

Burkholderia cepacia Produces a Hemolysin That Is Capable of Inducing Apoptosis and Degranulation of Mammalian Phagocytes

MICHAEL L. HUTCHISON,* IAN R. POXTON, AND JOHN R. W. GOVAN

Department of Medical Microbiology, University of Edinburgh Medical School, Edinburgh EH8 9AG, Scotland

Received 9 June 1997/Returned for modification 3 September 1997/Accepted 28 January 1998

Burkholderia cepacia is an opportunistic pathogen that has become a major threat to individuals with cystic fibrosis (CF). In approximately 20% of patients, pulmonary colonization with *B. cepacia* leads to cepacia syndrome, a fatal fulminating pneumonia sometimes associated with septicemia. It has been reported that culture filtrates of clinically derived strains of *B. cepacia* are hemolytic. In this study, we have characterized a factor which contributes to this hemolytic activity and is secreted from *B. cepacia* J2315, a representative of the virulent and highly transmissible strain belonging to the recently described genomovar III grouping. Biochemical data from the described purification method for this hemolysin allows us to hypothesize that the toxin is a lipopeptide. As demonstrated for other lipopeptide toxins, the hemolysin from *B. cepacia* was surface active and lowered the surface tension of high-pressure liquid chromatography-grade water from 72.96 to 29.8 mN m⁻¹. Similar to reports for other pore-forming cytotoxins, low concentrations of the hemolysin were able to induce nucleosomal degradation consistent with apoptosis in human neutrophils and the mouse-derived macrophage-type cell line J774.2. Exposure of human neutrophils to higher concentrations of toxin resulted in increased activities of the neutrophil degranulation markers cathepsin G and elastase. Based on the results obtained in this study, we suggest a role that allows *B. cepacia* to thwart the immune response and a model of the events that may contribute to the severe inflammatory response in the lungs of CF patients.

Cystic fibrosis (CF) is the most common inherited disease of Caucasian populations and results from a defective chloride channel called the CF transmembrane regulator (29, 31). Impaired CF transmembrane regulator function results in a high NaCl concentration and reduced volume of the airway fluid covering the apical surface of lung epithelia (37). Evidence from recent studies suggests that the susceptibility of the lungs of CF patients to respiratory infections results from a combination of sticky mucus which impairs mucociliary clearance, high NaCl concentrations which reduce bactericidal activity of airway surface fluid, and an increase in the number of epithelial receptors for pathogenic bacteria (5, 37, 39).

Most individuals with CF will experience a characteristic age-related pattern of pulmonary colonizations and intermittent exacerbations involving *Staphylococcus aureus* and *Haemophilus influenzae* in infancy and early childhood, followed inexorably in adolescence by *Pseudomonas aeruginosa* (7, 11, 16). However, in parallel with the improved life expectancy of CF individuals (6), the spectrum of microbial pathogens has also evolved. Since the mid-1980s, three factors have combined to make *Burkholderia (Pseudomonas) cepacia* a major threat to patients with in North America and Europe (36, 40): (i) the organism's innate resistance to most antibiotics (28); (ii) cross-infection due to patient-to-patient or nosocomial spread (10, 18); and (iii) the rapid and unexpected clinical decline in approximately 20% of colonized patients, associated with acute necrotizing pneumonia, sometimes accompanied by septicemia (9, 11, 17). *B. cepacia* colonization is less common than *P. aeruginosa* colonization but can nevertheless infect upward of 40% of CF patients during epidemic spread (11). *B. cepacia*

colonization therefore has a considerable impact on CF patients, since social ostracism is associated with the draconian segregation policies implemented to reduce patient-to-patient spread. In addition, life expectancy, which at present averages nearly 40 years for patients free of *P. aeruginosa* and *B. cepacia*, is reduced to approximately 20 years for individuals colonized by *B. cepacia* (6).

To date, most research on the role of *B. cepacia* in human infections has focused on the mechanisms responsible for its innate antibiotic resistance or on the use of molecular fingerprinting techniques to clarify the epidemiology of acquisition of infection (19, 22). The bacterial and host factors which determine the clinical outcome of *B. cepacia* colonization are unclear (4, 8, 26, 43). Accumulated evidence indicates that *B. cepacia* can invade and survive intracellularly within airway epithelia (2, 3, 9). Putative virulence factors identified in *B. cepacia* isolates include proteases (24), lipopolysaccharide (34), pili and mucin-binding adhesins (38, 39), and heat-labile hemolysin which has both phospholipase C and sphingomyelinase activities (42). Hemolytic and phospholipase C expression in *B. cepacia* appears to be complex (42). Previous reports indicate that up to 40% of isolates exhibit beta-hemolytic activity when erythrocytes from various animals are tested (42); however, unlike the phospholipase C activity of *P. aeruginosa*, the phospholipase activity of *B. cepacia* does not correlate with hemolytic activity (25, 42). A surprising result from our laboratory is that *B. cepacia* lipopolysaccharide stimulates tumor necrosis factor alpha and other proinflammatory cytokines 10 times more strongly than does lipopolysaccharide from *P. aeruginosa* (34). Cell-free supernatants of *B. cepacia* have also been shown to induce interleukin-8 from cultured human A549 lung epithelial cells (27).

We report the purification and activity of a hemolysin obtained from *B. cepacia* J2315. Our findings show that this factor can cause apoptosis of human neutrophils, can cause an in-

* Corresponding author. Mailing address: Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland. Phone: (44) 131 6503165. Fax: (44) 131 6506531. E-mail: Mikeh@srv1.med.ed.ac.uk.

crease in neutrophil degranulation markers, and causes hemolysis of erythrocytes. The clinical implications of the role of the toxin are speculatively discussed, since the strain is a representative of the virulent and highly transmissible ET/12 lineage belonging to the recently described genomovar III group of *B. cepacia* (9, 30, 41).

