Burkholderia cenocepacia utilizes ferritin as an iron source

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Burkholderia cenocepacia is a member of the *Burkholderia cepacia* complex, a group of genetically similar species that inhabit a number of environmental niches, including the lungs of patients with cystic fibrosis (CF). To colonize the lung, this bacterium requires a source of iron to satisfy its nutritional requirements for this important metal. Because of the high potential for damage in lung tissue resulting from oxygen–iron interactions, this metal is sequestered by a number of mechanisms that render it potentially unavailable to invading micro-organisms. Such mechanisms include the intracellular and extracellular presence of the iron-binding protein ferritin. Ferritin has a highly stable macromolecular structure and may contain up to 4500 iron atoms per molecule. To date, there has been no known report of a pathogenic bacterial species that directly utilizes iron sequestered by this macromolecule. To examine the ability of ferritin to support growth of *B. cenocepacia* J2315, iron-deficient media were supplemented with different concentrations of ferritin and the growth kinetics characterized over a 40 h period. The results indicated that *B. cenocepacia* J2315 utilizes iron bound by ferritin. Further studies examining the mechanisms of iron uptake from ferritin indicated that iron utilization results from a proteolytic degradation of this otherwise stable macromolecular structure. Since it is known that the ferritin concentration is significantly higher in the CF lung than in healthy lungs, this novel iron-acquisition mechanism may contribute to infection by *B. cenocepacia* in people with CF.

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INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) contains nine genetically distinct but phenotypically similar species (Mahenthiralingam *et al.*, 2005). In addition, the Bcc also contains a number of genomovars for which a designated species name has yet to be assigned, pending definitive biochemical tests (Mahenthiralingam *et al.*, 2005). Originally identified as phytopathogens (Burkholder, 1950), this group of organisms has become a focus of attention as human pathogens following isolation from the respiratory tracts of patients that are immunocompromised or have cystic fibrosis (CF) (Rosenstein & Hall, 1980; Isles *et al.*, 1984; Thomassen *et al.*, 1985). In the early 1980s, the incidence of *B. cepacia* infection in CF clinics increased dramatically. This, coupled with factors such as the innate resistance of the organism to multiple antibiotics, poor clinical prognosis, and high degree of person-to person transmission, led to fundamental changes in both hospital infection control policies and the social behaviour of CF patients (Govan *et al.*, 1993; Walters & Smith, 1993; Lewin *et al.*, 1993; Prince, 1986;

Simpson *et al.*, 1994). While initial attempts to stem the increasing prevalence of Bcc infections involved cohorting infected patients, complete segregation of all patients is becoming the norm (Vonberg & Gastmeier, 2005). This has resulted in a significant decrease in the acquisition of these pathogens. However, new infections still occur and suggest an environmental reservoir of the Bcc.

While representatives of all nine Bcc species have been isolated from CF patients, *B. cenocepacia* is the major CF pathogen (Mahenthiralingam *et al.*, 2005). Within this species there are several highly clonal lineages, of which one, ET12, was responsible for epidemics across the northern USA and Europe in the 1990s (Govan *et al.*, 1993; Johnson *et al.*, 1994; Pitt *et al.*, 1996). Infection with an isolate from the ET12 lineage is associated with poor prognosis consistent with ‘cepacia syndrome’, a rapid and fatal decline in lung function characterized by a necrotizing pneumonia with fever, bacteraemia, elevation of erythrocyte sedimentation rate and leukocytosis (Isles *et al.*, 1984). While there have been numerous reports detailing various potential virulence factors, such as proteases, haemolysins, pili and siderophores (Kooi *et al.*, 1994; Nakazawa *et al.*, 1987; Hutchison *et al.*, 1998; Sokol, 1986; Darling *et al.*, 1998), the specific molecular mechanisms that allow this pathogen to

Abbreviations: AEBSEF, 4-(2-aminoethyl)benzenesulfonyl fluoride; Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; EDDHA, ethylenediamine-*N,N'*bis(o-hydroxyphenyl)acetic acid.

