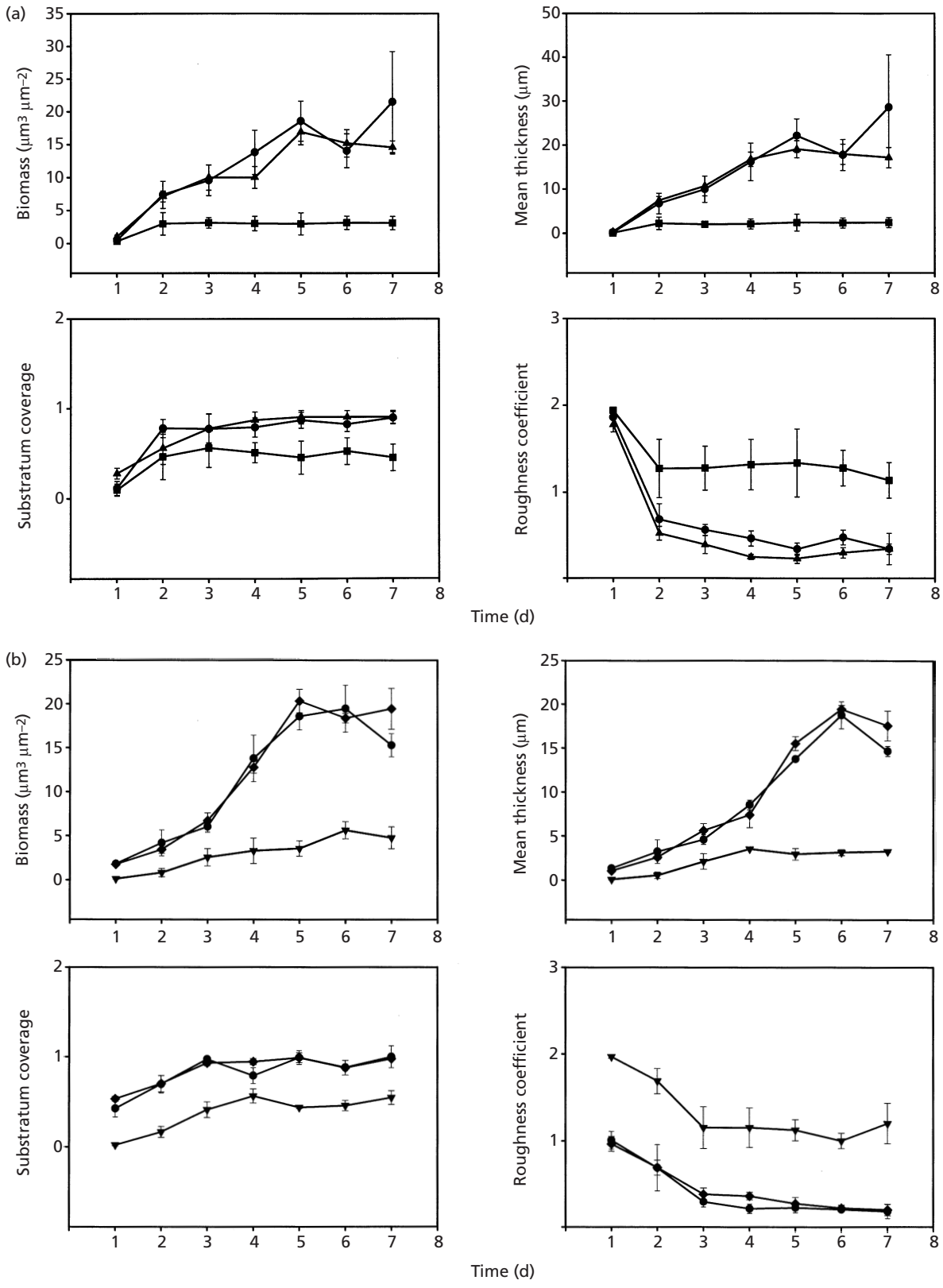

Fig. 5. Quantification of biofilm structures. Biofilms of wild-type H111 (●), and (a) the *cepI* mutant H111-I in the presence (▲) or absence (■) of 200 nM C8-HSL, and (b) the *cepR* mutant H111-R (▼) and the complemented *cepR* mutant H111-R(pBAH27) (◆) were grown in artificial flow cells. CLSM pictures were taken daily for 1 week and these pictures were analysed by the computer program COMSTAT (Heydorn *et al.*, 2000). Parameters calculated were biomass, mean biofilm thickness, substratum coverage and roughness coefficient. Mean values for seven independent CLSM pictures are shown with standard deviations.

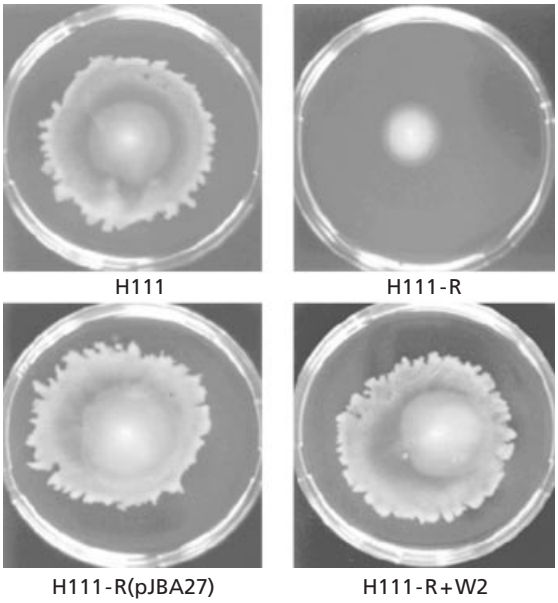

Fig. 6. Swarming motility of *B. cepacia* H111 is quorum-sensing-regulated. Swarming of wild-type H111, the *cepR* mutant H111-R in the presence or absence of the biosurfactant serrawettin W2 (1 μg ml^{-1}) and the complemented *cepR* mutant H111-R(pBAH27) was tested on minimal medium solidified with 0·4% agar and supplemented with 0·1% Casamino acids. All strains exhibited limited swimming motility at the point of inoculation under the assay conditions used.

of the substratum to form microcolonies. Finally, these microcolonies undergo a differentiation process which leads to the development of a typical three-dimensional biofilm architecture.

For *P. aeruginosa*, it has been shown that aggregation of the cells to microcolonies is dependent on twitching motility, a special form of surface translocation that depends on type IV pili (O'Toole & Kolter, 1998b). For *V. cholerae* El Tor and *E. coli*, it has been suggested that flagella-driven motility is not only important for initial attachment of cells to the substratum but also for translocation along the surface in a process that leads to the formation of microcolonies (Pratt & Kolter, 1998; Watnick & Kolter, 1999). The importance of motility for biofilm formation, together with the fact that different forms of bacterial motility, including swimming of *Yersinia pseudotuberculosis* (Atkinson *et al.*, 1999), twitching of *P. aeruginosa* (Glessner *et al.*, 1999) and swarming of *Serratia liquefaciens* (Eberl *et al.*, 1996) and *P. aeruginosa* (Köhler *et al.*, 2000) are quorum-sensing-regulated, prompted us to investigate whether the *cep* system of *B. cepacia* H111 is involved in the control of motility.

When cells of *B. cepacia* H111 are point-inoculated into AB minimal medium which is supplemented with 10 mM citrate and solidified with 0·3% agar, they swim through the water channels in the agar giving rise to typical chemotactic rings. Swimming behaviour of the *cep* mutants and the wild-type was completely indistinguishable, indicating that swimming motility is not quorum-sensing-regulated (data not shown). We also tested the strain for twitching motility under various conditions, but were unable to demonstrate this form of motility for strain H111. However, during the course of these experiments we observed that, when medium containing 0·4% agar was supplemented with 0·1% Casamino acids, cells also spread as a thin layer on the top of the agar surface. Microscopic inspection revealed that the cells migrate in a co-ordinated fashion that is characteristic of swarming motility (for reviews see Allison & Hughes, 1991; Harshey, 1994; Eberl *et al.*, 1999). The migration front of the expanding colony is preceded by a visible layer of slime-like material giving the colony a glistening appearance, a phenomenon that is typical for this form of motility. After incubation for 36 h, *B. cepacia* H111 colonized the entire surface of the agar plate (Fig. 6). By contrast, the two *cep* mutants were unable to swarm. Moreover, the mutants were also deficient in the production of extracellular slime. Addition of 200 nM C8-HSL to the medium restored both swarming motility and slime production of mutant H111-I (data not shown). Both phenotypes were also restored when mutant H111-R was complemented with plasmid pBAH27 (*cepR*$^{+}$) (Fig. 6). These results show that swarming motility of *B. cepacia* H111 is under control of the *cep* quorum-sensing system. Previously, it has been shown that AHL-mediated cell–cell communication is also required for swarming motility of *S. liquefaciens* MG1 (Eberl *et al.*, 1996) and *P. aeruginosa* (Köhler *et al.*, 2000). In both bacteria the quorum-sensing systems control the production of biosurfactants, namely rhamnolipids in the case of *P. aeruginosa* and serrawettin W2 in the case of *S. liquefaciens*, which are essential for swarming motility (Ochsner & Reiser, 1995; Lindum *et al.*, 1998; Köhler *et al.*, 2000). A *S. liquefaciens* mutant defective in the synthesis of AHL molecules is unable to swarm unless the medium is supplemented with either AHLs or a compound capable of lowering the surface tension of the medium such as serrawettin W2, surfactin or trace amounts of SDS (Lindum *et al.*, 1998; Eberl *et al.*, 1999). We therefore tested the two *cep* mutants of *B. cepacia* for their ability to swarm on low-agar plates supplemented with either surfactin or serrawettin W2. The two *cep* mutants swarmed on this medium (Fig. 6 and data not shown), suggesting that production of a biosurfactant in *B. cepacia* H111 is controlled by the *cep* quorum-sensing system, which in turn is required for swarming motility of this bacterium.

Swarming motility is not required for biofilm formation

For *Vibrio parahaemolyticus*, it has been demonstrated that swarming plays an important role in attachment and colonization of chitinaceous shells of crustaceans (Belas & Colwell, 1982). To investigate whether swarming motility is involved in the process of biofilm formation by *B. cepacia*, we tested the wild-type and the

two *cep* mutants for their abilities to form biofilms in minimal medium supplemented with different amounts of surfactin and serrawettin. The presence of high concentrations of the surfactants in the medium completely prevented the cells from attaching to the surface of the microtitre dishes. Importantly, the surfactant concentrations used did not influence the growth rates of the strains in liquid medium (data not shown). At lower concentrations, which were sufficiently high to completely restore swarming behaviour of the mutants, the two surfactants very weakly (less than 10 % increase in A_{570}) but reproducibly stimulated biofilm formation by the *cep* mutants while their presence did not affect biofilm formation by the wild-type. However, these results suggest that swarming motility *per se* is unlikely to play a major role in biofilm formation.

DISCUSSION

AHL-dependent communication systems provide bacteria with a regulatory mechanism that enables individual cells to sense their own population density. In response to the size of the population, i.e. when a certain critical mass, the 'quorum' has been attained, cells collectively induce the expression of particular phenotypic traits, which are not observable with individual cells. Hence, quorum-sensing can be viewed as an example of primitive multicellular behaviour. In nature, bacteria are normally associated with surfaces, on which they form highly structured biofilms (Costerton *et al*., 1995; Davey & O'Toole, 2000; Tolker-Nielsen & Molin, 2000; O'Toole *et al*., 2000). Bacteria living in biofilms are embedded in a matrix of extracellular polymeric substances and thus cell densities are obviously extremely high in these surface-attached communities. By contrast, bacteria growing planktonically, for example in the water column of aquatic systems, only rarely reach high cell densities. It is therefore conceivable that quorum-sensing is a particularly valuable mechanism for gene regulation in biofilms. In support of this view, it has been recently demonstrated that AHL molecules are present both in natural biofilms growing on submerged stones in a river and in biofilms formed on urethral catheters (McLean *et al*., 1997; Stickler *et al*., 1998). Moreover, for *P. aeruginosa* 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* only forms flat and undifferentiated biofilms, suggesting that the *las* system is in some way required for the maturation of biofilms. Importantly, the *lasI* mutant biofilm exhibited greater sensitivity to the biocide SDS in comparison to the wild-type biofilm. The results presented for biofilm formation by *B. cepacia* H111 are fully consistent with this hypothesis. Mutants defective in the *cep* quorum-sensing system form microcolonies on a glass surface that are indistinguishable from those formed by the wild-type, indicating that the early events of biofilm formation are unaffected by the mutations. Similar to the *lasI* mutant of *P. aeruginosa*, however, these microcolonies are unable to differentiate and thus failed to develop into a mature biofilm.

During an attempt to identify *cep*-regulated factors required for biofilm formation by *B. cepacia*, we noticed that the strain's ability to swarm on suitable surfaces is quorum-sensing-regulated. Swarming motility is an intrinsically surface-dependent mode of translocation, which, to the best of our knowledge, has not been reported earlier for members of the genus *Burkholderia* but has been described for many other bacteria (Allison & Hughes, 1991; Harshey, 1994). Swarming motility of quorum-sensing-defective mutants of *B. cepacia* H111 could be fully restored by supplementing the media with different surfactants. We therefore propose that the *cep* system of *B. cepacia* controls the production of a biosurfactant, which is required for swarming motility of the strain. Noteworthy in this context is the recent finding that another genomovar III *B. cepacia* strain (J2315) produces a lipopeptide of unknown structure which exhibits strong surface-active properties (Hutchison *et al*., 1998). Our results are reminiscent of the situation found with *S. liquefaciens* and *P. aeruginosa*. These two bacteria have been demonstrated to employ quorum-sensing systems to control the synthesis of the surface-active compounds serrawettin W2 and rhamnolipids, respectively (Ochsner & Reiser, 1995; Lindum *et al*., 1998). Since the ability to swarm is strictly dependent on the production of biosurfactants, swarming motility is a quorum-sensing-regulated phenomenon in both bacteria.

The addition of surfactants to the medium, at concentrations sufficiently high to restore swarming motility of the *cep* mutants to the level of the wild-type, only weakly, if at all, increased biofilm formation by the *B. cepacia* H111 *cep* mutants. This suggests that locomotion via swarming motility is not required for biofilm formation. On the other hand, the *cep*-regulated production of the biosurfactant itself may affect biofilm formation as previous results have demonstrated that various surface-active compounds have the capability of regulating the attachment and detachment of bacteria to and from surfaces (Rosenberg & Ron, 1999; Ahimou *et al*., 2000). There are several possible explanations as to why our attempts to substitute the missing biosurfactant with surfactin, serrawettin W2 or SDS failed to restore biofilm formation by the *cep* mutants: (i) the physical properties of the surfactants used and the one produced by the strain are substantially different, (ii) production of the surfactant has to follow a specific temporal and/or spatial expression pattern within the biofilm, or (iii) other *cep*-regulated, as yet unidentified, factors may be required for biofilm formation. Work currently under way aims to test these possibilities.

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Anhang 2

Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111

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eingereicht

Genetic Analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111

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Running title: biofilm formation in *B. cepacia*

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Summary

Burkholderia cepacia and *Pseudomonas aeruginosa* often co-exist as mixed biofilms in the lungs of patients suffering from cystic fibrosis (CF). Here we report the isolation of thirteen random mini-Tn*5* insertion mutants of *B. cepacia* H111 that are defective in biofilm formation on a polystyrene surface. We show that the screening procedure used in this study is biased towards mutants defective in the late stages of biofilm development. A detailed quantitative analysis of the biofilm structures formed by wild type and mutant strains revealed that the isolated mutants are impaired in their abilities to develop a typical three-dimensional biofilm structure. Molecular investigations showed that the genes required for biofilm maturation fall into several classes: (i) genes encoding for surface proteins, (ii) genes involved in the biogenesis and maintenance of an integral outer membrane, and (iii) genes encoding regulatory factors. It is shown that three of the regulatory mutants produce greatly reduced amounts of *N*-octanoylhomoserine lactone (C8-HSL). This compound serves as the major signal molecule of the *cep* quorum-sensing system. As this density dependent regulatory system is involved in the regulation of biofilm maturation we investigated the interplay between the three regulatory genes and the quorum-sensing cascade. The results of these investigations show that the identified genes encode for regulatory elements that are positioned upstream of the *cep* system, indicating that the quorum-sensing system of *B. cepacia* is a major checkpoint for biofilm formation.

Introduction

Burkholderia cepacia, a Gram-negative bacterium, which was first described as a phytopathogen, associated with a soft rot of onion bulbs (Burkholder, 1950), has been recognized as an important pulmonary pathogen in patients with cystic fibrosis (CF) since the late 1970s (Isles *et al*., 1984). CF, the most common lethal inherited disease among the Caucasian population, results from a defective chloride channel called the CF transmembrane regulator (CFTR) (Koch and Høiby, 1993; Tümmler and Kiewitz, 1999). Impaired CFTR function leads to the production of a sticky dehydrated mucus in the ducts of exocrine glands, e.g. the airways of the lungs. The increased mucus viscosity impairs mucociliary and alveolar clearing, which, in turn, facilitates colonization of the lung epithelium by opportunistic pathogenic bacteria (Govan and Deretic, 1996; Hutchison and Govan, 1999). The major pathogen in adult CF patients is *Pseudomonas aeruginosa*, but over the past two decades *B. cepacia* emerged as another important pathogen, with prevalence rates in some CF centres of up to 40% (Johansen *et al*., 1998). *B. cepacia* is usually acquired late in the course of the disease, and the clinical outcome of a colonization is variable and unpredictable, ranging from asymptomatic carriage to a fulminant and fatal pneumonia, the so-called 'cepacia syndrome' (Isles *et al.*, 1984). Two of the major problems arising from infection of CF patients with *B. cepacia* are its high intrinsic resistance against antibiotics and biocides impeding effective medical treatment and its high transmissability between patients.

In the past few years biofilms have become accepted as being the predominant natural life form for most bacteria (O´Toole *et al*., 2000; Tolker-Nielsen and Molin, 2000; Watnick and Kolter, 2000). This has enormous implications as bacteria growing in biofilms are in an altered physiological state when compared to planktonic bacteria on which most research was focused until recently. Of particular importance in the medical field is a dramatically increased resistance to antibiotics and host defences when bacteria grow in biofilms (Xu *et al*., 2000; Stewart and Costerton, 2001). As a consequence, biofilms are often associated with persistent and chronic infections including periodontitis, otitis media, biliary tract infection, and endocarditis (Costerton *et al*., 1999). Strong evidence has accumulated that *P. aeruginosa* cells exist as a biofilm in the lungs of CF patients (Høiby 1974; Singh *et al*., 2000; Ciofu *et al*., 2001). Infection with *B. cepacia* normally occurs in patients already colonized with *P. aeruginosa* and it has been suggested that *P. aeruginosa* exoproducts modify epithelial cell surfaces, thereby exposing receptors and facilitating *B. cepacia* attachment (Saiman *et al*., 1990; Sajjan *et al*., 2000). These observations, together with the chronic nature of lung infections in CF suggest that the two species form mixed biofilms in the CF lung.

Owing to the increasing appreciation of the significance of the biofilm lifestyle, genetic studies on the molecular mechanisms underlying biofilm formation have been initiated in a number of bacteria, including *Escherichia coli* (Pratt and Kolter, 1998), *Pseudomonas fluorescens* (O´Toole and Kolter, 1998a), *P. aeruginosa* (O´Toole and Kolter, 1998b), *Vibrio cholerae* (Watnick and Kolter, 1999), and *Streptococcus gordonii* (Loo *et al.*, 2000). These

investigations were made possible through the development of a simple microtitre dish-based biofilm assay. We have recently used this assay to screen a *B. cepacia* random transposon insertion library for mutants defective in biofilm development (Huber *et al*., 2001). One of the isolated mutants was shown to bear the transposon within the *cepR* gene, which encodes a LuxR-type transcriptional regulator. Together with the *cepI* AHL synthase gene, *cepR* comprises the *cep* quorum-sensing system of *B. cepacia* (Lewenza *et al*., 1999). More detailed investigations revealed that the *cep* cell-cell communication system is not involved in the regulation of initial cell attachment but rather controls the maturation of the biofilm. Here we report the molecular analysis of the remaining biofilm deficient mutants obtained in the initial screen.

Results

Isolation of mutants defective in biofilm formation

The suicide vector pUT (de Lorenzo and Timmis, 1994) was used to deliver the hybrid transposon mini-Tn*5* Km2-*luxCDABE* (Winson *et al.*, 1998a) into the *B. cepacia* H111 chromosome. A collection of 5000 random insertion mutants was screened for their ability to form biofilms in polystyrene microtitre dishes. In our routine assay cells were grown in AB minimal medium supplemented with 10 mM citrate (ABC medium) for 48 h at 30°C. In this screen a total of eighteen mutants defective in biofilm formation were obtained.

