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Alterations in the formation of lipopolysaccharide and membrane vesicles on the surface of *Pseudomonas aeruginosa* PAO1 under oxygen stress conditions

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It has been postulated that phenotypic variation in the relative expression of two chemically distinct types of lipopolysaccharide (LPS), a serotype-specific LPS (B-band) and a common antigen LPS (A-band) in *Pseudomonas aeruginosa* is an important mechanism enabling this opportunistic pathogen to alter its surface characteristics to mediate adhesion and to survive under extreme conditions. To further investigate this, the relative expression levels of the two distinct types of LPS in *P. aeruginosa* PAO1 were investigated with cells grown in a chemostat at different dissolved oxygen tensions (pO_2). The A-band LPS was constitutively expressed as pO_2 was increased from nearly zero to 350 % of air saturation. In contrast, the B-band LPS showed a remarkable increase with increased pO_2. Almost no B-band LPS was found in cells grown at a pO_2 of less than 3 % of air saturation. Electron microscopic examination of cells revealed increased formation of membrane vesicles (MVs) on the surface of *P. aeruginosa* PAO1 under oxygen stress conditions. The toxicity of the supernatant of *P. aeruginosa* cultures to the growth of a hybridoma cell line significantly increased in samples taken from oxygen-stressed steady-state cultures. Furthermore, studies of adhesion in a continuous-flow biofilm culture revealed an increased adhesiveness for hydrophilic surfaces in *P. aeruginosa* PAO1 grown at a higher pO_2. The oxygen-dependent alterations of cell-surface components and properties observed in this work provide a possible explanation for the emergence of *P. aeruginosa* lacking the B-band LPS in chronically infected cystic fibrosis patients. The results are also useful for understanding the processes involved in the formation of MVs in *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa can produce two chemically distinct types of lipopolysaccharide (LPS), a serotype-specific LPS (B-band) and a common antigen LPS (A-band). These LPS molecules are the main antigens and the source of endotoxic activity of *P. aeruginosa*. LPS also plays a prominent role in infections of the lungs of cystic fibrosis (CF) patients (Pier, 1998). Resistance to phagocytosis and inflammatory responses in chronic infections of CF patients are assisted by LPS (Berger, 2002). Furthermore, Makin & Beveridge (1996) postulated that phenotypic variation in the relative expression of A- and B-band LPS in *P. aeruginosa* is an important mechanism enabling this opportunistic pathogen to alter its surface characteristics to mediate adhesion and to survive under extreme conditions. During infection of the lungs of CF patients, wild-type *P. aeruginosa* undergoes phenotypic alterations, resulting in a gradual loss of B-band LPS (Hancock *et al.*, 1983; Knirel *et al.*, 2001). The conditions causing this phenotypic change are not clear.

A phenomenon related to the formation of B-band LPS in *P. aeruginosa* is the occurrence of membrane vesicles (MVs) on the cell surface under certain conditions (Beveridge, 1999). MVs of *P. aeruginosa* contain mainly B-band LPS and play important roles in the release of many virulence factors (Beveridge, 1999). The conditions that induce the occurrence and discharge of MVs in *P. aeruginosa* are not well understood, with the exception of the effect of the aminoglycoside antibiotic gentamicin (Kaduragamuwa & Beveridge, 1995).

Several physical (e.g. temperature) and chemical (e.g. varying pH, and high phosphate, NaCl, $MgCl_2$, glycerol and sucrose concentrations) conditions have been reported to cause alterations in the formation of LPS in *P. aeruginosa* PAO1 (Kropinski *et al.*, 1987; McGroarty & Rivera, 1990; Makin & Beveridge, 1996). However, the possible effects of oxygen availability or oxidative stress on the formation of

Abbreviations: CF, cystic fibrosis; MV, membrane vesicle; TEM, transmission electron microscopy.

