INFECTION AND IMMUNITY, Feb. 1989, p. 344–350
0019-9567/89/020344-07$02.00/0
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Vol. 57, No. 2

Surface Characteristics of *Pseudomonas aeruginosa* Grown in a Chamber Implant Model in Mice and Rats

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Received 8 August 1988/Accepted 22 October 1988

Pseudomonas aeruginosa **PAO1 was grown in vivo in chambers implanted into the peritoneums of mice and rats. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of extracts of bacterial cells taken from the chambers and washed to remove loosely bound host proteins revealed the presence of the major outer membrane proteins D2, E, F, G, and H2. Western immunoblotting with specific antisera confirmed the presence of porin protein F and lipoprotein H2. However, there was no apparent induction of the phosphate starvation-inducible porin P or the divalent cation starvation-inducible protein H1. Small amounts of proteins with molecular weights similar to those of the iron-regulated outer membrane proteins were found in cells grown in vivo; however, their presence could not be confirmed immunologically. The presence of pili and flagella on the cells grown in vivo was demonstrated by electron microscopy and Western immunoblotting. A consistent alteration in the lipopolysaccharide banding pattern was observed after growth in vivo. Compared with cells of strain PAO1 grown in vitro, cells grown in vivo appeared to lack a series of high-molecular-weight O-antigen-containing lipopolysaccharide bands and gained a new series of lower-molecular-weight lipopolysaccharide bands. This alteration in the lipopolysaccharide after growth in vivo did not affect the O-antigen serotype or the resistance of the bacteria to serum.**

Knowledge of the properties of pathogenic organisms such as *Pseudomonas aeruginosa* when they are growing in the human host is derived largely from observations made on freshly isolated organisms (2, 3, 7, 23) or from extrapolations from experiments on in vitro-grown organisms (17, 26). To recreate the circumstances of infections in the human host, animal models have been developed which simulate human *Pseudomonas* infections, including burn wound sepsis (38), eye infections (4), infections of the neutropenic host (8), and acute (5) and chronic (40) lung infections. While models simulating localized infections, such as those of the eyes and lungs, theoretically allow the recovery of the infecting organism for further studies, models simulating systemic *Pseudomonas* infections which result in the death of the animal do not. Furthermore, only partial recovery of organisms can be obtained from animal models or human infections, leading to the possible conclusion that one is selecting a subpopulation of in vivo-grown bacteria. To examine the properties of in vivo-grown *P. aeruginosa*, we have adopted a simple chamber implant animal model system (10) for the growth of *P. aeruginosa* in the peritoneums of mice (22) and rats (this study). The model involves implanting plastic chambers containing the bacteria into the peritoneums of laboratory animals. The plastic chambers are sealed at both ends with 0.22-μm-pore-size membrane filters, and the entire chamber culture can be removed from the animal for analysis at any time up to 6 months after implantation. The bacteria in the chamber grow logarithmically, after a short lag period, to a density of approximately 10^9 organisms per ml. Subsequently, they maintain this level for many months, and thus demonstrate features of a chronic contained infection (22). We have demonstrated previously that this model supports the growth of isolates of both mucoid and nonmucoid strains of *P. aeruginosa* from patients with cystic fibrosis (22). In the present study we examined the surface characteristics of a standard laboratory strain of *P. aeruginosa* grown in this in vivo model and compared them with those of the same strain grown in vitro. The surface characteristics examined included the outer membrane protein profile, the lipopolysaccharide (LPS) profile, and the presence of surface appendages. The data suggest that while the outer membrane protein profile of in vivo-grown *P. aeruginosa* resembles that of *P. aeruginosa* grown in vitro in rich media, the LPS profile is somewhat different. Flagella and pili were seen on *P. aeruginosa* grown in vivo.

