Proteomic identification and characterization of bacterial factors associated with *Burkholderia cenocepacia* survival in a murine host

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Burkholderia cenocepacia is a member of the *Burkholderia cepacia* complex, a diverse family of Gram-negative bacteria that are serious respiratory pathogens in immunocompromised patients and individuals with cystic fibrosis. To identify putative bacterial virulence determinants, proteomic profiles were compared between two *B. cenocepacia* isolates that demonstrated differential persistence in a mouse model of pulmonary infection; clinical isolate C1394 is rapidly cleared from the murine lung whereas the strain variant, C1394mp2, persists. Two-dimensional (2D) gel electrophoresis was used to identify candidate proteins involved in *B. cenocepacia* survival in a susceptible host. The 2D proteome of the persistent isolate (C1394mp2) revealed loss of an alkyl hydroperoxide reductase subunit C (AhpC) protein spot and increased production of flagellin proteins. Loss of AhpC expression in C1394mp2 correlated with enhanced susceptibility to oxidative stress. C1394mp2 expressed increased flagellin production and enhanced swimming motility, traits that were subject to regulation by heat and low pH. Together, these results revealed differential expression and stress regulation of putative virulence determinants associated with *B. cenocepacia* persistence in a susceptible host.

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INTRODUCTION

Burkholderia cenocepacia is a member of the *Burkholderia cepacia* complex (BCC), a diverse family of Gram-negative bacterial species recognized for their evolving taxonomy, metabolic diversity and recent emergence as opportunistic human pathogens, particularly in individuals with cystic fibrosis (CF). CF is a life-threatening, congenital disease affecting one in 2500 live-borne Caucasians, rendering them susceptible to chronic bacterial lung infection. Colonization by BCC is particularly problematic because these organisms are capable of patient-to-patient spread, and lung infection can result in rapid clinical deterioration with fatal septicaemia (Govan & Deretic, 1996; LiPuma, 1998). Furthermore, BCC organisms are resistant to multiple antibiotics, enabling them to establish infection and persist in the CF lung (Aaron *et al.*, 2000; Chernish & Aaron, 2003; Govan *et al.*, 1996). Although all nine genomovars within the BCC have been isolated from CF patients, infections by

B. cenocepacia are more prevalent and are most frequently associated with epidemic outbreaks and high rates of mortality (Mahenthiralingam *et al.*, 2002, 2005; Speert *et al.*, 2002).

Mechanisms of BCC virulence have been demonstrated by its invasion of eukaryotic cells and acute inflammation culminating in death in murine models of infection (Chu *et al.*, 2002; Martin & Mohr, 2000; Schwab *et al.*, 2002; Sokol *et al.*, 2003; Urban *et al.*, 2004). Several putative virulence factors have also been identified in the BCC, including a type III secretion system, cable pilus, siderophores and haemolysin (Hutchison *et al.*, 1998; Sajjan *et al.*, 1995; Tomich *et al.*, 2003; Visser *et al.*, 2004). The role of these factors in human disease has not yet been demonstrated, and their expression is variable among CF isolates of *B. cenocepacia*. Despite such variability, previous studies have differentiated *B. cenocepacia* isolates from other BCC species, such as *Burkholderia multivorans*, by their differential virulence in patients with CF (Aris *et al.*, 2001; Mahenthiralingam *et al.*, 2001). Furthermore, different species in the BCC demonstrate distinct patterns of virulence in a murine model of pulmonary infection (Chu *et al.*, 2002). In this model, *B. multivorans* persists in the lung of immunosuppressed BALB/c mice for up to 16 days without causing illness whereas *B. cenocepacia* is rapidly cleared but induces a pronounced host response that results in substantial toxicity and death in some animals. These results demonstrate

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Abbreviations: AhpC, alkyl hydroperoxide reductase subunit C; ASL, airway surface liquid; BCC, *Burkholderia cepacia* complex; CF, cystic fibrosis; EPS, exopolysaccharide; IM, inner membrane; OM, outer membrane; RNI, reactive nitrogen intermediates.

pathogenic differences among species and prompted us to investigate bacterial determinants of virulence in these important CF pulmonary pathogens.

