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Production of the antifungal compound pyrrolnitrin is quorum sensing-regulated in members of the *Burkholderia cepacia* complex

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Summary

Members of the genus *Burkholderia* are known for their ability to suppress soil-borne fungal pathogens by the production of various antibiotic compounds. In this study we investigated the role of *N*-acylhomoserine lactone (AHL)-dependent quorum sensing (QS) in the expression of antifungal traits. Using a quorum quenching approach, that is, by heterologous expression of the *Bacillus* sp. AiiA lactonase, we show that expression of antifungal activities is AHL-dependent in the large majority of the investigated strains belonging to various *Burkholderia* species. We demonstrate that in certain strains of *Burkholderia ambifaria*, *Burkholderia pyrrocinia* and *Burkholderia lata*, one of the QS-regulated antifungal agents is pyrrolnitrin (prn), a common broad-spectrum antibiotic that is also produced by some *Pseudomonas* and *Serratia* species. To investigate the underlying molecular mechanisms of AHL-dependent prn production in better detail, we inactivated the AHL synthase *cepI* as well as *cepR*, which encodes the cognate AHL receptor protein, in *B. lata* 383. Both QS mutants no longer produced prn as assessed by gas chromatography-mass spectrometry analysis and as a consequence were unable to inhibit growth of *Rhizoctonia solani*. Using fusions of the *lacZ* gene to the promoter of the *prnABCD* operon, which directs the synthesis of prn, we demonstrate that expression of prn is positively regulated by CepR at the level of transcription.

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Introduction

Because of public concerns about the use of pesticides and the common trend in agriculture towards greater sustainability the interest in biological control of plant pathogens has increased considerably (Raaijmakers *et al*., 2002; 2008; Haas and Défago, 2005). Moreover, for a number of plant diseases chemical solutions are ineffective or even not available (Roberts *et al*., 2005). A large number of microorganisms, including strains of the genera *Bacillus*, *Streptomyces*, *Serratia*, *Pseudomonas* as well as non-pathogenic strains of *Fusarium* sp., have been reported to exhibit biocontrol activities and some of these are used in commercial applications (Thomashow, 1996; Berg, 2000; Cao *et al*., 2005). Biocontrol strains have a direct positive effect on plant health through three possible mechanisms: (i) effective and competitive colonization of the rhizosphere (Paulitz, 2000), (ii) production of antibiotics that inhibit growth of the pathogen (O'Sullivan and O'Gara, 1992) and (iii) induction of systemic resistance in the host plant that increases the resistance to a broad spectrum of pathogens (Pieterse *et al*., 1996).

Many soil-borne plant diseases caused by fungi and oomycetes can be controlled by strains of the genus *Burkholderia*. Some of the most studied examples include biocontrol of damping-off and soil-borne diseases caused by *Pythium* spp., *Rhizoctonia solani*, and *Fusarium* spp. (Parke and Gurian-Sherman, 2001; Coenye and Vandamme, 2003; Compant *et al*., 2008). Although the beneficial effects of *Burkholderia* sp. include diverse mechanisms, the secretion of allelochemicals is of particular relevance for biocontrol activities of these organisms (for a recent review see Vial *et al*., 2007). Compounds produced by *Burkholderia* sp. that have been shown to exhibit antifungal activity include lipopeptides (Kang *et al*., 1998), cepaciamides A and B (Ying *et al*., 1996), cepacidines (Lee *et al*., 1994), siderophores (Thomas, 2007), altericidin (Kirinuki *et al*., 1984), pyrrolnitrin (Arima *et al*., 1964), glidobactins (Shoji *et al*., 1990;

Schellenberg *et al*., 2007), phenazines (Cartwright *et al*., 1995) and 2-hydroxymethyl-chroman-4-one (Kang *et al*., 2004).

The genus *Burkholderia* contains more than 50 validly described species, which occupy remarkably diverse ecological niches and have been isolated from soil, water, plants, insects, fungi and industrial settings (Coenye and Vandamme, 2003; Mahenthiralingam *et al*., 2005; 2008). Many *Burkholderia* species exhibiting antimicrobial activity and therefore can potentially be used to protect commercially important crops, particularly against fungal diseases, belong to the so-called *Burkholderia cepacia* complex (Bcc) (Vandamme *et al*., 1997). The Bcc comprises a phenotypically heterogeneous group of at least 17 species: *B. cepacia*, *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia dolosa*, *Burkholderia ambifaria*, *Burkholderia anthina* and *Burkholderia pyrrocinia* (Mahenthiralingam *et al*., 2005), in addition to *Burkholderia ubonensis*, *Burkholderia latens*, *Burkholderia diffusa*, *Burkholderia arboris*, *Burkholderia seminalis* and *Burkholderia metallica* (Vanlaere *et al*., 2008), *Burkholderia lata* and *Burkholderia contaminans* (Vanlaere *et al*., 2009) as well as several other potentially novel taxonomic groups (Mahenthiralingam *et al*., 2008).

The use of Bcc strains in commercial applications, however, has been severely limited by the US Environmental Protection Agency, as Bcc species have also emerged as opportunistic pathogens of humans, particularly those with cystic fibrosis (CF; Parke and Gurian-Sherman, 2001; Vandamme *et al*., 2007a; Mahenthiralingam *et al*., 2008). All Bcc species have been isolated from diverse environments as well as from CF sputa and at present no prediction of the pathogenic potential of strains on the basis of its phylogenetic status is possible (Baldwin *et al*., 2007; Mahenthiralingam *et al*., 2008).

In recent years, it has become evident that bacteria do not only exist as individual cells but often coordinate their activities and act in a concerted manner similar to multicellular organisms. Many Gram-negative bacteria have been shown to produce *N*-acylhomoserine lactone (AHL) signal molecules, which are utilized by the bacteria to monitor their own population densities in a process known as 'quorum sensing' (QS; Fuqua *et al*., 1996; Whitehead *et al*., 2001). These regulatory systems have been identified in many Gram-negative species, in which they regulate a wide variety of functions, often in connection with virulence, surface colonization, symbiosis and the production of antimicrobial compounds.

Most *Burkholderia* species investigated so far produce *N*-octanoylhomoserine lactones (C8-HSL), which is synthesized by the AHL synthase CepI. As the cell density increases, C8-HSL accumulates in the growth medium until a critical threshold concentration is attained. At this point C8-HSL binds to its cognate LuxR-type receptor protein CepR, which, in turn, leads to the induction or repression of target genes. Previous work has identified several QS-regulated functions in strains of the genus *Burkholderia*, including the production of extracellular proteases, chitinases, a polygalacturonase and siderophores, swarming motility and biofilm formation (for reviews see Eberl, 2006; Sokol *et al*., 2007). Initial evidence that AHL-dependent QS is also important for biocontrol activity in a Bcc strain, namely *Burkholderia ambifaria* BC-F, was presented by Zhou and colleagues (2003). In this study the authors demonstrated that inactivation of *cepI* or *cepR* resulted in a marked reduction of *in vitro* inhibition of the fungal plant pathogens *Pythium ultimum, R. solani* and *Fusarium oxysporum* f.sp. *lycopersici*. As a consequence, the mutants also lost the ability to suppress damping-off of cucumber caused by *P. ultimum*. However, neither has the AHL-controlled antifungal compound been identified nor were the molecular mechanisms of QS-dependent control of biocontrol activity analysed. The purpose of this study was to investigate the role of QS in the regulation of antifungal agents in members of the genus *Burkholderia*. We demonstrate that several Bcc species produce pyrrolnitrin (prn) and show that transcription of the *prnABCD* operon, which directs prn biosynthesis, is CepR-regulated.

Results

Antifungal activity of various Burkholderia *strains is dependent on AHL-dependent QS systems*

In agreement with previous reports (Cartwright *et al*., 1995; Holmes *et al*., 1998; Coenye *et al*., 2001; Roberts *et al*., 2005) we observed that many *Burkholderia* strains exhibit strong antifungal activity against the oomycete *P. ultimum* and/or the basidiomycete *R. solani* (Table 1). As all tested strains produced AHL signal molecules, we next wished to test whether QS is involved in the regulation of the production of antifungal compounds. To this end we heterologously expressed the *Bacillus* sp. strain 240B1 AHL lactonase AiiA in the respective *Burkholderia* strains by transferring plasmid pMLBAD-aiiA. Previous work has shown that the AiiA lactonase specifically degrades AHL molecules indepent of the length of the acyl side-chain (Dong *et al*., 2000) and that transfer of pMLBAD-aiiA into various *Burkholderia* strains rendered them AHL-negative (Wopperer *et al*., 2006). Intriguingly, expression of AiiA not only eliminated accumulation of AHL signal molecules but also reduced or abolished antifungal activities (Table 1). Importantly, the presence of the expression vector without the *aiiA* gene did not affect the

Table 1. Antifungal activities of *Burkholderia* strains and their AiiA-expressing derivates against *P. ultimum* and *R. solani* on Malt agar.

