A Quorum-Quenching Approach To Investigate the Conservation of Quorum-Sensing-Regulated Functions within the *Burkholderia cepacia* Complex

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Taxonomic studies of the past few years have shown that the *Burkholderia cepacia* complex, a heterogeneous group of *B. cepacia*-like organisms, consists of at least nine species. *B. cepacia* complex strains are ubiquitously distributed in nature and have been used for biocontrol, bioremediation, and plant growth promotion purposes. At the same time, *B. cepacia* complex strains have emerged as important opportunistic pathogens of humans, particularly those with cystic fibrosis. All *B. cepacia* complex species investigated thus far use quorum-sensing (QS) systems that rely on *N*-acylhomoserine lactone (AHL) signal molecules to express certain functions, including the production of extracellular proteases, swarming motility, biofilm formation, and pathogenicity, in a population-density-dependent manner. In this study we constructed a broad-host-range plasmid that allowed the heterologous expression of the *Bacillus* sp. strain 240B1 AiiA lactonase, which hydrolyzes the lactone ring of various AHL signal molecules, in all described *B. cepacia* complex species. We show that expression of AiiA abolished or greatly reduced the accumulation of AHL molecules in the culture supernatants of all tested *B. cepacia* complex strains. Phenotypic characterization of wild-type and transgenic strains revealed that protease production, swarming motility, biofilm formation, and *Caenorhabditis elegans* killing efficiency was regulated by AHL in the large majority of strains investigated.

In 1992, Yabuuchi et al. (53) established the genus *Burkholderia* to accommodate most of the former rRNA group II pseudomonads. Today, the genus comprises over 30 species, which inhabit diverse ecological niches and have been isolated from soil, water, plants, insects, industrial sources, hospital environments, and infected humans (7). A group of *Burkholderia cepacia*-like bacteria, referred to as the *B. cepacia* complex, has attracted particular attention for two reasons: (i) several *B. cepacia* complex strains have an enormous biotechnological potential and have been used for bioremediation of recalcitrant xenobiotics, plant growth promotion, and biocontrol purposes, and (ii) *B. cepacia* complex strains have emerged as problematic opportunistic pathogens in patients with cystic fibrosis (CF) patients and immunocompromised individuals (8, 22, 35, 49). Polyphasic-taxonomic studies have revealed that the *B. cepacia* complex comprises a very heterogeneous group of at least nine species, which share a high degree of 16S rRNA gene sequence similarity (98 to 100%) and only moderate levels of DNA-DNA hybridization: *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* (7). The clinical outcomes of CF patients infected with *B. cepacia* complex strains are variable and unpredictable, ranging from asymptomatic carriage to a fulminant and fatal pneumonia (“*cepacia* syndrome”) (28). Although all nine *B. cepacia* complex species have been iso-

lated from CF patients, *B. multivorans* and *B. cenocepacia* are most commonly found in clinical samples (7, 35). An increasing body of evidence suggests that the very same strains that occur in the environment are also capable of infecting CF patients, and thus no judgment on the pathogenic potential of a strain can be made solely on the basis of its phylogenetic status.

Many gram-negative bacteria use cell-to-cell communication systems relying on *N*-acylhomoserine lactone (AHL) signal molecules to express certain phenotypic traits in a density-dependent manner, a phenomenon referred to as quorum sensing (QS) (16, 19, 52). Systematic surveys revealed that all *B. cepacia* complex species produce AHL signal molecules (21, 27, 34). The first QS genes in a *B. cepacia* complex strain were identified in a screen for transposon insertion mutants that hyperproduced siderophores on chrome azurol S agar (31). That study showed that the QS system of *B. cenocepacia* consists of the AHL synthase CepI, which directs the synthesis of *N*-octanoylhomoserine lactone (C8-HSL) and, as a minor by-product, *N*-hexanoylhomoserine lactone (C6-HSL), and the transcriptional regulator CepR. At low population densities, cells produce a basal level of AHLs via the activity of the CepI synthase. As the cell density increases, the diffusible AHL signal molecule accumulates in the growth medium until a critical threshold concentration has been reached, upon which the signal molecules bind to the cognate LuxR-type receptor protein CepR, leading to the induction or repression of target genes. CepR controls the tight expression of *cepI* through binding to a *lux* box-like sequence that partially overlaps the –35 region of the putative *cepI* promoter (32, 51), creating a classic autoinduction loop. QS systems that are highly homologous to the CepI/CepR

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TABLE 1. Phenotypes affected by heterologous expression of the *aiiA* lactonase in Bcc strains