As unspecific growth defects indirectly decrease biofilm formation we determined the growth rates of the mutants and the wild type in ABC minimal medium (data not shown). Five mutants exhibited reduced growth rates and these were excluded from this study. The remaining thirteen mutants were to different degrees defective in biofilm formation with a 15% to 70% reduction of adhered biomass when compared to the wild type (Fig. 1). In a recent report O'Toole and Kolter (1998a) showed that the biofilm formation defects of some *Pseudomonas fluorescens* mutants could be restored by varying the growth conditions. We therefore examined the influence of different media, including rich LB medium and AB minimal medium supplemented with either glucose (0.2%), citrate (10 mM), or Casamino acids (0.1%), on biofilm formation of the mutants. Although medium composition weakly influences the general ability of *B. cepacia* to form biofilms on a polystyrene surface, none of the mutants could be rescued for their biofilm formation defect (data not shown).

Genetic characterization of the biofilm defective mutants

Southern blot analysis of the thirteen mutants using a DIG-labeled 600 bp DNA fragment derived from the kanamycin resistance gene of mini-Tn*5* Km2-*luxCDABE* as a probe showed that single copies of the hybrid transposon had inserted at different chromosomal locations (data not shown). Furthermore, we observed that with *Sph*I-digested chromosomal DNAs the probe hybridised to restriction fragments of similar sizes in the cases of mutants m13 and m15 and m17 and m20, respectively.

The DNA sequence flanking each mini-Tn*5* insertion was determined in order to identify the gene disrupted in the respective mutant. This was accomplished in two ways. To identify the DNA sequence adjacent to the I-end of the mini-Tn*5* transposon, chromosomal DNA was digested with *Sph*I and cloned into the vector pGEM-3Zf(+) cut with the same enzyme. By selecting for transposon-encoded kanamycin resistance, plasmids containing 1.5 kb of the transposon together with 0.2 to 7.5 kb of chromosomal DNA were obtained. In addition, the DNAs flanking the O-end of the transposon were amplified by arbitrary PCR (see Experimental procedures for details). This PCR technique gave rise to DNA fragments with sizes ranging from approximately 400 to 1000 bp and these were inserted into the vector

pCR2.1-TOPO. The DNA sequences flanking each mini-Tn*5* insertion were determined and analysed using the BLASTX program (Altschul *et al*., 1990), which translates the DNA sequences in all six reading frames and compares these predicted protein sequences with those in GenBank.

The results of these analyses are summarized in Table 2. Only for one mutant, m14, no significant match could be found with any sequences in the database. In the case of mutant m19 the deduced amino acid sequence of the inactivated gene is highly homologous to the hypothetical protein Slr1507 of *Synechocystis* sp. and the hypothetical protein SC6A5.15 of *Streptomyces coelicolor*. The functions of these proteins are unknown. In the remaining mutants, the insertion had taken place in genes with known homologues in other bacteria. Mutants m13 and m15 bear the transposon within the same gene (*bap*). However, as this protein contains numerous repeated amino acid motives it was not possible to precisely determine the transposon insertion points in the two mutants. Furthermore, in mutants m17 and m20 the transposon had inserted at different positions of the *yciR* gene.

Analysis of cell surface proteins

Previous work has demonstrated that cell surface proteins are particularly important for biofilm formation (O´Toole and Kolter, 1998a; Prigent-Combaret *et al*., 2000; Espinosa-Urgel *et al*., 2000) and thus it appeared reasonable to assume that some of the mutants isolated are defective in the biosynthesis of such proteins. We therefore analysed the surface proteins of *B. cepacia* H111 and the various mutants by SDS–PAGE (Fig. 2).

Two non-fimbrial adhesins with molecular weights of 37 kDa and 66 kDa are important for binding of *B. cepacia* cells to respiratory epithelial cells. The 37 kDa protein was shown to correspond to *B. cepacia* porin C while the identity of the 66 kDa outer membrane protein is unknown (Saiman *et al*., 1990). For *B. cepacia* H111 we could detect a weak band of 37 kDa among the surface proteins while a 66 kDa band was missing (Fig. 2). More importantly, the composition of cell surface proteins was altered in a number of mutants. Most notably, a small ~19 kDa protein, which is abundant in the wild type, is missing in the *cepR* mutant m64, which was included in these experiments as a control, and is greatly reduced in strains MA18 and MA24. In the latter two strains, the band intensity of a ~20 kDa protein is markedly increased when compared with the wild type. In addition, one band of ~22 kD is absent in the two mutants and this band is also missing in m19. Several minor differences were evident when the membrane protein patterns of mutants m7 and m15 were compared with the one of *B. cepacia* H111. The protein profiles of the remaining mutants were found to be unchanged.

Biofilm architecture of selected mutants

In order to investigate biofilm formation of selected mutants in better detail, we employed artificial flow cells, which allow to follow biofilm development on a glass surface under highly defined conditions in real time (Christensen *et al*., 1999). Furthermore, the use of flow

cells enabled us to study the effects of the various mutations on biofilm formation in a different biofilm setting (continuous versus static culture) and on a different surface (glass versus polystyrene). To determine the three-dimensional structures of the biofilms formed by the mutants confocal laser scanning microscopy (CLSM) was employed. We have chosen six mutants, which exhibited great defects in the micotitre assays, for further investigations in flow cells: m7, m15, m16, m17, MA2, and MA19. For this analysis it was necessary to tag the *B. cepacia* H111 wild type and the mutants with the green fluorescent protein (GFP). To this end, we inserted a $P_{A1/04/03}$-*gfp*-T0-T1 cassette (Andersen *et al*., 1998) into the broad host range cloning vector pBBR1MCS-5 (Kovach *et al*., 1995). The resulting plasmid, pBAH8, was transferred to the strains by a triparental mating procedure.

Parallel flow chambers were inoculated with the GFP-tagged strains and biofilm development was monitored on a daily basis for three days. Visual inspection revealed that all mutants tested formed biofilms that were significantly different from the wild type and a wide spectrum of biofilm structures was observed (Fig. 3). Biofilm formation of the *B. cepacia* H111 wild type proceeds in two steps (Huber *et al*., 2001): after initial surface attachment characteristic microcolonies are formed, later cells start to colonize the void space between these colonies to eventually cover the entire surface. By contrast, no microcolonies were formed by mutant m7 and the cells uniformly covered the surface giving rise to totally unstructured biofilms (Fig. 3). Likewise, microcolonies were missing in the loose biofilms formed by mutant m17. Typical wild type microcolonies were only rarely observed in MA19 biofilms. Instead, this mutant formed extremely thick and compact aggregates next to virtually uncolonized surface areas. The tendency of mutant m16 to form microcolonies was found to be slightly increased. More obviously, a large number of highly elongated cells were detected within the biofilm. Completely different structures were observed with the biofilms formed by mutants m15 and MA2. Cells of these two mutants were rarely found outside of microcolonies, which grew considerably larger than those formed by the wild type. As a consequence, these biofilms were comprised of noncoherent cell aggregates that failed to cover the entire surface during the course of the experiment.

In order to quantify the observed differences in biofilm structures, we employed the software package COMSTAT (Heydorn *et al*., 2000). This program provides ten image analysis features for quantifying three-dimensional image stacks acquired by CLSM. Out of these ten parameters, we chose five for the comparison of wild type and mutant biofilms, namely biomass, substratum coverage, maximum thickness, surface to volume ratio, and roughness coefficient. The results of this analysis for biofilms formed three days after inoculation of the flow chambers are summarized in Fig. 4. In agreement with the microtitre dish assays (Fig. 1) and the visual impression, the values for total biomass were reduced for the mutant biofilms. In addition, none of the mutants is capable of colonizing the surface as efficiently as the wild type, a fact that is reflected by lower values for substratum coverage. The mutant biofilms vary significantly with respect to the maximum thickness. While the maximum thickness of

biofilms formed by mutants MA2 and m17 is similar to the one of the wild type, this parameter is greatly reduced in the case of m7 and m16 biofilms. Increased values for maximum thickness were recorded for biofilms formed by mutants m15 and MA19. Likely, this is a consequence of the tendencies of these two mutants to form very high microcolonies (Fig. 3). Surface to volume ratios and roughness coefficients for mutant biofilms are in all cases higher than the respective coefficients of the wild type biofilm. Surface to volume ratio is a measure for the portion of the biofilm exposed to the nutrient flow and the roughness coefficient specifies the variance in biofilm thickness. The increased values of these two parameters therefore indicate that mutant biofilms are more heterogenous than the biofilms formed by the wild type. This finding is particularly obvious for those biofilms that consist mainly of distinct cell aggregates with large uncolonized areas between them as observed with mutants m15, MA2, MA19, and, to a lesser extent, m17. In the case of mutant m16 protruding cell filaments appear to increase biofilm heterogeneity. Although mutant m7 appears to form a very uniform and undifferentiated biofilm when inspected by CLSM, the parameters for heterogeneity were found to be increased when the biofilm was analysed by COMSTAT. A more detailed microscopic investigation revealed that quite large uncolonized areas are present within the otherwise homogenous biofilm, possibly arising from sloughing of cells. These holes are the cause for the increased values of surface to volume ratio and roughness coefficient.

Mutants m17, MA18, and MA24 are defective in quorum sensing

Previous work has established that the *cep* quorum-sensing system of *B. cepacia* is involved in the regulation of biofilm maturation (Huber *et al*., 2001). This density-dependent regulatory system consists of the *N*-acyl homoserine lactone (AHL) synthase CepI, which directs the synthesis of *N*-octanoylhomoserine lactone (C8-HSL) and, as a minor product, *N*-hexanoylhomoserine lactone (C6-HSL) (Gotschlich *et al*., 2001), and CepR, which after binding of C8-HSL is thought to activate or repress transcription of target genes. Beside biofilm maturation, the *cep* system positively regulates production of extracellular proteolytic and chitinolytic activity and represses synthesis of the siderophore ornibactin (Lewenza *et al*., 1999; Huber *et al*., 2001; Lewenza and Sokol, 2001).

We therefore tested the mutants for AHL signal molecule production. Three mutants, m17, MA18, and MA24, produced greatly reduced amounts of AHLs (Fig. 5). Hence, the biofilm formation defects observed with these three mutants may be a direct consequence of the strains' incapability to produce AHLs. To address this issue in better detail we attempted to restore the defects by the addition of 200 nM C8-HSL to the medium. However, the presence or absence of C8-HSL neither affected protease production, a phenotype that is strictly regulated by the *cep* system, nor biofilm formation (data not shown). By contrast, both phenotypes were completely restored when the *cepI* mutant H111-I was used as a control in these experiments.

Since CepR tightly controls expression of *cepI* (Huber *et al*., 2001; Lewenza and Sokol, 2001) the mutants could also be impaired in their ability to express CepR. To test this possibility we introduced plasmid pBAH27, which carries the *cepR* gene transcribed from the P*lac* promoter of the vector, into mutants m17 and MA18. The presence of the plasmid in the two mutants did not restore the biofilm formation defects, even after the addition of 200 nM C8-HSL to the medium. Interestingly, the presence of the plasmid stimulated production of extracellular proteolytic activity in the case of mutant m17, but not of MA18 (data not shown).

These results suggest that the genes that had been inactivated in the two mutants (*suhB* and *yciR*) encode for factors, which either affect the amount of functional CepR protein in the cell or directly interfere with the expression of target genes. We were unable to perform this complementation experiment with mutant MA24 since it was not possible to transfer plasmid pBAH27 into this strain. Further work will be required to elucidate the role of the MA24 mutation for quorum sensing in *B. cepacia*.

Discussion

Motility and biofilm formation

Evidence that accumulated over the past few years established that the formation of a mature biofilm on an abiotic surface proceeds through an ordered series of steps (for reviews see Watnick and Kolter, 2000; O'Toole *et al.*, 2000). In this model three developmental steps are distinguished: (i) initial attachment of individual cells to a surface, (ii) aggregation of these cells to microcolonies, and (iii) differentiation of the microcolonies into a mature biofilm. For a number of bacteria it has been demonstrated that flagella-driven swimming motility plays an important role for the initial stages of biofilm development, in particular for the initial attachment of cells to the surface and the formation of microcolonies. However, only one of the mutants (m16) isolated in this study was nonmotile. The screening procedure used appears to be biased towards mutants defective in the late stages of biofilm development. We speculated that due to the long incubation time of the microtitre dishes (48 h) factors required for initial surface attachment, such as motility, may be less important for biofilm formation. To test this hypothesis we compared the time course of biofilm development of the wild type with the one of a *motA* mutant strain. The *motA* lesion does not inhibit flagellum biosynthesis but renders the cells non-motile (Blair and Berg, 1990). In accordance with results reported for *E. coli* (Pratt and Kolter, 1998), we observed that within the first eight hours the wild type formed significantly thicker biofilms than the *motA* mutant (Fig. 6). Thereafter, however, the differences in surface associated biomass diminished and after 48 hours the biofilms of the mutant and the wild type were virtually indistinguishable. This result supports the view that the screen used in this study favours the identification of mutants defective in the late steps of biofilm formation. Conceivably, these factors are particularly relevant for biofilm maturation, i.e. the development of a typical three-dimensional biofilm structure.

Genetic characterization of biofilm defective mutants

Apart from two mutants, m14 and m19, which bear the transposon in genes showing no significant homology to previously characterized ones, we could assign at least putative functions to the inactivated genes and these fall into several categories:

Cell surface proteins

In the two mutants m13 and m15 the transposon had interrupted a gene that is highly homologous to the *mus-20* locus of *P. putida* KT2440 (Espinosa-Urgel *et al*., 2000), the *bap* gene of *Staphylococcus aureus* (Cucarella *et al*., 2001), and the *esp* gene of *Enterococcus faecalis* (Toledo-Arana *et al*., 2001). The *mus-20* locus (mutants unattached to seeds) was demonstrated to be required for the adhesion of *P. putida* KT2440 to corn seeds while *bap* (biofilm associated protein) and *esp* (Enterococcal surface protein) were shown to be involved in biofilm formation on inert surfaces. In agreement with previous studies showing that both

esp and *bap* mutants exhibit a decreased hydrophobicity (Toledo-Arana *et al*., 2001), we observed that the ability of mutant m15 to adhere to *n*-hexadecane is significantly reduced when compared to the wild type (Fig. 7). Furthermore, both mutants have a rough colony morphology (data not shown), which may indicate that a cell surface-associated factor is altered in these mutants. In fact, Mus-20, Bap, and Esp are predicted to be cell surface proteins. Conversely to our observation, loss of the Bap protein in *S. aureus* results in a transformation of the rough colony morphology into the smooth morphology (Cucarella *et al*., 2001). Inactivation of *bap* in *B. cepacia* H111 causes a dramatic change in the structure of the biofilm formed by the strain. Mutant m15 grows in large but well-separated cell aggregates on the glass surface of the flow chambers giving rise to porous and disconnected biofilms.

Outer membrane composition and cell shape

Mutant m7 is mutated in *tolA*. TolA is a component of the Tol multiprotein complex, which is involved in the biogenesis and maintenance of an integral outer membrane in various Gram-negative bacteria (Lazzaroni *et al*., 1999; Llamas *et al*., 2000). Recently, it has been proposed that TolA of *E. coli* modulates the surface expression of the O antigen by an involvement in the processing of its subunits, possibly during the process of membrane translocation of O antigen or at the subsequent stages of LPS assembly on the periplasmic side of the plasma membrane (Gaspar *et al*., 2000). Mutations in *tol* genes generally result in cells, which leak periplasmic and outer membrane proteins to the extracellular medium and which exhibit a markedly increased sensitivity to various agents such as detergents, quaternary compounds, and some antibiotics (Llamas *et al*., 2000). In full agreement with these data we observed that the *B. cepacia tolA* mutant m7 is hypersensitive to the detergents SDS and Triton X-100 (data not shown). In addition, for *tol* mutants of *P. putida* altered cell morphology, bleb formation, and reduced motility has been reported (Llamas *et al*., 2000). Microscopic inspection revealed that, in comparison to the wild type, m7 cells are often elongated or grow in short chains (Fig. 8). Swimming behaviour of the *B. cepacia tolA* mutant and the wild type was completely indistinguishable.

The biofilm formed by the m7 mutant does not contain microcolonies, rather the cells uniformly cover the surface and do not organize in any observable structure. Likely, this loss of biofilm structure is a consequence of an alteration of the outer cell membrane.

Mutant MA19 bears the transposon in *rodA*. In *E. coli* two morphogenic proteins are involved in the maintenance of the cylindrical shape of the cells during elongation: RodA and the penicillin-binding protein 2 (PBP2). Together with the *rlpA* gene, which encodes a lipoprotein of unknown function (Takase *et al*., 1987), they constitute the *pbpA-rodA-lrpA* operon (Matsuzawa *et al*., 1989). Both RodA and PBP2 are required for murein synthesis during elongation of the cell wall and a mutation of either of the genes leads to the generation of

spherical cells (Begg and Donachie, 1985). Consistent with these data, we observed that cells of mutant MA19 have a coccoid morphology (Fig. 8).
A simple explanation for the alteration of the biofilm structure observed with the *rodA* mutant MA19 could be that the package density of coccoid cells in the microcolonies is probably higher than the one of rod-shaped cells. However, since *rodA* is involved in the synthesis of the cell envelope it is also possible that an alteration of the surface property of the cell is responsible for the observed defect.

Using DNA microarrays Whiteley *et al*. (2001) showed that about 0.5% of the genes in *P. aeruginosa* are activated and about 0.5% are repressed when cells grow in biofilms. Interestingly, not only the *tolA* gene was among the differentially up-regulated genes but also the *mreC* gene, which encodes for a rod shape-determining protein. These data support the view that cell shape and cell surface composition are crucial factors for biofilm development.

Type II secretion
Strain MA2 is mutated in *gspE*, which encodes a component of the type II secretion machinery, which is part of a two step secretion pathway, known as the general or type II secretion pathway (Pugsley, 1993). Proteins utilizing this pathway are first secreted by the aid of amino-terminal signal sequences of precursors across the inner membrane and then in a second step from the periplasmic space via the type II translocation machinery across the outer membrane. The deduced amino acid sequence of the sequenced part of the *B. cepacia* H111 *gspE* gene is highly homologous to GspE from *B. pseudomallei*, which utilizes the general pathway to secrete protease, lipase, and phospholipase (DeShazer *et al*., 1999). Consistent with these data we found that MA2 no longer produces extracellular proteolytic and lipolytic activity (data not shown).
At present it is unclear in which way the type II secretion system is involved in biofilm formation of *B. cepacia*. As it has been shown recently that a surface-localized protein that is part of a high-affinity manganese uptake ABC transporter system functions as an adhesin (Kolenbrander *et al*., 1998) it appears possible that some components of the secretion machinery itself could be important for biofilm formation. However, we cannot rule out the possibility that the type II secretion machinery is involved in the export of a yet unknown protein required for biofilm development. Noteworthy in this context is the fact that the components of the type II secretion pathway are similar to proteins that are required for assembly of type IV pili in various Gram-negative bacteria, suggesting that the two systems have a common evolutionary origin (Lory, 1998). In *P. aeruginosa* a prepilin peptidase is required for both biogenesis of the type II secretion machinery and the assembly of type IV pili (Nunn *et al*., 1990). Work currently under way aims at testing whether GspE is involved in pilus biogenesis of *B. cepacia* H111.

Recombination – Phase switching?

B. cepacia m16 is mutated in *recR*. Together with RecO and RecF, RecR is involved in recombinational DNA repair and the initiation of the SOS response (Kuzminov, 1999). RecR and RecO are believed to form a protein complex that stimulates RecA protein binding to ssDNA coated with SSB (single stranded DNA binding protein). *E. coli* mutants with defects in the *recFOR* pathway show an increased sensitivity to DNA damaging agents and UV light (Kuzminov, 1999; Keller *et al*., 2001). Consistently, we observed that mutant m16 is markedly more sensitive to UV light than the wild type (data nor shown).

A functional overlap between DNA repair and biofilm formation may not be obvious at first sight. However, RecA also plays an important role in global gene regulation. In *Serratia marcescens* and *Erwinia carotovora* expression of extracellular proteins require *recA* (Zink *et al*., 1985; Ball *et al*., 1990). Furthermore, RecA-dependent DNA rearrangements are responsible for enterotoxin gene amplification in *Vibrio cholerae* (Goldberg and Mekalanos, 1986) and for pilus antigenic variation in *Neisseria gonorrhoeae* (Koomey *et al*., 1987). Most interestingly, *recA* mutants of *V. cholerae* were shown to be defective in adherence and colonization of animal intestines, an observation that was attributed to the strain´s inability to perform intragenic recombination required for expression of colonizing factors (Kumar *et al*., 1994).

Regulators

Two mutants, m17 and m20, bear the transposon in a gene that shows the highest homology to *yciR* of *E.coli*. To date, no function could be assigned to YciR but the protein contains a GGDEF motif (Hecht and Newton, 1995; Aldridge and Jenal, 1999), which is also present in more than 50 proteins from Gram-positive and Gram-negative bacteria. Since this domain is often found in combination with various response regulator receiver domains it has been proposed that proteins containing this domain are all members of a signal transduction system whose mode of action remains to be elucidated (Croft *et al.*, 2000). Most interesting in the context of this study is the recent finding that the GGDEF domain-containing protein AdrA of *Salmonella typhimurium* is involved in the regulation of cellulose biosynthesis, which is required for biofilm formation and the aggregative behaviour of the organism (Römling *et al*., 2000; Zogaj *et al*., 2001). The fact that mutant m17 only formed thin biofilms without developing microcolonies may support the view that the mutant is defective for a factor promoting auto-aggregation of cells.