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MATERIALS AND METHODS

Bacterial strains and in vitro growth conditions. The standard laboratory strain of *P. aeruginosa* PAO1 (smooth, nonmucoid) was used throughout this study (13). Other strains of *P. aeruginosa* used in this study were strain AK1012, a rough, LPS-altered derivative of PAO1 (17), which was used to raise antibodies to outer membrane proteins free of contaminating anti-LPS antibodies, and PA103, a Habs serotype 11 sputum isolate from B. Iglewski, University of Rochester (Rochester, N.Y.). Organisms were grown in vitro to the stationary phase in 10 ml of Proteose Peptone No. 2 broth (PP2; Difco Laboratories, Detroit, Mich.) in a 250-ml flask that was shaken at 250 rpm and kept at 37°C. The iron-deficient medium used for the induction of iron-regulated outer membrane proteins was the succinate minimal medium described by Meyer et al. (24), which was subjected to 8-hydroxyquinoline extraction of iron (14).

Laboratory animals. B6.D2/F1 mice (weight, 25 to 35 g) and Wistar rats (weight, 225 to 250 g) were used throughout this study. The animals were bred in our animal facility and maintained under normal laboratory conditions with free access to food and water.

In vivo growth conditions. Chambers for implantation into mice were constructed from 1-ml polypropylene syringe barrels as described previously (10). Chambers for implantation into rats were similarly constructed from 3-ml polypropylene syringe barrels. The *P. aeruginosa* culture used to inoculate the chambers was grown overnight in PP2 and

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diluted in physiological saline, to give a chamber inoculum of approximately 10^4 bacteria per ml. Chambers for implantation into mice received a volume of 100 μl of the diluted culture, while chambers for rats received 500 μl. The animals were anesthesized by intraperitoneal injection of sodium pentobarbitol (Somnotol; M.T.C. Pharmaceuticals, Mississauga, Ontario, Canada) at 0.06 mg/g of body weight for mice and 0.14 mg/g of body weight for rats. Four chambers were implanted into each animal through a small longitudinal incision in the abdomen. The chambers were removed after 2 to 3 days, at which stage the bacterial cultures had reached their maximal density of 10^8 to 10^9 cells per ml (22; N. Kelly, unpublished data for rats). Growth to maximal density was necessary to obtain sufficient numbers of organisms for the experiments described here.

Preparation of whole-cell sonicates. Whole-cell sonicates were prepared from *P. aeruginosa* PAO1 grown in vitro or in mice or rats in vivo. Total cell counts were performed microscopically, and the in vitro-grown culture was diluted in 0.9% sodium chloride in 10 mM sodium HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 6.8; saline buffer) to equal the concentration of the in vivo-grown culture. The cultures were washed 3 times in saline buffer by centrifugation for 3 min in a microfuge or for 5 min at 10,000 rpm in a centrifuge (RC-5B; Ivan Sorvall, Inc., Norwalk, Conn.), to remove host-associated material from the in vivo-grown cultures. Total counts performed before and after the washing procedure revealed minimal bacterial loss during washing. The washed bacterial cells were suspended at a 10-fold higher concentration in 2% sodium dodecyl sulfate (SDS) in sodium HEPES buffer (pH 6.8), frozen and thawed to aid cell breakage, and sonicated 4 times for 30 s each time on ice. The cell sonicate was concentrated twofold by lyophilization in a concentrator (Speed Vac; Savant Instruments, Inc., Hicksville, N.Y.), and the protein concentration was estimated by the method of Sandermann and Strominger (34). Two milliliters of in vivo-grown culture, obtained from 20 chambers implanted into five mice or four chambers implanted into one rat, consistently yielded 200 to 230 μg of bacterial protein.

Preparation of cell envelopes. Bacterial cell envelopes were prepared from 20 ml of *P. aeruginosa* grown in rats and a similar volume of *P. aeruginosa* grown in PP2. The cultures were washed twice in saline buffer and twice in 2% Triton X-100 in sodium HEPES buffer (pH 6.8) and suspended in 4 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 50 μg of DNase per ml. The cells were broken by two passages through a French press at 20,000 lb/in^2. Cellular debris was removed by centrifugation at 3,000 × *g* for 10 min, and the supernatant was centrifuged for 1 h at 160,000 × *g* at 4°C in the presence of 5 mM $MgCl_2$. The cell envelope pellet was suspended in distilled water.

