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Effect of nutrient limitation on adhesion characteristics of *Pseudomonas aeruginosa*

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B.A. COWELL, M.D.P. WILLCOX, B. HERBERT AND R.P. SCHNEIDER. 1999. *Pseudomonas aeruginosa* causes a variety of diseases in humans including lung and ocular infections. Infections of the cornea are usually associated with wearing contact lenses and can result in loss of vision. This study aimed to determine the effect of carbon or nitrogen limitation on the adhesion to contact lenses of a strain of *Ps. aeruginosa* isolated from contact lens-related corneal inflammation. Cells were grown in a continuous culture apparatus in varying levels of glucose or ammonia to effect nutrient limitation. Adhesion to contact lenses was measured as total counts and viable counts. The cell surface hydrophobicity and charge were measured using adhesion to surface-modified Sepharose. Changes in lipopolysaccharide were determined using 1D SDS-PAGE and changes in cell-surface proteins were measured using 2D gel electrophoresis. The more the cultures were nitrogen limited, the greater the increase in adhesion to unworn hydrogel contact lenses $0{\cdot}3 \times 10^3$–$2{\cdot}2 \times 10^3$ cells/mm^2 on Etafilon A lenses. Cells that were carbon limited showed a greater increase in adhesion to contact lenses when the lenses had been coated in artificial tears. It appeared that lipopolysccharide may have been involved in the constitutive adhesion to unworn lenses that occurred during C-limitation, whereas changes in the outer membrane proteins contributed to the increased adhesion under nitrogen limitation, or the change in adhesion that occurred to carbon-limited cells using contact lenses coated in artificial tears. Nine cell-surface proteins appeared during nitrogen limitation with kDa/pI of 75/4·8, 4·9, 5·0; 62/5·6; 89/6·5; 38/6·4; 28/1·5; 18/6·4; 12/4·5. Any or all of these may have been involved in the increased adhesion and further experiments are underway to examine this possibility.

INTRODUCTION

Corneal infection is a relatively rare but severe and potentially sight-threatening complication of contact lens wear, affecting 1 in 2800 users of daily wear contact lenses, and 1 in 500 users of extended wear contact lenses per year (Poggio *et al.* 1989). Although a relatively rare disease, the large number of contact lens wearers worldwide ($>$ 60 million) results in a significant number of eyes affected every year. Bacterial keratitis is one of the most destructive diseases of the cornea and is characterized by extensive ulceration and dissolution of the corneal stroma (Kernacki and Berk 1995). *Pseudomonas aeruginosa* is the most commonly isolated pathogen from ulcerative incidents (Cohen *et al.* 1991) and it is also implicated in inflammatory episodes of contact lens wear such as contact lens-induced acute red eye (CLARE, Holden *et al.* 1996). CLARE differs from infection as the causative bacteria adhere only to the contact lens and not to the cornea. CLARE is an acute response manifesting only during the overnight wear of lenses, causing acute pain, corneal infiltration, tearing, photophobia and reddening of the conjunctiva (Holden *et al.* 1996).

Adhesion to contact lenses is hypothesized to be the first

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step in ocular pathogenesis. The precise mechanisms involved in *Ps. aeruginosa* adhesion to lenses has not been determined, but involvement of surface structures such as pili and lipopolysaccharide have been suggested (Fletcher *et al*. 1993a,b). Little research has been conducted on the effect of physiological variability on adhesion of *Ps. aeruginosa* in the ocular environment. This study proposed to investigate the effect of limitations of growth conditions on the surface properties of *Ps. aeruginosa*, in particular the effect on adhesion to hydrogel lenses. The structure and composition of the Gram-negative cell surface is reported to change markedly in response to changes in the environment (Costerton *et al*. 1985; Rosenberg and Kjelleberg 1986; Allison and Goldsbrough 1994). Nutrient limitation of elements such as carbon and nitrogen may have profound effects on the cell surface composition of bacteria. Using continuous culture techniques, Allison and Nolan (1994) found peptidoglycan synthesis in *Ps. aeruginosa* to be affected by growth rate, but to different extents, during carbon and nitrogen limitation. Nitrogen limitation has profound effects on the production of proteins, especially those regulated by σ^{54} (Merrick 1993). Knowledge of the physiological variability of the surface of *Ps. aeruginosa* is essential for understanding the mechanism of this bacterium's adhesion to hydrogel lenses. Elucidation of the structures involved may lead to improved strategies to prevent lens contamination and subsequent ocular disease.

Table 1 Defined medium for growth of *Pseudomonas aeruginosa*

Constituent	Concentration
NaCl	145 mmol l^{-1}
KCl	20 mmol l^{-1}
$CaCl_2$	0·7 mmol l^{-1}
$MgSO_4$	0·44 mmol l^{-1}
Glucose	5·55 mmol l^{-1}
NH_4Cl	10 mmol l^{-1}
Sodium phosphate buffer, pH 7·4	20 mmol l^{-1}
α-Aminobenzoic acid	50 μg l^{-1}
Pyridoxine-HCl (B_6)	100 μg l^{-1}
Thiamine-HCl	50 μg l^{-1}
Riboflavin	50 μg l^{-1}
Nicotinic acid	50 μg l^{-1}
D-Ca pantothenate	50 μg l^{-1}
Lipoic acid	50 μg l^{-1}
Nicotinamide	50 μg l^{-1}
Cyanocobalamin (B_{12})	50 μg l^{-1}
Biotin	20 μg l^{-1}
Folic acid	20 μg l^{-1}
$FeCl_3.6H_2O$	8 mg l^{-1}
$ZnCl_2$	70 μg l^{-1}
$MnCl_2.4H_2O$	100 μg l^{-1}
$CoCl_2.6H_2O$	120 μg l^{-1}
$NiCl_2.6H_2O$	25 μg l^{-1}
$CuCl_2.2H_2O$	15 μg l^{-1}
H_3BO_3	100 μg l^{-1}
$Na_2MoO_4.2H_2O$	25 μg l^{-1}

