Relative contribution of *Prevotella intermedia* and *Pseudomonas aeruginosa* to lung pathology in airways of patients with cystic fibrosis

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**ABSTRACT**

**Background** Patients with cystic fibrosis (CF) with *Pseudomonas aeruginosa* lung infections produce endobronchial mucus plugs allowing growth of obligate anaerobes including *Prevotella* spp. Whether obligate anaerobes contribute to the pathophysiology of CF lung disease is unknown.

**Methods** The virulence of *Prevotella intermedia* and *Ps aeruginosa* was investigated in vitro and in mice, antibodies against *P intermedia* in CF sera were assessed and a culture-independent detection method for *P intermedia*/*P nigrescens* in CF sputum was tested.

**Results** *P intermedia* reached cell numbers of $10^7$ colony-forming units (CFU)/ml sputum. The majority of patients with CF (16/17; 94.1%) produced antibodies against two immunoreactive antigens of *P intermedia*. Culture supernatants, fluids, collected from $10^6$ *P intermedia* cells, were more cytotoxic to respiratory epithelial cells in vitro and inflammatory in mouse lungs than respective fluids from aerobically grown *Ps aeruginosa*, while fluids from aerobically grown *Ps aeruginosa* had the highest cytotoxicity and inflammation. Both pathological effects were largely reduced when culture supernatants from $10^5$ cells of either species were used. *P intermedia* cells (~$10^5$CFU/lung) did not induce mortality in the agar beads lung infection mouse model, while *Ps aeruginosa* cells caused death in 30% of mice due to rapid multiplication. A *P intermedia*/*P nigrescens*-specific PNA probe was significantly more sensitive than culture-dependent diagnostic assays to detect these strict anaerobes.

**Conclusion** *Ps aeruginosa* and *P intermedia* become significantly virulent in vitro and in vivo when cell numbers exceed $10^6$ CFU/lung.

**INTRODUCTION**

In patients with cystic fibrosis (CF), chloride retention as a consequence of defective CF transmembrane conductance regulator and increased sodium and water absorption causes an abnormally high viscosity of the mucus layer on the respiratory epithelium.1 2 The resulting impaired mucociliary clearance initiates a vicious circle of chronic infection and inflammation in the airways of patients with CF.3 Epidemiological data from a large patient registry have shown that the facultative anaerobic bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the dominating pathogens in CF lung disease.4 Both pathogens form biofilms under hypoxic conditions,5 6 thereby resisting host defence and antibiotic therapy.7 8 The persistence of facultative anaerobic bacteria results in a continuous influx of neutrophils into the airway lumen9 and, particularly in the absence of effective antibiotic therapy, large hypoxic mucus plugs are formed which lead to considerable airway obstruction.

Rapid consumption of oxygen by facultative anaerobic pathogens8 and by the large number of luminal neutrophils10 may favour substantial growth of obligate anaerobes. With the exception of two early reports11 12 obligate anaerobes in CF have only recently gained increased interest. Many obligate anaerobe species have been detected in CF sputum samples.13–20 High cell numbers of these anaerobes have ruled out the notion that the presence of these microorganisms in sputum is derived from upper airway contamination.16 17 The oral cavity has been regarded as a ‘stepping stone’ for lung colonisation and infection for certain bacterial anaerobic species.16

However, the important question—whether obligate anaerobes contribute to the pathophysiology of lung disease in CF—is still unresolved. In addition, it remains to be investigated whether widely used culture-dependent identification methods for obligate anaerobes correctly reflect the presence of obligate anaerobes in CF sputum.

We have addressed these issues by focusing on *Prevotella intermedia*. This Gram-negative black-pigmented anaerobic rod has been repeatedly detected in CF airway specimens.17–20 We comparatively investigated the virulence of *P intermedia* and *Ps aeruginosa* in vitro and in mice, assessed antibodies against *P intermedia* in CF serum and tested a culture-independent detection method for *P intermedia*, *P nigrescens* and *P melaninogenica* in CF sputum.

**METHODS**

**Patients**

Serum and sputum samples and lung function data were collected from 17 patients with CF attending the CF centre of the University Children’s Clinic, Universitätsklinikum Tübingen, Tübingen, Germany. Patients were eligible if they were infected with *Ps aeruginosa* and were sputum producers. To obtain a representative sputum
sample, at least 2 ml of purulent sputum had to be produced by
the patients during routine visits to the CF centre in Tübingen.
Serum samples from 30 healthy individuals were also collected.
Healthy individuals were recruited from members of staff at
the University Hospital of Tübingen. In addition, four patients with
CF who attended the CF centre of the Service de Pédieatrie,
Centre Hospitalier Lyon-Sud, Pierre-Benite, France were studied.21

Identification of Prevotella species
Bronchopulmonary samples for bacteriological investigations
including sputum, bronchial aspiration, protected catheter
brushing, mucosal biopsies and bronchial lavages were processed
by routine methods as described in the online supplement.
Prevotella species were identified using API 32A, a fluorescently
labelled 15-mer PNA probe by in situ hybridisation, MALDI-
TOF-MS and sequencing (for details see online supplement).22 23

