Desulfurization of mucin by *Pseudomonas aeruginosa*: influence of sulfate in the lungs of cystic fibrosis patients

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*Pseudomonas aeruginosa* is a common cause of chronic respiratory infection in cystic fibrosis (CF) patients. Infection is established within the lung epithelial mucus layer through adhesion to mucins. Terminal residues on mucin oligosaccharide chains are highly sulfated and sialylated, which increases their resistance to degradation by bacterial enzymes. However, a number of microbes, including *P. aeruginosa*, display mucin sulfatase activity. Using ion chromatography, the levels of sulfation on different respiratory mucins and the availability of inorganic sulfate to pathogens in sputum from CF patients were quantified. The ability of clinical isolates of *P. aeruginosa* to desulfate mucin was tested by providing mucin as a sole sulfur source for growth. All tested *P. aeruginosa* strains isolated from the lungs of CF patients were able to use human respiratory mucin as a source of sulfur for growth, whereas other non-clinical species of the genus *Pseudomonas* were not. However, measured levels of inorganic sulfate in sputum from CF patients suggested that bacteria resident in the lung have sufficient inorganic sulfur for growth and are unlikely to require access to mucin sulfur as a sulfur source during chronic infection. This was confirmed when expression of sulfate-repressed *P. aeruginosa* genes *atsK* and *msuE* was found to be repressed in the sputum of CF patients, which was detected by using quantitative RT-PCR. These results indicate that sulfate starvation is unlikely to occur in pathogens residing in the sputum of CF patients and, therefore, mucin desulfation may have an alternative purpose in the association between *P. aeruginosa* and the airways of CF patients.

INTRODUCTION

Changes in respiratory secretions have long been implicated in obstructive respiratory diseases such as asthma, chronic obstructive pulmonary disease and cystic fibrosis (CF), although the cause of these changes is often poorly understood. One notable change in lung secretions is the increased sulfation level that has been reported for mucins in the sputum of CF patients (Boat et al., 1976a; Chace et al., 1983; Davril et al., 1999; Xia et al., 2005). A positive correlation has been suggested between the level of sputum sulfation and the severity of disease in CF patients (Chace et al., 1983) and increased glycan sulfation has been proposed to occur as a response to bacterial infection, possibly to protect the underlying glycoprotein from enzymic degradation by bacteria (Corfield et al., 1993; Tsai et al., 1992, 1995). This theory has been difficult to study due to the very high rate of bacterial infection in CF patients but mucin oligosaccharides are more resistant to degradation and utilization by *P. aeruginosa* when sulfated (Chance & Mawhinney, 2000) and mucin in bronchoalveolar lavage (BAL) samples from artificially ventilated patients with ventilator-associated pneumonia has a fivefold higher sulfation level than mucin from artificially ventilated patients without pneumonia (Dennesen et al., 2003). Interestingly, a study using a radiolabelled xenograft model showed that tissue originating from the bronchi of CF patients produced significantly more sulfated mucus glycoprotein than tissue originating from healthy bronchi (Zhang et al., 1995), suggesting that oversulfation may be inherent in CF patients and at least partly independent of chronic bacterial infection.

The possession of mucin-degrading enzymes by pathogenic bacteria isolated from almost all mucosal surfaces in the human body indicates the rich source of nutrients that mucin can provide (Berry et al., 2002; Rho et al., 2005;
Roberton et al., 2005; Smith et al., 1994; Wickström et al., 2009), whilst also suggesting a mechanism by which bacteria may penetrate the protective mucus layer to gain access to the underlying epithelial cells. Mucin sulfatase activity has been identified in microbes associated with most mucosal layers, including the enteric bacteria of the genera *Bacteroides* and *Prevotella* (Tsai et al., 1991; Wright et al., 2000), oral streptococci (Jansen et al., 1997; Smalley et al., 1994), vaginal bacteria (Roberton et al., 2005) and the respiratory pathogens *Pseudomonas aeruginosa* and *Burkholderia cepacia* (Jansen et al., 1999; Tralau et al., 2007). Desulfation of the terminal sugars of mucin constitutes the first step in mucin degradation to provide carbon for growth but organisms like *P. aeruginosa* can also utilize human mucin as a source of sulfur (Tralau et al., 2007). Inorganic sulfate is the preferred sulfur source for *P. aeruginosa* (Hummerjohann et al., 1998) but if this is limited, *P. aeruginosa* is able to desulfurize a range of organosulfur compounds through expression of a range of loci involved in organosulfur utilization (Kertesz, 2004). Mucin desulfation may, therefore, be an alternative option for sulfur acquisition in the lung environment. Little is known about the biological significance or prevalence of mucin sulfatase activity in *P. aeruginosa*, although Jansen et al. (1999) found that the majority of CF-associated *P. aeruginosa* and *B. cepacia* lung isolates tested displayed mucin sulfatase activity and that this was independent of the presence of arylsulfatase activity.

