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The CepIR quorum-sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections

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The *cepIR* genes encode an *N*-acyl homoserine lactone (AHL)-dependent quorum-sensing system consisting of an AHL synthase that directs the synthesis of *N*-octanoyl-L-homoserine lactone (OHL) and *N*-hexanoyl-L-homoserine lactone and a transcriptional regulator. The virulence of *cepIR* mutants was examined in two animal models. Rats were infected with agar beads containing *Burkholderia cenocepacia* K56-2, K56-I2 (*cepI*::Tp^r) or K56-R2 (*cepR*::Tn*5*-OT182). At 10 days post-infection, the extent of lung histopathological changes was significantly lower in lungs infected with K56-I2 or K56-R2 compared to the parent strain. Intranasal infections were performed in *Cftr*$^{(-/-)}$ mice and their wild-type siblings. K56-2 was more virulent in both groups of mice. K56-I2 was the least virulent strain and was not invasive in the *Cftr*$^{(-/-)}$ mice. OHL was readily detected in lung homogenates from *Cftr*$^{(-/-)}$ mice infected with K56-2 but was only detected at levels slightly above background in a few mice infected with K56-I2. Lung homogenates from mice infected with K56-2 had significantly higher levels of the inflammatory mediators murine macrophage inflammatory protein-2, *KC/N51*, interleukin-1β and interleukin-6 than those from K56-I2-infected animals. These studies indicate that a functional CepIR quorum-sensing system contributes to the severity of *B. cenocepacia* infections. A zinc metalloprotease gene (*zmpA*) was shown to be regulated by CepR and may be one of the factors that accounts for the difference in virulence between the *cepI* mutant and the parent strain.

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INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane regulator (*cftr*) that encodes a chloride channel. CF is the most common lethal inherited disease in Caucasians. Absence or impaired function of CFTR leads to alterations in the lung environment including altered fluid and ion fluxes in respiratory epithelial cells, excessive mucous production in the airways, reduced mucociliary clearance and increased colonization of the lung by opportunistic pathogens [for reviews, see Rajan & Saiman (2002), Ratjen & Doring (2003), Schwiebert *et al.* (1998) and Tatterson *et al.* (2001)].

Although the major pathogen responsible for morbidity and mortality in CF patients is *Pseudomonas aeruginosa* (Govan & Deretic, 1996; Govan *et al.*, 1996; Mahenthiralingam *et al.*, 2002; Rajan & Saiman, 2002; Tatterson *et al.*, 2001), in the past 20 years *Burkholderia cepacia* has emerged as an important pulmonary pathogen in this patient population (Mahenthiralingam *et al.*, 2002; Mohr *et al.*, 2001; Speert *et al.*, 2002). Chronic colonization with *B. cepacia* is of a great concern in the CF community due to patient-to-patient transmissibility and inherent multi-drug resistance that makes eradication of *B. cepacia* almost impossible. Moreover, colonization with *B. cepacia* has been correlated with a poor clinical outcome sometimes resulting in death. The complexity of *B. cepacia* infections in CF patients has also increased due to the determination that the group of organisms that infect CF patients is not a single species but rather a group of at least nine closely related species or genomovars that are now referred to as the *B. cepacia* complex (Coenye *et al.*, 2001; Vandamme *et al.*, 2002). *Burkholderia cenocepacia* (Vandamme *et al.*, 2003), formerly *B. cepacia* genomovar III, is the most common species of the *B. cepacia* complex that has been reported in CF infections. The majority of strains identified as epidemic or

Abbreviations: AHL, *N*-acyl homoserine lactone; CF, cystic fibrosis; *cftr*, cystic fibrosis transmembrane regulator; IL-1β, interleukin-1β; IL-6, interleukin-6; MIP-2, murine macrophage inflammatory protein-2; KC, *KC/N51*; OHL, *N*-octanoyl-homoserine lactone; p.i., post-infection; TNFα, tumour necrosis factor α.

transmissible strains belong to this genomovar (LiPuma *et al.*, 2001; Mahenthiralingam *et al.*, 2002).

Several Gram-negative bacteria employ quorum-sensing-mediated cell-signalling systems that regulate various virulence factors in response to cell density (de Kievit & Iglewski, 2000; Williams *et al.*, 2000). The LuxIR family of quorum-sensing systems is composed of *N*-acyl homoserine lactone (AHL) synthases and transcriptional regulators that may either activate or repress target genes. The AHLs are freely diffusible and bind to transcriptional regulators when they reach a sufficient threshold concentration generally correlating with cell density. The *cepIR* quorum-sensing system, originally identified in *B. cenocepacia*, is widely distributed throughout the *B. cepacia* complex (Gotschlich *et al.*, 2001; Lewenza *et al.*, 1999; Lutter *et al.*, 2001). CepI is an AHL synthase that directs the synthesis of *N*-octanoyl-homoserine lactone (OHL) and minor amounts of *N*-hexanoyl-homoserine lactone. CepR encodes a transcriptional regulator that has both positive and negative regulatory properties. The CepIR quorum-sensing system has been shown to regulate expression of extracellular proteases, swarming motility and biofilm production (Huber *et al.*, 2001; Lewenza *et al.*, 1999, 2002). CepR has been shown to negatively regulate its own expression as well as the biosynthesis of the siderophore ornibactin via the *pvdA* gene (Lewenza & Sokol, 2001). Riedel *et al.* (2003) using a proteomics approach demonstrated that a number of proteins are differentially expressed between *B. cenocepacia* strain H111 and a *cepI* mutant. N-terminal amino acid sequence analysis identified a few proteins up-regulated in the wild-type strain including a putative superoxide dismutase, a peroxidase and a possible ABC transporter system. In *B. cepacia* genomovar I, CepR has been shown to positively regulate a secreted polygalacturonase involved in onion-rot pathogenicity and to have a negative effect on the expression of the stationary-phase sigma factor, *rpoS* (Aguilar *et al.*, 2003).

