**Klebsiella pneumoniae** MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces

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The type 3 fimbriae of *Klebsiella pneumoniae* are comprised of the major fimbrial subunit (MrkA) and the adhesin (MrkD) that has previously been shown to mediate binding to collagen. The ability of adhesive and non-adhesive derivatives of *K. pneumoniae* to form biofilms on collagen-coated surfaces in continuous-flow chambers was investigated. Unlike biofilm formation on abiotic plastic surfaces, the presence of the MrkD adhesin was necessary for growth on collagen-coated surfaces. Fimbriate strains lacking the MrkD adhesin did not efficiently adhere to and grow on these surfaces. Similarly, purified human extracellular matrix and the extracellular matrix formed by human bronchial epithelial cells grown *in vitro* provided a suitable substrate for MrkD-mediated biofilm formation, whereas direct binding to the respiratory cells was not observed. Type 3 fimbriae may therefore have two roles in the early stages of adherence and growth on in-dwelling devices such as endotracheal tubes. The MrkA polypeptide could facilitate adsorption to abiotic polymers of recently implanted devices and the MrkD adhesin could enable bacteria to adhere to and grow on polymers coated with host-derived proteins.

**INTRODUCTION**

The role of bacterial fimbriae in the production and formation of biofilms has been described for several species (Costerton *et al.*, 1987, 1999; Watnick & Kolter, 2000). Type 1 fimbriae and the associated FimH adhesin of *Escherichia coli* have been shown to influence biofilm formation *in vitro* on plastic surfaces in static batch culture conditions and in a hydrodynamic environment, respectively (Pratt & Kolter, 1998; Schembri & Klemm, 2001). Similarly, the type IV pili of *Pseudomonas aeruginosa* have been demonstrated to influence biofilm formation by these bacteria (O’Toole & Kolter, 1998). Also, the pili produced by *Vibrio cholerae* have been shown to play a role in biofilm production (Watnick & Kolter, 1999). The development of biofilms by pathogenic bacteria is believed to play an important role in facilitating evasion of host defence mechanisms, communication between bacterial cells and protection against antibiotic action. Recent data indicate that the expression of some genes in sessile, biofilm-producing bacteria may be different to that of planktonic cells (Whitely *et al.*, 2001). Consequently, the production of bacterial biofilms appears to be dependent on multiple genetic factors. Therefore, surface components of the bacterial cell that increase the efficiency of biofilm formation are likely to play a significant role in the establishment of infection by pathogens.

We have recently demonstrated that the type 3 fimbriae of *Klebsiella pneumoniae* influence the development of biofilms in plastic, continuous flow-through chambers (Langstraat *et al.*, 2001). The type 3 fimbriae are expressed by several species of opportunistic pathogens and are characterized by their ability to mediate agglutination, *in vitro*, of treated erythrocytes (Clegg *et al.*, 1994; Old & Adegbola, 1985; Old *et al.*, 1985). The MrkA polypeptide comprises the major structural component of type 3 fimbriae and is polymerized to form the fimbrial shaft. The MrkD protein is believed to function as the type 3 fimbrial adhesin and mediates binding to extracellular matrix (ECM) proteins such as collagen molecules (Schurtz *et al.*, 1994; Schurtz Sebghati *et al.*, 1998; Tarkkanen *et al.*, 1990, 1992). The efficient development of biofilms by *K. pneumoniae* on plastic surfaces was independent of the presence of the MrkD adhesin, but facilitated by the presence of the fimbrial shaft on the bacterial surface. The collagen-binding MrkD molecule appears to play little role in the development of biofilms on these plastic surfaces, whereas the MrkA shaft protein is an important contributing factor. Therefore, bacterial binding leading to colonization and biofilm formation on plastic devices such as endotracheal tubes may be more efficient by type 3 fimbriate bacteria. However, in-dwelling devices such as catheters and tubes are coated over time, *in situ*, with host-derived material (Donlan, 2001; Francois *et al.*, 1998). Consequently, specific receptor–ligand binding, such as the MrkD–collagen
interaction, could also play a role in biofilm development during infection. Also, MrkD-mediated binding to extra-cellular matrices is likely to have a role in colonization of damaged epithelial surfaces.

