The effect of recombinant human lactoferrin on growth and the antibiotic susceptibility of the cystic fibrosis pathogen \textit{Burkholderia cepacia} complex when cultured planktonically or as biofilms

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\textbf{Objectives:} The cystic fibrosis (CF) pathogen \textit{Burkholderia cepacia} complex (Bcc) is innately resistant to antibiotics and the development of effective therapeutic strategies to treat these infections is a major challenge. The objectives of this study were to investigate the effects of recombinant human lactoferrin (rHL) on planktonic and biofilm cultures of Bcc organisms and to establish whether lactoferrin alters the susceptibility of these cultures to a range of antibiotic therapies.

\textbf{Methods:} Planktonic and biofilm cultures of strains representative of three species of Bcc, \textit{Burkholderia multivorans}, \textit{Burkholderia cenocepacia} and \textit{Burkholderia dolosa}, were exposed to 0–900 mg/L lactoferrin over 0–48 h. Growth was determined using both spectrophotometric and plate counting methods. The ability of these strains to form and develop biofilms \textit{in vitro} was also examined. Antimicrobial susceptibility in the presence/absence of lactoferrin was assessed using conventional MICs and a modified method was used to determine biofilm susceptibility to various antibiotics.

\textbf{Results:} We found that physiological concentrations of lactoferrin inhibited the growth of both planktonic and biofilm cultures of Bcc \textit{in vitro}. The addition of lactoferrin to rifampicin enhanced the antibiotic susceptibility of the Bcc strains when grown planktonically and as biofilms.

\textbf{Conclusions:} The present study demonstrates the growth inhibitory and antibiotic activity of rHL against different species of Bcc. Furthermore, the enhanced susceptibility of both planktonic and biofilm cultures to rifampicin in the presence of lactoferrin offers the potential for novel uses of antibiotics in combination with lactoferrin to treat Bcc infections in CF patients.

Keywords: bacterial biofilms, biofilm inhibitory concentrations, rifampicin

\textbf{Introduction}

Chronic bacterial colonization of the major airways and associated inflammation are the major cause of morbidity and mortality in patients with cystic fibrosis (CF). \textit{Burkholderia cepacia}, a Gram-negative bacterium found in the environment, has recently emerged as an opportunistic pathogen in CF patients.\textsuperscript{1} \textit{B. cepacia} complex (Bcc) consists of at least 10 species that are phenotypically related but can be distinguished genetically.\textsuperscript{2} Although all these species have been isolated from CF patients, two of them, \textit{Burkholderia multivorans} (formerly genomovar II) and \textit{Burkholderia cenocepacia} (formerly genomovar III),\textsuperscript{2,4} are the most prevalent among CF patients. Bcc organisms are inherently resistant to antimicrobial treatment\textsuperscript{2} and are frequently associated with rapid decline in CF patients. In addition, it has been reported that Bcc organisms...
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form biofilms both in vitro and in vivo. The ability of Bcc organisms to form and develop biofilms may contribute to their resistant nature to antimicrobial agents and offer further protection from host defence.

Lactoferrin, an iron-binding glycoprotein, is one of the most abundant antimicrobial proteins found in airway secretions. It is also found in many other external secretions such as tears and saliva and is a major component of neutrophil-specific granules. Human lactoferrin has been found at high concentrations (~0.9 g/L) in the sputa of CF patients colonized with Pseudomonas aeruginosa. Many studies have indicated that lactoferrin plays an important role in human defence mechanisms against bacteria, fungi and viruses. Lactoferrin displays both bacteriostatic activity through its ability to sequester iron which is essential for microbial growth and bactericidal activity by the direct binding of lactoferrin to bacterial cell wall components such as lipopolysaccharide (LPS), causing changes in the cell wall permeability and thus increasing killing of the bacteria by other antimicrobials. Lactoferrin has been shown to inhibit the attachment of bacteria to cells and intracellular invasion. A recent report of Singh et al. demonstrated that subinhibitory concentrations of lactoferrin inhibited the formation and development of P. aeruginosa biofilms. Another study reported that cleavage of lactoferrin by cathepsin, a lysosomal protease found in the lungs of CF patients, resulted in loss of both its antimicrobial and antibiofilm activities against P. aeruginosa. In addition, the presence of lactoferrin has been demonstrated to increase the susceptibility of various bacteria to a range of different antibiotics.

Materials and methods

Bacterial strains

B. multivorans strains, LMG 13010 and ATCC 17616, B. cenocacei strains, J2315 and BC7, and B. dolosa strains, LMG 18941 and LMG 18943, were obtained from the BCCM/LMG, Belgium Coordinated Collections of Microorganisms/Laboratorium Microbiologie Ghent. These strains were all from the panel of Bcc strains identified by the International Burkholderia cepacia Working Group. Unless otherwise stated, the strains were routinely cultured on BSCA or Luria–Bertani (LB) broth at 37°C.