proliferate in the CF lung, but not healthy lungs, have yet to be elucidated. Presumably, the ability to colonize the CF lung relates to a physiological aspect of the environment favouring growth, such as the nutritional availability of an essential moiety. Our previous experience studying the haem and iron acquisition of *Haemophilus influenzae*, also a CF lung pathogen, suggests that iron availability may play a role in the colonization of the CF lung by *B. cenocepacia*. While it is known that *B. cenocepacia* produces siderophores, the role of the siderophores in obtaining iron from physiological iron sources to support growth in humans has not been examined. Our laboratory has identified and characterized a potentially secreted RTX toxin that may play a role in cytolysis (Whitby *et al.*, 2006), releasing intracellular iron sources that may satisfy the demand for iron.

Iron metabolism of the lung differs markedly from that of the rest of the body (Gutteridge *et al.*, 2001). Due to the increased possibility of iron-mediated oxidative damage to tissues at this site, free iron is rapidly sequestered and removed. Each day, the respiratory tract of humans is exposed to approximately 10 µg of soluble, airborne iron (Turi *et al.*, 2004). The risk of damage to the lung is further enhanced by the high partial pressure of oxygen and presence of inhaled micro-organisms. The main proteins involved in iron sequestration include transferrin, lactoferrin and ferritin. In the CF lung, ferritin is significantly upregulated in comparison with normal healthy lungs (Stites *et al.*, 1998, 1999; Turi *et al.*, 2004). Ferritins are a superfamily of roughly spherical, shell-like iron-storage proteins that are ubiquitous in both prokaryotes and eukaryotes. In higher organisms, each molecule consists of 24 subunits assembled into a hollow sphere with numerous pores allowing iron ingress and egress. The subunits can be either heavy or light (H or L type), having molecular masses of ~21 or ~19 kDa, respectively (Liu & Theil, 2005). The ferritins from individual tissues vary with respect to the individual number of H- or L-type subunits. Each subunit joins to its neighbouring subunits through non-covalent interactions and packs to form a hollow sphere ~8 nm in diameter, with walls that are ~1 nm thick and a combined molecular mass of approximately 474 000 kDa. Each ferritin molecule can hold approximately 4500 iron atoms, either as a sequestered form of iron or as a bioavailable store for later utilization. As such, ferritin represents a concentrated source of iron for the growth of micro-organisms. However, the protein is extremely stable, being resistant to pH levels as low as 2.0, as well as being highly thermostable and resistant to most proteases (Crichton, 1969).

Previous investigations of lung iron metabolism have shown that the CF lung has a very high ferritin level. In the study of Stites *et al.* (1999), ferritin levels in bronchial alveolar lavage (BAL) specimens of healthy humans were ~9 ng ml⁻¹, while in CF patients, the mean BAL ferritin level was 70-fold higher (mean 647 ng ml⁻¹, range 200–1200 ng ml⁻¹). In contrast, there was no apparent increase in transferrin levels between the two groups in the study. In a separate study by

Reid *et al.* (2004), evaluating the complex role of iron-regulatory cytokines and iron metabolism in the CF lung, CF sputum ferritin was recorded as ranging between 890 and 6980 ng ml⁻¹. A significant increase in ferritin levels was associated with infection of the CF lung by *Pseudomonas aeruginosa*. This study indicated that both the pro-inflammatory cytokine IL-1β and TNF-α were upregulated in the lung at the onset of infection, which increased intracellular iron homeostasis, with the net result of increasing levels of total iron and ferritin within the infected lung. Interestingly, the study also indicated that transition from an acute to a stable condition corresponded with a decrease in the total iron and ferritin levels (Reid *et al.*, 2004). However, patients with clinical decline had no associated decrease in iron and ferritin levels. Taken together, these studies show that there is sufficient ferritin in the lung to act as a potential source of iron for bacteria able to utilize it, and that the inflammatory response in the CF lung further elevates the available ferritin-bound iron.