Strain ^a	Source	C8-HSL concn (nM) ^b		Biofilm formation (mean OD ₅₇₀ ± SD) ^c		Proteolytic activity ^d		AidA production ^d		Pathogenicity ^e	
		Wild type	AiiA ⁺	Wild type	AiiA ⁺	Wild type	AiiA ⁺	Wild type	AiiA ⁺	Wild type	AiiA ⁺
<i>B. cepacia</i>											
LMG 1222 ^T	<i>Allium cepa</i> , United States	50–500	ND	1.73 ± 0.58	0.74 ± 0.25	(+)	–	+	–	3 (20–100)	0
LMG 6963	Soil, Australia	50–500	ND	1.20 ± 0.60	0.60 ± 0.10	+	–	–	–	3 (20–100)	0
LMG 6988	Leg wound, Sweden	<5	ND	0.30 ± 0.05	NB	+	–	+	–	3 (0)	0
R-18194	Forest soil, Trinidad	50–500	ND	0.66 ± 0.01	0.16 ± 0.02	+	–	+	–	2 (100–150)	0
LMG 6993	Soil, Trinidad	<5	ND	0.09 ± 0.02	0.06 ± 0.02	+	(+)	+	+	3 (0)	0
<i>B. multivorans</i>											
H107	CF patient, Germany	50–500	ND	0.90 ± 0.30	0.70 ± 0.30	–	–	+	(+)	0	0
R-654	CF patient, Canada	<5	ND	NB	NB	–	–	(+)	–	2 (50–100)	0
R-6275	CF patient, Germany	<5	ND	0.22 ± 0.02	NB	–	–	–	–	0	0
R-6278	CF patient, Germany	<5	ND	0.23 ± 0.01	NB	–	–	+	–	0	0
LMG 18825	CF patient, United Kingdom	<5	ND	0.34 ± 0.06	0.12 ± 0.06	–	–	+	–	0	0
LMG 18945	CF patient, United Kingdom	<5	ND	0.07 ± 0.01	NB	–	–	+	–	0	0
LMG 18822	CF patient, Canada	50–500	5–50	0.70 ± 0.38	0.50 ± 0.18	–	–	–	–	0	0
<i>B. cenocepacia</i>											
H111	CF patient, Germany	>500	ND	0.35 ± 0.05	NB	+	(+)	+	–	3 (0)	0
R-6274	CF patient, Germany	>500	ND	0.34 ± 0.05	0.17 ± 0.04	+	–	+	+	3 (0)	0
LMG 6981	Bronchial washings	50–500	ND	0.33 ± 0.01	0.12 ± 0.08	+	–	–	–	0	0
LMG 14271	CF patient, Belgium	50–500	ND	0.16 ± 0.02	0.08 ± 0.07	+	–	+	–	0	0
LMG 16656	CF patient, United Kingdom	50–500	ND	NB	NB	–	–	(+)	–	1 (20–50)	0
LMG 16659	CF patient, United Kingdom	>500	ND	0.13 ± 0.03	NB	–	–	+	–	0	0
R-6108	CF patient, New Zealand	<5	ND	0.09 ± 0.01	0.37 ± 0.23	+	–	–	–	0	0
R-651	CF patient, Canada	<5	ND	0.35 ± 0.09	0.58 ± 0.15	–	–	+	–	1 (100–200)	0
<i>B. stabilis</i>											
R-6270	CF patient, Germany	5–50	ND	0.20 ± 0.09	0.08 ± 0.03	+	–	–	–	3 (0)	0
R-6272	CF patient, Germany	5–50	ND	0.19 ± 0.02	0.08 ± 0.03	(+)	–	–	–	0	0
R-6273	CF patient, Germany	>500	ND	2.80 ± 1.26	0.70 ± 0.24	+	–	–	–	1 (>200)	0
R-6276	CF patient, Germany	>500	5–50	4.20 ± 0.90	0.70 ± 0.08	+	–	+	–	3 (0–10)	0
R-10033	CF patient, Germany	5–50	ND	2.22 ± 1.15	0.71 ± 0.04	+	–	–	–	3 (0)	0
R-6279	CF patient, Germany	5–50	ND	2.40 ± 1.30	0.40 ± 0.30	+	–	+	–	3 (0)	0
R-6281	Water outlet, Germany	5–50	ND	2.15 ± 0.59	0.43 ± 0.23	+	–	+	–	0	0
R-6280	CF patient, Germany	5–50	ND	2.92 ± 0.70	0.70 ± 0.15	+	–	–	–	3 (0)	0
LMG 6997	Ear, Sweden	5–50	ND	2.17 ± 0.50	0.50 ± 0.09	+	–	+	–	3 (0–10)	0
LMG 14291	CF patient, Belgium	5–50	ND	1.00 ± 0.30	0.70 ± 0.30	–	–	+	–	3 (0)	0
R-3338	CF patient, Germany	5–50	ND	1.82 ± 0.90	0.54 ± 0.15	–	–	+	–	0	0
<i>B. vietnamiensis</i>											
LMG 6998	Blood, Sweden	<5	ND	0.16 ± 0.02	NB	–	–	+	–	1 (100–200)	0
LMG 6999 ^f	Neck abscess, Sweden	50–500	ND	0.70 ± 0.20	0.55 ± 0.10	–	–	+	–	0	0
LMG 10929 ^{1f}	<i>Oryza sativa</i> , rhizosphere, Vietnam	50–500	ND	0.30 ± 0.14	0.50 ± 0.30	–	–	–	–	0	0
LMG 18836 ^f	Septic granulomatosis disease, Canada	50–500	ND	0.60 ± 0.08	0.90 ± 0.20	–	–	–	–	1 (>200)	0
R-1808 ^f	Industrial waste treatment facility, United States	<5	ND	0.55 ± 0.08	0.13 ± 0.03	–	–	–	–	1 (>200)	0
R-921 ^f	CF patient, Sweden	5–50	ND	1.00 ± 0.40	0.60 ± 0.10	–	–	+	–	ND	0
<i>B. dolosa</i>											
LMG 21820	CF patient, United Kingdom	5–50	ND	1.40 ± 1.15	1.00 ± 0.18	–	–	+	–	0	0
LMG 21443	<i>Ahyrcarpus glutaceus</i> , root nodule, Senegal	5–50	ND	1.50 ± 0.60	0.90 ± 0.20	–	–	+	–	3 (0–10)	0
<i>B. ambifaria</i>											
LMG 17828	Corn roots, United States	50–500	ND	1.00 ± 0.30	2.70 ± 0.90	–	–	+	–	3 (0–10)	0
LMG 19182 ^{1f}	Pea rhizosphere, United States	50–500	ND	2.00 ± 0.30	1.30 ± 0.90	(+)	–	–	–	2 (0–20)	0
LMG 19467	CF patient, Australia	<5	ND	1.80 ± 0.47	1.08 ± 0.49	(+)	–	–	–	3 (0)	0
<i>B. anthina</i>											
LMG 20983	CF patient, sputum, United Kingdom	50–500	ND	1.30 ± 0.03	0.90 ± 0.10	–	–	–	–	3 (0–10)	0
LMG 21821	CF patient, United States	<5	ND	3.18 ± 0.20	1.00 ± 0.2	+	–	–	–	3 (0–10)	0