A *B. cepacia* genomovar I *cepI* mutant was less virulent in an onion-rot model as evident by attenuated tissue maceration compared to the wild-type. Complementation of the *cepI* mutant with *cepIR in trans* enhanced the virulence above that of the wild-type level indicating a role for the *cepIR* quorum-sensing mechanism in onion-rot pathogenicity (Aguilar *et al.*, 2003). A functional *cepIR* system was also shown to be required for efficient killing of the nematode *Caenorhabditis elegans* by *B. cenocepacia* H111 (Kothe *et al.*, 2003). Nematode killing appears to involve the production of a diffusible extracellular toxin, which has not yet been identified in the *B. cepacia* complex.

Several animal models have been developed to investigate the virulence of the *B. cepacia* complex in respiratory infections. These include agar bead models in rats and mice (Cieri *et al.*, 2002; Sokol *et al.*, 1999, 2000) and agar beads or intranasal infection models in CF mice (Davidson *et al.*, 1995; Sajjan *et al.*, 2001). The chronic agar bead model (Cash *et al.*, 1979) originally developed to mimic *P. aeruginosa* respiratory infections in CF patients has been used to demonstrate that iron acquisition via the siderophore ornibactin is necessary for persistence of *B. cenocepacia* chronic infections (Sokol *et al.*, 1999, 2000). Recently, we have used this model to demonstrate that a zinc metalloprotease contributes to the virulence of some strains of *B. cenocepacia* (Corbett *et al.*, 2003). A modified agar bead model in mice has been used to compare invasiveness of members of the *B. cepacia* complex during acute infections (Cieri *et al.*, 2002) and to demonstrate that Type III secretion is important for virulence (Tomich *et al.*, 2003). CFTR-deficient (knockout) mice have also been used to study the pathogenesis of lung disease in experimental infections due to *B. cepacia*. Chronic models of *B. cepacia* infection have been developed in CFTR-deficient mice by repeated intranasal inoculation or repeated exposure to aerosolized organisms (Davidson *et al.*, 1995; Sajjan *et al.*, 2001). Recently, a CFTR-deficient mouse model has been developed that does not require repeated inoculation of *B. cenocepacia*. Short-term infections can be established in 10- to 12-week-old mice following a single intranasal inoculation (Sajjan *et al.*, 2002). In the present study, we report that the CepIR quorum-sensing system contributes to the pathogenesis of *B. cenocepacia* strain K56-2 respiratory infections, using two different animal models, the short-term intranasal colonization mouse model and the chronic agar bead infection model in rats.

METHODS

Strains, growth conditions and DNA manipulations. The bacterial strains and plasmids used in this study are listed in Table 1. DNA manipulations were performed using standard techniques as described by Sambrook *et al.* (1989). *B. cenocepacia* cultures were routinely grown at 37 °C in Luria–Bertani broth (LB) (Invitrogen Life Technologies) or on LB solidified with 1·5 % agar unless otherwise noted. Medium was supplemented with 200 μg tetracycline ml^{-1} or 100 μg trimethoprim ml^{-1} to select for the introduction of plasmids into *B. cenocepacia*. OHL was extracted from cultures grown in trypticase soy broth (TSB; Difco Laboratories). Cultures were grown overnight in TSBD-C medium (Ohman *et al.*, 1980) for infections using the agar bead model and in TSB medium for mouse infections. Protease activity was monitored using D-BHI-milk medium (Sokol *et al.*, 1979).

Agrobacterium tumefaciens strains were routinely grown at 30 °C in LB or on LB solidified with 1·5 % agar. Medium was supplemented with 25 μg kanamycin ml^{-1}, 4·5 μg tetracycline ml^{-1}, 50 μg spectinomycin ml^{-1} or 10 μg rifampicin ml^{-1} as required. When necessary, liquid medium was supplemented with 3 μg tetracycline ml^{-1}. For preparation of *A. tumefaciens* electrocompetent cells, cultures were grown in MG/L broth (Chilton *et al.*, 1974) and prepared as described by Cangelosi *et al.* (1991). Plasmids were introduced into *A. tumefaciens* as described previously (Cangelosi *et al.*, 1991).