MATERIALS AND METHODS

Bacterial preparation

Pseudomonas aeruginosa (Paer1) was isolated from a contact lens at the time of a CLARE response. The strain was maintained at −70 °C on 25% glycerol. A loopful of this culture was inoculated into 50 ml defined medium (Table 1) and grown overnight at 37 °C before inoculation in a 1 litre continuous culture vessel containing the same medium. The salts and pH of the defined medium were adjusted to values reported for open eye tears (Cowell *et al*. 1997). The reactors were operated at a dilution rate of 0·3 h^{-1}, aerated at 1 litre min^{-1} and allowed to reach steady state before cells were sampled. The steady state was attained when the biomass measured after approximately 10 volume changes of medium was constant. The ammonium concentration was varied to achieve conditions of carbon and nitrogen limitation. The culture was found to be carbon limited at a C : N ratio of 5 : 1 and 5 : 2, and nitrogen limited at C : N = 20 : 1. These limitations were compared by analysis of glucose and ammonium concentrations in the medium.

After sampling, bacteria were washed three times in phosphate-buffered saline (PBS, 8 g l^{-1} NaCl, 0·2 g l^{-1} KCl, 0·2 g l^{-1} KH_2PO_4, 1·15 g l^{-1} Na_2HPO_4, pH 7·4) before resuspension to $O.D._{600}$ = 1·0 (approximately 4×10^8 cfu ml^{-1}) prior to assay.

Adhesion assays

Paer1 was aliquoted (1·0 ml) into wells of a 24 well plate (Nunc, Roskilde, Denmark). Etafilcon A (medium water content, ionic lens, ACUVUE™, Vistakon, Johnson & Johnson Ltd, Jacksonville, FL, USA) or Polymacon (low water content, non-ionic lens, SeeQuence2™, Bausch and Lomb, Rochester, NY, USA) lenses were added to wells and incubated at 37 °C for 10 min. Lenses were removed and washed three times with 2 ml PBS with 30 s shaking during each wash. Bacteria attached to lenses were visualized by staining with 1 ml crystal violet (10% w/v) for 1 min. Excess stain was removed by rinsing three times in PBS. The stained lenses were then mounted on microscope slides with coverslips for counting by light microscopy. Five grids (0·005625 mm^2) per lens were counted and triplicate lenses for each treatment were assayed. The adhesion assay was repeated on a separate day, i.e. adhesion was assayed twice

for each C : N ratio. Counts were expressed as mean numbers of bacteria per $mm^2 \pm$ S.D.

Adhesion to lenses coated in artificial tear components was also assayed for cultures that were carbon (C : N = 5 : 2) or nitrogen (C : N = 20 : 1) limited. Both total and viable (cultivable) counts of adhered bacteria were estimated on Etafilcon A lenses. Etafilcon A lenses were incubated overnight at 4 °C in artificial tear fluid (ATF, closed eye formulation, Table 2), then rinsed three times in PBS before assay. Carbon or nitrogen limited *Ps. aeruginosa* was added to lenses as described previously and total adhesion determined using crystal violet staining and light microscopy. Viable (or cultivable) bacteria were determined on additional lenses by lens maceration to release adhered bacteria. Polymacon lenses are resistant to lens maceration and therefore, only Etafilcon A lenses were assayed. Lenses were placed in a Bijou bottle containing 1 ml PBS and a small magnetic stirring bar (octagonal cross-section, 0·5″ × 0·125″; Sigma) and stirred at maximum speed for 1 h at ambient temperature which was sufficient for lens disintegration. Serial dilutions were then made according to the technique of Miles and Misra (1938), and aliquots (20 μl) plated out on nutrient agar (Oxoid). After incubation overnight at 37 °C, viable bacteria were determined and results expressed as colony-forming units/mm^2 after calculation of the surface area of the lens (307·9 mm^2).

Retention on Sepharose columns

The method of Smyth *et al*. (1978) was adapted to determine hydrophobic and ionic interactions with Sepharose gels.

Table 2 Composition of artificial tear fluid

Tear component	Concentration (mg ml^{-1})*	
	Open eye	Closed eye
Proteins and glycoproteins		
Lysozyme (chicken egg white)	1·9	1·9
Lactoferrin (bovine colostrum)	1·8	1·8
α-Acid glycoprotein (bovine serum)	0·5	0·5
Albumin (human serum)	0·02	1·2
Mucin (bovine submaxillary gland)	0·15	0·15
γ-Globulins (human serum)	0·1	12·7
Lipids		
Cholesterol lineolate	0·024	0·024
Linalyl acetate	0·02	0·02
Triolein	0·016	0·016
Oleic acid propyl ester	0·012	0·012
Dicaproin	0·0032	0·0032
Undecylenic acid, sodium salt	0·003	0·003
Cholesterol	0·0016	0·0016
Salts		
Sodium chloride	6·626	6·08
Potassium chloride	1·716	1·57
Sodium bicarbonate	1·376	1·26
Lactic acid	0·27	0·27
Calcium chloride, dihydrate	0·147	0·147
Sodium dihydrogen phosphate, monohydrate	0·1	0·1
Buffer		
3-(N-Morpholino) propanesulphonic acid (MOPS)	4·18	4·18
Pooled human serum	0	2% (*v*/*v*)
pH	7·5	7·4

* Open eye formulation from Mirejovsky *et al.* (1991). Closed eye formulation modified from Mirejorsky *et al.* (1991) according to protein concentrations given by Sack *et al.* (1992) using the γ-globulin fraction to mimic increases seen in levels of sIgA during sleep.