SDS-PAGE. The whole-cell and envelope proteins of *P. aeruginosa* were analysed by SDS-polyacrylamide gel electrophoresis (PAGE), after solubilization at 88°C for 10 min in the presence of 2-mercaptoethanol, as described by Hancock and Carey (13). Outer membrane proteins were identified on the basis of their migration pattern compared with a standard preparation of *P. aeruginosa* PAO1 outer membrane.

The LPS profile of whole-cell lysates prepared by the method of Hitchcock and Brown (19) was examined by electrophoretic separation on a 15% acrylamide gel containing 0.5% SDS by the method of Peterson and McGroarty (31), with the addition of 0.2 M EDTA to the sample buffer to reduce LPS aggregation. The LPS bands were visualized by periodate treatment and silver staining (39).

Western immunoblotting. Proteins were transferred from gels to nitrocellulose paper overnight at 10 mA, with an additional 3-h transfer at 750 mA for the high-molecular-weight iron-regulated outer membrane proteins. Western immunoblotting was performed as described previously (16, 26, 36).

Protein H1-specific antiserum. Polyclonal antibody to purified outer membrane protein H1 was prepared as follows. Protein H1 was purified from outer membranes of strain AK1012, a rough, LPS-altered derivative of strain PAO1 (17). Cells were grown at 30°C in Mg^{2+}-deficient BM2 succinate medium (27), to induce expression of H1, and outer membranes were prepared, without lysozyme treatment, by sucrose gradient centrifugation (13). Outer membranes were extracted once with Triton X-100 and twice with Triton X-100–EDTA, as described previously (15), and the insoluble pellet was then washed twice in deionized water and solubilized at 22°C in 2% SDS–20 mM Tris hydrochloride (pH 8.0). After ultracentrifugation at 15°C, the supernatant (3 mg of protein) was applied to a 1.5-mm-thick preparative SDS–14% polyacrylamide gel as described previously (30). Under these conditions protein H1 runs as a heat-unmodified band with an apparent molecular weight of 18,000 (13). This band was excised and the protein was electroeluted as described previously (30), with 0.1% SDS–25 mM Tris hydrochloride (pH 8.3) used as the electroelution buffer. Samples obtained in this way (about 0.6 mg of protein) appeared pure when analyzed on SDS-polyacrylamide gels. When solubilized at 22°C, bands with apparent molecular weights of 21,000 (heat modified, or H1*) and 18,000 were observed, suggesting that some of the protein was denatured during the procedure. No LPS O antigen was detected in the protein H1 preparations when analyzed on gels that were silver stained for LPS (39). A total of 380 μg of protein H1 was administered to a female New Zealand White rabbit in a series of subcutaneous injections over a period of 11 weeks. The first injection included 50% Freund complete adjuvant and the second included 50% Freund incomplete adjuvant. When the titer against SDS-solubilized outer membranes (16) reached 10^7, as determined by enzyme-linked immunosorbent assay, the rabbit was exsanguinated by cardiac puncture and the serum was extracted and stored at −70°C. Contaminating antibodies directed against outer membrane proteins F and H2 were removed by passing the serum over a protein F-Sepharose affinity column (32) that was kindly provided by K. Poole. The serum fraction that remained unbound to the column was then adsorbed to whole PAO1 cells as described by Poole and Hancock (32). The resulting adsorbed, affinity-purified serum reacted with both 18,000- and 21,000-molecular-weight protein H1 bands on Western immunoblots, when the serum was used at a 1/25 dilution, but it did not recognize any other components of *P. aeruginosa* cell lysates.

Antibodies to other *P. aeruginosa* proteins and rat proteins. Monoclonal antibodies specific for the outer membrane proteins porin F (MA5-8) and lipoprotein H2 (MA1-6) and polyclonal antibody to the monomeric form of the phosphate starvation-induced protein P were prepared as described previously (18, 25, 32).