In mutant MA18 the transposon interrupted the *suhB* gene. The *suhB* gene of *E. coli* was shown to encode a protein of 268 amino acids that possesses inositol monophosphatase activity (Chen and Roberts, 2000). However, mutant alleles of the *E. coli suhB* gene have appeared under a variety of conditions as extragenic suppressors for various unrelated temperature-sensitve mutations, including the heat shock response mutation (*rpoH15*) (Yano

et al., 1990), the DNA replication mutation (*dnaB121*) (Chang *et al*., 1991), and the protein secretion mutation (*secY24*) (Shiba *et al*., 1984). It has been suggested that SuhB participates in the post-transcriptional control of gene expression by modulating mRNA decay (Inada and Nakamura, 1995).

In mutant MA24 the transposon has interrupted a gene, *yciL*, the deduced amino acid sequence of which is homologous to pseudouridine synthases from different bacteria (Charette and Gray, 2000). Interestingly, a *P. aeruginosa* strain with a lesion in the pseudouridine synthase gene *truB* is defective in several parameters related to osmotic stress and is also unable to grow on solid, but not liquid, media at 43°C (Sage *et al*. 1997).

The cep *quorum-sensing system: a central checkpoint for biofilm development?*
It has been shown that that the *cep* quorum-sensing system is involved in the regulation of the late events of biofilm development (Huber *et al*., 2001). Here we show that three mutants, m17, MA18, and MA24, which were isolated on the basis of their biofilm formation defects, produce greatly reduced amounts of C8-HSL. External addition of C8-HSL to the growth medium did neither restore biofilm formation nor protease production. Since expression of *cepI* and thus C8-HSL production is CepR-dependent, we also attempted to complement the defects by providing a functional *cepR* allele on a plasmid (pBAH27). Noteworthy is the fact that we could not perform this complementation experiment with mutant MA24 as, for reasons that are unclear, we were unable to transfer pBBR-based plasmids into this strain. The presence of plasmid pBAH27 in mutants m17 and MA18 did neither restore AHL production (data not shown) nor biofilm formation, while this was the case when the plasmid was introduced into a defined *B. cepacia cepR* mutant (data not shown; Huber *et al*., 2001). Interestingly, provision of *cepR in trans* partially rescued the protease production defect of mutant m17 but not of MA18, indicating the existence of multiple regulatory layers within the *cep* quorum-sensing cascade.

In conclusion, our results provide evidence that *yciR*, *suhB*, and *yciL* encode higher level regulators of the *cep* quorum-sensing system (Fig. 9). More specifically, our data suggest that YciR and SuhB either affect the concentration or the activity status of CepR. At present it is unclear whether these regulators also interfere with the expression of genes outside of the *cep* regulon. In any case, we show that the *cep* quorum-sensing system is a major checkpoint for biofilm formation in *B. cepacia*.

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Experimental procedures

Strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, the strains were grown at 37°C in modified Luria-Bertani (LB) broth (Andersen *et al*., 1998) or AB minimal medium (Clark and Maaløe, 1967) supplemented with 10 mM citrate. Solid media were routinely solidified with 1.5% agar. Growth media for examination of swimming motility contained 0.3% (w/v) agar. Antibiotics were added as required at final concentrations of 100 µg ml^{-1} ampicillin, 10 µg ml^{-1} tetracycline, 20 µg ml^{-1} gentamicin, and 10 µg ml^{-1} choramphenicol. Kanamycin was used at 50 µg ml^{-1} for *E. coli* and 100 µg ml^{-1} for *B. cepacia*. Growth of liquid cultures was monitored spectrophotometrically by an Ultrospec Plus spectrophotometer (Pharmacia) by measurement of optical density at 600 nm (OD_{600}).

Conjugative plasmid transfer

Plasmids were delivered to *B. cepacia* by tri-parental mating as described previously (de Lorenzo and Timmis, 1994). Briefly, donor and recipient strains as well as the helper strain *E. coli* HB101 (pRK600) were grown at 37°C overnight in 5 ml LB supplied with the appropriate antibiotics. Following subculturing to an OD_{600} of 0.9, the cells from 2 ml of culture were harvested, washed, and resuspended in 200 µl LB. Donor and helper cells (100 µl each) were mixed and incubated for 30 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 agar plates. After overnight incubation at 37°C, the cells were scraped off and were resuspended in 1 ml 0.9% NaCl. Serial dilutions were plated on LB medium containing antibiotics for counter-selection of donor, helper and untransformed recipient cells.

DNA manipulations and nucleotide sequencing

Cloning, restriction enzyme analysis, and transformation of *E. coli* were performed essentially as described previously (Sambrook *et al*., 1989). Polymerase chain reaction (PCR) was performed using the TaKaRa rTaq DNA polymerase (TaKaRa Shuzo). Plasmid DNA was isolated with the QIAprep Spin Miniprep kit and chromosomal DNA from *B. cepacia* was purified using the DNeasy Tissue kit. DNA fragments were purified after PCR and from agarose gels with the QIAquick PCR Purification Kit and with the QIAquick Gel Extraction kit, respectively (all kits are from QIAGEN).

DNA sequences flanking transposon mutants were determined by cloning as well as by arbitrary PCR. To ascertain the DNA sequence adjacent to the I-end of the mini-Tn*5* transposon, chromosomal DNA was digested with *Sph*I and cloned into the vector pGEM-3Zf(+) digested with the same enzyme. Following transformation of *E. coli* XL1-Blue, kanamycin resistant clones were isolated. For amplification of DNA sequences flanking the O-end of the transposon a slightly modified version of an arbitrary PCR protocol described previously (O´Toole and Kolter, 1998a; Pratt and Kolter, 1998) was employed. Briefly, we performed two rounds of PCR amplification using arbitrary primers to prime from the chromosome and primers specific to the mini-Tn*5* transposon. Primers used in the first round were ARB6 (5´-GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC-3´) and luxCext2 (5´-AGTCATTCAATATTGGCAGG-3´). First-round reaction conditions were (i) 5 min at 95°C; (ii) 6 x [30 s at 95°C, 30 s at 30°C, 1 min at 72°C]; (iii) 30 x [30 s at 95°C, 30 s at 45°C, 1 min at 72°C]; (iv) 5 min at 72°C. The second round of PCR amplification used 5µl purified first round PCR product and the following primers: ARB2 (5´-GGCCACGCGTCGACTAGTAC-3´) and luxCint2 (5´-GGATTGCACTAAATCATCAC-3´). Second-round reaction conditions were (i) 30 x [30 s at 95°C, 30 s at 45°C, 1 min at 72°C];

(ii) 5 min at 72°C. The PCR products were purified from an agarose gel and ligated into the vector pCR 2.1-TOPO.
Sequencing was performed by dideoxynucleotide chain determination in a LI-COR 4200 DNA sequencer. Primer 5'-CAGATCTGATCAAGAGACAG-3' was used for sequencing the DNA flanking the I-end of the mini-Tn5, primer 5´-CACTTGTGTATAAGAGTCAG-3´ was used for the O-end.
For flow chamber experiments, the strains were tagged with the green fluorescent protein (GFP). For this purpose, we constructed plasmid pBAH8 by cloning the $P_{A1/04/03}$-RBSII-*gfp*mut3*-T_0-T_1 cassette from pJBA28 (Andersen *et al.*, 1998) as a 2 kb fragment into the cloning vector pBBR1MCS-5. The plasmid was delivered into respective target strains by conjugative transfer as described above, selecting transconjugants on LB medium containing gentamicin and tetracycline.

Construction of a B. cepacia *H111 mutant bank*

The hybrid transposon mini-Tn*5* Km2-*luxCDABE* was randomly inserted into the chromosome of *B. cepacia* H111 by the triparental mating procedure described above. Transconjugants were selected on LB medium containing kanamycin and tetracycline. These random insertion mutants were picked and grown in 150 µl LB medium in the wells of polypropylene MicroWell dishes (Nunc). For storage 75 µl of 50% glycerol were added and the dishes frozen at – 80°C.

Screen for mutants defective in biofilm formation

Biofilm formation in polystyrene microtitre dishes was assayed essentially as described previously (O`Toole and Kolter, 1998a; Pratt and Kolter, 1998) with a few modifications (Huber *et al*., 2001). Cells were grown in the wells of the microtitre dishes in 100 µl AB medium supplemented with 10 mM citrate for 48 hours at 30°C. Thereafter, the medium was removed, and 100 µl of a 1% (w/v) aqueous solution of crystal violet (CV) was added. Following staining at room temperature for 20 min, the dye was removed and the wells were washed thoroughly. For quantification of attached cells the CV was solubilized in DMSO and the absorbance was determined at 570 nm.

Detection of AHLs

Production of AHLs was investigated by cross-streaking the strains against *E. coli* MT102 containing the bioluminescent plasmid sensor pSB403 (Winson *et al*., 1998b). 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.* After over night incubation at 30°C, bioluminescence was detected with the highly sensitive photon counting camera C2400-40 (Hamamatsu Photonics K. K.).
For quantification of AHL signal molecules 100 µl of filter-sterilized supernatants of cultures grown in LB medium to an OD_{600} of 3.0 were added to 100 µl of an exponential culture of *E. coli* MT102 (pSB403) in the wells of a FluoroNunc Polysorp microtitre dish. Following incubation at 30°C for 6 h bioluminescence was measured with a Lambda Fluoro 320 Plus reader (Bio-Tek Instruments).

Cell surface hydrophobicity

The cell surface hydrophobicities of *B. cepacia* strains were determined as described previously (Rosenberg *et al*., 1980) with a few modifications. Cells were either grown over night (stationary phase) or to early exponential phase (OD_{600} = 0.5) in LB. After harvesting and washing cells two times in PUM buffer (22.2 g K_2HPO_4 x 3 H_2O, 7.26 g KH_2PO_4, 1.8 g

urea, 0.2 g $MgSO_4$ x 7 H_2O ad 1 l; pH 7.1), cells were suspended in PUM buffer to an OD_{600} of 1.0. 160 µl of *n*-hexadecane (Merck) were added to 1.6 ml of cells in glass test tubes. Following 10 min preincubation at 30°C, tubes were uniformly shaken for 90 s and the aqueous phase was allowed to separate from the hexadecane for 15 min. The OD_{600} of the aqueous phase was determined and the bacterial adhesion to hydrocarbon (BATH) was calculated as $[1 - (OD_2/OD_1)] \times 100$ with OD_1 = cell density at the beginning of the experiment and OD_2 = cell density after incubation of cells with hydrocarbon.

Extraction of cell surface proteins

Membrane proteins were isolated using the protocol of Kawai *et al*. (1998) with minor modifications. Cells from 2 ml of a culture grown to an OD_{600} of 3 in LB were harvested, washed in PBS buffer (Harlow and Lane, 1988), and resuspended in 400 µl 0.3% sodium dodecyl sulfate (SDS). After incubation at room temperature for 3 min and centrifugation at 13,000 rpm for 15 min, the supernatants containing the cell surface proteins were collected. Protein concentrations were determined colourimetrically with the help of Coomassie Plus Protein Assay Reagent (Pierce) according to the manufacturer´s instructions. Samples (1.3 µg) were separated on a 12% SDS-polyacrylamide gel (SDS-PAGE) (Laemmli, 1970) and protein bands were visualized by silver staining (Harlow and Lane, 1998).

Monitoring of biofilm formation by confocal laser scanning microscopy (CLSM) and image analysis

Biofilms were grown in artificial flow cells supplied with AB medium and containing 1 mM glucose (ABG). The flow system was assembled and prepared as described previously (Christensen *et al*., 1999). The substratum consisted of a microscope glass coverslip (Knittel Gläser). Over night cultures in ABG medium were subcultured to an OD_{600} of 0.7 before they were diluted in 0.9% NaCl to an OD_{600} of 0.1. 300 µl of these dilutions were used to inoculate the flow channels. Medium flow was kept at a constant rate of 0.7 mm/s by a Watson-Marlow 205S peristaltic pump. Incubation temperature was 30°C.
Microscopic inspection and image acquisition were performed on a confocal laser scanning microscope (TCS4D, Leica Lasertechnik) equipped with a 63x/1.32-0.6 oil objective. For statistical evaluation of biofilm structures, a 40x /0.75 air objective was used. 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) running on a Silicon Graphics Indigo 2 workstation.
For statistical evaluation of biofilm structures, two independent rounds of biofilm experiments were performed, and in each round, each strain was grown in two separate channels. Seven image stacks were taken of each channel every 24 h for seven days after inoculation. These images were analysed by the computer program COMSTAT, which comprises various features for quantifying three-dimensional biofilm image stacks (Heydorn *et al.*, 2000). The parameters used for characterization of biofilm architectures included biomass, substratum coverage, maximum thickness, surface to volume ratio, and roughness coefficient.

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Table 1. Bacterial strains and plasmids used in this study

Strains	Description	Source or reference
E. coli		
HB101	*recA thi pro leu hsdR⁻M⁺* Sm^R	Kessler *et al*., 1992
MT102	*araD139*, (*ara-leu*)*7697* Δ*lac thi hsdR*	Laboratory collection
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´*proAB lacI*^{q} *ZΔM15* Tn*10* (Tet^R)]	Stratagene
B. cepacia		
H111	CF isolate from a patient at the Medizinische Hochschule Hannover (1993), genomovar III	Römling *et al*., 1994; Gotschlich *et al*., 2001
m64	*cepR*::Tn*5* Km2-*luxCDABE* derivative of H111	Huber *et al*., 2001
plasmids		
pBAH8	pBBR1MCS-5 containing $P_{A1/04/03}$-*gfp mut3*-T_o-T_1	this study
pBAH27	pBBR1MCS-5 containing the *cepR* gene of *B. cepacia* H111	Huber *et al*., 2001
pBBR1MCS-5	Gm^R; broad host range cloning vector	Kovach *et al*., 1995
pCR 2.1-TOPO	Ap^R, Km^R; cloning vector	Invitrogen
pGEM-3Zf(+)	Ap^R; multicopy cloning vector	Promega
pRK600	Cm^R; ColE1*oriV*; RK-2Mob$^+$ RK2-Tra$^+$; helper plasmid in triparental matings	de Lorenzo and Timmis, 1994
pSB403	Tc^R; *luxRI´*::*luxCDABE* transcriptional fusion; bioluminescent AHL sensor plasmid	Winson *et al*., 1998b
pUT mini-Tn*5* Km2-*luxCDABE*	Km^R; delivery vector for miniTn*5* Km2-*luxCDABE*	Winson *et al*., 1998a

Table 2: Genetic identification of biofilm-defective mutants of *B. cepacia* H111.

Mutant	mutated gene (organism)[a]	accession number	positives	gene product	putative role in biofilm formation
m7	*tolA* (*P. aeruginosa*)	NP_249662	80/133 (60%)	Membrane protein	Cell surface composition
m13/m15	*bap* (*Staphylococcus aureus*)	AAK38834	101/211 (48%)/ 110/232 (47%)	Biofilm-associated surface protein	Surface protein
m14	no matches	-	-	-	Unknown
m16	*recR* (*Yersinia pestis*)	NP_406598	68/122 (56%)	Recombination protein	Phase switching?
m17/m20	*yciR* (*E. coli*)	P77334	185/271 (68%) 102/199 (51%)	Probable membrane protein	Quorum-sensing regulator
m19	hyp. protein slr1507 (*Synechocystis* sp.)	NP_441334	149/214 (70%)	-	Unknown
MA2	*gspE* (*B. cepacia*)	BAB18789	117/126 (93%)	General secretory pathway protein E	Protein secretion
MA18	*suhB* (*Azotobacter vinelandii*)	T44277	88/121 (73%)	Suppressor protein	Quorum-sensing regulator
MA19	*rodA* (*Vibrio cholerae*)	NP_230596	37/73 (51%)	Rod shape determining protein	Cell shape
MA24	*yciL* (*E. coli*)	H64874	42/54 (78%)	Probable pseudouridylate synthase	Quorum-sensing regulator

a The gene with the highest similarity is shown.

Fig. 1: Biofilm formation of the wild type H111 and various transposon insertion mutants in polystyrene microtitre dishes. Strains were grown in AB minimal medium supplemented with 10 mM citrate. After incubation for 48 h at 30°C planktonic cells were removed and attached cells were stained with crystal violet. Error bars represent the standard deviation of the mean for six independent wells.

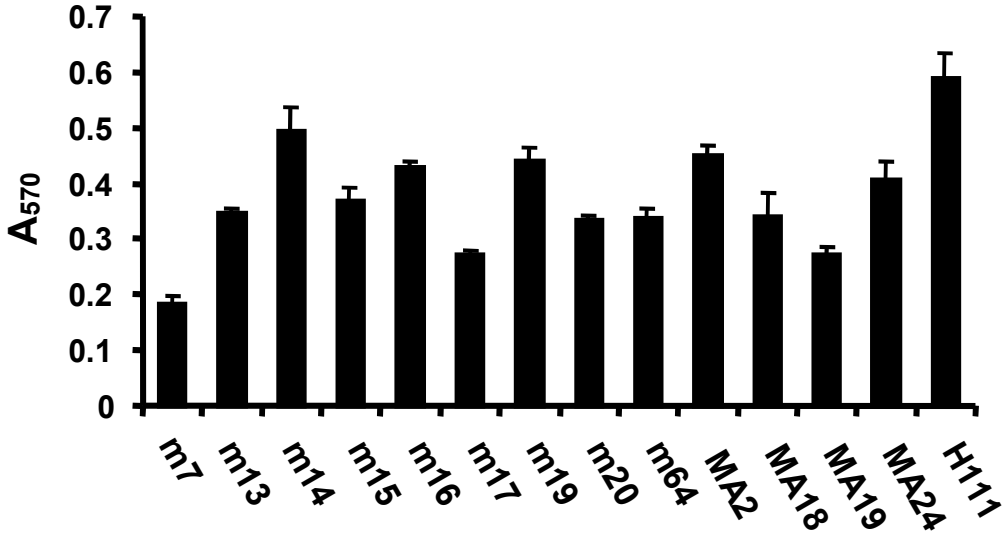


Fig. 2: Surface proteins of *B. cepacia* H111 and various biofilm-defective mutants. Surface proteins were extracted by treatment of cells with 0.3% SDS and samples were separated on a 12% SDS-PAGE. Bands showing reduced intensity in the mutants compared to the wild type H111 are marked with black arrows; bands with increased intensity in the mutants are indicated with open arrows. Positions of molecular weight markers are indicated in kilodaltons.

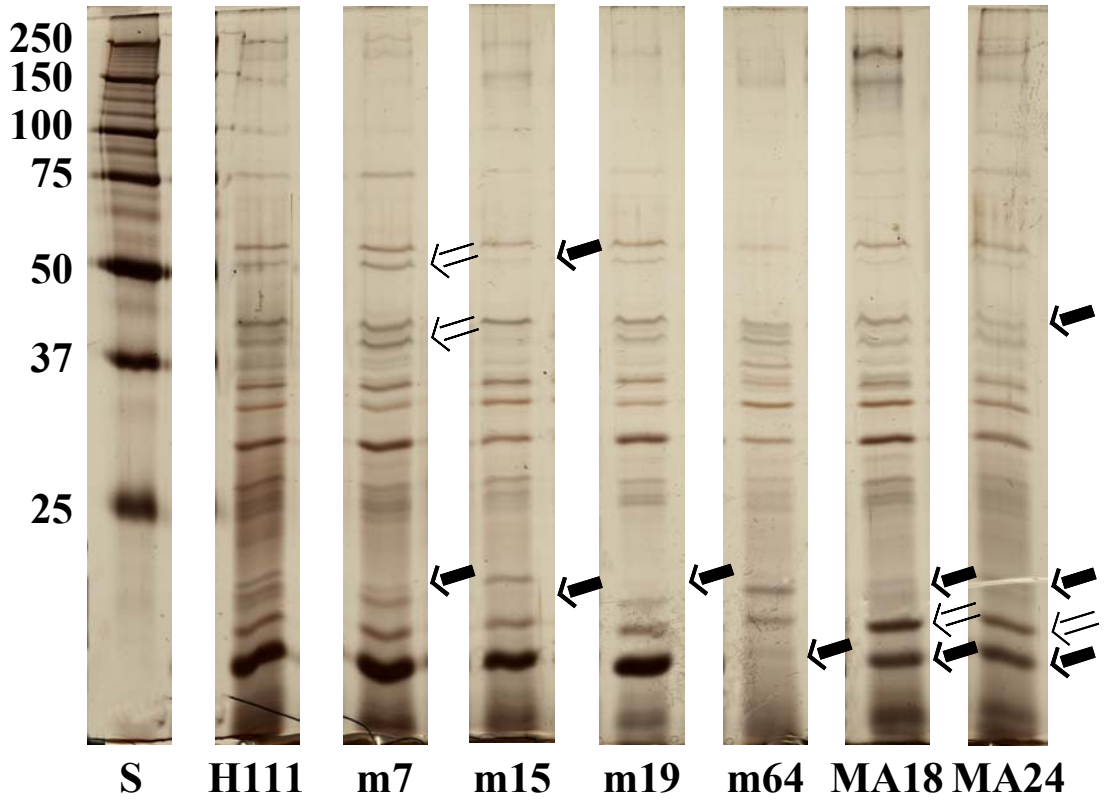


Fig. 3: Biofilm structures of *B. cepacia* H111 and different biofilm-defective mutants. Flow chambers were inoculated with *gfp*-tagged derivatives of the wild type H111 and selected mutants. CLSM pictures were taken 24, 48, and 72 h after inoculation. The larger central plots are simulated fluorescence projections. Shown in the right and lower frames are vertical sections through the biofilms.