Construction of a *traI–luxCDABE* reporter. To construct pMV26, primers traI pro2 (5′-TTCTGGTGTTGGTATTGGTC-3′) and traIproIn2 (5′-AGTAGTTCGCCAGTTAATAG-3′) were used to amplify −143 to +68 of the *traI* promoter region from pCF372, a *traI–lacZ* fusion (Fuqua & Winans, 1996). The Psa origin of replication was also amplified from pCF372 using primers Psa-PacF

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strain		
B. cenocepacia		
K56-2	CF respiratory isolate, genomovar III, BCESM$^+$, CblA$^+$	Mahenthiralingam *et al.* (2000)
K56-R2	*cepR*::Tn*5*-OT182 derivative of K56-2, Tc^r	Lewenza *et al.* (1999)
K56-I2	*cepI*::*tmp* derivative of K56-2, Tp^r	Lewenza *et al.* (1999)
A. tumefaciens		
A136	Ti plasmidless host, Rf^r	Watson *et al.* (1975)
Plasmid		
pCF218	IncP, *traR*, Tc^r	Zhu *et al.* (1998)
pCF372	*traI–lacZ*, Sp^r	Fuqua & Winans (1996)
pCR2.1TOPO	Cloning vector for PCR products, Ap^r, Km^r	Invitrogen
pCR*traI*	pCR2.1TOPO with 269 bp fragment from pCF372 containing *traI* promoter, Ap^r, Km^r	This study
pCRPsa	pCR2.1TOPO with 2·4 kb fragment from pCF372 containing Psa origin of replication, Ap^r, Km^r	This study
pCS26-*Pac*	Promoterless *luxCDABE* vector, Km^r	Bjarnason *et al.* (2003)
pCS26-Psa	pCS26-Pac with the pSC101 origin of replication replaced by the 2·4 kb *Pac*I fragment from pCRPsa, Km^r	This study
pMV26	pCS26-Psa with a 351 bp *Bam*HI–*Xho*I fragment from pCR*traI* containing the *traI* promoter, Km^r	This study
pUCP26	Broad-host-range vector, IncP pRO1600 *ori*, Tc^r	West *et al.* (1994)
pUCP28T	Broad-host-range vector, IncP OriT, pRO1600 *ori*, Tp^r	Schweizer *et al.* (1996)
pZ1918G	Source of *lacZ* reporter, Ap^r, Gm^r	H. P. Schweizer, Colorado State University
pSG206	pUCP26 containing *zmpA* on a 2·6 kb *Pst*I fragment with a *Sma*I fragment from pZ1918G inserted in the *Stu*I site of *zmpA*, Tc^r	This study
pSG208	pUCP28T containing *zmpA* on a 2·6 kb *Pst*I fragment with a *Sma*I fragment from pZ1918G inserted in the *Stu*I site of *zmpA*, Tp^r	This study

(5′-CGCTCATAGGGCTTAATTAACCAACGTTTTA-3′) and Psa-PacR (5′-CATACTACAATTAATTAACAGAGCCATC-3′), which incorporate *Pac*I restriction sites. The pSC101 origin of replication in the promoterless *luxCDABE* vector pCS26-*Pac* (Bjarnason *et al.*, 2003) was replaced with a 2·41 kb Psa origin *Pac*I fragment to allow for replication in *A. tumefaciens*, forming pCS26-Psa. A 351 bp *Bam*HI–*Xho*I pCRtraI fragment containing the *traI* promoter was then cloned into pCS26-Psa to form pMV26.

Agar bead model. Groups of 20 male Sprague–Dawley rats (150–170 g; Charles River Canada) were tracheostomized under anaesthesia and inoculated with approximately $1{\cdot}5 \times 10^5$ c.f.u. of K56-2 or the mutant strains embedded in agar beads as described previously (Cash *et al.*, 1979). On days 3 and 10 post-infection (p.i.), the lungs from five animals per group were removed aseptically and homogenized (Polytron Homogenizer; Brinkman Instruments) in 3 ml of PBS, serially diluted and plated onto trypticase soy agar and incubated at 37 °C to determine the number of bacteria present in the lung. Lung homogenates from K56-R2- or K56-I2-infected animals were also plated onto trypticase soy agar containing tetracycline (300 μg ml^{-1}) or trimethoprim (100 μg ml^{-1}), respectively, to confirm that the mutations were stable throughout the course of the infections. In a representative experiment, approximately 50 colonies from each animal were also tested for protease production on skim milk agar to confirm that the *cepI* or *cepR* phenotype was maintained during the infection. Lungs from five additional animals per group were examined for quantitative histopathological changes as described previously (Sokol & Woods, 1984) with the following modifications. The lung sections were scanned using an Epson 1650 scanner, and areas of inflammation were digitized with SCION IMAGE software (http://www.scioncorp.com) and reported as the percentage of lung inflammation.

Intranasal infection model. Liquid-fed *Cftr*$^{(-/-)}$ knockout mice (Kent *et al.*, 1996) and their littermate wild-type *Cftr*$^{(+/+)}$ controls, age 10–12 weeks, were infected with approximately 1×10^8 c.f.u. of K56-2, K56-I2 or K56-R2 in 40 μl PBS. The inoculum was instilled drop-wise intranasally and allowed to be aspirated into the lungs as described previously (Sajjan *et al.*, 2001). Mice were observed for 3 days. At 72 h p.i., lungs and spleens were harvested, weighed and homogenized as described previously to determine the number of bacteria present (Sajjan *et al.*, 2001). If mice died prior to the 72 h, the lungs and spleens were recovered, homogenized and plated. One millilitre of lung homogenate from each mouse was immediately mixed with complete protease inhibitor cocktail (Roche Diagnostics), centrifuged and the supernatant removed and stored at −70 °C for cytokine assays. *Cftr*$^{(-/-)}$ mice were also infected with approximately 5×10^6 c.f.u. of K56-2 or K56-I2. Mice were observed for 5 days, after which lungs and spleens were harvested as above.