In this study, the ability of CF-associated *P. aeruginosa* isolates to grow with mucin as a sulfur source *in vitro* was related to the levels of mucin sulfation and to the levels of available inorganic sulfate in the lungs of CF patients.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. Clinical strains of *P. aeruginosa* isolated from patients with CF were sourced originally from Liverpool (UK) and Wythenshawe (Manchester, UK) CF clinics and were provided by J. Govan (University of Edinburgh Medical School, Edinburgh, UK). Clinical and laboratory strains were routinely cultured on Luria–Bertani medium at 37 °C. Liquid cultures were incubated with shaking at 200 r.p.m. Where defined medium was required, *P. aeruginosa* was grown in MMAA medium, comprising 50 mM Tris/HCl (pH 7.3), 20 mM NH₄Cl, 0.5 mM MgCl₂, 25 mM sodium succinate or sodium acetate, 20 mM NaCl, 20 mM KCl, 20 mM KP and trace elements (Thurnheer et al., 1986), plus non-S amino acids each at a final concentration of 4 μg ml⁻¹ (Beil et al., 1995). Sulfur sources were added as required, including sulfate or cyclamate (500 μM), pig gastric mucin (PGM; 2 mg ml⁻¹) or human mucin 3B (MUC3B; 0.154 mg ml⁻¹). When MMAA was required as a solid medium, molecular-grade agarose was washed three times in 5 vols Milli-Q water before being added to the medium at a final concentration of 0.75 % (w/v).

Bacterial growth curves were produced by using a Synergy H1 microplate reader (Biotek) with 96-well, flat-bottomed microtitre plates (Greiner Bio-One) with 200 μl medium per well. Growth was measured as OD₆₀₀ every 10 min over a period of 24–72 h.

**Mucin preparation and purification.** Unfractionated human polymeric salivary mucins (MUC5B) were prepared from pooled fresh saliva provided by healthy volunteers by using a caesium chloride isopycnic gradient method (Davies et al., 2012). Pooled saliva was added to an equal volume of 8 M guanidinium chloride and mixed overnight at 4 °C. Caesium chloride was added (1.4 g ml⁻¹ final density) and the mixture was centrifuged at 147 000 g for 65 h at 15 °C. Mucin-containing gradient fractions were identified by periodic acid–Schiff (PAS) stain (adapted from Fairbanks et al., 1971; Kirkham et al., 2002) and then pooled and dialysed into 0.5 M guanidinium chloride. After a second caesium chloride density-gradient centrifugation (1.5 g ml⁻¹ caesium chloride, 65 h at 147 000 g, 15 °C), mucin-rich fractions were pooled and dialysed into 4 M guanidinium chloride and purified by size-exclusion chromatography on a Sepharose CL-2B column (90 × 5 cm; GE Healthcare Life Sciences), equilibrated and run with 4 M guanidinium chloride at 2 ml min⁻¹. Mucin-containing fractions were analysed by SDS-PAGE with Coomassie blue staining to confirm the absence of other contaminating proteins and then lyophilized. Before use, salivary mucin was resuspended in ultrapure water at a

### Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td><em>P. aeruginosa</em> strain:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Laboratory strain</td>
<td>Holloway (1955)</td>
</tr>
<tr>
<td>49211</td>
<td>Sporadic CF isolate, non-mucoid</td>
<td>Liverpool</td>
</tr>
<tr>
<td>59032</td>
<td>Sporadic CF isolate, non-mucoid</td>
<td>Liverpool</td>
</tr>
<tr>
<td>59039</td>
<td>Sporadic CF isolate, mucoid</td>
<td>Liverpool</td>
</tr>
<tr>
<td>59079</td>
<td>Sporadic CF isolate, non-mucoid</td>
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</tr>
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<td>C3425</td>
<td>Manchester epidemic strain, non-mucoid</td>
<td>Kenna <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>E2703</td>
<td>Sporadic CF isolate, non-mucoid</td>
<td>Wythenshawe</td>
</tr>
<tr>
<td>E601</td>
<td>Sporadic CF isolate, non-mucoid</td>
<td>Jansen <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>7NSK2</td>
<td>Environmental isolate from barley rhizosphere</td>
<td>Iswandi <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>PA114</td>
<td>Environmental isolate from swimming goggles</td>
<td>J. Manos, Sydney</td>
</tr>
<tr>
<td><em>P. putida</em> S-313</td>
<td></td>
<td>Zürrer <em>et al.</em> (1987)</td>
</tr>
<tr>
<td><em>P. fluorescens</em> CHA0</td>
<td></td>
<td>Keel <em>et al.</em> (1989)</td>
</tr>
<tr>
<td><em>P. syringae</em> pv. <em>tomato</em> DC3000</td>
<td></td>
<td>Barbanti <em>et al.</em> (2011)</td>
</tr>
<tr>
<td><em>E. coli</em> S17-1</td>
<td>Tp⁺ Sm⁺ recA, thi, pro, hsdR RP4: 2-TcMu: Km Tn7 ·pir</td>
<td>Simon <em>et al.</em> (1983)</td>
</tr>
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final concentration of 1 mg ml\(^{-1}\) and filter sterilized through a 0.22 µm syringe filter (Whatman).