	Antifungal activity[a]			
	P. ultimum		*R. solani*	
Strain	Wild type	AiiA	Wild type	AiiA
B. cepacia LMG 1222	++	–	+	–
B. cenocepacia H111	+	–	–	–
B. stabilis R 6270	+	–	–	–
B. vietnamensis LMG 10929	+	–	+	–
B. ambifaria LMG 19182	–	–	+	–
B. ambifaria LMG 19467	(+)	–	++	–
B. pyrrocinia LMG 21822	–	–	+	–
B. pyrrocinia LMG 21823	–	–	+	–
B. lata 383	++	+	+	+
B. caryophylii LMG 2155	+	–	++	–
B. phenazinium LMG 2247	+	–	++	–
B. phenazinium S12	+	–	n.t.	n.t.
B. phenazinium S20	++	–	n.t.	n.t.
B. phenanzinium S32	++	–	n.t.	n.t.
B. bryophila 1S5	+	+	+	–
B. megapolitana A1	+	+	+	–

a. Inhibition zone in dual culture assays: – represents 0 mm, + 1–4 mm, ++ 5–10 mm wide zone of inhibition (radius).
n.t., not tested.

antifungal activities of the strains (data not shown). These results suggest that the AHL-dependent QS systems operating in these *Burkholderia* strains control the synthesis of antifungal agents.

Identifying prn as one of the QS-regulated antifungal compounds in Burkholderia *spp.*

Previous work has demonstrated that various *Burkholderia* strains produce a wide range of antifungal compounds (for a recent review see Vial *et al*., 2007). Although the structures of many of these antifungals have been determined, very little is known about the genes required for their synthesis. To identify the genes responsible for the production of the antifungal compounds in the tested *Burkholderia* strains, we performed PCR assays using primer pairs that have previously been used to amplify genes required for the synthesis of pyoluteorin (plt), phenazine (phz), 2,4-diacetylphloroglucinol (phl) and prn, antifungal compounds produced by many fluorescent pseudomonads and some other bacterial species (Dwivedi and Johri, 2003; Haas and Keel, 2003). Only with the primer pair targeting a conserved region within the *prnD* gene, which is part of the *prnABCD* operon that directs the synthesis of prn, amplification products were obtained with some of the strains (Table 2), while with none of the strains tested amplicons were obtained with the other primer pairs (data not shown). Interestingly, all PCR-positive strains belonged to the Bcc. Sequence analysis of the 786 bp amplicons of strains of *B. cepacia*, *B. cenocepacia*, *B. ambifaria*, *B. pyrrocinia* and *B. ubonensis* unambiguously confirmed the presence of the *prnD* gene in these strains. Moreover, hybridization of a macroarray carrying chromosomal DNAs (Baldwin *et al*., 2004) of 228 strains belonging to various *Burkholderia* species (16 Bcc taxonomic groups and 15 other *Burkholderia* species) and other closely related genera (see Table S1) using a *prnD* probe confirmed our PCR results; hybridization showed that only certain strains of *B. cepacia* (3), *B. cenocepacia* (3 IIIA and 6 IIIB), *B. ambifaria* (2), *B. pyrrocinia* (4), and Bcc novel Groups Bcc 4 (1) and Bcc 6 (2) contain a homologous *prnD* gene (see Table S2), while none of the strains of other species gave positive hybridization results. Among these positive Bcc isolates, the *prnD* gene was present in strains from clinical infection (10 CF strains and 4 from other infections) as well as in environmental isolates (7). These data indicate that within at least 10% of the investigated Bcc isolates (21/204 strains, Tables S1 and S2) prn may play a role in their antifungal activities.

Prn is produced by different Bcc species in an AHL-dependent manner

To test whether prn is in fact produced by the identified *prnD*-positive strains we extracted cultures of *B. lata* 383 (formerly *B. cepacia* Group K 383; Vanlaere *et al*., 2009), *B. cenocepacia* PC184, *B. ambifaria* LMG 19467, *B. pyrrocinia* LMG 21823 and *B. ubonensis* LMG 20358 and analysed the extracts by thin-layer chromatography (TLC). For detection of compounds with antifungal activities the TLC plates were overlaid with soft agar seeded with *R. solani*. Growth inhibitory spots with mobilities indistinguishable from the one of synthetic prn (R_f value of

Table 2. Bacterial strains and plasmids used.

Strain or plasmid	Characteristics	*prnD*[a]	Source or reference
Bacterial strain			
B. lata 383	Forest soil, Trinidad	+	Stanier *et al*. (1966)
B. lata 383-I	*cepI::dhfr* mutant of 383; Trm^r	+	This study
B. lata 383-R	*cepR::dhfr* mutant of 383; Trm^r	+	This study
B. lata 383-P	*prnA::dhfr* mutant of 383; Trm^r	+	This study
B. cepacia MC 0353	Environmental isolate	+	Baldwin *et al*. (2004)
B. cepacia TA (BCC240)	Clinical non-CF isolate, USA	+	Baldwin *et al*. (2004)
B. cepacia IST444	CF-patient, Portugal	+	Cunha *et al*. (2003)
B. cepacia LMG 1222	Type strain, *Allium cepa*, USA	+	Yabuuchi *et al*. (1992)
B. cenocepacia PC184	CF-patient, USA	+	Mahenthiralingam *et al*. (2000)
B. cenocepacia R-10741	Environmental isolate	+	Baldwin *et al*. (2004)
B. cenocepacia C1518	CF-patient, UK	+	Baldwin *et al*. (2004)
B. cenocepacia H111	CF-patient, Germany	–	Römling *et al*. (1994)
B. cenocepacia H111-I	*cepI::npt* mutant of H111; Km^r	–	Huber *et al*. (2001)
B. cenocepacia H111-R	*cepR::npt* mutant of H111; Km^r	–	Huber *et al*. (2001)
B. stabilis R 6270	CF-patient, Germany	–	Gotschlich *et al*. (2001)
B. vietnamensis LMG 10929	*Oryza sativa*, rhizosphere, Vietnam	–	Gillis *et al*. (1995)
B. ambifaria LMG 19467	CF-patient, Australia	+	Coeyne *et al*. (2001)
B. ambifaria AU 1366	CF-patient, USA	+	Baldwin *et al*. (2005)
B. ambifaria LMG 19182	Type strain, =AMMD, Pea rhizosphere, USA	+	Coeyne *et al*. (2001)
B. pyrrocinia LMG 21823	Water, UK	+	Vandamme *et al*. (1997)
B. pyrrocinia LMG 21822	Soil, USA	+	Vandamme *et al*. (1997)
B. pyrrocinia LMG 14191	Soil, Japan	+	Vandamme *et al*. (1997)
B. ubonensis LMG 20358	Soil, Thailand	+	Yabuuchi *et al*. (2000)
B. caryophylii LMG 2155	*Dianthus caryophyllus*, USA	–	Yabuuchi *et al*. (1992)
B. phenazinium LMG 2247	Soil enriched with threonine	–	Bell and Turner (1973)
B. phenazinium S12	Isolate from *Sphagnum rubellum*	–	Opelt and Berg (2004)
B. phenazinium S20	Isolate from *S. rubellum*	–	Opelt and Berg (2004)
B. phenanzinium S32	Isolate from *S. rubellum*	–	Opelt and Berg (2004)
B. bryophila 1S5	Isolate from *S. rubellum*	–	Vandamme *et al*. (2007b)
B. megapolitana A1	Isolate from *Aulacomnium palustre*	–	Vandamme *et al*. (2007b)
Pseudomonas putida F117	AHL-negative derivate of IsoF; *ppuI*		Steidle *et al*. (2001)
Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q} ZΔM15 Tn*10* (Tet^r)]		Stratagene
E. coli MT102	*araD139(ara-leu)7696 Δlac thi hsdR*		Laboratory collection
E. coli HB101	*recA thi pro leu hsdR-M*$^+$ Smr		Kessler *et al*. (1992)
E. coli OP50	Bacterial food source for *Caenorhabditis elegans*		Brenner (1974)
Plasmid			
pBBR1MCS	Broad-host-range cloning vector, Cm^r		Kovach *et al*. (1994)
pSU11	Broad-host-range *lacZ*-based promoter probe vector; Gm^r		S. Uehlinger, unpublished
pRN1	P_{prn1}–*lacZ* on pSU11; Gm^r		This study
pRN2	P_{prn2}–*lacZ* on pSU11; Gm^r		This study
pRNC3A	P_{prnC3A}–*lacZ* on pSU11; Gm^r		This study
pRNA17C	$P_{prnA17C}$–*lacZ* on pSU11; Gm^r		This study
pRNΔ	$P_{prnΔ}$–*lacZ* on pSU11; Gm^r		This study
pAS-C8	P_{cepI}-*gfp*(ASV)-P_{lac}-*cepR*; C8-HSL biosensor; Gm^r		Steidle *et al*. (2001)
pSB403	Bioluminescent broad-host-range AHL biosensor; Tet^r		Winson *et al*. (1998)
pRK600	ColE1 RK2-Mob$^+$ RK2-Tra$^+$ helper plasmid; Cm^r		Kessler *et al*. (1992)
pEX18Gm	gene replacement vector; Gm^r		Hoang *et al*. (1998)
pEX-ko	pEX18Gm containing the *dhfr* gene, Trm^r		This study
pEX-ko-cepI	Integration vector pEX18Gm carrying *dhfr* and $cepI'_{383}$		This study
pEX-ko-cepR	Integration vector pEX18Gm carrying *dhfr* and $cepR'_{383}$		This study
pEX-ko-prnA	Integration vector pEX18Gm carrying *dhfr* and $prnA'_{383}$		This study
pMLBAD-aiiA	Broad-host-range vector carrying *araC*-P_{BAD}-*aiiA* for expression of AiiA; Trm^r		Wopperer *et al*. (2006)
pBAH27	pBBRMCS-5 containing the *cepR* of *B. cenocepacia* H111, Gm^r		Huber *et al*. (2001)
pBBR-cepR	pBBR1MCS containing the *cepR* gene of *B. cenocepacia* H111, Cm^r		This study