Polyclonal antibody to the PAO1 pilin protein (which, coincidentally, detected flagellin protein) was a gift from W. E. Paranchych (Department of Biochemistry, University of Alberta, Canada).

Attempts were made to raise antisera to the iron-regulated

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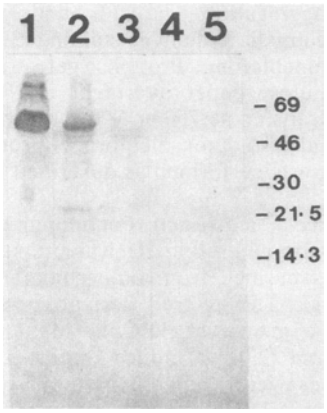

FIG. 1. Western immunoblot of proteins from rat chambers containing PAO1 cells reacted with anti-rat serum. Lane 1, Sonicated rat chamber fluid supernatant without bacteria (110 μg); lane 2, supernatant from the first washing of the chamber-grown cells, without bacteria (100 μg); lane 3, washed sonicated chamber-grown cells (66 μg); lane 4, washed sonicated cells grown in vitro in PP2 (60 μg); lane 5, PAO1 outer membrane (18 μg). The running positions of Rainbow protein molecular weight markers (in thousands; Amersham, Oakville, Ontario, Canada) are indicated to the right of the figure.

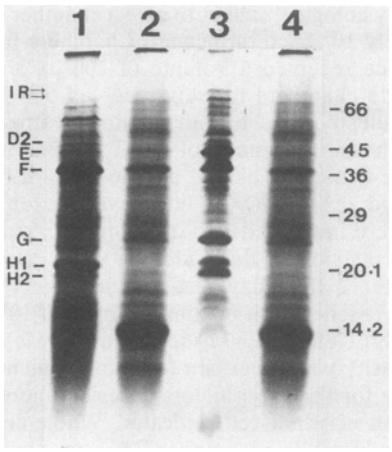

FIG. 2. SDS-polyacrylamide gel electrophoretogram of envelope preparations of PAO1 grown in vitro and in vivo. Lane 1, Cells grown to the stationary phase in vitro (50 μg of protein); lane 2, cells grown for 3 days in vivo in rat chambers (50 μg); lane 3, outer membrane of cells grown under iron-deficient conditions (see text) in vitro (25 μg); lane 4, an overexposure of lane 2 showing putative iron starvation-induced outer membrane proteins (indicate by arrows). The positions of the major outer membrane proteins, including the putative iron-regulated outer membrane proteins (IR) present in lane 3, are indicated on the left-hand side of the figure. The running positions of protein molecular weight markers (in thousands) are indicated on the right.

outer membrane proteins by purifying them from SDS-polyacrylamide gels and immunizing rabbits essentially as described above for protein H1-specific antiserum. Anti-rat whole serum (Sigma Chemical Co., St. Louis, Mo.) was used to detect native rat proteins. Serum samples from patients with cystic fibrosis (CF8, CF9, CF10, and CF22), which were previously shown to react strongly with major outer membrane proteins (enzyme-linked immunosorbent assay titer, 10^5 to 10^8 [16]), were a gift from D. Speert (Children's Hospital, Vancouver, British Columbia, Canada).

Electron microscopy. In vivo-grown bacteria were prepared for electron microscopic visualization of surface appendages by the method of Speert et al. (37), with the exception that uranyl acetate was used in place of phosphotungstic acid. The samples were examined by Nancy Martin by using an electron microscope (STEM; Zeiss) in the transmission mode.

Serum sensitivity testing. *P. aeruginosa* was tested for its sensitivity to human serum as described previously (17).

RESULTS

Preparation of in vivo-grown bacterial cells free of host protein. In this study *P. aeruginosa* cultures were grown in chambers in both mice (22) and rats, and the cell surface characteristics proved to be similar in the two hosts. The use of rats (this study) allowed us to isolate larger numbers of in vivo-grown organisms.