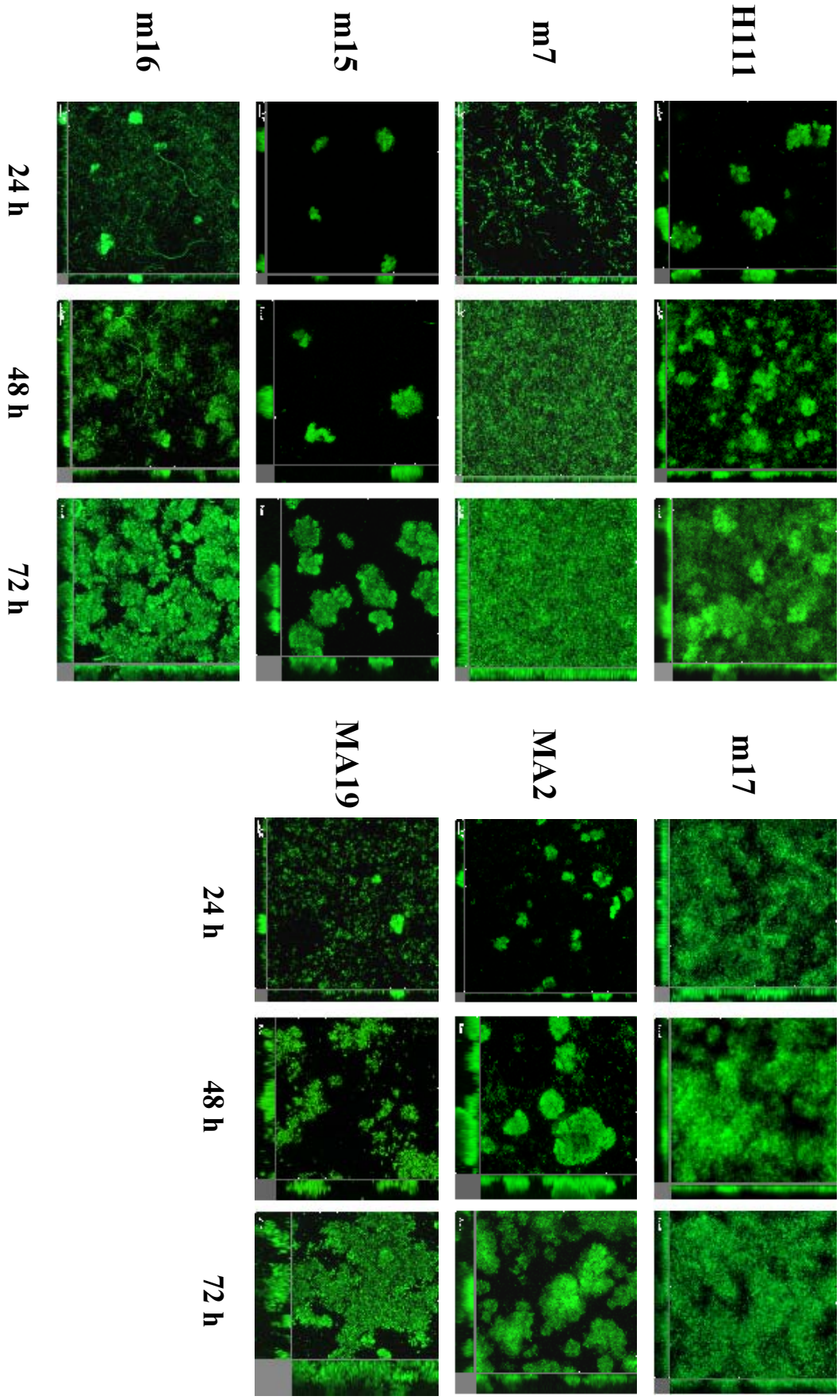

H111
m7
m15
m16
m17
MA2
MA19
24 h
48 h
72 h

Fig. 4: Quantification of biofilm structures. Biofilms of the wild type H111 and the mutants m7, m15, m16, m17, MA2, and MA19 were grown in artificial flow cells. CLSM pictures were taken 72 h after inoculation and these pictures were analysed by the computer program COMSTAT (Heydorn *et al*., 2000). Parameters calculated are biomass, substratum coverage, maximum biofilm thickness, surface to volume ratio, and roughness coefficient. Mean values for seven independent CLSM pictures are shown with standard deviations. The data are presented as relative values (mutant/wild type) with those of the wild type arbitrarily set to 1.

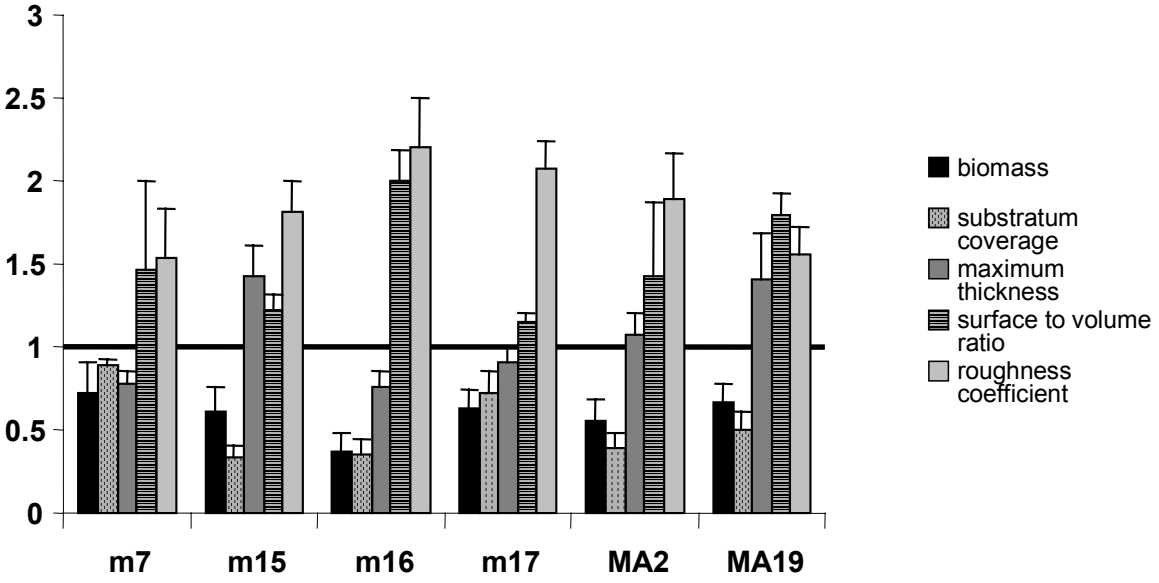


Fig. 5: Quantification of AHL production of *B. cepacia* mutants. Strains were grown in LB to an OD_{600} of 3.0. 100 µl of filter-sterilized culture supernatant of each mutant was added to 100 µl of an exponential culture of the monitor strain *E. coli* MT102 (pSB403) in the wells of a microtitre plate. Luminescence was measured after 6 h incubation at 30°C. C8-HSL and C6-HSL were used at a concentration of 44 nM and 100 nM, respectively. Mean values for three wells are shown with standard deviations.

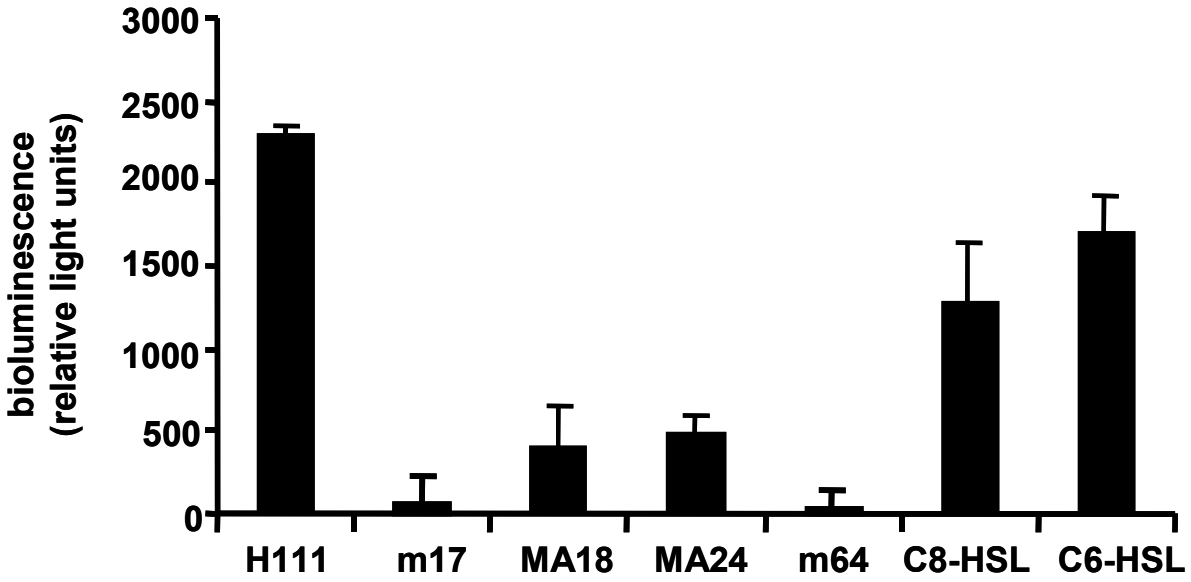


Fig. 6: Motility promotes initiation of biofilm formation but is not required for biofilm maturation. The time courses of biofilm formation by the *B. cepacia* H111 wild type and a *motA* mutant were determined. Overnight cultures of the respective strains were adjusted to an OD_{600} of 0.2 in ABC minimal medium and were then inoculated into the wells of polystyrene microtitre dishes. Biofilms were incubated for 2h, 4h, 6h, 8h, 24h, and 48h, before planktonic cells were removed and attached cells were stained with crystal violet. Error bars represent the standard deviation of the mean for six independent wells.

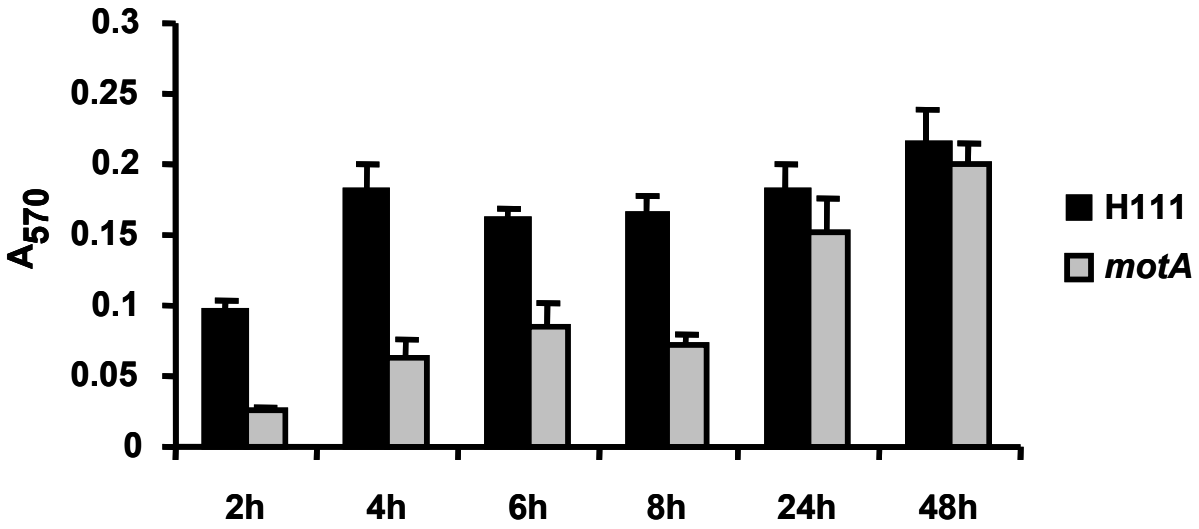


Fig. 7: Mutant m15 exhibits a decreased hydrophobicity. Mutant m15 and the wild type H111 were grown to early exponential (black columns) or stationary (grey columns) phase, respectively. Aqueous bacterial suspensions were mixed with *n*-hexadecane as described in the Experimental procedures. BATH (bacterial adhesion to hydrocarbon) is a measure for the percentage of cells adhering to the hydrophobic phase after the subsequent phase separation.

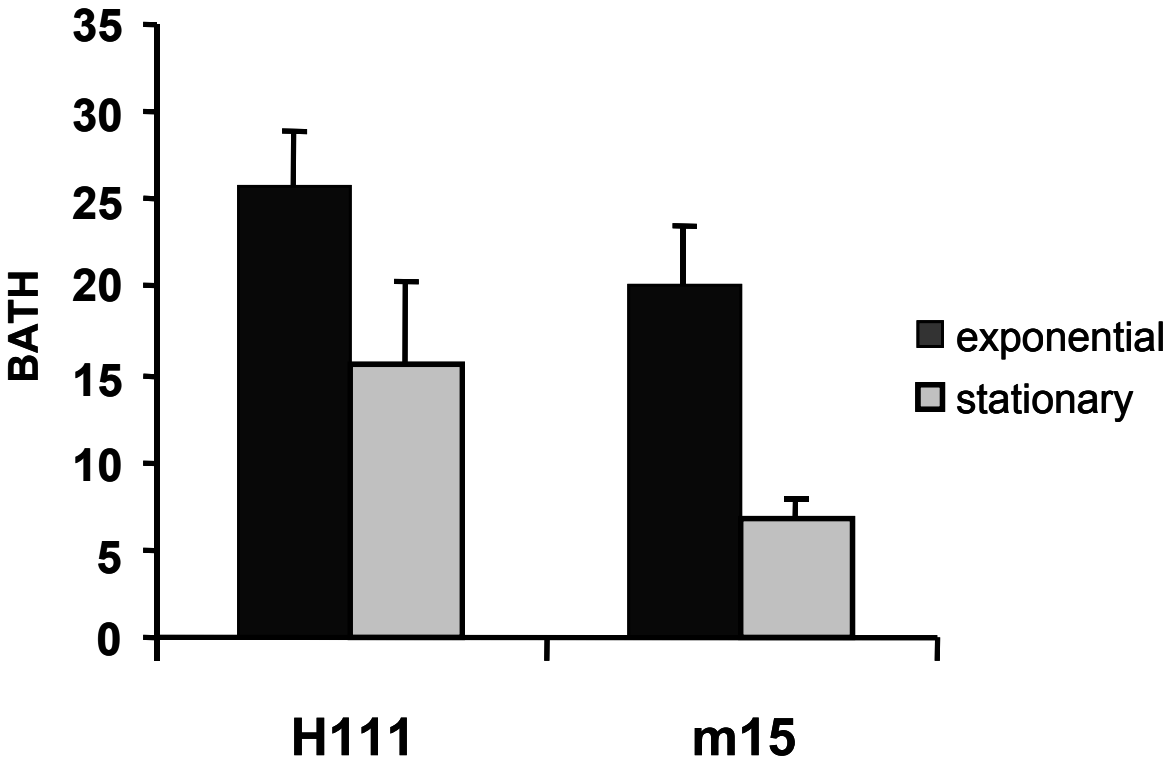


Fig. 8: Mutants m7 and MA19 exhibit aberrant cell morphologies. Phase-contrast micrographs were taken with a Carl Zeiss Axioplan microscope with a 100x objective lens. The bar represents 2 µm.

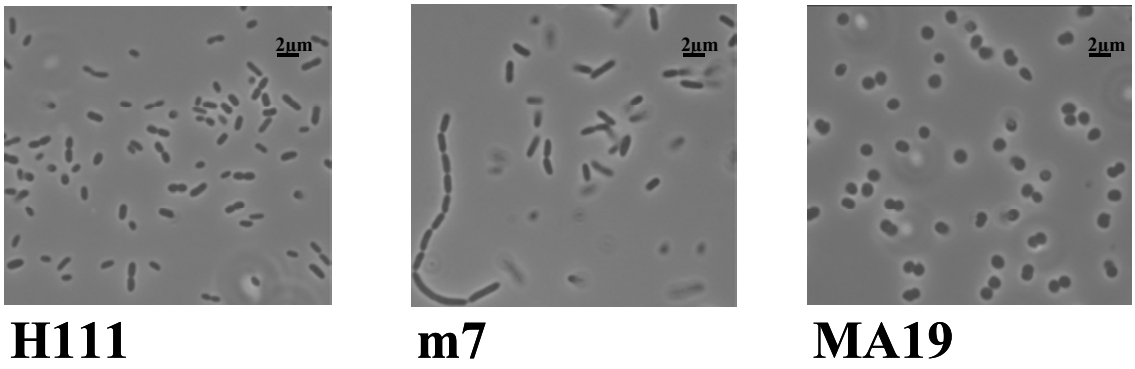
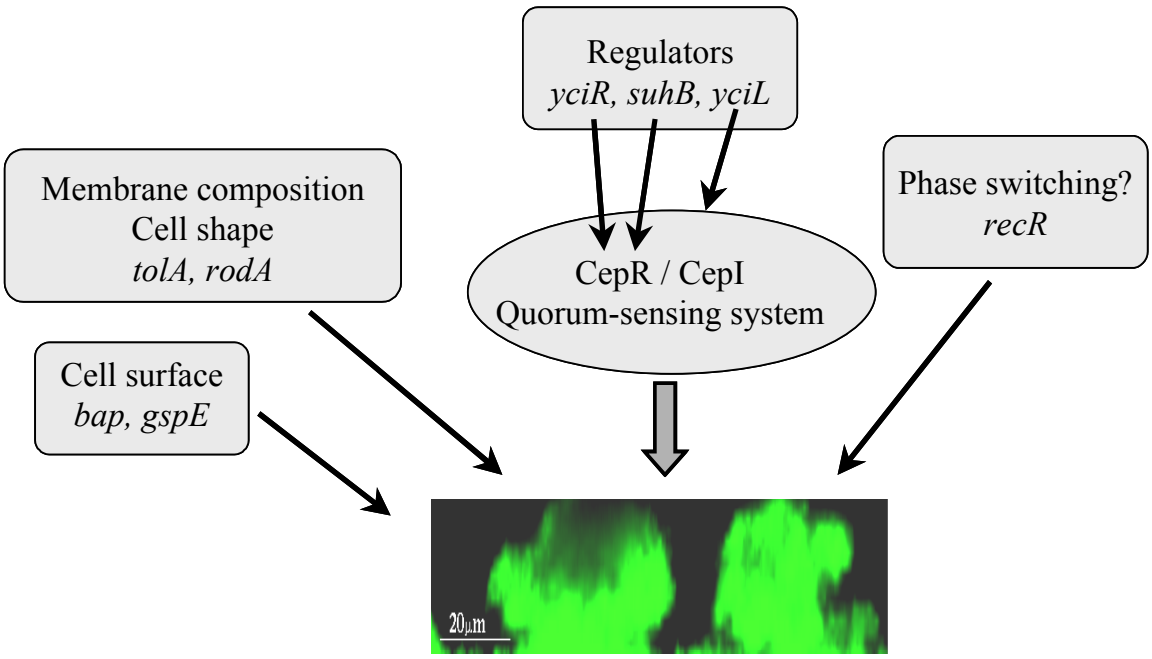


Fig. 9: A genetic model for biofilm development of *B. cepacia*. Our genetic analyses show that the genes required for biofilm maturation fall into several classes: (i) genes affecting cell surface protein composition (*bap, gspE*), (ii) genes involved in the biogenesis and maintenance of an integral outer membrane (*tolA, rodA*), and (iii) genes encoding regulatory elements (*yciR, suhB, yciL, recR*). The *recR* gene may promote DNA rearrangements that are required for a phase switching mechanism that leads to the expression of genes important for biofilm formation. The three regulatory genes *yciR, suhB,* and *yciL* are higher level regulators of the *cep* quorum-sensing system, which, in turn, is essential for biofilm maturation.