a. The presence of *prnD* was determined by PCR and/or Southern blot analysis.

Table 3. Inhibition zones of prn extracted from cultures of Bcc strains and their QS-quenched derivates.

	Inhibition zone[a]	
Strains	Wild type	AiiA
B. lata 383	47 ± 7	8 ± 5
B. ambifaria LMG 19467	42 ± 8	0 ± 0
B. pyrrocinia LMG 21823	32 ± 5	0 ± 0
B. cenocepacia PC184	0 ± 0	n.t.
B. ubonensis LMG 20358	0 ± 0	n.t.

a. Diameters (in mm) of inhibition zones were measured after 96 h of incubation of *R. solani*-overlayed TLC plates.
n.t., not tested.

0.64) were observed with the supernatants of *B. lata* 383, *B. ambifaria* LMG 19467 and *B. pyrrocinia* LMG 21823 (Table 3; Fig. S1) but not of *B. cenocepacia* PC184 and *B. ubonensis* LMG 20358 (Table 3). To investigate the influence of QS on prn production in these strains we also analysed extracts of the respective QS-quenched derivates. Prn production of the transconjugants was found to be abolished or greatly reduced, suggesting that production of this antifungal compound is AHL-dependent.

Construction and characterization of defined QS and prn mutants of B. lata 383

To investigate the role of QS in prn synthesis and the importance of prn in suppressing fungal plant pathogens in better detail, we inactivated the genes *cepI*, *cepR* and *prnA* in *B. lata* 383 (see *Experimental procedures* for details). We have chosen this strain because it produces AHLs as well as prn and, most importantly, its genome sequence has been determined (GenBank CP000150, CP000151, CP000152). The genetic structures of the *cepI*, *cepR* and *prnA* mutants, which were designated 383-I, 383-R and 383-P respectively, were confirmed by PCR and Southern blot analysis (data not shown).

Cross-streaking experiments as well as quantitative AHL bioassays using different sensor strains revealed that mutants 383-I and 383-R no longer produced AHL signal molecules, suggesting that the CepIR system is the only AHL-dependent QS system in this strain. In agreement with this finding we were unable to identify an additional *luxI* homologue in the genome sequence of strain 383. AHL production of the *cepR* mutant was rescued when the highly homologous *cepR* gene of *B. cenocepacia* H111 (97% aa identity) was provided *in trans* on plasmid pBBR-cepR. Expectedly, production of AHL signal molecules was unaffected in the *prnA* mutant 383-P.

Previous studies have demonstrated that the production of siderophores, pigments and extracellular proteolytic activity is QS-regulated in many members of the genus *Burkholderia* (Wopperer *et al.*, 2006). We therefore tested the *B. lata* 383 wild-type strain and the two mutants 383-I and 383-R for these phenotypes. Using CAS indicator plates for assessing synthesis of siderophores no difference between the wild type and the two QS mutants was observed. Proteolytic activity of 383-I and 383-R on skim milk plates was found to be greatly reduced relative to the wild type. Importantly, proteolytic activities of the mutants could be restored to the level of the wild type when strain 383-I was grown in the presence of 200 nM C8-HSL or when mutant 383-R was complemented with *cepR*H111 (Table 4). We also noticed that the wild-type strain produced a purple pigment when grown on PIA medium while both QS mutants did not. Pigmentation of the complemented *cepR* mutant appeared to be even stronger than the one observed with the wild-type strain (Fig. 1). These experiments demonstrate that proteolytic activity and pigment production but not synthesis of siderophores are QS-controlled in *B. lata* 383.

Table 4. Phenotypic characterization of *B. lata* 383 wild type, *cepIR* and *prnA* mutants.

Strains	Genotype	C8-HSL (RFU)[a]	Protease activity[b]	Antagonistic activity towards *Rhizoctonia solani*[c]		Antagonistic activity towards *Pythium ultimum*[c]		Siderophore activity[d]
				Malt agar	KMB	Malt agar	KMB	
B. lata 383	wt	1327 ± 106	++	+	+	++	+	++
B. lata 383-I	*cepI*⁻	74 ± 2	+	–	–	++	+	++
B. lata 383-R	*cepR*⁻	73 ± 4	–	–	–	++	+	++
B. lata 383-I+ C8-HSL	*cepI*⁻	n.a.		++	+	+	+	+
B. lata 383-R(pBBR-cepR)	*cepR*⁻	831 ± 21	++	+	+	++	+	++
B. lata 383-P	*prnA*⁻	1155 ± 64	++	–	–	++	+	++

a. Presence of C8-HSL in culture supernatants were determined by the aid of the GFP-based monitor strain *P. putida* F117(pAS-C8). Means of relative fluorescence units (RFU) ± standard deviation from two experiments with four technical replicates each are shown. Values below 80 RFU represent the uninduced background of the biosensor.
b. Protease activity was determined on skim milk agar plates after 48 h of incubation at 37°C. ++, clear signal; +, weak signal; –, no signal.
c. Inhibition zone in dual culture assays: – represents 0 mm, + 1–4 mm, ++ 5–10 mm wide zone of inhibition (radius).
d. CAS agar zones were measured after 48 h of incubation at 37°C. + represents 1–3 mm, ++ 4–8 mm diameter.
n.a., not applicable.

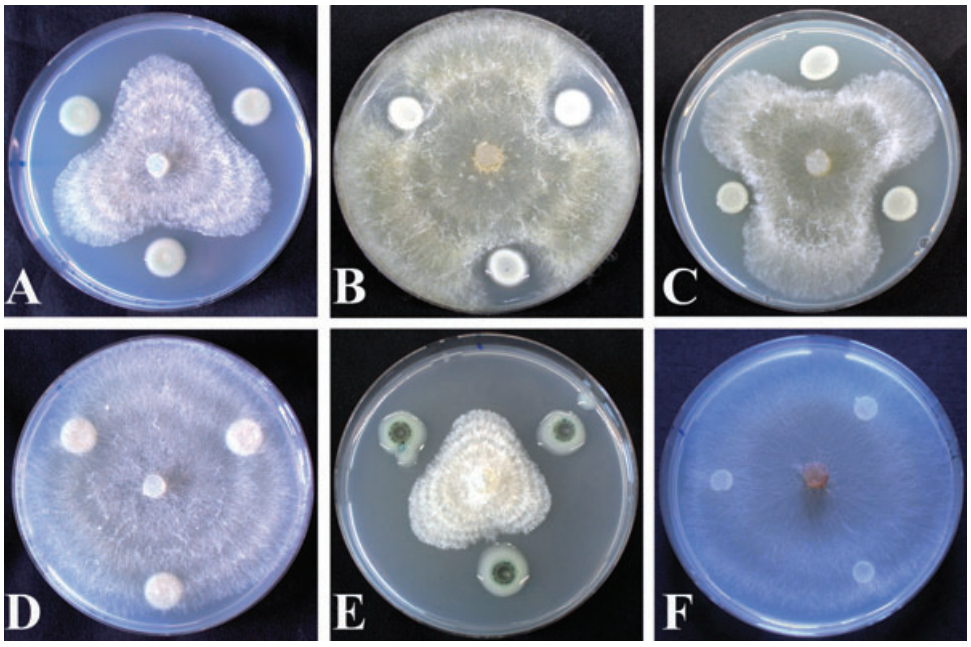

Fig. 1. Antifungal activities of the *B. lata* 383 wild type (A), the *cepI* mutant 383-I in the absence (B) or presence of 200 nM C8-HSL (C), the *cepR* mutant 383-R (D), the complemented *cepR* mutant 383-R (pBBR-cepR) (E) and the *prnA* mutant 383-P (F) against *R. solani* on malt agar. Pictures were taken after 96 h of incubation.