MATERIALS AND METHODS

Purification of hemolysin. *B. cepacia* J2315 was grown at 37°C in 3% (wt/vol) glucose–1% (wt/vol) yeast extract–1% (wt/vol) tryptone with vigorous shaking (300 rpm; Gallenkamp orbital incubator). After 72 h of culture the cells were killed by the addition of isopropanol to 50% (vol/vol) and the pH of the medium was lowered to pH 4.0 by addition of concentrated HCl. Cellular debris was removed by centrifugation (4,000 × g for 15 min), and the solvent was removed by flash evaporation at 45°C. Dowex 1 anion-exchange chromatography was used for further purification as described previously (13). The column eluate was extracted three times with acetone, and the aqueous phases were pooled and freeze-dried before being further purified by high-pressure liquid chromatography (HPLC).

HPLC purification of hemolysin. Highly purified hemolysin was prepared by reverse-phase HPLC with Gilson 712 series chromatographic equipment (Gilson Plc.). A linear acetonitrile gradient of 0 to 100% (vol/vol) containing 0.1% (vol/vol) trifluoroacetic acid was used at a flow rate of 1.3 ml min⁻¹ to purify the hemolysin to a single peak. A semipreparative column with C₁₈ as the active group (Beckman 5-μm-pore-size Ultrasphere ODS; 150 by 10 mm [internal dimensions]) was used with detection at 215 nm. Aliquots (1 ml) of crudely purified toxin containing ~0.5 mg (dry weight) of material were injected for purification.

Erythrocyte assays for hemolytic activity. Assays were conducted with heparinized horse erythrocytes (0.2 ml) washed in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 0.9% NaCl [pH 7.0]) prior to suspension in 100 ml of PBS. The concentration of whole blood cells (1 to 2 μl ml⁻¹) was adjusted with PBS to an optical density at 600 nm between 0.09 and 0.1 in a final volume of 1 ml. Hemolysin was used from a 1-mg-ml⁻¹ stock solution and added to blood cells to a final concentration of 0.5 to 8 μg ml⁻¹ at 22°C. After mixing for 1 to 2 s, the rate of lysis was monitored spectrophotometrically at 600 nm. Each data point was the result of at least six individual replicates performed as two separate trials.

Osmotic protection assay. Osmotic protection assays were performed with solid agar plates (PBS containing 20 ml of 1% [wt/vol] agar and 2% [vol/vol] whole horse blood). Osmotic protectants added to the plates were polyethylene glycol 1450 (PEG 1450), PEG 1000, raffinose, lactose, sucrose, glucose, and arabinose (14). All protectants were added to a concentration of 500 μM. An aliquot (5 μg in 10 μl) of HPLC-pure toxin was spotted several times on each plate before incubation at 37°C for 3 h.

Scanning electron microscopy. Sample preparation was carried out as described previously (33) with minor modifications. The sample was fixed in 3% (vol/vol) glutaraldehyde–0.1 M sodium cacodylate buffer (pH 7.4) for a minimum of 3 h. Prior to dehydration in acetone, samples were stored in 1% (vol/vol) osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h. Critical-point drying was performed with CO₂, and the sample was sputter coated with 20-nm gold-palladium (60:40 [wt/wt]). The samples were observed with a Philips 505 scanning electron microscope.

Culturing of J774.2. The mouse-derived macrophage-type cell line J774.2 was grown in 75-cm² culture flasks (Greiner Labortechnik) in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 10 μg each of streptomycin and penicillin (Sigma) ml⁻¹. Incubation was carried out at 37°C with 5% (vol/vol) CO₂.

Isolation of neutrophils. Neutrophils were isolated from citrated fresh whole blood. An aliquot (50 ml) was centrifuged (1,000 × g for 20 min at 25°C), and the plasma was discarded. The pellet was resuspended in 0.25 volume of 6% (wt/vol) dextran–0.9% (wt/vol) NaCl, and 50% (vol/vol) PBS (pH 7.0) was added. After sedimentation for 45 min, the upper layer was layered onto 6 ml of Histopaque (Sigma) and the neutrophils were pelleted by centrifugation (1,200 × g for 30 min). Contaminating erythrocytes were lysed by resuspension in distilled H₂O and neutrophils stored on ice in Hanks buffered salt solution containing 0.1% (wt/vol) dextrose.

Apoptosis assay. Cells were used as 100-μl aliquots containing 5 × 10⁶ cells. Cultured J774.2 macrophages and freshly isolated human neutrophils were exposed to HPLC-pure toxin (1 μg) for 8 h at 37°C, in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, under 5% (vol/vol) CO₂. The cells were pelleted (3,000 × g for 10 min), washed in PBS, and resuspended at 2 × 10⁷ ml⁻¹ in 10 mM EDTA–50 mM Tris–0.5% (wt/vol) sodium lauryl sarcosinate. Protein was removed by extraction with aqueous phenol (pH 7.4)-chloroform-isoamyl alcohol (25:25:1). Nucleic acids were precipitated by addition of 1 ml of ethanol and centrifugation (14,000 × g for 30 min). RNA was removed by addition of 1 μg of DNase-free RNase per μg of DNA. DNA was resolved by agarose gel electrophoresis (1.2% [wt/vol] agarose) and stained with ethidium bromide.

Degranulation assay. Neutrophils were isolated from fresh human blood as described previously, and aliquots of cells (5 × 10⁵ cells) were incubated with various quantities of HPLC-pure toxin. Substrates of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide were used at 1 mM for determination of cathepsin G and elastase activities, respectively. Incubation was carried out typically for 2 h with gentle (50 rpm) agitation at 37°C. Generation of the cleavage product, *p*-nitroanilide, was determined spectrophotometrically at 410 nm.

Determination of antimicrobial activity. The MIC of the toxin for three CF isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was determined by twofold dilution performed in microtiter wells. The tested optical density of both bacterial types at 600 nm (OD₆₀₀) was 0.01. The range of toxin concentrations tested was 5 mg ml⁻¹ to 2.5 μg ml⁻¹.