Hydrophobic interactions were determined from retention on hydrophobic sepharoses (octyl and phenyl), and ionic interactions were determined by retention on cation exchange (CM) and anion exchange (DEAE) Sepharose columns.

Sepharose 6-B, octyl-, phenyl-, CM- and DEAE-Sepharose were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Columns were constructed by loading 1 ml (approximately 5 cm) of each Sepharose gel into Pasteur pipettes plugged with glass wool. The void volume determined using methylene blue was 0·5 ml. Columns were equilibrated with 5 ml PBS. Bacterial suspensions (0·5 ml, $O.D._{600} = 1·0$) were added to the columns and the first eluant (0·5 ml) discarded. A second aliquot of bacteria (0·5 ml) was added to the column and the eluant collected with the first wash of 0·5 ml PBS. Three additional washes (1 ml) were collected and the absorbance at 600 nm of each wash determined. Percentage of cells retained on each gel, and percentage of cells subsequently desorbed in the next three washes, were determined and from these data, the net retention (percentage of original inoculum) of bacteria on each Sepharose type was calculated.

Electron microscopy

Carbon- and nitrogen-limited organisms (C : N = 5 : 2 and C : N = 20 : 1, respectively) were examined for visual changes of the cell surface using negative staining and electron microscopy. Bacteria were harvested, washed and resuspended in PBS as described above. Bacteria were floated onto copper grids, fixed with 2·5% glutaraldehyde in 0·1 mol l^{-1} sodium phosphate buffer (pH 7·0), then stained with 1% sodium silica tungstate in sodium phosphate buffer (pH 7·0). After washing grids in distilled water, transmission electron microscopy was performed on a Phillips EM400 microscope and cells photographed.

Outer membrane protein preparation

The method was based on that described by Carnoy *et al.* (1994). Paer1 was harvested from continuous culture (1 litre), centrifuged at 2000 *g* for 10 min and washed three times with PBS before resuspension to a final volume of 100 ml in 50 mmol l^{-1} sodium citrate buffer (sodium citrate/citric acid, pH 4·5) containing 0·1% (w/v) Zwittergent (Calbiochem, La Jolla, CA, USA), 1 mmol l^{-1} PMSF (phenylmethyl-sulphonyl fluoride, Boehringer Mannheim) and 10 mmol l^{-1} EDTA (ethylenediaminetetraacetic acid, Sigma). The reaction mixture was incubated for 25 min at 45 °C with occasional mixing. Bacteria were then pelleted by centrifugation at 3200 *g* for 2 h at 4 °C. The supernatant fluid containing the extracted proteins was dialysed overnight against distilled water containing 0·02% (w/v) sodium azide. Proteins were concentrated using Centriprep-10 concentrators (10 000 molecular weight cut-off, Amicon, Beverly, MA, USA).

In preparation for the first dimension isoelectric focusing (IEF), 1 ml of dialysed *Ps. aeruginosa* membrane extract was freeze-dried and resuspended in 1 ml of 8 mol l^{-1} urea, 4% CHAPS and 100 mmol l^{-1} dithiothreitol. Individual 18 cm pH 4–10 Immobiline DryStrips were rehydrated in 500 μl of the protein solution in 2 ml plastic tissue culture pipettes. Rehydration was allowed to proceed at room temperature for 24 h. The IEF was carried out using a Pharmacia Multiphor II with a DryStrip Kit; power was supplied using a Consort 5000 V power supply and cooling water at 20 °C was supplied by a Pharmacia Multitemp III. The settings used for IEF were 300 V for 5 h, 1000 V for 5 h, 2500 V for 5 h and a final phase of 5000 V up to a maximum of 500 000 V h^{-1}.

After IEF, the strips were stored at – 80 °C until required for the second dimension. Prior to the second dimension run, the strips were equilibrated according to the method of Gorg *et al.* (1987). Second dimension gels were run using the Investigator System from Oxford Glycosystems (Abingdon, UK). The gels were 2 mm thick and 8–18% T gradients, and were cross-linked with PDA at 2·5% C. The gel buffer was 0·12 mol l^{-1} Tris/acetate, pH 6. Anode buffer consisted of 45 mmol l^{-1} Tris/acetate, pH 6·6, 0·1% (w/v) SDS. Cathode buffer was composed of 80 mmol l^{-1} Tricine/Tris pH 7·1, 0·1% (w/v) SDS, 0·001% (w/v) Bromophenol blue. The equilibrated IEF strips were embedded on the top of the SDS-PAGE gel using molten 1% (w/v) agarose in cathode buffer. Pharmacia low molecular weight standards were loaded in a single lane at one side of each gel. Gels were run at constant power of 4 watts per gel for 2 h and then 12 watts per gel until the Bromophenol blue front had traversed the gel.

The completed second dimension gels were stained using a Silver diamine stain (Bjellqvist *et al.* 1993). The silver stained gels were scanned using a Molecular Dynamics Personal Densitometer SI address and the images were aligned and matched using MELANIE II 2-D PAGE analysis software from Bio-Rad (Hercules, CA, USA).

