BACTERIAL PATHOGENICITY

A novel mucin-sulphatase activity found in *Burkholderia cepacia* and *Pseudomonas aeruginosa*

HUGO-JAN JANSEN, C. ANTHONY HART*, JONATHAN M. RHODES†, JONATHAN R. SAUNDERS‡ and JOHN W. SMALLEY

Departments of Clinical Dental Sciences, *Medical Microbiology and †Medicine and ‡School of Biological Sciences, University of Liverpool, Liverpool L69 3GA

Lung infections due to *Burkholderia cepacia* and *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF) are common, are associated with respiratory morbidity and are a cause of mortality. Respiratory mucin in CF patients is highly sulphated, which increases its resistance to bacterial degradation. Desulphation increases the susceptibility of mucin to degradation by bacterial glycosidases and proteinases, and subsequent deglycosylation may facilitate bacterial colonisation by increasing available substrates and binding sites. This study determined whether clinical and environmental strains of *B. cepacia* and *P. aeruginosa* had the ability to desulphate mucin. Mucin-sulphatase activity was tested by incubating bacterial cell suspensions with ³⁵S-sulphated mucins purified from LS174T and HT29-MTX human colon carcinoma cell lines. These mucins were also used to test for differences in substrate specificities. Mucin-sulphatase activity was detected in all nine *B. cepacia* strains and in four of six *P. aeruginosa* strains. There was strain variability in the level of mucin-sulphatase activity. Aryl-sulphatase activities of *Pseudomonas* isolates (determined with methylumbelliferyl sulphate) were *c.* 20-fold higher than those of *B. cepacia* strains, and were independent of mucin-sulphatase activity. This is the first report to demonstrate desulphation of mucin by *B. cepacia* and *P. aeruginosa*. It is concluded that *B. cepacia* and *P. aeruginosa* produce one or more cell-bound glycosulphatase(s), in addition to aryl-sulphatase activity. Mucin-sulphatase activity of *B. cepacia* and *P. aeruginosa* may contribute to their association with airway infections in patients with cystic fibrosis.

Introduction

Burkholderia cepacia and *Pseudomonas aeruginosa* are opportunist pathogens that frequently colonise the respiratory tract of patients whose mucociliary clearance is defective [1, 2]. The most striking of such diseases is cystic fibrosis (CF). CF is characterised by production of a highly viscid respiratory mucin which results in impaired mucociliary clearance, generalised mucus obstruction and colonisation of the airways by *P. aeruginosa*. The initial colonisation is with non-mucoid *P. aeruginosa*, but mucoid variants emerge later and are associated with chronic pulmonary infection [1]. The

reasons why these organisms colonise and persist in the respiratory tract are not fully understood. Although the prevalence of *B. cepacia* (7%) [2] is not as high as that of *P. aeruginosa* (77%) [3], it is increasing and epidemic spread of highly transmissible strains has been reported [4, 5]. The clinical course of *B. cepacia* infection is variable, but *c.* 20% of patients succumb to the so-called 'cepacia syndrome', a rapidly fatal clinical deterioration with necrotising pneumonia and bacteraemia [2].

The severity of CF correlates with increased acidity of tracheo-bronchial mucin from such patients [6]. Part of the increased acidity is caused by mucin over-sulphation [7, 8], possibly due to perturbations in intracellular sulphate activation or the transfer of activated sulphate to the mucin molecule [9], and part is the result of degradation of neutral mucin oligosaccharides by *P. aeruginosa* [10]. The sulphate esters impart a strong negative charge, which influences the physical properties of the mucus [11].

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Corresponding author: Professor C. A. Hart (e-mail: cahmm@liverpool.ac.uk). Present address: Department of Medical Microbiology, Royal Liverpool Hospital, Duncan Building, PO Box 147, Liverpool L69 3BX.