Bacteria grown in chambers implanted into mice and rats were solubilized, and extracts were applied to SDS-polyacrylamide gels. The large amounts of host proteins associated with these unwashed in vivo-grown bacteria obscured the bacterial proteins (Fig. 1, lane 1). Thus, the bacterial cells were washed 3 times in physiological saline made up in sodium HEPES buffer (pH 6.8), a buffering solution which we have found to be least injurious to the bacterial cells. Most of the host proteins were removed from the bacteria in the first wash (Fig. 1, lane 2). The apparent lack of bands corresponding to bacterial cell proteins in the washing supernatants (data not shown) indicated that the washing procedures did not selectively extract any major bacterial cell proteins. After three washes only small amounts of rat proteins were associated with washed cells, as detected by using antisera to rat serum proteins (Fig. 1, lane 3). Comparisons by SDS-PAGE of the washed in vivo-grown cells and their envelopes with a similarly washed preparation of in vitro-grown cells revealed the presence of one major protein species with a molecular weight of 14,000 that was only associated with in vivo-grown cells (Fig. 2, lane 2). This protein was not a major component of peritoneal fluids but reacted, albeit weakly, with antisera to whole rat serum. Thus, we presume that this is a host protein, and based on its mobility and comigration with egg white lysozyme on SDS-polyacrylamide gels, we propose that it is lysozyme. In addition to this band, a 66,000-molecular-weight band was found to be associated with in vivo-grown cells in small to moderate amounts (Fig. 1, lane 3). This protein was probably host albumin based on its prominence in unwashed cells, its reactivity with antisera to whole rat serum, and its apparent molecular weight (Fig. 1, lane 1).

Outer membrane proteins of *P. aeruginosa* grown in vivo. A comparison on SDS-polyacrylamide gels of the protein profile of sonicated washed mouse- and rat-grown *P. aeruginosa* PAO1 cells (data not shown) and of an envelope fraction prepared from rat-grown cells with a standard outer membrane preparation of strain PAO1 allowed the identification of outer membrane proteins D2, E, F, G, and H2 (Fig. 2, lane 2). However, proteins H1 and P were not evident. In addition, strain PAO1 cells contained very small amounts of three high-molecular-weight protein bands which were thought to correlate with protein bands that were present in an outer membrane fraction prepared from PAO1 grown under iron-deficient conditions (Fig. 2, lanes 3 and 4). Because of the apparent low expression of these iron-regulated outer membrane proteins, we considered the possibility that carry-over of iron from the chamber inoculum

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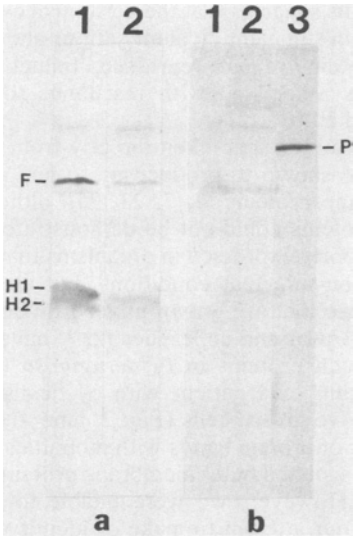

FIG. 3. Western immunoblots of washed cell sonicates of strain PAO1 grown in vitro and in vivo and reacted with antisera to major outer membrane proteins. Lane 1, Cells grown in PP2 in vitro (70 μg); lane 2, cells grown in mouse chambers (70 μg); lane 3, purified protein P monomer (5 μg). The blot in panel a was probed with pooled antibodies raised against outer membrane proteins F (monoclonal antibody MA5-8), H1 (polyclonal serum), and H2 (monoclonal antibody MA1-6). The blot in panel b was probed with antibodies to the monomer form of protein P (polyclonal serum). The proteins were transferred to nitrocellulose after electrophoresis through a 12% polyacrylamide gel.