PGM (type III; Sigma) was rehydrated before use in ultrapure water to a concentration of 20 mg ml\(^{-1}\) by vigorous shaking for 30 min. Insoluble material was removed by centrifugation (10 000 g, 30 min, 4 °C) and the supernatant was dialysed at 4 °C against 2 l ultrapure water for 24 h with a minimum of four water changes. The dialysed mucin was diluted to 4 mg ml\(^{-1}\) in ultrapure water before sterile filtration through a 0.22 µm GD/X syringe filter (Whatman).

To investigate the structural properties of PGM, partial degradation was carried out by sonication and proteinase K digestion. For sonication, rehydrated PGM was cooled on ice for 30 min after removal of insoluble material by centrifugation. It was then subjected to tip-probe sonication with a tapered microtip three times for 30 s each, with 1 min on ice between each cycle. Proteinase K digestion was carried out after dialysis. Agarose-immobilized proteinase K (Sigma) was washed three times in 5 vols sterile ultrapure water before use. Proteinase K–agarose (30 mg dry weight; 38 U g\(^{-1}\) (Sigma) was washed three times in 5 vols sterile ultrapure water for 24 h with a minimum of four water changes. The dialysed agarose-immobilized enzyme was removed by centrifugation (10 000 g, 10 min, 4 °C). The supernatant was removed and centrifuged as before to remove remaining traces of immobilized enzyme.

Human mucin 5AC (MUC5AC) and high- and low-charged MUC5B were purified from the human intestinal cell line HT-29 and saliva, respectively, as described previously (Kirkham et al., 2002).

**CF sputum collection and processing.** A total of 36 sputum samples were collected in sterile pots by expectoration from 29 adult CF patients (eight female and 21 male, mean age 31 ± 10 years) attending the CF clinic at the Royal Prince Alfred Hospital, Sydney, Australia, on two separate occasions. All sputum samples were provided anonymously and each was assigned a unique number to correlate with medical data recorded at the time of specimen collection. Seven repeat samples were collected from randomly selected patients 2 weeks or 5 months after the first collection. The samples were stored immediately at 4 °C and then transferred to −80 °C within 1–3 h of collection. All patients gave written informed consent for analysis of their sputum samples for the research project.

For analysis, the samples were thawed on ice and weighed. Free saliva was removed from the sputum by brief washing with 5 vols sterile PBS (agitated by inversion four to six times). The washed sputum was liquefied by addition of an equal volume of Sputasol solution (0.1% DTT, 0.78% sodium chloride, 0.02% potassium chloride, 0.112% disodium hydrogen phosphate, 0.02% potassium dihydrogen phosphate; Oxoid) and incubated at 37 °C for 20–30 min, with shaking and periodic vortexing. Sputasol treatment was necessary in order to handle the highly viscous sputum and preliminary investigations confirmed that the presence of DTT in Sputasol did not affect the measured inorganic sulfate content in the sample. The liquefied sputum samples were stored at −80 °C until further use.

**DNA and RNA extractions from sputum and bacterial cells.** For DNA isolation, aliquots of processed CF sputum (0.2–1 ml) were centrifuged at 10 000 g for 5 min to pellet cell material. Total DNA was extracted from cell material using an ISOLATE Genomic DNA Mini kit (Bioline) according to the manufacturer’s instructions and eluted in 50 µl sterile water.

For RNA extraction, liquefied sputum (0.5–1 ml) was added to 1 ml TrisSure reagent (BioLine) and mixed by repeated vortexing for 30 s until the sample had become homogeneous. The homogenate was incubated at room temperature for 5 min and extracted once with chloroform (0.2 ml). RNA was then purified from the aqueous phase using an ISOLATE RNA Mini kit (Bioline), first mixing with 4 vols Lysis Buffer R and then, following the manufacturer’s instructions, applying the sample in a number of portions to each spin column. The column was washed as recommended by the manufacturer and the RNA was eluted in 30 µl RNase-free water. To confirm the complete removal of DNA, the RNA samples were used as template in PCRs with the universal 16S rRNA gene primers 27F and 1492R (Lane, 1991).

RNA was isolated from exponential-phase bacterial cells using RNeasy extraction spin columns (Qiagen), followed by DNase I treatment and repurification with the RNeasy system, eluting in 40 µl RNase-free water.

**PCR and RT-PCR.** PCR was carried out in an S1000 thermocycler (Bio-Rad) using 0.5 U MangoTaq DNA polymerase in 1 x MangoTaq reaction buffer (Bioline) with 1.5 mM MgCl\(_2\), 200 µM dNTPs, 10 pmol each primer (Table 2; Integrated DNA Technologies), 5% (v/v) DMSO and 50 ng DNA template in a 25 µl reaction. PCR cycling conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, the annealing temperature for 30 s and 72 °C for 1 min kb\(^{-1}\), with a final extension at 72 °C for 5 min. Where the extension step was longer than 3 min, the temperature was reduced to 68 °C.

For cDNA synthesis, 1–4 µg RNA was added to 625 µl each dNTP (Bioline) and 5 µM random hexamer primers (NEB) in a total volume of 32 µl in RNase-free water. This was incubated at 70 °C for 5 min and then at 4 °C for 2 min. RT buffer (NEB) was added to a final concentration of 1 x, along with 40 U RNase inhibitor (NEB) and 400 U Moloney murine leukaemia virus reverse transcriptase (NEB) in a final volume of 40 µl. The reaction was incubated at 25 °C for 10 min, 42 °C for 1 h and 90 °C for 10 min and then stored at 4 °C.