Mature Biofilm

Anhang 3

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

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

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***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

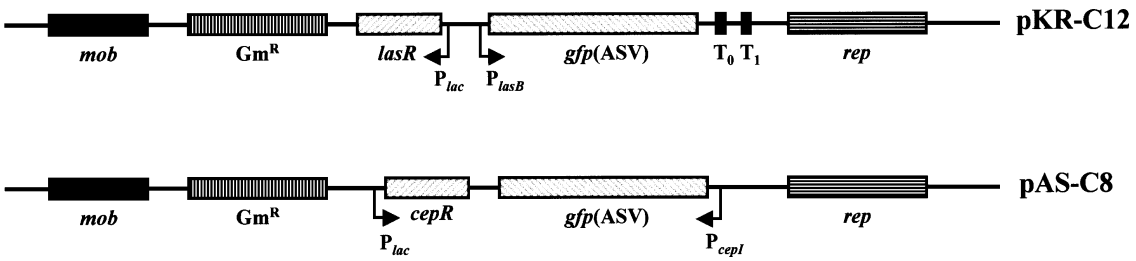

Fig. 1. Schematic drawings (not to scale) of the *las*- and *cep*-based AHL sensor plasmids used in this study. Plasmid pKR-C12 contains a *lasB–gfp*(ASV) translational fusion together with the *lasR* gene placed under control of P_{lac} on the broad-host-range plasmid pBBR1MCS-5. Plasmid pAS-C8 contains 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

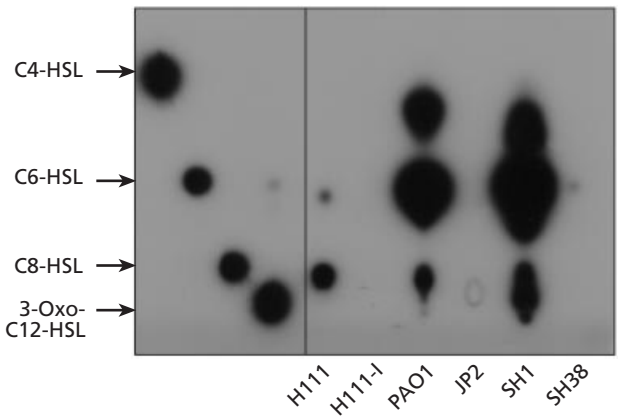

Fig. 2. TLC analysis of AHLs produced by strains of *P. aeruginosa* and *B. cepacia* used in this study. Samples were chromatographed on C_{18} reversed-phase thin-layer plates, developed with methanol/water (60:40, v/v) and spots were visualized by overlaying the TLC plates with *E. coli* MT102 harbouring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

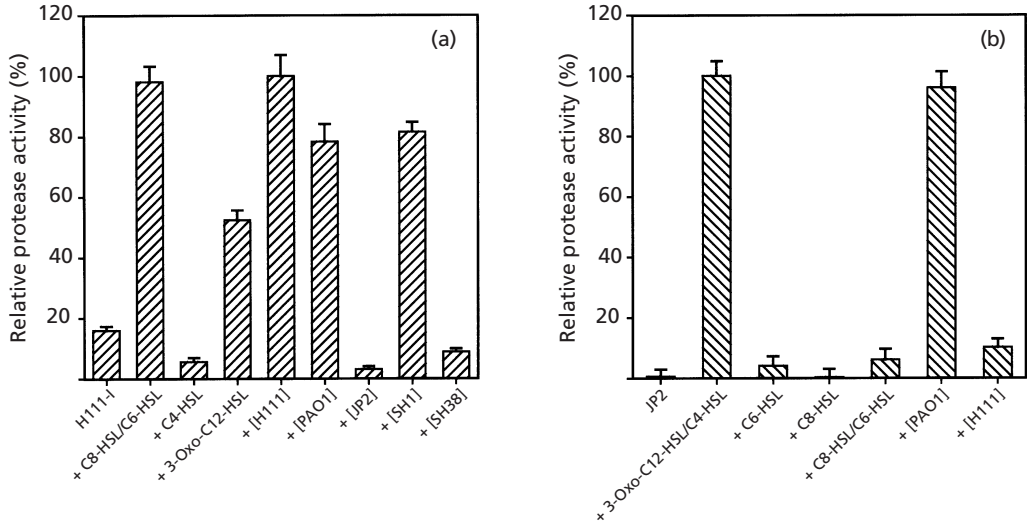

Fig. 3. Proteolytic activities of culture supernatants of (a) *B. cepacia* H111-I and (b) *P. aeruginosa* PAO1-JP2. The strains were grown in the absence or presence of different AHL molecules (at concentrations of 1 μM) or of dichloromethane extracts of supernatants prepared from cultures of the *P. 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,

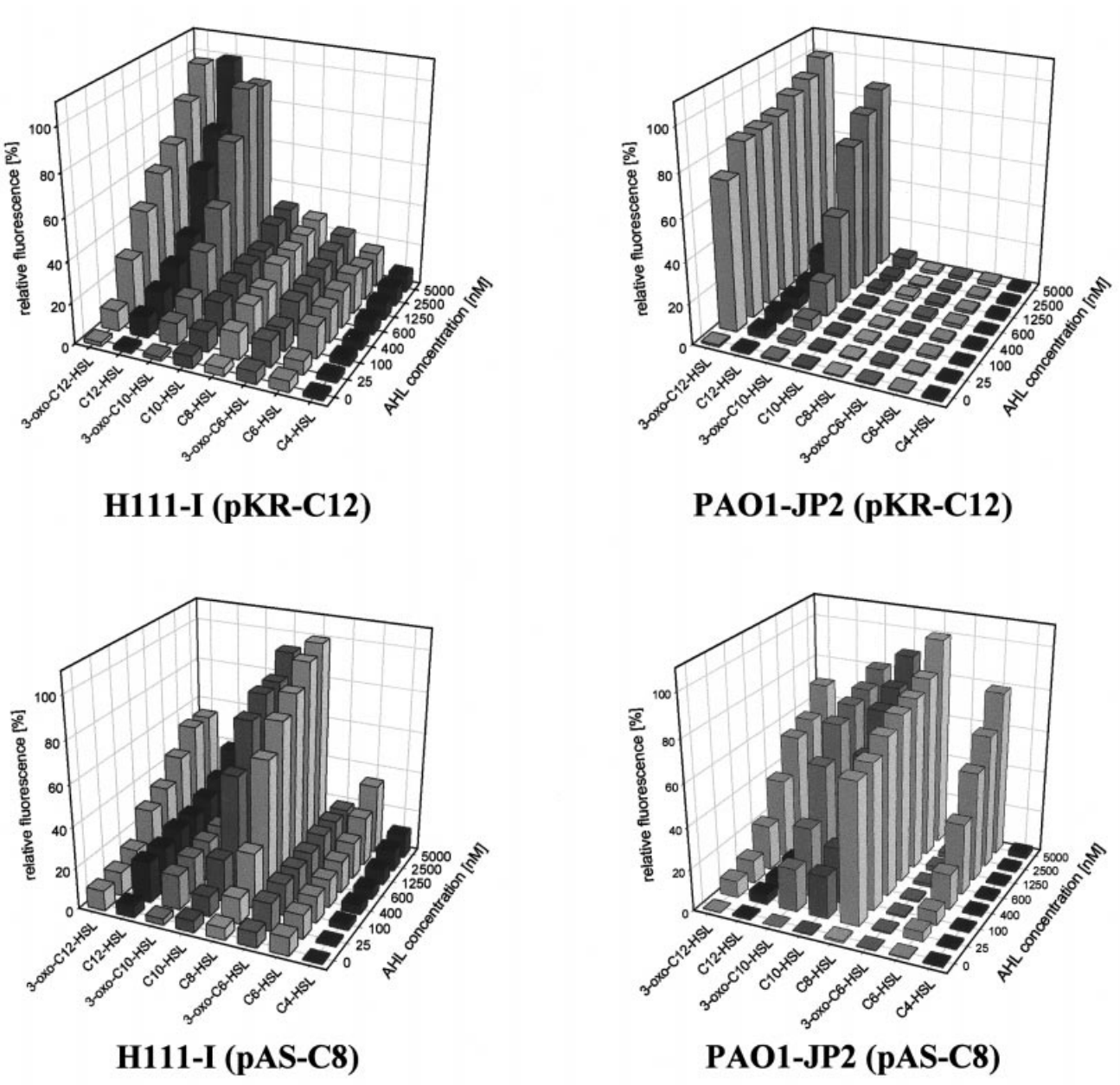

Fig. 4. Characterization of the AHL monitor strains. The monitor strains were grown in the presence of different AHL compounds at concentrations of 0–5000 nM. Green fluorescence of the cultures was recorded by a microtitre plate reader at a wavelength of 515 nm. Maximal induction levels of the monitor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

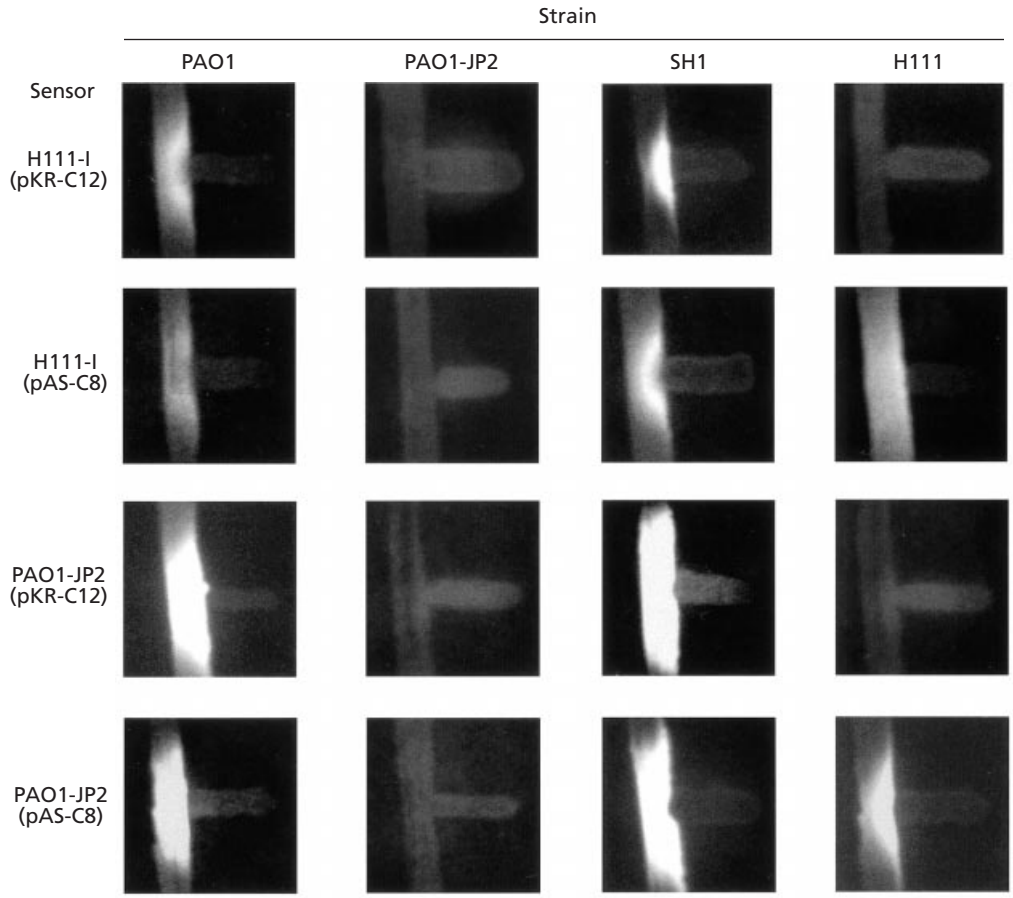

Fig. 5. Activation of the AHL monitor strains in cross-streak experiments. The four monitor strains H111-I(pKR-C12), H111-I(pAS-C8), PAO1-JP2(pKR-C12), PAO1-JP2(pAS-C8) and the test strains PAO1, PAO1-JP2, SH1 and H111 were cross-streaked on LB agar plates as described in Methods. Following 24 h of incubation 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

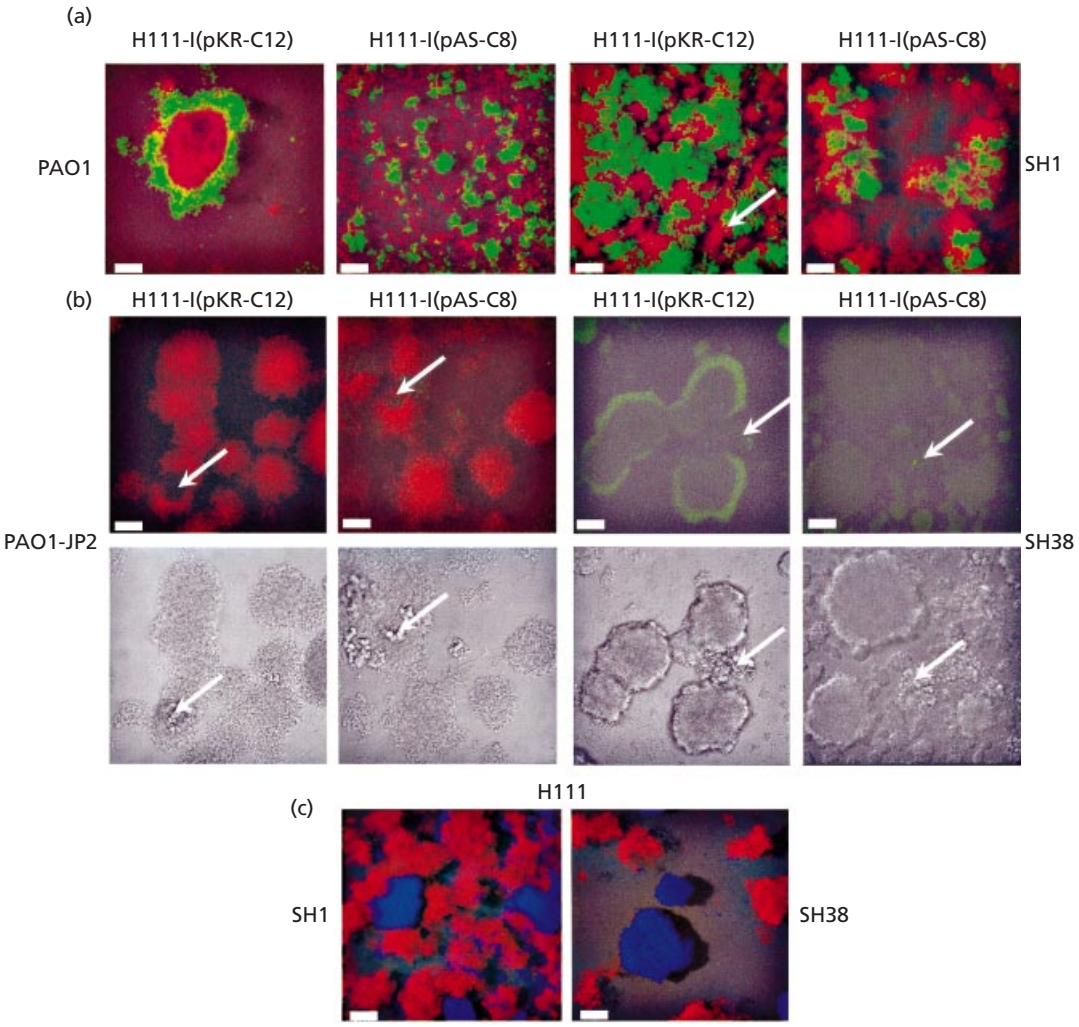

Fig. 6. Intergeneric communication between *P. aeruginosa* and *B. cepacia* in biofilms cultured in flow chambers. (a) Mixed biofilms of H111-I harbouring the sensor plasmids pKR-C12 (left panel) or pAS-C8 (right panel), and *dsred*-tagged derivatives of PAO1 and SH1. Perception of AHL molecules by the *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

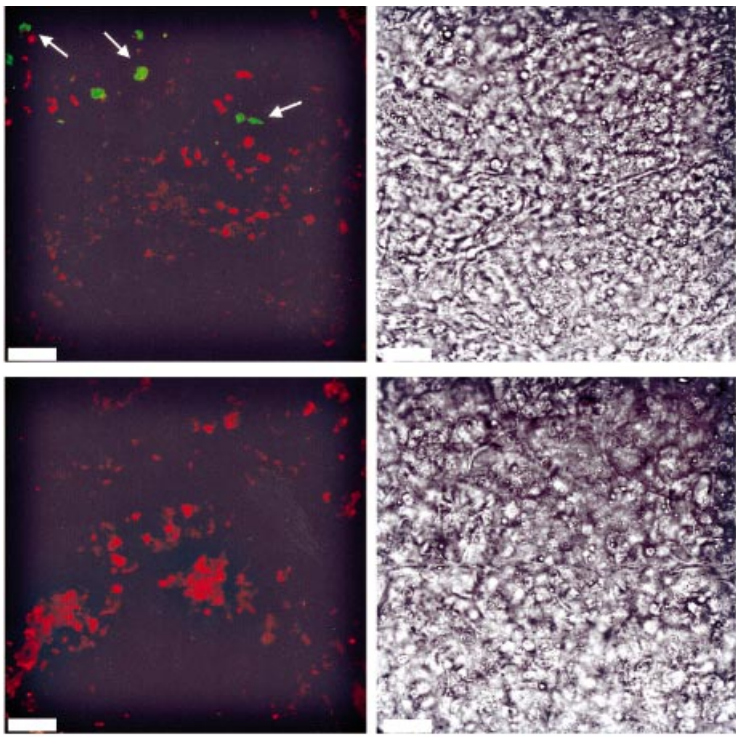

Fig. 7. Intergeneric communication between *P. aeruginosa* and *B. cepacia* in mouse lung tissue. Simulated fluorescence projections generated by CSLM of mouse tissue infected with alginate beads containing a mixture of H111-I(pAS-C8) and *dsred*-tagged derivatives of SH1 (top panel) and PAO1-JP2 (bottom 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.

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Anhang 4

Synthesis of multiple *N*-acyl-homoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex

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Synthesis of Multiple *N*-Acylhomoserine Lactones is Wide-spread Among the Members of the *Burkholderia cepacia* Complex

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Summary

Seventy strains of the *Burkholderia cepacia* complex, which currently comprises six genomic species, were tested for their ability to produce *N*-acylhomoserine lactone (AHL) signal molecules. Using thin layer chromatography in conjunction with a range of AHL biosensors, we show that most strains primarily produce two AHLs, namely *N*-octanoylhomoserine lactone (C8-HSL) and *N*-hexanoylhomoserine lactone (C6-HSL). Furthermore, some strains belonging to *B. vietnamiensis* (genomovar V) produce additional long chain AHL molecules with acyl chains ranging from C10 to C14. For *B. vietnamiensis* R-921 the structure of the most abundant long chain AHL was confirmed as *N*-decanoylhomoserine lactone (C10-HSL) by liquid chromatography - mass spectrometry (LC-MS) in combination with total chemical synthesis. Interestingly, a number of strains, most notably all representatives of *B. multivorans* (genomovar II), did not produce AHLs at least under the growth conditions used in this study. All strains were also screened for the production of extracellular lipase, chitinase, protease, and siderophores. However, no correlation between the AHL production and the synthesis of these exoproducts was apparent. Southern blot analysis showed that all the *B. cepacia* complex strains investigated, including the AHL-negative strains, possess genes homologous to the C8-HSL synthase *cepI* and to *cepR*, which encodes the cognate receptor protein. The nucleotide sequence of the *cepI* and *cepR* genes from one representative strain from each of the six genomovars was determined. Furthermore, the *cepI* genes from the different genomovars were expressed in *Escherichia coli* and it is demonstrated that all genes encode functional proteins that direct the synthesis of C8-HSL and C6-HSL. Given that *cepI* from the *B. multivorans* strain encodes a functional AHL synthase, yet detectable levels of AHLs were not produced by the wild-type, this suggests that additional regulatory functions may be present in members of this genomovar that negatively affect expression of *cepI*.

Key words: quorum sensing – *Burkholderia cepacia* – exoenzymes – *cepIR*

Introduction

In recent years *Burkholderia cepacia* has emerged as an important pathogen in patients with cystic fibrosis (CF) (GOVAN and DERETIC, 1996; GOVAN et al., 1996). Infection with *B. cepacia* often occurs in patients who are already colonized with *Pseudomonas aeruginosa*, the leading cause of chronic lung infection in CF patients. Co-colonization can result in three clinical outcomes: asymptomatic carriage, slow and continuous decline in lung function, or, for approximately 20 percent of the patients, fulminant and fatal pneumonia (ISLES et al., 1984). Taxonomic studies of *B. cepacia*-like bacteria revealed that they comprise a very heterogeneous group of strains, which have been collectively designated the *B. cepacia* complex (VANDAMME et al., 1997). This complex comprises a cluster of five genomic species, originally referred to as *B. cepacia* genomovars I through V.

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A sixth genomovar was recently described (COENYE et al., in press). Binomial species names have been assigned to those genomovars that can be identified by using routine diagnostic or PRC-based tests: *B. multivorans* (genomovar II), *B. stabilis* (genomovar IV), and *B. vietnamiensis* (genomovar V) (GILLIS et al. 1995; VANDAMME et al., 1997; 2000). It has been suggested that members of the different genomovars may vary greatly in both their pathogenic potential and their transmissibility (VANDAMME et al., 1997; MAHENTHIRALINGAM et al., 2000).