Preparation of lipopolysaccharide extracts

Lipopolysaccharide was extracted from suspensions of *Ps. aeruginosa* using the hot water/phenol extraction method described by Westphal and Jann (1965). Carbon- and nitrogen-limited Paer1 cells were harvested (50 ml) from continuous culture and washed three times in PBS prior to resuspension in distilled water. The bacterial suspension was incubated at 68 °C prior to extraction. An equal volume of phenol (68 °C) was added and vortexed. The suspension was incubated for 20 min at 68 °C, then removed and cooled at 4 °C to aid separation of the two phases. The aqueous phase

was then carefully removed and the procedure repeated. The aqueous phases were pooled and dialysed extensively against 4 l distilled water at 4 °C for 2 d (changing water after 1 d) or until all traces of phenol odour had disappeared. The amount of LPS was measured spectrophotometrically using a Coatest® endotoxin kit (Chromogenix AB, Möindal, Sweden). The LPS was fractionated using SDS-PAGE (10% acrylamide) and the bands of LPS were visualized by silver staining. Approximately 0·58 μg LPS of each preparation was loaded onto the gel.

Competition of bacterial adhesion with lipopolysaccharide preparation

To determine the effect of lipopolysaccharide on adhesion of *Ps. aeruginosa* to hydrogel lenses, phenol extracts of carbon- and nitrogen-limited cell suspensions prepared as described above were used as crude preparations of lipopolysaccharide. In these adhesion assays, 0·5 ml nitrogen-limited bacterial suspension was added to 0·5 ml LPS preparation (carbon- or nitrogen-limited extracts, $3{\cdot}4 \times 10^5$ EU (endotoxin units) ml^{-1}) and incubated with Etafilcon A lenses. Adhesion was compared to the control where 0·5 ml PBS was added as a replacement for the LPS preparation.

Statistics

Adhesion data for carbon and nitrogen limited cells were not normally distributed and therefore were analysed non-parametrically with the Mann–Whitney V-Wilcoxon Rank Sum test. Competition of adhesion with lipopolysaccharide data was analysed using Bonferroni's test. Retention on Sepharose was analysed using the Student's *t*-test.

RESULTS

Effect of nutrient limitation on adhesion of *Ps. aeruginosa* to hydrogel lenses

Under conditions of carbon limitation (C : N = 5 : 2), *Ps. aeruginosa* adhered poorly to both lens types (Etafilcon A and Polymacon; Fig. 1; 266·7 ± 322·6 and 314·1 ± 312·1 cells/mm^2, respectively). As the degree of nitrogen limitation was progressively increased, adhesion to both lens types increased ($P < 0{\cdot}05$). A notable effect was observed at a C : N of 20 : 1 (nitrogen limitation) where cells adhered in significantly larger numbers to Polymacon than to Etafilcon A lenses ($P < 0{\cdot}05$). Bacteria were not observed to clump on lenses in any of the assays.

There was no significant difference between total adhesion of carbon-limited and nitrogen-limited *Ps. aeruginosa* to ATF coated Etafilcon A lenses (Fig. 2). There was a significant ($P < 0{\cdot}05$) increase in adhesion of carbon-limited cells (viable or total counts) to the ATF-coated lenses compared with uncoated lenses.

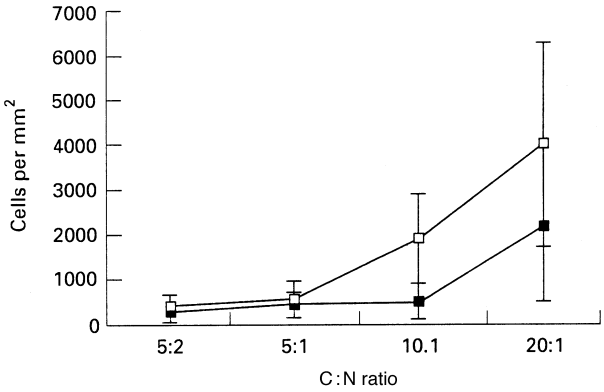

Fig. 1 Effect of carbon/nitrogen limitation on adhesion of *Pseudomonas aeruginosa* to hydrogel lenses. (■), Etafilcon A; (□), Polymacon

Electron microscopy

Both carbon- and nitrogen-limited cells expressed flagella and fimbriae and no differences in cell-surface structures were evident using transmission electron microscopy (Fig. 3). Resolution of these photographs is limited to relatively large components and therefore, may not indicate more subtle differences between the cell types responsible for their different adhesive capabilities.

Effect of nutrient limitation on retention of *Ps. aeruginosa* on Sepharose columns

Retention of *Ps. aeruginosa* on Sepharose 6-B increased significantly when C : N was increased from 5 : 2 to 5 : 1 ($P < 0{\cdot}05$, Table 3). Further changes in C : N did not result in additional alteration of retention of *Ps. aeruginosa* ($P > 0{\cdot}05$). Sepharose 6-B is a slightly polar matrix often used by researchers as a control for substituted sepharoses such as octyl Sepharose in hydrophobicity assessments. *Pseudomonas aeruginosa* was almost totally retained on DEAE Sepharose. Retention of cells on CM Sepharose increased with each increase of the C : N ratio up to 10 : 1 indicating an overall increase in positively-charged groups on the bacterial surface. However, these effects were reversed when C : N was further increased to 20 : 1. There was also a gradual increase in retention of cells on octyl Sepharose when C : N changed from 5 : 2 to 10 : 1 ($P < 0{\cdot}05$). This trend was then reversed such that the same number of cells was retained at C : N = 20 : 1 as observed for C : N = 5 : 1 ($P > 0{\cdot}05$). On phenyl Sepharose, cells grown in media with a C : N ratio of 5 : 2 and 5 : 1 were retained in similar numbers, which was