During the course of our studies investigating the role of type 3 fimbriae in biofilm development on abiotic plastic surfaces we observed that fimbriate bacteria were limited in their ability to form biofilms on glass surfaces. Consequently, coverslips coated with ECM or collagen should provide a good surface to investigate the role of MrkA and MrkD proteins of the type 3 fimbriae on biofilm formation in continuous-flow chambers coated with these substances.

**METHODS**

**Bacterial strains and plasmids.** *K. pneumoniae* IA565 is a clinical isolate expressing type 3 fimbriae (Fim+) that mediate adherence (Adh+) to collagen and treated erythrocytes as described previously (Hornick et al., 1995). *K. pneumoniae* IApc35 is a mrkD mutant of strain IA565 that is phenotypically fimbriate (Fim+), but lacks adhesive ability (Adh+) and its construction and characterization has been reported elsewhere (Schurtz Sebghati et al., 1998; Tarkkanen et al., 1997). The non-fimbriate (Fim-), non-adhesive (Adh-) strain, IAAT3, is a mrkD mutant of *K. pneumoniae* IApc35 and its construction has been described previously (Tarkkanen et al., 1997). All strains were grown on GCAA medium for optimal expression of type 3 fimbriae and cultures were incubated at 37 °C for 18-24 h. *K. pneumoniae* IAAT3(pFK12) possesses the plasmid carrying the complete mrk gene cluster and is restored in type 3 fimbrial expression and adhesiveness (Allen et al., 1991; Gerlach & Clegg, 1988; Langstraat et al., 2001). To monitor biofilm development, bacteria expressing green fluorescent protein (GFP) were used and these strains have been described elsewhere (Langstraat et al., 2001).

**Detection of type 3 fimbriae.** The ability of bacteria to produce type 3 fimbriae was determined using monospecific antisera against purified fimbriae as reported previously (Schurtz et al., 1994). Haemagglutinating activity of *K. pneumoniae* strains was determined using tanned erythrocytes according to the method of Old et al. (1985). Bacterial binding to collagen was performed using the ELISA described in detail previously (Schurtz Sebghati et al., 1998). Purified human placenta collagen (types V and IV) and ECM were purchased from BD Biosciences and coating concentrations were determined by standard techniques (Korhonen et al., 1997; Kukkonen et al., 1993; Tarkkanen et al., 1997).

**Biofilm formation on uncoated glass surfaces.** The once flow-through continuous culture system, developed by Parsek & Greenberg (1999), was used to assay biofilm development on glass slides that are affixed to the top of the flow cell. Biofilm development on the glass slides incubated at 37 °C using GCAA broth (diluted 1:50) as the medium was examined at 6, 24, 48 and 72 h post-inoculation. Following incubation the glass coverslips were gently removed from the flow cell, ensuring that the correct orientation of the coverslip was maintained, and the coverslips were placed on top of a clean, sterile flow-through chamber. In this way fluorescence from bacteria growing on the plastic surfaces of the biofilm chamber could be eliminated. Subsequently, imaging of the biofilm was performed using a Bio-Rad MRC600 confocal microscope and images are presented as composite sections through the x-y planes of these sections as described in detail elsewhere (Langstraat et al., 2001; Parsek & Greenberg, 1999). The complete area of the slides exposed to the biofilm chamber was examined by confocal microscopy and representative sections were analysed to calculate the depth of biofilm formed.

**Biofilm formation on collagen- and cellular-coated coverslips.** To examine biofilm formation on treated slides the following modifications to the procedure described above were performed. Collagen-coated glass or plastic coverslips were prepared as described previously using optimal coating concentrations of collagen solution or human ECM (Schurtz et al., 1994). Collagen coating (0.005 mg ml⁻¹) was performed overnight at 4 °C in carbonate buffer by incubating the flow cell filled with coating solution, and before inoculation the cell was flushed with PBS to remove excess unbound collagen. When using human ECM the coating concentration was 0.109 mg ml⁻¹.