Lungs were graded for severity of inflammation by determining the ratio of total wet weight of lungs to body weight. A ratio of less than 0·02 was associated with normal or mild inflammation, 0·021–0·03 indicated moderate inflammation and greater than 0·03 (range 0·031–0·05) indicated severe inflammation. Lungs graded as severe had white patches and were more friable indicating consolidation of the lung upon gross pathology examination. Normal lungs appeared pink with more elasticity. Lungs with small patches of consolidation were classified as mild or moderate. The gross pathology observations were found to correlate with the lung-to-body weight ratios.

Cytokine assays. Murine tumour necrosis factor α (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), interferon γ (IFN-γ) (Biosource International), *KC/N51* (KC) and macrophage inflammatory protein-2 (MIP-2) (R&D Systems) were measured by ELISA. Lung homogenates were diluted 1 : 10, 1 : 50 or 1 : 100 in diluent buffer and assayed in duplicate according to the manufacturers' instructions. The limits of detection for the cytokine assays were less than 2·0 pg ml^{-1} for KC, less than 1·5 pg ml^{-1} for MIP-2, less than 7 pg ml^{-1} for IL-1β, less than 1 pg ml^{-1} for IFN-γ and less than 3 pg ml^{-1} for IL-6 and TNFα.

OHL extraction and purification. AHLs were extracted from 20 h cultures of K56-I2(pSL225), which carries *cepI* on a high-copy plasmid, with equal volumes of acidified ethyl acetate and dried as described previously (Lewenza *et al.*, 1999). To purify OHL, the dried extract from a 500 ml culture was resuspended in 2 ml of deionized water and subjected to reversed-phase FPLC using a Sephasil Peptide C18 12 μm ST 4·6/250 column fitted to the AKTA Explorer 900 FPLC system (Amersham Pharmacia Biotech). Analysis of the resulting chromatogram was performed using the UNICORN version 3.12.02 program (Amersham Pharmacia Biotech). The AHLs were separated with a 0–100 % acetonitrile gradient at a flow rate of 1·0 ml min^{-1}, and the eluent was analysed using an *Agrobacterium* TLC assay (Lutter *et al.*, 2001; Shaw *et al.*, 1997) to identify fractions containing OHL and *N*-hexanoyl-homoserine lactone. The concentration of OHL was estimated by comparison with an OHL standard (Fluka) using the same overlay assay. Fractions containing OHL were pooled and stored at −20 °C.

Detection of OHL in lung tissue. OHL was extracted from mouse or rat lung homogenates as described with modifications (Erickson *et al.*, 2002). Two-hundred microlitres of mouse lung or 2 ml of rat lung homogenate was extracted three times with dichloromethane (2 : 1, v/v). Samples were centrifuged (2000 r.p.m., 10 min) and the organic layer was removed and pooled. Solvent was removed by evaporation and the residue was resuspended in 10 μl acetonitrile. An overnight culture of *A. tumefaciens* A136 (pCF218)(pMV26) was used for detection of OHL. Assays were performed in 96-well microtitre plates (Costar; Corning). Ten microlitres of extract, 10 μl of the *A. tumefaciens* A136(pCF218)(pMV26) culture and 80 μl LB were mixed, incubated at 30 °C with shaking and luminescence was measured at selected time intervals using a Wallac Trilux luminescence counter (Perkin-Elmer Life Sciences). Lung homogenates from uninfected animals were used to determine background levels of luminescence. Synthetic OHL (Fluka) was used to prepare a standard curve.

Construction of a *zmpA*::*lacZ* fusion. The *zmpA*::*lacZ* fusion was constructed by inserting the *Sma*I fragment of pZ1918G containing a *lacZ*–Gm^r cassette (Schweizer, 1993) into the *Stu*I site of *zmpA* contained on a 2·6 kb *Pst*I fragment cloned into pUCP28T (Schweizer *et al.*, 1996). This plasmid was designated pSG208. The same *zmpA*::*lacZ* fusion was subsequently cloned into pUCP26 (Schweizer *et al.*, 1996) and designated pSG206. Expression of the fusion was monitored by measuring β-galactosidase activity as described previously (Platt *et al.*, 1972).

RESULTS

Effects of *cepIR* mutations on virulence in a chronic respiratory infection model

Sprague–Dawley rats were infected with agar beads containing K56-2, K56-I2 or K56-R2 to establish chronic respiratory infections (Cash *et al.*, 1979; Sokol *et al.*, 1999). On days 3 and 10 p.i., lungs were removed and subjected to quantitative bacteriology and histopathological analysis. Although the number of bacteria recovered from the lungs of animals infected with the mutants compared to the parent strain was slightly lower on both day 3 and day 10 (Table 2) the difference was not significant. The numbers of bacteria in the lungs increased by at least 1·5

Table 2. Effect of *cepI* or *cepR* mutations on quantitative bacteriology in the agar bead infection model

Results are shown as the mean ± SD ($\times 10^6$), and are based on five animals per group for day 3 p.i. and four animals per group for day 10 p.i.

Day p.i.	*B. cenocepacia* strain:		
	K56-2	K56-I2	K56-R2
3	8·6 ± 8·8	7·8 ± 3·9	5·1 ± 9·0
10	7·4 ± 7·4	5·9 ± 5·3	6·1 ± 7·5

Table 3. Effect of *cepI* or *cepR* mutations on histopathology in the agar bead infection model

Results are shown as the mean percentage inflammation ± SD.