Antibiotics

Five antibiotics representing agents from the β-lactam, aminoglycoside, rifampicin and fluoroquinolone classes used to clinically treat CF infections were used in these studies. Amikacin, rifampicin and tobramycin were purchased from Sigma (Dorset, UK). Ceftazidime (FortumTM, GlaxoSmithKline) and ciprofloxacin (Ciproxin®, Bayer) were obtained commercially. rHL was kindly provided by Agennix (TX, USA).

Measurement of growth of Bcc strains in the presence of rHL

Growth studies were performed as follows: overnight cultures of Bcc strains were diluted and 1 × 10^8 bacteria were added to LB broth containing varying concentrations of rHL (0, 50, 100, 500 and 900 mg/L). Cultures were incubated at 37°C, shaking for 0, 4, 24 and 48 h. Bacterial growth was assessed using both spectrophotometric and plate methods. The optical density (OD) of each culture was read at 600 nm. In addition, each culture was serially diluted and 50 µL plated out on LB agar plates and incubated at 37°C for 48 h. Bacterial density (cfu/mL) was then determined.

Determination of MICs by broth microdilution

MICs were determined by broth microdilution. Briefly, overnight cultures (10 mL) of Bcc strains were grown to mid-log phase by inoculating in LB broth (100 mL) and incubating at 37°C until the OD at 600 nm (OD₆₀₀) reached approximately 0.6. Strains were then diluted to 1 × 10^8 cfu/mL and seeded at 100 µL/well in 96-well plates. Stock solutions of each antibiotic to be tested were serially diluted using cation-adjusted Mueller–Hinton broth (CAMHB) (100 µL/well). Samples were incubated with the respective antibiotic in the presence or absence of 900 mg/L rHL at 37°C for 24 h, shaking and the OD₆₀₀ read. The MIC was read as the concentration of antibiotic that inhibited visible growth of the strain. The MIC for each antibiotic/strain was carried out in triplicate in three independent experiments. A significant change was considered to be a shift in susceptibility of more than two doubling dilutions in either direction.

Biofilm formation assay

Biofilm formation was assessed by determining the ability of Bcc strains to form biofilms in microtitre plates in vitro, as described previously. The bacterial concentration (cfu/mL) was determined for each strain and strains were diluted to 10^6 cfu/mL. Bacteria were seeded at 10^6 cfu/mL, 100 µL/well in a 96-well polypropylene microtitre plate (Costar #3790; Fisher) in media containing a range of concentrations of rHL (0, 20, 50, 100, 500 and 900 mg/L) and incubated at 37°C for 24 and 48 h. Wells containing sterile growth
medium were used as negative controls. Following incubation, biofilm quantification was determined, as described previously.\textsuperscript{23}

\textbf{Determination of the effect of rHL on established Bcc biofilms}

The effect of rHL on established biofilms was assessed by allowing Bcc strains to form biofilms on pegs in a modified 96-well plate, as described previously.\textsuperscript{24} Briefly, overnight cultures of Bcc were diluted to $1 \times 10^5 \text{ cfu/mL}$ and seeded at 100 $\mu$L/well in 96-well plates. The bacterial biofilms were formed by immersing the pegs of a modified polystyrene microtitre lid (Nunc TSP system, Naperville, IL, USA) into the biofilm growth plate and incubating at 37°C for 24 h. The pegs were rinsed with sterile water and placed onto 96-well plates containing different concentrations of rHL (0, 20, 50, 100, 500 and 900 mg/L) and incubated at 37°C for 24 h without agitation. Following incubation, Bcc biofilms were washed three times with sterile water. Biofilms on the pegs were stained with 0.1% (w/v) crystal violet for 30 min, washed again three times with sterile water and allowed to air-dry for 60 min. The crystal violet-stained Bcc biofilm was solubilized using 95% (v/v) ethanol and the absorbance read at 590 nm. Biofilm development assays were carried out in three independent experiments in triplicate for each strain/rHL concentration.