Antifungal activity of B. lata *383 against* R. solani *is dependent on QS-controlled synthesis of prn*

We next tested the wild-type strain *B. lata* 383 and the two QS-deficient mutants for their antifungal activities against *R. solani*, against which prn is particularly effective (Homma *et al*., 1989). While strong inhibition of fungal growth was observed for the wild-type strain both QS mutants were overgrown by the fungus after 96 h of incubation (Table 4; Fig. 1A, B and D). Importantly, antifungal activities could be restored to the level of the wild type by the external addition of 200 nM C8-HSL to 383-I or complementation of 383-R with pBBR-cepR (Fig. 1C and E). Like the QS mutants the *prnA* mutant 383-P was unable to inhibit growth of *R. solani* (Fig. 1F), suggesting that production of prn might be responsible for the observed antifungal activity.

To unequivocally demonstrate that *B. lata* 383 is producing prn in an AHL-dependent manner we analysed acetonitril extracts of cultures of the wild-type 383, the AiiA-expressing transconjugant and the mutants 383-I, 383-R and 383-P in an *in vitro* inhibition assay as well as by gas chromatography-mass spectrometry (GC-MS). The extracts of the wild-type strain (Fig. 2A) exhibited strong antifungal activity against *R. solani* while extracts of the transconjugant and the QS-mutants (Fig. 2B, C and D) as well as the solvent control (Fig. 2E) did not influence growth of the fungus. In concordance with the TLC analysis we were able to identify prn in the extract of the wild-type 383 but not in the extract of the *prnA* mutant by GC-MS

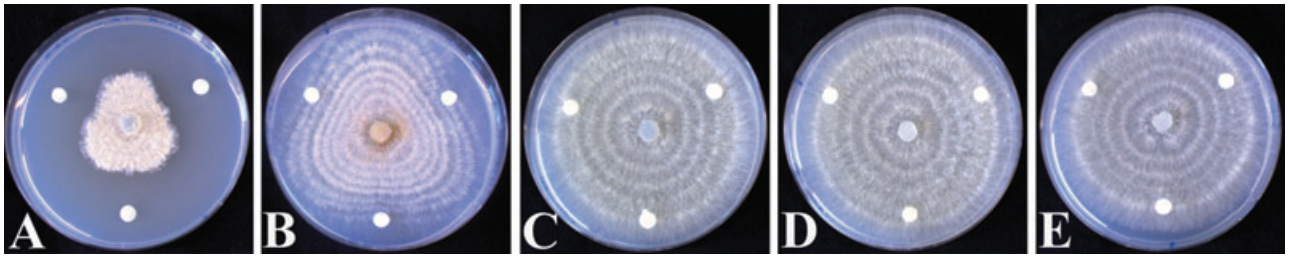

Fig. 2. Antifungal activities of acetonitril extracts of cultures of the wild-type *B. lata* 383 (A), the quorum-quenched derivative of 383 (B), the *cepI* mutant 383-I (C) and the *cepR* mutant 383-R (D). As a control acetonitril was also tested (E). Three sterile filter discs soaked with 50 µl extract or pure acetonitril were placed onto MA agar plates, which were inoculated with *R. solani* pads placed in the centre. Pictures were taken after 96 h of incubation.

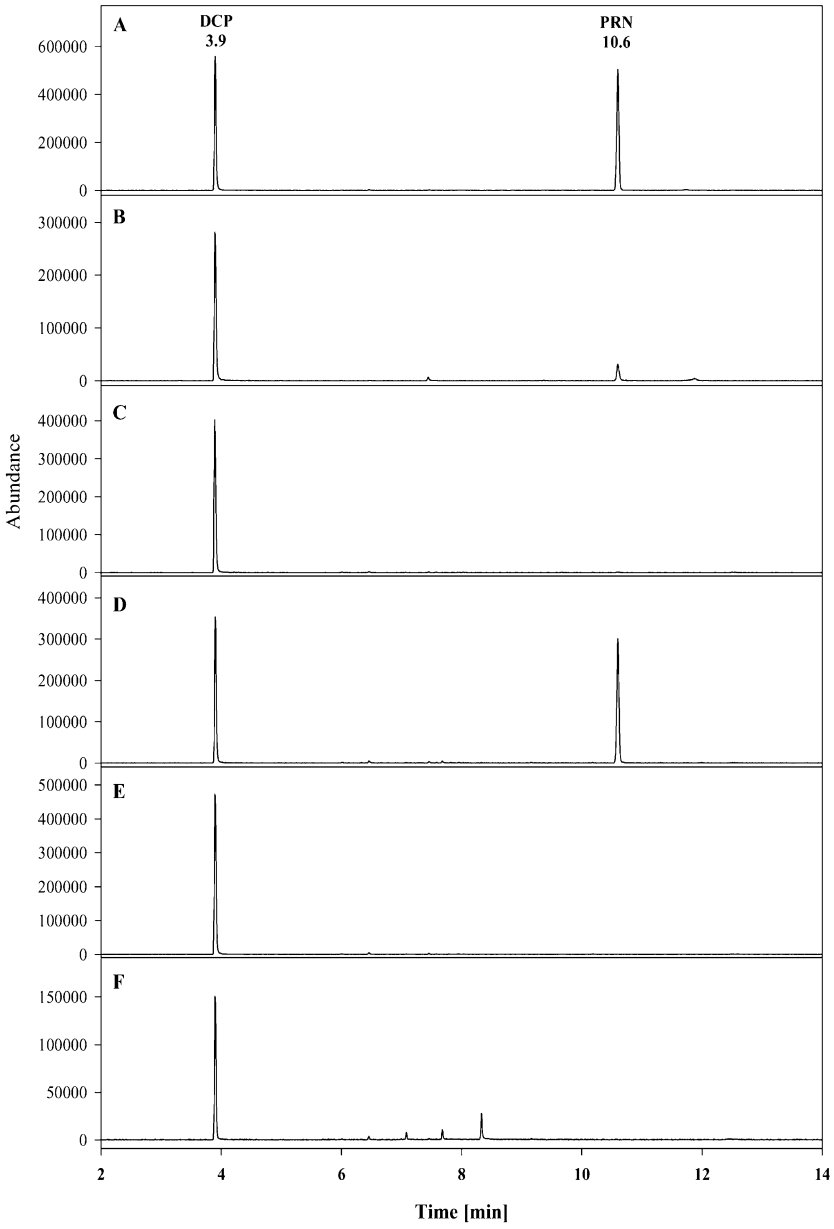

Fig. 3. GC-MS analysis of acetonitril extracts of cultures of the wild-type *B. lata* 383 (A), the quorum-quenched derivative of 383 (B), the *cepI* mutant 383-I in the absence (C) and presence of 200 nM C8-HSL (D), the *cepR* mutant 383-R (E) and *prnA* mutant 383-P (F). The prn standard had a retention time of 10.6 min. The fragment ion $m/z = 166$ was used to identify prn (PRN) and dichlorophenole (DCP).

(Fig. 3). The amounts of prn present in extracts of the two QS mutants and the transconjugant were found to be greatly reduced relative to the wild-type strain. However, addition of 200 nM C8-HSL to the growth medium restored prn production of mutant 383-I to the level of the wild type. These results clearly show that production of prn is under the control of the CepIR QS system of *B. lata* 383.

Burkholderia lata 383 not only suppressed growth of *R. solani* but also of *P. ultimum*. To our surprise neither the QS mutants 383-I and 383-R nor the *prnA* mutant 383-P showed reduced antifungal activity against *P. ultimum* (Table 4). These data suggest that in addition to prn another, yet unidentified, antifungal compound is produced by *B. lata* 383 in a QS-independent manner.

