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Bovine lactoferrin interacts with cable pili of *Burkholderia cenocepacia*

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Abstract In this study we evaluated the ability of lactoferrin, the most abundant antimicrobial protein in airway secretions, to bind the surface structures of a *Burkholderia* strain cystic fibrosis-isolated. *Burkholderia cenocepacia* is a gram-negative bacterium involved as respiratory pathogen in cystic fibrosis patient infections. This bacterium possesses filamentous structures, named cable pili that have been proposed as virulence factors because of their ability to bind to respiratory epithelia and mucin. Previously, we demonstrated that bovine lactoferrin was able to influence the efficiency of invasion of different iron-regulated morphological forms of *B. cenocepacia*. Bovine lactoferrin showed to efficiently inhibit invasion of alveolar epithelial cells by free-living bacteria or iron-induced aggregates or biofilm. Results of the present study demonstrate that bovine lactoferrin is also able to specifically bind to *B. cenocepacia* cells and show that cable pili are involved in this interaction. The attachment of bovine lactoferrin to pili led to a reduced binding of bacterial cells to mucin. Since cable pili are implicated in mediating the bacterial interactions with mucin and epithelial cells, lactoferrin binding to these structures could play an important role in neutralizing bacterial infection in cystic fibrosis patients.

Keywords Bovine lactoferrin · *Burkholderia cenocepacia* · Cable pili

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Introduction

Burkholderia cenocepacia, a member of *B. cepacia* complex (Bcc), is a gram-negative bacterium emerged as a serious respiratory pathogen in cystic fibrosis (CF) patients (Govan and Deretic 1996). Infection is often associated with severe pulmonary inflammation, and some patients develop a fatal necrotizing pneumonia and sepsis ('*cepacia syndrome*'), that causes 30% of fatal clinical outcome (Isles et al. 1984; Tablan et al. 1985). The rapid decline in CF patients is favoured also by the antimicrobial resistance developed by Bcc organisms (LiPuma et al. 2001). Intrinsic resistance to antibiotics may also enable Bcc strains to persist in the lungs

of CF patients, but the factors that establish infection are still undefined. Putative virulence determinants that have been described include cable pili, lipopolysaccharide (LPS), extracellular protease, lipase, hemolysin, a melanin-like pigment, and siderophores (LiPuma 1998; Mohr et al. 2001). The roles of these factors in lung infections in CF disease remain to be clarified, as their presence does not necessarily correlate with the severity of disease (LiPuma 1998; Speert 2001).

In the last years, several papers have been focused on bacterial interaction with CF epithelial cells. In fact, one of the most important microbial factors facilitating colonization and infection may be adhesion to host tissues, which seems to be mediated by bacterial pili (Kuehn et al. 1992; Goldstein et al. 1995). Molecular studies have identified at least five different structural pilus types; one of these has been implicated in the enhanced transmissibility of one clone of *B. cepacia* (Goldstein et al. 1995). This clone expressed the specific cable-like pilus (*Clb*) that is the only genetically well characterised putative virulence factor associated with an epidemic *B. cepacia* strain type (Sajjan and Forstner 1992, 1993; Sajjan et al. 1995). Cable pili have been proposed to facilitate binding to respiratory epithelia and mucin (Tomich and Mohr 2003, 2004) and may also play a role in mediating *B. cenocepacia* cell–cell interactions (Sajjan and Forstner 1992).

Lactoferrin (Lf) is a glycoprotein isolated from milk. It is a major component of neutrophil-specific granules and, together with lysozyme, is the most abundant antimicrobial protein in airway secretions (0.1–1 mg/ml). Bacteriostatic and bactericidal activity of lactoferrin against a broad range of gram-positive and gram-negative bacteria have been reported (Arnold et al. 1977; Orsi 2004; Valenti et al. 2004). This activity was attributed to its chelating ability towards ferric ions which are essential bacterial nutrients. Lactoferrin has also been shown to bind to lipid A (Appelmelk et al. 1994) and porins (Erdei et al. 1994), and to induce LPS release from the bacterial cell wall (Ellison et al. 1988; Ellison 1994). Moreover, several studies have demonstrated the inhibitory effect of lactoferrin on the interaction between pathogenic bacteria and host cells in vitro (Izhar et al. 1982; Longhi et al. 1993; Conte et al. 1999; Ling and Schryvers 2006).

Previously, we demonstrated that bovine lactoferrin (bLf) was able to influence the efficiency of invasion of different iron-regulated morphological forms of *B. cenocepacia*; bLf showed to efficiently inhibit invasion of alveolar epithelial cells by free-living, aggregates or biofilm bacterial forms iron-induced (Berlutti et al. 2008). To further investigate the inhibitory action of bLf against *B. cenocepacia*, we focused this study on the examination of the interaction between bLf and *B. cenocepacia*, by testing the ability of bLf to bind to bacterial surface. Results obtained indicated that bLf was able to bind to cable pili of *B. cenocepacia* and that this interaction caused the inhibition of bacteria binding to mucin.