Sulphate is thereby likely to contribute significantly to the rheological properties of CF mucin. The increased tenacity of CF mucins impedes mucociliary clearance. Host defence at the epithelial surface is decreased by a raised NaCl concentration as a result of cystic fibrosis transmembrane regulator (CTFR)-associated defective Cl⁻ transport across the airway epithelia [12]. Hampered mucus clearance and decreased host defence both strongly favour bacterial colonisation, but only if bacteria can find sufficient substrates to metabolise and sites for adherence.

In general, mucin is most heavily sulphated at bodily sites such as the mouth and the colon where there is a rich normal flora [13]. This sulphation protects the mucin from bacterial degradation unless the bacteria also have mucin-sulphatase activity [14]. Desulphation increases the susceptibility of mucin to breakdown by bacterial glycosidases and proteinases as it exposes formerly inaccessible oligosaccharides and, ultimately, the protein core [13]. The released carbohydrates and amino acids are likely to stimulate bacterial growth by analogy with mucin degradation by consortia of oral [15, 16] or colonic [17, 18] micro-organisms. Sulphate in mucin can be found along the oligosaccharide side chains bound to terminal and internal galactose, N-acetylglucosamine and, occasionally, N-acetylgalactosamine residues [19–25]. Both *P. aeruginosa* and *B. cepacia* have been reported to recognise and adhere to di- and tri-saccharide structures that are part of mucin side chains [26–28]. However, these antigenic structures may be shielded by other carbohydrate residues, sialic acid, or sulphate, and thus are not readily available for adhesion. Partial deglycosylation of mucin, of which desulphation is thought to be a first step, may promote bacterial colonisation by increasing available substrate and exposing cryptic binding sites.

In this study, clinical and environmental strains of *B. cepacia* and *P. aeruginosa* were investigated to determine the prevalence of mucin-sulphatase activity among these two respiratory pathogens.

Materials and methods

Bacterial strains

Non-mucoid strains of *B. cepacia* and *P. aeruginosa* (Table 1) were obtained from the collection of the Department of Medical Microbiology, University of Liverpool and comprised mostly isolates from previous studies [3, 29, 30]. All strains were identified by standard biochemical techniques and the API 20 NE system (bioMérieux) and confirmed by the Central Public Health Laboratory (61 Colindale Avenue, London). Environmental strains of *B. cepacia* were described further by Butler *et al.* [29]. *B. cepacia* epidemic isolates were from different patients from different centres and confirmed to be separate strains by SDS-PAGE of whole protein and pyrolysis mass spectroscopy [30].

Bacterial growth conditions

Strains of *B. cepacia* and *P. aeruginosa* and the aryl-sulphatase negative control strain *Escherichia coli* K12 that were tested for aryl-sulphatase activity and sulphatase activity against LS174T mucin were grown in triplicate on blood agar containing defibrinated horse blood 5% v/v, for 24 h at 37°C, harvested with sterile swabs, washed once in 0.1 M Tris, 0.15 M NaCl, pH 7.0 (Tris-saline) and resuspended in 5 ml of Tris-saline. Strains of *B. cepacia* that were tested for sulphatase activity against HT29-MTX mucin were grown on five plates of blood agar for 24 h at 37°C. Mucin-sulphatase positive control strain *Bacteroides thetaiotaomicron* NCTC 10582 was grown anaerobically on 10 plates of blood agar for 48 h. The protein content of bacterial cell suspensions was estimated by the biuret bicinchoninic acid (BCA) protein assay (Sigma) with bovine serum albumin as a standard.