LPS and MVs in *P. aeruginosa* have not been investigated. Oxygen availability is one of the important influences in biofilms (Xu *et al.*, 1998), the preferred growth mode of *P. aeruginosa*, especially in the lung of CF patients (Costerton *et al.*, 1999). In a recent study, we showed that *P. aeruginosa* PAO1 possesses a remarkable ability to decrease the transport of oxygen in liquid culture, causing oxygen limitation or microaerobic conditions in the milieu (Sabra *et al.* 2002). Oxygen limitation markedly affected growth and the formation of virulence factors and this may play an important role in the defence of this pathogen against oxidative stress. In this work, we investigated the effects of dissolved oxygen tensions (pO_2) on the formation of LPS and MVs on the surface of *P. aeruginosa* PAO1 grown in a continuous chemostat culture under clearly defined conditions. Adhesion to hydrophilic surface (biofilm formation) of cells grown under different pO_2 tensions and the overall toxicity of *P. aeruginosa* PAO1 culture supernatant for a mammalian cell line were also examined.

METHODS

Micro-organism and cultivation conditions. The *P. aeruginosa* strain used in this study was the clinical strain PAO1 (NCCB 2425). The modified minimal medium described by Sabra *et al.* (2002) was used. Computer-controlled continuous culture was performed as described previously (Sabra *et al.*, 2000). A glucose-limited chemostat culture (with 1·5 g glucose l^{-1} in feed medium) was carried out at a dilution rate (D) of 0·20 h^{-1}. pO_2 was controlled at various levels (between 1 and 350 % of air saturation) by mixing nitrogen and pure oxygen in the inlet gas using a proportional-integral (PI) controller through a real-time on-line computer control system as described by Sabra *et al.* (2000).

Continuous-flow biofilm culture. The adhesion of PAO1 cells from steady states of the chemostat culture at different pO_2 levels was determined using clean glass tubes as substratum in a continuous flow biofilm culture as shown in Fig. 1. A cell suspension (8 ml with an identical initial OD_{600} of 0·4) from the steady-state culture was poured into the glass tubes and incubated for 4 h at 37 °C. The tubes were then rinsed with a 1 : 10 dilution of glucose minimal medium (Sabra *et al.*, 2002) for 20 h at 37 °C at a dilution rate as high as 10 h^{-1} to prevent planktonic growth. The tubes were bubbled with pure oxygen. They were then washed with phosphate buffer solution (39 mM KH_2PO_4, 61 mM K_2HPO_4, pH 7·4) and frozen and thawed several times in the same buffer to resuspend the biofilm. The OD_{600} and protein content of the resuspended culture were measured as an indication of adhesion and biofilm formation.

LPS analysis and immunoblotting. Cells from different steady states of the chemostat culture were harvested and dried and the LPSs were prepared from 300 mg dried cells using the proteinase K method described by Hitchcock & Brown (1983). The LPSs were precipitated with ethanol (96 %) containing 0·37 M $MgCl_2$, then washed with 95 % ethanol and extracted three times with chloroform/methanol (2 : 1, v/v) and finally dried in a vacuum. LPS was resuspended in 10 ml Tris/HCl buffer and the total sugar was analysed after acid hydrolysis as described by Dubois *et al.* (1956). Samples with the same amount of LPSs as determined by the analysis of total sugar were then analysed by electrophoresis in 14 % (w/v) polyacrylamide gels. LPSs were visualized by silver staining (Fomsgaard *et al.*, 1990). When required, LPS samples were transferred from polyacrylamide gels to nitrocellulose using the method of Makin & Beveridge (1996). Immunoblots were probed with mAb MF15-4 (specific for serotype 05) or mAb NIF10 (specific for A-band), kindly provided by Dr J. S. Lam (University of Guelph, Canada) (diluted to 1 : 50 in PBS containing 5 %, w/v, skim milk), and visualized using horseradish-peroxidase-conjugated goat anti-mouse antibodies (diluted to 1 : 1000 in PBS containing 5 %, w/v, skim milk) (Burrows & Lam, 1999).