Materials and methods

Bacterial strain and growth conditions

Burkholderia cenocepacia strain PV1 was previously characterized (ET12 lineage, genomovar IIIA, *cblA* positive, BCESM negative) (Petrucca et al. 2003). Bacteria were maintained in Brain Heart Infusion (BHI) (Difco Laboratories, MD, USA) with glycerol (25%) at −80°C and checked for purity on *Burkholderia cepacia* medium (BCM) (Oxoid LTD, England). In all experiments, Chemically Defined Medium (CDM) (Bühler et al. 1998), a medium with low iron concentration, slightly modified, was used. To favour cable expression pili, CDM (pH 6.5), was adjusted with 200 mM NaCl, according to Tomich and Mohr (2004).

Bovine lactoferrins

Lactoferrin from bovine milk (bLf) was purchased from Morinaga Milk Industries (Zama City, Japan) and deprived of endotoxin as previously described (Pietrantoni et al. 2006). BLf purity was checked according to Siciliano et al. (1999). Apo-bLf was prepared by the depletion of iron in 0.1 M citric acid, pH 2.3, containing 500 mg/l disodium EDTA, followed by exhaustive dialysis against deionized water. Iron saturated-bLF was obtained by adding adequate molar ratio of ferric ions according procedure described by Berlutti et al. (2008). The iron saturation

rates were approximately 4.5% for apo-bLf, 19.4% for native bLf, and 95.6% for iron saturated-bLf.

Anti-bacterial activity of lactoferrins

Burkholderia cenocepacia PV1 cells were cultured overnight at 37°C in CDM and then inoculated (approx 1×10^7 cells per ml) in medium with different concentrations of apo-, native, and iron-saturated bLf (from 2 to 0.0625 mg/ml). Samples were incubated overnight at 37°C with shaking (120 rpm) and the detection of the number of viable bacteria was performed by colony forming units (CFU) counts on TSA plates. To verify a putative bacteriostatic effect of lactoferrins, lactoferrin-treated bacteria were washed and allowed to growth to further 4 h in absence of the compounds.

Light microscopy visualization of aggregates

For light microscopy, stationary-phase cultures were used to inoculate (1:10 dilution) glass vials with different concentrations of lactoferrins in CDM and incubated for 4 h at 37°C with shaking. To visualize aggregates, bacterial cells were gently seeded onto glass slides and stained with May-Grunwald-Giemsa. Samples were examined by an Leitz Dialux 20 microscope at a magnification of 1000×.

Scanning electron microscopy (SEM) of aggregates

For SEM, cultures of PV1 cells growth with or without different lactoferrin concentrations were used to inoculate glass slides coated with polylisine. Control and treated cells were then fixed in cacodylate-buffered 2.5% glutaraldehyde, post-fixed in cacodylate-buffered 1% OsO_4 and dehydrated in a graded series of alcohols. After Critical Point Drying, samples were mounted onto SEM stubs with silver dag, gold sputtered, and examined by a Cambridge SE360 scanning electron microscope.

Transmission electron microscopy

For pili visualization, *B. cenocepacia* strain grown on TSA plates was inoculated in CDM with bLf (1 mg/ml) or CDM alone and grown for 4 h with shaking. The cultures were removed and allowed to settle for 8 h at room temperature without shaking. Aliquots were taken from both settled and unsettled cultures and applied onto formvar-coated grids. Excess liquid was removed by blotting. The bacterial cells were stained with an aqueous solution of 0.5% uranyl acetate and analysed by a Philips 208S transmission electron microscope at 80 kV.

For electron microscopy localization of lactoferrin, the copper-coated grids with bacteria were inverted, floated for 1 h on a drop of 10% Bovine Serum Albumin (BSA) to blocking non-specific sites and then on gold-lactoferrin (Sigma, USA) (1:20 dilution) for 1.5 h at room temperature. After extensive washing with Phosphate Buffered Saline (PBS), pH 7.4, containing 1% BSA, specimens were counter-stained with 0.5% uranyl acetate and examined with a Philips 208S transmission electron microscope at 80 kV.

Dissociation of bLf-bacterium complex

The specificity of bLf binding to bacterial cells was evaluated by treatment of bLf-bacterium complex with NaCl 2 M. Ninety-six-well plates were coated overnight at room temperature with bLf (1 mg/ml) in PBS. After blocking with 3% BSA in PBS, wells were incubated for 1 h in a humid chamber at 37°C with *B. cenocepacia* cells (grown overnight in CDM medium). 50 µl aliquots of bacterial suspensions (obtained by dilution of stationary cultures to an OD600 of 1.0) were added to lactoferrin-coated wells. Plates were washed three times with PBS wash buffer or with wash buffer supplemented with NaCl to final concentration of 2 M. The second of three washes was left in the wells for 15 min. The bound bacteria were released by the addition 0.25% Triton X-100 and enumerated by plating serial dilutions on TSA plates. Binding assays were performed in triplicate, with two independent enumerations for each well.

Semipure pili preparation

Pili enriched samples was obtained as described by Urban et al. (2005) with minor modifications. Briefly, bacteria, grown in CDM overnight with shaking, were suspended in 0.15% sodium chloride. Extracts containing pili sheared from the bacterial surface were prepared by incubation of the bacterial

suspension at 60°C for 20 min with shaking, followed by centrifugation. Supernatant was collected and immediately mixed with complete protease inhibitor cocktail (Sigma, USA) and stored at −20°C.