Mucin

³⁵SO₃-labelled human colonic mucin was obtained after incorporation of ³⁵SO₄²⁻ into mucins secreted by

Table 1. Source and description of strains used in this study

Organism	Strain	Source	Description
<i>B. cepacia</i>	D505	Liverpool	Cepacia syndrome, epidemic
<i>B. cepacia</i>	D538	Liverpool	Cepacia syndrome, epidemic
<i>B. cepacia</i>	E195	Sheffield	CF isolate, epidemic
<i>B. cepacia</i>	E241	Edinburgh	CF isolate, epidemic
<i>B. cepacia</i>	E242	Edinburgh	CF isolate, non-epidemic
<i>B. cepacia</i>	E243	Edinburgh	Environmental strain
<i>B. cepacia</i>	E244	Edinburgh	Environmental strain
<i>B. cepacia</i>	E245	Edinburgh	Environmental strain
<i>B. cepacia</i>	E246	Edinburgh	Environmental strain
<i>P. aeruginosa</i>	E546	Liverpool	CF isolate
<i>P. aeruginosa</i>	E551	Liverpool	CF isolate
<i>P. aeruginosa</i>	E567	Liverpool	CF isolate
<i>P. aeruginosa</i>	E575	Liverpool	CF isolate
<i>P. aeruginosa</i>	E601	Liverpool	CF isolate
<i>P. aeruginosa</i>	PAO-1	BW Holloway, LaFroke	Laboratory strain

the human colon carcinoma cell lines LS174T and HT29-MTX. Cells were grown in Dulbecco's Modified Eagle's Medium (Life Sciences) supplemented with 2 mM L-glutamine, penicillin 100 U/ml, streptomycin 100 µg/ml and fetal calf serum (10% v/v for HT29-MTX; 15% v/v for LS174T) (DMEM) at 37°C, in air with CO₂ 5%. Spent medium was replaced with fresh DMEM on alternate days. After cells had been confluent for 7 days, they were incubated for 72 h in low-sulphate DMEM (Life Sciences) with 50 µCi/ml Na₂[³⁵S]O₄ (NEN). Low-sulphate DMEM contains a quarter of the sulphate concentration in normal DMEM and was used to promote ³⁵SO₄²⁻ incorporation [31]. ³⁵S-labelled-sulphated mucins were purified from cells and cell culture medium by gel filtration on Sepharose CL-4B (Pharmacia) and Superose-6 FPLC (Pharmacia) as described previously [32]. Mucin-containing fractions were pooled, lyophilised and de-salted on prepacked gravity-fed PD10-Sephadex G25M columns (5 × 1.5 cm; Pharmacia) equilibrated in 0.1 M Tris-HCl, pH 7.0.

Mucin-sulphatase assay

Mucin-sulphatase (MS) activity was measured by the release of ³⁵SO₄²⁻ after incubation of bacterial cells from replicate cultures of *B. cepacia*, *P. aeruginosa* and *E. coli* with ³⁵S-sulphated mucin by a method slightly modified from that previously described by Smalley *et al.* [33]. Samples of bacterial cell suspensions containing *c.* 1 mg of whole-cell protein were incubated at 37°C with 11 000 dpm (0.005 µCi) of ³⁵SO₄-labelled mucin in a final volume of 2.5 ml of 0.1 M Tris, pH 7.0. Incubation was stopped after 48 h by boiling for 3 min. Bacterial cells were centrifuged and the supernate was passed through PD10-Sephadex G25M columns (5 × 1.5 cm). The mucin substrate eluted in the void volume (3.5 ml) whilst cleaved ³⁵SO₄²⁻ was washed from the column with a further 6 ml of Tris buffer and precipitated by addition of 1 ml of 0.1 M BaCl₂ and 0.9 ml of 0.1 M K₂SO₄. This method precipitates free sulphate but not sulphated monosaccharides [34]. The precipitate was washed once, resuspended in 0.5 ml of Tris buffer, and radioactivity was quantified by β-scintillation counting. *Bact. thetaiotaomicron* [14] and a faecal homogenate, prepared from pooled faeces from six patients with ulcerative colitis [34], served as positive controls (*n* = 8); 13 samples of ³⁵S-sulphated mucin in Tris buffer were used as negative controls to assess spontaneous background hydrolysis of sulphate.