We previously reported *B. cenocepacia* adaptation to a murine host after sequential passages of a clinical strain in a pulmonary infection model (Chung *et al.*, 2003). The parent *B. cenocepacia* strain is rapidly cleared from the murine lung whereas the passaged strain variant persists. Comparative analyses between these two clonal isolates identified a colonial morphology change, increased piliation and enhanced exopolysaccharide (EPS) production as potential factors for persistence *in vivo*. Our observations highlighted a potential role for colonial morphology in differentiating *B. cenocepacia* strains, particularly those that cause persistent rather than transient infections. Further analyses of these colonial variants were undertaken to identify the protein differences responsible for such diverse infection outcomes. In the current study we present differences in alkyl hydroperoxide reductase subunit C (AhpC) and flagellin expression as detected by two-dimensional (2D) gel electrophoresis. Our observations on differential expression of potential virulence determinants between these two strain variants form the basis of this report.

METHODS

Bacterial strains and growth conditions. *B. cenocepacia* strain C1394 is a clinical isolate recovered from an outbreak among CF patients in Manchester, England (Mahenthiralingam *et al.*, 2000). Strain variant C1394mp2 was recovered from sequential passage of C1394 through a mouse model of pulmonary infection as previously described (Chung *et al.*, 2003). Clinical strains of *Enterococcus faecalis* and *Staphylococcus aureus* were obtained from our repository of isolates recovered from CF patients in Canadian CF clinics. Bacteria were stored at -70°C in Mueller–Hinton broth with 8.0% (v/v) DMSO and cultured at 37°C on blood agar plates (PML Microbiologicals) or Luria–Bertani (LB) agar. Liquid cultures were grown shaking in LB broth (pH 7 or 5) for 16 h at 37°C or 42°C .

Extraction of inner- and outer-membrane proteins. Membrane proteins were isolated by modification of the method of Hancock & Nikaido (1978). Four hundred millilitre bacterial cultures were grown shaking in LB broth. Bacteria were centrifuged at 7000 g for 15 min at 4°C and resuspended in 6 ml cold 10 mM Tris/HCl (pH 8) containing 20% (w/v) sucrose. Suspensions were frozen at -20°C and thawed to assist cell breakage, treated with 50 μg DNase (Roche) ml^{-1} and passed twice through a French pressure cell at 69 MPa. Unbroken cells were removed by centrifugation at 1000 g for 10 min at 4°C . Four millilitres of the supernatant [in 20% (w/v) sucrose] was layered onto a two-step sucrose density gradient containing 4 ml of 70% (w/v) sucrose and 4 ml of 50% (w/v) sucrose. Samples were centrifuged without braking at 100 000 g in an SW41 rotor overnight at 4°C . The inner-membrane (IM) fraction lay between the 20 and 50% sucrose layers while the outer-membrane (OM) fraction was between the 50 and 70% sucrose layers. The fractions were collected separately, diluted in two to three volumes of distilled water, centrifuged at 17 000 g for 1 h, and resuspended in 100 μl distilled water. Proteins were quantified using the Bradford and Lowry assay. Approximately 15 μg protein was mixed with Laemmli buffer, boiled, electrophoresed on SDS-12.5% PAGE gels then stained with 0.02% (w/v) Coomassie brilliant blue in 25% (v/v) ethanol and 10% (v/v) acetic acid.

Whole-cell protein extractions. Bacteria were grown at 37°C or 42°C in 100 ml LB broth. Bacteria were centrifuged at 4500 g at 4°C , washed once with cold PBS then resuspended in 1 ml cold lysis buffer [5 mM EDTA and 1 mM PMSF (Sigma) in PBS]. This suspension was added to 500 μl washed glass beads (0.1 mm diameter) in a 2 ml tube. Cells were lysed with a mini bead-beater (Biospec Products) for three pulses of 1 min with 20 s intervals on ice to prevent overheating. Lysates were centrifuged at 14 000 g for 3 min at 4°C , and the supernatant was collected. The pellet was resuspended in 1 ml lysis buffer, disrupted by bead-beating for 1 min, centrifuged, and the supernatants collected and pooled. Protein concentrations were determined using the BCA microassay kit (Pierce) and 150 μg aliquots were frozen at -20°C .