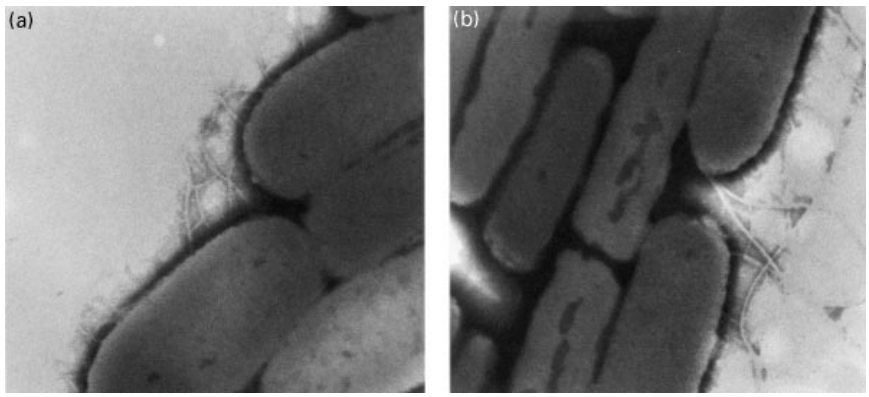

Fig. 2 TEM of *Pseudomonas aeruginosa* (Paer 1). (a) C : N = 5 : 2; (b) C : N = 20 : 1. Magnification 27 500 ×

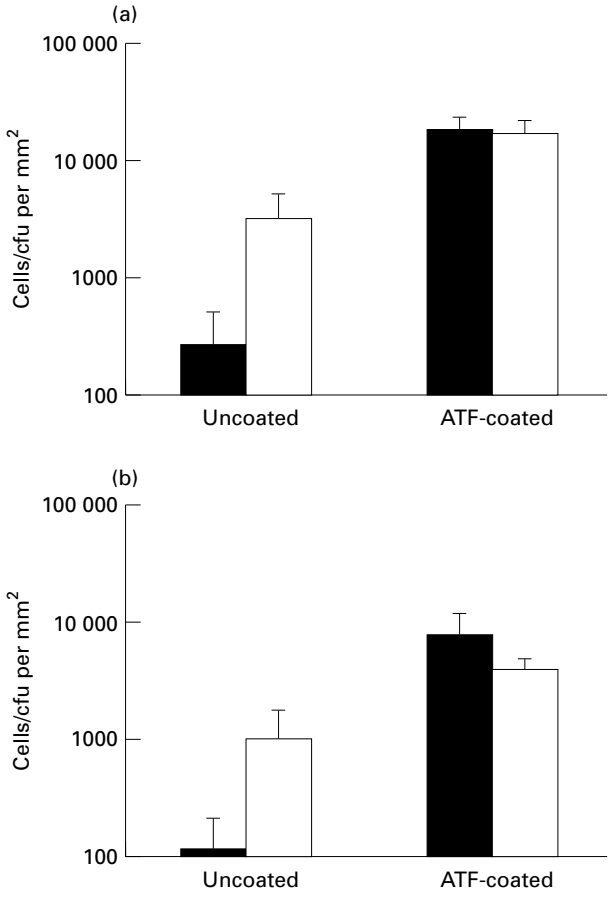

Fig. 3 Effect of carbon/nitrogen limitation on adhesion of *Pseudomonas aeruginosa* to Etafilcon A lenses coated with artificial tear components. (a) Total counts; (b) viable counts. (■) C : N = 5 : 2; (□) C : N = 20 : 1

about 16·8% lower than the similar values recorded for C : N = 10 : 1 and 20 : 1. There was an overall significant increase in retention ($P < 0{\cdot}05$) between cells grown at C : N = 5 : 2 and 20 : 1. These results suggested that the outer surface of *Ps. aeruginosa* was predominantly negative in nature, but it also contained regions of positive charge and hydrophobic character. It also indicated that the C : N ratio significantly modified charged and hydrophobic groups. However, the changes of cell surface properties associated with alterations of the C : N ratio measured with column chromatography did not correlate with adhesion of the cells to hydrogel lenses.

Effect of nutrient limitation on outer membrane components of *Ps. aeruginosa*

The outer membrane proteins of nitrogen-limited *Ps. aeruginosa* differed from those of carbon-limited cells (Fig. 4). The major differences are highlighted by arrows in the figure. Three proteins of approximate molecular weights (MWt) and isoelectric points (pI) of 26 kDa/4·9 pI, 48 kDa/5·6 pI and 46 kDa/5·7 pI were released from carbon-limited cells but were absent in nitrogen-limited cells. More proteins (75 kDa/4·8 pI; 75 kDa/4·9 pI; 75 kDa/5·0 pI; 62 kDa/5·6 pI; 59 kDa/6·3 pI; 38 kDa/6·4 pI; 28 kDa/9·5 pI; 18 kDa/6·4 pI; 12 kDa/4·5 pI) were released from nitrogen-limited cells than carbon-limited cells. Table 4 indicates possible identities of proteins revealed by examination of the literature for membrane proteins of *Ps. aeruginosa*.