For quantitative PCR, approximately 5 ng cDNA was used as template in 5 µl reactions with LightCycler 480 SYBR Green 1 Master Mix (Roche). Reactions were set up in triplicate using a Biomek NX\(^\text{®}\) Laboratory Automation Workstation (Beckman Coulter) in 384-well plates (Roche). Cycles were run on a LightCycler 480 (Roche Applied Science) with pre-amplification at 95 °C for 5 min, followed by amplification for 40 cycles of 95 °C for 10 s, 62 °C for 20 s and 72 °C for 10 s.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>msuE1</td>
<td>CAAGCACCCGTGGTCGACCTG</td>
<td>This work</td>
</tr>
<tr>
<td>msuE2</td>
<td>TAGTTGTCGAAGTCGGCCCTC</td>
<td>This work</td>
</tr>
<tr>
<td>rpsL-R</td>
<td>CGGCTGTGCTTGCAGGTGTGGA</td>
<td>Dumas et al. (2006)</td>
</tr>
<tr>
<td>SyAtsK_for</td>
<td>TGCACAGCAACGAAGTACGACT</td>
<td>This work</td>
</tr>
<tr>
<td>SyAtsK_rev</td>
<td>CGTAGACGTCGAGGTGACA</td>
<td>This work</td>
</tr>
</tbody>
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for 5 s. Determination of melting curves was carried out by incubation at 95 °C for 5 s and 65 °C for 1 min and then ramping up to 97 °C at a ramp rate of 0.06 °C s⁻¹. Single fluorescence acquisitions were carried out during each extension step and 10 acquisitions were taken per °C during the melting curve. Data were analysed using the LightCycler 480 SW 1.5 software (Roche).

**Denaturing gradient gel electrophoresis (DGGE).** DNA amplification was carried out using 1 µl boiled bacterial cells (single colony, 100 °C, 5 min) or 0.1–2.0 µl purified sputum cDNA as template, 200 µM each dNTP (Bioline), 50 pmol each of primers dgge_341F (containing a GC clamp) and 518r (Muyzer et al., 1993), 5 µL Kappa Robust 3G DNA polymerase and 1 × Kappa buffer A (Geneworks) in 25 µl reactions. The PCR cycle used consisted of initial denaturation at 95 °C for 5 min, followed by 10 cycles of 95 °C for 30 s, 60 °C for 30 s (reducing by 1 °C per cycle) and 68 °C for 30 s, with a further 20 cycles of 95 °C for 30 s, 50 °C for 30 s and 68 °C for 30 s, followed by a final elongation step at 68 °C for 5 min.

DGGE was performed with a DCode 16 × 16 cm gel system (Bio-Rad) as described previously (Cunliffe & Kertesz, 2006) with a denaturant gradient of 30–60 %, which was poured with a gradient mixer. The gel was loaded (200 ng per sample) and electrophoresis was conducted at 60 °C for a total of 1008 Vh (constant voltage of 63 V for 16 h). Gels were stained with 2 µl SYBR Gold stain (Invitrogen) in 20 ml 1 × TAE for 30 min in the dark and then washed briefly with distilled water. Gel image analysis was carried out using the Quantity One V4.6.9 software (Bio-Rad). The bands of interest were excised and sequenced as described previously (Cunliffe & Kertesz, 2006).

**Quantification of sulfate by HPLC.** To measure the organic sulfate content in mucin and sputum preparations, sulfate was released by acid hydrolysis. Mucin or sputum samples (200–500 µl) were added to an equal volume of 200 mM HCl and incubated at 100 °C overnight in 1.5 ml screw-cap tubes. After cooling, the samples were neutralized by the addition of NaOH and further diluted 1:10 in water. Samples were centrifuged at 10,000 g for 5 min to remove insoluble material and sulfate in the supernatants was determined by ion chromatography.

The HPLC system used for ion chromatography consisted of a Dionex system controlled by Chromeleon software (Dionex), with an IonPac AS14/AG14 anion-exchange column (4 × 250 mm column, 9 µm particle size), using conductimetric detection with an ED10 electrochemical detector coupled to an ASRS300 micromembrane suppressor (Dionex). Analytes were eluted with 3.5 mM Na₂CO₃/1 mM NaHCO₃ with a flow rate of 1.2 ml min⁻¹, at 25 °C. Glassware used for sulfate quantification was washed with 3 M HCl overnight and rinsed in ultrapure water before use.

**RESULTS**

**Sulfation level in purified salivary mucin**

MUC5B was prepared from the pooled saliva of healthy volunteers and hydrolysed to allow measurement of mucin sulfation levels by HPLC. As a comparison, the sulfate content was also measured in purified solutions of the three dominant soluble polymeric mucins in human airways, high- and low-charge MUC5B glycoforms and MUC5AC. All mucins were sulfated to the same order of magnitude (Fig. 1), although the high-charge MUC5B glycoform displayed the highest degree of sulfation at almost 500 µmol sulfate (g mucin)⁻¹ (dry weight). The unfractionated MUC5B mucins had a mean of 195 µmol sulfate (g mucin)⁻¹. This equated to a range of 1.9–4.8 % (w/w) organic sulfate in the mucins tested. The non-fractionated MUC5B was expected to have a sulfation level intermediate between those of the purified charge forms (Fig. 1), and the lower sulfation level observed for the unfractionated sample probably reflects the fact that it was prepared from different individuals from the purified mucins, as the mucin composition of saliva is altered in response to environmental stimuli (Davis, 1997). Preliminary testing showed that total salivary sulfate levels varied widely among individuals (data not shown).