2D gel electrophoresis. All equipment for IEF (Ettan IPGphor IEF unit) and 2D-PAGE (Ettan Dalt six electrophoresis unit) were purchased from Amersham Biosciences (GE Healthcare) except where indicated. Whole-cell protein extracts (150 μg) were incubated with 50 μl DNase/RNase solution [1 mg DNase I and 1 mg RNase A (Roche) ml^{-1}] for 1 h on ice. Proteins were concentrated by ultrafiltration at a molecular mass cut-off of 10 000 Da. Concentrated proteins were resuspended in 450 μl rehydration solution [8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) carrier ampholytes, 0.002% (w/v) bromophenol blue and 0.3% (w/v) DTT] for 1 h at 4°C and applied to 24 cm long immobilized pH 4–7 gradient (IPG) strips. The IPG strips were rehydrated overnight with the solubilized proteins (450 μl) at 20°C under mineral oil. IEF followed rehydration for a total of 99.7 kVh at 20°C . Focused strips were equilibrated in buffer [50 mM Tris/HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; 2% (w/v) SDS; 1% (w/v) DTT] for 30 min then placed atop second dimension high-tensile-strength slab gels containing 12.5% acrylamide (Duracryl, Genomic Solutions). Gels were electrophoresed overnight with a broad-range molecular mass marker (Bio-Rad) at 1.5 mA per gel and stained with silver nitrate (Rabilloud, 1999; Shevchenko *et al.*, 1996). Protein spots were excised in a laminar flow hood with methanol-cleaned instruments and stored in 1% acetic acid.

MALDI-TOF MS, liquid chromatography (LC)-MS/MS analysis and sequencing. Protein spots excised from 2D-PAGE gels were sent for processing and analysis at the Genome BC Proteomics Centre at the University of Victoria, BC. In-gel digestion was performed with 400 ng modified porcine trypsin (Promega, #V5111) for 4 h at 37°C . Peptides were extracted from the gel piece using 30 μl of 10% (v/v) formic acid and spotted onto MALDI-TOF plates with α -cyano-4-hydroxycinnamic acid (CHCA) matrix using C18 ZipTips. The peptide mass data were submitted to MASCOT (http://www.matrixscience.com/search_form_select.html) for identification. Samples were further processed by LC-MS/MS if there were no confident search results. Samples were separated by one-dimensional (1D) reversed-phase chromatography using Analyst software (Applied Biosystems) controlled Ultimate gradient pumps, SwitchOS II and FAMOS Autosampler (LC Packings/Dionex) with a 75 μm i.d. $\times$ 15 cm PepMap C18 3 μm , 100 Å nanocolumn (LC Packings/Dionex). Peptides were eluted and ionized by electrospray into a PE Sciex Qstar Pulsar I Quadrupole TOF MS. The MS/MS data obtained were submitted to ProID (proprietary Applied Biosystems software) for bioinformatics analysis of public protein databases (NCBI) and identification.

Western blotting. Whole-cell and membrane proteins were separated by electrophoresis in a 12.5% SDS-polyacrylamide gel and flagellin protein was detected by immunoblotting. The flagellin-specific rabbit polyclonal antiserum used was raised against purified *Burkholderia pseudomallei* flagellin and was a generous gift from Dr Don Woods (University of Calgary). Separated proteins were electroblotted onto PVDF membrane (Bio-Rad) for 1 h at 100 V. The membrane was rinsed with distilled water and with wash buffer

[25 mM Tris; 137 mM NaCl, pH 7.5; 0.1% (v/v) Tween 20] and then incubated in blocking buffer [wash buffer containing 5% (w/v) BSA] for 1 h at room temperature. After incubation, the membrane was rinsed in wash buffer three times for 5 min then incubated overnight at 4 °C with the primary flagellin-specific antiserum at a dilution of 1:10 000 in blocking buffer. The membrane was rinsed again in wash buffer as above and incubated for 1 h at room temperature with secondary antibody [Phototope-horseradish peroxidase (HRP)-linked anti-rabbit immunoglobulin G; Cell Signalling] at a 1:2000 dilution in blocking buffer. Proteins were detected and developed using the HRP Western Blot Detection System (Cell Signalling), following the manufacturer's instructions. Protein standards included a prestained broad-range molecular mass marker (Bio-Rad) and the biotinylated protein ladder provided by the detection system (Cell Signalling).