Determination of the ability of *B. cepacia* to grow anaerobically. Test cultures of *B. cepacia* and positive controls of *P. aeruginosa* were cultured on the defined medium described by Malka et al. (23) with minor modifications: 0.5% (wt/vol) dextrose was used as the sole carbon source, 1% (wt/vol) potassium nitrate was used as a medium supplement, and agar was used to a final concentration of 1.5% (wt/vol). The plates were incubated in an anaerobic chamber (Don Whitley Scientific, Mark III, Shipley, United Kingdom) in an atmosphere of 80% N₂–10% CO₂–10% H₂.

RESULTS

Production and purification of hemolysin by *B. cepacia*. *B. cepacia* J2315 cultured in a medium rich in glucose with fast shaking secreted a substance that was capable of lysing horse and human erythrocytes. Aeration and medium composition were important for production of the lytic substance, since it was not produced on medium containing less than 1% glucose and production fell sharply at lower shake speeds (data not shown). The purification scheme used for the toxin was informative. The compound is stable, remains soluble at low pH, and is amphipathic in nature since it partitions into butanol. Furthermore, the toxin does not bind to the Dowex chromatography resin, which indicates a net positive charge at low pH. During the purification of the hemolytic activity, the samples were tested at each stage for the ability to lyse whole blood in isotonic agar. Small amounts of lysis were occasionally observed at some stages, which we explain by traces of solvent in these waste fractions. This small amount of nonspecific activity was insignificant compared to the majority of activity contained in the fractions eventually used for HPLC. Final separation by HPLC was used to purify the hemolysin to a high degree. Figure 1a shows a typical HPLC trace of the crudely purified material. The peaks marked A and B appeared reproducibly at 56 and 57% (vol/vol) acetonitrile. Both peaks were hemolytic on 2% (vol/vol) isotonic blood agar and had the ability to inhibit the growth of *S. aureus*. Fractions outside the two peaks had little or no hemolytic activity. No differences were observed in the hemolytic ability of these two peaks. The average percentage of the total area of peaks A and B from three separate batches of toxin, calculated by using the Gilson 712 integration module, was 5.48% (standard error [SE] 1.03%; *n* = 20) and 21.69% (SE 2.63%; *n* = 20), respectively. The minor peak, peak A, was not included in the fractions of toxin saved for freeze-drying; all subsequent work was performed with only peak B. After freeze-drying, reanalysis by HPLC of the purified material (Fig. 1b) showed a hemolysin peak area of >97.7% of the total trace area. A total weight of 5.2 mg of toxin was purified by HPLC.

Surface-active properties of the toxin. Throughout the purification procedure, a pronounced foaming of hemolytic preparations was observed. These detergent properties were quantified by the drop-weight method originally described by Harkins and Brown (12). The surface tension of solutions of toxin up to 10 mg ml⁻¹ was determined (Fig. 2). Above this concentration, solutions of toxin became cloudy, and short-term (>20 min) storage resulted in the formation of a precipitate. At 10 mg ml⁻¹, the surface tension of the freshly pre-

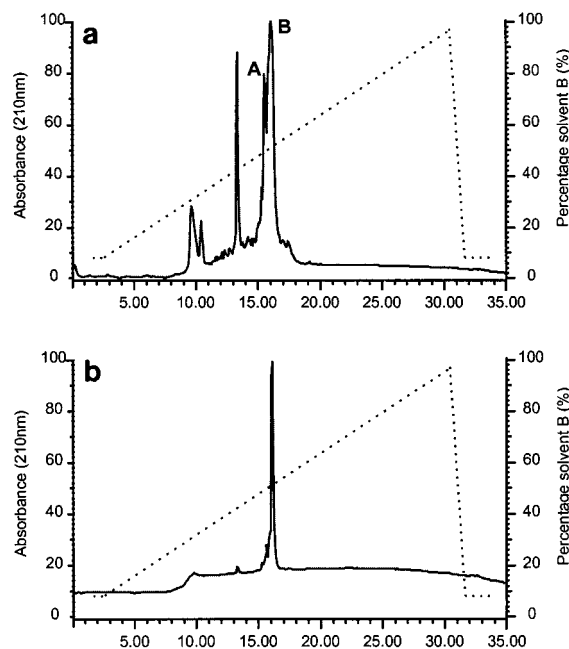


FIG. 1. (a) Reverse-phase HPLC trace showing peak separation of hemolysin-containing culture filtrates of *B. cepacia* J2315. Peaks with hemolytic activity are labelled A and B. Partial purification prior to HPLC was as described in Materials and Methods. (b) Peak B was used for all subsequent experiments and reanalysed by HPLC to test purity. The concentration of solvent B (acetonitrile in 0.1% [vol/vol] trifluoroacetic acid) is shown by the broken line and the right-hand y axis. Detection was carried out at 210 nm with a flow rate of 1.3 ml min^{-1} .

pared toxin solution was determined as 29.78 mN m^{-1} . This compares to the surface tension of HPLC-grade water, which was measured as 72.96 mN m^{-1} . Thus, *B. cepacia* hemolysin displays strong surface-active properties and could be considered a powerful biosurfactant. The critical micellar concentration of the toxin and the γ_{CMC} were determined graphically from Fig. 2, by the method of Sheppard and Mulligan (35), to be 12.6 mg ml^{-1} and 42.2 mN m^{-1} , respectively.

Electron microscopy. Typical electron micrographs showing the effect of J2315 hemolysin on human erythrocytes are shown in Fig. 3. Scanning electron microscopy revealed differences in the appearance of the cells compared to PBS-treated controls. The hemolysin-treated cells were generally smaller and had a spiked morphology consistent with crenation. In addition, there appeared to be larger amounts of cellular debris in the background of the toxin-exposed samples.

