Carbohydrate sulfation effects on growth of *Pseudomonas aeruginosa*

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***Pseudomonas aeruginosa* is a key player in the pathology and morbidity of cystic fibrosis. Chronic obstructive pulmonary disease, which results from the most common and severe mutations in this genetic disorder, typically includes chronic infection with *P. aeruginosa* which, even with rugged antibiotic and physical therapy regimens, is rarely eradicated. It is not known whether the increased oligosaccharide sulfation characteristic of cystic fibrosis tracheobronchial mucins plays a role in the survival of *P. aeruginosa* in the airway. In this study, sulfated monosaccharides were synthesized and tested for their effects on the growth of clinical isolates and laboratory strains of this organism when supplied as the sole carbon source *in vitro*. Carbohydrate sulfation was observed to reduce, but not prohibit, growth of *P. aeruginosa* on carbohydrates normally utilized in their nonsulfated form. The various sulfated sugars employed as the sole carbon source gave characteristic and consistent growth profiles and maximum growth values across the strains tested. *P. aeruginosa* isolates from patients with cystic fibrosis often express a mucoid phenotype, which is thought to contribute to their ability to survive in harsh conditions. Carbohydrate sulfation effects on growth did not differ significantly between mucoid and nonmucoid strains. These results suggest that the additional sulfation of tracheobronchial mucin documented in cystic fibrosis may in fact contribute to the mucin's resistance to utilization by *P. aeruginosa* and potentially other pathogens, providing an additional level of host protection, and limiting the available nutrient pool and thereby bacterial growth.**

Keywords: *Pseudomonas aeruginosa*, growth, sulfate, mucin, cystic fibrosis

INTRODUCTION

Pseudomonas aeruginosa is regarded as an opportunistic human pathogen, capable of the elaboration of many environmentally regulated virulence factors (i.e. mucoid exopolysaccharide and bacteriocidal pigments) that aid in its survival in stressful situations (Buret & Cripps, 1993). Under normal conditions, this organism is rapidly cleared from the human airway by the mucociliary clearance mechanism (MCM). Mucous glycoproteins are the major macromolecular components ($> 1.5 \times 10^6$ Da) of the MCM, serving to trap inhaled debris

and infectious agents and assist in their removal (Gupta & Jentoft, 1992; Widdicomb, 1995; Woodward *et al.*, 1982). In cystic fibrosis (CF) however, airway mucus is dehydrated, pulmonary clearance is diminished, mucous glycoproteins are increased in O-sulfation and chronic respiratory tract infection with mucoid *P. aeruginosa* is common (Amerongen *et al.*, 1998; Davril *et al.*, 1999; Gerken & Gupta, 1993; Jansen *et al.*, 1999). Recent studies report a correlation between mucin character and the severity of bacterial infection in patients with chronic obstructive pulmonary disease, including CF (Davril *et al.*, 1999). It has also been demonstrated that purified CF mucin and some of its constituents are chemotactic for *P. aeruginosa* (Nelson *et al.*, 1990). It is not clear whether the structural differences in CF mucous glycoproteins in some way function to enhance *P. aeruginosa* survival in the CF airway.

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Abbreviation: CF, cystic fibrosis.

With impaired mucociliary clearance in CF, respiratory mucins may serve not only to bind bacteria, but also to provide nourishment for entrapped organisms. Approximately 70–80 % of the mass of these normally protective structures is carbohydrate, consisting of heterologous oligosaccharide chains attached via O-glycosidic linkages of *N*-acetylgalactosamine to serine or threonine residues of the protein backbone (Boat *et al.*, 1976; Roussel *et al.*, 1975). The principle sugars of mucin oligosaccharide chains are galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid and fucose. In the absence of disease, a small percentage of the oligosaccharide chains possess sulfate esters, whereas in CF there is generally a significantly higher level of oligosaccharide sulfation (Boat *et al.*, 1976; Chace *et al.*, 1983; Cheng *et al.*, 1989; Scharfman *et al.*, 1996). Structural analysis of these sulfated mucins has revealed terminal galactose 3-sulfate and galactose 6-sulfate, and internal galactose 6-sulfate and *N*-acetylglucosamine 6-sulfate residues (Chance & Mawhinney, 1996; Mawhinney *et al.*, 1987, 1992a, b; Mawhinney & Chance, 1994). Whilst mucinases have been found in a number of pathogenic bacteria, (Schneider & Parker, 1982; Prizont & Reed, 1991; Smith *et al.*, 1994), implying that mucins may be a potential source of nutrients for these organisms, it is suggested that sulfation may confer a protection to mucins against degradative enzymes of bacteria (Amerongen *et al.*, 1998). In studies of growth of *Pseudomonas fluorescens* with glucose 6-sulfate as the sole carbon source in a minimal salts solution, Fitzgerald & Dodgson (1971a, b) demonstrated that over time this organism was able to grow on this sugar sulfate by an undefined mechanism. The identification of mucin sulfatase activity from *Helicobacter pylori* (Slomiany *et al.*, 1992), *Porphyromonas gingivalis* (Slomiany *et al.*, 1993) and recently *Burkholderia cepacia* and *P. aeruginosa* (Jansen *et al.*, 1999) suggests that these adaptive organisms may in fact also be capable of utilization of sulfated sugars, including sulfomucins.