Sulfation levels of commercial PGMs were measured for comparison purposes and were found to be highly variable, with up to a fivefold difference in sulfate levels measured between different batches (data not shown). The physical nature of freeze-dried mucin and the heterogeneity of the commercial product probably contributed to this variability. The mean PGM sulfation level was ~50 µmol sulfate (g mucin)⁻¹.

**Growth of CF-associated P. aeruginosa isolates with mucin as a sulfur source**

To determine the prevalence of mucin sulfatase activity in clinical P. aeruginosa strains, a range of CF-associated isolates were tested for their ability to utilize mucin as a sole sulfur source. Mucin was added to culture medium as a sole sulfur source. Mucin was added to culture medium as a sole sulfur source. Mucin was added to culture medium as a sole sulfur source. Mucin was added to culture medium as a sole sulfur source.
could be utilized. The total organic sulfate content of the medium after addition of mucin was quantified by HPLC before use. Growth of seven *P. aeruginosa* isolates from the lungs of CF patients (strains C3425, E2703, E601, 49211, 59032, 59039 and 59079) were compared with the laboratory strain PAO1, two environmental strains (7NSK2 and PA114), other pseudomonads (*Pseudomonas fluorescens, Pseudomonas putida* and *Pseudomonas syringae*) and *Escherichia coli* (Fig. 2). Background growth on medium with no added sulfur was measured as a control.

All CF-associated *P. aeruginosa* isolates and laboratory strain PAO1 were able to utilize >70% of the organically bound sulfate in MUC5B for growth, with the majority of strains using 100% (calculated as growth yield relative to growth with an equivalent amount of inorganic sulfate and confirmed by the absence of residual organic sulfate in the growth medium at the end of the experiment) (Fig. 2). The environmental *P. aeruginosa* strains and the other species tested were able to use significantly less (*P*<0.01) of the available mucin sulfur. PAO1 was utilized to a much lesser extent as a sulfur source by all *P. aeruginosa* isolates, with 20–55% utilization being observed. There was no significant difference in utilization of PGM between *P. aeruginosa* and the other species (*P*=0.13), as *P. fluorescens* and *P. putida* were also able to utilize about 40% of PGM-bound sulfate for growth. *P. syringae* and *E. coli*, however, were not able to grow without an additional sulfur source. The growth rates (μ) with mucins as the sulfur source were ~30% slower with PGM than with MUC5B (μ=0.005–0.011 h⁻¹ in a microplate assay), and considerably longer than with inorganic sulfate (μ=0.15–0.64 h⁻¹). In none of the strains did mucin inhibit growth in the presence of cyclamate or inorganic sulfate, provided as an alternative sulfur source (data not shown).

In both healthy lungs and the lungs of CF patients, the predominant sulfated sugars in mucin are N-acetyl-D-glucosamine 6-sulfate (GlcNAc-6 S), galactose 6-sulfate (Gal-6 S) and galactose 3-sulfate (Xia et al., 2005). *P. aeruginosa* PAO1 was able to fully desulfate both GlcNAc-6 S and Gal-6 S when they were provided as the sole sulfur source for growth; the growth rates observed with these sulfated sugars (μ=0.35 and 0.21 h⁻¹, respectively) were >20-fold faster than with mucin.

Organic and inorganic sulfate levels in PGM medium supernatants were quantified before and after growth of *P. aeruginosa* PAO1 to verify that the observed growth could be attributed to desulfation of mucin. After inoculation with *P. aeruginosa* PAO1 and incubation for 5 days, 76% of the organic mucin-bound sulfate in the medium remained. No spontaneous sulfate release from mucin was observed after incubation of uninoculated samples under the same conditions.

The incomplete desulphation of PGM observed for *P. aeruginosa* could in principle be due to steric hindrance caused by the physical conformation of the substrate mucin or to *P. aeruginosa* lacking an enzyme with appropriate sulfate ester specificity. To test this, PGM was partially degraded before use, either by sonication or by incubation with agarose-immobilized proteinase K at 37 °C, or by a combination of these treatments. None of the three treatments led to hydrolytic release of sulfate or oligosaccharides (tested by PAS staining), nor did they cause gross fragmentation of the mucin backbone, observable by SDS-PAGE with Coomassie or PAS staining (data not shown). Proteinase K treatment caused a small amount of proteolytic cleavage after 21 h incubation, observed as a slight reduction in intensity of the main bands on an SDS-PAGE gel. Growth of *P. aeruginosa* with PGM that had been subjected to combined sonication and proteolysis led to a twofold increased growth yield compared with untreated PGM in the proportion of mucin sulfur available for growth, suggesting that the structural conformation of the mucin is at least partially responsible for preventing complete desulfation of PGM. Over 50% of the bound sulfate still remained unavailable for utilization by *P. aeruginosa* as a sulfur source, which may represent inaccessible mucin sulfur, or other sulfated glyconjugates present in the commercial PGM sample.