Quantification of hemolytic activity. A liquid blood cell assay was used to quantify the hemolytic properties of the toxin. The rate of lysis was nearly linear between the $3\text{-}\mu\text{g ml}^{-1}$ threshold required for measurable activity and the upper concentration tested of $8 \mu\text{g ml}^{-1}$ (Fig. 4). Since the effect was dose dependent, the maximal rate of lysis of $0.06 \text{ OD}_{600} \text{ unit min}^{-1}$ was recorded at $8 \mu\text{g ml}^{-1}$. Above this concentration, lysis was too rapid for the rate to be measured accurately. The osmotic protection assays did not show a diminished rate of lysis on solid agar blood plates. Both the size of the zone of clearing after 3 h and the rate of lysis were investigated. Since the osmotic protectants chosen offer protection against colloid-osmotic cellular lysis induced by pores with radii of 1.2 nm, our results suggests that the pores formed by *B. cepacia* hemolysin have a viscometric radius considerably larger than 1.2 nm.

Hemolysin-induced DNA degradation. Exposure of freshly purified human neutrophils to the toxin for 6 h gave rise to nucleosomal degradation that is consistent with apoptosis. Both the human neutrophils and mouse macrophage cell types (results not shown) gave a ladder pattern of cut DNA with fragment sizes increasing in increments of approximately 200 bp (Fig. 5). These banding patterns have become the biochemical hallmark of programmed cell death.

Inhibition of *S. aureus* by *B. cepacia* hemolysin. MIC determinations for three clinical isolates of *S. aureus* and *P. aeruginosa* were undertaken with a minimum of nine replicates. *P. aeruginosa* was sensitive to the crudely purified hemolysin but required a high concentration of the toxin ($125 \mu\text{g ml}^{-1}$) (SE 0; $n = 9$) for all three strains for complete inhibition. All three strains of *S. aureus* were sensitive to the crude toxin preparation at concentrations between $16 \mu\text{g ml}^{-1}$ (SE 0; $n = 9$) and $64 \mu\text{g ml}^{-1}$ (SE 0; $n = 9$). The effect of a small repeat trial of HPLC-purified toxin was to lower the MICs for the strains. All strains of *P. aeruginosa* were inhibited by $64 \mu\text{g ml}^{-1}$ (SE 0; $n = 3$); *S. aureus* strains were sensitive at between 8 and $16 \mu\text{g ml}^{-1}$ (SE 0; $n = 3$). Interestingly, *B. cepacia* J2315 showed no detectable inhibition by crude preparations of toxin at concentrations up to 5 mg ml^{-1} .

Activation and degranulation of human neutrophils. Human neutrophils isolated from fresh blood and exposed to the hemolysin became activated and released both cathepsin G and leukocyte elastase into the assay medium (Fig. 6). The rate of release of these degranulation markers was toxin dose dependent, and the effect was measurable at concentrations as low as $100 \text{ ng of HPLC-pure toxin ml}^{-1}$ with a 2-h incubation period. The activities were recorded as separate trials performed with samples from six separate blood donors with three replicates per data point. The range of maximal activities was 65 to $29.5 \text{ pmol converted s}^{-1} \mu\text{g of toxin}^{-1}$ for leukocyte elastase and 55 to $140 \text{ pmol converted s}^{-1} \mu\text{g of toxin}^{-1}$ for cathepsin G, which tends to suggest that different host leukocytes react differently or have different susceptibilities to the hemolysin.

***B. cepacia* is a strict aerobe.** No visible growth was observed when *B. cepacia* was cultured on modified Malka media under anaerobic conditions and the plates were incubated for up to 1 week. Although no visible growth was observed, viable bacteria

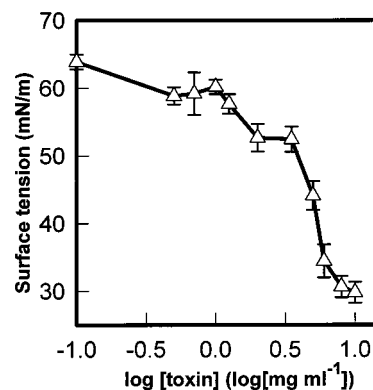


FIG. 2. Quantification of the surface-active properties of the hemolysin. The surface tension of solutions of HPLC-purified toxin was determined by the drop-weight method originally described by Harkins and Brown (12), as described in Materials and Methods. All points are the result of at least 10 separate replicates. Error bars are \pm the standard deviation of at least 10 replicates.