This study was designed to test whether carbohydrate sulfation affects the *in vitro* growth of clinical isolates and laboratory strains of *P. aeruginosa* in an environment of limited nutrients, as might be experienced in the CF airway.

METHODS

Bacterial strains. *P. aeruginosa* strains were isolated and identified from sputum samples of patients with CF at the University of Missouri Hospital and Clinics. *P. aeruginosa* isolates represented major colonizing organisms for these patients. Clinical strains were further verified as *P. aeruginosa* in the research laboratory by assays for glucose and lactose oxidation/fermentation, cytochrome oxidase activity, ONPG activity, growth on acetamide, growth at 42 °C and polymyxin B sensitivity (Koneman *et al.*, 1992). Initial phenotypes of clinical isolates were recorded and are presented in Table 1. Multiple isolates from an individual patient sputum sample are indicated by a common laboratory identifier, i.e. CF 8314-1 and CF 8314-2; whilst phenotypically distinct, these strains show few differences by restriction fragment analyses of the

genomic DNA, suggesting epidemiological relatedness (Loutit & Tompkins, 1991). Isolates were also observed for the presence or absence of visible pigment and/or fluorescent pigment at 366 nm following growth on trypticase soy agar or acetamide agar. Laboratory strain PAO1 (Holloway *et al.*, 1979) was kindly provided by Alice Prince, Columbia University, NY, USA. Laboratory standard strain ATCC 39018, a non-toxic exotoxin A derivative of PAO1, was purchased from the American Type Culture Collection, Manassas, VA, USA, as was ATCC 33467, an oxidase-negative, nonmucoid sputum isolate. Clinical isolates and laboratory strains were immediately propagated in Difco trypticase soy broth at 30 °C, and stored frozen with 40 % (v/v) glycerol at –80 °C. Fresh stock cultures were prepared by plating freezer stocks onto Difco trypticase soy agar and MacConkey agar plates, with careful attention to maintenance of original phenotype.

Experimental sugars. Carbohydrates employed in these investigations of *P. aeruginosa* growth are listed in Table 2. Common monosaccharides were purchased from Pfanstiehl Laboratories. Sulfated monosaccharides and sulfated methyl glycosides were synthesized with modifications to previously described procedures (Ball & Jones, 1958; Ball, 1966; Bocker *et al.*, 1992; Lloyd, 1960; Whistler *et al.*, 1963). 6-O-Sulfated monosaccharides (20 g) were routinely derivatized with chlorosulfonic acid in a 1:1 molar ratio of derivatization reagent to starting sugar. Chlorosulfonic acid in chloroform (1:1, v/v), maintained at –60 °C, was slowly added to the desired sugar dissolved in 100 ml pyridine, with the reaction solution being maintained at 10–15 °C throughout the addition. Following the destruction of excess derivatization reagent and removal of solvents by addition of water and rotoevaporation, the sulfated product was removed from the neutral starting material by passage through a Rohm and Haas Amberlite IRN 78 anion exchange resin column (hydroxide form; 2.5 × 45 cm), at 4 °C, which was then washed with 4 bed vols deionized water. Sulfated monosaccharides were eluted with 0.025 M H₂SO₄ at 4 °C. The effluent was kept on ice, neutralized with barium carbonate, and after the addition of several pieces of dry ice, the insoluble inorganic barium salts were removed by filtration. Sugar sulfates were then directly converted to their potassium salts by passage through an Amberlite IRN 77 column in the potassium form. After volume reduction by rotoevaporation, sulfated sugars were either lyophilized, or precipitated by the addition of 4 vols ethanol and dried *in vacuo*. Synthetic monosaccharide sulfate purity and site of sulfation were evaluated by proton and C-13 NMR on a Bruker AM-X500 MHz NMR spectrometer, by comparison with data from the literature and with authentic laboratory standards. Verification of salt form of the sulfated monosaccharides was determined by inductively coupled plasma (ICP) analysis employing a Fisons sequential inductively coupled plasma spectrophotometer. Authentic sugar 6-sulfates were also synthesized in stepwise fashion by selectively blocking the C-6 hydroxyl group by tritylation with triphenylmethyl chloride, followed by peracetylation of the tritylated product (Tally, 1963). Following detritylation, the selectively blocked sugar was sulfated as described above, deacetylated and then purified as above. Authentic 3-O-sulfated glucose and galactose were generated by sulfation, as described above, of the 1,2,5,6-diisopropylidene glucopyranose or the 1,2-isopropylidene-4,6-ethylidene galactopyranose, respectively (Ball, 1966; Ball & Jones, 1958). After sulfation, protecting groups were removed by mild acid hydrolysis (Ball, 1966), and the purified potassium salts of the 3-O-sulfated sugars were generated as above.