<i>B. pyrocinia</i> LMG 21822 LMG 21823	Cornfield soil, United States	<5	ND	2.11 ± 1.3	0.80 ± 0.50	+	–	–	3 (0–10)	0
	Water, United States	<5	ND	0.40 ± 0.3	0.70 ± 0.20	+	–	(+)	–	3 (0–10)

^a Strains were obtained from the Laboratorium voor Microbiologie Gent culture Collection, Universiteit Gent, Belgium (LMG), the Research Collection, Peter Vandamme, Gent, Belgium (R), or the Medizinische Hochschule Hannover, Hannover, Germany (H).

^b CS-HSL concentrations of culture supernatants were determined along the growth curves by the aid of the GFP-based monitor strain *P. putida* F117(pAS-C8). Since the AHL concentrations varied depending on the growth phase, the concentration ranges are shown. <5 nM, only unambiguously detectable in 100-fold concentrated dichloromethane extracts of culture supernatants; ND, not detectable in 100-fold-concentrated extracts of supernatants.

^c Biofilm formation was measured in polystyrene microtitre dishes by staining attached cells with CV. All values were corrected for background staining of the wells. NB, no biofilm (*A*₅₇₀ values of <0.05 were considered biofilm negative).

^d Protease activity was determined on skim milk agar plates after 48 h of incubation at 37°C. Strains were tested for *AidA* by Western blotting. +, clear signal; (+), weak signal; –, no signal.

^e Pathogenicity score as described in Materials and Methods. Values in parentheses are numbers of worms at day 5 postinfection. In the case of the transconjugants, more than 200 nematodes were observed.

^f Production of C10-HSL was assessed by the aid of the AHL biosensor *P. putida* F117(pKR-C12), which is highly sensitive for long-chain AHLs.

system of *B. cenocepacia* were subsequently identified in all *B. cepacia* complex species investigated thus far (1, 21, 34, 54). Several strains of *B. vietnamiensis* also produce *N*-decanoyl-homoserine lactone (C10-HSL) in addition to C8- and C6-HSL via a second QS system that consists of BviI and BviR (9, 21, 34). Whether the BviI/BviR system interacts with the CepI/CepR system, which is also present in most of these *B. vietnamiensis* strains, is currently unknown.

A comparison of two-dimensional protein patterns of the *B. cenocepacia* strain H111 and its isogenic *cepI* mutant showed that ca. 5% of the proteome was downregulated and 1% up-regulated in the absence of CepI. Thus, the CepI/CepR system is a global regulatory system that can activate and repress expression of proteins (42). Amino-terminal sequence analysis identified several proteins that were downregulated in the *cepI* mutant, including a putative superoxide dismutase, a peroxidase, a FimA homologue, and a predicted ABC transporter system. The phenotypic characterization of defined QS mutants revealed that the CepI/CepR system positively regulates the expression of extracellular proteases, chitinases, swarming motility, and biofilm formation and represses synthesis of the siderophore ornibactin (1, 25, 31, 32). For *B. cenocepacia* H111, the CepI/CepR system is required to form biofilms on abiotic surfaces (25). Also, the CepI/CepR system contributes to the virulence of *B. cenocepacia* in a wide range of hosts, including the plant alfalfa (4), the nematode *Caenorhabditis elegans* (29), and murine species (45).

The present study was initiated to investigate how conserved the QS regulons in the different *B. cepacia* complex species are. Specifically, we wanted to determine whether the expression of previously identified QS-regulated functions, including production of extracellular proteases, swarming motility, biofilm formation, and nematode pathogenicity, are AHL regulated in the different *B. cepacia* complex species. Large-scale analyses, which otherwise would require the systematic construction of knockout mutants in the respective AHL synthases of the each strain, were facilitated by the construction of a heterologous expression system of the *Bacillus* sp. strain 240B1 AiiA lactonase. This provided us with a functional quorum-quenching approach for the screening of a large number of *B. cepacia* complex strains.

MATERIALS AND METHODS

Strains and growth conditions. *Burkholderia* strains used in the present study are listed in Table 1. Unless otherwise stated, the strains were grown at 37°C in modified Luria-Bertani (LB) medium (2) or AB minimal medium (6) supplemented with 10 mM citrate. Solid media were routinely solidified with 1.4% agar. Growth media for examination of swarming motility contained 0.4% (wt/vol) agar (15). Antibiotics were added as required at final concentrations of 20 µg/ml for gentamicin and 10 µg/ml for chloramphenicol. Trimethoprim was used at 50 µg/ml for *Escherichia coli* XL1-Blue and at 100 µg/ml for *B. cepacia* complex strains when required. Growth of liquid cultures was monitored spectrophotometrically with an Ultrospec 3100 Pro spectrophotometer (Biochrom, Ltd., Cambridge, England) by measurement of optical density at 600 nm.

DNA manipulations. Cloning, restriction enzyme analysis, and transformation of *Escherichia coli* were performed essentially as described previously (43). PCR was performed with TaKaRa rTaq DNA polymerase (TaKaRa Shuzo, Shiga, Japan) or Pwo DNA polymerase (Roche, Mannheim, Germany). Plasmid DNA was isolated with the QIAprep Spin Miniprep kit, and chromosomal DNA was purified with the DNeasy tissue kit. DNA fragments were purified from agarose gels by using the QIAquick gel extraction kit (all kits are from QIAGEN, Hilden, Germany).