Expression of prn is controlled by the CepIR QS system at the level of transcription

Sequence analysis of the *prnABCD* promoter region identified a 20 bp imperfect palindrome with high similarity to *cep* box sequences (Fig. 4A). This sequence is adjacent to the −35 site of a putative promoter consensus sequence, suggesting that transcription of *prnABCD* may be positively regulated by the CepR/C8-HSL complex. This finding prompted us to construct a transcriptional fusion of the *prnABCD* promoter region to the promoterless *lacZ* gene on vector pSU11. The resulting plasmid, pRN1, was transferred to the *B. cenocepacia* wild-type H111, the *cepI* mutant H111-I and the *cepR* mutant

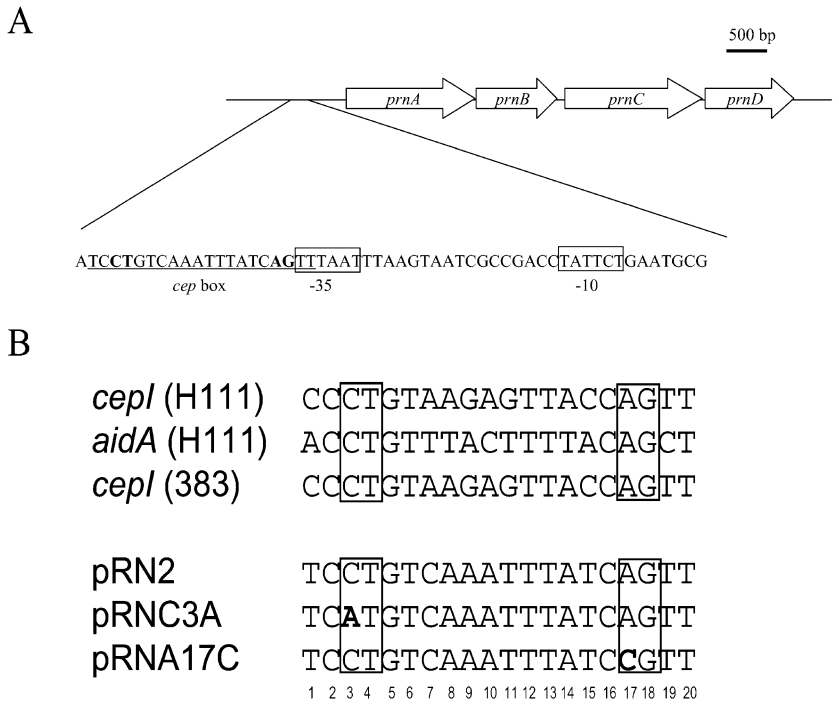

Fig. 4. A. Genetic map of the *prnABCD* operon of *B. lata* 383. Genes are represented by open arrows pointing in the direction of transcription. Shown below is the nucleotide sequence of the *prnABCD* upstream region of *B. lata* 383. The putative –35 and –10 promoter sequences are boxed and the *cep* box sequence is underlined. The minimal consensus sequence defined for operators of QS-regulated genes in *P. aeruginosa* (Whiteley and Greenberg, 2001) is shown in boldface.
B. Nucleotide sequence alignment of previously identified CepR binding sites in the promoter regions of *cepI* and *aidA* with the putative *cep* box upstream of *prnABCD*. Substituted nucleotides in the mutated *cep* boxes are shown in boldface and the minimal consensus sequence is boxed.

H111-R. Due to the intrinsic resistance of strain 383 to various antibiotics we decided to use *B. cenocepacia* H111 in these experiments, as it belongs to the Bcc, does not harbour the *prnABCD* genes (Table 2) and, like *B. lata* 383, only contains the CepIR QS system (Huber *et al.*, 2004). Promoter activity of the recombinant strains was followed by measuring β-galactosidase activity along the growth curve. In the wild-type strain the *prnABCD* promoter was induced in the stationary phase. Promoter activity was at least 20-fold reduced in the *cepI* and *cepR* mutant background of H111-I and H111-R respectively. No activation of the *prnABCD* promoter was observed in the stationary phase (Fig. 5). Addition of 200 nM C8-HSL to the medium increased β-galactosidase activity of H111-I(pRN1) to about 50% of the level observed in the wild-type background. Expectedly, no activation of the promoter was observed when cultures of H111-R(pRN1) were amended with 200 nM C8-HSL.

To further analyse the importance of the *cep* box in the *prnABCD* promoter region we introduced single nucleotide substitutions at two positions in the palindrome present on plasmid pRN2. The two nucleotides are highly conserved and have been suggested to be crucial for binding of LuxR-type proteins (Egland and Greenberg, 1999). On plasmid pRNC3A the C3 of the palindrome was exchanged against A and on plasmid pRNA17C the A17 was exchanged against C (Fig. 4B). In addition, we deleted the entire *cep* box, giving rise to plasmid pRNΔ. The three constructs were transferred to *B. cenocepacia* H111 and promoter activities were determined by measuring β-galactosidase activity along the growth curve (Fig. 6). Deletion of the *cep* box as well as the introduction of a mutation at position 3 or 17 dramatically reduced β-galactosidase activity, demonstrating that this sequence is essential for the activation of the *prnABCD* promoter. These data provide strong evidence that the CepR/C8-HSL complex may bind to this palindromic sequence and thereby activates transcription of the *prnABCD* operon.

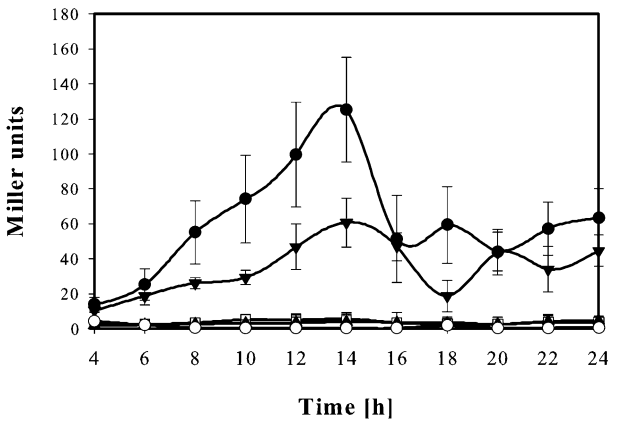

Fig. 5. QS controls activity of the *prnABCD* promoter. Growth and β-galactosidase activity was monitored throughout the growth curve. The transcriptional fusion P_{prn1}–*lacZ* was measured in the wild-type *B. cenocepacia* H111 (●); in the *cepI* mutant H111-I in the absence (●) or presence of 200 nM C8-HSL (▼), the *cepR* mutant H111-R in the absence (□) or presence of 200 nM C8-HSL (▲). β-Galactosidase activity of the empty promoter probe vector pSU11 (○) was measured as a control. Data represent mean values ± standard deviation of three independent experiments.

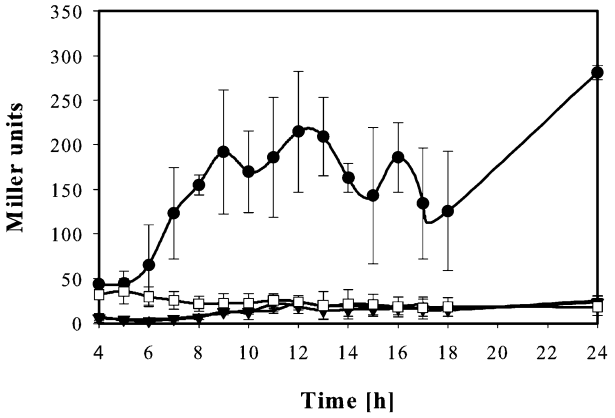

Fig. 6. The *cep* box upstream of the *prnABCD* operon is essential for promoter ativity. Effects of single-base-substitution *cep* box mutations or deletion of the *cep* box on the *prnABCD* promoter ativity was determined. Growth and β-galactosidase activity of *B. cenocepacia* H111 harbouring pRN2 (●), pRNC3A (□), pRNA17C (▼) and pRNΔ (+) was monitored. Data represent mean values ± standard deviation of three independent experiments.