Media and growth conditions. For evaluation of the effects of

Table 1. *P. aeruginosa* strains and their phenotypes

Laboratory identifier	Characteristics
Laboratory strains	
PAO1	Nonmucoid, motile, green diffusible pigment
ATCC 39018	Nonmucoid, motile, green diffusible pigment
ATCC 33467	Nonmucoid, nonmotile, green diffusible pigment
CF sputum isolates	
CF 6935	Mucoid, nonmotile
CF 7201-1	Nonmucoid, nonmotile, brown diffusible pigment
CF 7202-2	Mucoid, nonmotile, green diffusible pigment
CF 8314-1	Nonmucoid, nonmotile, green diffusible pigment
CF 8314-2	Mucoid, nonmotile
CF 8981-1	Nonmucoid, nonmotile
CF 8981-2	Mucoid, nonmotile, brown diffusible pigment
CF 9965	Mucoid, nonmotile
CF 10095	Mucoid, nonmotile
CF 12227	Mucoid, nonmotile
CF 12630	Mucoid, nonmotile, green diffusible pigment
CF 12631	Mucoid, nonmotile, green diffusible pigment
CF 21165	Mucoid, nonmotile

sulfation of the sole carbon source on the growth of *P. aeruginosa* *in vitro*, these studies employed a modified version of the liquid minimal medium described by Fitzgerald & Dodgson (1971a, b) in studies of bacterial adaptation to growth on glucose sulfate. Minimal medium pH 7, containing 22 mM KH_2PO_4 , 40 mM K_2HPO_4 , 19 mM NH_4Cl , 17 mM NaCl and 1.5 mM MgCl_2 , was supplemented with the designated amount of monosaccharide, monosaccharide sulfate (potassium form) or water. Media containing non-sulfated sugar were additionally supplemented with 56 mM K_2SO_4 . Media was filter-sterilized (0.22 μm) and dispensed into sterile 16 $\times$ 100 mm glass culture tubes. Prior to inoculation of test media, bacteria were grown overnight in minimal medium containing 56 mM glucose, in shaking culture (240–250 r.p.m.) at 35 °C, and the cells were then washed by centrifugation of the liquid culture at 2400 g for 15 min at 8 °C, resuspension in minimal medium minus sugars, MgCl_2 and K_2SO_4 , and centrifugation and resuspension as above. Cells were inoculated into experimental media to an OD_{600} of approximately 0.02 ($\sim 4 \times 10^7$ c.f.u. ml^{-1}). Optical densities were determined directly for each culture tube. Test cultures and media controls for each condition (without sugar and/or without bacteria) were incubated in shaking culture at 35 °C.

For initial growth experiments, laboratory standard ATCC 39018 was incubated for 1 week in minimal medium containing 56 mM glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, mannose, fucose or no sugar at all. Growth was measured as described below. The effect of carbohydrate concentration on *P. aeruginosa* growth in minimal medium was assessed for a panel of eight strains (see Fig. 2) by culture as described above, for up to 5 d with 56, 5.6 and 0.56 mM glucose-supplemented minimal medium.