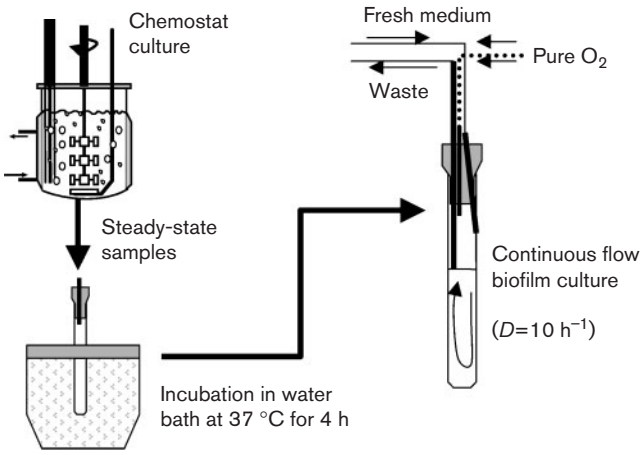

Fig. 1. Experimental set-up for the study of adhesion and biofilm formation in a continuous flow glass tube culture with cells from steady states of a glucose-limited chemostat culture at a constant dilution rate but different pO_2. The cells from steady states of the chemostat culture were first incubated in the water bath for 4 h before a continuous flow of fresh medium at a very high dilution rate was started to remove planktonic cells from the biofilm culture.

Electron microscopy. Cells from both microaerobic and oxidative stress steady states of the chemostat cultures were prepared for transmission electron microscopy (TEM) as described by Yakimov *et al.* (1998) and surface charge was detected using cationic colloidal thorium, as described by Winkler *et al.* (2001).

Overall cytotoxicity assay. A toxicity assay was performed using cell-free supernatant from steady states of the chemostat culture at a constant growth rate, but different pO_2 levels. The cytotoxicity was tested by applying different dilutions of the supernatant to the hybridoma cell line HyGPD YK-1-1 cultured in RPMI medium (Gibco-BRL) in freshly seeded 24-well plates. The plates were incubated at 37 °C under 5 % CO_2 and 95 % relative humidity for 30 h. The proportion of hybridoma cells that survived was determined by staining with tetrazolium dye (MTT) as described by Denizot & Lang (1986). Untreated cell cultures were used as controls.

Other analytical methods. Glucose concentrations were determined with immobilized glucose oxidase using a glucose analyser (Biochemistry Analyser; Yellow Springs Instruments). Alginate concentrations were determined in the culture supernatants as described previously (Sabra *et al.*, 2002). Total extracellular protein content was determined in the cell-free supernatant by the Lowry method. The mannuronic acid concentration was determined using an isocratic HPLC system equipped with an HPX-87H column (Bio-Rad) and a differential refractive index detector. As a mobile phase, 0·005 M H_2SO_4 at a flow rate of 0·6 ml min^{-1} was used at a working temperature of 60 °C.

RESULTS

Expression of A- and B-band LPSs in cells grown in chemostat culture at different pO_2 levels

As variations in growth rate, pH and nutrient concentrations are inherent in many batch culture systems and these conditions may cause phenotypic changes in *P. aeruginosa*, we used a pH-controlled chemostat culture at a constant dilution rate (approximating a constant growth rate) with pO_2 controlled at levels ranging from 1 to 350 % of air saturation. Glucose-limited chemostat culture was used to avoid rhamnolipid production, which is known to be mainly produced under carbon-sufficient conditions (Guerra-Santos *et al.*, 1986; Chayabutra *et al.*, 2001) and can induce removal of LPS from *P. aeruginosa* (Al-Tahhan *et al.*, 2000). The chemostat culture with a constant dilution rate ($0{\cdot}2\ h^{-1}$) had a relatively constant biomass concentration ($0{\cdot}40\ g\ l^{-1}$) in steady states with varying pO_2 levels.