PCR and RFLP analysis. Flagellin (*fliC*) gene amplification with oligonucleotide primers BC4 (CTGGTCGCACAGCAGAACCTGA-AC) and BCR12 (ACATGTTTCGCGTTTCCTG) was carried out as previously described (Hales *et al.*, 1998). Each reaction contained 40 ng genomic DNA template, 200 nM each primer, 100 µM dNTPs, 1 × *Taq* buffer (Invitrogen), 2.5 mM MgCl₂ and 2.5 U *Taq* polymerase (Invitrogen). PCR was performed for 30 cycles consisting of 1 min at 94 °C, 1 min at 56 °C and 2 min at 72 °C, followed by a final extension at 72 °C for 10 min. Flagellin gene PCR-amplified products (5 µl) were digested with restriction endonucleases *Hae*III and *Msp*I, or *Pst*I as previously described (Hales *et al.*, 1998; Tungpradabkul *et al.*, 1999; Winstanley *et al.*, 2001). Reactions were carried out in volumes of 20 µl under the conditions recommended by the supplier (New England Biolabs).

Motility assays. Motility was tested by spotting a single colony onto plates of LB adjusted to pH 7 and containing 0.3% (w/v) agar. For acid induction of motility, LB was adjusted to pH 5. For high-salt effects on motility, LB was made with 0.5 or 1 M NaCl and adjusted to pH 7. Motility was assessed by measuring bacterial colony diameter following incubation at 37 °C for 48 h.

Oxidative stress assays. Disc inhibition assays were used to test sensitivity to hydrogen peroxide and cumene hydroperoxide. Bacteria were grown in LB broth overnight and adjusted to 10⁸ c.f.u. ml⁻¹ in LB broth. One millilitre of this bacterial suspension was added to 9 ml molten LB agar, mixed, and dispensed into Petri plates. Following solidification of agar, paper discs (6 mm) were soaked with 6 µl of 30% (v/v) hydrogen peroxide or 15 µl of 10% (w/v) cumene hydroperoxide and placed on the surface of agar plates. To test for sensitivity to reactive nitrogen intermediates (RNI), bacteria were grown in LB broth (pH 5) containing sodium nitrite for 16 h at 37 °C. Following incubation, cultures were diluted and plated onto LB agar quadrant plates for viable counts.

RESULTS

Proteomic analysis of *B. cenocepacia* strain C1394 and its variant C1394mp2 reveals differential AhpC protein expression

We have previously reported *B. cenocepacia* persistence in a mouse model of pulmonary infection and described piliation and EPS production as potential cell-surface factors that may enhance bacterial survival in a host (Chung *et al.*, 2003). A proteomic approach was taken to further analyse the differences between *B. cenocepacia* strain C1394 (non-persistent parent strain) and its persistent variant C1394mp2. 2D gel electrophoresis was used to

identify candidate proteins that may contribute to *B. cenocepacia* adaptation and persistence in the murine lung. All protein differences between strains C1394 and C1394mp2 were detected in at least three separate protein extractions of both isolates, and 2D-PAGE was performed at least twice with each extraction. Fig. 1 shows representative silver-stained gels of the whole-cell protein profiles of C1394 and C1394mp2 separated along a pH gradient of 4–7, with highlighted areas containing major reproducible spot differences (gain or loss) between the parent and variant. One of the major proteomic differences observed between the two isolates was a ~20 000 Da protein spot, C1. This spot was detected in C1394 but was not present in C1394mp2 (Fig. 1). The LC-MS/MS analysis of spot C1 yielded the amino acid sequences shown in Table 1. Due to the incomplete annotation of the *B. cenocepacia* genome at the time of LC-MS/MS analysis, spot C1 matched to the putative AhpC protein of *Burkholderia xenovorans* LB400. The AhpC protein is among a number of antioxidant enzymes expressed by bacteria under oxidative stress, particularly in the presence of organic peroxides, hydrogen peroxide and RNI (Chen *et al.*, 1998; Hofmann *et al.*, 2002; Loprasert *et al.*, 2003; Springer *et al.*, 2001). To examine the role of AhpC in *B. cenocepacia*, strains C1394 and C1394mp2 were evaluated for their capacity to withstand various oxidative stresses. C1394mp2 was consistently more sensitive than C1394 to treatment with hydrogen peroxide and cumene hydroperoxide at 37 °C (Fig. 2a). Although sodium nitrite in acidic LB broth reduced survival of both isolates, C1394mp2 was significantly more susceptible than C1394 to RNI in the form of acidified nitrite (Fig. 2b). Hence the absence of AhpC protein in C1394mp2 may be responsible for enhanced susceptibility to oxidative stress.