To determine the effects of carbohydrate sulfation on *P. aeruginosa* growth curves and maximum optical densities, one laboratory standard and two clinical isolates, one nonmucoid

and one mucoid, were subjected to culture for 1 week in minimal medium supplemented with 56 mM sulfated or nonsulfated monosaccharides or methyl glycosides as the sole carbon source. Sulfated sugars were also screened for their possible inhibition or enhancement of growth of these three strains on neutral sugars by supplementation of nonsulfated sugar test media with its sulfated analogue. Additionally, possible inhibition of growth by sulfated sugars was tested by application of filter discs impregnated with sulfated sugars to trypticase soy agar plates inoculated with *P. aeruginosa*.

To assess whether sulfation of glucose affected its utilization across a wide variety of strains of *P. aeruginosa*, a panel of 12 clinical isolates and 2 laboratory standard strains of *P. aeruginosa* were cultured with glucose 6-sulfate or glucose as the sole carbon source. Similarly, to further investigate the growth of *P. aeruginosa* on (or adaptation to growth on) sugars commonly found at the termini of tracheobronchial mucin oligosaccharides, this panel of 14 strains was screened for growth in minimal medium with galactose, galactose 3-sulfate or galactose 6-sulfate as the sole carbon source. To minimize any negative effects of manipulation of cells prior to assay for growth on galactose and galactose sulfates, test media was inoculated directly with 50 μl aliquots of overnight cultures. Growth contributed by residual glucose in the inocula was determined by parallel inoculation and incubation in medium with no other carbon source.

Assessment of growth. OD_{600} measurements were made throughout the culture period of every original, independent, test culture tube and all control tubes, employing a Milton Roy Genesys II spectrophotometer (1 cm path length). Specific growth rates were not determined as growth on the test substrates was generally not exponential (linear with semilog analysis of optical density vs time) in this minimal media. Optical density maxima were used to compare growth on various substrates and with various strains of bacteria, and were assumed to reflect maximum growth as preliminary studies yielded the highest viable cell counts at the optical

density maxima. Data are expressed as mean OD₆₀₀ ± SEM for each culture condition and for each bacterial strain. Cell counts (c.f.u. ml⁻¹) and phenotypes were determined at the time of inoculation and approximately at the culture maximum optical density by plating serial dilutions of test culture aliquots onto trypticase soy agar plates. Culture phenotypes were recorded from these plates after 48 h incubation at 35 °C.

Statistical analysis. Statistical significance was assessed by one-tailed *t*-test at a confidence interval of 0.05 (Voelker & Orton, 1993). A two-sample *t*-test for comparing two means was employed to compare mean optical density maxima for bacteria in media with sulfated sugar as the sole carbon source with parallel cultures in media containing the analogous nonsulfated sugar or glucose.

RESULTS

Initial characteristics of *P. aeruginosa* strains

All clinical *P. aeruginosa* isolates employed in these studies were small Gram-negative rods, nonfermentative, cytochrome-oxidase-positive, ONPG-negative, polymyxin-B-sensitive and capable of aerobic growth on basal oxidative/fermentative medium with glucose or galactose, on acetamide agar and at 42 °C on trypticase soy agar. *P. aeruginosa* from sputa of patients with CF varied widely in their phenotypes. Phenotypes of patient and laboratory strains are presented in Table 1. Many of the CF isolates produced the classic green pigmentation characteristic of *P. aeruginosa*, much like the laboratory standards PAO1 and ATCC 39018. Typical of isolates from CF sputa, many of the isolates obtained during this investigation exhibited a mucoid phenotype. Non-mucoid CF isolates were generally nonmotile upon isolation. Bacterial experimental stock cultures, prepared fresh from frozen stocks, tended to retain the features of the original isolate. Laboratory strains PAO1 and ATCC 39018, as expected for *P. aeruginosa* not derived from patients with CF, were nonmucoid, motile

and were indistinguishable from one another by their colony morphology.

Growth of *P. aeruginosa* with various monosaccharides as the sole carbon source

Growth of *P. aeruginosa* was first assessed with several neutral sugars or sialic acid as the sole carbon source in a minimal medium, intended here to parallel the conditions in the airway of patients with CF where nutrient availability may be quite limited. As shown in Fig. 1, *P. aeruginosa* strain ATCC 39018 readily grew on glucose in minimal medium, and, given time, achieved equally significant growth on *N*-acetylglucosamine. Other sugars potentially available in the airway, such as galactose, *N*-acetylgalactosamine, mannose, sialic acid and fucose, did not support marked growth under these conditions.