The LPS profiles of *P. aeruginosa* PAO1 grown at varying pO_2 levels are shown in Fig. 2. Two major LPS bands were visible by PAGE at pO_2 levels greater than 3 %, but only one band was detected under microaerobic or oxygen-limited conditions (Fig. 2a). The band of higher mobility corresponded to A-band LPS, while the more slowly migrating band probably contains B-band LPS (McGroarty & Rivera, 1990). When higher LPS concentrations were electrophoresed, the typical ladder pattern of the high-molecular-mass O-polymer-containing LPS was detected (Fig. 2b). Generally, at oxygen-limited conditions, the LPS banding pattern changed significantly, especially with respect to the high-molecular-mass O-polymer-containing molecules. Furthermore, immunoblotting with mAb NIF10, which is specific for the common antigen, indicated that the size distribution of A-band LPS was minimally affected by the pO_2 level (Fig. 2c). However, the reaction with B-band-specific mAb MF15-4 was more intense under conditions of oxidative stress (Fig. 2d). A very weak B-band LPS reaction was detected by immunostaining in cells grown under oxygen-limited conditions.

Formation of MVs on the surface of *P. aeruginosa* PAO1 and increased toxicity under conditions of oxidative stress

TEM was used to examine the surface ultrastructure of PAO1 cells grown in a chemostat under oxygen-limited ($pO_2 \sim 0$ %) and oxygen stress conditions ($pO_2 \sim 350$ %). Under oxygen-limited conditions, MVs were seldom observed (Fig. 3a). However, under conditions of oxygen stress MVs were observed on the surface of most of the cells (Fig. 3b). Blebs dissociated from the outer membrane of cells were also seen. HPLC analysis of the culture supernatants revealed a threefold increase in the concentration of mannuronic acid residues when the pO_2 level increased from 0 to 350 % (Fig. 4). Mannuronic acid is the main component of B-band LPS and may mainly originate from the dissociated MVs.

The enhanced formation and release of MVs in *P. aeruginosa* have been mainly studied in cells exposed to gentamicin (Kaduragamuwa & Beveridge, 1995). The MVs shown in Fig. 3 have a diameter in the range of 24–50 nm, which is much smaller than the MVs (50–150 nm) induced by gentamicin. In addition to their size, oxygen-induced MVs may also differ from gentamicin-induced MVs in their components and function. Kadurugamuwa & Beveridge (1995) showed that the excretion of different virulence factors is associated with MVs.

Toxicity of diluted cell-free supernatants of *P. aeruginosa* PAO1 cultures grown under different pO_2 levels for a hybridoma cell line was assessed (Fig. 5). At dilutions below 1 : 8, the viability of hybridoma cells was greatly reduced after treatment with supernatants from cultures grown under conditions of oxidative stress. This concords with the observations of increased formation of MVs under

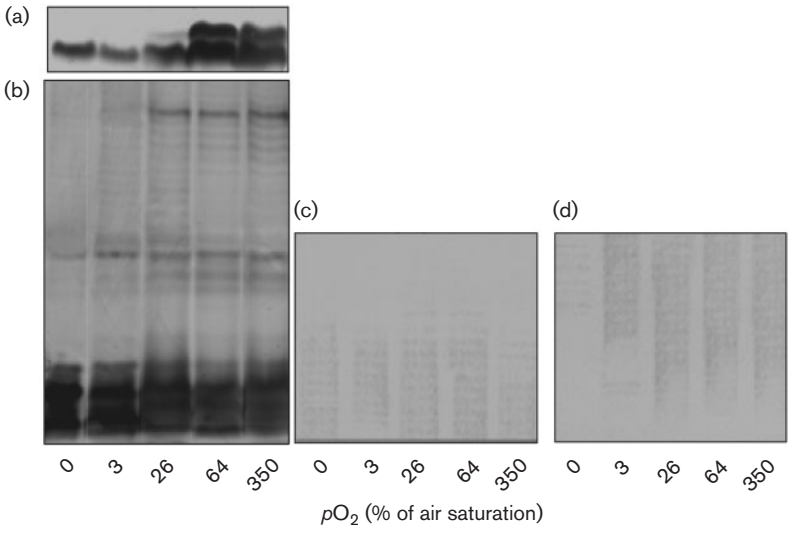

Fig. 2. LPS analysis by PAGE and silver staining with different loadings of LPS samples: (a) 5 μl resuspended solution of LPS and (b) 15 μl of the same solution from *P. aeruginosa* PAO1 cells grown at different pO_2 in chemostat cultures (cf. Fig. 1). Fractionated LPS from PAGE analysis (loading: 25 μl LPS solution) was transferred to nitrocellulose and probed with (c) A-band- or (d) B-band-specific antibodies.