The C1394mp2 proteome displays more flagellin protein spots encoded by the type II *fliC* gene

Three protein spots (M1, M2 and M3), positioned between 40 000 and 50 000 Da, were observed in the C1394mp2 proteome; however, only spot M2 was sporadically or scarcely detected in the C1394 proteome (Fig. 3). Spots M1 and M2 yielded sequences that matched *B. cepacia* flagellin whereas spot M3 was identified as type II flagellin (Table 1). While two flagellin types have been described for bacteria from the BCC, the unclassified BCC flagellin identified for spots M1 and M2 is closer in sequence to the type II flagellin (83% identity) than to type I (72% identity) (Hales *et al.*, 1998). The BCC *fliC* gene encodes the type II flagellin, which reportedly has a molecular mass of 45 kDa and exhibits considerable heterogeneity among strains as determined by RFLP (Hales *et al.*, 1998). We used previously described PCR/RFLP methods to determine whether C1394 and C1394mp2 carried the same *fliC* gene (Tungpradabkul *et al.*, 1999; Winstanley *et al.*, 2001). Our results showed identical PCR products (1.0 kb band) and restriction patterns for both C1394 and C1394mp2, indicating that, by these methods, there was no obvious difference between the *fliC* genes of C1394 and C1394mp2 (data not shown).

Fig. 1. Silver-stained 2D gels of C1394 (a) and C1394mp2 (b) grown in LB broth at 37 °C and extracted at stationary phase. Whole-cell proteins (150 µg) were separated on IPG strips with a pH gradient of 4–7, followed by separation on SDS-12.5% PAGE. Boxed areas indicate zones of significant protein spot differences between C1394 and C1394mp2 that were detected and later analysed by LC-MS/MS. The black ellipse indicates a large protein spot difference that was not reproducible in subsequent experiments. Magnification of the smaller boxed area shows protein spot C1, identified as a putative AhpC protein (c, d). Spot C1 was present in C1394 (c) and absent in C1394mp2 (d).

C1394mp2 produces more flagellin protein and is more motile than the parent strain C1394

The type II flagellin of the BCC is reported to have 77.5 % identity to the amino acid sequence of the flagellin protein of *B. pseudomallei*, the causative agent of melioidosis and a species closely related to the BCC (Hales *et al.*, 1998). To verify the differential expression of the BCC flagellin protein in C1394 and C1394mp2, rabbit polyclonal antiserum raised against the *B. pseudomallei* flagellin was used to probe Western blots with separated protein extracts from both isolates (Fig. 4). Immunoblots detected a ~45 000 Da band (arrow) that was more concentrated in the whole-cell protein extracts of C1394mp2 than in the parent C1394, suggesting enhanced expression of this protein in the former isolate. In addition, other bands below the 45 000 Da protein were highlighted with the antiserum in the whole-cell protein extracts of C1394mp2. It is possible that these bands correspond to the extra 2D-PAGE spots identified as flagellin (Fig. 3). Motility was assessed to determine if the differential expression of flagellin had a biological effect.

C1394mp2 appeared more motile: the diameter of the zone of motility was almost twice that of C1394 (C1394, 4 mm; C1394mp2, 8 mm; Fig. 5a).

Heat and low pH affect flagellin production and motility of C1394

We previously reported heat induction (by growth at 42 °C) of the shiny colonial morphology in C1394, rendering it similar in colonial appearance to the persistent variant, C1394mp2 (Chung *et al.*, 2003). This suggests stress regulation of the cell surface determinants contributing to the shiny colonial morphology. These findings prompted the investigation of protein expression of both isolates at 42 °C. Both isolates grew at similar but considerably lower rates at 42 °C than at 37 °C, a phenomenon that reduced the recovery of protein mass for 2D-PAGE analysis. Therefore, protein profiles of the IMs and OM were analysed and resolved by 1D-PAGE to enhance detection of structural differences responsible for the matte and shiny colonial morphologies of C1394 and C1394mp2, respectively.