Influence of substrate concentration on the growth of *P. aeruginosa*

The influence of carbohydrate concentration on *P. aeruginosa* growth in minimal medium was assessed for a panel of eight strains with glucose as the sole carbon source and the results are presented in Fig. 2. Culture growth profiles were concentration-dependent for all tested strains, with the exception of isolate CF 8981-1 which did not grow on minimal medium with glucose at any concentration. Laboratory strains PAO1 and ATCC 39018 showed almost identical concentration-dependent growth curves, whilst clinical isolates varied markedly in their glucose growth profiles. Comparing the mean maximal growth for each glucose concentration for nonmucoid and mucoid *P. aeruginosa* strains as groups showed no statistically significant differences between these expressed phenotypes. Mean nonmucoid strain maximum optical densities with 56 mM, 5.6 mM and 0.56 mM glucose, were 1.223 ± 0.021, 0.480 ± 0.001 and 0.283 ± 0.001, and mean maximal values for mucoid strains were 1.15 ± 0.095, 0.460 ± 0.003 and 0.280 ± 0.001, respectively. Optical density measurements and cell counts (not shown) produced parallel results.

Effects of carbohydrate sulfation on the growth of *P. aeruginosa*

The influence of carbohydrate sulfation on the growth of *P. aeruginosa* was initially evaluated for three strains: ATCC 39018 as a nonmucoid, motile, laboratory standard, and CF 8314-1 and CF 8314-2 as representative nonmucoid and mucoid CF sputum isolates, respectively. Growth curves of these strains in minimal medium with 56 mM glucose, glucose 3-sulfate, *N*-acetylglucosamine, *N*-acetylglucosamine 6-sulfate, or with no sugar are presented in Fig. 3. Growth on glucose was similar for these three strains. *N*-acetylglucosamine growth curves were comparable for the two nonmucoid strains, whilst the mucoid isolate showed a steeper

Fig. 1. Growth of *P. aeruginosa* ATCC 39018 in minimal medium with 56 mM sugars as the sole carbon source. ●, Glucose; ▲, galactose; ■, *N*-acetylglucosamine; △, *N*-acetylgalactosamine; □, *N*-acetylneuraminic acid; ○, mannose; ▽, fucose. Data are means ± SEM of up to eight independent cultures (in cases of very low SEM, error bars are covered by symbols).

Fig. 2. Influence of glucose concentration in minimal medium on growth curves of clinical and laboratory strains of *P. aeruginosa*. ●, 56 mM glucose; ■, 5.6 mM glucose; ▲, 0.56 mM glucose. (a) ATCC 39018, (b) CF 8314-1, (c) CF 8314-2, (d) CF 8981-1, (e) PAO1, (f) CF 8981-2, (g) CF 10095, (h) ATCC 33467. Data are means $\pm$ SEM of three independent cultures.

Fig. 3. Influence of carbohydrate sulfation on growth curves of clinical and laboratory strains of *P. aeruginosa*. ●, Glucose; ○, glucose 3-sulfate; ■, N-acetylglucosamine; □, N-acetylglucosamine 6-sulfate; ▽, minimal medium with no carbon source. (a) ATCC 39018, nonmucoid; (b) CF 8314-1, nonmucoid; (c) CF 8314-2, mucoid. Data are means $\pm$ SEM of up to six independent cultures.

slope, quite similar to its glucose curve after about a 12 h lag. Glucose 3-sulfate and N-acetylglucosamine 6-sulfate supported markedly reduced growth relative to the nonsulfated analogues, with only limited immediate growth of these strains and then maintenance of the characteristic optical density for the remaining duration of the experiment. Mean optical densities of cultures and cell counts (not shown) for cultures with no carbon source indicated only slow decreases in population over the experimental period. Of note, in preliminary tests, the addition of free potassium sulfate to media supplemented with sulfated monosaccharides had no effect on growth curves.

The effects of carbohydrate sulfation on growth of these three strains of *P. aeruginosa* for a panel of synthetic sulfated monosaccharides and methyl glycosides and their nonsulfated analogues, are reflected in the maximum optical densities obtained over an 8 d culture period and are presented in Table 2. As above, glucose 3-sulfate consistently supported significantly less growth than glucose. Glucose 6-sulfate supported even further reduced growth. Optical densities for all three strains on both glucose sulfates were significantly increased over those of cultures with no carbon source ($P < 0.025$) and over initial inocula optical densities, indicating some growth on these substrates. N-Acetylglucosamine 6-

Table 2. Effects of carbohydrate sulfation on the growth of *P. aeruginosa* strains in minimal medium

Results are means $\pm$ SEM of up to 12 independent cultures (value of *n* shown in parentheses).