Discussion

Prn is a broad-spectrum antifungal metabolite that was first isolated from *B. pyrrocinia* (formerly *Pseudomonas pyrrocinia*; Arima *et al*., 1964). It has been shown that prn inhibits the fungal respiratory chains and is active against various fungi belonging to the *Basiomycota*, *Deuteromycota* and *Ascomycota* (Tripathi and Gottlieb, 1969). Prn has been used as an antimicotic topical antibiotic in human medicine, particularly against dermatophytic fungi of the genus *Trichophyton*, and synthetic analogues of prn have been developed for use as agricultural fungicides (Ligon *et al*., 2000). Prn is produced by many strains of the genera *Burkholderia* and *Pseudomonas* as well as by *Myxococcus fulvus* and *Serratia plymuthica* (Gerth *et al*., 1982; Kalbe *et al*., 1996; Hammer *et al*., 1999; Haas and Défago, 2005). The four genes required for the biosynthesis of prn from the precursor tryptophan are organized in the *prnABCD* operon (Lively *et al*., 1966; Chang *et al*., 1981).

In this study we performed an extensive survey on the distribution of the *prnD* gene within the genus *Burkholderia (*15 non-Bcc species and 16 Bcc taxonomic groups; Table S1) in 228 isolates employing both Southern blot analysis (Baldwin *et al*., 2004) and PCR assays using a primer pair that specifically targets *prnD*. In agreement with previous studies we found that strains of *B. pyrrocinia*, *B. cepacia* (Garbeva *et al*., 2004; Mendes *et al*., 2007) and *B. cenocepacia* (Seo and Tsuchiya, 2004; Mendes *et al*., 2007) harbour the *prnABCD* genes. In addition, we also detected the operon for the first time in different strains of *B. ambifaria,* in *B. ubonensis* LMG 20358, *B. lata* 383 and novel Bcc Groups Bcc 4 and Bcc 6. In the case of *B. ambifaria* LMG 19467 we were able to unambiguously identify prn in cultures grown in LB for 2 days, while we could not detect prn in cultures of *B. ubonensis* LMG 20358 or *B. cenocepacia* PC184 (Table 3; Fig. S2).

Homology searches within the NCBI database confirmed our findings and revealed the presence of the *prnABCD* operon in the genomes of *B. ambifaria* MC40-6 (GenBank CP001026) and *B. ambifaria* AMMD (GenBank CP000441). This homology search also revealed the presence of the *prnABCD* operon in many strains of the primary human pathogen *Burkholderia pseudomallei* and the closely related species *Burkholderia thailandensis* TXDOH (GenBank NZ_ABBD01000026) and *Burkholderia oklahomensis* EO147 (GenBank NZ_ABBF01000648), but not in *Burkholderia mallei*, another species that is closely related to *B. pseudomallei*. Strains belonging to these species have not been included in our genetic screen.

In summary, our data indicate that the *prnABCD* operon is only present in the genomes of eight Bcc species/novel taxa, and three species of the *B. pseudomallei* group. Phylogenetic analysis of the *Burkholderia* 16S rRNA gene sequence demonstrates the presence of at least two evolutionary lineages (Coenye and Vandamme, 2003), and as the Bcc and *B. pseudomallei* groups form one of those clusters, our data suggest that the *prnABCD* operon distribution is restricted to one arm of the genus *Burkholderia*. Recently, five novel Bcc species, *B. latens* sp. nov., *B. diffusa* sp. nov., *B. arboris* sp. nov., *B. seminalis* sp. nov. and *B. metallica* sp. nov., have been described (Vanlaere *et al*., 2008); in this study we were able to test *B. diffusa* and *B. arboris* isolates; however, *prnD* was absent (Tables S1 and S2). Vanlaere and colleagues (2009) have also recently added *B. lata* and *B. contaminans* to the Bcc and we have shown QS-regulated prn expression in *B. lata* 383*,* but absence of the *prnABCD* operon in *B. contaminans* (Tables S1 and S2). Additional work will be required to fully test these novel Bcc species for the presence of the *prnABCD* biosynthesis genes and prn production.

Previous studies have shown that in some bacteria AHL-dependent QS systems control the production of antifungal compounds. The best investigated example is *Pseudomonas aureofaciens* strain 30-84, which is used as a biocontrol agent to protect wheat from take-all disease caused by *Gaeumannomyces graminis* var. *tritici.* When this strain is present in the rhizosphere of wheat the severity of the disease is strongly reduced due to the production of phenazine antibiotics, which are active against the ascomycete fungus. Synthesis of these antibiotic compounds is regulated by the PhzI/PhzR QS system that utilizes the AHL molecule *N*-hexanoylhomoserine lactone (Pierson *et al*., 1994; Wood *et al*., 1997). PhzR-dependent expression of phenazines

appears to be highly conserved among pseudomonads, as it has also been reported for *Pseudomonas fluorescens* 2-79 (Khan *et al*., 2005) and *Pseudomonas chlororaphis* PCL1391 (Chin-A-Woeng *et al*., 2000). In *Pseudomonas* sp. M18 production of the polyketide compound pyoluteorin that is active against several oomycete fungi including the seed- and root-rot pathogen *P. ultimum* is regulated by the *N*-butanoylhomoserine lactone-dependent RhlI/RhlR system (Yan *et al*., 2007). In a recent study Liu and colleagues (2007) presented evidence that production of prn in *S. plymuthica* HRO-C48 is AHL-dependent. However, the underlying molecular mechanism(s) of how the QS circuitry affects prn production in this organism has not yet been elucidated.

Here we have demonstrated that heterologous expression of the AiiA lactonase in *B. ambifaria* LMG 19467 and *B. pyrrocinia* LMG 21823 abolishes production of prn, suggesting an essential role of QS in the control of the biosynthesis of this antifungal metabolite. To investigate the role of AHL-mediated signalling in prn production in better detail, we inactivated the *cepI* and *cepR* genes in the sequenced strain *B. lata* 383 and showed that these mutants no longer produced prn. Complementation of the *cepR* mutant as well as growth of the *cepI* mutant in medium supplemented with C8-HSL restored prn production to the level of the wild type. Moreover, we identified a *cep* box sequence adjacent to the −35 site of the putative *prnABCD* promoter. Deletion of the *cep* box or single base substitutions at positions that are highly conserved among *cep* box sequences (C3A or A17C, see Fig. 4B) dramatically reduced the promoter activity. These results are in full agreement with mutational analyses of the *Vibrio fischeri lux* box, which demonstrated that nucleotides 3–5 and 16–18 are critical for promoter activity (Egland and Greenberg, 1999; Antunes *et al*., 2008). In conclusion, our data provide strong evidence that binding of the CepR/C8-HSL complex to the identified *cep* box activates transcription of the *prnABCD* operon in *B. lata* 383.

A multi-alignment of the *prnABCD* promoter regions of various Bcc strains revealed the presence of *cep* box sequences in all cases (Figs S3 and S4). However, the number as well as the positions of the identified *cep* boxes varied greatly. The promoter regions of *B. lata* 383 and *B. cepacia* LT4-12-W (GenBank AF161183) share a very high degree of homology and contain only one CepR-binding site about 100 bp upstream of the translational start site of the *prnA* gene. A single *cep* box sequence could also be identified in the *prnABCD* promoter regions of *B. pyrrocinia* (GenBank AF161186), *B. cenocepacia* MCO-3 (GenBank NC_010512.1) and *B. ubonensis* Bu (GenBank NZ_ABBE01000764), whereas two and three sequences were found to be present in the promoter regions of *B. cenocepacia* PC184 (GenBank CH482379) and two *B. ambifaria* strains (MC40-6 and AMMD) respectively. In most cases the identified *cep* boxes partially overlap with the −35 regions of putative promoter sequences. Although the exact role of these putative *cep* boxes in the regulation of prn synthesis in the various strains remains to be elucidated it, together with the results of our QS-quenching experiments, supports the idea that CepR-dependent transcriptional control of *prnABCD* expression is highly conserved within the Bcc.

The multi-alignment also revealed another interesting finding. Each of the two *cep* boxes identified upstream of the *prnABCD* operon of *B. cenocepacia* PC184 carries a single point mutation: a C-to-A mutation at position 3 in the first and an A-to-C mutation at position 17 in the second *cep* box (Fig. S5). As these positions are critical for *cep* box function, it may explain why this strain does not produce prn (Table 3; Fig. S2).

Interestingly, no *cep* box could be identified upstream of the *prnABCD* operon in *B. thailandensis* and *B. oklahomensis*. In *B. pseudomallei* a sequence with very modest overall homology to other *cep* boxes could be identified (Figs S5 and S6). However, no obvious promoter consensus sequences were found in the vicinity of this putative CepR binding site. These data suggest that prn production in strains of the *B. pseudomallei* group, which yet remains to be demonstrated, may not be AHL-dependent.