In a separate series of experiments, semiconfluent monolayers of a human bronchial epithelial (HBE) cell line were also used to coat the coverslips. Chambers were inoculated with 2 x 10⁵ cells and incubated under 5 % CO₂ for 24 h at 37 °C in Dulbecco's Modified Eagle Medium supplemented with 10 % fetal bovine serum and 25 μg chloramphenicol ml⁻¹. Under these conditions the coverslips are coated with a semiconfluent monolayer of HBE cells. Following bacterial inoculation the flow rate of the chamber was set at 130 μl min⁻¹ and the tissue culture fluid plus 10 % fetal bovine serum was used as culture medium that was supplemented with appropriate antibiotics. For the biofilm assays the HBE cells were stained with 1 μM cell tracker orange (Molecular Probes) according to the manufacturer’s instructions.

Finally, to examine the ability of *K. pneumoniae* strains to form biofilms on the ECM produced in vitro by respiratory cells, the HBE cells were grown as a confluent monolayer on the slides of the biofilm chamber. Inoculation of the chamber with the HBE cells was as described above and the cells were grown for 48 h. Under these conditions the HBE cells were observed to form a continuous and confluent monolayer when examined by microscopy. Subsequently, the cells were removed from the slides by treatment in situ with 25 mM EGTA as described in detail earlier (Hornick et al., 1995). This treatment results in effective removal of the HBE cells without damaging the matrix laid down by these cells. The ability of GFP-labelled bacteria to form biofilms on this matrix was determined as described above.

The imaging of the biofilms on coated coverslips was performed as described above for untreated surfaces. The biofilm development was monitored at 6, 24 and 48 h post-inoculation for the ECM- and collagen-coated slides, and 24 h for the HBE-coated slides.

**Bacterial adherence to HBE cells grown in vitro.** Examination of the binding of *K. pneumoniae* strains to HBE cells in vitro was performed as described previously (Hornick et al., 1995).

**RESULTS**

**Biofilm formation on glass coverslips.** Previously we have demonstrated that the MrkD adhesin of the type 3 fimbriae was not required for efficient biofilm formation on abiotic plastic surfaces (Langstraat et al., 2001). However, examination of glass slides used in the flow cell indicated very little bacterial growth. Consequently, *K. pneumoniae* IA565 (Fim+ Adh+) and its derivative strains, IApc35 (Fim+ Adh-) and IAAT3 (Fim- Adh-), were further analysed for their ability to form biofilms on glass surfaces. For all three strains no bacterial growth on the glass of the flow cell was observed even after 72 h...
incubation. This is in contrast to the extensive biofilm formed on the plastic surfaces, previously described by our group (Langstraat et al., 2001), by the fimbriate strains after 24 h incubation.

**Bacterial growth on human ECM- and collagen-coated surfaces**

Since we have previously demonstrated that the MrkD adhesin mediates binding in vitro to extracellular matrices and collagen (Langstraat et al., 2001; Schurtz Sebghati et al., 1998), we investigated the ability of *K. pneumoniae* strains to grow on human ECM-coated surfaces in a continuous culture system. Optimal coating concentrations of matrix and collagen on the coverslips have been determined previously (Schurtz et al., 1994; Tarkkanen et al., 1990) and these conditions were used to coat the coverslips of the flow-through chambers. Careful removal of the coverslips from the chambers enabled us to examine growth of the GFP-producing bacteria on these surfaces in the absence of fluorescence due to growth on any uncoated plastic of the chambers. After 6 h incubation patchy areas of growth of *K. pneumoniae* IA565 were observed on the ECM-coated slides and the maximum depth of the biofilm formed in these regions was approximately 12.5 μm (Fig. 1). No significant growth of *K. pneumoniae* IApc35 or IAΔT3 was observed on the slides after 6 h incubation.