Carbon source	Maximum OD ₆₀₀		
	ATCC 39018	CF 8314-1	CF 8314-2
Galactose	0.195 $\pm$ 0.211 (12)	0.159 $\pm$ 0.180 (12)	0.153 $\pm$ 0.204 (12)
Galactose 3-sulfate	0.123 $\pm$ 0.117 (6)	0.021 $\pm$ 0.001 (6)	0.027 $\pm$ 0.019 (6)
Galactose 6-sulfate	0.046 $\pm$ 0.003 (6)	0.044 $\pm$ 0.001 (5)	0.041 $\pm$ 0.001 (6)
Glucose	1.663 $\pm$ 0.250 (12)	1.571 $\pm$ 0.234 (12)	1.614 $\pm$ 0.292 (12)
Glucose 3-sulfate	0.264 $\pm$ 0.005 (6)	0.312 $\pm$ 0.006 (6)	0.287 $\pm$ 0.004 (6)
Glucose 6-sulfate	0.071 $\pm$ 0.001 (6)	0.068 $\pm$ 0.002 (6)	0.084 $\pm$ 0.002 (6)
Mannose	0.197 $\pm$ 0.006 (3)	0.192 $\pm$ 0.002 (3)	0.112 $\pm$ 0.001 (3)
Mannose 6-sulfate	0.093 $\pm$ 0.017 (6)	0.063 $\pm$ 0.002 (6)	0.051 $\pm$ 0.005 (6)
N-Acetylglucosamine	1.112 $\pm$ 0.050 (3)	1.308 $\pm$ 0.014 (3)	1.555 $\pm$ 0.145 (3)
N-Acetylglucosamine 6-sulfate	0.189 $\pm$ 0.002 (6)	0.197 $\pm$ 0.002 (6)	0.147 $\pm$ 0.002 (6)
α -Methylgalactose	0.024 $\pm$ 0.002 (3)	0.020 $\pm$ 0.001 (3)	0.020 $\pm$ 0.001 (3)
α -Methylgalactose 6-sulfate	0.051 $\pm$ 0.001 (6)	0.054 $\pm$ 0.001 (6)	0.054 $\pm$ 0.003 (6)
β -Methylgalactose	0.038 $\pm$ 0.043 (6)	0.181 $\pm$ 0.360 (6)	0.028 $\pm$ 0.014 (6)
β -Methylgalactose 6-sulfate	0.048 $\pm$ 0.002 (6)	0.051 $\pm$ 0.001 (6)	0.052 $\pm$ 0.003 (6)
α -Methylglucose	0.019 $\pm$ 0.003 (3)	0.015 $\pm$ 0.001 (3)	0.023 $\pm$ 0.001 (3)
α -Methylglucose 6-sulfate	0.022 $\pm$ 0.002 (6)	0.020 $\pm$ 0.002 (6)	0.023 $\pm$ 0.002 (6)
β -Methylglucose	0.019 $\pm$ 0.003 (3)	0.017 $\pm$ 0.001 (3)	0.024 $\pm$ 0.004 (3)
β -Methylglucose 6-sulfate	0.038 $\pm$ 0.002 (6)	0.039 $\pm$ 0.001 (6)	0.031 $\pm$ 0.001 (6)

sulfate maximum growth was also significantly less than maximum growth on its nonsulfated analogue or glucose. The mean maximum optical density for growth on N-acetylglucosamine sulfate across the strains tested was 0.178, intermediate between the glucose 3-sulfate and glucose 6-sulfate values. Whilst mannose-supplemented medium supported low levels of growth, the analogous sulfated sugar medium supported even less growth. Similarly, growth was generally limited on galactose and was even less on galactose derivatives. α -Methylgalactose 6-sulfate, β -methylgalactose 6-sulfate and β -methylglucose 6-sulfate appeared to support very low levels of growth of *P. aeruginosa*, though values did not reflect statistically significant increases over initial inocula measurements ($P > 0.2$). Strains differed somewhat in their mean maximum optical densities for given test sugars, though generally measurements were within 20 % of one another with small standard deviations. The wider SEMs of cultures with galactose, galactose 3-sulfate, and β -methylgalactose reflects a duality of responses observed with these sugars. Greater than 80 % of independent cultures in galactose and > 60 % of cultures in galactose 3-sulfate and β -methylgalactose expressed relatively little to no growth on these substrates, with mean maximum optical densities of 0.099 ± 0.028 , 0.019 ± 0.002 and 0.021 ± 0.003 , respectively. The remaining cultures displayed increased optical densities after varying times, suggesting a possible adaptive response to culture with galactose and galactose derivatives as the sole carbon sources. In experiments assessing the effects of the presence of