Day p.i.	*B. cenocepacia* strain:		
	K56-2	K56-I2	K56-R2
3	46·2 ± 10·5*	42·4 ± 11·6*	40·5 ± 4·3*
10	24·7 ± 1·2†	18·6 ± 2·7*‡	18·5 ± 1·9†‡

*Five animals per group.
†Four animals per group.
‡Significantly different from K56-2 ($P<0·01$, ANOVA, Dunnett multiple comparisons test).

logs by day 3 and persisted for the 10-day period. These data indicate that mutations in either *cepI* or *cepR* do not affect the ability of K56-2 to establish and maintain chronic infections in this model.

Despite the fact that there was no significant difference in the number of bacteria present in the lungs, the extent of lung histopathological changes on day 10 p.i. was significantly lower in rats infected with either K56-I2 or K56-R2 compared to the parent strain (Table 3). There were no qualitative differences in the pathological changes observed (data not shown). These data indicate that the *cepIR* system contributes to the maximum virulence of strain K56-2 probably by regulating production of extracellular virulence factors that result in increased lung injury.

Effects of *cepIR* mutations on virulence in a mouse intranasal infection model

$Cftr^{(-/-)}$ mice or their $Cftr^{(+/+)}$ littermate controls were infected intranasally with approximately 1×10^8 c.f.u. of K56-2, K56-I2 or K56-R2. Strain K56-2 was more virulent than K56-I2 and K56-R2 in the wild-type mice since both mice infected with the parent strain died by day 3 p.i. (Table 4). The lungs of these mice when examined for gross pathology changes appeared to have severe inflammation whereas only one mouse infected with K56-I2 and none of the mice infected with K56-R2 had severe lung inflammation. Bacteria were cultured from spleens of both surviving mice infected with K56-2 but from only one of the K56-I2-infected mice.

K56-2 was also more virulent than K56-I2 in the $Cftr^{(-/-)}$ mice (Table 4). Although none of these mice died during the 3-day period, two of three appeared ill and had severe lung inflammation. All three had bacteria present in the spleen. K56-R2 was also virulent in these mice, with invasion and severe lung inflammation evident in two of three mice. One of the K56-R2-infected mice that died, interestingly, had only mild lung inflammation and no bacteria recovered from the spleen. In contrast to the parent and the *cepR* mutant, infection with K56-I2 resulted in only mild or moderate lung inflammation in four of five mice, and the infection was not invasive in any of the $Cftr^{(-/-)}$ mice. There were fewer bacteria recovered from the lungs of mice infected with K56-I2 than the other strains.

The virulence of K56-2 and K56-I2 was also compared in $Cftr^{(-/-)}$ mice using a lower inoculum than in the previous experiment. Mice were infected with approximately 5×10^6 c.f.u. and observed for 5 days, since the mice tolerated this dose better than the higher inoculum. There were insufficient wild-type littermate controls available so they were not included in this experiment. K56-I2 was

Table 4. Comparison of virulence of *B. cenocepacia* K56-2, K56-I2 and K56-R2 in CF and wild-type mice infected intranasally with 1×10^8 c.f.u.

Mouse type	*B. cenocepacia* strain	No. mice per group	No. dead by day 3 p.i.	No. with severe inflammation*	C.f.u. per lung (mean ± SD)	No. with bacteria in spleen
$Cftr^{(+/+)}$	K56-2	2	2	2	$>10^8$	2
	K56-I2	3	1	1	$>10^8$	1
	K56-R2	2	0	0	$1 \times 10^5 \pm 3 \times 10^4$	0
$Cftr^{(-/-)}$	K56-2	3	0	2	$>10^9$	3
	K56-I2	5	1	1	$4 \times 10^7 \pm 3{\cdot}8 \times 10^7$	0
	K56-R2	3	2	2	$9{\cdot}8 \times 10^8 \pm 1 \times 10^9$	2

*Severe inflammation was graded based on the ratio of wet weight of lungs to body weight and by gross pathology observations of lung consolidation.

Table 5. Comparison of virulence of *B. cenocepacia* K56-2 and K56-I2 in CF mice infected intranasally with 5×10^6 c.f.u.

Strain	No. mice per group	No. dead by day 5 p.i.	No. with severe inflammation	Log c.f.u. per lung (mean ± SD)	No. with bacteria in spleen
K56-2	4	1	3	8·0 ± 0·6*	3
K56-I2	5	0	0	5·3 ± 1·3†	0

*Mouse dead on day 5 not included in mean.
†Significantly different from K56-2 ($P < 0{\cdot}02$, unpaired *t*-test).

significantly less virulent than the parent strain as evident by the approximately 3-log difference in bacteria recovered from the lungs and the degree of lung inflammation. The absence of bacteria recovered from the spleen indicated that K56-I2 was not invasive in any of the $Cftr^{(-/-)}$ mice (Table 5).

To confirm that differences in virulence in this model between K56-2 and K56-I2 were due to the absence of OHL production, lung homogenates from the infected animals were extracted with dichloromethane and analysed for OHL using a *traI–luxCDABE* reporter system in *A. tumefaciens*. This reporter system is able to detect femtomolar levels of OHL (M. B. Visser, C. E. Chambers & P. A. Sokol, unpublished observations). OHL was detected in lung homogenates from all three $Cftr^{(-/-)}$ mice infected with 1×10^8 c.f.u. of K56-2 but was not detectable in the four mice infected with K56-I2 or the one surviving mouse infected with K56-R2. The mean ± SD c.p.m. value minus the background for the K56-2-infected animals was 30 714 ± 26 067 (range = 5087–57 200) at 8 h, which was the time point with the highest activity in this assay. If all of this activity was due to OHL, the concentration would be approximately 0·0025 pM as determined from a standard curve prepared with synthetic OHL. The c.p.m. values for all the K56-I2-infected lungs were below those obtained with the uninfected control (13 573 c.p.m.) and the vector control strain (19 391 c.p.m.).