Previous studies have shown that bacteria can acquire iron from a number of iron-binding proteins, including transferrin, lactoferrin, haemopexin, haemoglobin, haemoglobin and haemoglobin/haptoglobin complex, and human serum albumin (Morton & Williams, 1989; Morton & Stull, 2004; Jin *et al.*, 1999; Stull, 1987). However, no pathogens have been reported to directly acquire iron from ferritin. Evidence suggests indirect, host-mediated effects. For example, *Neisseria meningitidis* accelerates the degradation of ferritin via modulation of the host-cell machinery (Larson *et al.*, 2004).

To further understand host-pathogen interaction in the lung, we examined the ability of ferritin, at the physiological levels observed in CF patients, to support growth of *B. cenocepacia* J2315, and examined the molecular mechanism involved in iron acquisition from this metalloprotein complex.

METHODS

Bacterial strains and growth. *B. cenocepacia* strains J2315 (BcJ2315) (ATCC BAA-245), Pc 76 (ATCC 17765), Pc77 (ATCC 25608), Pc78 (ATCC 25609), Pc79 (ATCC 25610), Pc83 (ATCC 35130) and Pc84 (ATCC 35254) were obtained from the American Type Culture Collection, and isolates Pc856 (LMG 12614) and Pc857 (LMG 12615) were obtained from the Laboratorium voor Mikrobiologie (LMG). *B. cenocepacia* isolates K56-2, Bc7 and AU1051 were kindly supplied by P. Sokol, University of Calgary, J. Goldberg, University of Virginia, and J. LiPuma, University of Michigan, Ann Arbor, respectively. All *B. cenocepacia* isolates were routinely maintained on Luria-Bertani (LB) agar plates. *H. influenzae* strain Rd KW20 (ATCC 51907), used as a control in some experiments, was routinely maintained on chocolate agar with bacitracin (BBL) at 37 °C.

Iron sources and protease inhibitors. Human and equine ferritin, and equine haemin, were purchased from Sigma. Biotinylated ferritin was purchased from EY laboratories. Haemin was prepared as a 1 mg ml⁻¹ stock solution, as described elsewhere (Poje & Redfield, 2003).

Ethylenediamine-*N,N'*bis(*o*-hydroxyphenyl)acetic acid (EDDHA) was deferrated and prepared as described by Rogers (1973). Protease inhibitors were purchased from Sigma. Protease inhibitor cocktail (Sigma, product no. P2714), containing a mixture of water-soluble protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic and metalloproteases, contained 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin and sodium EDTA. The cocktail was resuspended in water to give a final concentration, with respect to leupeptin, of 10 μM . Individual protease inhibitors were purchased from Sigma and suspended in water to final concentrations of 2.0 M AEBSF, 1.30 mM bestatin, 1.4 mM E64, 1.0 mM leupeptin and 300 μM aprotinin. Each stock solution was filter-sterilized prior to use. A standard inoculum of BcJ2315 was prepared in LB/EDDHA with 10 μg ferritin ml^{-1} and divided into several 4 ml aliquots, to which 25, 50 or 100 μl of the protease cocktail was added, and samples were then analysed for growth (see Methods, Growth curves, below). In addition, a similar set of samples was prepared with the non-proteinaceous iron source haem (10 μg ml^{-1}) and compared to ferritin as sole iron source. In experiments examining the individual protease inhibitors, 4 ml aliquots of BcJ2315 were prepared and the inhibitors added in 1, 5 and 10 μl quantities.