Cloning of the *aiiA* gene on broad-host-range expression vectors. The *aiiA* gene (GenBank accession number AF196486) of *Bacillus* sp. strain 240B1 (14) was amplified by using the primer pair *aiiA*-F (5'-GCCATGGATCCATGACAGTAAAGAGCT-3') and *aiiA*-R (5'-CCAAGCTTGGAGCTGACTGGGTGAAG-3') (the introduced restriction sites *Nco*I and *Hind*III are underlined). The amplicon was digested with *Hind*III and *Nco*I and ligated directionally into the broad-host-range expression vectors pMLBAD and pMLS7 (30) cut with the same enzyme, yielding pMLBAD-*aiiA* and pMLS7-*aiiA*, respectively.

Conjugative plasmid transfer. Plasmids were delivered to *B. cepacia* complex strains by triparental mating as described previously (13). Briefly, donor and recipient strains, as well as the helper strain *E. coli* HB101(pRK600), were grown at 37°C overnight in 5 ml of LB medium supplied with the appropriate antibiotics. After subculture to an optical density at 600 nm of 0.9, the cells from 2 ml of culture were harvested, washed, and resuspended in 500 µl of LB medium. Donor and helper cells (100 µl each) were mixed and incubated for 10 min at room temperature. Then, 200 µl of the recipient cells was added, and the mixture was spot inoculated onto the surfaces of preheated LB agar plates. After overnight incubation at 37°C, the cells were plated on PIA medium (Becton Dickinson Biosciences, Sparks, MD) containing antibiotics for counter selection of donor, helper, and untransformed recipient cells.

Detection and quantification of AHLs. AHL production was investigated by cross-streaking the strains against two fluorescent AHL biosensors: *Pseudomonas putida* F117(pKR-C12) and *P. putida* F177(pAS-C8) (46). The AHL monitor plasmid pKR-C12 contains a P_{lasB} -*gfp*(ASV) translational fusion, together with the *lasR* gene placed under control of P_{lac} . This sensor strain is highly sensitive for 3-oxo-C12- and 3-oxo-C10-HSL. Plasmid pAS-C8 was constructed from components of the *cep* system of *B. cenocepacia* H111 and contains a P_{cepR} -*gfp*(ASV) translational fusion together with the *cepR* gene transcribed from the P_{lac} promoter of the broad-host-range plasmid pBBR1MCS-5. This sensor plasmid responds very efficiently to C8-HSL and only with low efficiency to other AHL molecules. After overnight incubation of cross-streaking plates at 30°C, fluorescence was detected by illumination with blue light by using an HQ 480/40 filter (AHF-Analysentechnik, Tübingen, Germany) in combination with a halogen lamp (Volpi, Schlieren, Switzerland) as a light source in a dark box equipped with a light-sensitive camera (Hamamatsu Photonics, Herrsching, Germany) with a Pentax CCTV camera lens and an HQ 535/20 filter. Since most *Burkholderia* species produce C8-HSL (21, 34), we used *P. putida* F117(pAS-C8), which is highly specific for this signal molecule, for quantification. To this end, 5 µl of filter-sterilized culture supernatants was added to 50 µl of sterile distilled water and 50 µl of an exponential culture of the sensor strain in the wells of a FluoroNunc Polysorp microtiter dish (Nunc, Wiesbaden, Germany). After incubation at 30°C for 12 h expression of the *gfp* reporter gene was measured with a Lambda Fluoro 320 Plus reader (Bio-Tek Instruments, Winooski, VT). AHL concentrations were determined by comparing fluorescence signal intensities with a standard curve generated with defined concentrations of pure C8-HSL.

Phenotypic characterization of wild-type and transconjugant strains. Biofilm formation in polystyrene microtiter dishes was assayed essentially as described previously (39, 40) with a few modifications. Cells were grown in the wells of the microtiter dishes in 100 µl of AB medium supplemented with 10 mM citrate for 48 h at 30°C. Thereafter, the medium was removed, and 100 µl of a 1% (wt/vol) aqueous solution of crystal violet (CV) was added. After 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 an 800:120 (vol/vol) mixture of ethanol and dimethyl sulfoxide, and the absorbance was determined at 570 nm. The ability to form a swarming colony was tested by point-inoculating strains into ABC minimal medium supplemented with 0.1% Casamino Acids and solidified with 0.4% agar as described previously (25). Proteolytic activity was determined by streaking strains on LB agar supplemented with 2% skim milk.

Detection of AidA. For Western blotting, whole-cell proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Eschborn, Germany). The membrane was probed with the anti-AidA antibodies (27), and detection reactions were performed with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma, Steinheim, Germany) according to the recommendations of the manufacturer (Roche, Mannheim, Germany).