SDS-PAGE, western-blot, and far-western-blot

Enriched pili samples were subjected to SDS-PAGE as described by Laemmli (1970) under denaturing or non-denaturing conditions on 10% acrylamide gel. Separated proteins were then transferred from gel to nitrocellulose membranes (Bio-Rad, California, USA), using a Semidry Transfer Cell apparatus (Trans-blot, Bio-Rad, California, USA) according to manufacturer's protocol. After transfer, excess sites on the membranes were blocked with 5% skim-milk solution for 1 h, followed by washing with PBS/0.05% Tween-20 (washing solution).

For far-western blot, strips were incubated for 90 min with washing solution containing 1 mg/ml bLf. After extensive washing, membranes were first incubated with rabbit polyclonal anti-bovine lactoferrin antibodies (Hycult Biotechnology, Netherlands) (1:2,500 dilution) in a washing solution for 1 h, washed again, and then incubated for 1 h with goat peroxidase-labelled anti-rabbit IgG antibodies (Bio-Rad, California, USA) (1:3,000 dilution in washing solution). Staining was achieved by using TMB substrate kit for peroxidase (Vector Laboratories, Inc., CA), according to manufacturer's instructions. All incubations were carried out at room temperature. Non-specific anti-bLf antibody binding to bacterial proteins was verified by incubating nitrocellulose strips with anti-bLf rabbit antibodies (Hycult Biotechnology, Netherlands) followed by horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (Bio-Rad, California, USA).

For western-blot, strips were incubated with rabbit polyclonal antibodies anti-cblA (Abcam plc, Cambridge, UK) for 1 h at room temperature. After washing with PBS-0.5% Tween-20, anti-rabbit (Bio-Rad, California, USA) HRP-conjugated antibodies were added and incubated for 1 h at room temperature. Following extensive washing, staining was achieved by using TMB substrate kit for peroxidase (Vector Laboratories, Inc., CA) according to manufacturer's instructions.

Co-precipitation of cable pili and lactoferrin

Enriched cable pili preparations (20 μg) were incubated with bLf (1 mg/ml final concentration) in RIPA buffer (140 mM NaCl, 20 mM Tris–HCl pH 7.8, 1% NP-40, 0.5% Nadeoxycolate, 1 mg/ml BSA, 0.5% PMSF, 0.5% anti-protease cocktail) overnight at 4°C with gentle shaking. Then, rabbit anti-bLf antibodies (1 μg/ml) were added. After overnight incubation at 4°C with gentle agitation, Protein A/G-Sepharose beads (10 μg) (Santa Cruz Biotechnology, Inc., USA) were added and reacted at 4°C for 4 h. The beads with bound antibody molecules were recovered by centrifugation at 2,500×*g* for 5 min, washed four times in RIPA buffer, and resuspended in 20 μl of SDS-PAGE sample buffer. The suspension was subjected to 10% SDS-PAGE and western blotting as above described. The blots were visualized with rabbit anti-cblA antibodies followed by anti-rabbit HRP-conjugated IgG. Staining was achieved by using TMB substrate kit for peroxidase (Vector Laboratories, Inc., CA) according to manufacturer's instructions.

Mucin adherence assay

Porcine gastric mucin (Sigma–Aldrich, USA) was dissolved at a concentration of 50 μg/ml in water, filter-sterilised, and 50-μl aliquots were added to wells of a 96-well microtitre plate. After incubation at 37°C for 18 h, 50-μl aliquots of bacteria treated or not treated with lactoferrin as above described, were added to mucin-coated wells and incubated for 30 min. Wells were then washed 10 times with PBS to remove unbound bacteria, and the bound bacteria were released by the addition of 0.25% Triton X-100. The bound bacteria were enumerated by plating serial dilutions on TSA. Mucin adherence assays were performed in triplicate, with two independent enumerations for each well.

Statistical analysis

Statistical analysis was performed by Student's t test for unpaired data. Data were expressed as the mean and SD and P-values of <0.05 were considered significant.

Results

Anti-bacterial activity of lactoferrins

In order to detect the anti-bacterial activity of all lactoferrins, bacteria were grown for 24 h in presence of native, apo-, and iron saturated-lactoferrin. Apo- and iron saturated-lactoferrins did not show any bactericidal activity at the tested concentrations; on the contrary, native lactoferrin displayed a little inhibitory activity on bacterial growth. To discriminate the bacteriostatic or bactericidal effect of lactoferrins, lactoferrin bacteria treated were washed and allowed to growth to further 4 h in absence of the compounds. Results showed in Fig. 1 correlated with a bacteriostatic effect of native bLf.

Aggregation assays

After incubation of *B. cenocepacia* with different concentration of lactoferrins, we observed different extent of aggregation respect to the control cultures. This aggregation was not due to iron content carried by lactoferrin since the aggregates were visible also in apo-lactoferrin, that was iron deprived, and native lactoferrin (19.4% iron-saturated) treated cultures. A dose-dependent relationship with increased lactoferrin concentrations was observed. Majority of aggregates was visible at 2 mg/ml lactoferrin concentration decreasing in size and number with reduced concentration of lactoferrins. While culture without lactoferrins remains turbid after prolonged incubation (24 h), treated cultures were clear with the majority of bacterial cells settled on the bottom tube and with a number of aggregates flocculating on the suspension.