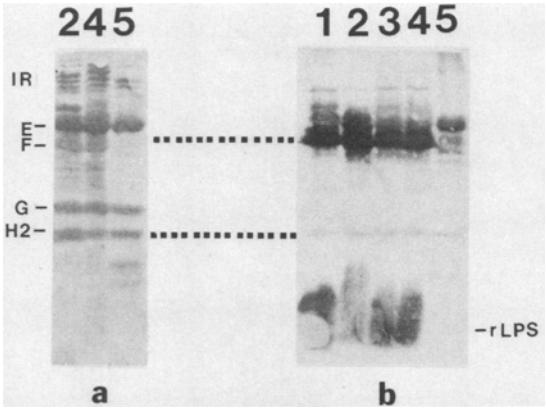

FIG. 4. Outer membranes of *P. aeruginosa* strains probed with serum from patients with cystic fibrosis. Lane 1, PAO1 grown under iron-sufficient conditions; lanes 2, PAO1 grown under iron-deficient conditions; lane 3, PA103 grown under iron-sufficient conditions; lanes 4, PA103 grown under iron-deficient conditions; lanes 5, partially purified outer membrane of the rough strain AK1012 grown under iron-deficient conditions. Each lane contained approximately 20 μg of protein. The blot in panel a was stained with amido black to show the transfer of proteins from the gel. The blot in panel b was reacted with serum sample CF22 from a patient with cystic fibrosis (17). The running positions of the major outer membrane proteins and the iron-regulated outer membrane proteins (IR) are indicated to the left of the figure, and the running position of the LPS rough core-lipid A molecules (rLPS) is indicated to the right of the figure.

was preventing their induction. However, the inoculum contained only 13 nmol of iron per chamber (as measured by atomic adsorption spectrometry). Furthermore, the chambers remained permeable to fibronectin (molecular weight, 220,000) (J. L. Kluftinger, N. M. Kelly, and R. E. W. Hancock, manuscript in preparation) and albumin (Fig. 1, lane 1) after implantation, and thus presumably were permeable to iron and transferrin. Thus, we consider it unlikely that carry-over of iron explained the apparent low expression of iron-regulated outer membrane proteins.

To confirm the presence of both the major (13) and inducible outer membrane proteins, whole-cell sonicates of in vivo- and in vitro-grown strain PAO1 were electrophoretically transferred to nitrocellulose filters for immunoblotting. Using specific polyclonal antisera, we probed for outer membrane proteins H1, which was induced in vitro under conditions of divalent cation depletion (27), and P, which was induced in vitro under conditions of P_i deficiency (32) (Fig. 3). Neither protein was found in detectable levels in the in vivo-grown cells. The presence of the major outer membrane proteins F and H2 in in vivo-grown cells was confirmed by Western immunoblotting by using specific monoclonal antibodies (Fig. 3a).

In a similar manner, we wished to probe for the iron-regulated outer membrane proteins using immunological techniques. Antisera were prepared by using selectively solubilized, gel-excised, iron-regulated outer membrane proteins as antigens. However, the resultant antibody preparations reacted very weakly with only one of the four iron-regulated outer membrane proteins in strains AK1012 and PAO1 grown in vitro under iron-deficient conditions and failed to identify any iron-regulated proteins in the in vivo-grown PAO1 cell sonicate (data not shown). We also tried to use high-titer serum samples from patients with cystic fibrosis as a source of antibodies to the iron-regulated outer membrane proteins (2). However, in our hands serum samples from four patients with cystic fibrosis (CF8, CF9, CF10, and CF22 [16]) failed to react with the iron-regulated outer membrane proteins of *P. aeruginosa* laboratory strains PAO1 or PA103, despite their strong immunoblotting patterns with other major outer membrane proteins (Fig. 4; similar results were obtained for serum samples CF8, CF9, and CF10).

Surface appendages of *P. aeruginosa* grown in vivo. *P. aeruginosa* cells removed from chambers that were implanted in mice and rats displayed good motility when viewed under a light microscope. Western immunoblotting with a polyclonal preparation containing antibodies to both flagellin and pilin proteins demonstrated the presence of both of these protein species in whole-cell sonicates of strain PAO1 grown in mice and rats (data not shown). Electron microscopy of mouse-grown strain PAO1 cells demonstrated that fully assembled flagella and pili were present on the surface of the cells (data not shown).