For lipopolysaccharide, the sample from carbon-limited cells contained $3{\cdot}44 \times 10^5$ EU ml^{-1} of endotoxin and the nitrogen-limited sample, $3{\cdot}42 \times 10^5$ EU ml^{-1} of endotoxin (both approximately 29 μg ml^{-1} LPS). Examination of phenol extracts of *Ps. aeruginosa* using SDS-PAGE indicated no differences in the LPS of carbon- and nitrogen-limited cells (Fig. 5).

Addition of the LPS extract into the contact lens adhesion assay system decreased adhesion of *Ps. aeruginosa*, suggesting that LPS is involved in adhesion of *Ps. aeruginosa* to hydrogel lenses (Fig. 6). Nitrogen-limited LPS reduced adhesion by 66% while carbon-limited LPS reduced adhesion by 45%. Both carbon- and nitrogen-limited LPS significantly

Table 3 Percentage retention of bacterial cells on Sepharose columns

	C : N ratio			
	5 : 2	5 : 1	10 : 1	20 : 1
Sepharose 4-B	12·64 ± 0·96	33·13 ± 5·29	31·38 ± 1·78	33·23 ± 1·74
Octyl	52·26 ± 4·88	69·51 ± 6·80	82·69 ± 1·62	72·50 ± 4·11
Phenyl	72·28 ± 4·13	69·17 ± 6·13	86·29 ± 2·53	83·80 ± 1·55
Diethylaminoethyl	98·89 ± 0·54	99·19 ± 0·88	99·56 ± 0·15	99·69 ± 0·18
Carboxymethyl	19·61 ± 0·77	20·76 ± 9·05	32·83 ± 0·52	15·00 ± 1·69

Bacteria were applied to columns containing the appropriate sepharose types, the optical density of eluants determined after application of phosphate-buffered saline and the % retention estimated from the optical density of the eluants compared with the initial optical density of the bacterial suspension. Results expressed as mean ± standard deviation.

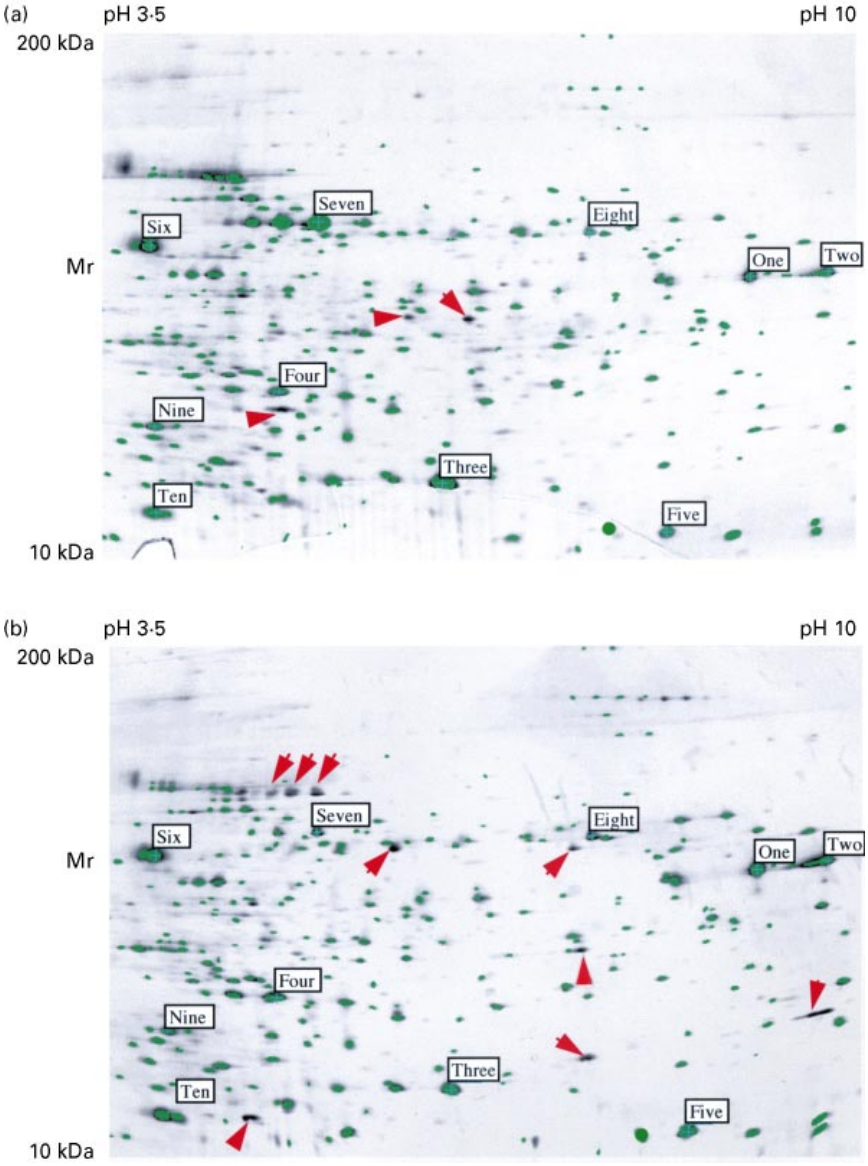

Fig. 4 2D gel electrophoresis of the outer membrane proteins of *Pseudomonas aeruginosa*. Arrows indicate appearance or differences in proteins between C-limited (a) or N-limited (b). Numbers indicate the proteins used to align the gels from C-limited and N-limited growths of Paer l

Table 4 Possible identities of the outer membrane proteins of *Pseudomonas aeruginosa* Paer 1 that were differentially regulated during growth in either carbon or nitrogen limited conditions