These results were visualized by light and scanning electron microscopy observations (Fig. 2). Microscopic examination of cultures treated with lactoferrins revealed the presence of bacterial aggregates [Fig. 2b (light microscopy) and d (scanning microscopy)]. Some aggregates were also observed in the cultures of untreated cells [Fig. 2a (light microscopy) and c (scanning microscopy)], but they appeared to be more less numerous and smaller in size than those observed in lactoferrin-treated cultures.

Since lactoferrin activity was not affected by iron saturation or by removal of iron ions, we decided to use in following experiments only the native form of lactoferrin.

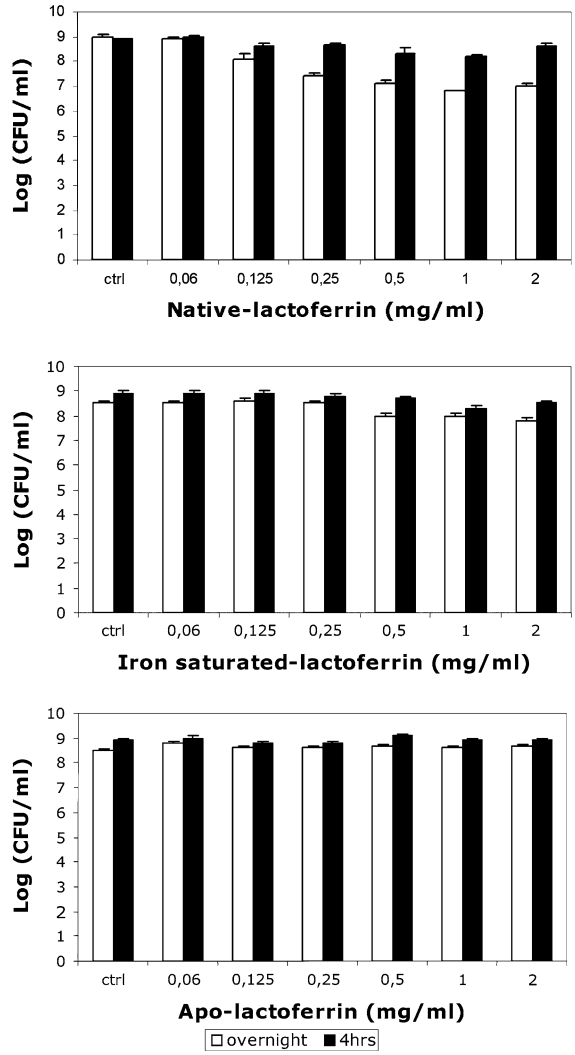

Fig. 1 Anti-bacterial activity of lactoferrins. Bacterial cells treated with increased concentrations of native, iron-saturated and apo lactoferrins. To verify bacteriostatic effect of lactoferrins, bacterial cultures were washed after overnight incubation and grown for additional 4 h. Data represent the mean of three independent experiments ±SD

Dissociation of bLf-bacterium complex

Since lactoferrin is a highly charged molecule (Baker and Baker 2005), the role of charge in binding with *B. cenocepacia* was investigated. When bLf-treated bacteria were washed with 2 M NaCl, no effect was seen with binding to lactoferrin (Table 1). This implies that *B. cenocepacia* binding to lactoferrin was not due to non-specific electrostatic interactions

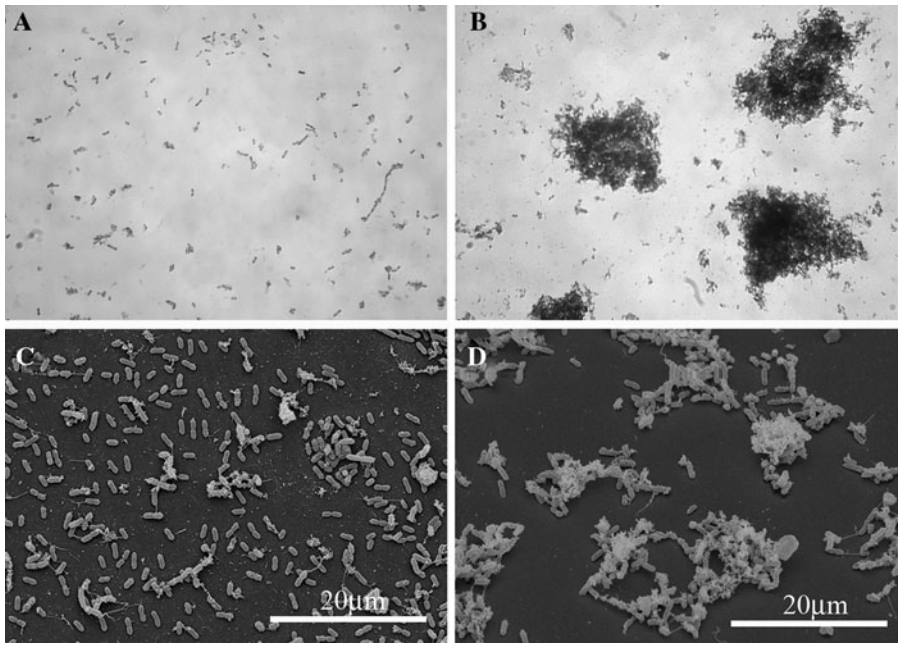

Fig. 2 Light and scanning electron microscopic (SEM) visualization of aggregates. Bacterial cultures incubated with lactoferrins for 4 h and examined for aggregates by May-Grunwald-Giemsa (**a** and **b**) and SEM (**c** and **d**). Micrograph represents untreated bacteria (**a** and **c**) and aggregates of bacteria treated with native lactoferrin (**b** and **d**)