In animals infected with 1×10^6 c.f.u., OHL activity was detected in three of three animals infected with K56-2 with a mean ± SD c.p.m. value minus the background of 91 796 ± 69 497 (range = 38 532–170 409) at 15 h, which was the time of maximum activity in this assay. Some activity was detected in three of five animals infected with K56-I2, although the values obtained were much lower than in animals infected with K56-2 and ranged from 5344 to 29 102 c.p.m. (mean ± SD = 9804 ± 12 322). Due to the large standard deviation in these groups of animals the differences were not quite significant. Although the c.p.m. values appear higher in the animals infected with 5×10^6 than in animals infected with 1×10^8 c.f.u. the actual amount of OHL detected is lower. The background c.p.m. value for extracts from uninfected lung in this assay was 38 289, therefore the c.p.m. values from the K56-I2-infected lungs were not even twice the background level. The mean c.p.m. values detected are equivalent to less than 0·0012 pmol OHL.

The levels of selected cytokines were also measured in lung homogenates from the surviving mice. There were no significant differences between the amounts of KC, MIP-2, TNFα, IL-1β or IL-6 in the lungs from $Cftr^{(-/-)}$ mice infected with 1×10^8 c.f.u. of K56-2 or K56-I2 (data not shown). In the mice receiving the lower inoculum, however, the levels of KC, MIP-2, IL-1β and IL-6 were significantly higher in lungs from animals infected with K56-2 compared to K56-I2 (Table 6). There was no difference in the amount of TNFα. Interestingly, the levels of IFN-γ were higher in mice infected with K56-I2 than in mice infected with K56-2. It was not possible to compare cytokine levels in $Cftr^{(-/-)}$ and $Cftr^{(+/+)}$ mice infected with K56-2 due to the high mortality rate; however, we were able to compare cytokine levels in these mice infected with 1×10^8 c.f.u. of K56-I2. The levels of MIP-2, IL-6 and TNFα were significantly higher in lungs from $Cftr^{(-/-)}$ mice than from $Cftr^{(+/+)}$ mice (Table 7).

Effect of *cepI* or *cepR* mutations on protease expression

A zinc metalloprotease gene (*zmpA*) which encodes the previously described PSCP protease (McKevitt *et al.*, 1989)

Table 6. Cytokine levels in lungs of $Cftr^{(-/-)}$ mice infected with 5×10^6 c.f.u. of *B. cenocepacia* K56-2 or K56-I2

Results are expressed as the mean log values ± SD.

Cytokine	Cytokine level [ng (g lung tissue)$^{-1}$] in mice infected with:	
	K56-2	K56-I2
KC	1·7 ± 0·3	0·6 ± 0·5*
MIP-2	2·0 ± 0·5	0·1 ± 1·5*
IL-6	1·4 ± 0·2	0·7 ± 0·5*
IL-1β	2·2 ± 0·1	1·4 ± 0·2*
TNFα	1·0 ± 0·5	0·5 ± 0·3
IFN-γ	0·8 ± 0·1	1·3 ± 0·4*

*Significantly different from K56-2 ($P < 0{\cdot}05$, unpaired *t*-test with Welch's correction).

Table 7. Comparison of cytokine levels in $Cftr^{(-/-)}$ and $Cftr^{(+/+)}$ mice infected with 1×10^8 c.f.u. of *B. cenocepacia* K56-I2

Results are expressed as the mean log values ± SD.

Cytokine	Cytokine level [ng (g lung tissue)$^{-1}$] in:	
	$Cftr^{(-/-)}$	$Cftr^{(+/+)}$
KC	1·9 ± 0·5	1·2 ± 0·3
MIP-2	2·2 ± 0·5	1·2 ± 0·03*
IL-6	1·7 ± 0·5	0·7 ± 0·03*
IL-1β	1·9 ± 0·3	1·7 ± 0·1
TNFα	1·2 ± 0·3	0·45 ± 0·1*
IFN-γ	1·6 ± 0·8	0·7 ± 0·2

*Significantly different from $Cftr^{(-/-)}$ ($P < 0·05$, unpaired *t*-test with Welch's correction).

has recently been cloned and characterized in our laboratory (Corbett *et al.*, 2003). A K56-2 *zmpA* mutant was less persistent and caused less lung pathology than the wild-type strain in the rat agar bead infection model. Since the CepIR quorum-sensing system has previously been shown to positively regulate extracellular protease activity, the effects of *cepI* or *cepR* mutations on expression of *zmpA* in K56-2 were examined. Transcriptional fusions were constructed by inserting a promoterless *lacZ* cassette into the *zmpA* gene. Expression of the *zmpA*::*lacZ* fusion in K56-I2, K56-R2 and K56-2 was examined over 24 h of growth with and without the addition of OHL (Fig. 1). There was a significant decrease in *zmpA*::*lacZ* expression in the mutants compared to the parent strain, which was restored by the addition of OHL to the K56-I2 cultures. Addition of OHL to K56-I2(pSG206) resulted in an approximately sixfold increase in β-galactosidase activity at 18 h but did not restore expression to parental levels. The K56-R2(pSG208) culture did not respond to the addition of OHL. Interestingly, addition of OHL to K56-2(pSG208) resulted in a twofold increase in expression, suggesting that the production of OHL by the wild-type *cepI* gene did not saturate the regulatory mechanism governing the expression of *zmpA*. These experiments indicate that *zmpA* is regulated by CepIR and that differences in protease expression may be one of the factors that contribute to the differences in virulence between these mutants and the parent strain, although it is likely that a number of virulence factors are involved.