\textbf{Determination of biofilm inhibitory concentrations by biofilm antibiotic susceptibility assay}

Biofilm antibiotic susceptibility assays were performed, as described previously,\textsuperscript{24} using a 96-well plate format allowing biofilms to form on pegs. Bcc strains were cultured overnight in LB broth (10 mL), inoculated into LB broth (100 mL) and then grown at 37°C until an OD\textsubscript{600} of approximately 0.6 was reached. Cultures were diluted to give a final concentration of $1 \times 10^5 \text{ cfu/mL}$ and 100 $\mu$L was transferred to wells of flat-bottom 96-well microtitre plate (Nunc). Bcc biofilms were formed on pegs following 24 h of incubation. The biofilms were then incubated with each antibiotic (serially diluted) in the presence or absence of rHL (900 mg/L) for a further 24 h. Following incubation with antibiotics, the pegs were rinsed three times with sterile water and placed in a fresh 96-well plate containing 100 $\mu$L of antibiotic-free CAMHB. The bacteria were removed from the pegs by sonicating the plates for 5 min on high with a Decon FS1 006 sonicator. The peg lids were discarded and replaced with standard lids. The OD\textsubscript{600} was measured before and after incubation at 37°C for 6 h. Biofilm susceptibility assays were carried out in three independent experiments in triplicate for each antibiotic/strain. An OD\textsubscript{600} value of <0.05 was regarded as absence of biofilm. The biofilm inhibitory concentration (BIC) value was read as the concentration of antibiotic that inhibited visible growth of the strain. A significant change was considered to be a shift in susceptibility of more than two doubling dilutions in either direction.

\textbf{Statistical analysis}

Values are expressed as mean ± SD of three separate experiments, carried out in triplicate. Statistical analysis was performed using one-way analysis of variance using the Holm–Sidak test. $P$ values less than 0.05 were considered significant.

\textbf{Results}

\textbf{Effects of rHL on growth of Bcc strains}

Many commercially available lactoferrin preparations are of relatively low purity and have undefined iron saturation levels. To overcome these issues, rHL with a purity level of 96% and an iron saturation level of 7.2% was chosen for these studies.

The bacteriostatic and bacteriocidal effect of lactoferrin has been previously reported for other bacteria, such as Escherichia coli. We investigated the effect of rHL on the growth of three different Bcc strains, representative of three species. OD readings of cultures demonstrated significant growth inhibition for all three Bcc strains in the presence of 500 and 900 mg/L rHL at 24 and 48 h (Figure 1a, c and e). Growth was also monitored by culture methods and the cfu/mL were determined at various time points (Figure 1b, d and f). High concentrations of rHL (500 and 900 mg/L) demonstrated a bacteriocidal effect on B. dolosa LMG 18941 (Figure 1f). However, in LMG 13010 and BC7 cultures (Figure 1b and d), it is evident that the cells recovered in culture without lactoferrin, indicating a bacteriostatic rather than a bacteriocidal effect. Lower concentrations of rHL (20, 50 and 100 mg/L) did not have either a bacteriocidal or bacteriostatic effect on the three Bcc strains tested (data not shown). In summary, physiological concentrations of rHL were bacteriocidal for the B. dolosa strain and bacteriostatic for both the B. multivorans and B. cenocepacia strains.

\textbf{Effect of rHL on MICs for Bcc strains}

A summary of the MIC data for the five antibiotics tested in the presence or absence of 900 mg/L rHL is presented in Table 1. Antibiotic susceptibility to rifampicin was enhanced for all the strains in the presence of lactoferrin. All six Bcc strains were not susceptible to rifampicin on conventional MIC tests. However, all but two strains (ATCC 17616 and LMG 18943) had significantly lower MIC values in the presence of rHL (4–8-fold).

The two aminoglycosides tested, amikacin and tobramycin, had comparable MIC values in the presence of 900 mg/L rHL, indicating that rHL does not affect the susceptibility of the Bcc organisms to aminoglycosides. Cefazidime showed no significant difference between the MIC values when incubated with rHL, except for the B. cenocepacia strain, BC7. When this strain was incubated with rHL, the MIC of cefazidime was significantly lower (16-fold) (Table 1). When the Bcc strains were incubated with ciprofloxacin, a fluoroquinolone, no change in susceptibility of the Bcc strains to form biofilms in vitro was investigated. Bcc strains were incubated in the presence of differing concentrations of rHL for 24 and 48 h and biofilms were allowed to form. Following 24 h of incubation, there was a significant decrease in the biofilm formation in the presence of both 500 and 900 mg/L rHL for three of the six Bcc strains tested ($P = 0.018$ for B. multivorans LMG 13010, $P = 0.023$ for B. cenocepacia BC7 and $P = 0.02$ for B. dolosa LMG 18941) (Figure 2a, d and e).
The remaining three Bcc strains (\textit{B. multivorans} ATCC 17616, \textit{B. cenocepacia} J2315 and \textit{B. dolosa} LMG 18943) also demonstrated decreased biofilm formation in the presence of higher concentrations of rHL when compared with their respective controls (Figures 2b, c and f); however these results were not statistically significant. Furthermore, the antibiofilm activity of rHL...
measured after 24 h of incubation was not observed following 48 h of incubation (Figure 2a–f). This finding indicates that all the Bcc strains were capable of recovering from the antibiofilm activity of the rHL and at 48 h, the Bcc biofilms in the presence of rHL were comparable with those of controls.