Aryl-sulphatase assay

Aryl-sulphatase (AS) activity was assayed by release of 4-methylumbelliferone (MUF) after incubation of bacterial cells (*c.* 60 µg protein) from triplicate cultures of *B. cepacia*, *P. aeruginosa* and *Bact. thetaiotaomicron* at 37°C with 170 µM 4-methylumbelliferyl sulphate in a final volume of 200 µl of 0.1 M Tris, pH 7.0. Incubation

was stopped after 24 h by boiling for 3 min, cellular debris was centrifuged and the volume adjusted to 3 ml with 2.2 mM NaOH to a final pH of 10.3 (the pH optimum of fluorescence intensity of MUF [35]). The amount of released sulphate was calculated from the concentration of free MUF as detected with a Model 1000 Fluorescence Spectrophotometer (excitation 363 nm, emission 450 nm; Perkin Elmer). Free MUF (Sigma) was used as a standard. Strains of *P. aeruginosa* [36] and faecal homogenate from patients with ulcerative colitis were used as positive controls; *E. coli* K12 and Tris buffer were negative controls. Suspensions of bacterial cells in Tris buffer served to control for autofluorescence of bacterial products.

Statistical analysis

The data are expressed as means and SEM and amounts of sulphate released by bacterial strains were compared with that released by the Tris buffer control. Differences were analysed by the one-tailed Student's *t* test and differences at *p* < 0.05 were considered to be significant.

Results

With an assay in which ³⁵SO₄²⁻ was cleaved from ³⁵S-labelled human colonic mucin and precipitated with BaCl₂, mucin-sulphatase activity was shown to be associated with whole cells of *B. cepacia* and *P. aeruginosa* clinical and environmental isolates. All isolates of *B. cepacia* possessed MS activity against one or both ³⁵S-mucins (Fig. 1). Four of nine isolates were active against either LS174T or HT29-MTX mucin; the other five isolates were active against both. Sulphatase activity against LS174T-derived mucin was detected in four of the six *P. aeruginosa* isolates. Inter-strain MS activity of the test isolates varied, but MS activity was usually similar to or greater than that displayed by the positive control, *Bact. thetaiotaomicron*. Of the MS-positive isolates, the average release of radioactivity minus that of the Tris negative control for *B. cepacia*, as a percentage of the total available, was 2.8% SD 3.0% (*n* = 7, range 0.5–9%) for LS174T mucin and 4.3% SD 2.6% (*n* = 7, range 2–10%) for HT29-MTX mucin. The average sulphatase desulphation of LS174T mucin by *P. aeruginosa* (2.5% SD 0.7, *n* = 4) was similar to that of *B. cepacia*, but the range of inter-strain MS activity was smaller (2–3.5%). The faecal homogenate liberated 38% of radiolabelled sulphate from LS174T mucin, but only 5.5% from HT29-MTX mucin. Clinical isolates of *B. cepacia* did not appear to have MS activities different from those of environmental strains, as was the case for epidemic versus non-epidemic isolates. Furthermore, sulphatase activity against LS174T-derived mucin was detected in *E. coli*.

AS activity was demonstrated in all isolates of *B. cepacia* and *P. aeruginosa* (Fig. 2). The activities of

Fig. 1. Mucin-sulphatase activity of *B. cepacia* and *P. aeruginosa* strains against ^{35}S -labelled mucins. Activity was determined by release of $^{35}\text{SO}_4^{2-}$ after incubation of whole bacterial cells with ^{35}S -sulphated mucin from human colon carcinoma LS174T ($\square$) and HT29-MTX ($\blacksquare$) cell lines at 37°C for 48 h. *Bact. thetaiotaomicron* and a faecal homogenate from patients with ulcerative colitis were used as positive controls. Tris buffer was used as a negative control. Results are expressed as mean and SEM dpm of released ^{35}S -sulphate and differences between bacterial strains versus the negative control were statistically significant ($p < 0.05$) unless indicated (*).

the *B. cepacia* isolates were all similar, but were c. 20-fold lower than those of *P. aeruginosa*.