**Sulfate and organosulfate content of sputum from CF patients**

To determine the availability of both inorganic sulfate and bound organic sulfate to bacteria growing in the lungs of CF patients, levels of sulfate were determined before and after hydrolysis in sputum samples taken from 29 CF patients. Growth was measured as maximum OD 600 reached after 3 days of incubation in MMAA medium containing MUC5B (0.154 mg ml⁻¹, 30 μM sulfur; filled bars) or PGM (2 mg ml⁻¹, 100 μM sulfur; open bars), as a percentage of the maximum OD reached in the absence of an added sulfur source and show means ± SEM (n=6) from two independent experiments.
patients. Specimens varied widely in quantity and purulence, and the lung function of patients, measured by the percentage forced expiratory volume in 1 s (FEV1%pred.), ranged from 19 to 95% of the predicted value for the individual's age and height. Inorganic sulfate levels were in the micromolar range in the sputum, with a mean of 400 nmol sulfate (g sputum)$^{-1}$, with the lowest recorded measurement being 84 nmol sulfate (g sputum)$^{-1}$ (Fig. 3).

If it is uniformly distributed within the mucus layer, sulfur should therefore be freely accessible to bacteria in the form of inorganic sulfate and should not be a limiting factor for microbial growth. Organic sulfate, measured after acid hydrolysis of sputum samples, was more varied than inorganic sulfate measurements, with a range of 0–1100 nmol (g sputum)$^{-1}$. No significant correlation was apparent between inorganic and organic sulfate levels in the sputum. In addition, no correlation was evident between sulfate level (inorganic, organic or total sulfate) and lung function (FEV1%pred.; Fig. 4), hospitalization within 7 days of specimen collection, age or gender (not shown), using either absolute measurements or using the percentage of sulfate measured in each sample attributed to inorganic and organic sulfate.

To assess whether changes in disease severity were related to changed levels of mucin sulfur for individual patients, a second sputum specimen was collected from seven of the individuals 2 weeks or 5 months after the initial specimen collection. A weak correlation was observed between the change in organic sulfate level and the change in the individuals' FEV1%pred. between collection dates (data not shown; $P=0.173$), suggesting that decreased lung function may be weakly associated with an increase in sputum sulfation level.

**Fig. 3.** Concentration of inorganic and organic sulfate in sputum. Inorganic and organic sulfate content was determined by ion chromatography before and after hydrolysis, respectively. The distribution of sputum sulfate measurements is indicated as the median (thick line), interquartile range (outer extents of boxes) and minimum/maximum data points (error bars). No readings were classified as outliers, all falling within 1.5 interquartile ranges from the median.

**Fig. 4.** Relationship between sputum organic sulfate levels and lung function (measured as FEV1%pred.) in 29 individuals with CF. Organic sulfate was calculated from inorganic and total sulfate levels measured by HPLC from homogenized sputum specimens. No significant correlation was observed ($P=0.74$).

**Expression of sulfate-repressed P. aeruginosa genes in the lungs of CF patients**

The availability of inorganic sulfate to bacteria residing in the lungs of CF patients was investigated by measuring expression of known bacterial sulfate-repressed genes in sputum. Community analysis of the microbial population in the sputum samples confirmed that >90% of the patients harboured a P. aeruginosa infection and many also contained further characteristic organisms associated with CF, including Haemophilus influenzae, Staphylococcus aureus and Stenotrophomonas maltophilia (Fig. 5). There was no difference in organic sulfate level ($P>0.5$ in independent $t$-tests) between sputum samples in which P. aeruginosa was the predominant species ($n=8$) and those

**Fig. 5.** DGGE separation of 16S rRNA gene products from 11 CF sputum DNA samples. Markers correspond to H. influenzae (HI), S. aureus (SA), P. aeruginosa (PA) and Stenotrophomonas maltophilia (SM). The three asterisked bands were sequenced and were most similar to Streptococcus sp., Capnocytophaga sp. (both 100% coverage/100% nucleic acid identity) and an uncultured skin bacterium recovered from the microbiome (90% coverage/97% nucleic acid identity).
in which it was not dominant \((n=3)\) or not present \((n=2)\;\) data not shown), as determined by DGGE band intensity. The presence of \(P.\) \textit{aeruginosa} in the sputum samples allowed us to quantify expression of \(P.\) \textit{aeruginosa} genes known to be sulfate repressed \textit{in vitro} (Tralau \textit{et al.}, 2007) to determine whether sufficient inorganic sulfate was sensed by the bacteria to repress expression of genes involved in desulfurization of organosulfur compounds as part of the general sulfate-starvation response. Total cDNA synthesized from RNA from sputum of CF patients was used as template for quantitative RT-PCR, targeting the \(P.\) \textit{aeruginosa} \textit{atsK} gene (alkylsulfatase, PAO193 gene) and \textit{msuE} gene (methanesulfonatase component, PA2357 gene), which are sulfate repressed \textit{in vitro} (Tralau \textit{et al.}, 2007). Expression levels were normalized to that of the reference gene \textit{rpsL} and compared with expression levels found for \textit{in vitro} cultures grown with inorganic sulfate or with cyclamate as an organic sulfur source. Expression of \textit{msuE} was below the detectable level for the culture grown with sulfate as a sulfur source and in all of the sputum samples. Relative expression of \textit{atsK} in sputum was below the level of detection in cells grown in the presence of inorganic sulfate in all five specimens tested (Fig. 6). This suggests that inorganic sulfate levels in sputum are sufficient to repress the \(P.\) \textit{aeruginosa} sulfate starvation response during chronic infection in the lung.