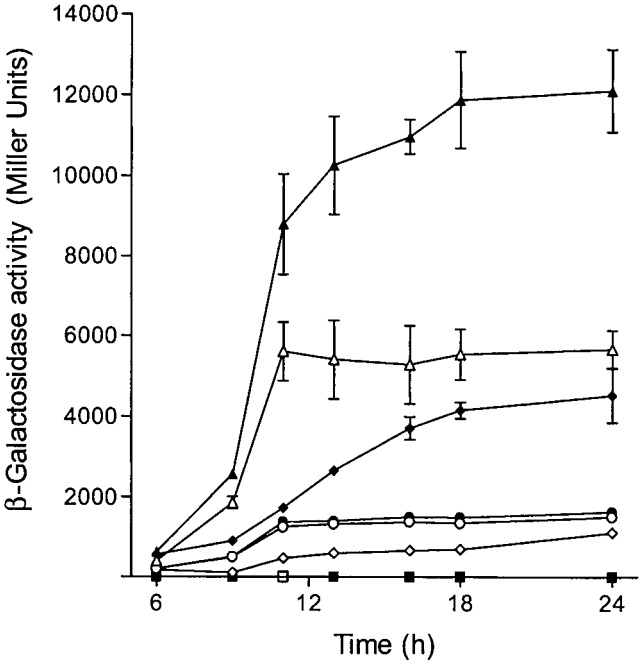

Fig. 1. Expression of *zmpA*::*lacZ* fusions in K56-2, K56-I2 and K56-R2. Overnight cultures were diluted 1 : 200 in 10 ml of fresh medium. Cultures were grown in the presence (solid symbols) or in the absence (open symbols) of 5 nM OHL, and β-galactosidase activity was determined at selected intervals. K56-2(pSG208), triangles; K56-I2(pSG206), diamonds; K56-R2(pSG208), circles; K56-2(pUCP28T), squares.

DISCUSSION

Using both chronic and acute infection models, we have demonstrated a role for the CepIR quorum-sensing system in the pathogenesis of *B. cenocepacia* lung disease. Rats infected with K56-I2 or K56-R2 were found to have similar numbers of bacteria recovered from the lungs to K56-2-infected animals, indicating that CepIR is not required for establishment or persistence in this model. A significant decrease in lung histopathology despite similar numbers of bacteria in the lungs could be due to decreased expression of extracellular virulence factors by the *cepI* and *cepR* mutant. One factor that has been shown to play a role in the virulence of K56-2 in lung injury in this model is the zinc metalloprotease PSCP (Corbett *et al.*, 2003). In this study, we have determined that the *zmpA* gene encoding this protease is regulated by *cepIR* and therefore may be one of the factors that accounts for the difference in virulence between the mutants and the parent strain.

A similar difference in histopathology, quantified by measuring the size of lung abscesses, was reported between *P. aeruginosa* PAO1 and a *rhlI lasI* double mutant in the agar bead model (Wu *et al.*, 2001). The decrease in the extent of histopathological changes in lungs infected with the mutant compared to the parent was significant on days 14 and 28 p.i. but not day 7. These data, together with this study, suggest that in both *P. aeruginosa* and *B. cenocepacia* infections, quorum-sensing mechanisms have a greater role during the later stages of lung infection in this model. *rhlI* and *lasI* mutants have also been shown to be less virulent in an acute pulmonary infection model in neonatal mice (Pearson *et al.*, 2000). In this study both single and double mutants in *rhlI* or *lasI* were shown to cause pneumonia and bacteraemia less frequently than the parent strain.

In the agar bead model the bacteria are introduced directly into the lung through the trachea, bypassing the normal route of colonization. The mechanical entrapment in the beads prevents their clearance from the lung. CepR-regulated virulence factors potentially involved in adherence and colonization may not contribute to the difference in virulence observed between the *cepR* or *cepI* mutants and the parent strain in this model. Although infections with some strains of the *B. cepacia* complex in C57/Black 6 mice led to splenic invasion (Cieri *et al.*, 2002), we have not recovered strain K56-2 from the spleens of infected rats (data not shown), suggesting that the infection remains confined to the lungs.

The intranasal infection model may be able to detect differences in virulence due to mutations that affect the ability to colonize mucosal surfaces or invade the bloodstream. K56-2 was lethal in the wild-type mice infected with 1×10^8 c.f.u., whereas the *cepR* mutant was less virulent and was not recovered from the spleen indicating that it did not result in an invasive infection. Interestingly, the CF mice tolerated K56-2 better than the wild-type mice. Although the mice infected with K56-2 had greater than 10^9 c.f.u. in the lung and had bacteria in the spleen, no mice died by day 3 p.i. Mice infected with the *cepI* mutant had approximately 2 logs less bacteria in the lungs and no bacteria in the spleen. The number of K56-I2-infected mice with severe lung inflammation was also less than in mice infected with K56-2 or K56-R2. Although this experiment suggested that the *cepI* mutant may be less virulent in CF mice, the degree of virulence due to the high inoculum made it difficult to distinguish differences between the strains. When CF mice were infected with 5×10^6 c.f.u., there was a marked difference in virulence between K56-2 and K56-I2 as indicated by the lack of invasion and the recovery of approximately 3 logs fewer bacteria from the lungs. K56-R2 was not examined at the lower inoculum due to the limited availability of $Cftr^{(-/-)}$ mice of the appropriate age.