The LPS profile of *P. aeruginosa* grown in vivo. Whole-cell lysis of mouse-, rat-, and broth-grown PAO1 cells followed by protease digestion (19), SDS-PAGE, and periodate treatment and silver staining allowed visualization of the LPS banding pattern. Compared with the in vitro-grown strain PAO1, in vivo-grown cells appeared to be missing a series of higher-molecular-weight O-antigen repeat units and gained a new series of lower-molecular-weight O-antigen repeat units (Fig. 5). This alteration in pattern was reproducible and occurred for *P. aeruginosa* PAO1 grown in both mice and rats. The effect of this alteration in LPS-banding pattern on the serotype specificity and susceptibility to the bactericidal effects of human serum was investigated. Using a monoclonal antibody, MA1-8, which was specific for the serotype 5 O antigen of *P. aeruginosa* PAO1 (18), in a Western immunoblot assay, we found that the O-antigen serotype specificity

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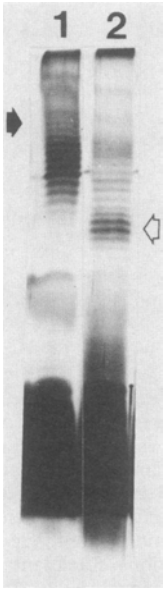

FIG. 5. SDS-polyacrylamide (15%) gel electrophoretogram of lysates of PAO1 cells grown in vitro and in vivo stained for LPS. Lane 1, Cells grown in vitro in PP2; lane 2, cells grown in mouse chambers. Arrows indicate the bands that were present in strain PAO1 grown in vitro but that were missing from strain PAO1 grown in vivo (closed arrow), and vice versa (open arrow).

of strain PAO1 was retained when the organism was grown in vivo. Using two different systems to assay for serum sensitivity, we determined that strain PAO1 grown in mice retains its resistance to the bactericidal effects of normal human serum. Both in vitro- and in vivo-grown PAO1 exhibited >100% survival after a 1-h exposure to 50% serum, and both grew in 40% serum by 18 h.

DISCUSSION

In this study we attempted to assess the influence of growth in vivo on the surface characteristics of *P. aeruginosa* PAO1. The choice of this strain was predicated by the substantial body of knowledge about its physiology, genetics, and outer membrane characteristics in vitro (21, 28, 29). In addition, we reported on the adaption of an in vivo growth model, the peritoneal chamber implant model, for the full recovery of in vivo-grown organisms substantially free of host proteins. It should be mentioned that host cells are excluded from the bacterial chambers. We perceived this as an advantage, in that it decreased potential complications caused by bacterial-host cell interactions, including selective removal of bacterial cells by adherence and phagocytosis. Thus, this model, while not precisely mimicking infection, can be used to demonstrate bacterial cell properties while cells are growing on available nutrients in vivo.

Using this model, we found that some of the major outer membrane proteins previously identified under in vitro growth conditions, including proteins D2, E, F, G, and H2, were also expressed when the organism was grown in laboratory animals. This confirms in part our immunofluorescent identification of proteins F and H2 in lung sections from autopsy samples of patients with cystic fibrosis (36). The absence of detectable levels of outer membrane proteins H1 (induced under conditions of low divalent cations) and P (induced under conditions of low phosphate) in the in vivo-grown cells suggests that the peritoneal cavities of mice and rats contain sufficient divalent cations and phosphate so that these proteins remain repressed. Induction of protein H1 in vitro is associated with resistance to polycationic antibiotics and EDTA (27).

A variety of organisms taken directly from human infection sites were shown to produce up to five iron-regulated outer membrane proteins (2, 7, 23, 35), although in some cases such proteins could not be demonstrated (23). These proteins are poorly expressed in organisms that are grown in vitro under iron-sufficient conditions, but are strongly expressed in those that are grown under iron-deficient conditions (6, 14). Anwar and colleagues (2, 7) have reported the presence of such proteins in *P. aeruginosa* taken directly from the sputum of a patient with cystic fibrosis. In our studies of in vivo-grown cells (Fig. 2, lane 4), we observed small amounts of protein bands with mobilities equivalent to those of iron-regulated outer membrane proteins of *P. aeruginosa* PAO1. However, we were unable to confirm their identity since our attempt to make or identify an antiserum specific for the iron-regulated proteins of strain PAO1 was unsuccessful.