**DISCUSSION**

The ability of \(P.\) \textit{aeruginosa} to desulfate a range of organosulfur compounds is well established and a number of specialized sulfatase enzymes have been characterized (Beil \textit{et al.}, 1995; Fitzgerald & Kight, 1977; Hageluken \textit{et al.}, 2006; Lucas \textit{et al.}, 1972). These include a mucin sulfatase activity that could potentially be important for survival in the lung environment (Jansen \textit{et al.}, 1999), as enhanced levels of mucin sulfation have been associated with CF and mucin oligosaccharides are more resistant to degradation and utilization by \(P.\) \textit{aeruginosa} when sulfated (Chance & Mawhinney, 2000). Here, we report that the ability to use human MUC5B as a source of sulfur for growth is common among \(P.\) \textit{aeruginosa} strains isolated from the lungs of CF patients but is not found among other non-clinical species of \textit{Pseudomonas}. However, measured levels of inorganic sulfate in sputum from CF patients suggested that bacteria resident in the lung have sufficient inorganic sulfate for growth and are unlikely to access mucin sulfur as a sulfur source; this was confirmed when expression of \(P.\) \textit{aeruginosa} sulfatase and sulfonatase genes was found to be repressed in CF sputum.

Early reports of increased sulfation in CF sputum were based on colorimetric sulfate determination using sodium rhodizonate (Chace \textit{et al.}, 1983) or benzidine (Boat \textit{et al.}, 1976a). Later studies have focused on sulfated glycoprotein fractions generated from mucin, either using mAbs directed against \(\text{SO}_3^-\text{-3Gal}\beta1-3\text{GlcNAc}\) (Dennesen \textit{et al.}, 2003), an epitope that is expressed exclusively on sulfated mucins (Veerman \textit{et al.}, 1997), or HPLC analysis of sulfate released by hydrolysis from these fractions, a method that is significantly more sensitive than the colorimetric methods, with a limit of detection of \(\sim 1\) \(\mu\)g sulfate in a sample \(\pm 5\%\) error (Terho & Hartiala, 1971), compared with 9 ng sulfate per sample using HPLC methods. The present study measured total organic sulfate by hydrolysis and ion chromatography and confirmed the results by measuring bioavailable sulfur as growth yields relative to inorganic sulfate. The total organic sulfation levels in hydrolysed mucus glycoprotein fractions from CF patients, measured by Lo-Guidice \textit{et al.} (1994), ranged from 370 to 469 \(\mu\)mol g\(^{-1}\), while Davril \textit{et al.} (1999) observed 3.1 \%(w/w) sulfate of purified bronchial mucin from CF patients, both of which are consistent with the values reported here.

Higher levels of sulfation, along with increased sialylation, are thought to occur on the high-charge glycoform of MUC5B, contributing to the increased charge density (Kirkham \textit{et al.}, 2002). Our results clearly show that high-charge MUC5B had almost twofold higher sulfation than low-charge MUC5B (Fig. 1). The importance of this for CF patients is uncertain. Although increased amounts of high-charge mucins have been associated with increased disease severity (Chace \textit{et al.}, 1983), other studies have reported a relative decrease in the ratio of high- to low-charge MUC5B during pulmonary exacerbation in individual CF patients compared with a stable period (Davies \textit{et al.}, 1999). In the current study, we were unable to demonstrate a clear correlation between disease severity and overall sputum sulfate content, measured per gram of expectorated sputum, although a weak trend was observed between changes in the organic sulfation level of sputum and FEV1%pred. when compared in individual patients over...
time (data not shown). However, the measure of disease severity in this study was limited to only one measure of lung function, FEV1%pred., which measures the individual’s expiratory volume as a percentage of the expected volume for their age and height. By contrast, Chace et al. (1983) used a more comprehensive measure, scoring patients on a clinical scale of 0–100 that covered many aspects of health (Doershuk et al., 1964).