AS activity in *B. cepacia*, *P. aeruginosa*, *Bact. thetaiotaomicron* and the (AS-negative) *E. coli* control strain did not correlate with the presence of MS activity, indicating that the activities are mediated by different enzymes. As suspensions of washed bacterial cells were used, the mucin- and aryl-sulphatases detected are likely to be cell-wall-associated or localised in the periplasmic space.

Discussion

This study demonstrated for the first time that *B. cepacia* and *P. aeruginosa* can desulphate mucin. MS activity with *P. aeruginosa* has not been reported previously and is mediated by an enzyme that differs from the aryl- [36], choline- [37] and alkyl- [38] sulphatase activities that have been known for some time. No sulphatase activity of any kind has been demonstrated before in *B. cepacia*. The present study further indicates that, although the level of MS activity

Fig. 2. Aryl-sulphatase activity of *B. cepacia* and *P. aeruginosa* strains. Activity was determined by release of sulphate, yielding free methylumbelliferone, after incubation of washed bacterial cells with methylumbelliferyl sulphate for 24 h at 37°C . A faecal homogenate from patients with ulcerative colitis acted as positive control. Negative controls were *E. coli* and Tris-buffer. Results are expressed as mean and SEM ($n = 3$) of released sulphate and differences between bacterial strains versus the Tris buffer control were statistically significant ($p < 0.05$) unless indicated (*).

may vary considerably between isolates, this ability is found commonly in the two genera and they are relatively active as compared with control organisms from the gut such as *Bact. thetaiotaomicron* and *E. coli*.

The role of sulphomucins and the importance of mucin desulphation in the gut was reviewed recently [13]. In colonic mucin, sulphate is ester-linked to C-3 and C-6 of galactose and *N*-acetylglucosamine residues [19, 39]. As sulphate is found on the same positions and residues in human tracheobronchial and CF-derived mucin [20–22] it seems likely that the sulphatases detected in *B. cepacia* and *P. aeruginosa* can also degrade mucin from these sources.

To ensure a maximum release of sulphate from the substrate, an excess of the bacterial suspension in relation to available substrate was used in both MS and AS assays and incubation times were relatively long compared with other studies [33, 34, 40]. Hence, sulphatase activity is expressed as the release of absolute amounts of sulphate, rather than release of sulphate/mg of cell protein, or release of sulphate/mg/min.

Approximately 38% of radiolabelled sulphate was released from the LS174T mucin by the faecal homogenate. This is similar to the 30% of sulphate released by the faecal homogenate from mucin from cultured human colonic biopsies [34] and the 33% of sulphate released from rat gastric mucin by a purified glycosulphatase from *Prevotella* sp. RS2 [41]. However, the sulphate release from HT29-MTX mucin by the faecal homogenate was seven-fold lower, suggesting that there is substrate heterogeneity. The differences in sulphatase activity between test isolates and controls observed for mucins from the two cell lines also indicate substrate heterogeneity. This implies that mucin secreted by the LS174T cell line contains different sulphated oligosaccharide structures from mucin produced by the HT29-MTX cell line. Unfortunately, biochemical data to characterise these differences are not yet available. However, LS174T produces a mucin with the protein core (apomucin) predominantly encoded by the *MUC2* gene [42], whilst the majority of apomucins secreted by HT29-MTX are encoded by *MUC5A/C* and *MUC5B* [43], and mucin glycosylation is modulated by the amino-acid sequence of the protein core [44]. Lower sulphate release from HT29-MTX mucin by faecal homogenate may be due to the substrate specificity of the glycosulphatases not matching the remaining sulphated sugars in the mucin oligosaccharide chains.