Recent work has established that many gram-negative bacteria employ sophisticated intercellular communication systems that rely on *N*-acylhomoserine lactone (AHL) molecules to control the expression of multiple target genes (FUQUA et al., 1996; HARDMAN et al., 1998; EBERL, 1999). AHL signal molecules enable the bacteria to monitor their own population density in a process known as 'quorum sensing'. 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 leads to the induction/repression of target genes. To date, AHL-dependent quorum sensing circuits have been identified in a wide range of gram-negative bacteria in which they regulate a variety of phenotypes and processes. In many cases these phenotypes are closely associated with the pathogenicity of the organism (WILLIAMS et al., 2000). Conceivably, pathogenic bacteria utilize quorum sensing to ensure virulence factor production is kept to a minimum at low cell densities to reduce the risk of premature detection by host defenses. However, once a sufficient population density is reached, a rapid and concerted attack is achieved which will overwhelm the host before it is able to mount an efficient response.

Recently, it has been demonstrated that *B. cepacia* genomovar III K56-2 employs a quorum sensing system, *cepRI*, to control production of an extracellular protease and the siderophore ornibactin (LEWENZA et al., 1999). The *cepI* gene product directs the synthesis of *N*-octanoylhomoserine lactone (C8-HSL) while *cepR* encodes for the cognate transcriptional regulator protein. Upon binding of C8-HSL to CepR, it is thought that the CepR-C8-HSL complex then regulates transcription of target genes. Accordingly, the *cep* system was shown to positively regulate protease production yet to repress ornibactin synthesis.

This study was initiated to investigate how the ability to produce AHLs is distributed among members of the different *B. cepacia* genomovars. Furthermore, we phenotypically characterized the various strains with respect to the production of protease, lipase, chitinase, and siderophores in order to assess whether AHL synthesis is associated with the production of extracellular factors.

Materials and Methods

Bacterial strains and growth conditions

All strains studied and their sources are listed in Table 1. Most strains were obtained from the Belgian Coordinated Collections of Microorganisms/Laboratorium Microbiologie Gent (BCCM/LMG) and the collection of the Medizinische Hochschule Hannover (Germany). In addition, a few strains from the University of Nottingham (United Kingdom) were included. The selected strains cover all six genomic species currently comprising the *B. cepacia* complex and two additional isolates that represent a novel *B. cepacia* complex genomovar (VANDAMME, unpublished data). Bacteriological purity was checked by plating, Gram staining and examining living cells by phase contrast microscopy. Strains were routinely grown aerobically at 30 °C in Luria-Bertani (LB) medium (BERTANI, 1951) or for AHL analyses in AB minimal medium (CLARK and MAALØE, 1967) supplemented with 10 mM citrate. The AHL sensor strains *E. coli* MT102 (pSB403), *E. coli* MT102 (pSB1075) (WINSON et al., 1998) and *Chromobacterium violaceum* CV026 (MCCLEAN et al., 1997) were grown aerobically at 30 °C in LB medium. For *E. coli* MT102 (pSB403) and *E. coli* MT102 (pSB1075) the media were supplemented with 10 µg/ml tetracycline and 100 µg/ml ampicillin respectively (Sigma, Deisenhofen, Germany).

Detection and characterization of AHLs

Production of AHLs by the various *B. cepacia* strains was investigated by the aid of different biosensors: the bioluminescent plasmid sensors pSB403 and pSB1075 (WINSON et al., 1998), and *C. violaceum* CV026 (MCCLEAN et al., 1997). Each of these sensors is capable of detecting a certain range of AHL molecules according to the specificity of the respective LuxR homologue. The sensor plasmid pSB403 contains the *Vibrio 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 *V. fischeri* relies on *N*-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL) and the sensor plasmid consequently exhibits the highest sensitivity for this AHL molecule and closely related AHLs such as C6-HSL. Likewise, the pSB1075 sensor was derived from the *lasIR* operon of *P. aeruginosa* which utilizes *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL) and therefore exhibits the highest sensitivity for this long-chain AHL. As pSB1075 was found to be not significantly more sensitive for 3-oxo-C12-HSL than pSB403 albeit more specific (GEISENBERGER et al., 2000), the latter was used for routine analyses. Bioluminescence was either detected with a highly sensitive photon counting camera C2400-40 (Hamamatsu Photonics K. K., Herrsching, Germany) or by exposure of an X-ray film (X-Ray-90, AGFA-Geveart, München, Germany). The sensor strain CV026 is a violaceine-negative mutant of *C. violaceum*, in which production of the purple pigment can be restored by addition of AHLs. This sensor is most sensitive for *N*-hexanoylhomoserine lactone (C6-HSL) and for *N*-butanoylhomoserine lactone (C4-HSL; MCCLEAN et al., 1997).

Initially, to assay for AHL production, strains were cross-streaked vertically against a horizontal streak of *E. coli* MT102 (pSB403), *E. coli* MT102 (pSB1075), and CV026 on LB agar plates and observed for activation of the sensor strain following overnight incubation at 30 °C. For more detailed analysis the AHL molecules were extracted from spent culture supernatants of the strains, separated by thin-layer chromatography (TLC) and AHL spots were then visualized by overlaying the TLC plates with soft agar seeded with an appropriate sensor strainas has been described previously (SHAW et al., 1997;

Table 1. List of strains studied with corresponding phenotypic characteristics (AHL-, exoenzyme- and siderophore-production, *cepIR* hybridisation signals).

Accession no. from BCCM/LMG Culture Collection	Strain name[a]	Source[b]	Production of[c]					Hybridisation signals (kb)	
			AHL	Protease	Chitinase	Lipase	Siderophores	*cepI*	*cepR*
B. cepacia Gv. I (6)			6	3	5	6	4		
LMG 6963		Soil (Australia, 1968)	+	+	+	+	+	2.4	2.4/1.9
LMG 1222^T		*Allium cepa* (USA, 1948)	+	+	+	+	+	n.d.	n.d.
LMG 14087		Wound swab (UK, 1988)	+	+	+	+	+	4.3	4.3
LMG 14095		CF patient (UK, 1974)	+	–	+	+	–	4.3	4.3
LMG 16663	C1963	CF patient (UK)	+	–	–	+	–	n.d.	n.d.
LMG 18821	CEP509	CF patient (Australia, 1997)	+	–	+	+	+	2.4	2.4/1.9
B. multivorans (16)			0	0	0	16	12		
LMG 13010^T		CF patient (Belgium, 1992)	–	–	–	+	+	n.d.	n.d.
LMG 16660	C1576	CF patient (UK, 1995), Glasgow epidemic reference	–	–	–	+	+	2.0	2.0/1.2
LMG 16665	C1962	Brain abscess (UK, 1995)	–	–	–	+	–	2.0	n.d.
LMG 17588	CEP144	Soil (USA, 1996)	–	–	–	+	+	n.d.	n.d.
LMG 18822	C5393	CF patient (Canada, 1996)	–	–	–	+	+	2.0	2.0/1.2
LMG 18824	FC0147	CGD patient (USA, 1998)	–	–	–	+	+	n.d.	n.d.
LMG 18825	CF-A1-1	CF patient (UK, 1998), epidemic	–	–	–	+	+	n.d.	n.d.
LMG 18945	97/35	CF patient (UK, 1998)	–	–	–	+	–	2.0	2.0/1.2
R-139	C1524	CF patient (UK)	–	–	–	+	+	2.0	2.0/1.2
R-654	C4297	CF patient (Canada)	–	–	–	+	–	2.0	2.0/1.2
R-1914	C1991	CF patient (UK)	–	–	–	+	–	2.0	2.0/1.2
R-6268	H59	CF patient (Germany)	–	–	–	+	+	2.0	2.0/1.2
R-6269	H115	CF patient (Germany)	–	–	–	+	+	2.4	2.4
R-6275	H158	CF patient (Germany)	–	–	–	+	+	2.4	n.d.
R-6278	H174	CF patient (Germany)	–	–	–	+	+	2.0	n.d.
R-6284	H191	CF patient (Germany, 1996)	–	–	–	+	+	2.0	2.0/1.2
B. cepacia Gv. III (17)				13	11	16	16	14	
LMG 6981		Bronchial washing (1985)	+	+	+	+	+	2.4	2.4/1.9
LMG 6988		Leg wound (Sweden, 1972)	+	+	+	+	+	3.2	3.2/1.9
LMG 6993		Soil (Trinidad, 1960)	+	–	+	+	+	4.3	4.3
LMG 12615	81/92	CF patient (UK, 1992), ET12 lineage	+	–	+	+	+	2.4	2.4/1.9
LMG 14271	A3P	CF patient (Belgium, 1993)	+	+	+	+	+	2.4	2.4/1.9
LMG 16654	J415	CF patient (UK, 1984)	+	+	–	–	–	n.d.	n.d.
LMG 16655	C1335	CF patient (UK, 1988)	–	–	+	+	+	4.3	4.3
LMG 16657	C1340	CF patient (UK)	+	–	+	+	–	2.4/3.2	2.4/1.9/3.2
LMG 16659	C1394	CF patient (UK, 1995), Manchester epidemic reference	+	–	+	+	+	2.4	2.4/1.9
LMG 18826	BC7	CF patient (Canada, 1997), epidemic	–	+	+	+	+	2.4	2.4/1.9
LMG 18827	C5424	CF patient (Canada, 1996), epidemic	–	+	+	+	+	2.4	2.4/1.9
LMG 18863	K56-2	CF patient (Canada, 1999), epidemic	+	+	+	+	+	2.4	2.4/1.9
R-651	C1257	CF patient (Canada), epidemic	–	+	+	+	+	2.4	2.4/1.9
R-6108	AD0979	CF patient (New Zealand), ET12 lineage	+	–	+	+	–	2.4	2.4/1.9
R-6274	H147	CF patient (Germany)	+	+	+	+	+	2.4	2.4/1.9
R-6282	H111	CF patient (Germany, 1993)	+	+	+	+	+	2.4	2.4/7.5
R-6285	BC2a	CF patient	+	+	+	+	+	2.4	2.4/1.9
B. stabilis (15)			13	9	15	15	4		
LMG 6997		Ear (Sweden, 1974)	+	+	+	+	–	2.4	2.4/1.9
LMG 7000		Blood (Sweden, 1983)	+	+	+	+	–	2.4	2.4/1.9
LMG 14291	B4P	CF patient (Belgium, 1993)	+	–	+	+	+	2.4	2.4/1.9

Table 1. (Continued).

Accession no. from BCCM/LMG Culture Collection	Strain name[a]	Source[b]	Production of[c]					Hybridisation signals (kb)	
			AHL	Protease	Chitinase	Lipase	Siderophores	*cepI*	*cepR*
LMG 18138	E20	CF patient (Belgium, 1995)	–	–	+	+	+	2.4	2.4/1.9
R-136	J1750	CF patient (USA)	+	+	+	+	–	n.d.	n.d.
R-3338	M71-40	CF patient (Germany, 1997)	+	–	+	+	+	2.4/3.2	2.4/1.9/3.2
R-6270	H118	CF patient (Germany)	+	–	+	+	–	2.4	2.4/1.9
R-6272	H134	CF patient (Germany)	+	–	+	+	–	2.4	2.4/1.9
R-6273	H145	CF patient (Germany)	+	+	+	+	–	2.4	2.4/1.9
R-6276	H162	CF patient (Germany)	+	+	+	+	–	2.4	2.4/1.9
R-6277	H173	CF patient (Germany)	–	–	+	+	–	2.4	2.4/1.9
R-6279	H177	CF patient (Germany)	+	+	+	+	–	2.4	2.4/1.9
R-6280	H193	CF patient (Germany)	+	+	+	+	–	2.4	2.4/1.9
R-6281	H177E	Water outlet (Germany)	+	+	+	+	+	2.4	2.4/1.9
R-10033	H175	CF patient (Germany, 1996)	+	+	+	+	–	2.4	2.4/1.9
B. vietnamiensis (9)			9	0	0	8	9		
LMG 6998		Blood (Sweden, 1978)	+[d]	–	–	–	+	n.d.	n.d.
LMG 6999		Neck abscess, child (Sweden, 1980)	+[d]	–	–	+	+	n.d.	n.d.
LMG 10929[T]		Rice rhizosphere (Vietnam, 1990)	+[d]	–	–	+	+	2.8	2.8/2.5
LMG 16232		CF patient (Sweden, 1993)	+	–	–	+	+	2.1	2.1/2.5
LMG 18835	CEP40	CF patient (USA, 1997)	+	–	–	+	+	n.d.	n.d.
LMG 18836	FC0441	CGD patient (Canada, 1997)	+	–	–	+	+	n.d.	n.d.
R-128	J1697	CF patient (USA)	+[d]	–	–	+	+	2.1	2.1/2.7
R-723	PC30	CF patient (USA)	+	–	–	+	+	2.1	2.1/2.7
R-921		CF patient (Sweden)	+[d]	–	–	+	+	2.1	2.1/2.7
B. cepacia Gv. VI (5)			5	0	5	5	5		
LMG 18941	FC348	CF patient (USA, 1996)	+	–	+	+	+	2.4	2.4/2.8
LMG 18942	PC534	CF patient (USA, 1997)	+	–	+	+	+	2.4	2.4/2.8
LMG 18943	AU0645	CF patient (USA, 1998)	+	–	+	+	+	2.4	2.4/2.8
LMG 18944	PC688	CF patient (USA, 1997)	+	–	+	+	+	2.4	2.4/2.8
LMG 18946	AU0018	CF patient (USA, 1997)	+	–	+	+	+	2.4	2.4/2.8
B. cepacia complex, novel Gv. (2)			2	0	0	2	0		
LMG 16670	J2552	*C. palmata* rhizosphere (UK)	+	–	–	+	–	n.d.	n.d.
R-3976	J2863	Soil	+	–	–	+	–	n.d.	n.d.

H – Medizinische Hochschule Hannover, Hannover, Germany; LMG – Laboratorium Microbiologie Gent Culture Collection, Universiteit Gent, Ghent, Belgium; R – Research collection, Peter Vandamme, Belgium; CF – cystic fibrosis; CGD – chronic granulomatous disease; Gv. – genomovar; n.d. – not determined

[T] type strain

[a] Numbers in parantheses indicate numbers of strains examined

[b] if known

[c] Numbers in the field are the numbers of strains giving a positive result

[d] strains producing additionally C10-HSL

+, strains positive; –, strains negative

MCCLEAN et al., 1997; GEISENBERGER et al., 2000). Routinely, AHLs were extracted twice with dichloromethane (250:100 supernatant/dichloromethane) from 250 ml sterile-filtered supernatants of *B. cepacia* cultures grown in AB minimal medium containing 10 mM citrate at 30 °C to OD_{600} 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 KgaA, 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 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 compari-

son of mobilities (R_f-values) relative to those for the synthetic AHL standards, which were either purchased from Fluka Chemie AG (Buchs, Switzerland) or were synthesized as previously described (CÁMARA et al., 1998; CHHABRA et al., 1993). AHL concentrations were estimated by comparing the sizes of the TLC spots with those from a dilution row of the respective AHL reference substances.

Isolation, purification, and chemical characterization of AHLs

AHLs were purified and characterized as described by BAINTON et al. (1992) and CÁMARA et al. (1993). Essentially, spent supernatants from cultures of *B. cepacia*, which were grown in AB medium supplemented with 10 mM citrate to an OD_{600} of 1.0, were extracted with dichloromethane and the residue resuspended in 250 µl of acetonitrile. Crude extracts were then analysed by HPLC-mass spectrometry (LC-MS) (Micromass Instruments, Manchester, United Kingdom) using an acetonitrile in water linear gradient (20% to 100%) over a 32 min period at a flow rate of 0.7 ml/min and monitored at 200 nm. This technique couples the resolving power of C_8 reverse phase HPLC (Kromasil 5C8 [250 mm × 4.6 mm] column: Hichrom, Reading, United Kingdom) directly with mass spectrometry such that the mass of the molecular ion (M+H) and its major component fragments can be determined for a compound with a given retention time. Samples eluting from the HPLC column were ionised by positive-ion atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) and were analysed at two cone voltages, 18 eV and 28 eV. The spectra obtained were compared with those of the synthetic AHL standard subjected to the same LC-MS conditions.

Analysis of extracellular products

Exoenzyme activities were examined by streaking the strains on appropriate indicator plates. LB agar plates supplemented with 2% skim milk powder (Merck, Darmstadt, Germany) or 0.037% ethylene glycol chitin and 0.01% trypan blue (both Sigma, Deisenhofen, Germany) were used for determination of proteolytic and chitinolytic activity, respectively. Lipolytic activity was investigated on plates containing Tributyrin agar base and 1% glycerol tributyrate (both Merck, Darmstadt, Germany). After 48 h of incubation at 30 °C the plates were inspected. The production of the relevant exoenzyme was indicated by the presence of zones of clearing, surrounding bacterial growth, due to substrate degradation. Siderophore activity was determined using Chromazurol S (CAS) agar plates which contain a highly coloured iron-dye complex (SCHWYN and NEILANDS, 1987). In the presence of siderophores, the release of free iron is accompanied by a colour change from blue to orange.

Southern hybridisation

Genomic DNA was isolated with the DNeasy™ Tissue Kit (QIAGEN, Hilden, Germany) and digested overnight with the restriction endonuclease *Pst*I (MBI Fermentas, St. Leon-Rot, Germany). The digested DNA was then transferred to a positively charged nylon membrane Biodyne® Plus Membrane (Pall, Port Washington, NY) using a PosiBlot® pressure blotter (Stratagene, La Jolla, CA, USA). Probes specific for *cepI* and *cepR* were generated by PCR. The primer pairs cepI-1V 5′-CCCTGTAAGAGTTACCAGTT-3′ and cepI-1R 5′-GATATCGATCCAGCACGCCACGAC-3′ at 56 °C annealing temperature and cepR-3V 5′-CTGGATGGCGCACTACCAGGC-3′ and cepR-3R 5′-ACGTGGAAGTTGACCGTCCGC-3′ at 60 °C annealing temperature, were used to generate a 600 bp *cepI* and a 450 bp *cepR* specific probe, respectively. After purification with QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) the PCR products were labelled, by random priming, using Klenow polymerase and digoxigenin-dUTP DNA labelling and detection kit (Roche, Mannheim, Germany) in accordance with the manufacturer′s instructions. Approximate sizes of hybridising fragments were assessed by reference to co-migrating *Hin*dIII-digested lambda DNA (Roche, Mannheim, Germany). The positions of PCR primers used in this study are depicted in Fig. 1.

Cloning and sequence analysis of *cepI* and *cepR*

The entire *cep* loci from one representative of each *Burkholderia* genomovar were amplified by whole cell PCR using the primer pair cepIR-Ba1V 5′-GGAAACGCGCGTTCCGGCTCAGGCGGCGATAGC-3′ and cepIR-Ba2R 5′-CGTGAGCTGGACCGCGAAGGAAGCGGAGCC-3′, which were derived from the *B. cepacia* K56-2 *cepIR* nucleotide sequence (LEWENZA et al., 1999), at 62 °C annealing temperature. The resulting 2.16 kb PCR products were cloned into the vector pCR2.1® by using the TOPO™ TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. After isolation of recombinant plasmid DNA with the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany), double-strand DNA sequencing was performed with the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) in a PTC-100 Programmable Thermo Controller (MJ Research Inc., Watertown, Mass., USA) and a LI-COR® 4200

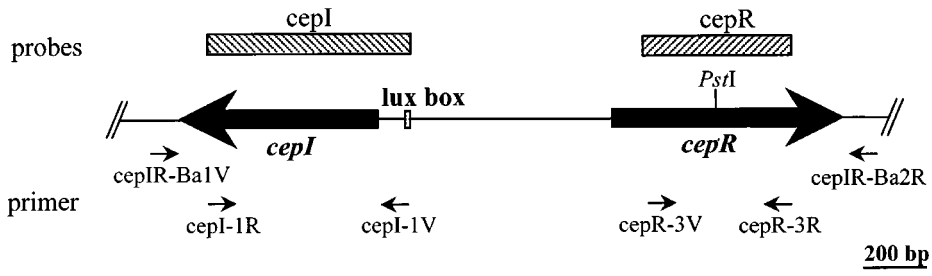


Fig. 1. Physical and genetic map of the *cep* region. The arrows represent the position and orientation of the *cepI* and *cepR* genes. The shaded bar designates the position of a *lux* box sequence in the promoter region of *cepI*. The location of a unique *Pst*I restriction site present in the *cepR* gene of most strains is shown. The positions of primers used for PCR are depicted by arrows and the two probes used for Southern blot analysis are indicated.

DNA sequencer (LI-COR Inc., Lincoln, USA). Initial sequencing was performed using M13 universal sequencing primers after which specifically designed primers, from the sequence data obtained, were used for successive reactions spanning the entire fragment length of both strands. The nucleotide sequence assembly and analysis was performed with DNAMAN software (Lynnon BioSoft Copyright© Version 4.13, Quebec, Canada). The BLASTN and BLASTX programs (MADDEN et al., 1996) were used to search the nonredundant sequence database for homologous sequences.