It is interesting to note that although both the *cepI* and *cepR* mutant of *B. lata* 383 were unable to suppress growth of *R. solani* they still exhibited antifungal activity against the plant pathogen *P. ultimum* (Table 4). In accordance with this result we observed that the prn-negative strain *B. lata* 383-P, in which the *prnA* gene has been inactivated, was still capable of inhibiting growth of *P. ultimum* but not of *R. solani*. This indicates that prn plays a key role in controlling *R. solani* and confirms previous reports demonstrating that this fungus is particularly sensitive for prn, whereas *P. ultimum* is resistant (Homma *et al*., 1989; Hill *et al*., 1994; Hwang *et al*., 2002). The fact that *B. lata* 383-P still suppresses growth of *P. ultimum* suggests that this strain produces another antifungal factor, whose expression is independent of the CepI/CepR QS system. Additional work will be required to identify the structure of this antifungal agent.

In this study we have shown that many *Burkholderia* strains belonging to different species of the Bcc produce prn in an AHL-dependent manner. However, it is important to note that the *prnABCD* operon is only present in some of the strains exhibiting antifungal activity. Although the antifungal metabolites produced by strains not synthesizing prn remain to be identified, we could, by employing a QS-quenching approach, demonstrate that their production is in most cases AHL-regulated. This suggests that QS-dependent regulation of the synthesis of antifungal agents is a widespread mechanism within the genus

Burkholderia, a knowledge that may be of great value for the identification of the responsible antifungal compounds by comparative metabolomics of culture supernatants.

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown aerobically in modified Luria–Bertani (LB) medium containing 4 g of NaCl l^{-1} instead of 10 g of NaCl l^{-1} at 30°C (*Pseudomonas putida*) or 37°C (*Escherichia, Burkholderia*). Solid media contained 15 g of agar l^{-1} (Conda, Madrid, Spain). For selection of *Burkholderia* strains *Pseudomonas* isolation agar (PIA, Becton Dickinson, Sparks, USA) was used. Antibiotics were added as required at final concentrations of 20 μg ml^{-1} of tetracycline, 20 μg ml^{-1} of gentamicin, 50 μg ml^{-1} of kanamycin and 20 μg ml^{-1} chloramphenicol. Trimethoprim was used at 50 μg ml^{-1} for *Escherichia coli* XL1-Blue and at 100 μg ml^{-1} for Bcc strains. For supplementation of 383-I or H111-I 200 nM C8-HSL (Fluka, Buchs, Switzerland) were added to the media. Antifungal activity was scored after 4 days of cocultivation on King's medium B (KMB, King *et al*., 1954), Malt agar (15 g l^{-1} Becton Dickinson Malt extract agar, Sparks, USA and 12 g l^{-1} agar), Potato dextrose agar (Becton Dickinson, Sparks, USA) or Waksman medium at 22°C in the dark (Berg *et al*., 2002). For identification of prn by TLC or GC-MS, bacterial strains were grown 2 days at 30°C in the dark on LB medium. Growth of liquid cultures was monitored spectrophotometrically by an Ultrospec 3100 Pro spectrophotometer (Biochrom, Cambridge, England) by measurement of optical density at 600 nm.

DNA manipulations and plasmids

Cloning, restriction enzyme analysis, agarose gel electrophoresis, sequencing and transformation of *E. coli* were performed essentially as described by Sambrook and colleagues (1989). PCR was performed using the TaKaRa rTaqDNA polymerase (TaKaRa Shuzo, Shiga, Japan). Plasmid DNA was isolated with the QIAprep Spin Miniprep kit and chromosomal DNA from Bcc strains 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, Hilden, Germany).

For complementation of *B. lata* 383-R the *cepR* gene of *B. cenocepacia* H111 was isolated as an 873 bp KpnI fragment of plasmid pBAH27 (Huber *et al*., 2001) and cloned into the broad-host-range plasmid pBBR1MCS cut with the same enzyme. In the resulting plasmid, pBBR-cepR, the *cepR* gene is transcribed from the *lacZ* promoter of the plasmid.

A transcriptional fusion of the *prnABCD* promoter of *B. lata* 383 with *lacZ* was constructed as follows: a 311 bp XhoI-HindIII fragment of the promoter region was amplified with the primer pair Pprn1-XhoI-f (5′-GACCTCGAGCGTATATTGGGCGTGCAA-3′) and Pprn1-HindIII-r (5′-GACAAGCTTACCGCCCACGATGACGAT-3′) (the introduced XhoI and HindIII restrictions sites are underlined). The XhoI and HindIII digested DNA fragment was then inserted into the compatible sites of the promoter-probe vector pSU11 (S. Uehlinger, unpublished), yielding plasmid pRN1.

To assess the role of the cep box we constructed a set of transcriptional PprnABCD–lacZ fusions.

Using primers that were completely or partially complementary to the *cep* box upstream of *prnA* we introduced point mutations within the *prnABCD* promoter. Primer pair Pprn2-XhoI-f (5′-GACCTCGAGCCTGTCAAATTTATCAGTTTAA-3′) and Pprn2-HindIII-r (5′-GACAAGCTTGGGATCGCCTCGGACTCG-3) amplified the wild-type fragment without any sequence alteration. The use of primers PprnC3A-XhoI-f (5′-GACCTCGAGC**A**TGTCAAATTTATCAGTTTAA-3′), and PprnA17C-XhoI-f (5′-GACCTCGAGCCTGTCAAATTTATC**C**GTTTAA-3′) in combination with Pprn2-HindIII-r introduced a C3 to A3 and A17 to C17 nucleotide substitution (bold) within the *cep* box respectively. A *prnABCD* promoter fragment lacking the *cep* box was amplified using the primer pair PprnΔ-XhoI-f (5′-GACCTCGAGGTTTAATTTAAGTAATCGCCGACC-3′) and PprnΔ-HindIII-r (5′-GACAAGCTTGCAAATTCGGGATGGTCGCC-3′). The introduced restriction sites (underlined) were used to clone the promoter fragments site-directed into the vector pSU11, yielding pRN2, pRNC3A, pRNA17C and pRNΔ. The constructs were verified by sequencing.

Conjugative plasmid transfer

Plasmids were delivered to *B. lata* 383, its mutants and two further Bcc strains by triparental mating as described by De Lorenzo and 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 15 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 PIA medium containing antibiotics for counter selection of donor, helper and untransformed recipient cells.

PCR screen for the presence of prnD *in* Burkholderia *strains*

To screen for the presence of the *prnABCD* operon in various *Burkholderia* strains, a PCR assay employing the primer pair PRND1 (5′-GGGGCGGGCCGTGGTGATGGA-3′) and PRND2 (5′-YCCCGCSGCCTGYCTGGTCTG-3′) as described by De Souza and Raaijmakers (2003) was employed.

Southern blot hybridization

A screen of Bcc strains for *prn*D was performed with an array containing genomic DNA (Baldwin *et al*., 2004) from 228 isolates of *Burkholderia* species (including type and refer-

ence strains) and other closely related genera (see Table S1 for all species and strains present on the DNA arrays). Identity of the Bcc species represented on the genomic DNA array was determined by *recA* gene analysis and multilocus sequence typing (Baldwin *et al*., 2005; Mahenthiralingam *et al*., 2008). Hybridizations were performed according to standard procedures. High-stringent conditions comprised prehybridization for 4 h at 68°C, hybridization for 12 h at 68°C, washing of membranes twice for 5 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) – 0.15% SDS at room temperature and twice for 30 min with 0.5× SSC-0.1% SDS at 68°C. Prn probes were generated by random primed labelling of amplified *prnD* fragments with digoxigenin dUTP after PCR using the DIG labelling mix (Roche, Mannheim, Germany). The 786 bp fragment amplified from *B. lata* 383 DNA was used to generate the prn probe. Hybridized probes were immunodetected according to the protocols provided by the supplier.

Construction of B. lata *383* cepI*,* cepR *and* prnA *mutant strains*

For the construction of a *B. lata* 383 *cepI* mutant, two DNA fragments were PCR-amplified: the *dhfr* gene, which confers resistance to trimethoprim, from plasmid pMLBAD (Lefebre and Valvano, 2002) using the primer pair Dhfr-f (5′-AGCGGATCCGGTCTG ACGCTCAGTGGAACG-3′) and Dhfr-r (5′-ACGGGATCCGCTTAGGCCACACGTTCAAG-3′) (introduced BamHI restriction sites are underlined) and an internal DNA fragment of *cepI* of *B. lata* 383 using the primer pair cepI-ko-f (5′-CGAAAGCTTCGAGCGCGACCAGTTCGA TCGCGA-3′) and cepI-ko-r (5′-ACGAAGCTTCCGTCCAC CTGCTTCGGCGG-3′) (introduced HindIII restriction sites are underlined). The 647 bp BamHI fragment and the 390 bp HindIII DNA fragment were successively cloned into the vector pEX18Gm, which was cut with the respective enzymes, and transformed into *E. coli* XL1-Blue. The final construct, designated pEX-ko-cepI, was transferred to *B. lata* 383 via triparental mating and integrants were selected on PIA medium containing trimethoprim. One mutant, which was designated *B. lata* 383-I, was chosen and the correct genetic structure of the strain was verified by Southern blot analysis, PCR and sequencing.