**Fig. 1.** Production of a biofilm on the surfaces of human ECM-coated coverslips. Scanning confocal laser microscopy composite analysis (x–y plane) of biofilm images formed by GFP-producing bacteria after 6, 24 and 48 h incubation. The maximum depth (33 μm) of biofilm was exhibited by strain IA565 after 6–24 h incubation. The maximum depth of patchy biofilm growth formed by strain IApc35 (middle row) was approximately 8 μm.
Following incubation for 24 h the fimbriate and adhesive strain, IA565, formed an extensive biofilm covering most of the coverslip surface (Fig. 1) and the maximum biofilm depth was determined to be 33 μm, almost three times greater than that observed after 6 h incubation. After 24 h incubation *K. pneumoniae* IApc35 (Fim⁺ Adh⁻) demonstrated no growth on the collagen as determined by the lack of fluorescence associated with the treated coverslips (Fig. 1). The non-fimbriate strain, IAΔF3, did not grow on the collagen-treated surfaces after 24 h incubation.

At 48 h post-inoculation of the chambers, strain IA565 continued to exhibit dense growth on the collagen-coated surfaces, although the maximum depth of the biofilm was approximately 25·5 μm which is less than that observed at 24 h. However, even after 3 days incubation the fimbriate but non-adhesive strain, IApc35, still exhibited only limited growth and a similar reduction in the depth of biofilm was observed. The non-fimbriate and non-adhesive strain was not observed to grow on the collagen-coated slides under any conditions of incubation.

Fig. 2 demonstrates the time-course of biofilm development by *K. pneumoniae* IA565 on human ECM. Initial bacterial growth was limited during the first 16 h of incubation. Subsequently, extensive growth occurred over the following 16 h with the formation of characteristic pillars of bacterial growth. A real-time video of GFP-labelled *K. pneumoniae* IA565 growing over a 32 h time period on human ECM is available as supplementary data with the online version of this paper at http://mic.sgmjournals.org.

Similar results were observed using coverslips coated with collagen (Table 1). Only *K. pneumoniae* IA565 developed extensive growth over 48 h incubation. Both *K. pneumoniae* IApc35 and IAΔF3 exhibited little or no growth over the complete area of the coverslips during the incubation period.

### Growth of *K. pneumoniae* IAΔF3(pFK12) on coated surfaces

*K. pneumoniae* IAΔF3 is a *mrkB* mutant of strain IA565 and has been shown by our group to be a constitutively non-fimbriate strain (Tarkkanen et al., 1997). The plasmid pFK12 contains the complete *mrk* gene cluster of strain IA565 and transformants of the *mrkB* mutant are fully restored to type 3 fimbrial expression (Tarkkanen et al., 1997). Fig. 3 shows the ability of *K. pneumoniae* IAΔF3(pFK12) to grow as a biofilm on chambers coated with the ECM. Unlike the untransformed strain, type 3...
fimbriate transformants efficiently colonized and subsequently grew on the surfaces coated with the ECM. Similarly, the pFK12 transformants were able to form biofilms over a 24 h period on chambers coated with collagen. However, the strongly fimbriate transformants did not grow on untreated glass surfaces in the biofilm chambers. The ability of the *K. pneumoniae* strains to grow in the presence of airway epithelial cells was determined. HBE cells were grown to semiconfluency to detect biofilm formation with bacteria, the following results were obtained. Both *K. pneumoniae* IApc35 (Fim− Adh−) and IAΔT3 (Fim− Adh−) failed to grow over a 24 h incubation period with no observable fluorescence on any region of the coverslips. In contrast, *K. pneumoniae* IA565 formed an extensive biofilm after 24 h incubation at 37°C (Fig. 5). These images are representative of biofilm formation on the complete matrix-covered slides. These experiments were repeated three times to ensure that the images generated are consistent with bacterial growth at different locations on the slides. The red fluorescence represents single HBE cells that were occasionally observed to remain attached to the slides after treatment with EGTA. Transformation of *K. pneumoniae* IApc35 with pFK12 to restore adhesiveness also resulted in the ability of transformants to grow on the HBE-derived matrix.