Table 1. LC-MS/MS identification of differentially expressed proteins of C1394 and C1394mp2

Isoelectric points (pI) and molecular mass (M_r) were estimated by 2D-PAGE experiments.

Spot ID	Amino acid sequences obtained from LC-MS/MS	Observed pI/ M_r	ID by LC-MS/MS (accession no.)	Description
C1	GELIDQLGVR, ATFIVDPDNTIQHVSNNLVGR	4.6/20.0	Putative AhpC, <i>B. xenovorans</i> (gi91783507)	Present in C1394
M1	INSAADDAAGLAISTR, NQVLQQAGISVLAQANSLPQQVLK	4.3/47.0	Flagellin, <i>B. cepacia</i> (gi4210944)	Present in C1394mp2
M2	INSAADDAAGLAISTR, NQVLQQAGISVLAQANSLPQQVLK	4.9/47.0	Flagellin, <i>B. cepacia</i> (gi4210944)	Enhanced in C1394mp2
M3	NQVLQQAGISVLAQANSLPQQVLK	4.4/40.0	Type II flagellin, <i>B. cepacia</i> (gi2935155)	Present in C1394mp2

Fig. 2. Assays for sensitivity to hydrogen peroxide, cumene hydroperoxide and sodium nitrite. Discs soaked in hydrogen peroxide (6 μ l, 30%) or cumene hydroperoxide (CH; 15 μ l, 10%) were placed on agar containing either C1394 or C1394mp2 and incubated for 24 h at 37 °C (a). *S. aureus* and *E. faecalis* were used as catalase-positive and -negative controls, respectively. Survival of C1394 (black bars) and C1394mp2 (grey bars) exposed to indicated concentrations of sodium nitrite at pH 5 for 16 h in LB at 37 °C (b). Results are the mean $\pm$ SEM of duplicates or triplicates in two experiments.

Fig. 6(a) shows a $\sim$ 45 000 Da band that was the most significant difference between the two isolates grown at 37 °C and was more prominent in the IM protein extracts of C1394mp2. The $\sim$ 45 000 Da band was excised, sequenced, and identified by LC-MS/MS, yielding the following amino acid sequence: INSAADDAAGLAISTR. This sequence matched with the BCC flagellin protein (accession no. gi4210944) and its enhanced expression in C1394mp2 correlates with the 2D proteome of C1394mp2. However, at 42 °C, C1394 and C1394mp2 appeared to have equivalent amounts of flagellin whereas at 37 °C, flagellin protein production was more apparent in C1394mp2 (Fig. 6a). Immunoblots also confirmed equal amounts of flagellin protein in the OM protein extracts of C1394 and C1394mp2 grown at 42 °C (Fig. 6b). Compared to flagellin levels at 37 °C, flagellin production at 42 °C appeared to be suppressed in C1394mp2 and enhanced in C1394. The slow growth at 42 °C obscured detection of any possible motility difference between C1394 and C1394mp2.

Fig. 3. 2D gel detection of protein spots M1, M2 and M3 identified as flagellin. Spot M2 was not as apparent in C1394 (a) as in C1394mp2 (b). Spots M1 and M3 were detected only in C1394mp2 (b).

Other environmental stresses were tested to determine their effect on flagellin production and motility. Salt concentration and acidification have been implicated in altering the airway surface liquid (ASL) of the CF lung, thereby enhancing colonization by CF pathogens (Poschet *et al.*, 2002; Smith *et al.*, 1996; Zabner *et al.*, 1998). Motility agar was made with different concentrations of NaCl or adjusted to pH 5. Growth of C1394 and C1394mp2 was impeded at 0.5 M and 1.0 M NaCl, but growth rates were not affected by acidic conditions. Isolates grown on motility agar with 0.5 M NaCl showed the same degrees of motility as observed when grown on normal motility medium (pH 7; Fig. 5b).