sulfated carbohydrates on the utilization of their non-sulfated carbohydrate analogues, no inhibitory or stimulatory effects on *P. aeruginosa* growth were observed (data not shown). Similarly, no zone of inhibition of growth was noted surrounding sulfated-sugar-impregnated disks applied to cultures plated on trypticase soy agar.

Survey of effects of glucose and galactose sulfation on growth of a variety of *P. aeruginosa* strains

A panel of 14 strains of *P. aeruginosa*, subjected to culture with glucose 6-sulfate, galactose 3-sulfate, galactose 6-sulfate, their nonsulfated analogues, or no sugar at all, generally demonstrated comparable substrate utilization profiles to those reported above. Growth on glucose and glucose 6-sulfate paralleled previous results, with glucose 6-sulfate supporting little growth for all strains (maximum optical densities ranged between 0.046 and 0.092). As presented in Table 3, galactose supported intermediate growth with the exceptions of two clinical isolates, CF 8981-1, which showed no growth on galactose or glucose, and CF 6935, which showed significant growth on galactose, reaching an optical density of 0.845 by day 8. As seen previously, galactose 3-sulfate and galactose 6-sulfate supported minimal to no growth of clinical isolates or laboratory strains of *P. aeruginosa*. Removing the cell washing step, with its inherent cell manipulation, prior to inoculation did not improve utilization of galactose and galactose sulfates. For this panel of bacteria, the maximum optical

Table 3. Growth characteristics of laboratory strains and clinical isolates of *P. aeruginosa* cultured in minimal medium with galactose, galactose 3-sulfate, galactose 6-sulfate or glucose as the sole carbon source

Results were obtained during 8 d shaking incubation period at 35 °C ($n = 1$). Values include a mean of 0.083 ± 0.015 contributed by growth from glucose in the inoculum, as determined by parallel inoculation into medium with no additional carbon source, and excluding CF 8981-1 which showed no growth on glucose.

Laboratory identifier	Maximum OD ₆₀₀			
	Glucose	Galactose	Galactose 3-sulfate	Galactose 6-sulfate
PAO1	1.754	0.261	0.096	0.128
ATCC 39018	1.745	0.253	0.079	0.105
CF 6935	1.590	0.845	0.107	0.127
CF 7201-1	1.606	0.303	0.097	0.140
CF 7202-2	1.803	0.248	0.081	0.139
CF 8314-1	1.753	0.273	0.107	0.148
CF 8314-2	1.618	0.259	0.087	0.151
CF 8981-1	0.006	0.006	0.015	0.020
CF 8981-2	1.527	0.195	0.064	0.083
CF 9965	1.338	0.296	0.109	0.142
CF 12227	1.783	0.287	0.099	0.134
CF 12630	1.579	0.284	0.118	0.149
CF 12631	1.676	0.253	0.083	0.133
CF 21165	1.535	0.273	0.183	0.156

densities of galactose 6-sulfate cultures, with the exception of one isolate, CF 21165, although not statistically different from cultures with no test sugar, were consistently slightly higher than cultures with galactose 3-sulfate. Interestingly, isolate CF 8981-1, which showed no growth on glucose or galactose, showed a gradual small increase in optical density and cell number when cultured with galactose 3-sulfate and galactose 6-sulfate. Comparisons of nonmucoid and mucoid cultures as groups yielded no significant differences in growth on sulfated or on nonsulfated sugars ($P > 0.2$) based on maximum optical densities of the *P. aeruginosa* strains in this survey.

Whilst the effects of sulfated sugars on the mucoid character of *P. aeruginosa* were not quantitatively determined in this study (in terms of uronic acid analyses or changes in lipopolysaccharide chemotype), plating of these 14 strains following the prolonged incubation period provided generalized observations. Culture in minimal medium with glucose, glucose 3-sulfate or glucose 6-sulfate as the sole carbon source each showed trends toward diminishing mucoid character for those strains initially expressing the mucoid phenotype, whereas nonmucoid strains showed no change. Following prolonged culture on galactose and galactose sulfates, the original mucoid character was retained for some mucoid strains, and diminished for others. In no case was an enhancement of the mucoid character observed by growth on the sulfated sugars or the nonsulfated analogues employed in this study.