**DISCUSSION**

Many of the fimbrial types synthesized using the chaperone-usher pathway of assembly are comprised of at least two structural protein components that play a direct role in determining the architecture and receptor-binding specificity of the fimbriae (Hultgren & Normark, 1991; Hultgren et al., 1991). The MrkA polypeptide of the *K. pneumoniae* type 3 fimbriae is the major subunit, whereas the MrkD adhesin mediates binding, *in vitro*, to collagen (Allen et al., 1991; Clegg et al., 1994). Fimbriae from a variety of bacterial species have been shown by numerous groups to facilitate biofilm formation *in vitro* using several different detection

**Table 1. Adherence and biofilm-forming properties of K. pneumoniae and its derivatives on collagen-coated slides**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant phenotype</th>
<th>Biofilm on collagen-coated surfaces</th>
<th>Biofilm in intercellular spaces of HBE cells</th>
</tr>
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<tbody>
<tr>
<td>6 h incubation</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IA565</td>
<td>MrkAD</td>
<td>+ (19)</td>
<td>+</td>
</tr>
<tr>
<td>IApc35</td>
<td>MrkA</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IAΔT3</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>24 h incubation</td>
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<tr>
<td>IA565</td>
<td>MrkAD</td>
<td>+ + (45)</td>
<td>+ +</td>
</tr>
<tr>
<td>IApc35</td>
<td>MrkA</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IAΔT3</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>48 h incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA565</td>
<td>MrkAD</td>
<td>+ + (36)</td>
<td>+ +</td>
</tr>
<tr>
<td>IApc35</td>
<td>MrkA</td>
<td>−</td>
<td>−</td>
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<tr>
<td>IAΔT3</td>
<td>−</td>
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+++; Bacterial growth over the whole surface of the coverslip; +, patchy areas of bacterial growth throughout the coverslip; −, no bacteria or only isolated single bacteria observed. Numbers in parentheses indicate the mean depth (μm) of the biofilm.

After 24 h incubation *K. pneumoniae* IA565 was observed to form patchy areas of biofilm in the regions where the HBE cells were clustered. The bacteria were localized to regions adjacent to HBE cells at sites where these cells are most likely to deposit the highest concentrations of an ECM. Strain IApc35 (Fim− Adh−) did not form areas of biofilm growth and were occasionally observed as single bacteria among the HBE cells and strain IAΔT3 (Fim− Adh−) did not grow on the coverslips even though viable clusters of HBE cells were present. Fig. 4 shows the characteristic growth of *K. pneumoniae* IA565 adjacent to the cultured HBE cells. As reported previously by our group (Hornick *et al.*, 1995), conventional binding assays in which the bacteria are incubated with HBE cells for 60 min indicated no extensive localization of large numbers of bacteria to the margins of the epithelial cells.