Nematode killing assays. Nematode killing assays were performed essentially as described by Köthe et al. (29). Briefly, overnight cultures of the *B. cepacia* complex strains and transconjugants were adjusted to a density of about 1.3×10^4 to 1.5×10^4 CFU/ml, and 100 µl of the suspensions was plated on six-well plates containing nematode growth medium (NGM II) for slow killing assays. After 24 h of incubation at 37°C a bacterial lawn was formed and approximately

25 hypochlorite-synchronized L4 larvae of *C. elegans* Bristol N2 (obtained from the *Caenorhabditis* Genetics Centre, University of Minnesota, Minneapolis) were used to inoculate the plates. The actual number of worms was determined by using a Stemi SV6 microscope (Zeiss, Oberkochen, Germany) at a magnification of $\times 50$. Plates were then incubated at 20°C and scored for live worms; nematodes were considered dead when they failed to respond to touch. The percentage of live worms and their morphological appearance was registered after 2 days. After 5 days, the total number of nematodes, including parental and progeny nematodes (if existing), was scored. All experiments were carried out at least five times, and *E. coli* OP50 was used as a negative control in the assays. According to Cardona et al. (5), we considered a strain of the *B. cepacia* complex to be pathogenic for *C. elegans* if one of the following criteria was met: (i) a sick appearance at day 2, including reduced locomotive capacity and the presence of a distended intestine; (ii) percentage of live worms after 2 days of $\leq 50\%$; and (iii) total number of worms after 5 days of ≤ 100 . For differentiating mild from severe infections, the presence of one, two, or three of these criteria was scored as 1, 2, or 3, respectively (Table 1). A strain was considered pathogenic when at least one criterion was observed. A strain was described as nonpathogenic when no symptoms of disease were observed during the course of the infection experiment (pathogenicity score 0).

RESULTS

Cloning of the *Bacillus* sp. strain 240B1 *aiiA* gene on broad-host-range expression vectors. To express the *aiiA* lactonase gene in different *B. cepacia* complex strains, we PCR amplified the *Bacillus* sp. strain 240B1 gene as described in Materials and Methods and cloned it directionally into the broad-host-range expression vectors pMLBAD and pMLS7 (30), yielding pMLBAD-*aiiA* and pMLS7-*aiiA*, respectively. In pMLBAD-*aiiA*, the *aiiA* gene is transcribed from the P_{BAD} promoter of *E. coli*, and thus expression is inducible with arabinose. On pMLS7-*aiiA* transcription of *aiiA* is driven by the constitutive S7 ribosomal promoter of *Burkholderia xenovorans* LB400.

Heterologous expression of AiiA in *B. cepacia* complex strains reduces AHL accumulation. To test the functionality of the constructs and the applicability of the quorum-quenching approach, we conjugated pMLBAD-*aiiA* and pMLS7-*aiiA* into *B. cenocepacia* H111. Measurements of the C8-HSL concentrations of the culture supernatant of wild-type and transconjugant strains along the growth curve showed that the presence of either construct greatly diminished the accumulation of signal molecules (Fig. 1). However, we observed that *B. cenocepacia* H111(pMLS7-*aiiA*) produced small amounts of C8-HSL in the late exponential growth phase, presumably because the activity of the S7 ribosomal promoter is reduced upon entry into stationary phase. Because of this finding and the observation that conjugative transfer frequencies of plasmid pMLS7-*aiiA* were generally much lower than with pMLBAD-*aiiA*, we decided to use the latter plasmid in all subsequent quorum-quenching experiments. The plasmid was found to be stably maintained in *B. cenocepacia* H111 for at least 80 generations without antibiotic selection (data not shown). Since expression of *aiiA* on pMLBAD-*aiiA* is controllable by the amount of arabinose, we tested the effects of various concentrations of the inducer on AHL production of *B. cenocepacia* H111(pMLBAD-*aiiA*). At a concentration of 0.02% production of signal molecules was reduced to a level below the detection limit of the AHL bioassay (Fig. 2). Thus, we routinely used 0.02% arabinose in all further experiments. In the presence of 0.2% glucose, which is known to repress the P_{BAD} promoter, AHL concentrations in the supernatants were increased, albeit not to the levels of the wild-type strains,

FIG. 1. Heterologous expression of AiiA in *B. cenocepacia* H111 reduces accumulation of C8-HSL. The wild-type H111 (●), the *cepI* mutant H111-I (○), and the transconjugants H111(pMLBAD) (□), H111(pMLBAD-aiiA) (■), and H111(pMLS7-aiiA) (▲) were grown in LB medium, and samples were taken along the growth curves. C8-HSL concentrations of the spent culture supernatants were determined by the aid of the green fluorescent protein-based biosensor *P. putida* F117(pAS-C8) and are expressed as relative fluorescence units (rfu). All values are the means $\pm$ the standard deviations from three independent experiments.

indicating that the P_{BAD} promoter is somewhat leaky in *B. cenocepacia*.

Transfer of plasmid pMLBAD-aiiA into various *B. cepacia* complex strains caused a dramatic reduction of AHL accumulation in virtually all transconjugants (Table 1). The amounts of C8-HSL produced by the different wild-type strains varied over a wide range of concentrations (<5 nM to >500 nM; Table 1). Many strains produced C8-HSL in concentrations that were easily detectable by the aid of the AHL biosensor *P. putida* F117(pAS-C8) directly in cell-free culture supernatant samples. However, we noticed that, in agreement with an earlier study (21), most *B. multivorans* and *B. pyrocinia* strains and a few strains of other *B. cepacia* complex species produced only very small amounts of C8-HSL, which could only be unambiguously detected when concentrated dichloromethane extracts of supernatants were analyzed. Although the reason for these differences is unclear, it has been speculated that additional regulatory factors may be present in these strains that silence *cepI* expression and derepression only occurs when certain, yet unknown, environmental conditions prevail (54). The heterologous expression of the lactonase abolished signal molecule accumulation independently of the AHL amounts produced by the strains. The only two exceptions were the transconjugants of *B. multivorans* LMG 18822 and *B. stabilis* R-6276, which produced approximately 10-fold reduced amounts of C8-HSL relative to the parent strain.