Construction of a defined *cepR* and *prnA* mutant was performed essentially as described for the *cepI* mutant, except that a 324 bp HindIII DNA fragment within c*epR* and a 406 bp HindIII DNA fragment within *prnA* were amplified using the primers cepR-ko-f (ACGAAGCTTACTGCTGTTACGGCAT TCGCGTCCCGCTGC) and cepR-ko-r (ACGAAGCTTTGT TGATCTCGGCCGGCGTCAGCCGGTCGG), and prnA-ko-f (5′-ACGAAGCTTGCGGGAGTGGATGCCCCAGGTGAAC GGCGC-3′) and prnA-ko-r (5′-ACGAAGCTTGCTGGAG ATGAAGCCACGCTCGTTCAGGCG-3′) respectively, and inserted into pEX-ko. The genetic structures of the two mutants generated, 383-R and 383-P, were verified by Southern blot analysis, PCR and sequencing.

Detection and quantitative analysis of AHLs

Production of AHLs was investigated by the aid of the biosensor *P. putida* F117(pAS-C8), which is most sensitive for C8-HSL (Steidle *et al*., 2001). For quantification of AHLs 5 µl of filter-sterilized culture supernatants from cultures grown to an OD_{600} of 5 was added to 45 µl of distilled water and 50 µl of an exponentially growing culture of *P. putida* F117(pAS-C8) in the wells of a FluoroNunc Polysorp microtiter dish (Nunc, Wiesbaden, Germany). After 12 h of incubation at 30°C expression of the *gfp* reporter gene was measured with a Lambda Fluoro 320 Plus reader (Bio-Tek Instruments, Winooski, VT). Experiments were performed twice with four technical repicates each.

Phenotypic characterization of Burkholderia *strains*

Siderophore activity was measured by CAS assays (Schwyn and Neilands, 1987). On CAS agar, siderophores remove iron from the CAS dye complex, resulting in a blue-to-orange colour change around the colonies, which was recorded after 2 days of incubation at 37°C. Proteolytic activity was determined by streaking strains on LB agar supplemented with 2% skim milk. The plates were incubated for 2 days at 37°C and examined for clearing zones around the colonies.

Measurement of β-galactosidase activity

Samples were taken along the growth curves and cells were permeabilized by toluene. Determination of β-galactosidase activity was performed as described by Miller (1972).

Antifungal activity in vitro

Antagonistic activities of bacterial strains against *P. ultimum* and *R. solani* were assayed either on KMB, Potato dextrose agar or Malt agar by spotting 10 µl stationary-phase cultures of selected strains at three positions on the plate. Following incubation for one night at 37°C a 5-mm-diameter fungal inoculum, which was cut from a fungal culture plate, was placed onto the centre of the plate. Plates were incubated at 22°C in the dark and inhibition zones were recorded after 4 days. All experiments were repeated at least twice. The fungi (*R. solani* Kühn AG and *P. timum* 67-1) were obtained from the culture collection of the Phytopathology group of the Institute of Plant Sciences (Federal Institute of Technology, Zurich, Switzerland).

Extraction and identification of prn by GC-MS and TLC

For detection of prn by TLC 1 g bacterial cells were extracted with 20 ml acetone (Fluka). After rotary evaporation at 45°C cells were extracted by shaking in 10 ml chloroform (Fluka). As water increases the photosensitivity of prn only pure solvents were used (Sako *et al*., 2002). The dried extracts were dissolved in 1 ml acetone. A volume of 100 µl extract and 500 ng HPLC-graded prn (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) as a standard were applied to precoated silica gel plates (20 cm × 20 cm, silica gel 60 F254, Merck, Germany) and separated by using chloroform-acetone (9:1 v/v). The corresponding prn spots were detected by overlaying the TLC plates with Waksman agar seeded with *R. solani*. Antagonistic activity of extracts was registered after 4 days

by measuring the inhibition zone. The TLC analysis was conducted at least twice for every strain.

The GC-MS analysis was used to analyse the presence and amount of prn in wild type, QS mutant and transconjugant strains AiiA$^+$. To this end, 1–2 g of bacterial cells was sonicated for 15 min in 5 ml acetonitril (anhydrous, Fluka). After filtration the filtrate was evaporated to dryness at 40°C and 30 mbar. The residue was resuspended in half the mg cell amount acetonitril (μl) and centrifuged for 10 min at 500 *g*. Three microlitres of 3,5-dichlorophenol (10 μg ml^{-1}) was added to 300 μl supernatant as an internal standard. Analysis of the extracts was conducted using a combined GC-MS (Fison Instruments, GC 8000 Top, MD 800). Using a DB 1301 capillary column (30 m, 0.32 mm diameter, film thickness 0.25 μm; J and W Scientific) under following conditions. Time programme: 150°C for 1 min; increase temperature at a rate of 15°C min^{-1} until 250°C; isocratic for 10 min at 250°C (total time: 17.67 min); injector temperature: 250°C; injection volume: 1 μl; carrier gas: helium; source temperature: 200°C; pressure 50 kPa; ionization: EI+ ionization energy: 70 eV; detector voltage: 400 V; full scan modus, fragment ion $m/z = 166$; retention times: DCP 3.90 min, PYR 10.60 min.

To determine the activity of extracts a bioassay against *R. solani* was used. To this end, sterile filter discs soaked with 50 μl extract were placed at three positions onto Malt and KMB agar plates with a pad of *R. solani* in the centre. After 6 days of incubation at 20°C in the dark inhibition zones around the discs were measured.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. TLC analysis of prn extracted of cultures of *B. lata* 383 (A), *B. ambifaria* LMG 19467 (C), *B. pyrrocinia* LMG 21823 (E) and their respective AiiA-expressing derivates (B, D, F). Overlaying with Waksman agar seeded with *R. solani* was used to detect antifungal activity. The 500 ng HPLC-graded prn (PRN) was included as reference compound.

Fig. S2. GC-MS analysis of acetonitril extracts from *B. ambifaria* LMG 19467 (A), LMG 19467 AiiA$^+$ (B), *B. pyrrocinia* LMG 21823 (C), LMG 21823 AiiA$^+$ (D), *B. ubonensis* LMG 20358 (E) and *B. cenocepacia* PC184 (F). The fragment ion $m/z = 166$ was used to identify pyrrolnitrin (PRN) and dichlorophenole (DCP). The prn standard showed a retention time of 10.6 min.

Fig. S3. Sequence alignment of the *prnABCD* promoter regions of *B. cepacia* (AF161183) and *B. lata* 383. Sequences on green background represent the 20 bp *cep* box sequence. The translation start ATG is marked in grey.

Fig. S4. Multi-aligned sequences of the *prnABCD* promoter regions of *B. pyrrocinia* (AF161186), *B. ambifaria* AMMD, *B. ambifaria* MC40-6, *B. cenocepacia* PC184, *B. cenocepacia* MCO-3 and *B. ubonensis* Bu. Sequences on green background represent a possible first, on violet a possible second, on blue a possible third and on orange a possibly non-functional *cep* box sequence. The translation start ATG is marked in grey.

Fig. S5. Multi-aligned *cep* box sequences. Red nucleotides represent the minimal consensus defined for operators of QS-regulated genes in *P. aeruginosa* (Whiteley and Greenberg, 2001). Numbers behind strains represent the possible first (1), second (2) and third (3) *cep* box sequence upstream of *prnA.*

Fig. S6. Alignment of *prnABCD* promoter sequences of *B. pseudomallei* 1655, *B. thailandensis* TXDOH and *B. oklahomensis* EO147. The sequence on green bachground represents a putative *cep* box sequence upstream of *prnA.* The translation start ATG is marked in grey.

Table S1. All 228 strains present on the macroarrays. Genomic DNA of various strains of the Bcc, other *Burkholderia* species and representatives of other genera were spotted on a membrane.

Table S2. Strains on the macrorray that gave a positive hybridization signal in Southern blots using the *prnD* gene as a probe.

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