Heterologous expression of *cepI* in *E. coli*

The *cepI* genes from representative strains of the different genomovars were amplified using the primer pair cepI-R-Exp1 5′-CCCAAGCTTCCGGCTCAGGCGGCGATAG-3′ and cepI-V-Exp1 5′-TGCGGATCCAGACCTTCGTTCACGAGG-3′ (*Hin*dIII and *Bam*HI restriction sites are underlined). The resulting 0.6 kb DNA fragments were digested with *Hin*dIII and *Bam*HI, inserted into the expression vector QIA*express*® pQE-32 (QIAGEN, Hilden, Germany) cut with the same enzymes, and electroporated (DOWER et al., 1988) into *E. coli* XL1-Blue (BULLOCK et al., 1987). Cultures were grown in LB medium supplemented with 100 µg/ml ampicillin (Sigma, Deisenhofen, Germany) at 37 °C and expression of *cepI* was induced at OD_{600} 0.2 by the addition of 1-isopropyl-ß-D-1-thiogalactopyranoside (IPTG) (Merck KgaA, Darmstadt, Germany) to a final concentration of 2 mM. When the cultures reached OD_{600} 1.0 (approximately four hours after induction), cell free supernatants were extracted and the presence of AHLs analysed by TLC in conjunction with AHL biosensor strains as described above.

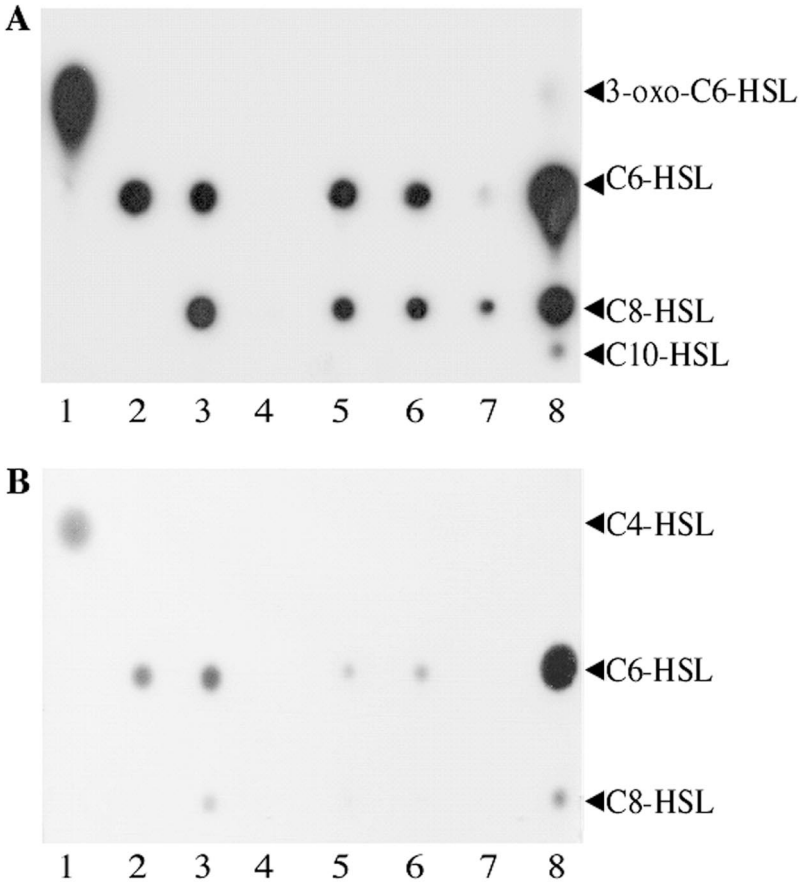
Results

Production of AHLs by members of different *Burkholderia* genomovars

To investigate whether the production of AHLs is widespread among members of the different *Burkholderia* genomovars, extracts of spent culture supernatants were analysed by TLC in conjunction with AHL biosensors (Table 1). Since growth conditions had a pronounced effect on AHL profiles (both the nature and levels of AHLs produced) efforts were made to cultivate cells under standardized conditions with respect to medium composition, oxygen supply, temperature as well as the optical density at which cultures were harvested (OD_{600} 1.0). For detection of AHLs on TLC plates we routinely used the bioluminescent sensor plasmid pSB403, which is capable of detecting a broad spectrum of AHL molecules (WINSON et al., 1998). In cases where pSB403 failed to detect AHLs, additional biosensors, which exhibit different specificities and sensitivities were employed (see Materials). Out of a total of 70 strains tested 47 were positive for AHL production (Table 1). All strains of genomovars I (6) and VI (5) and of *B. vietnamiensis* (9), most strains of *B. stabilis* (13/15), and both isolates of the novel genomovar (R-3976 and LMG 16670) synthesized AHL molecules. In sharp contrast, however, AHLs were not detected for any of the 16 *B. multivorans* strains tested.

As illustrated in Fig. 2 , for most strains two AHLs were detected which, on the basis of their mobility relative to synthetic AHL standards and their differential activation of various AHL-biosensors, were tentatively identified as C8-HSL and C6-HSL. In all cases C8-HSL was produced in larger amounts than C6-HSL (at least 10-fold) but the molar ratio was strain dependent. This is in good agreement with a previous report, in which the presence of C8-HSL in spent culture supernatants of *B. cepacia* K56-2 (a representative of genomovar III) was confirmed by chemical analyses (LEWENZA et al., 1999).

In the supernatants of five strains of *B. vietnamiensis* (LMG 6998, LMG 6999, LMG 10929, R-128, R-921) additional AHL



◄ **Fig. 2.** Identification of AHLs produced by selected strains from the different *Burkholderia* genomovars. AHLs extracted from cell-free culture supernatants were separated by TLC and detected using an overlay of agar seeded with (A) *E. coli* MT102 (pSB403) and (B) *C. violaceum* CV026, respectively. Lane 3, LMG 1222^T (Gv. I); lane 4, LMG 16660 (Gv. II); lane 5, H111 (Gv. III); lane 6, LMG 18943 (Gv. VI); lane 7, LMG 14291 (Gv. IV); lane 8, LMG 10929^T (Gv. V). The loaded volumes of extracts are equivalent to 5 ml (lane 3, 5, 8), 10 ml (lane 7), 30 ml (lane 4), and 40 ml (lane 6) of culture, respectively. 3-oxo-C6-HSL and C6-HSL standards are included in lanes 1 and 2.

molecules with acyl side chains longer than C8 were detected. Using the plasmid sensor pSB403 for AHL detection, the most prominent spot exhibited mobility properties similar to C10-HSL (Fig. 2). To identify the structures of these additional AHL molecules the extracts of culture supernatants of *B. vietnamiensis* R-921 were analysed by LC-MS. This analysis confirmed that this strain, beside C6- and C8-HSL, also produces large amounts of C10-HSL (Fig. 3). Lower amounts of *N*-(3-oxodecanoyl)-homoserine lactone (3-oxo-C10-HSL), *N*-dodecanoylhomoserine lactone (C12-HSL), and *N*-tetradecanoylhomoserine lactone (C14-HSL) were also detected (data not shown).

The amounts of AHLs produced by the various strains were found to vary dramatically. For example, while the concentration of C8-HSL in the supernatants of most strains of genomovar III was very low, in the range of 10-50 nM, for another representative of this genomovar, H111, approximately 100-fold higher levels were determined. Thus, the failure to detect AHLs for some of the *Burkholderia* strains may simply be a matter of sensitivity. As no AHLs were detected under routine growth conditions, and because, as noted earlier, growth conditions can greatly affect AHL production, we wondered whether AHL production, in these apparently AHL negative strains, was in fact growth medium dependent. Consequently, we tested for AHL production following growth of two selected strains (LMG 13010^T and LMG 18826) in a variety of media. Neither substituting the glucose of AB minimal medium with either glycerol, succinate, citrate, or Casamino acids nor growing the cells in rich medium such as LB or brain heart infusion (Difco, Augsburg, Germany), resulted in the production of detectable levels of AHLs. Furthermore, in these experiments spent culture supernatants, taken at various time points along the growth curve, were analysed to rule out the possibility that AHLs were only produced in a particular phase of growth and were perhaps degraded by the time the culture had reached an OD_{600} ~ 1.0 (data not shown).

Production of extracellular enzymes and siderophores by members of different *Burkholderia* genomovars

For the genomovar III strain *B. cepacia* K56–2 it has been demonstrated previously that the production of an extracellular protease and the siderophore ornibactin is regulated by the *cep* quorum sensing system (LEWENZA et al., 1999). To investigate whether AHL production correlates with the production of extracellular protease, lipase, chitinase, or siderophores we tested the strains on appropriate indicator plates as described in the Materials and Methods section. The results are shown in Table 1.

Three out of six genomovar I members, eleven out of seventeen genomovar III members and nine out of fifteen *B. stabilis* strains were found to produce protease(s). However, none of the other strains showed proteolytic activity. Chitinase production was demonstrated for all strains of *B. stabilis*, genomovar III (except for LMG 16654) and VI and for most strains of genomovar I (5/6). In contrast, all strains of *B. multivorans* and *B. vietnamiensis* as well as the two isolates representing a novel genomovar were negative for chitinase production. Except for *B. vietnamiensis* LMG 6998 and genomovar III LMG 16654 all strains produced lipase. Siderophore activity, as assessed on CAS indicator plates, was observed for all strains of genomovar VI and *B. vietnamiensis* and for most strains of *B. multivorans* (12/16), genomovar I (4/6) and III (14/17). None of the genomovar VI, nor the two isolates representing a novel genomovar, and only four of the 15 tested *B. stabilis* strains exhibited siderophore activity.

The *cepI* and *cepR* genes are present in all strains of the *Burkholderia* complex

The observation that some *Burkholderia* strains did not produce detectable amounts of AHLs prompted us to investigate whether these strains carry the *cep* genes. For this purpose we performed Southern blot experiments using DNA fragments containing part of the *cepI* or *cepR* gene, respectively, as probes. Surprisingly, both probes gave rise to hybridisation signals with all 53 strains tested (Table 1), irrespective of whether the strains produced AHLs or not.

Fig. 4 shows the results of the Southern blot analysis for representative strains from each genomovar using *Pst*I digested chromosomal DNA. While for most strains of genomovar III and IV and two of four tested genomovar I strains the cepI probe hybridised to chromosomal fragments of approximately 2.4 kb, for strains belonging to genomovar II, V, and VI bands of approximately 2 kb, 2.1 kb, and 2.4 kb, respectively were seen (see Table 1). When probing with the cepR probe, two hybridisation bands were detected for most of the strains (46/53). This is as expected due to the presence of a *Pst*I restriction site within *cepR* (Fig. 2; LEWENZA et al. 1999). Consequently, in addition to the hybridisation bands that were also detected by the cepI probe, the following bands were also typically observed by the cepR probe: 1.9 kb for strains of genomovar I (2/4), III (13/16), and IV (14/14), 1.2 kb for *B. multivorans* strains (8/9), 2.7 kb for *B. vietnamiensis* strains (3/5), and 2.8 kb for strains of genomovar VI (5/5).

Interestingly, with five strains belonging to the genomovars I, II, or III only a single hybridisation signal was observed when probed with cepR, indicating the absence of a *Pst*I site within *cepR* in these strains (Fig. 4). Also of note is the finding that in two strains, LMG 16657 (genomovar III) and M71-40 (genomovar IV), one additional band at 3.2 kb was detected with both probes, indicating the presence of an additional *cep* locus (data not shown).

Sequence analysis of the *cepI* and *cepR* genes of strains from different *Burkholderia* genomovars

Since the Southern blot analysis indicated that all *B. cepacia* strains investigated (including those that were AHL-negative) carry genes homologous to *cepI* and *cepR*, the nucleotide sequence of these genes in one

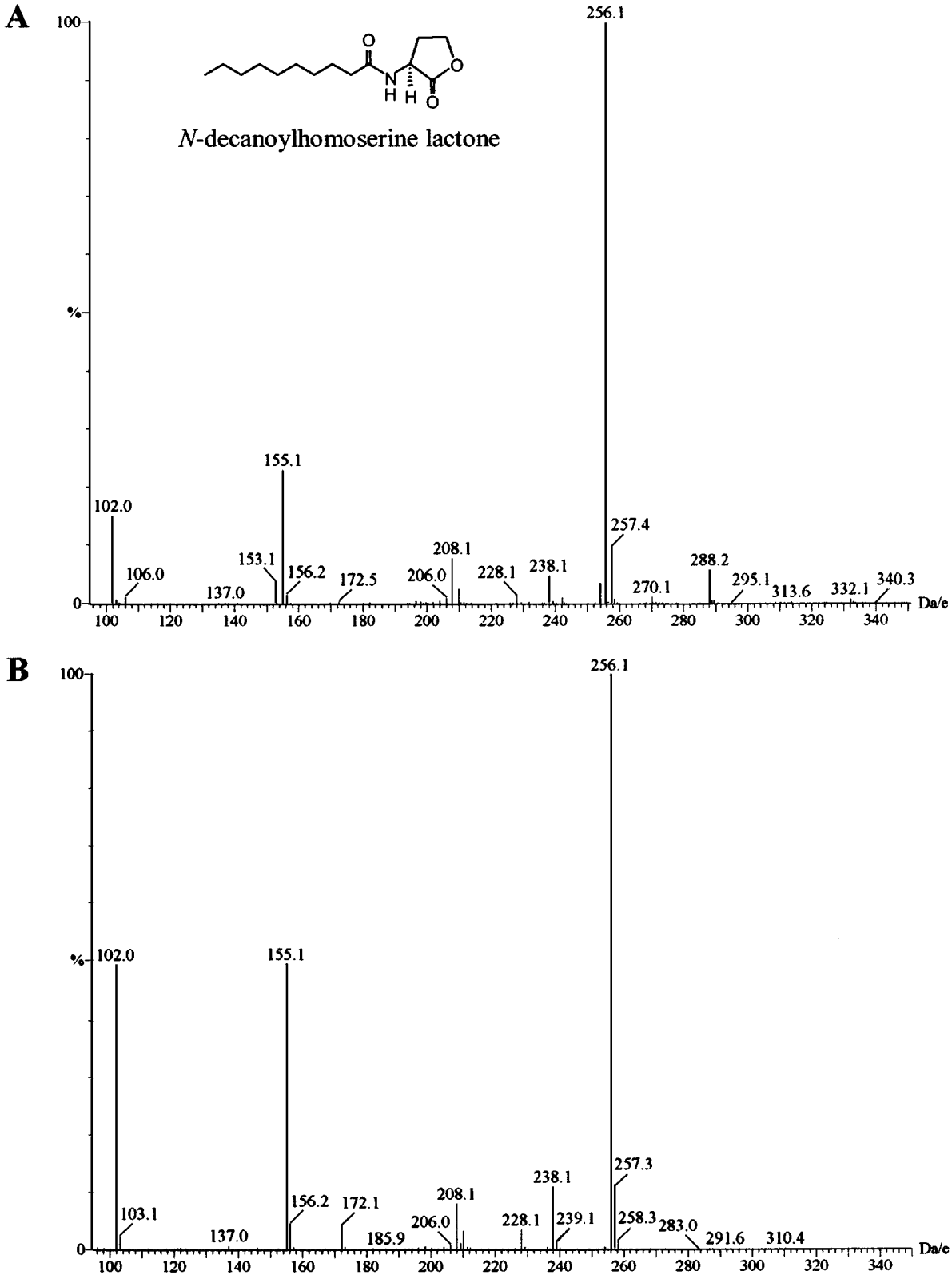


Fig. 3. Mass spectrum (LC-MS) of a compound [(M + H) 256 peak] purified from spent culture supernatant of *B. vietnamiensis* R-921 (B) is indistinguishable from that of synthetic C10-HSL (A).

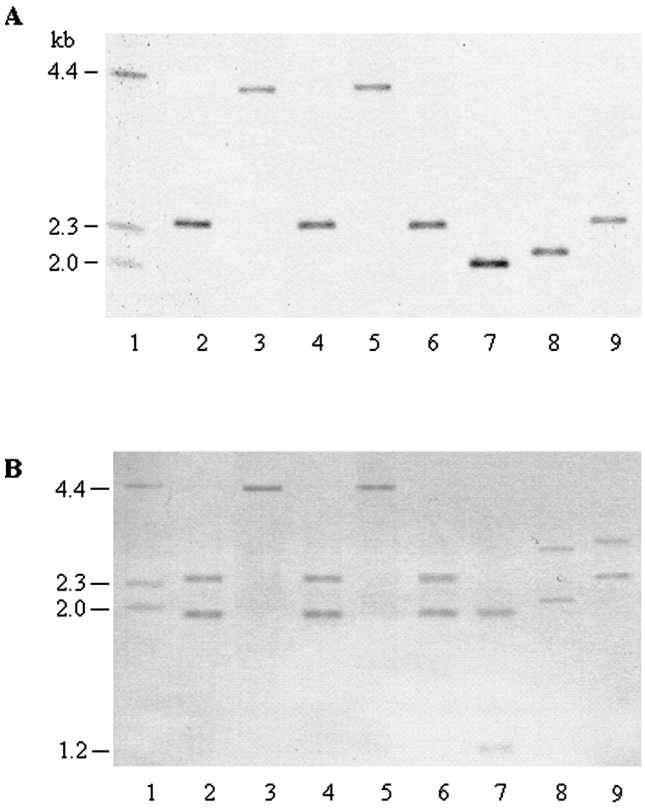


Fig. 4. Southern hybridisation of *Pst*I-digested chromosomal DNAs of different *Burkholderia* strains from different genomovars using (A) a *cepI*- and (B) a *cepR*-specific probe. Lane 2, LMG 18821 (Gv. I); lane 3, LMG 14087 (Gv. I); lane 4, K56-2 (= LMG 18863, Gv. III); lane 5, LMG 16655 (Gv. III); lane 6, LMG 14291 (Gv. IV); lane 7, LMG 16660 (Gv. II); lane 8, R-921 (Gv. V); lane 9, LMG 18943 (Gv. VI). Lane 1 contains *Hin*dIII-digested lambda DNA as reference.

representative strain from *Burkholderia* genomovars I through VI was determined. For this purpose the entire *cep* loci of the strains LMG 1222^T, LMG 16660, H111, LMG 14291, R-921, and LMG 18943 were amplified by PCR and cloned into the vector pCR2.1-TOPO. Sequence analyses of *cepI* and *cepR* from the different genomovars revealed a very high degree of conservation (Fig. 5). The deduced amino acid sequences of the CepI proteins from the different genomovars are identical at 145 of 202 residues (Fig. 5A). The CepR proteins are even more conserved, with 211 of the total 239 residues being identical (Fig. 5B).

With only one exception, the deduced CepI proteins from all of the genomovars contained each of the ten amino acids that have been shown to be completely conserved among members of the LuxI family (PARSEK et al., 1997). The exception was a glutamic acid to aspartic acid (E → D) substitution at position 43 of CepI of *B. multivorans* LMG 16660 (Fig. 5A). To investigate whether this amino acid substitution is conserved in all members of genomovar II, the sequence of *cepI* from four additional strains (LMG 18822, R-139, LMG 17588, and H191) was determined. The deduced CepI sequence from all these strains also showed the same E43 → D substitution, indicating that this alteration is indeed characteristic for strains of *B. multivorans* (Fig. 6).

Seven amino acids with complete conservation among members of the LuxR family of transcriptional regulators have been identified (FUQUA et al., 1996). As has been previously noticed for the genomovar III strain K56-2 (LEWENZA et al., 1999), one of these highly conserved residues, the proline at position 77, was substituted by a serine in the deduced CepR sequences from all of the genomovars. Thus, the P77 → S mutation appears to be characteristic for the entire *B. cepacia* complex. However, the substitution of the conserved glutamic acid for aspartic acid (E → D) at position 180, which was reported for strain K56-2, was not found in any of the sequences determined in this study and hence appears to be specific for this strain (Fig. 5B).

Heterologous expression of CepI from different genomovars in *E. coli*

To determine whether the sequence variations of the CepI proteins, in particular the E43 → D substitution in *B. multivorans*, could account for the observed differences in AHL levels produced, we expressed *cepI* in a heterologous *E. coli* host. This was accomplished by cloning the *cepI* gene from each of the six genomovars into the expression vector pQE-32. Cells harbouring the respective expression plasmids were grown to an optical density of 0.2 OD_{600} and CepI expression was then induced by the addition of 2 mM IPTG. AHLs were extracted from the spent culture supernatants and analysed by TLC in conjunction with AHL biosensors. In all cases, expression of *cepI* from the different genomovars in *E. coli* resulted in the production of C8-HSL and C6-HSL (Fig. 7). From these data we conclude that *cepI* from strains of *B. multivorans* codes for a functional protein and AHL synthesis is not affected by the E43 → D substitution. These results suggest that, at least under the conditions used in this study, *cepI* may not be expressed in *B. multivorans*.