**Biofilm formation on HBE-derived matrices in the absence of cells**

We have previously described the inability of fimbriate strains of *K. pneumoniae* to adhere *in vitro* to confluent monolayers of HBE cells (Hornick *et al.*, 1995). Consistent with this observation is the lack of biofilm formation on HBE-coated coverslips in the chambers by any of the *Klebsiella* strains (data not shown). However, if the HBE cells were removed from the coverslips prior to infection with bacteria, the following results were obtained. Both *K. pneumoniae* IApc35 (Fim+ Adh−) and IAΔT3 (Fim− Adh−) failed to grow over a 24 h incubation period with no observable fluorescence on any region of the coverslips. In contrast, *K. pneumoniae* IA565 formed an extensive biofilm after 24 h incubation at 37°C (Fig. 5). These images are representative of biofilm formation over the complete matrix-covered slides. These experiments were repeated three times to ensure that the images generated are consistent with bacterial growth at different locations on the slides. The red fluorescence represents single HBE cells that were occasionally observed to remain attached to the slides after treatment with EGTA. Transformation of *K. pneumoniae* IApc35 with pFK12 to restore adhesiveness also resulted in the ability of transformants to grow on the HBE-derived matrix.
systems (O’Toole & Kolter, 1998; Parsek & Greenberg, 1999; Schembri & Klemm, 2001). For *E. coli* type 1 fimbriae it has been demonstrated that the FimH adhesin plays a role in biofilm formation by fimbriate bacteria on abiotic surfaces (Schembri & Klemm, 2001; Watnick & Kolter, 2000). Using the type 3 fimbrial system of *K. pneumoniae*, we recently reported that the MrkA subunit facilitated biofilm formation on untreated plastic surfaces of flow-through chambers that enable biofilm morphology to be examined (Langstraat et al., 2001). However, the MrkD adhesin did not appear to play a role in this assay system as the fimbriate but non-adhesive strain, *K. pneumoniae* IApc35, could establish a biofilm on abiotic plastic surfaces as rapidly as the wild-type strain. Therefore, in the once flow-through chambers using *K. pneumoniae*, the adhesin molecule of the type 3 fimbriae was not necessary for efficient biofilm formation on untreated surfaces. During these investigations we observed that fimbriate bacteria did not adhere to glass coverslips if these coverslips were used to cover the plastic biofilm chambers. Consequently, we have used treated coverslips in the flow-through chambers to investigate whether the MrkD/collagen interaction plays any role in facilitating biofilm formation *in vitro*.

Coating of the coverslip surfaces with human ECM or purified collagen allows for the efficient formation of biofilms by fimbriate bacteria bearing the MrkD adhesin. Over a prolonged period of incubation (48 h or longer) we observed that the depth of the biofilm mass did decrease. This may be due to removal of distal layers of bacteria by the constant flushing of medium through the chambers once a maximum depth is formed after approximately 24 h. Also, we found that it was necessary to remove the coverslips from the chambers to view biofilm formation since, as we previously reported, GFP-producing fimbriate bacteria grow and efficiently adhere to the poorly coated plastic surfaces of the chamber and interfere with our observations of the collagen-coated surface. The coverslips were removed to ensure minimal disturbance of the biofilm that was present, but biofilms formed after 48 h incubation may be more fragile than those of earlier time points. It is clear, however, that the fimbriate strain lacking the MrkD adhesin did not form biofilms on the collagen as efficiently as the wild-type strain.

It is known that *K. pneumoniae* type 3 fimbriae do not mediate adherence to confluent monolayers of HBE cells (Hornick et al., 1992, 1995). In addition, we have demonstrated that these fimbriae facilitate binding to the basement membrane of human lung tissue sections (Hornick et al., 1992). Consequently, we decided to investigate whether the MrkD adhesin would enable *K. pneumoniae* biofilms to be formed on the ECM produced by HBE cells grown *in vitro* on the coverslips of the chambers. In one series of experiments we ensured that the HBE cells were not grown to confluency so that extracellular material produced by the cells would be exposed on the glass surfaces. The location of the biofilm produced by *K. pneumoniae* IA565 under these conditions is consistent with the interaction of the fimbrial adhesin with the ECM. Large areas of bacterial growth were associated with clusters of HBE cells where the ECM is most likely to be exposed to the bacteria. As previously reported, adherence assays using HBE cells grown in a similar way indicated poor direct binding to the HBE cells. Consequently, we propose that the large areas of bacterial growth around the cells in the chambers is due to MrkD-mediated binding to the ECM by small numbers of fimbriate bacteria. Subsequently, these bacteria grow and establish biofilm formation in these regions. The non-adhesive strains were not able to grow on the slides under these conditions. Although we did not directly demonstrate that the MrkD protein interacts with any specific type of collagen in the ECM of the HBE cells, it is known that collagen is an integral