Observed outer membrane protein (nutrient limited condition) kDa/pI	Possible identity kDa/pI	Possible function of homologous protein
48/5·6 (Carbon limited)	OprB (48/?)[1,2]	Glycerol and glucose regulated transport protein
	Mucin-binding protein (48/?)[3,4]	Mucin binding
	OprP (48/?)[5]	Phosphate transport
46/5·7 (Carbon limited)	Opr (46/?)[5]	Glucose porin
	OprD (45·5/?)[5]	Basic amino acid/imipenem/gluconate transport
	Mucin-binding protein (46/?)[3,4]	Mucin binding
26/4·9 (Carbon limited)	Opr (25/?)[5]	Unknown
	Mucin-binding protein (25/?)[3]	Mucin binding
75/4·8, 4·9, 5·0 (Nitrogen limited)	Mucin-binding protein (77–85/?)[4]	Mucin binding
	OprC (73/?)[6]	Copper-binding protein
	Iron regulated outer membrane protein (77–87)[5]	Fe^{3+}-siderophore binding/transport
62/5·6 (Nitrogen limited)	PilB (62/5·6)[7]	Type II secretion of type IV fimbriae
59/6·3 (Nitrogen limited)	PilS (59/6·3)[8]	Sensory component of two-component regulatory system
38/6·4 (Nitrogen limited)	OprF (38/?)[9]	Major outer membrane protein
	PilC (38–45/?)[7]	Pilin assembly/maturation
28/9·5 (Nitrogen limited)	Mucin-binding protein (28/?)[3]	Mucin binding
	PilD (28–32/?)[7]	Pilin assembly/maturation
18/6·4 (Nitrogen limited)	OprL (18/?)[9]	Peptidoglycan-associated outer membrane lipoprotein
	Mucin-binding protein (18/?)[3]	Mucin binding
12/4·5 (Nitrogen limited)	PilZ (13/?)[10]	Fimbrial biosynthesis
	Mucin-binding protein[3]	Mucin binding
	Ferripyochelin binding protein (9–14/?)[5]	Ferripyochelin binding/transport

[1,2] (Williams *et al*. 1994; Wylie and Worobec 1994). [3,4] (Carnoy *et al*. 1994; Scharfman *et al*. 1996). [5] (Hancock *et al*. 1990). [6] (Yoneyama and Nakae 1996). [7] (Nunn *et al*. 1990). [8] (Hobbs *et al*. 1993). [9] (Lim *et al*. 1997). [10] (Alm *et al*. 1997).
?, pI values not known or not reported.
The observed outer membrane protein kDa/pI was estimated from Fig. 4.
Opr, suggested name for outer membrane proteins of *Ps. aeruginosa* (Hancock *et al*. 1990).
Pil, designates protein involved in synthesis or secretion of pili/fimbriae.

decreased adhesion to Etafilcon A lenses ($P < 0{\cdot}05$). There was no statistically significant difference between the effects of the two extracts ($P > 0{\cdot}05$).

DISCUSSION

This study has demonstrated that altering the nutrient limitation in which cells of *Ps. aeruginosa* were grown significantly altered the adhesion profile of strains and the types of proteins that could be extracted from cells. Adhesion of cells to uncoated contact lenses increased with nitrogen limitation, with nine new proteins being demonstrated after 2D gel electrophoresis. One or more of these proteins may be involved in adhesion to contact lenses. The increased adhesion was not the result of non-specific factors such as changes in cell surface hydrophobicity or charge, as these factors did not change in relation to the changes in adhesion. Cell-surface hydrophobicity, as measured by retention on either octyl- or phenyl-Sepharose, peaked at nitrogen limitations of 10 : 1 whereas adhesion peaked at a nitrogen limitation of 20 : 1. The negative charge on the cell surface did not alter significantly during nutrient limitation, and the cell-surface positive charge peaked at a nitrogen limitation of 10 : 1.

The expression of pili/fimbriae would be expected to change under nitrogen limitation as the expression of σ^{54} (RpoN) is increased (Merrick 1993). However, in the present study, pili/fimbriae as revealed by electron microscopy did not alter during nutrient limitation. This suggests that the

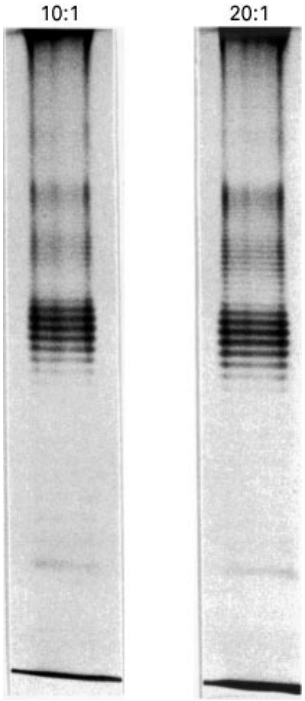

Fig. 5 Lipopolysaccharide extracts of *Pseudomonas aeruginosa*

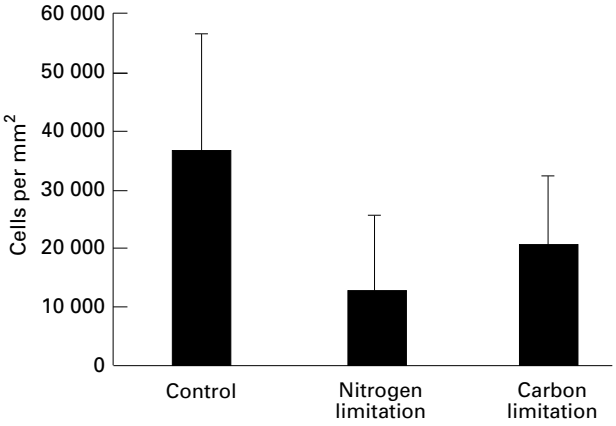

Fig. 6 Competition of *Pseudomonas aeruginosa* with free lipopolysaccharide in adhesion to Etafilcon A lenses