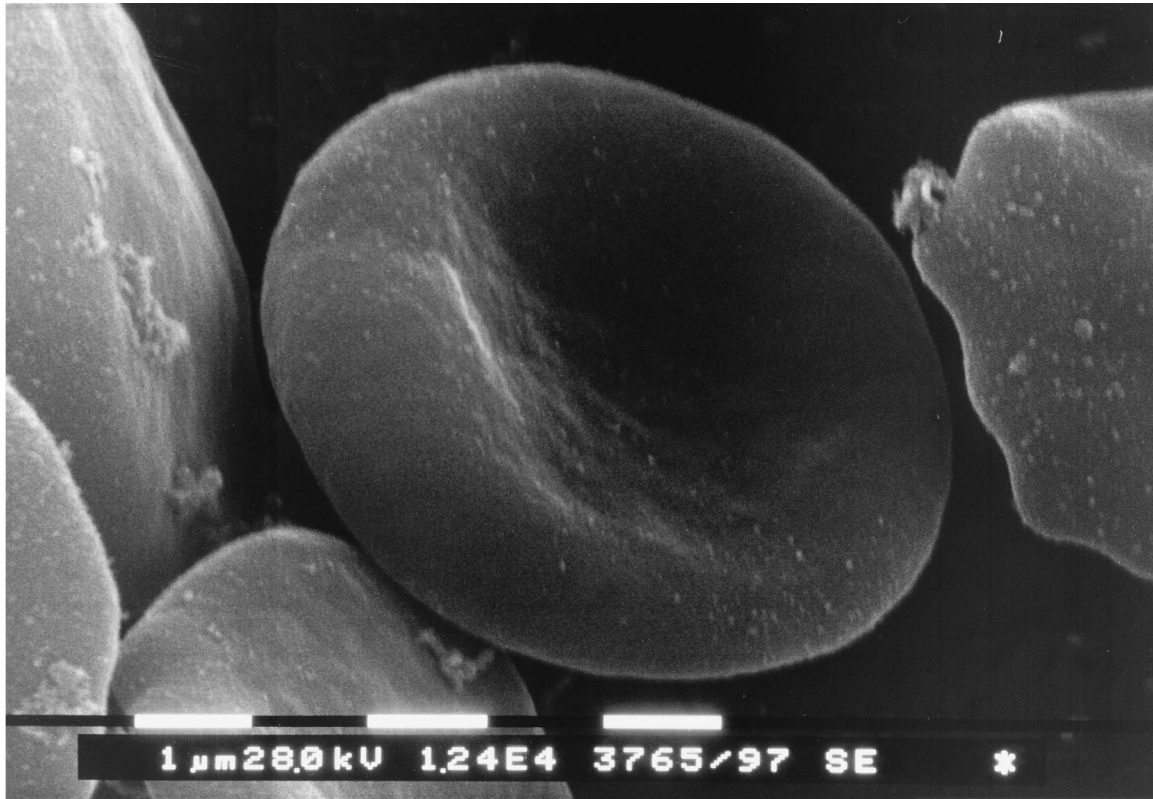
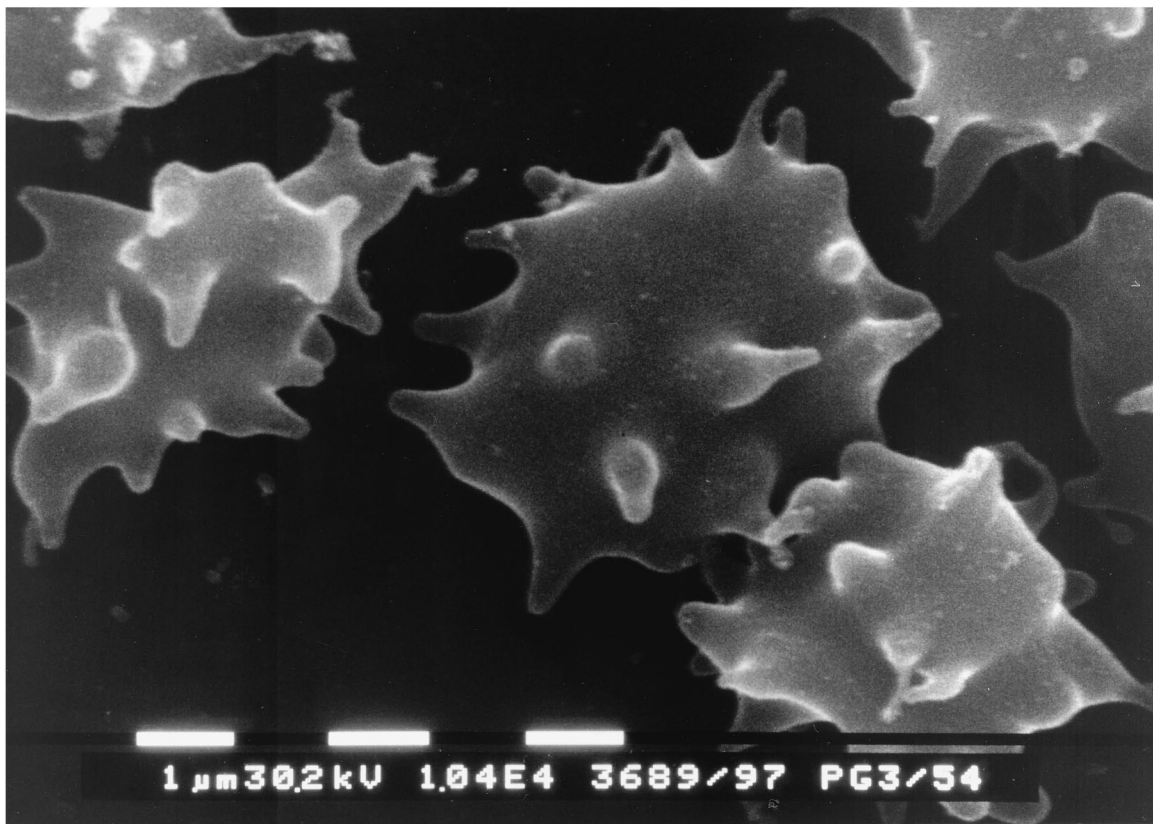
A**B**

FIG. 3. Effect of *B. cepacia* hemolysin on fresh human erythrocytes. (A) Control cells washed in PBS. (B) Cells exposed to 5 μ g of hemolysin. Both samples were incubated for 1 h at 37°C. The horizontal white bars in the lower half of the plates are 10^{-6} m each.

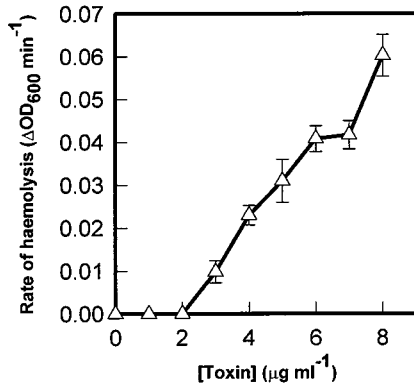


FIG. 4. Effect of HPLC-purified *B. cepacia* hemolysin on erythrocytes. Suspensions of cells were mixed with aliquots of toxin, and the rates of lysis were calculated by measuring the time-dependent decrease in OD₆₀₀. Each data point was the result of at least six individual replicate determinations performed as two separate trials.

could be cultured from the agar surface at the end of 7 days of anaerobic incubation.