The in vivo-grown *P. aeruginosa* PAO1 expressed both pilin and flagellin proteins and assembled both proteins to give rise to pili and flagella. The flagellin protein has been previously noted as being immunogenic, eliciting an antibody response in patients with cystic fibrosis and burns (2, 3) and in rabbits hyperimmunized with outer membrane preparations of *P. aeruginosa* (26). The experiments reported by Holder et al. (20) suggest that flagellin protein is a good vaccine candidate or, alternatively, that anti-flagellar antiserum could be an immunotherapeutic agent for the elimination of *P. aeruginosa* infections of a systemic nature.

The major difference noted for *P. aeruginosa* PAO1 grown in vivo when compared with its in vitro-grown counterpart related to the length of the LPS O-antigen chain (Fig. 5). The LPS of gram-negative bacteria, when separated on an SDS-PAGE system, forms a ladderlike pattern in which each band represents the rough core of LPS substituted with a different number of O-antigen repeat units (12). This heterogeneity in the molecular size of the LPS is seen after the in vitro growth of *P. aeruginosa* strains, including strain PAO1, for which approximately 90% of the LPS molecules contain no O-antigen side chain (rough LPS), while the other 10% of LPS molecules contain various multiples of the O-antigen trisaccharide unit attached to the rough core-lipid A portion of LPS (smooth LPS) (33). Using SDS-PAGE for the separation of the LPS molecules according to size, we found that the in vivo-grown PAO1 was missing a series of high-molecular-weight (longer chain length) smooth LPS molecules and gained a series of lower-molecular-weight (shorter chain length) rough LPS molecules compared with the same strain grown in vitro (Fig. 5). Functions which have been attributed to the LPS of *P. aeruginosa* include roles in the resistance and susceptibility of cells to antibiotics, human complement, and phagocytosis and an involvement in virulence (1, 9, 11, 16, 29). In addition, the LPS O antigen is one of the major immunogenic determinants on the bacterial cell surface and forms the basis for strain identification by serotyping (9, 17, 26). When checked in an immunoblot assay with monoclonal antibody specific for the serotype 5 O antigen of *P. aeruginosa* PAO1 (17), the in vivo-grown cells were found to express the same serotype as that expressed in vitro. Moreover, we found that the alteration in the size of the LPS molecules expressed on the surface of strain PAO1

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when grown in vivo did not change its resistance to the bactericidal effects of normal human serum.

In conclusion, we found substantial similarities between the cell surface characteristics of *P. aeruginosa* PAO1 grown in vitro on laboratory media and in vivo on available nutrients supplied by the host. Nevertheless, a major difference was observed in the distribution of smooth LPS species. We have since found that in vivo-grown bacteria demonstrate enhanced uptake of compounds via the hydrophobic pathway, are resistant to killing by tobramycin during the mid-log phase of growth (N. M. Kelly, E. Rawling, and R. E. W. Hancock, manuscript in preparation), and demonstrate altered susceptibility to nonopsonic phagocytosis (22). The relationship between these functional alterations and the structural alterations observed here is being investigated.

ACKNOWLEDGMENTS

We acknowledge the financial assistance of the Medical Research Council of Canada. N.M.K. was a recipient of a fellowship from the Canadian Cystic Fibrosis Foundation. A.B. was a recipient of an overseas studentship from the Science and Engineering Research Council (United Kingdom).

We thank Nancy Martin for doing the electron microscopy, Gordon Crockford for attempting to raise antibodies to iron-regulated outer membrane proteins, David Speert for providing sera from patients with cystic fibrosis, Bill Paranchych for providing pilin and anti-pilin antisera, and Eileen Rawling for testing the susceptibility of our strains to serum.

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