Table 1 Effect of NaCl on the binding of bLf to the *B. cenocepacia* cells

	Adherent bacteria (log CFU/ml)	Dissociation (%)
PV1 + bLf (1 mg/ml)	4.5 ± 0.5	–
PV1 + bLf + NaCl (2 M)	4.3 ± 0.4	4.4

Note: Data represent the mean of three separate experiments ±SD

but is likely to be due to specific interactions with bacterial surface.

Electron microscopy of cable pili

Since aggregation phenotype has been observed in *B. cenocepacia* cells not expressing cable pili (Tomich and Mohr 2003), we perform electron microscopic observations of bacterial cells settled without shaking in order to detect cable pili. Positive staining of supernatants of control bacterial cells allowing settled for 8 h without shaking showed numerous peritrichously expressed cable pili on the bacterial cell surface (Fig. 3). The outwardly projecting pili appeared to tether adjacent cells together and mediate cell-to-cell interactions (Fig. 3b). The bottom sample appeared with low bacteria showing non-piliated surfaces that form aggregates (Fig. 3a). Supernatant sample of lactoferrin treated bacteria showed a low amount of both piliated or not piliated bacteria together with numerous free cable pili (Fig. 3d), whereas bacteria observed on the bottom tube were in most part aggregate and heavy piliated (Fig. 3c).

In order to verify if lactoferrin-mediated aggregation of *B. cenocepacia* cells was due to lactoferrin binding to cable pili, we perform an electron microscopy localization assay with colloidal gold lactoferrin. As shown in Fig. 4, gold lactoferrin was found to be attached to surface pili. The gold particles were distributed over the pili tracing the typical cable distribution (Fig. 4b, c).

SDS-PAGE of *B. cenocepacia* cable pili and lactoferrin blotting assay

Extracted cable pili of *B. cenocepacia* cells were subjected to SDS-PAGE and electroblotted onto nitrocellulose. After transfer, protein bands were incubated sequentially with native lactoferrin,

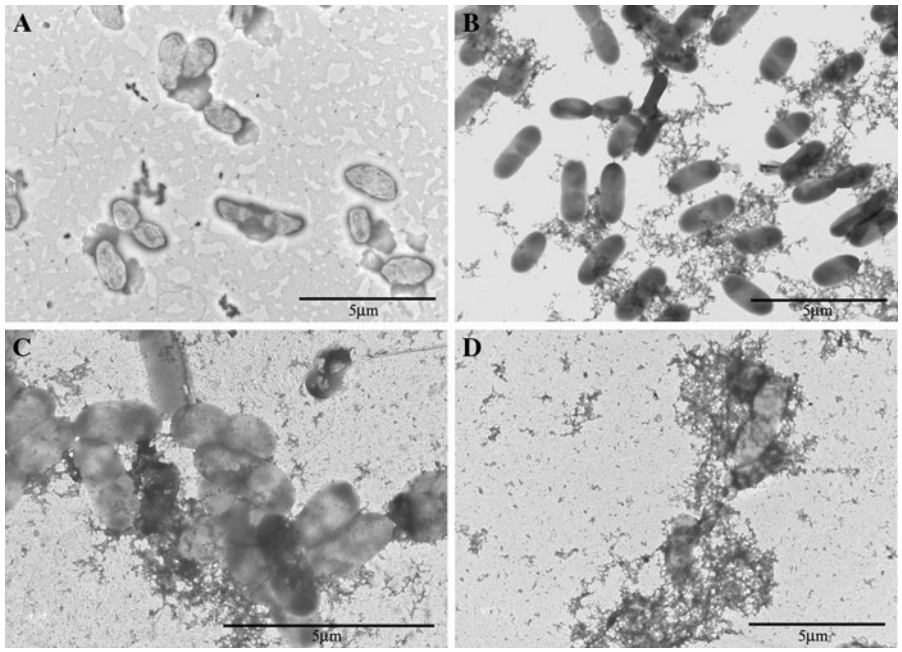

Fig. 3 Transmission electron microscopy (TEM) of *top* and *bottom* bacterial cultures. Electron microscopic visualization of control bacterial cells and native bLf-treated bacteria after 8 h settling. Positive staining of *bottom* (**a** for control and **c** for bLf-treated cells) and *top* (**b** for control and **d** for bLf-treated cells) samples