Growth curves. Growth studies were performed as follows, using the Bioscreen C Microbiology Reader (Oy Growth Curves AB). BcJ2315 was grown for 12–14 h on LB agar and used to inoculate 10 ml LB broth containing 100 μM EDDHA, which was incubated for 4 h at 37 °C with shaking (175 r.p.m.). Preliminary studies indicated that BcJ2315 was iron starved in LB broth with the addition of 100 μM EDDHA. The 4 h cultures were pelleted by centrifugation and resuspended to an OD_{605} of 0.5 in fresh LB broth. One millilitre of the bacterial suspension was diluted with 5 ml LB, and this final bacterial suspension was used to inoculate fresh LB (0.1%, v/v, inoculum) to give an approximate initial concentration of 200 000 c.f.u. ml^{-1} in media containing various defined supplements. Growth curves were performed in 300 μl volumes with five replicates for each growth condition in each individual experiment. Experiments were repeated a minimum of three times. OD_{600} measurements were obtained at 30 min intervals with the Bioscreen C set to incubate at 37 °C with constant shaking (machine setting at 'low'). Growth curves with *H. influenzae* were performed identically, except that the inoculum was prepared as previously described (Morton *et al.*, 2004). For each condition examined, growth curves were repeated a minimum of three times. Data from a single experiment (mean of five replicates) are displayed in each figure.

Ferritin degradation assay. A standard inoculum of BcJ2315 was prepared as described above and used to seed a 60 ml culture of LB. Following 48 h growth, the bacteria were pelleted and the culture media filter-sterilized. Biotinylated horse ferritin was added to a final concentration of 5 μg ml^{-1} and the culture split into three aliquots of 20 ml. One aliquot was immediately processed to capture the biotinylated ferritin by mixing with 25 μl streptavidin-linked agarose beads, with shaking for 1 h. The beads were pelleted by centrifugation for 5 min at 13 000 g, the supernatant was aspirated, and the beads were washed once with 1 ml PBS. After a second centrifugation to collect the beads, the supernatant was removed and the beads were mixed with 25 μl SDS-PAGE loading buffer prior to applying the sample to an SDS-PAGE gel. To one of the remaining two samples, AEBSF was added to a final concentration of 200 μM , and an equal volume of sterile water was added to the remaining sample. The latter two samples were incubated at 37 °C for 48 h, after which time biotinylated ferritin, and any degradation products, were captured as described above. All samples were analysed in adjacent wells of a 12% polyacrylamide gel.

Comparative genomics. We examined the preliminary BcJ2315 predicted genes (http://www.sanger.ac.uk/Projects/B_cenocepacia/) for putative extracellular serine proteases. The analysis was performed

using the program HMMER (<http://hmmer.wustl.edu>) to search the Pfam sequence database (Bateman *et al.*, 2004). In particular, the search focused on proteins within the Peptidase_S8 family (PF00082), which includes a number of well-described extracellular serine proteases related to subtilase from *Bacillus subtilis* (Smith *et al.*, 1966). Predicted proteins with significant matches to serine protease families were then examined to determine whether an autotransporter domain (PF03797) or a signal peptide (<http://www.cbs.dtu.dk/services/SignalP/>) was present.

Statistical comparisons. The Kruskal–Wallis test was used to compare growth between separate conditions. In some cases the analysis was performed over the entire growth curve and in others only over the period of active growth, as specified above. Analyses were performed using Analyse-It for Microsoft Excel, version 1.71 (Analyse-It Software). $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Selection of iron-starved media

Initial experiments were performed to establish a suitable medium for the growth of *B. cenocepacia* that could be manipulated to provide iron-replete and -deplete growth conditions. Previously we have grown *B. cenocepacia* in LB broth, and this was used with increasing concentrations of EDDHA as an iron chelator. Since BcJ2315 produces siderophores and utilizes siderophore-bound iron, it was not clear if this chelator would limit iron availability within the media. To avoid variation in iron content among different batches of LB, a single large batch of LB broth was prepared and used throughout these experiments. A standard inoculum of BcJ2315 was prepared and used to inoculate LB broth containing 100, 200, 400, 600, 800 and 1000 μg EDDHA ml^{-1} , and the growth profile determined over a 40 h incubation period (Fig. 1). An EDDHA concentration of 600 μg ml^{-1} inhibited the growth rate and final concentration of BcJ2315 in LB broth sufficiently to permit the assessment of the utilization of any potential exogenous iron sources. To ensure that the depression of growth by EDDHA was not due to toxic effects, the experiment was repeated with 150 mM iron (FeCl_2) added to LB with 600 μg EDDHA ml^{-1} and the growth of BcJ2315 observed. The resulting growth curves (data not shown) indicated that addition of the supplemental iron source returned growth to the same level as that observed in EDDHA-free media. Thus, a concentration of 600 μg EDDHA ml^{-1} was chosen for all further growth studies.