P. aeruginosa has few glycosidase activities [45], but no data are available for *B. cepacia*. In colonic mucins, the oligosaccharide side chains are densely packed and may vary in length from 2 to 12 residues and sulphate is linked to galactose and N-acetylglucosamine along the chains [46]. The low sulphate release by bacterial samples is presumably due to steric hindrance of the neighbouring oligosaccharide side chains impeding the access of glycosulphatases to the carbohydrate side chains in the absence of bacterial glycosidases, particularly when sulphate is linked to carbohydrate residues within the side chain, or to non-matching substrate specificities of the glycosulphatases.

The *B. cepacia* and *P. aeruginosa* mucin- and arylsulphatases detected are likely to be localised either on the cell wall or in the periplasmic space, as is the case for the glycosulphatase of *Prevotella* sp. RS2 [41] and MS activities in oral streptococci [33]. It is likely that adhesion to the mucin molecules is a prerequisite for desulphation. Bacterial adherence to CF mucin has been reported for *P. aeruginosa* and *B. cepacia* [27, 47]. In addition to cell-bound activities, *Streptococcus salivarius* and *S. gordonii* produce extracellular MS activities [48], and mucin-specific glycosulphatases are found extracellularly in *Bact. thetaiotaomicron* [14], *Helicobacter pylori* [49], *Ruminococcus torques* [50] and *Bifidobacterium* sp. [50].

Desulphation increases susceptibility of mucin to degradation by bacterial glycosidases and proteinases

[13]. *P. aeruginosa* can break down neutral mucins in the sputum of CF patients [10]. It produces proteinases, some glycosidases [45] and a putative neuraminidase that is active against mucins [51], although the latter activity may be rare [52]. Desulphation and desialylation of the acidic mucins will increase the amount of the neutral mucin fraction degradable by the organism. In addition, this activity will expose formerly inaccessible oligosaccharides to glycosidases of its own and other infecting bacteria such as *S. pneumoniae* and *Staphylococcus aureus*, thus accelerating mucin breakdown.

Desialylation greatly decreases adhesion of *P. aeruginosa* to CF salivary mucin, not only indicating that sialic acid plays an important role in binding [53], but also that desialylation may slow further colonisation. Moreover, *P. aeruginosa* recognises and adheres to type 1 (Gal β 1-3GlcNAc) and type 2 (Gal β 1-4GlcNAc) backbone structures of mucin carbohydrate side chains [26, 28], which are abundant in tracheobronchial [20, 21], colonic [19] and salivary [25] mucins. Other neutral oligosaccharide structures, including asialo GM1 and asialo GM2, are recognised by both *P. aeruginosa* and *B. cepacia* [28]. The disaccharides GlcNAc β 1-3-GalNAc or GlcNAc β 1-6-GalNAc found, for example, in core 3 and core 4 mucin oligosaccharide configurations [27] may also be binding receptors for *B. cepacia*. However, these structures, such as the Gal β (1-3)-GlcNAc backbone which is a major site of sulphation of colonic mucins [39, 54], may be shielded by other carbohydrate residues, sialic acid, or sulphate, and thus not be readily accessible for adhesion. The results of the above studies [26, 28, 53] indicate that various adhesins with different carbohydrate specificities exist on *P. aeruginosa*. Conflicting data about receptor involvement in the binding of *P. aeruginosa* to mucin were reported by Sajjan and co-workers [47]. They found that *P. aeruginosa* bound non-specifically to CF mucin, intestinal mucin, bovine serum albumin, casein and other structurally unrelated proteins by hydrophobic interactions. Partial deglycosylation of mucin may facilitate bacterial colonisation by exposing cryptic binding sites for *B. cepacia* and possibly *P. aeruginosa*. Furthermore, the released sugars are likely to promote bacterial growth, including the growth of those that metabolise carbohydrates but do not have glycosidase activities themselves, by analogy with mucin degradation by consortia of oral [15, 16] or colonic [17, 18] micro-organisms.

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