The inorganic sulfate content of sputum from CF patients was also measured to give an indication of the biological level of inorganic sulfate in the lungs and to determine whether sulfate starvation occurs in vivo. Inorganic sulfate was detected in all specimens, ranging from 84 to 900 nmol sulfate (g sputum)^–1. This equates to micromolar levels, which suggests that bacterial growth in the lung is probably not limited by inorganic sulfate, provided the sulfate is evenly distributed throughout the lung. However, it is important to distinguish between overall sulfate levels in sputum and the level of sulfate available at the site of infection that is readily available for utilization by pathogens. Gene expression studies to determine whether CF-associated P. aeruginosa cells residing in sputum are able to sense the available inorganic sulfate showed that P. aeruginosa genes atsK and msuE, which are repressed by the presence of sulfate in vitro, were expressed in sputum at low levels, which were comparable to those seen during growth in vitro with inorganic sulfate in minimal medium. These results indicate that, on a population level, sulfate starvation does not occur in sputum around established P. aeruginosa biofilms in the lung. However, active mucin desulfation may still occur, as this may be induced not for sulfur acquisition but in order to reveal cryptic mucin-binding sites or to remove sulfate groups that cause steric hindrance for enzymes that degrade mucin as part of the carbon cycle. To confirm that mucin desulfation is not required for sulfur acquisition at any stage of infection in the lung environment, it would be necessary to study sputum sulfate levels in individuals not infected by P. aeruginosa and upon first detection of P. aeruginosa-positive sputum. Additional confirmation could be achieved through the study of BAL samples. Although more invasive, BAL samples may provide a clearer representation of sulfate levels within the lower respiratory tract. Expectorated sputum has the advantage of representing many areas of the bronchial tree but naturally contains older, excess mucus and shed cells, whereas BAL would reflect the environment in closer proximity to the lung epithelium within alveolar compartments.

Due to its availability and low cost, PGM is often used as a substitute for human mucins in growth and degradation studies (Aristoteli & Willcox, 2003; Macfarlane & Gibson, 1991; Poncz et al., 1988; Tralau et al., 2007). Early studies of PGM concluded that its general structure and chemical composition are similar to those of human gastric and respiratory mucins (Boat et al., 1976b; Scawen & Allen, 1977; Sheehan et al., 1986). However, more recent studies of mucins from a variety of species and epithelial locations have highlighted differences both between individual mucins and within overall mucus composition and this is reflected in the results obtained here and in previous studies on Pseudomonas mucin desulfation (Jansen et al., 1999; Tralau et al., 2007). The bound sulfate level in PGM is approximately fourfold lower than that for MUC5B (Fig. 1) but the macromolecular composition of commercial PGM has not been defined and there are considerable differences in size, density, glycosylation and sulfation of mucin preparations isolated from different areas of the pig gastric mucosa (Nordman et al., 1997). Commercial preparations are, therefore, likely to be heterogeneous mixtures and may contain not only sulfated mucin but also other sulfated glycoconjugates. The differences in mucin structure and mucus composition may go some way to explaining the inconsistency seen in the ability of P. aeruginosa strains to desulfate mucin in previous studies.

The inability of P. putida, P. fluorescens and P. syringae to utilize sulfate from human MUC5B suggests either a lack of appropriate functional genes or lack of the regulatory pathways involved. In contrast, the ability of the clinical P. aeruginosa isolates tested to desulfate MUC5B further supports the theory that these strains possess a mucin-specific sulfatase, which can be employed in the context of human pathogenesis. Interestingly, non-clinical P. aeruginosa strains isolated from water and soil environments showed a highly reduced ability to utilize MUC5B sulfate (Fig. 2). As environmental strains provide the pool from which CF patients acquire new infections, this suggests that either incoming strains acquire the relevant genes by horizontal gene transfer within the lung or the lung environment itself is important in activating the relevant genes. The ability of P. fluorescens and P. putida to partially desulfate PGM has not been investigated further but could indicate the presence of different sulfatase enzymes to those used for MUC5B desulfation or altered specificity of similar enzymes. Alternative enzymes active against O-linked sulfated esters or other organosulfur compounds found in the environment may have limited activity against mucin but this remains to be tested.

Chance & Mawhinney (2000) reported that sulfated sugars supported significantly less growth of P. aeruginosa than their non-sulfated analogues when provided as a sole carbon source. However, in the present study, two of the same sulfated sugars (GlcNAc-6 S and Gal-6 S), which are the most abundant sulfated oligosaccharides on MUC5AC and MUC5B in respiratory mucus (Xia et al., 2005), were completely desulfated by P. aeruginosa during growth with succinate as the carbon source. The short lag times and generation times of cultures grown with these sugar sulfates suggest that, under sulfate-limited conditions, desulfation enzymes are expressed, despite the absence of whole mucin, and can efficiently hydrolyse the sugar–sulfate ester linkage, releasing sulfate for bacterial growth. The slower growth rate and longer lag time observed with mucins indicate the steric effects of rich glycosylation, which are likely to play a large part in rate limitation for growth (Kozarsky et al., 1988).
The data presented here suggest that mucin sulfatase activity is common among clinical P. aeruginosa isolates but not in other non-clinical species of Pseudomonas. However, the level of inorganic sulfate in the sputum of CF patients is sufficient to repress the P. aeruginosa sulfate-starvation response and mucin is, therefore, unlikely to be utilized by bacteria as a sulfur source in the lung, although mucin desulfation activity may be used to reveal cryptic binding sites or targets for enzymes that degrade mucin to provide carbon for bacterial growth. Further studies into the mode of action by which P. aeruginosa is able to desulfate mucin will shed more light on the role of this activity in CF-associated lung infections.

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