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**Fig. 3.** Growth of *K. pneumoniae* IAΔT3 (a) and IAΔT3(pFK12) (b) on human ECM-coated surfaces after 24 h incubation. Scanning confocal laser microscopy composite analysis of biofilm image of an x–y series through a depth of 32 μm.
component of the matrix synthesized by cells in vitro (Fenwick et al., 2001; Hastie et al., 2002; Yurchenko & O’Rear, 1994). However, the composition of the ECM produced by the HBE cells has not been investigated in detail. The ability of the MrkD adhesin to facilitate adherence and subsequent growth on ECms was confirmed using commercially available, purified human ECM. Extensive and complete biofilm formation on this material was only observed using the strain expressing the MrkD molecule.

As indicated above, we believe that the MrkD adhesin does not mediate direct bacterial binding to the HBE cell

**Fig. 4.** Scanning confocal laser microscopy of the growth of *K. pneumoniae* IA565 (a) and IApc35 (b) growing on and near HBE cells after 24 h incubation at 37 °C. The HBE cells are labelled with cell-tracker orange. Large areas of the GFP-producing and fimbriate bacteria (IA565) are located in close proximity to the HBE cells and exhibit a yellow fluorescence. The non-fimbriate strain (IApc35) does not adhere or grow around the HBE cells. Viable, uninfected HBE cells (c) are stained red by the cell-tracker dye surrounded by a pale green fluorescent substrate.

**Fig. 5.** Biofilm formation by MrkD-positive or -negative bacteria on an HBE-derived matrix following removal of HBE cells by EGTA. (a) The confluent monolayer of HBE cells are efficiently removed by EGTA treatment leaving single isolated cells (red) on the slide. (b) The fimbriate but non-adhesive strain, *K. pneumoniae* IApc35, does not grow following 24 h incubation. (c) Extensive biofilm formation by *K. pneumoniae* IA565 on the matrix after 24 h at 37 °C.
membranes in the chambers. However, to confirm that the MrkD adhesin binds to the HBE-generated matrix it was possible to remove a confluent layer of HBE cells from the coverslips to expose any material laid down by the HBE cells. In this case extensive biofilm formation was only observed with MrkD-positive bacteria. The ability of MrkD-possessing fimbriae to grow on the underlying matrix of epithelial cells cultured in vitro strongly suggests that disruption of an intact epithelial surface is necessary for type 3 fimbriate bacteria to grow on host surfaces. This is consistent with the clinical observations associated with nosocomially acquired infections due to K. pneumoniae (Craven et al., 1990; Duma, 1985; Riser & Noone, 1981).

The use of the once flow-through chambers coated with matrix proteins or as a support for culturing cells from a relevant host provides an excellent model system to investigate the interaction of K. pneumoniae with human cells and tissues in a dynamic environment. In this respect it has advantages over static and closed systems, but does differ from the natural site of infection by presenting the bacteria to host material in a fluid environment rather than at an air/mucosal surface interface located in the respiratory tract. However, the ability to investigate the stages of adherence, colonization and growth on host-derived tissues and matrices will facilitate a molecular analysis of gene expression in K. pneumoniae.

In humans, susceptibility to infections of the airways by the opportunist K. pneumoniae is most frequently associated with predisposing factors. During secondary infections, exfoliated and denuded epithelial surfaces may expose collagenous receptors that enable MrkD-mediated adherence of K. pneumoniae. Subsequent growth at these sites in the form of biofilms could enable the bacteria to avoid efficient killing by alveolar macrophages. For patients with in-dwelling endotracheal tubes the type 3 fimbriae may have a dual role in the infective process. Immediately after insertion of these devices the hydrophobic nature of the type 3 fimbrial shaft could facilitate attachment to the polymer surfaces of these tubes with subsequent growth on the device. Also, it has been demonstrated that, with time, these tubes are coated, in situ, with host-derived material (Donlan, 2001; Francois et al., 1998). Therefore, a second role of the type 3 fimbriae in these types of infection may involve a specific receptor ligand interaction involving MrkD-mediated adherence to matrix proteins covering the tubes.

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