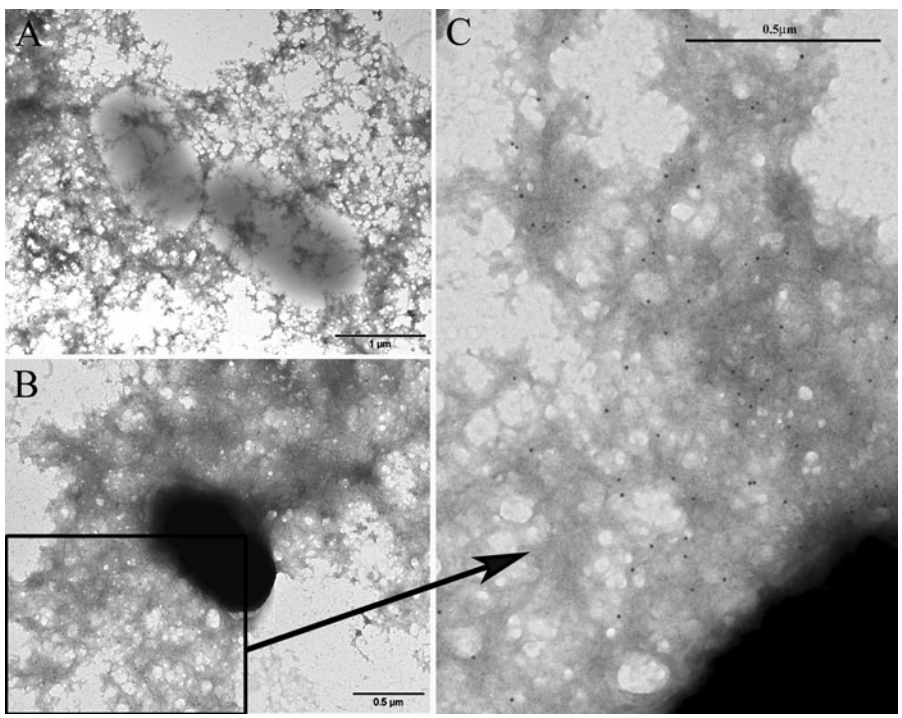

Fig. 4 Transmission electron microscopy localization of bLf on bacterial surface. Gold lactoferrin binding to bacterial cells tracking the typical cable distribution (**b** and **c**). **a** Bacteria untreated with gold lactoferrin

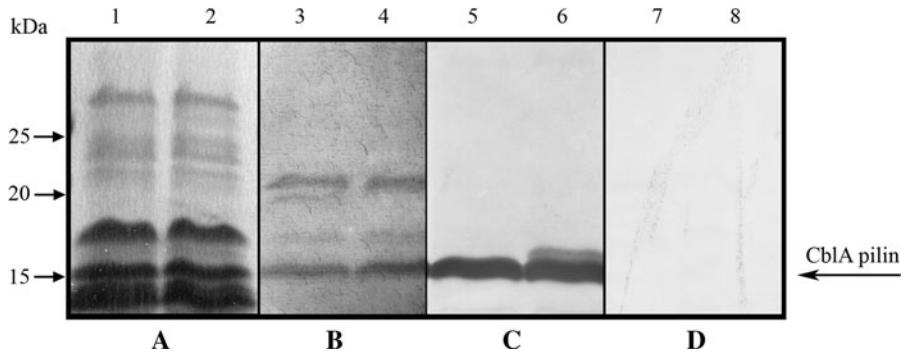

Fig. 5 SDS-PAGE of cable pili and lactoferrin blotting assay. Electrophoretic separation of semipure cable pili under denaturing (lanes 2, 4, 6, 8) or non-denaturing conditions (lanes 1, 3, 5, 7) and electroblotting (lanes 3–8) onto nitrocellulose. Lactoferrin blotting assay performed with native bLf and specific lactoferrin antiserum (panel **b**) and western-blot for cable pilin detected by anti-cblA antibodies (panel **c**). Non-specific anti-bLf antibody binding to bacterial proteins was showed in panel **d**. Panel **a** represents SDS-PAGE of semipure pili preparation

antibodies to lactoferrin, and secondary conjugated antibodies as described in "Materials and Methods". Cable pilin was detected by anti-cblA antibodies.

As shown in Fig. 5, an abundant protein of approximately 15 kDa, corresponding in size to CblA pilin, was detected in the semi-pure pilin fraction, obtained with cable pili extraction procedure, although other positive bands were also observed (Fig. 5a). Blot probed with anti-cblA specific antibodies confirms its identity as CblA pilin (Fig. 5c). Lactoferrin overlays of the same pilin preparations indicated that the band of 15 kDa corresponding to cable pilin was bound by bLf both in denaturing or non-denaturing conditions (Fig. 5b). BLf appeared to bind also a protein band of about 22-kDa that could represent the cable pili adhesin distributed along the shaft of the pili that was isolated together with CblA pilin during the extraction procedure of pili proteins.

Co-precipitation of cable pili and lactoferrin

To further confirm bLf interaction with CblA pilin, semi-pure cable pilin preparations were incubated with lactoferrin. Protein complex were then recovered by using a polyclonal serum against lactoferrin and after gel separation and blotting, pilin was detected by specific anti-CblA antibodies.