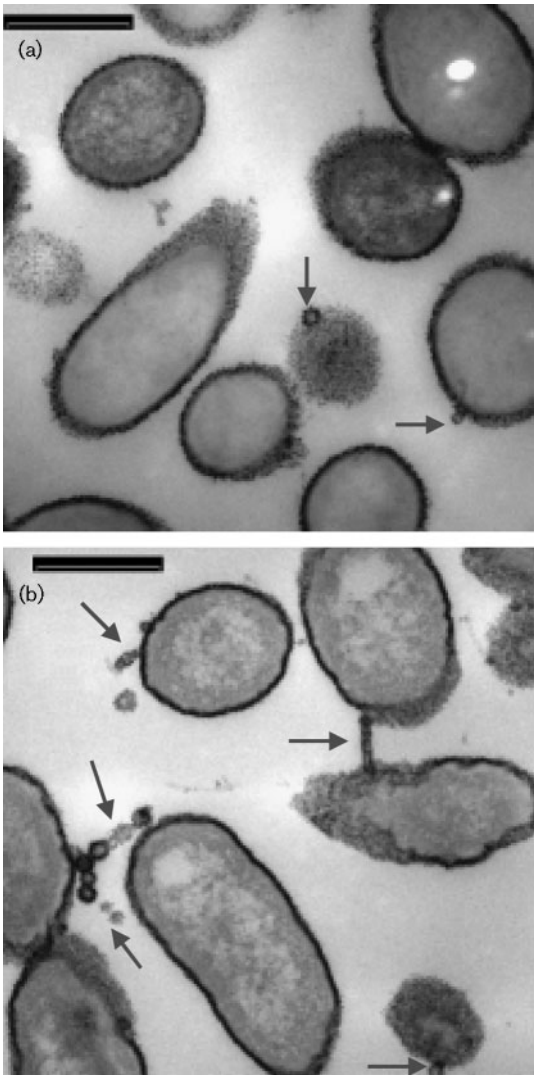

Fig. 3. Transmission electron micrographs of *P. aeruginosa* PAO1 grown under (a) oxygen limited ($pO_2 \sim 0$) and (b) oxidative stress ($pO_2 \sim 350$ % of air saturation) conditions. Arrows indicate the formation of outer-membrane vesicles. Bars, 500 nm.

oxidative conditions, as shown in Fig. 3, and the suggestion that the release of virulence factors is associated with the formation of MVs.

Adhesion of *P. aeruginosa* PAO1 grown in chemostat culture under controlled conditions to glass and the formation of biofilms

Bacterial adhesion to surfaces is one of the initial steps in biofilm formation (Costerton *et al.*, 1987). The adhesion of PAO1 cells from steady states of the chemostat culture at different pO_2 levels is indicated by the optical density values in Fig. 6. The protein concentration of the resuspended biofilm showed a similar trend (data not shown). The amount of adhesion was highly dependent on the dissolved oxygen tension in the chemostat culture. The

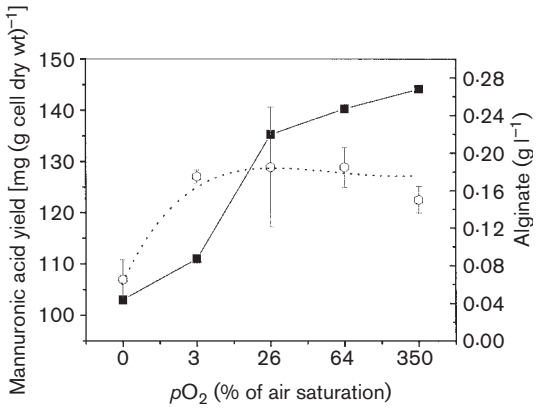

Fig. 4. Concentration of alginate (open hexagons) in cell-free supernatant and mannuronic acid yield (filled squares; concentration of free mannuronic acid) of *P. aeruginosa* PAO1 cultures grown in a chemostat at different pO_2 levels.