The effect of rHL on established Bcc biofilms

Having demonstrated the inhibitory effect of rHL on biofilm formation by Bcc strains, we subsequently investigated the effect of rHL on pre-formed Bcc biofilms. Bcc biofilms were allowed to form on pegs for 24 h and then incubated for a further 24 h in the presence of varying concentrations of rHL (0, 20, 50, 100, 500 and 900 mg/L). Consistent with the data on biofilm formation, in the presence of both 500 and 900 mg/L of rHL, Bcc biofilms did not mature further and were in fact disrupted in the presence of rHL (Figure 3a–f).

The effect of rHL on BICs of Bcc strains

The BIC data for the antibiotics tested in the presence or absence of 900 mg/L rHL are summarized in Table 1. Consistent with the MIC data, the most notable difference in the effect of rHL on the BICs of Bcc strains was observed for the antimicrobial agent rifampicin. Five of the six Bcc strains tested displayed significant increases in susceptibility to rifampicin in the presence of rHL when compared with the observed BIC in the absence of rHL. Indeed, in some cases, there was no biofilm detected when the Bcc strains (B. cenocepacia BC7 and B. dolosa LMG 18941) were incubated with both rifampicin and rHL (Table 1).

The susceptibility of all the Bcc strains to ciprofloxacin was comparable regardless of the presence of rHL (Table 1). However, one exception was for the B. dolosa strain LMG 18943, in which a significant increase in susceptibility (8-fold lower) to ciprofloxacin was observed in the presence of 900 mg/L rHL when compared with the standard MIC value of the agent.

The two B. dolosa strains (LMG 18941 and LMG 18943) exhibited significantly enhanced susceptibility to the aminoglycoside amikacin in the presence of 900 mg/L rHL (Table 1), although this increase in susceptibility was not observed for the other aminoglycoside, tobramycin. When the B. multivorans (LMG 13010 and ATCC 17616) and B. cenocepacia (J2315 and BC7) biofilms were incubated with either aminoglycoside in the presence of rHL, there were no notable differences in the observed BICs when compared with controls (Table 1).

Discussion

The iron-complexing ability of lactoferrin is central to its role as a growth inhibiting agent. Many microorganisms require iron for growth and their cellular structure loses its integrity under iron-deprived conditions and necrosis ensues. However, human lactoferrin has also been shown to promote the growth of one of the predominant bacteria of the gut of breast-fed infants, Bifidobacterium species.29 Our results indicate that human lactoferrin is bacteriostatic and not bacteriocidal against two of the three Bcc species (B. multivorans and B. cenocepacia). The recovery of growth in these cultures may be as a result of siderophore production, by absorption of haem iron or by direct removal of iron from lactoferrin by the Bcc organisms. In contrast to these two species, when B. dolosa was grown in the presence of rHL (500 and 900 mg/L), a significant bacteriocidal effect was evident.
which suggests that *B. dolosa* is more vulnerable to the effects of rHL when compared with the other Bcc species tested. *B. dolosa* is not as prevalent in CF as either *B. multivorans* or *B. cenocepacia*, which may in part be due to the high levels of lactoferrin in the CF lung. Inhibition of Bcc growth for all species examined occurred with lactoferrin in the physiological concentration range (500–900 mg/L) unlike the lower levels of 100 mg/L previously reported to be
effective in inhibiting growth of *P. aeruginosa*,\(^\text{16}\) providing further evidence of the fastidious nature of Bcc organisms.

Although infections with Bcc are less common than *P. aeruginosa* in CF patients, the consequences of Bcc infections are significantly worse.\(^\text{30}\) Clinically, these patients will have a poorer prognosis and their eligibility for lung transplant is a contentious issue. One of the most significant characteristics of Bcc is its broad resistance to antibiotics. The ability of Bcc strains to form biofilms *in vivo* in the CF lung may further contribute to their inherent antibiotic-resistant nature. The availability of iron