Growth of *B. cenocepacia* in media containing ferritin as sole available iron source

To evaluate the ability of BcJ2315 to utilize ferritin as sole iron source, we compared growth profiles in LB, LB/EDDHA and LB/EDDHA containing ferritin. Both human and horse ferritin were examined. Following analysis of the data, it was apparent that both human and horse ferritin supported identical growth of BcJ2315 (data not shown). Due to the ready availability of horse ferritin, and the high degree of homology with human ferritin (87 and 91% identity of light and heavy chains, respectively), horse

Fig. 1. Effect of EDDHA on the growth of BcJ2315. Growth in LB (●) and LB containing EDDHA at: 100 µg ml⁻¹ (○), 200 µg ml⁻¹ (▼), 400 µg ml⁻¹ (▽), 600 µg ml⁻¹ (■), 800 µg ml⁻¹ (□) or 1000 µg ml⁻¹ (◆). Error bars represent the standard deviation of the values from five duplicate wells.

ferritin was used for the detailed studies of concentration dependence and mechanism of utilization. Fig. 2 shows the growth profiles of BcJ2315 grown in the presence of various concentrations of horse ferritin as sole iron source. From these data it is clear that exogenous ferritin can be utilized by BcJ2315 as a sole iron source to support growth. At physiological levels of ferritin found in healthy human lungs (0.1 µg ml⁻¹) there was no apparent growth (data not shown). Examination of the resulting curves indicated that both maximal growth rate and maximal cell yield at the highest concentration of ferritin examined (20 µg ml⁻¹) were significantly reduced compared with iron-replete LB media. In addition, we observed an apparent extension of the lag period prior to growth. These experiments were

repeated with a further 12 distinct isolates of *B. cenocepacia*. Isolates Pc76, Pc78, Pc79, Pc83, Pc856, Pc857 (LMG 12615), K56-2, Bc7 and AU1051 all showed repression of growth by 600 µg EDDHA ml⁻¹ which was reversed by the addition of 10 µg ferritin ml⁻¹ (data not shown). Using these conditions, there was no growth with isolates Pc77 and Pc84. Further studies with the latter isolates indicated that they were more sensitive to the EDDHA concentration. Experiments repeated with 150 µg EDDHA ml⁻¹ showed a decrease in growth profiles and also showed that the growth rate was elevated by the addition of ferritin. In all cases, growth curves were similar to those determined for BcJ2315, shown in Fig. 2. Thus, all isolates of *B. cenocepacia* studied were able to use ferritin as a sole iron source.

Fig. 2. Growth of BcJ2315 with ferritin as the sole iron source. Growth in LB (●), LB/EDDHA (○), and LB/EDDHA containing horse ferritin at: 2.5 µg ml⁻¹ (▼), 5.0 µg ml⁻¹ (▽), 10.0 µg ml⁻¹ (■) or 20.0 µg ml⁻¹ (□). Error bars represent the standard deviation of the values from five duplicate wells.

To ensure that the perceived growth was entirely due to utilization of the iron contained within ferritin, and not due to iron contamination or ferritin autolysis, control experiments using *H. influenzae* were performed. *H. influenzae* strain Rd KW20 was unable to grow in brain heart infusion broth supplemented with protoporphyrin IX and ferritin as the sole iron source. This demonstrated that the ferritin was not contaminated with free iron, nor was it undergoing autolysis (data not shown).