Previous work has shown that in addition to C8- and C6-HSL, several *B. vietnamiensis* strains also produce *N*-decanoyl-homoserine lactone (C10-HSL) (9, 21, 34). To test whether pMLBAD-aiiA also degrades C10-HSL, we measured the AHL concentrations of the *B. vietnamiensis* wild-type and transconjugant supernatants by the aid of the biosensor *P. putida* F117(pKR-C12), which is highly sensitive for long-chain

FIG. 2. Effects of glucose and arabinose on C8-HSL degradation in *B. cenocepacia* H111 harboring plasmid pMLBAD-aiiA. The wild-type H111 and the transconjugant H111(pLBAD-aiiA) were grown in the presence of 0.2% glucose or 0.02% arabinose as follows: H111 plus 0.2% glucose (■), H111 plus 0.02% arabinose (●), H111(pLBAD-aiiA) plus 0.2% glucose (○), and H111(pLBAD-aiiA) plus 0.02% arabinose (□). Production of C8-HSL (expressed as relative fluorescence units [rfu]) was already reduced to the level of the background of the bioassay at a concentration of 0.02% arabinose. In the presence of 0.2% glucose, which is known to repress the P_{BAD} promoter, AHL concentration was increased albeit not to the level of the wild-type strain. All values are the means $\pm$ the standard deviations from three independent experiments.

AHLs. These measurements showed that the presence of pMLBAD-aiiA not only abolished C8-HSL but also C10-HSL (data not shown).

Lactonase expression in *B. cepacia* complex strains reduces extracellular proteolytic activity. Previous work has shown that the CepI/CepR system positively regulates production of extracellular proteases (1, 25, 31). To test whether expression of proteolytic activity is generally AHL-regulated in *B. cepacia* complex strains, we streaked wild-type and transconjugant strains onto LB agar plates supplemented with 2% skim milk. Clearing zones, which are indicative of protease activity, were observed for many of the wild-type *B. cepacia* complex strains after 48 h of incubation (Fig. 3). However, in agreement with an earlier study (21), strains from *B. multivorans*, *B. vietnamiensis*, and *B. dolosa* did not exhibit proteolytic activity. Heterologous expression of AiiA decreased proteolytic activity dramatically in all protease-positive strains (Table 1 and Fig. 3), indicating that the AHL-dependent regulation of this phenotype is highly conserved.

Pigment production in *B. cepacia* complex strains is AHL dependent. We noticed that *B. cepacia* LMG 1222^T, *B. cepacia* LMG 6963, *B. cepacia* LMG 6993, *B. cenocepacia* LMG 14271, and *B. cenocepacia* LMG 16656 produced pigments of different colors. Pigment production was greatly reduced in the respective transconjugants (Fig. 3 and data not shown). Although the function of these pigments in *Burkholderia* is unknown, our results suggest an involvement of quorum sensing in the regulation of their synthesis.

Swarming motility of *B. cepacia* complex strains is affected by *aiiA* expression. *B. cenocepacia* H111 is capable of swarming

FIG. 3. AHL degradation in *B. cepacia* complex strains reduces extracellular proteolytic activity and affects pigment production. Wild-type (Wt) and transconjugant strains were grown on LB agar plates supplemented with 2% skim milk for 48 h. Clearing zones are indicative of protease activity. Interestingly, expression of AiiA also abolished pigment production in strains C and E. Spots: A, *B. stabilis* R-6270; B, *B. cepacia* LMG 6993; C, *B. cepacia* LMG 6963; D, *B. cenocepacia* H111; E, *B. cepacia* LMG 1222^T; F, *B. cenocepacia* R-6274.

motility in a CepI/CepR-dependent manner (25). To test the wild-type strains for swarming motility, we point inoculated them into medium containing 0.4% agar. Under the conditions used, only very few strains, namely, *B. cenocepacia* H111, *B. cenocepacia* LMG 6981, *B. stabilis* R-6270, and *B. anthina* LMG 20983, swarmed. When AiiA was expressed in these strains, swarming motility was abolished (data not shown), indicating that a factor required for swarming is strictly QS regulated in these strains.

Role of QS in biofilm formation of *B. cepacia* complex strains. Biofilm formation of *B. cenocepacia* on abiotic surfaces was previously shown to require a functional CepI/CepR system (25, 47). We tested all strains included in the present study for their ability to form biofilms in polystyrene microtiter dishes. Dramatic differences in the biofilm formation capacity of the strains were observed (Table 1). Whereas some strains produced very thick biofilms, others were very poor colonizers of the polystyrene surface, at least under the assay conditions used. Expression of AiiA resulted in a reduction in biomass volume in many but not all strains. All transconjugants of strains from *B. stabilis*, *B. dolosa*, and *B. anthina* showed reduced biofilm formation capacity and most transconjugants from other *Burkholderia* species also showed defects in biofilm formation relative to their parent strains. Interestingly, in a few cases (*B. cenocepacia* R-6108, *B. cenocepacia* R-651, *B. vietnamiensis* LMG 10929^T, *B. vietnamiensis* LMG 18836, *B. ambifaria* LMG 17828, and *B. pyrrocinia* LMG 21823) increased biofilm formation was observed with the transconjugants, suggesting that in these strains QS may negatively regulate biofilm development. Only in *B. cepacia* LMG 6993, *B. cenocepacia* LMG 14271, *B. vietnamiensis* LMG 6999, and *B. dolosa* LMG 21820 biofilm formation was apparently unaffected by heterologous expression of AiiA, suggesting that in the large majority of *Burkholderia* strains QS is an important factor for surface colonization.