biofilm optical density was more than fourfold higher when cells were grown under conditions of oxygen stress compared to that seen when cells were grown under oxygen-limited conditions, indicating a decreased hydrophobicity of the cell surface under conditions of oxygen stress. Makin & Beveridge (1996) studied the relative cell-wall hydrophobicity of *P. aeruginosa* PAO1 strains lacking either A- or B-band LPS. They concluded that the relative hydrophilicity of the cells follows the order: $B^+A^- > A^+B^+ > A^-B^- > A^+B^-$. The increased formation of B-band LPS observed under oxidative conditions may therefore be at least partially responsible for the increase in adhesion indicated by the optical density values (Fig. 6). As shown in Fig. 4, mannuronic acid in the supernatants, which is a major component of B-band LPS, increased by about 1·4-fold as the pO_2 level increased from 0 to 350 %.

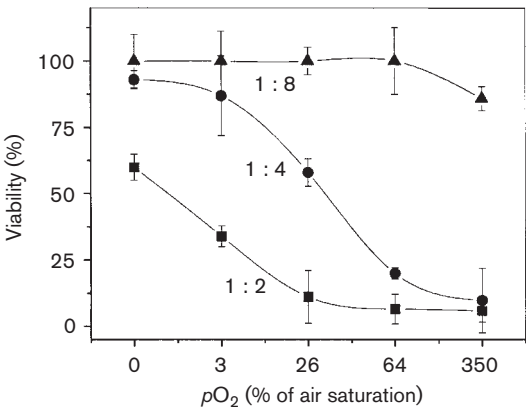

Fig. 5. Cytotoxicity assay with a culture of hybridoma cell line HyGPD YK-1-1 exposed to different dilutions of cell-free supernatants from *P. aeruginosa* PAO1 cultures grown in a chemostat at different pO_2 levels.

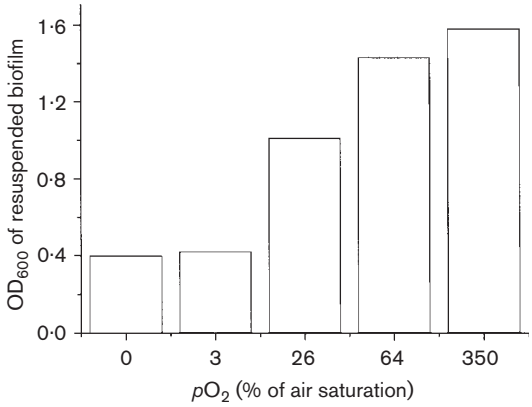

Fig. 6. OD_{600} of resuspended biofilms as affected by the pO_2 value at which the cells were previously grown in chemostat culture (cf. Fig. 1).

The increased formation of MVs, which mainly contain B-band LPS (Fig. 3), may well explain the increased content of mannuronic acid at higher pO_2 levels. On the other hand, alginate, which is often considered to be an important factor for adhesion and biofilm formation in *P. aeruginosa* (Boyd & Chakrabarty, 1995), was found to be less than $0{\cdot}2$ g l^{-1} and remained relatively constant in the chemostat cultures at different pO_2 levels (except for the steady state at $pO_2 = 0$) (Fig. 4). Thus, alginate cannot be responsible for the increased adhesion shown in Fig. 6.

DISCUSSION

The results presented in this work clearly show that the expression levels of the two distinct types of LPS, A-band and B-band, in *P. aeruginosa* PAO1 grown under defined conditions in a chemostat are significantly affected by pO_2 (Fig. 2). The A-band LPS was constitutively expressed as pO_2 increased from nearly 0 to 350 %. In contrast, expression of B-band LPS showed a remarkable increase with increased pO_2. Similar changes in LPS formation in response to changes in oxygen concentration have been shown for *P. syringae* grown in batch culture by Smith *et al.* (1994). These authors concluded that the changes in LPS affected the virulence of this plant pathogen. In this study, we further demonstrated that the enhanced formation of B-band LPS was related to increased formation of MVs on the surface of *P. aeruginosa* PAO1 (Fig. 3). These results extend our knowledge about the formation and release of MVs, which play an important role in the release of virulence factors in *P. aeruginosa* and have been mainly studied in cells exposed to gentamicin (Kaduragamuwa & Beveridge, 1995). MVs formed under oxygen stress have a smaller diameter (24–50 nm) than the MVs (50–150 nm) induced by gentamicin. In addition to the difference in size, oxygen-induced MVs may also differ from gentamicin-induced MVs in their components and toxicity.