B. cenocepacia obtains iron from ferritin by proteolysis

To further define the ability of BcJ2315 to utilize ferritin we examined the possible mechanism of acquisition. It is known that BcJ2315 produces siderophores that may assist in iron assimilation; however, it is unlikely that siderophores penetrate ferritin to release iron. One possible scenario is that the ferritin molecule is physically degraded by a proteolytic process. To examine this mechanism of iron release, we attempted to block ferritin-iron utilization by BcJ2315 using a broad-range protease inhibitor cocktail containing AEBSF, EDTA, bestatin, E64, leupeptin and aprotinin that targets serine, cysteine and metalloproteases. To control for possible toxic effects of the inhibitor cocktail, we also examined the growth of BcJ2315 in LB/EDDHA with haem as sole iron source. Previously, we have shown that haem satisfies the *B. cenocepacia* iron requirement (P. W. Whitby and others, unpublished observations); accordingly, haem was used, since it represents a non-proteinaceous iron source. The protease inhibitor cocktail had no significant effect on BcJ2315 growth in haem-supplemented conditions ($P=0.66$, $P=0.38$ or $P=0.12$ for haem supplemented with 25, 50 or 100 μl protease inhibitor cocktail, respectively). However, a statistically significant decrease in the growth of BcJ2315 with 10 μg ferritin ml^{-1} as the sole iron source was observed ($P=0.039$ for 25 μl and $P<0.0001$

for 50 and 100 μl protease inhibitor cocktail) (Fig. 3). Examination of the resulting curves indicated that the lag phase increased with increasing concentration of protease inhibitor cocktail, although the growth rate was unaffected. These results suggest that the cocktail prevented BcJ2315 from using ferritin-bound iron until a threshold level of *de novo* synthesized protease was achieved. Alternatively, ferritin may have been unstable under our conditions and EDTA present in the protease cocktail may have bound any released iron and changed the growth profiles. To examine the latter possibility, BcJ2315 was grown in LB/EDDHA/ferritin with an EDTA concentration equivalent to that used in the protease cocktail. No significant impact on the growth profile was observed (data not shown). Together these findings strongly suggest that the utilization of ferritin iron by BcJ2315 is protease-mediated.

To further investigate the apparently protease-dependent utilization of ferritin by BcJ2315, the effect of the specific protease inhibitors in the cocktail was examined. As in the experiments described above, non-specific effects of the protease inhibitors were also determined for BcJ2315 grown in LB/EDDHA supplemented with haem. None of the protease inhibitors showed an effect on growth in LB/EDDHA supplemented with haem across the concentrations examined (data not shown). With the exception of AEBSF, none of the inhibitors showed any effect on the growth profiles in media supplemented with ferritin as the sole iron source (data not shown). In contrast, increasing concentrations of AEBSF abrogated the ability of BcJ2315 to grow in medium supplemented with ferritin as iron source in a dose-dependent manner (Fig. 4). AEBSF is an irreversible inhibitor of serine proteases, and is stable in aqueous solution. Thus, the growth curves obtained using this compound are consistent with the inhibition of *de novo* synthesized protease until the level of protease inhibitor in

Fig. 3. Effect of a protease inhibitor cocktail on ferritin utilization by BcJ2315. Growth in LB/EDDHA (●), LB/EDDHA/ferritin (○), and LB/EDDHA/ferritin containing protease inhibitor cocktail solution at: 6.3 $\mu\text{l ml}^{-1}$ (▼), 12.5 $\mu\text{l ml}^{-1}$ (▽) or 25 $\mu\text{l ml}^{-1}$ (■). Error bars represent the standard deviation of the values from five duplicate wells.

Fig. 4. Effect of AEBF on ferritin utilization by BcJ2315. Growth in LB/EDDHA/ferritin (●), and LB/EDDHA/ferritin containing AEBF at: 50 µM (○), 250 µM (▼) or 500 µM (▽). Error bars represent the standard deviation of the values from five duplicate wells.

the medium is exhausted, at which point the bacteria utilize the available ferritin to satisfy their growth requirements.