DISCUSSION

Production of ion channel-forming toxins by animal and plant pathogens is common and has been widely documented (13, 15, 20, 21). In addition, there have been a number of reports of hemolytic activities by *B. cepacia*. Abe and Nakazawa (1) describe a hemolysin called cepalysin, isolated from a Japanese clinical strain, which forms pores with diameters of 20 to 30 nm. Vasil et al. (42) describe two separate hemolysins of 72 and 22 kDa from a U.S. clinical isolate. We believe that our hemolytic activity, from a British isolate, is distinct from both of these because we did not observe pores in the size range of 20 to 30 nm and polyacrylamide gel electrophoresis showed our activity to have a molecular mass of less than 3 kDa (results not shown). It remains to be shown that the *B. cepacia* hemolytic toxin we have described is secreted in vivo. However we speculate that conditions within the lungs of CF patients would be conducive to toxin synthesis. We observed that the synthesis and secretion of toxin activity are enhanced by the presence of oxygen, and it could be argued that oxygen may be limiting in congested areas of the lungs of these patients. At

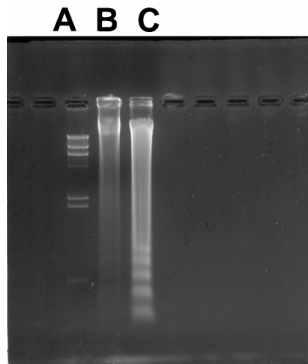


FIG. 5. Hemolysin-induced nucleosomal degradation. Freshly isolated neutrophils (5×10^6) were exposed to 1 μg of HPLC-pure toxin (lane B); control neutrophils were not exposed to the toxin (lane C). Molecular size standards (lane A) were generated by digestion of λ phage by *Hind*III and are 23.13, 9.41, 6.56, 4.36, 2.32, 2.03, and 0.56 kbp. DNA was resolved with a 1.2% (wt/vol) agarose gel.

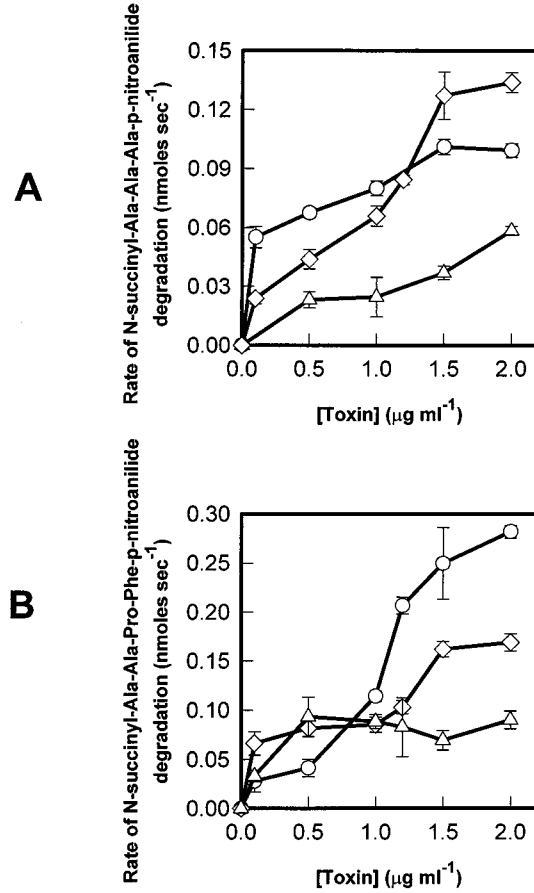


FIG. 6. Effect of *B. cepacia* hemolysin on the activity of neutrophil degradation marker enzymes. (A) Activity of granulocyte elastase. (B) Activity of cathepsin G. Each data set is obtained with samples from individual donors; each data point is the mean of at least three replicate determinations. Error bars are ±SE.

other sites, however, the concentration gradient of unbound oxygen between air in the lungs and the bloodstream would ensure a continual stream of oxygen across the epithelial surface. Other evidence for the availability of oxygen within the lungs of CF patients is that although *B. cepacia* is a strict aerobe and cannot utilize nitrate as an alternative electron acceptor, the organisms can be cultured from the CF pulmonary secretions at high concentrations, typically 10^8 CFU/ml. A priority for our future studies will be the analysis of *B. cepacia*-colonized patient sera for antibodies against the activity, allowing us to assign a clinical relevance to the hemolysin.

It has been speculated that the pathogenic potential of surface-active lipopeptides is associated with their detergent properties (15, 32). Generally, since it is difficult to estimate the concentrations of these bacterial toxins produced in human tissues or on a plant surface, their contribution to pathogenicity is difficult to determine precisely. Nevertheless, our study provides the first evidence of a potent surface-active agent produced by the virulent and highly transmissible *B. cepacia* lineage represented by strain J2315 and may provide clues to the pathogenic mechanisms which may operate in the lungs of CF patients. Our results demonstrated that very low concentrations of the *B. cepacia* toxin were required for cell damage. Preliminary studies with crude preparations of toxin also demonstrated an ability to form slightly anion-selective channels.

In the lungs of CF patients, low concentrations of lipopeptide could reduce the protective capacity of pulmonary neutrophils by causing pore formation and cellular apoptosis. This mechanism has already been proposed for other ion-channel forming toxins including staphylococcal α -toxin (20). However, although we were able to demonstrate that the *B. cepacia* lipopeptide caused degradation of DNA consistent with apoptosis, the hemolytic activity of the toxin suggests that pore formation may not be the major cause of the degradation. Data from our osmotic protection assays indicated that the viscometric radius of the toxin-induced channels is significantly greater than 1 nm. Close scrutiny of toxin-exposed cells under the scanning electron microscope, however, showed no pore-like structures on the surface of the erythrocyte membranes. Since the heavy-metal coating of the samples is likely to mask features smaller than 20 nm, it seems likely that the diameter of the toxin-induced pore is <20 nm. Alternatively, we do not rule out the possibility that the detergent properties of the hemolysin play a role in the mechanism of hemolysis.