DISCUSSION

To further our understanding of the structure–function relationships of mucins in health and in disease, we evaluated the effects of carbohydrate sulfation, as seen in CF respiratory mucin oligosaccharides (Chance & Mawhinney, 1996; Mawhinney *et al.*, 1987, 1992a, b; Mawhinney & Chance, 1994), on the growth of *P. aeruginosa*. The means of regulation of this mucin O-sulfation and what roles this modification plays in the characteristic chronic infection of CF patients with *P. aeruginosa* are not clear. It is not known whether these sugars may serve as a carbohydrate source or as a stimulus of bacterial virulence factors in this scenario, as inhaled *P. aeruginosa* survive and adapt to life in the CF respiratory tract.

This study indicates that many of the sugars composing mucins are not readily used to support growth of laboratory or clinical strains of *P. aeruginosa*, supporting the hypothesis that mucins by their structure are protected from bacterial degradation and therefore more able to perform their clearance role under normal conditions. Sulfation of these sugars appears to add another level of protection against degradation. Given time though, as expected in a stagnant airway such as is seen in CF, *P. aeruginosa* may utilize mucin-derived sugars. As observed here, with time, *N*-acetylglucosamine supports as much growth as glucose, possibly reflecting amidase/acetylase activity like that responsible for the hydrolysis of and growth on acetamide by this organism (Clarke & Slater, 1986). O-Sulfatase has

recently been described by Jansen *et al.* (1999) for several clinical *P. aeruginosa* isolates. Evidence of such activity was not observed in the current study, suggesting that under these minimal media conditions, the organisms tested here were incapable of removal of the sulfate ester or of internalization and usage of the intact sugar sulfates. Additional experiments would be necessary to ascertain whether the strains surveyed here express detectable sulfatase activity *in vivo* or under different *in vitro* conditions.

The capability of *P. aeruginosa* to survive in suboptimal conditions until a better means of survival becomes available was evidenced in these studies. Galactose and other sugars, which did not support rapid growth in these minimal medium-based experiments, did supply enough nutritive value for a low level of growth and maintenance of viability. The apparent improvement in utilization of available substrates noted for some strains on galactose and galactose derivatives suggests that *P. aeruginosa* has additional mechanisms which are inducible in times of need or through which a mutation proves beneficial. Unlike *P. fluorescens* in previous studies (Fitzgerald & Dodgson, 1971a, b), *P. aeruginosa*, under similar conditions, did not adapt to utilize sulfated sugars. Data revealed no statistically significant patterns of carbohydrate utilization among nonmucoid versus mucoid isolates, suggesting that improved carbohydrate metabolism may not necessarily be a general feature related to the prevalent mucoid phenotype in CF. Likewise, the growth of these strains of *P. aeruginosa* on sulfated and nonsulfated sugars common to mucin, as the sole carbon source, did not appear to promote conversion to the mucoid phenotype of nonmucoid strains or enhance the mucoid character of mucoid strains.

Whilst not directly part of this study, it should be noted that liberation of mucin carbohydrates for use as an energy source for bacteria presumably requires glycosidase activity. Although there are no reports of 'mucinase activity' from *P. aeruginosa*, as there are for other pathogens, (Prizant & Reed, 1991; Schneider & Parker, 1982; Smith *et al.*, 1994), there have been reports of β -galactosidase activity (Cybulski *et al.*, 1993; Vieu *et al.*, 1987), which could liberate terminal galactose residues from mucins as well as make accessible N-acetylglucosamine, which is often the penultimate sugar on mucin oligosaccharide chains. The inability of the methyl glycosides or sulfated methyl glycosides tested here to support significant growth of *P. aeruginosa* suggests that under these conditions of limited nutrients, glycosidases are not elaborated.

Conclusions

These data suggest that the increased tracheobronchial mucin oligosaccharide sulfation seen in CF may in fact serve to protect the mucins from degradation during prolonged contact with *P. aeruginosa*, and limit the bacterium's growth on the basis of substrate availability, regardless of its mucoid status. Additional detailed *in*

vitro research with clinical isolates and defined laboratory strains is required to assess whether other culture conditions may affect *P. aeruginosa* growth in the presence of sulfated mucins, before proceeding to the more complex analysis of *in vivo* *P. aeruginosa* responses to CF airway mucin sulfation.

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