Expression of AiiA in *B. cepacia* complex strains abolishes killing of *C. elegans*. Previous work has shown that the nematode *C. elegans* is a valuable model for studying pathogenesis of various *Burkholderia* species (5, 20, 38). In *B. cenocepacia* H111, it was shown that the wild type efficiently kills *C. elegans*, whereas mutants defective in QS are attenuated (29). However, the killing efficiencies of *B. cepacia* complex strains varied

over a wide range (5, 20), with strains killing all nematodes within 3 days and others being virtually nonpathogenic (Table 1). In agreement with a recent study, we noticed that all tested strains of *B. cepacia*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* and most strains of *B. stabilis* and *B. cenocepacia* are pathogenic, whereas only a few *B. multivorans* strains kill the nematode (5). Intriguingly, expression of the AiiA lactonase in virulent strains attenuated their pathogenicity, in most cases rendering the strain completely avirulent. These results provide clear evidence that expression of factors required for nematode killing are QS regulated.

Transconjugants are defective in the expression of AidA. Expression of the QS-regulated gene *aidA* in *B. cenocepacia* H111 is required for efficient killing of *C. elegans* (27). Although the exact role of AidA in pathogenesis of H111 is unclear, it has been suggested that this protein is important for persistence in the nematode gut (27). To test whether expression of AidA is generally QS-regulated, we performed Western blotting on overnight cultures of the wild-type and transconjugant strains with anti-AidA antibodies. These experiments revealed that not all strains tested positive for AidA killed *C. elegans* and not all strains that were pathogenic for the nematode expressed AidA, indicating that additional virulence factors may contribute to the differences in virulence between the strains. However, with two exceptions (*B. cepacia* LMG 6993 and *B. cenocepacia* R-6274), all strains expressing AiiA showed reduced or abolished expression of AidA (Fig. 4), indicating that expression of this protein is QS regulated in the large majority of *B. cepacia* complex strains.

FIG. 4. AHL degradation in *B. cepacia* complex strains reduces expression of AidA. Wild-type and transconjugant strains were grown in LB medium overnight, and 10- μ l samples were used for Western blotting with anti-AidA antibodies. Transconjugants (lanes labeled +) are shown in the lane next to the parental strain.

DISCUSSION

AHL-dependent QS systems appear to be particularly widespread among *B. cepacia* complex strains, since all *B. cepacia* complex species produce these signal molecules (21, 27, 34). Several QS-regulated functions in *B. cepacia* complex strains have been identified, including the production of extracellular proteases and siderophores, swarming motility, biofilm formation, and pathogenicity (for reviews, see references 18 and 50). However, these data are based on investigations of a few model bacteria, mainly strains of *B. cenocepacia*. We used a quorum-quenching approach to investigate whether AHL-dependent regulation of these functions is conserved within the entire spectrum of *B. cepacia* complex. This strategy, based on the enzymatic degradation of AHL signal molecules, was preferred over the systematic and tedious construction of QS mutants in each of the strains. Heterologous expression of AiiA was previously shown to reduce or even abolish expression of AHL-regulated functions in *Erwinia carotovora*, *Pseudomonas aeruginosa*, and *B. thailandensis* (14, 41, 48). In the present study, we show that heterologous expression of AiiA greatly reduced the amounts of AHL signal molecules and interfered with the expression of QS-regulated traits in various *B. cepacia* complex strains. In fact, the transconjugant of *B. cenocepacia* H111, which has been extensively investigated in our laboratory, was found to be virtually indistinguishable from the defined *cepI* mutant H111-I.

As previously reported, AiiA degraded both short- and long-acyl-chain AHLs effectively (14). Hence, the quorum-quenching approach rendered strains producing long-chain AHLs in addition to C8-HSL (strains of *B. vietnamiensis* and *B. ambifaria* LMG 19182) entirely AHL negative. This strategy is therefore highly valuable to circumvent the construction of double mutants in these strains, but it does not allow conclusions to be drawn on the individual contributions of the two QS systems present in these organisms or on possible interrelationships between them. The ET12 lineage of *B. cenocepacia* is another interesting group of strains utilizing two QS systems. In addition to the *CepI/CepR* system, these highly transmissible strains also harbor the *CciI/CciR* QS system, which was shown to be part of a 31.7-kb low-GC-content pathogenicity island that is unique to this *B. cenocepacia* lineage (3, 35). Recent work has demonstrated that the two systems do not operate independently of each other in that expression of *ccrI* was shown to require active *CepR* (36). Whereas a *ccrI* mutant exhibited increased proteolytic activity and reduced swarming motility relative to the parent strain, the phenotypes of the *cepI cciI* and *cepR cciR* double mutants were similar to *cepI* or *cepR* mutants, with less protease activity and impaired swarming motility.

AHL-dependent QS systems control the production of extracellular proteolytic activity in many gram-negative bacteria (16, 52). We observed that, in agreement with a previous study (21), strains from *B. dolosa*, *B. multivorans*, and *B. vietnamiensis* did not produce extracellular proteases under the conditions used in the present study. However, in all strains that tested positive for proteolytic activity on skim milk plates, the expression of AiiA greatly reduced this enzymatic activity. These data suggest that expression of proteolytic activity is a highly conserved QS-regulated phenotype in *B. cepacia* complex strains.

Recent work identified an extracellular zinc metalloprotease, ZmpA, in *B. cenocepacia*, which plays a role in virulence in both chronic and acute models of respiratory infection (45). Expression of this protease was shown to be regulated by *CepI/CepR* at the level of transcription (11). Since very little is known about the distribution of *zmpA* among the various *B. cepacia* complex species, it is currently unclear whether ZmpA or other extracellular proteases are QS regulated in *B. cepacia* complex species other than *B. cenocepacia*. A BLAST search in the genome sequences of *B. cepacia* complex species currently available in the NCBI database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) revealed that *zmpA* orthologues are present in *B. cepacia* R-18194 but not in *B. vietnamiensis* R-1808, which was found to be protease negative (Table 1). Moreover, the genomes of *B. pseudomallei*, *B. mallei*, and *B. thailandensis*, which do not belong to the *B. cepacia* complex, also harbor *zmpA* homologues, suggesting that this protease might be widespread among *Burkholderia* species.