Interestingly, heterologous expression of the CepI proteins in *E. coli* yielded one additional spot on the TLCs, which by comparison with R_f-values of synthetic AHL standards, seems most likely to be *N*-heptanoylhomoserine lactone (C7-HSL) (Fig. 7), which has recently been identified in spent culture supernatants of *Rhizobium leguminosarum* biovar viciae (LITHGOW et al., 2000). Another noteworthy result is the observation that the *B. vietnamiensis* R-921 *cepI* gene, when expressed in *E. coli*, directed the synthesis of C8-HSL and C6-HSL but not of long chain AHLs, which are also produced by the parent strain (Fig. 7). However, as only one hybridisation band was observed in Southern blots using the *cepI* probe, this suggests the gene encoding this putative second AHL synthase is significantly different from *cepI*.

A

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LMG 1222 (Gv. I)     1 MRTFVHEEGRLPHELAADLGRYRRRVFVEQLGWALPSANESFERDQFDRDDTVYVFARNA
C1576 (Gv. II)       1 MQTFVHEGRQLPMPQATELARYRHRVFVEQLGWTLPSADEGIDRDAFDHDDTVYVVARDG
H111 (Gv. III)       1 MQTFVHEEGRLPHELAADLGRYRRRVFVEQLGWALPSANVSFERDQFDRDDTVYVFARNA
K56-2 (Gv. III)      1 MQTFVHEEGRLPHELAADLGRYRRRVFVEQLGWALPSANESFERDQFDRDDTVYVFARNA
B4P (Gv. IV)         1 MRTFVHEEGRLPHELAADIGRYRRRVFVEQLGWALPSANESFERDQFDRDDTVYVFARNA
R-921 (Gv. V)        1 MRTFVHEEGRLPSELAAELGRYRRRVFIEQLGWALPSANERFEHDQFDRDDTVYVFARDA
AU0018 (Gv. VI)      1 MQTFVHEEGRLPYELAADLGRYRRRVFVEQLGWALPSANEAFERDQFDRDDTVYVMARNA
                         *.*****  .**   *... ***.***.*****.****    ..* **.****** **

LMG 1222 (Gv. I)    61 GGDMCGCARLLPTTRPYLLKSLFADLIAEDMPLPQSAAVWELSRFAATDDEGGPGNAEWA
C1576 (Gv. II)      61 SGEMCGCARLLPTTRPYLLETLFADLIAPDLPLPRSAAVWELSRFAASGADGGASGADWA
H111 (Gv. III)      61 DGDMCGCARLLPTTRPYLLKSLFADLIAEDMPLPQSAAVWELSRFAATDDEGGPGNAEWA
K56-2 (Gv. III)     61 DGDMCGCARLLPTTRPYLLKSLFADLVAEDMPLPQSAAVWELSRFAATDDEGGPGNAEWA
B4P (Gv. IV)        61 DGDMCGCARLLPTTRPYLLGSLFADLIAEDMPLPQSAAVWELSRFAATDDESGSGNAEWA
R-921 (Gv. V)       61 GGDVCGCARLLPTTRPYLLESLFADLIAEDVALPQSAAVWELSRFAATGDEGGAGNADWA
AU0018 (Gv. VI)     61 AGEMCGCARLLPTTQPYLLESLFADLVAQDVPLPKSAAVWELSRFAATADENGPGNAEWA
                        *..**********.**** .*****.* *. **.************.  . *  .*.**

LMG 1222 (Gv. I)   121 VRPMLAAVVECAAQLGARQLIGVTFASMERLFRRIGIHAHRAGPPKQVDGRLVVACWIDI
C1576 (Gv. II)     121 VRPMLAAVVACAAERGARQLIGVTFASMERLFRRIGVHAHRAGPPKQVDGRLVVACWIDL
H111 (Gv. III)     121 VRPMLAAVVECAAQLGARQLIGVTFASMERLFRRIGIHAHRAGPPKQVDGRLVVACWIDI
K56-2 (Gv. III)    121 VRPMLAAVVECAAQLGARQLIGVTFASMERLFRRIGIHAHRAGPPKQVDGRLVVACWIDI
B4P (Gv. IV)       121 VRPMLAAVVECAAQLGARQLIGVTFASMERLFRRIGIHAHRAGPPKQVDGRLVVACWIDI
R-921 (Gv. V)      121 VRPMLAVVVECAAQLGARQLIGVTFASMERLFRRIGVHAHRAGPPKQVDGRLVVACWIDI
AU0018 (Gv. VI)    121 VRPMLAAVVECAAQLGARQLIGVTFASMERLFRRIGVHAHRAGPPKQVDGRLVVACWIDI
                       ****** ** ***. ********************.**********************.

LMG 1222 (Gv. I)   181 DPQTFAALGIEPGQAARQAIAA
C1576 (Gv. II)     181 DPQTFAALGIEPERIARPAIAA
H111 (Gv. III)     181 DPQTFAALGIEPGQAARQAIAA
K56-2 (Gv. III)    181 DPQTFAALGIEPGQAARQAIAA
B4P (Gv. IV)       181 DPQTFAALGIEPGQASRQAIAA
R-921 (Gv. V)      181 DPQTFAALGITPGRAARQAIAA
AU0018 (Gv. VI)    181 DPQTFAALGIEPGPAARQAIAA
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Fig. 5. Comparison of the CepI (A) and CepR (B) sequences of one representative of each *Burkholderia* genomovar I through VI. The strains and corresponding GeneBank accession numbers are: LMG 1222^T (Gv. I; AF330012 and AF330018), LMG 16660 (Gv. II; AF330013 and AF330019), H111 (Gv. III; AF330014 and AF330020), LMG 14291 (Gv. IV; AF330015 and AF330021), R-921 (Gv. V; AF330016 and AF330022), and LMG 18943 (Gv. VI; AF330017 and AF330023). The sequences of the previously published Gv. III strain K56-2 (LEWENZA et al., 1999) were also included. Alignments were constructed by using the CLUSTAL V programme. Amino acid residues identical in all six sequences are indicated by asterisks, conserved residues by dots. PARSEK et al. (1997) identified ten residues that are identical in all members of the LuxI family and seven residues that are conserved in 90% of the homologs with the remaining residues being similar. These residues are indicated by black and grey shading, respectively. Likewise, FUQUA et al. (1996) identified seven residues that are identical in all members of the LuxR family and 21 residues that are conserved in 75–92% of the homologs and these residues are indicated by black and grey shading, respectively.

Discussion

We have demonstrated that most strains of the *B. cepacia* complex synthesize at least two AHLs, namely C8-HSL and C6-HSL, in a ratio of approximately 10:1. The levels of AHLs produced by the different strains varied dramatically with concentrations ranging from 10 nM to 1 µM for the more abundant C8-HSL. Moreover, with the biosensors used in this study no AHLs could be detected in the supernatants of some strains indicating that either no AHLs were produced or that the levels were below the limits of detection (about 1 nM for C8-HSL with the bioluminescent sensor plasmid pSB403). Most interestingly, all strains of *B. multivorans* fell into this group. However, Southern blot analysis demonstrated that all strains examined in this study (including the apparently AHL-negative strains) carry genes homologous to *cepI* and *cepR*. Sequence analysis of these two genes revealed a surprisingly high degree of conservation among the different genomovars. One notable difference, however, was an E → D substitution at position 43 in the CepI sequence of *B. multivorans*, which is highly conserved among all other members of the LuxI family. This substitution, however, did not appear to affect the AHL synthase activity of the protein, as when expressed in *E. coli*, both the nature and levels of the AHLs synthesized via CepI from *B. multivorans* were comparable to those produced by CepI from the other genomovars. Consequently, as *cepI* from *B. multivo-*

B

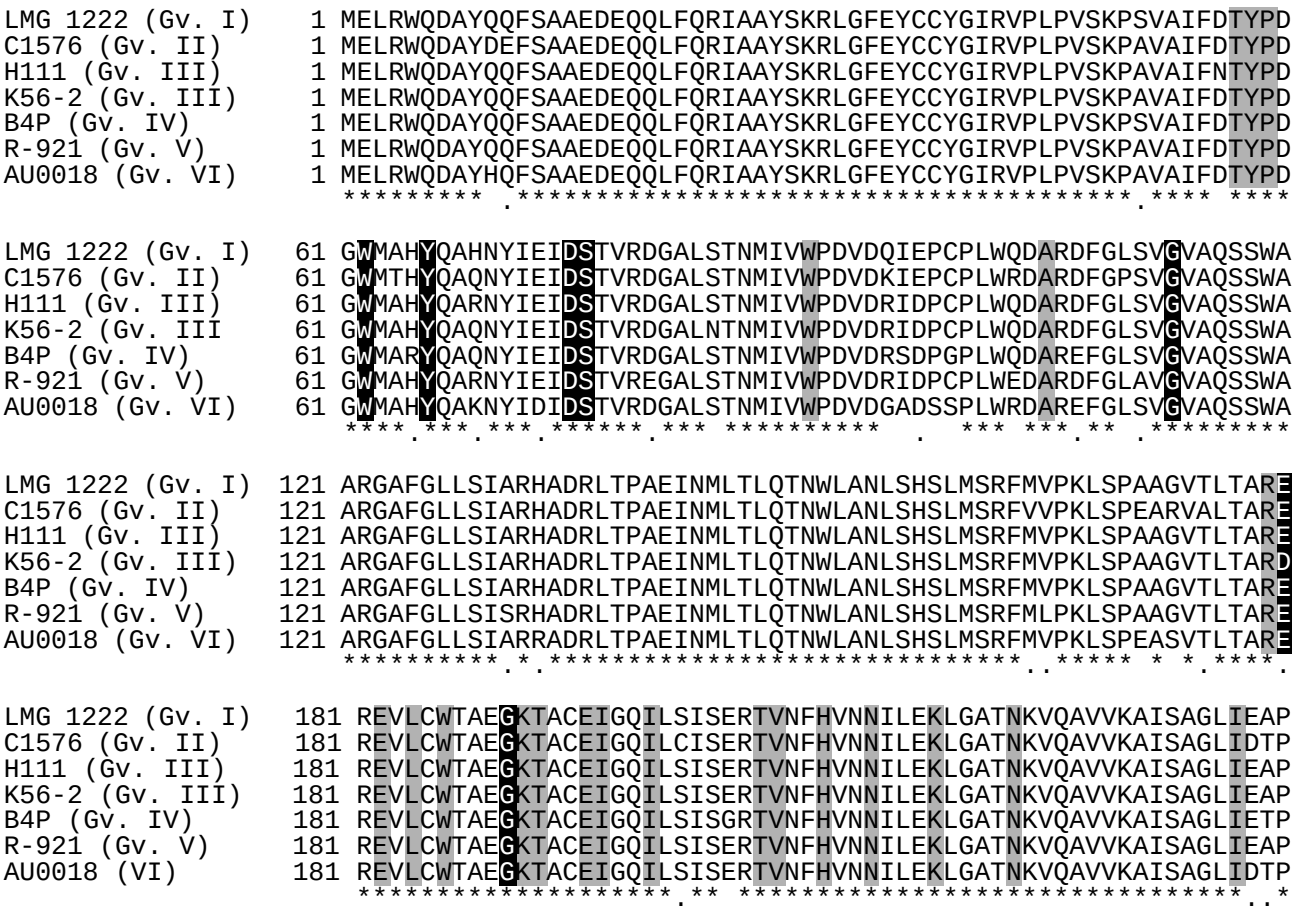

Fig. 5. (Legend see on the site before).

rans encodes a functional protein, the absence of AHL production in the wild-type may be due to no or very low levels of expression. In fact, when *cepI* from *B. multivorans* LMG 16660 was introduced into a defined genomovar III *cepI* mutant (strain H111), AHL production was restored (data not shown). Given that the *cepR* sequences in the two strains are almost identical (Fig. 5B) and that regions upstream of *cepI*, containing the promoter and *lux* box-like sequences, are highly conserved we suggest that additional regulatory factors may be present in *B. multivorans* that silence *cepI* expression. For *Agrobacterium tumefaciens* and *Ralstonia solanacearum*, additional external signals are required to activate AHL synthesis (FUQUA and WINANS, 1994; FLAVIER et al., 1997). It is, therefore, conceivable that despite employing a variety of growth media, the conditions used to cultivate *B. multivorans* were not appropriate for AHL synthesis.

Analysis of AHL profiles also revealed that most strains of *B. vietnamiensis* produced in addition to C6- and C8-HSL also long chain AHL molecules. For *B. vietnamiensis* R-921 the most abundant long chain AHL was identified as C10-HSL. 3-oxo-C10-, C12-, and C14-HSL were produced in lesser amounts. The combined results from Southern blot analysis and heterologous expression of *cepI* from *B. vietnamiensis* R-921 in *E. coli*, strongly suggest that a second AHL synthase gene, which is proposed to encode a protein that directs the synthesis of long chain AHLs, is present in this organism.

Since in many bacteria quorum sensing is involved in regulating the synthesis of extracellular enzymes and virulence factors (FUQUA et al., 1996; HARDMAN et al., 1998; EBERL, 1999; WILLIAMS et al., 2000) we tested all strains for the production of protease, lipase, chitinase and siderophores. Previously, it has been demonstrated that the production of protease and the siderophore, ornibactin, is regulated by the *cep* system in *B. cepacia* K56-2 (LEWENZA et al., 1999). However, no direct correlation between the production of AHLs and extracellular enzymes or siderophores was apparent. Lipase and siderophore activity did not correlate with the AHL production. None of the genomovar VI, the novel genomvar, *B. multivorans*, or *B. vietnamiensis* strains produced extracellular protease(s). However, while all *B. multivorans* strains were AHL-negative, all strains of genomovars I,

```
C1576          1 MQTFVHEGRQLPMPQATELARYRHRVFVEQLGWTLPSADEGIDRDAFDHDDTVYVVARDG
C5393          1 MQTFVHEGRQLPIAQATELARYRHRVFVEQLGWTLPSADEGIDRDAFDHDDTVYVVARDG
R-139          1 MQTFVHEGRQLPIAQATELARYRHRVFVEQLGWTLPSADEGIDRDAFDHDDTVYVVARDG
CEP144         1 MQTFVHEGRQLPIAQATELXRYRHRVFVEQLGWTLPSADEGIDRDAFDHDDTVYVVARDG
H191           1 MQTFVHEGRQLPMPQATDVARYRHRVFVEQLGWTLPSADEGIDRDAFDHDDTVYVAARDG
                 ************. ***.. ********************************** ****

C1576         61 SGEMCGCARLLPTTRPYLLETLFADLIAPDLPLPRSAAVWELSRFAASGADGGASGADWA
C5393         61 SGAMCSCARLLPTTRPYLLETLFADLIAPDLPLPRSAAVWELSRFAASGADGGASGADWA
R-139         61 SGAMCGCARLLPTTRPYLLETLFADLIAPDLPLPRSAAVWELSRFAASGADGGASGADWA
CEP144        61 SGEMWGCARLLPTTRPYLLETLFADLIAPDLPLPRSAAGWELLRFAASDADGGASGADWA
H191          61 SGAMCGCARLLPTTRPYLLETLFADLIAPDLPLPRSAAVWELSRFAASGADGGASGADWA
                 ** *  ******************************** *** ***** ***********

C1576        121 VRPMLAAVVACAAERGARQLIGVTFASMERLFRRIGVHAHRAGPPKQVDGRLVVACWIDL
C5393        121 VRPMLAAVVACAAERGARQLIGVTFASMERLFRRIGVHAHRAGPPKQVDGRLVVACWIDL
R-139        121 VRPMLAAVVACAAERGARQLIGVTFASKERLFRRIGVHAHRAGPPKQVDGRLVVACWIDL
CEP144       121 VRPMLAAVVACAAERGARQLIGVTFASMERLFRRIGVHAHRAGPPKQVDGRLVVACWIDL
H191         121 VRPMLAAVVACAAERGARQLIGVTFASMERLFRRIGVHAHRAGPPKQVDGRLVVACWIDL
                 *************************** ********************************

C1576        181 DPQTFAALGIEPERIARPAIAA
C5393        181 DPQTFAALGIEPERIARPAIAA
R-139        181 DPQTFAALGIEPERIARPAIAA
CEP144       181 DPQTFAALGIEPERIARPAIAA
H191         181 DPQTFAALGIEPERIARPAIAA
                 **********************
```

Fig. 6. Comparison of CepI sequences of five *B. multivorans* (Gv. II) strains. Amino acid residues identical in all five CepI sequences are indicated by asterisks, similar residues by dots. The characteristic E → D substitution at amino acid residue 43 is indicated by black shading. GeneBank accession numbers are: AF330024 (R-139), AF330025 (LMG 18822), AF330026 (LMG 17588), AF330027 (H191).

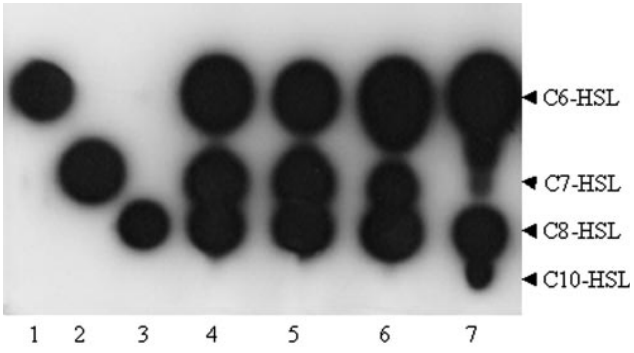

Fig. 7. Identification of AHLs produced by *E. coli* XL1-Blue expressing *cepI* genes from different *Burkholderia* genomovars, namely Gv. III (lane 4), Gv. II (lane 5), and Gv. V (lane 6). AHLs extracted from cell-free culture supernatants were separated by TLC and detected using an overlay of agar seeded with *E. coli* MT102 (pSB403). Lanes 4-6 contain extracts from *E. coli* XL1-Blue harbouring pQE cepI-H111 (Gv. III), pQE cepI-LMG 16660 (Gv. II), and pQE cepI-R-921 (Gv. V), respectively. Lane 7 contains an extract obtained from spent culture supernatant of strain R-921 grown in LB-medium. Synthetic AHL standards are included in lanes 1-3: C6-HSL, C7-HSL, and C8-HSL.

VI, the novel genomvar, and *B. vietnamiensis* produced AHL molecules. Addition of up to 500 nM C8-HSL to the test plates did not restore protease production in *B. multivorans* (data not shown), indicating that the apparent protease deficiency is not due to a lack of AHLs. Likewise, all strains of genomovar VII, *B. multivorans*, and *B. vietnamiensis* were negative in our assays for chitinolytic activity and the addition of C8-HSL to the test plates did not result in chitinase production in *B. multivorans*.

For the opportunistic pathogen *Pseudomonas aeruginosa*, which causes serious infections in immunocompromised individuals and is associated with chronic infections in cystic fibrosis patients, expression of a battery of virulence factors is controlled by two interlinked quorum sensing circuits (VAN DELDEN and IGLEWSKI, 1998; WILLIAMS et al., 2000). 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 quorum sensing defective mutants were substantially less virulent than the parent strains. We, therefore, speculated that the *B. cepacia* strains producing no, or very little, AHLs may be less pathogenic. However,

two isolates of the major transmissible lineage known as ET12, namely the genomovar III isolates LMG 18826 (SAJJAN et al., 1995) and LMG 18827 (MAHENTHIRALINGAM et al., 1996), did not produce any detectable AHLs. Another representative of this lineage, K56-2 (LMG 18863 in the present study [Table 1]), produced low levels of AHLs (about 20 nM; LEWENZA et al., 1999; this study). In contrast, the genomovar III strain H111, which only transiently colonized a CF patient, produced high levels of AHLs (about 500 nM; GEISENBERGER et al., 2000). None of the *B. multivorans* strains tested were AHL-positive in any of our assays, despite the fact that strain LMG 16660 was the index strain in an outbreak among 17 pediatric CF patients attending a treatment center in which five children died subsequent to infection (WHITEFORD et al., 1995). Moreover, the AHL negative strain *B. multivorans* LMG 18825 is a representative of an outbreak among four adult patients in Cardiff, Wales (MILLAR-JONES et al., 1998). Thus, from these clinical data there appears to be no direct link between AHL production and pathogenic potential. However, we have shown that solvent extracted sputa from CF patients colonized with *Burkholderia* activates AHL biosensors indicating that they are produced *in vivo* during infection (WILLIAMS et al., 2000). Therefore, we cannot rule out the possibility that strains which are apparently AHL negative under laboratory conditions do produce AHLs *in vivo* during the course of an infection. It will be of great importance to test some of the strains characterized in this study in an appropriate experimental animal infection model.

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Publikationsliste

(in Klammern angegeben ist der von Birgit Huber geleistete Beitrag zu der jeweiligen Publikation)

1. 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.

(der überwiegende Anteil an der experimentellen Arbeit sowie das Verfassen des Manuskripts zusammen mit L.E.)

2. Huber, B., Riedel, K., Givskov, M., Molin, S., Eberl, L. Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. *eingereicht*.

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

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4. Gotschlich, A., Huber, B., Geisenberger, O., Tögl, A., Steidle, A., Riedel, K., Hill, P., Tümmler, B., Vandamme, P., Middleton, B., Camara, M., Williams, P., Hardman, A., Eberl, L. (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.

(Untersuchung der extrazellulären Produkte der *B. cepacia* Isolate und vergleichende Sequenzanalyse der *cepI/R* Gene)

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