Using a sensitive reporter assay employing a *traI–luxCDABE* fusion that responds to OHL, we were able to detect OHL in lung homogenates of CF mice infected with K56-2 but not K56-I2 in animals inoculated with 1×10^8 c.f.u. We were able to detect OHL in the mice infected with the lower inoculum but were approaching the limits of sensitivity of the assay in these animals and c.p.m. values were close to background levels. We attempted to detect OHL in lung homogenates from rats infected with approximately 1×10^5 c.f.u. via the agar bead model but were not able to do so, presumably due to the lower numbers of bacteria present (data not shown). Expression of AHLs by *B. cepacia* or *P. aeruginosa* in lung infection models has previously been reported using co-infection experiments with reporter strains containing plasmids with green fluorescent protein (GFP) fusions that respond to AHLs (Riedel *et al.*, 2001; Wu *et al.*, 2001). In these studies, evidence of AHL production was demonstrated by visualization of the GFP-expressing reporter by confocal scanning microscopy. The development of the new *lux*-based reporter system makes it possible to quantify relative amounts of AHLs with sensitivity in the picomolar to femtomolar range depending on the AHL.

Sajjan *et al.* (2001) reported previously that infection with strain BC7 resulted in elevated levels of the chemokine KC in bronchoalveolar lavage fluid of CF mice compared to wild-type mice. No differences were observed in levels of TNFα or MIP-2. In the present study, infection with K56-I2 resulted in significantly higher levels of MIP-2, IL-6 and TNFα in lung homogenates from CF mice compared to wild-type but no differences in IL-1β or KC. The difference in cytokine profiles observed between the two studies may be due to strain variation, since K56-2 is generally more virulent than BC7 (unpublished observations), or to differences in the age of the mice. In a study comparing *P. aeruginosa* agar bead infections in CF and wild-type mice, elevated levels of TNFα, MIP-2 and KC were reported in bronchoalveolar lavage fluid from CF mice compared to the wild-type (van Heeckeren *et al.*, 1997), which also suggests that there is strain variation in the stimulation of inflammatory mediators.

Infection with K56-2 resulted in elevated levels of the cytokines MIP-2, IL-6, IL-1β and KC in the lungs compared to infection with K56-I2. The increase in pro-inflammatory cytokines correlates with the increased inflammation observed in these animals and may be due to either CepIR regulation of virulence factors, such as the zinc metalloprotease, or direct stimulation of the host response by AHLs. *N*-(3-Oxododecanoyl) homoserine lactone (OdDHL) was shown to induce the production of several inflammatory chemokines and cytokines when injected directly into the skin of mice, including MIP-2, MIP-1β, IL-6 and IL-1α (Smith *et al.*, 2002). It has also been shown to activate IL-8 production in human lung fibroblasts and epithelial cells (Smith *et al.*, 2001). OdDHL has also been reported to suppress leukocyte proliferation and inhibit lipopolysaccharide-induced secretion of TNFα and IL-12 (Chhabra *et al.*, 2003; Telford *et al.*, 1998). A comparison of a range of synthetic analogues of OdDHL suggests that AHLs with 11–13 carbon side chains are optimal for immune suppressive activity (Chhabra *et al.*, 2003). Although these studies suggest that long-chain AHLs have more activity than compounds with short side chains (Chhabra *et al.*, 2003; Smith *et al.*, 2002; Telford *et al.*, 1998), the potential for a direct effect of OHL or *N*-hexanoyl-homoserine lactone on inflammation cannot be ruled out.

This study together with those of Kothe *et al.* (2003) and Aguilar *et al.* (2003) indicate that the CepIR quorum-sensing system contributes to virulence in a wide range of hosts including plants, nematodes and murine species. Although the virulence factors regulated by CepR that play a role in virulence in the various models need to be determined, the importance of this cell-signalling mechanism in virulence has been established. The virulence of the *cepR* and *cepI* mutants was comparable in the agar bead

model and in the wild-type mice; however, the *cepR* mutant was more virulent than the *cepI* mutant in the *Cftr*$^{(-/-)}$ mice. Interestingly, a *cepR* mutant was also more virulent than the *cepI* mutant in the *C. elegans* model (Kothe *et al.*, 2003). CepR functions as both an activator and a repressor of target genes (Lewenza & Sokol, 2001; Riedel *et al.*, 2003) and therefore may be involved in both up-regulation and down-regulation of virulence factors. The importance of different complements of virulence factors in the model systems used may account for the observed differences in the virulence of the *cepR* mutant. For example, a *cepR* mutant produces elevated amounts of ornibactin which might contribute to its increased virulence. An additional LuxR homologue has been identified in the *B. cenocepacia* genome (A. Baldwin, P. A. Sokol, J. Parkhill & E. Mahenthiralingam, unpublished data) and this protein may compensate for the *cepR* mutation. This possibility is currently being investigated in our laboratory.

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