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**Figure 3.** Effect of rHL on established Bcc biofilms. \(10^6\) cfu/mL of Bcc strains LMG 13010 (a), ATCC 17616 (b), J2315 (c), BC7 (d), LMG 18941 (e) and LMG 18943 (f) were inoculated in 96-well plates fitted with modified lids. Bcc biofilms were allowed to form on pegs for 24 h. Following incubation, the biofilms were then incubated with varying concentrations of rHL (0–900 mg/L) for 24 h. Biofilm formation was quantified as described in the Materials and methods section. The data presented are the means and SD of three separate experiments carried out in triplicate. *\(P < 0.05\).*
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has been demonstrated to positively influence the ability of both *P. aeruginosa* and *B. cenocepacia* to form biofilms in vitro by inducing non-motile forms of the bacteria. The effects of lactoferrin on Bcc biofilms have not been reported in the literature and are the subject of this investigation. Unlike the reports for *P. aeruginosa*, which found that 20 mg/L lactoferrin could prevent biofilm formation, we observed that high concentrations (500 and 900 mg/L) of rHL were required to prevent Bcc biofilm formation. Comparable concentrations of rHL were found to retard planktonic growth of these strains, which is likely to contribute to the reduced biofilm formation levels in the presence of rHL. However, Bcc biofilms overcame the antibiofilm effects of rHL within 48 h. Factors such as lactoferrin saturation, increased production of siderophores, quorum-sensing molecules and increased exopolysaccharide levels may contribute to the restored biofilm formation at 48 h. As with the planktonic cultures, *B. dolosa* biofilms were more susceptible to rHL when compared with the other Bcc species (*B. multivorans* and *B. cenocepacia*), demonstrating a greater vulnerability of this species to the iron-binding protein.

The increased susceptibility to antibiotics in the presence of lactoferrin has been described previously for Bcc, other CF pathogens and also other organisms. In most of these studies, the effect of lactoferrin on increased sensitivity of the bacteria occurred with the antibiotic rifampicin, an antibiotic usually used in combination with another agent because of the high rate of resistant mutants, and not routinely used in the treatment of Bcc infections in CF patients. We found increased susceptibility for some strains from each of the three species of Bcc to rifampicin in the presence of rHL, which corroborates previous findings for this antibiotic. In a previous report, nine Bcc strains, two of which were identified as UK-epidemic strains, were examined, but no other information regarding the speciation of these strains was given. In this study, six strains representative of three different Bcc species were assessed, three of which showed increased susceptibility to rifampicin. Significantly, enhanced ceftazidime susceptibility was also observed against the *B. cenocepacia* epidemic strain BC7, which is of interest given the virulent nature of this strain. Overall, however, the lactoferrin effect on antibiotic efficacy in planktonic cultures is most evident with rifampicin. Given these MIC data and those previously published, rifampicin may have potential as an adjunctive therapy in the treatment of Bcc infections.

There are no reports in the literature regarding the effect of human lactoferrin on the antibiotic susceptibility of Bcc biofilms. To investigate the impact of iron depletion on Bcc biofilms, we exposed pre-formed biofilms to rHL and showed that Bcc biofilms were susceptible to the antibiotic effects of rHL at physiological concentrations. Subsequently, we examined the effect of rHL on the antibiotic susceptibility of Bcc biofilms and demonstrated that rHL enhances the antibiotic susceptibility of Bcc biofilms to certain antibiotics. Consistent with the findings for the MIC, rHL also enhanced the susceptibility of all Bcc biofilms to the agent rifampicin. For the other agents tested, there was no significant increase in antibiotic susceptibility in the presence of rHL. A study by Singh *et al.* examined whether iron chelation, which limits biofilm formation and development, could also affect an important feature of biofilm formation, namely, antimicrobial resistance. We found that, in some cases, the combined effect of rHL and antibiotic treatment completely removed the Bcc biofilm, e.g. for *B. dolosa* LMG 18941, in the absence of rHL, the BIC for amikacin was 32–64 mg/L, whereas in the presence of rHL, no biofilm was detected. These results suggest a possible synergistic effect between lactoferrin and the antibiotics in the elimination of Bcc biofilms.

Biofilm-based sensitivity testing better mimics the physiology of airway infection in CF patients. Studies examining biofilm susceptibility have demonstrated reduced susceptibility to a range of antimicrobial agents when compared with planktonic cultures. This is the first study to show that rHL enhances the rifampicin antibiotic susceptibility of Bcc biofilms formed by five strains, representative of three species within this complex. Given that both the MIC and BIC of rifampicin for all the Bcc strains tested were lower in the presence of lactoferrin suggests that this particular antibiotic in combination with lactoferrin has potential as an effective alternative for the treatment of Bcc infections in CF patients.

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Transparency declarations

None to declare.

References


Escherichia coli
Antimicrob Agents Chemother
Stenotrophomonas maltophilia

055B5 lipopolysaccharide.