To further examine the possibility of proteolytic cleavage of ferritin by BcJ2315, the effect of incubation of biotinylated ferritin with a growing culture of BcJ2315 over a 4-day period was analysed. Biotinylated ferritin was chosen for these experiments because this tag, on intact ferritin and/or its degradation products, facilitates purification using streptavidin-linked agarose beads. We first established that biotinylated ferritin supported growth of BcJ2315 in a manner similar to non-biotinylated ferritin (data not shown). Following purification of the biotinylated ferritin and any potential degradation products over a 4-day period, the samples were analysed on a 12% polyacrylamide gel and stained with Gelcode Blue Stain reagent (Pierce). Examination of the destained gel (Fig. 5) showed two distinct bands for the time zero sample. The lower band corresponded to approximately 20 kDa, the size of the ferritin subunits. The higher band presumably corresponded to native intact ferritin composed of 24 subunits, although this band did not correspond to the predicted molecular mass of the complex. This may reflect the partial denaturation of the complex and lack of resolution of the gel in this region. It is known that ferritins are resistant to high temperature, and the 5 min boiling step employed in sample preparation is probably insufficient to dissociate all the ferritin macromolecules. Examination of the band corresponding to the ferritin subunits showed a distinct decrease in band intensity over the duration of the experiment. Similarly, the high-molecular-mass complex showed a corresponding decrease in intensity with incubation time. Examination of the remainder of the gel indicated multiple, discrete degradation products located between the high- and low-molecular-mass bands that peaked in the 72 h sample. These may represent partially intact complexes that have

undergone limited proteolysis. The intensity of these bands decreased in the 96 h sample; however, a smaller, more diffuse band appeared at the bottom of the gel in this sample, corresponding to small fragments of protein. Control samples, containing sterile LB incubated over the same time period, showed no differences in the banding patterns over a 4-day period (data not shown). This experiment clearly shows the proteolytic degradation of ferritin by a growing culture of BcJ2315. To determine if the protease is

Fig. 5. Proteolytic degradation of ferritin by BcJ2315. Samples (1 ml) of a growing culture of BcJ2315 in LB/EDDHA with 20 µg biotinylated ferritin ml⁻¹ were taken at 0, 24, 48, 72 and 96 h (lanes A–E, respectively) and the ferritin was purified on agarose beads. Lane F contains molecular mass markers, sizes as indicated. The dark-staining band at the top corresponds to intact ferritin, while the second major band at approx. 20 kDa represents the subunit. Lanes D and E show partial degradation products (denoted with a line alongside lane D).

secreted or cell associated, the experiment was repeated with cell-free supernatant from a 48 h culture of BcJ2315 grown in LB. In the absence of AEBSF, degradation of ferritin was observed. Neither LB alone nor supernatant with added AEBSF showed ferritin degradation (data not shown), thus indicating that the proteolytic activity is secreted into the growth medium.

These results clearly indicate the role of a serine protease in the acquisition of iron from ferritin by BcJ2315. Presumably, since the EDDHA would bind any iron released into the medium, this must occur at or near the cell surface. With these two points as search criteria, we examined the genome of BcJ2315 to identify surface-associated serine proteases. The results of this study indicated that the genome of BcJ2315 contains four predicted proteins that are candidates for an extracellular serine protease. These are encoded by the putative ORFs BCAM0922, BCAM0957, BCAM1744 and BCAS0405. Each of these predicted proteins contained a putative signal peptide sequence, and comparison with the Pfam protein database revealed that all were homologous to the subtilase family of serine proteases (PF00082). Future studies will be performed to further elucidate the role, if any, of these proteases in the utilization of ferritin-bound iron.

In summary, this study shows that isolates of *B. cenocepacia* can use iron sequestered in ferritin in a protease-dependent manner. The protease(s) involved is a serine protease and is probably extracellular, being either secreted into the growth media or present on cell surfaces. The ability to use ferritin may contribute to the ability of *B. cenocepacia* to colonize and persist in the CF lung.

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