structural pilin gene was not involved in adhesion to contact lenses. Flagella were demonstrated to be important in *Ps. aeruginosa* binding to stainless steel by Stanley (1983). This adhesion mechanism does not appear to be critical to adhesion of Paer1 to hydrogel lenses as flagella were present on both carbon- and nitrogen-limited cells. A decrease in nitrogen limitation has been shown to decrease the expression of *algD*, the gene excoding GDP-mannose dehydrogenase (Delic-Attree *et al*. 1996), and hence, the synthesis of alginate. This would be expected to alter the net negative charge on the cell surface. The present investigation did not reveal an increase in the net negative cell-surface charge of strains as they were grown in progressively increasing nitrogen-limited conditions, demonstrating that the expected increases in alginate synthesis did not affect adhesion of strains to contact lenses. Research into alginate production by *Ps. aeruginosa* has shown that factors such as gluconate, magnesium, sodium chloride and also adhesion to a surface stimulate the synthesis of this compound (Chan *et al*. 1984; Hoyle *et al*. 1993). The adhesion protocol used in the present study was designed to minimize the effect of post-adhesion physiological responses by limiting the incubation time of lenses with bacteria to 10 min. Although nitrogen limitation has been shown to increase the production of mucoid colonies, this occurred only when nitrate was used as the limiting nitrogen source (Terry *et al*. 1991) and even then, the percentage of colonies that were mucoid was $< 0{\cdot}5\%$. Furthermore, the concentration of Mg^{2+} used in the current medium ($0{\cdot}44$ mmol l^{-1}) is less than that reported to stimulate mucoid colony formation (Chan *et al*. 1984). The amount or type of lipopolysaccharide on the cell surface of strains did not alter during changes in nutrient limitation. Although LPS has been shown to be involved in the adhesion of strains to contact lenses (Fletcher *et al*. 1993b), the data from the present study indicate that LPS may be involved in the low-level constitutive adhesion to contact lenses seen for strains grown under carbon limitation, and not for the increases seen with increasing nitrogen limitation.

Carbon limitation appeared to affect the adhesion of strains to ATF-coated contact lenses. Carbon-limited cells had a greater increase in numbers adhered to contact lenses coated with ATF than did nitrogen-limited cells for both viable and total adhesion. It would be expected that these changes could be the result of either the appearance of novel moieties or structures on the surface of carbon-limited cells, or an increase in the expression of existing moieties or structures. The only differences seen during this study that were likely to have mediated this change were the appearance of three proteins during carbon limitation that were not present during nitrogen limitation, and/or the upregulation of three proteins of apparent MWt/IEPs of 65 kDa/4·7IEP, 65 kDa/4·9IEP and 65 kDa/5·2IEP. The identity of these proteins is unknown. However, examination of the literature for *Pseudomonas* proteins which may be located on or near the cell surface in this molecular weight (65 kDa) and pI range (5·0 pI) indicated that the flagella M-ring protein (FliF), which has an MWt 64217 Da and pI 4·92 (Arora *et al*. 1996), was the closest match. This protein was probably not expressed on the surface and may only be involved in adhesion by allowing other molecules to localize at the surface (Arora *et al*. 1996). There is no indication in the literature that this molecule would have several isoelectric points (isoforms).

The three new proteins which appeared when cells were grown in carbon-limited conditions may have similarities to previously described adhesins or porins (Table 4), including mucin-binding proteins, seen after 1D gel electrophoresis of *Ps. aeruginosa*.

Nitrogen limitation resulted in the appearance of nine new proteins and this correlated with changes in adhesive potential. The new proteins may have been previously characterized surface proteins (Table 4) involved in adhesion, binding to iron-chelating compounds or sensory apparati. Further work is needed, for example N-terminal sequencing, to characterize these proteins and determine whether they are the proteins presumptively identified in Table 4.

Previous studies of adhesion of *Ps. aeruginosa* to hydrogel lenses have resulted in conflicting reports of different levels of adhesion to lens types (Duran *et al*. 1987; Lawin-Brussel *et al*. 1991; Cook *et al*. 1993; Stapleton *et al*. 1993). Different media and growth conditions were used in these studies to grow the bacteria. In the current study, it was demonstrated that growth conditions had a significant effect on the surface properties of *Ps. aeruginosa*. It is therefore likely that differences in the results reported in the literature are due to variability in cultivation methods. When *Ps. aeruginosa* was carbon-limited, no difference between adhesion to the two lens types was seen, but adhesion of nitrogen-limited cells to Etafilcon A and Polymacon lenses differed significantly. Consideration of the growth conditions is therefore required before adhesion results can be compared between research groups.

In conclusion, this study has demonstrated that growth in carbon- or nitrogen-limiting conditions profoundly affects the adhesive ability of *Ps. aeruginosa*. This change was not correlated with changes in cell-surface structural components such as flagella or pili/fimbriae, or with changes in cell-surface hydrophobicity or charge. There was a correlation between changes in adhesion and alterations in the types and number of cell-associated proteins that could be demonstrated by 2D gel electrophoresis. Further work is required to identify these proteins and determine their exact role in adhesion or resistance.

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