Fig. 4. Immunoblot detection of flagellin protein in C1394 and C1394mp2. Lanes contained 15 μ g whole-cell protein extracted from 16 h cultures grown at 37 °C, separated on SDS-12.5% PAGE gels, and immunoblotted with anti-flagellin polyclonal antibody. The arrow highlights position of the flagellin protein. Lanes: 1, C1394; 2, C1394mp2; L, biotinylated ladder.

Fig. 5. Motility assays with C1394 and C1394mp2. Zones of motility are demonstrated in swimming LB media at neutral pH (a) or graphed and compared with swimming LB media of different composition (b). Motility zones were measured after 48 h of incubation at 37 °C. Black bars are C1394 and grey bars are C1394mp2. Results are the mean $\pm$ SEM of duplicates in two experiments.

Elevated salt concentrations inhibited motility of both isolates. Under acidic conditions, C1394mp2 was more motile than C1394; however, motility was significantly enhanced in both isolates over that at pH 7 (Fig. 5b). Moreover, C1394 motility at pH 5 was comparable to that of C1394mp2 at pH 7. Immunoblots confirmed the increased production of the flagellin protein in whole-cell extracts of C1394 grown in acidic media (Fig. 6c). These results suggest that heat and low pH enhance flagellin production and motility of *B. cenocepacia*.

DISCUSSION

We previously described two clonal isolates of *B. cenocepacia* that differed in colonial morphology and in their capacity to persist in a mouse model of infection. Comparative analyses between isolates C1394 and C1394mp2 identified piliation and EPS production as potential cell surface factors that induced a matte to shiny colonial phenotype and facilitated *B. cenocepacia* persistence in the murine lung. These observations highlighted a correlation between colonial morphotypes and infection outcomes, similar to that between mucoid isolates of *Pseudomonas aeruginosa* and persistent infections in CF. Analysis of such colonial variants may aid in predicting which *B. cenocepacia* strains are most

Fig. 6. IM protein profiles of C1394 and C1394mp2 and immunoblot detection of flagellin production. In each section, lanes 1 and 3 are C1394; lanes 2 and 4 are C1394mp2. IM proteins were stained with Coomassie brilliant blue; arrows denote the flagellin band in C1394 and C1394mp2 grown at 37 °C and at 42 °C (a). Immunoblots detected flagellin in OM protein extracts of C1394 and C1394mp2 grown at 37 °C and at 42 °C (b). Immunoblot detection for flagellin was also performed on whole-cell lysates of isolates grown in LB broth, pH 7 or pH 5 (c). Each lane contained 15 μ g protein preparations and was separated on SDS-12.5% PAGE gel. L denotes the molecular mass marker (a) or biotinylated ladder (b, c).

likely to cause persistent rather than transient infections and elucidate critical virulence determinants. In this study, we performed proteomic analysis on colonial variants C1394 and C1394mp2 to identify candidate proteins involved in *B. cenocepacia* survival in a susceptible host. Flagellin proteins were more prominent in the 2D proteome of the persistent variant C1394mp2 whereas the AhpC protein was detected only in the non-persistent parent strain C1394. Because the current study focused on the presence or absence of 2D protein spots that may have represented only a small percentage of the total proteome, we did not identify quantitative protein differences that corresponded to the increased piliation or EPS production as previously described in C1394mp2 (Chung *et al.*, 2003). In addition, we did not use the same media that resulted in the detection of pili and EPS. We focused on proteins expressed in stationary phase from LB broth cultures grown at 37 °C to

mimic conditions used to prepare bacteria prior to animal infection. Consequently, our proteomic results were obtained from isolates grown in different media and growth conditions that may not have promoted or enhanced the production of pili and EPS or other putative virulence determinants.