Irrespective of the precise mechanism however, we show that DNA degradation characteristic of programmed cell death occurs when neutrophils are exposed to the toxin. It can be surmised that since phagocytes that have undergone apoptosis do not degranulate and release lysosomal contents, there would be a delayed exposure of potential bacterial pathogens to the bacteriocidal contents of the phagocytes and increased opportunity for small numbers of *B. cepacia* to become established. However, further studies involving inoculation of hemolysin-defective mutants into CF animal models are required to establish the contribution of the hemolysin to the pathogenicity of *B. cepacia*.

ACKNOWLEDGMENTS

This work was supported by the Cystic Fibrosis Trust (U.K.) project grant 416.

We thank C. Doherty (this laboratory) for the identification and provision of *P. aeruginosa* and *S. aureus* isolates and D. Apps (Department of Biochemistry, University of Edinburgh) for the use of HPLC equipment. We particularly acknowledge R. Ashley and S. Wyllie (Department of Biochemistry, University of Edinburgh) for investigating the channel-forming potential of the toxin.

REFERENCES

- Abe, M., and A. Nakazawa. 1994. Characterization of hemolytic and anti-fungal substance, cepalycin from *Pseudomonas cepacia*. *Microbiol. Immunol.* **38**:1-9.
- Burns, J. L. 1992. Characterization of invasion of epithelial cells by *Pseudomonas cepacia*. *Pediatr. Pulmonol. Suppl.* **8**:201. (Abstract.)
- Burns, J. L., M. Jonas, E. Y. Chi, D. K. Clark, A. Berger, and A. Griffith. 1996. Invasion of respiratory epithelial cells by *Burkholderia cepacia*. *Infect. Immun.* **64**:4054-4059.
- Butler, S. L., J. W. Nelson, and J. R. W. Govan. 1992. Cell surface characteristics of *Pseudomonas cepacia* isolates from patients with cystic fibrosis, abstr. TP8, p. 149. In Abstracts of the Eleventh International Cystic Fibrosis Congress.
- Debentzmann, S., P. Roger, and E. Puchelle. 1996. *Pseudomonas aeruginosa* adherence to remodeling respiratory epithelium. *Eur. Respir. J.* **9**:2145-2150.
- FitzSimmons, S. 1996. United States Cystic Fibrosis Foundation Database. Cystic Fibrosis Foundation, Bethesda, Md.
- Gilligan, P. H. 1991. Microbiology of airway disease in patients with cystic fibrosis. *Clin. Microbiol. Rev.* **4**:35-51.
- Goldman, D. A. and J. D. Klinger. 1986. *Pseudomonas cepacia*: biology, mechanisms of virulence, epidemiology. *J. Pediatr.* **108**:806-812.
- Govan, J. R. W., J. Hughes, and P. Vandamme. 1996. *Burkholderia cepacia*: medical, taxonomic and ecological issues. *J. Med. Microbiol.* **45**:395-407.
- Govan, J. R. W. G., P. H. Brown, J. Maddison, C. J. Doherty, J. W. Nelson, M. Dodd, A. P. Greening, and A. K. Webb. 1993. Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. *Lancet* **342**:15-19.
- Govan, J. R. W., and V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol. Rev.* **60**:539-574.
- Harkins, W. D., and F. E. Brown. 1919. The determination of the surface tension (free surface energy) and the weight of falling drops: the surface tension of water and benzene by the capillary height method. *J. Am. Chem. Soc.* **41**:449-503.
- Hutchison, M. L., and D. C. Gross. 1997. Lipopeptide phytotoxins produced by *Pseudomonas syringae* pv. *syringae*: comparison of the biosurfactant and ion channel-forming activities of syringopeptin and syringomycin. *Mol. Plant-Microbe Interact.* **10**:347-354.
- Hutchison, M. L., M. A. Tester, and D. C. Gross. 1995. Role of biosurfactant and ion channel-forming activities of syringomycin in transmembrane ion flux: a model for the mechanism of action in the plant-pathogen interaction. *Mol. Plant-Microbe Interact.* **8**:610-620.
- Hutchison, M. L., and K. Johnstone. 1993. Evidence for the involvement of the surface active properties of the extracellular toxin tolaasin in the manifestation of brown blotch disease symptoms by *Pseudomonas tolaasii* on *Agaricus bisporus*. *Physiol. Mol. Plant Pathol.* **42**:373-384.
- Immundo, L., J. Barasch, A. Prince, and Q. Al-Awqati. 1995. Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface. *Proc. Natl. Acad. Sci. USA* **92**:3019-3023.
- Isles, A., I. Maclusky, M. Corey, R. Gold, C. Prober, P. Fleming, and H. Levison. 1984. *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *J. Pediatr.* **104**:206-210.
- Jarvis, W. R., D. Olson, O. Tablan, and W. J. Martone. 1987. The epidemiology of nosocomial *Pseudomonas cepacia* infections: endemic infections. *Eur. J. Epidemiol.* **3**:233-236.
- Johnson, W. M., S. D. Tyler, and K. R. Rozee. 1994. Linkage analysis of geographic and clinical clusters in *Pseudomonas cepacia* infections by multilocus enzyme electrophoresis and ribotyping. *J. Clin. Microbiol.* **32**:924-930.
- Jonas, D., I. Waley, T. Berger, M. Liebraun, M. Palmer, and S. Bhakdi. 1994. Novel path to apoptosis: small transmembrane pores created by staphylococcal alpha-toxin in T lymphocytes evoke internucleosomal DNA degradation. *Infect. Immun.* **62**:1304-1312.
- Khelef, N., A. Zychlinsky, and N. Guiso. 1993. *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. *Infect. Immun.* **61**:4064-4071.
- Mahenthalingam, E., M. E. Campbell, D. A. Hendry, and D. P. Speert. 1996. Epidemiology of *Burkholderia cepacia* infection in patients with cystic fibrosis—analysis by randomly amplified polymorphic DNA-fingerprinting. *J. Clin. Microbiol.* **12**:2914-2920.
- Malka, R.-G., M. Poiret, and G. N. Cohen. 1970. The aspartate kinase of *Pseudomonas putida* regulation of synthesis and activity. *Biochim. Biophys. Acta* **206**:17-30.
- McKevitt, A. I., S. Bajaksouzian, J. D. Klinger, and D. E. Woods. 1989. Purification and characterization of an extracellular protease from *Pseudomonas cepacia*. *Infect. Immun.* **57**:771-778.
- Nakazawa, T., Y. Yamada, and M. Ishibashi. 1987. Characterisation of hemolysin in extracellular products of *Pseudomonas cepacia*. *J. Clin. Microbiol.* **25**:195-198.
- Nelson, J. W., S. L. Butler, D. Krieg, and J. R. W. Govan. 1984. Virulence factors of *Burkholderia cepacia*. *FEMS Immunol. Med. Microbiol.* **8**:89-98.
- Palfreyman, R. W., M. L. Watson, C. Eden, and A. W. Smith. 1997. Induction of biologically active interleukin-8 from lung epithelial cells by *Burkholderia (Pseudomonas) cepacia* products. *Infect. Immun.* **65**:617-622.
- Pitt, T. L., M. E. Kaufmann, P. S. Patel, L. C. A. Benga, S. Gaskin, and D. M. Livermore. 1996. Type characterisation and antibiotic susceptibility of *Burkholderia (Pseudomonas) cepacia* isolates from patients with cystic fibrosis in the United Kingdom and the Republic of Ireland. *J. Med. Microbiol.* **44**:203-210.
- Quinton, P. M., and M. M. Reddy. 1992. Control of CFTR chloride conductance by ATP levels through non-hydrolytic binding. *Nature* **360**:79-81.
- Reverts, H., P. Vandamme, A. Van Zeeboeck, K. De Boeck, M. J. Struelens, J. Verhagen, J. P. Ursi, G. Verschraegen, H. Franckx, A. Malfrout, I. Dab, and S. Lauwers. 1996. *Burkholderia (Pseudomonas) cepacia* and cystic fibrosis: the epidemiology in Belgium. *Acta Clin. Belg.* **51**:222-230.
- Riordan, J. R., J. M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.-L. Chou, M. L. Drumm, M. C. Iannuzzi, F. S. Collins, and L. C. Tsui. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**:1059-1065.
- Rosenberg, E. 1986. Microbial surfactants. *Crit. Rev. Biotechnol.* **3**:109-132.
- Sabitini, D. D., K. Bensch, and R. J. Barnett. 1962. New means of fixation for electron microscopy and histochemistry. *Anat. Rec.* **142**:274-279.
- Shaw, D., I. R., Poxton, and J. R. W. Govan. 1995. Biological activity of *Burkholderia (Pseudomonas) cepacia* lipopolysaccharide. *FEMS Immunol. Med. Microbiol.* **11**:99-106.
- Sheppard, J. D., and C. N. Mulligan. 1987. The production of surfactin by *Bacillus subtilis* grown on peat hydrolysate. *Appl. Microbiol. Biotechnol.* **27**:110-116.
- Simmonds, E. J., S. P. Conway, A. T. M. Ghoneim, H. Ross, and J. M. Littlewood. 1990. *Pseudomonas cepacia*: a new pathogen in patients with