As showed in Fig. 6, bLf was able to bind CblA pilin that was recovered in the protein complex precipitated by lactoferrin specific antiserum (Fig. 6, line C). None protein appeared in the absence of bLf (Fig. 6, line A) whereas pilin protein was visualized

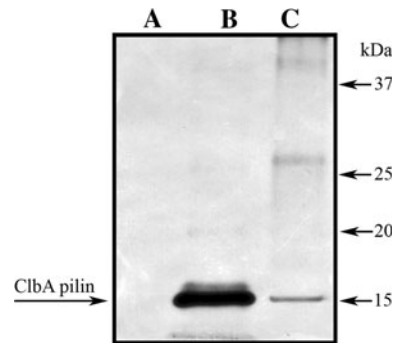

Fig. 6 Co-precipitation of cable pili and lactoferrin. Protein complex of pili and native bLf, precipitated by lactoferrin specific antiserum, was separated by SDS-PAGE and, after electroblotting, stained with anti-cblA pilin (lane C). Pilin protein was visualized by specific antibody in lane B whereas lane A showed that no protein complex was recovered in absence of lactoferrin

by anti CblA pilin antibodies (Fig. 6, line B). Normal rabbit serum alone was also used as a control but no proteins appeared (data not shown).

Mucin adherence

Previous studies have shown that cable pili can bind to mucin of both human and porcine origin (Sajjan and Forstner 1992; Sajjan et al. 1992). To examine the influence of bLf interaction with cable pili in binding to mucin, the ability of lactoferrin-treated PV1 cells to adhere to porcine mucin-coated microtitre plates was compared. BLf binding to cable pili appeared to inhibit bacteria-mucin interaction of

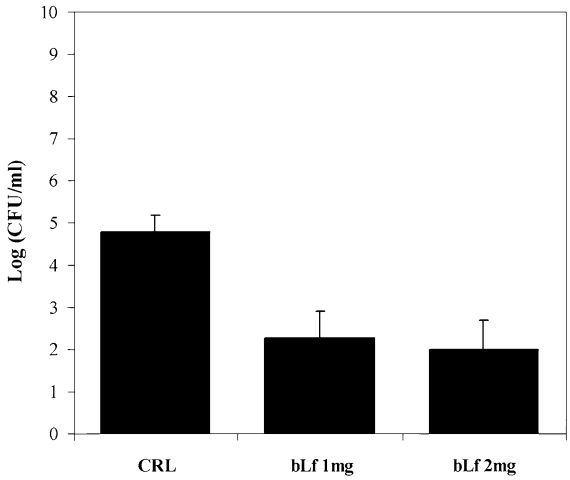

Fig. 7 Mucin adherence assay. Reduced adherence to mucin of bacterial cells treated with native lactoferrin at 1 and 2 mg/ml compared to untreated cultures. Log (CFU/ml) was calculated from three separated experiments and showed as mean ± SD

about 50% (2.26 ± 0.66 for bLf 1 mg/ml and 2 ± 0.7 for bLf 2 mg/ml, respectively) compared to untreated-bacteria (4.79 ± 0.4) (Fig. 7).

Discussion

B. cenocepacia colonisation, as well as patient-to-patient transmission of the microrganism, has been associated with the expression of filamentous cable pili (Clode et al. 2000; Sun et al. 1995). Cable-piliated strains of *B. cenocepacia* have been shown to bind mucins (Sajjan et al. 1992), glycoprotein components of the airway surface fluid, which are particularly abundant in the CF lung due to poor mucocilliary clearance (Govan and Deretic 1996). Additionally, cable-piliated strains have been shown to bind to cytokeratin 13 (CK13), a predominantly cytoplasmic protein that may become exposed on the surfaces of epithelia during the course of chronic infection in CF (Sajjan et al. 2000). More recently, *B. cenocepacia* cable pili have been demonstrated to act as initiator of cytotoxicity and apoptosis (Cheung et al. 2007).

Lactoferrin is thought to play a pivotal role in the prevention of infection in the host and its ability to sequester iron from potential pathogens has been regarded as an antimicrobial function (Bullen 1972). This antimicrobial activity might be due not simply to the removal of free iron ions from the environment but could involve the interaction of Lf with surface of microorganisms (Pettersson et al. 1994; Ochoa et al. 2006; Ling and Schryvers 2006).

In this study, we found that lactoferrin binds cable pili expressed on the bacterial surface providing an inhibitory effect on the *B. cenocepacia* activity. The binding seems to be due to the specific interaction between bLf and bacterial cells. Hydrophobic interactions, which could play an important role in lactoferrin binding to cable pili because of the cationic charge of bLf, were not found to influence the binding to bacterial pili, as judged by the total lack of effect of NaCl upon the binding. The interaction between bLf and pili led to an increased aggregation of bacterial cells, as showed by pellet formation at the bottom culture tubes after spontaneous settling. When incubated in the absence of shaking, we observed a difference between Lf-untreated and Lf-treated cultures of *B. cenocepacia*. This difference consisted in an increase in the rate of settling of the Lf-treated cultures. These aggregates were revealed also by microscopic observations and appeared on average until ten fold the size of those formed in untreated cultures.