Protein spot C1 was detected only in the C1394 proteome, and was identified as the AhpC protein of *B. xenovorans*. AhpC protein is among a number of antioxidant enzymes that are expressed by bacteria under oxidative stresses imposed by peroxides and RNI (Hofmann *et al.*, 2002; Springer *et al.*, 2001). Since proteins were extracted from stationary-phase cultures, AhpC expression in C1394 may have resulted from oxidative stresses encountered from metabolic respiration and oxygen depletion after exponential growth (McDougald *et al.*, 2002; Miller & Britigan, 1997). The absence of AhpC protein in C1394mp2 did not affect viability but it correlated with reduced oxidative stress resistance. Compared to C1394, the variant was more susceptible to cumene hydroperoxide and acidified nitrite, the latter representing RNI. Although C1394mp2 showed a small increase in sensitivity to hydrogen peroxide, this result was reproduced over several assays. This small difference might be explained by compensatory activity by other oxidative protection enzymes such as catalase, which primarily detoxifies hydrogen peroxide. It is not clear how lack of AhpC expression would facilitate C1394mp2 persistence in a susceptible host. It is possible that AhpC may not have an active role in C1394mp2 persistence in the murine host. Interestingly, studies with *Salmonella typhimurium* showed that AhpC was a potential target for the immune system, eliciting an inflammatory response in animal hosts (Taylor *et al.*, 1998). Further studies are required to determine whether up-regulation of AhpC expression in *B. cenocepacia* results in rapid clearance from the murine host.

BCC flagellin appeared as three spots (M1 to M3) of different pIs in the proteome of C1394mp2, whereas only one protein spot (M2) was occasionally detected in C1394. SDS-PAGE of IM proteins and immunoblots confirmed that there was greater production of flagellin by C1394mp2 than by the parent, C1394. It is possible that the two additional 2D-PAGE spots (M1 and M3) and extra bands highlighted in immunoblots may represent modified or degraded forms of the flagellin produced by C1394mp2. Two of the three flagellin spots detected by 2D-PAGE (M1 and M2) had similar molecular masses yet possessed different pIs, a finding that may indicate post-translational modifications such as phosphorylation or glycosylation (Winstanley & Morgan, 1997).

Environmental stimuli such as high salt concentrations impaired the growth and subsequently the motility of *B. cenocepacia*. However, an elevation in temperature resulted in increased flagellin production by C1394. Immunoblots detected more flagellin at 42 °C than at 37 °C, suggesting that flagellin production in *B. cenocepacia* is amplified in

response to heat stress. Flagellin production was also affected at low pH, conditions under which both C1394 and C1394mp2 had enhanced motility. Although C1394 remained less motile than C1394mp2, its motility at pH 5 was comparable to that of C1394mp2 at neutral pH. This increase in motility at pH 5 may be due to the fact that low external pH generates a large pH difference across the inner cell membrane which contributes to the proton potential that powers motility (Maurer *et al.*, 2005). However, immunoblots also confirmed enhanced flagellin production in C1394 grown in acidic media. Growth on acidic LB agar did not induce the shiny phenotype in C1394, suggesting that flagellin production alone does not regulate the colonial morphology of *B. cenocepacia*. Nonetheless, these results implicate pH as a potentially important environmental signal that may regulate and enhance flagellin synthesis and motility in *B. cenocepacia*. This observation may provide some insight into *B. cenocepacia* survival in the CF host. The ASL in the CF lung is reported to be more acidic than normal ASL, a fact which may contribute to thicker than normal mucus, and affect the bactericidal activity of innate defences (Coakley *et al.*, 2003; Fischer *et al.*, 2002; Poschet *et al.*, 2002). Since increased flagellin production and motility are associated with the persistent phenotype of C1394mp2, these features may confer enhanced survival in an acidic environment such as the CF lung. Previous studies have also described the role of BCC flagella in *in vitro* host cell invasion as well as infectivity and inflammation in a mouse model, implicating its importance in BCC pathogenesis (Tomich *et al.*, 2002; Urban *et al.*, 2004).

In summary, our proteomic analyses identified AhpC and flagellin as candidate proteins to further characterize *B. cenocepacia* survival in the murine lung. Lack of AhpC expression in C1394mp2 correlates with susceptibility to oxidative stress; however, further investigation is required to identify its potential role in *B. cenocepacia* persistence. Flagellin production in *B. cenocepacia* appeared to be subject to environmental regulation as demonstrated in other pathogens that adapt to multiple conditions. The heat and acidic induction of flagellin production suggests that motility is amplified in response to stress and may facilitate adaptation and survival in hostile conditions, particularly within the host. Like pili and EPS, enhanced production of flagellin appears to be associated with *B. cenocepacia* survival in the murine host. Whilst protein expression under *in vitro* conditions may not necessarily reflect all proteins that are expressed *in vivo*, our study highlights differences that warrant further investigation into stress-regulated proteins that contribute to *B. cenocepacia* persistence.

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