- cystic fibrosis referred to a large centre in the United Kingdom. Arch. Dis. Child. **65**:874–877.
37. **Smith, J. J., S. M. Travis, E. P. Greenberg, and M. J. Welsh.** 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell **85**:229–236.
38. **Sun, L., R.-Z. Jiang, S. Steinbach, A. Holmes, C. Campanelli, J. Forstner, U. Sajjan, Y. Tan, M. Riley, and R. Goldstein.** 1995. The emergence of a highly transmissible lineage of *cbl*⁺ *Pseudomonas* (*Burkholderia*) *cepacia* causing CF center epidemics in North America and Britain. Nat. Med. **1**:661–666.
39. **Sylvester, F. A., U. S. Sajjan, and J. F. Forstner.** 1996. *Burkholderia* (Basonym *Pseudomonas*) *cepacia* binding to lipid receptors. Infect. Immun. **64**:1420–1425.
40. **Thomassen, M. J., C. A. Demko, J. D. Klinger, and R. C. Stern.** 1985. *Pseudomonas cepacia* colonization among patients with cystic fibrosis: a new opportunist. Am. Rev. Respir. Dis. **131**:791–796.
41. **Vandamme, P., B. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauwers, M. Gillis, K. Kersters, and J. R. W. Govan.** 1997. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients; proposal of *Burkholderia multivorans* sp. nov. Int. J. Syst. Bacteriol. **47**:1188–1200.
42. **Vasil, M. L., D. P. Krieg, J. S. Kuhns, J. W. Ogle, V. D. Shortbridge, R. M. Ostroff, and A. I. Vasil.** 1990. Molecular analysis of hemolytic and phospholipase C activities of *Pseudomonas cepacia*. Infect. Immun. **58**:4020–4029.
43. **Wilkinson, S. G., and T. L. Pitt.** 1995. *Burkholderia* (*Pseudomonas*) *cepacia*: pathogenicity and resistance. Rev. Med. Microbiol. **6**:10–17.

Editor: R. N. Moore