We examined the toxicity of supernatants of *P. aeruginosa* cultures for a hybridoma cell line (Fig. 5). The toxicity significantly increased in samples taken from oxygen-stressed steady-state cultures. This correlates with the observed increase in MV formation. It may partially explain the observation that cells of *P. aeruginosa* in biofilms become less virulent (Xu *et al.*, 1998; Hassatt *et al.*, 1999), because in biofilms the environment is often predominantly microaerobic or oxygen-limited. However *P. aeruginosa* can secrete a number of extracellular virulence factors such as exoenzyme S, elastase, protease and phospholipase. Recently, we have shown differences in the release of virulence factors at varying pO_2 levels in pO_2-controlled batch cultures (Sabra *et al.*, 2002 and unpublished data). The release of some of the virulence factors, such as elastase, was found to be strongly enhanced under oxygen-limited conditions. More detailed investigations are needed to understand the influence of varying oxidative conditions on the pathogenicity and the release of different virulence factors in *P. aeruginosa*.

The differences in production of B-band LPS and MVs in *P. aeruginosa* at different oxygen tensions can also significantly affect its adhesion to surfaces and thus biofilm formation, as shown in continuous-flow biofilm culture (Fig. 1). *P. aeruginosa* PAO1 grown at higher pO_2 levels had an increased capacity to adhere to hydrophilic surfaces (Fig. 6). This increased adhesion was not due to increased formation of alginate, but rather oxygen-dependent alterations in cell-surface components and properties (e.g. B-band LPS). Previous studies have shown that clinical isolates of *P. aeruginosa* that initially infect the lungs of CF patients have the non-mucoid phenotype (without alginate) typical of environmental isolates (Tatterson *et al.*, 2001). The initial adhesion of *P. aeruginosa* to the lungs of CF patients must therefore be mediated by other factors. It would be of interest to examine if B-band LPS plays a role in the initial adhesion of *P. aeruginosa* under *in vivo* conditions.

Considerable effort has been devoted to understanding the emergence of *P. aeruginosa* lacking the B-band LPS in chronically infected CF patients. However, the specific molecular mechanisms responsible for this change have not been fully elucidated (Knirel *et al.*, 2001). Although B-band LPS may be necessary for initial attachment to hydrophilic surfaces in chronic infections, where a mature biofilm is normally formed in the lung, the expression of B-band LPS may be reduced due to the prevailing microaerobic environment in the biofilm (Xu *et al.*, 1998). Our work clearly shows that under such conditions the expression of B-band LPS is markedly diminished (Fig. 2). Thus, this study provides a possible explanation for the lack of B-band LPS in clinical isolates of *P. aeruginosa*.

Since the release of many virulence factors is known to be strongly associated with LPS structure (Michel *et al.*, 2000) and MVs (Kadurugamuwa & Beveridge, 1995; Beveridge, 1999), the alterations in the formation of LPS and MVs

caused by changes in oxygen tension deserve more attention in studies of the pathogenicity of this bacterium. The significance of this is emphasized by the recent discovery of a mechanism in *P. aeruginosa* to autogenously reduce oxygen availability in cultures under conditions of oxygen stress (Sabra *et al.*, 2002) and the importance of oxidative stress responses in many host–pathogen interactions (Nathan & Shilon, 2000). Furthermore, MVs have interesting potential medical applications in drug delivery and in development of novel vaccines. A detailed characterization of the induction of MVs by oxygen stress to determine the mechanisms involved in formation and discharge, their composition, function and potential applications is desirable.

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