Tomich and Mohr (2003) demonstrated that non-piliated strains of *B. cenocepacia* displayed higher aggregation than piliated bacteria. Cable pili interactions in *B. cenocepacia* appeared diffuse with bacteria tethered at a distance from one another allowing bacteria to remain in suspension. Based on these data, we hypothesized that aggregative effect of lactoferrin could be due to pili binding. Immunogold labelling of PV1 strain, growth in medium conditions favouring bacterial piliation, showed that bLf is able to bind pili on the bacterial surface, tracing the typical cable distribution. This binding ability of bLf was confirmed by lactoferrin blotting and co-precipitation assays in which lactoferrin showed to recognize and bind cable pilin protein. The *B. cenocepacia cbl* locus is comprised of at least five genes, designated *cblB*, *cblA*, *cblC*, *cblD* and *cblS*. The first four genes encode the structural and accessory components of the cable pilus biogenesis pathway (Sajjan et al. 2003). The *cblA* gene encodes the major structural subunit of cable pili (Sajjan et al. 1995), while *cblB*, *cblC* and *cblD* are predicted to encode the periplasmic chaperone, outer membrane usher, and minor

structural subunit, respectively (Sajjan et al. 2003). Lactoferrin appeared to bind cblA subunit in far-western blot assay together with another band that likely corresponds to the Cbl pilus associated 22-kDa adhesin. This protein of *B. cenocepacia* is located along shaft of pili and mediates binding to CK13 and mucin (Sajjan et al. 2000).

As bLf-treated cultures showed a trend to settle on the bottom tube and since it was reported that non-piliated strains displayed higher aggregation than piliated bacteria (Tomich and Mohr 2003), we performed electron microscopic observations of bacterial cells settled without shaking in order to detect cable pili sheared by lactoferrin. Microscopic observations of bottom and top samples of Lf-treated cultures as well as western blot of supernatant of the same cultures (data not shown) indicated that pili were retained by the bacterial cells treated with Lf. The aggregates appeared highly piliated and a low cblA subunit protein content was recovered in the supernatant of Lf-treated bacteria compared to control samples. Moreover, lactoferrin-mediated aggregates appeared more compact compared to control cultures and showed conserved cable pili. Thus, lactoferrin did not appeared to remove cable pili from the bacterial surface increasing autoaggregation of non-piliated bacteria; on the contrary, we speculate that Lf acts as a bridge among bacterial cells by binding cable pili.

Lactoferrin was demonstrated to bind also fimbrial subunits of *Escherichia coli* (Teraguchi et al. 1996; De Oliveira et al. 2001). It was observed that bovine lactoferrin strongly inhibited the hemagglutination activity of type 1 fimbriated *E. coli* and it agglutinated these bacteria (Teraguchi et al. 1996). In addition, lactoferrin was able to bind to CFA I fimbriae, indicating that this protein might act as a receptor analogue in the inhibition of adhesion of CFA I to the host cell (De Oliveira et al. 2001). Four of five components of the cable pilus biosynthetic apparatus of *B. cenocepacia* share high homology to components of the *E. coli* CS1 family of pilus assembly pathways. In particular, the amino acid sequence of CblA was found to be 72–74% similar to the major pilin subunits of CFA/I and CS pili, respectively. In *E. coli* bLf binding to fimbriae appeared mediated by the glycans of bovine lactoferrin that can serve as receptors for type 1 fimbrial lectin. In the same way, our results suggest that, about *B. cenocepacia* aggregation activity, lactoferrin could act with the same mechanism observed for *E. coli*. The protein showed to bind both cblA pilin and 22-kDa adhesin and, even though we cannot completely rule out the cooperation of polar hydrophobic bounds in this interaction, the binding seems to be specific and stable. It is possible that bLf binding to cable pili may favour autoaggregation, by preventing the occurrence of diffuse cell-to-cell interactions. In fact, it was reported that lactoferrin promote aggregation of other bacteria, such as *Streptococcus mutans* (Soukka et al. 1993), *E. coli* (Teraguchi et al. 1996), and *Clostridium* spp. (Tomita et al. 1998). This aggregation is believed to promote bacterial clearance as it reduces the number of exposed bacterial adhesins, and bacterial aggregates are likely to be more susceptible to body flows.

BLf binding to cable pili led not only to bacterial aggregation but also to reduced interaction of pili with mucin. It is known that *B. cenocepacia* is able to bind mucin and this interaction is mediated by cable pili. In particular, a 22-kDa adhesin located on surface-expressed pili (Sajjan and Forstner 1992) is responsible of bacterial binding to mucin and respiratory cells (Urban et al. 2005). The apical mucus layer in airway was found to play an important role in protecting the underlying epithelium. In CF patients, protective role of mucus is reduced because of its physical properties and/or its composition that cause an increased absorption of water and decreased fluid secretion. It was reported that (Sajjan et al. 2004) *B. cepacia* isolates from CF patients, mainly from those with the most severe lung damage, tended to bind more strongly to mucins. As the mucus blanket that normally covers the epithelial surfaces of the respiratory tract is poorly cleared from lungs of patients with CF, secreted mucin may provide a reservoir for mucin-binding *B. cepacia* strains, thereby facilitating colonization. In this contest, lactoferrin inhibitory activity exerted through the binding of cable pili could led to a reduction of bacterial colonization and permanence inside mucin layer, with consequent decreased access of bacteria to respiratory cells. In conclusion, our results indicate that lactoferrin could contribute to the protection against *B. cenocepacia* infection in patients with CF by blocking bacterial colonization, and support the possible use of this glycoprotein for prophylactic or therapeutic purposes.

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