Swarming motility is a special form of surface translocation that enables bacteria to move on top of certain surfaces in a highly coordinated manner (23). The swarming phenomenon involves cell differentiation, extensive flagellation, and contact between neighboring bacteria during migration. Previous work has provided evidence that in many bacteria swarming motility is QS controlled (12). More specifically, in *Serratia liquefaciens* and *P. aeruginosa* it was shown that AHL-dependent synthesis of the surface-active compounds serrawettin W2 and rhamnolipids, respectively, are required to form a swarming colony (33, 37). Likewise, the *CepI/CepR* system of *B. cenocepacia* is required for swarming motility, possibly by controlling the production of an as-yet-unidentified biosurfactant (25, 36). Only a very few strains were found to be capable of swarming under the conditions used in the present study, and these belonged to different *B. cepacia* complex species. Swarming motility is highly dependent on the nutritional status of the cells and the physical properties of the surface, and thus this assay is strongly dependent on the experimental settings (17, 23). Importantly, expression of AiiA abolished swarming in all strains that tested positive for this form of motility. Additional work will be required to investigate whether the inability of the transconjugants to swarm is due to the lack of biosurfactant production and, if so, what the chemical nature of the compound(s) in the various strains is. Interestingly, heterologous expression of AiiA in *B. thailandensis*, which harbors multiple QS systems and produces numerous AHL signal molecules, not only affected swarming but also twitching motility (48).

A role of AHL-mediated QS in biofilm formation has been demonstrated for various bacteria, including *P. aeruginosa*, *Aeromonas hydrophila*, *S. liquefaciens*, and *P. putida* (24). In *B. cenocepacia* it was shown that mutants defective in QS form only flat and undifferentiated biofilms on abiotic surfaces compared to the one of the wild type (25, 47). A detailed quantitative analysis of the biofilm architectures revealed that the *CepI/CepR* system is not involved in the regulation of initial cell attachment but rather controls the maturation of the biofilm (25, 26). However, the QS-regulated factors required for biofilm development have not yet been identified. Using a simple microtiter plate assay we showed that the biofilm-forming abilities of the various strains differed tremendously (Table 1). In agreement with previous work we observed that in the

majority of strains interference with cell-to-cell signaling reduced the biomass of the biofilm formed. In a few strains, however, the transconjugants expressing AiiA formed thicker biofilms than the parental strains, suggesting that in these strains QS represses biofilm development. Since only in a very few strains did the quorum-quenching approach not affect biofilm formation, we suggest that QS plays an important role in biofilm formation in *Burkholderia* species. In an earlier study in which biofilm formation and AHL production of a large number of *B. cepacia* complex strains was determined, a clear-cut correlation between the two phenotypes was not possible (10).

Recent work has established that the CepI/CepR QS system contributes to the virulence of *B. cenocepacia* in a wide range of hosts, including murine species (45), the plant alfalfa (4), and the nematode *Caenorhabditis elegans* (29), which has emerged as a highly valuable nonmammalian model for the study of bacterial pathogenicity (44). Depending on the medium used for growth, two different modes of nematode killing were observed with *B. cenocepacia* H111. On high-osmolarity medium the worms are killed within 24 h due to the action of a diffusible extracellular toxin ("fast killing"). In contrast, on nematode growth medium the killing occurs over the course of 1 to 3 days ("slow killing") and involves the accumulation of bacteria in the intestinal lumen of the worm and thus resembles an infection-like process. The CepI/CepR QS system of *B. cenocepacia* H111 is essential for killing of *C. elegans* under both slow-killing and fast-killing conditions (29). We have tested the virulence of all *B. cepacia* complex strains included in the present study in the *C. elegans* slow-killing pathogenesis model. Our data fully support the recent findings demonstrating that nematode pathogenicity is strain but not species specific (5). Most intriguingly, however, heterologous expression of AiiA in the virulent strains greatly attenuated their pathogenicity, suggesting that expression of functions required for nematode killing is strictly QS regulated in the various *B. cepacia* complex strains.

We have recently identified a novel QS-regulated virulence factor in *B. cenocepacia* H111, AidA, which plays an important role in nematode killing (27). Since AidA is required for slow killing of *C. elegans* but has little effect on fast killing, it has been suggested that the protein is essential for the accumulation of the bacteria in the nematode gut. Hence, AidA appears to be required for survival in the nematode intestine and thus for establishing an infection-like process rather than acting as a toxin. Using polyclonal antibodies directed against AidA, it was shown that many *B. cepacia* complex strains produce AidA (Table 1) (27). However, since some strains that tested positive for AidA did not kill the nematode and other strains that expressed the protein were avirulent, we conclude that additional virulence factors may exist that are responsible for the observed differences in nematode pathogenicity. Several studies using different experimental approaches have established that expression of AidA in *B. cenocepacia* and *B. cepacia* is tightly regulated by the CepI/CepR system (1, 27, 42, 51). Here we showed that with two exceptions expression of the AiiA lactonase reduced or abolished synthesis of AidA in all *B. cepacia* complex strains tested, indicating that AHL-dependent control of AiiA expression is highly conserved within the *B. cepacia* complex. In conclusion, our data suggest that although aidA is important for persisting in the nematode gut, other

virulence factors are required for killing of *C. elegans*. However, given that AHL-mediated QS is essential for nematode pathogenicity in all *B. cepacia* complex strains included in the present study, we propose that expression of these nematocidal determinants is also QS regulated. Work is currently under way to identify these virulence factors.

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