Antibody response to P intermedia antigens in CF serum
Serum samples from patients with CF were subjected to
western blotting and ELISA using isolated membrane proteins of
the P intermedia strain ATCC 25611 (for details see online supplement).24

Cytotoxicity assays
Culture supernatant fluids from Ps aeruginosa strain PAO1
grown under aerobic and anaerobic conditions and P intermedia
strain ATCC 25611 grown under anaerobic conditions were
incubated with the human alveolar type II cell line A549 for 1 h
at 37°C. Cytotoxicity was assessed using the Syto13/propidium
iodide viability test.25 Additionally, the culture supernatant
fluids were incubated for various time periods with purified
human polymorphonuclear leucocytes.26 Finally, the ability of
the P intermedia strain ATCC 25611 to degrade casein, elastin or
gelatin was assessed on agar plates with the respective
substrates (for details see online supplement).

Animal studies
For lung infection of C57Bl/6 mice with Ps aeruginosa or
P intermedia the agar bead model was used.27 Twenty-four
hours after challenge, lungs were excised and bacterial colony-
forming units (CFUs) were determined using routine methods.
Mice were also challenged with sterile culture supernatant
fluids from strain PAO1 grown anaerobically or aerobically,
undiluted culture supernatant fluids of P intermedia at compar-
able cell numbers did not have activity against these substrates
(data not shown).

Culture supernatant fluids from Ps aeruginosa grown anaero-
ically corresponding to 7.8×10^5 CFU had a low cytotoxic effect
(figure 1A, B). Culture supernatant fluids from aerobically
grown Ps aeruginosa were significantly more cytotoxic (p=1.53×10^{-9}-2.21×10^{-12}, χ^2 test/Fisher exact test; figure 1A, B). A 1:100 dilution produced cytotoxicity of 16.3% towards A549
cells. A similar result was obtained when the culture superna-
tant fluids were collected when the culture was grown aerobi-
cally to a cell number of 7.2×10^6 CFU/ml (cytotoxicity 13.5%).
This cytotoxicity is significantly lower than undiluted culture
supernatant fluids of P intermedia (p=8.06×10^{-40}-1.35×10^{-25}).
Undiluted culture supernatant fluids from anaerobically grown
Ps aeruginosa were significantly less cytotoxic than those from
P intermedia (p=9.08×10^{-40}-1.44×10^{-52}).

When purified human neutrophils were incubated with
undiluted culture supernatant fluids from P intermedia, flow
cytometry showed an increasing percentage of necrotic neutro-
philis with time, reaching ~50% dead cells after 60 min of
incubation, corroborating the results obtained with A549 cells
(figure 1C). Undiluted Ps aeruginosa culture supernatant fluids
from strain PAO1 grown anaerobically were significantly less
cytotoxic than culture supernatant fluids from P intermedia
(p=0.005) and killed only 22% of the cells (figure 1C). In
contrast, culture supernatant fluids from aerobically grown Ps
aeruginosa were highly cytotoxic and killed 99% of neutrophils
within 1 min of incubation (figure 1C). In further experiments
the inflammatory potential of culture supernatant fluids of
P intermedia and Ps aeruginosa were compared in mouse lungs
(figure 1D, E). P intermedia and Ps aeruginosa caused a signifi-
cantly higher influx of neutrophils (p<0.0025) (figure 1D) and
macrophages (p<0.025) (figure 1E) than anaerobically grown
Ps aeruginosa.

Collectively, these in vitro and animal data show that the
cytotoxicity of P intermedia and anaerobically grown Ps aerugi-
na is significantly different. They further reveal that 1:100
diluted culture supernatant fluids from both pathogens, equiva-
 lent to cell numbers of ~10^7 CFU and irrespective of growth
conditions, are markedly reduced.

Virulence of P intermedia in mouse lungs is dependent on high
growth rates
When groups of mice were challenged with 5×10^6 CFU
P intermedia embedded in agar beads, bacterial CFUs remained
stable after 24 h (figure 2A). None of the mice died 48 h after
infection. In contrast, agar bead-embedded Ps aeruginosa grew
1.1×10^6-1.5×10^10 CFU within 24 h after challenge (figure 2A)
and killed 4/12 (30%) of the mice after 48 h. Similar to their
differential capacity to induce mortality in mice, P intermedia
and Ps aeruginosa cells differed in their inflammatory potential in
mouse lungs (figure 2B, C). Washed P intermedia cells caused less

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influx of neutrophils (figure 2B) and macrophages (figure 2C) than Ps aeruginosa cells within 24 h of the challenge. Our in vitro data showed that P intermedia can grow outside agar beads when anaerobic growth conditions prevail and reach cell numbers of $2.1 \times 10^8 \pm 9.5 \times 10^6$ CFU/ml while, under aerobic growth conditions, cell numbers remained at $3.7 \times 10^6 \pm 4.9 \times 10^5$ CFU/ml.

Figure 1  Prevotella intermedia produces more extracellular toxins than Pseudomonas aeruginosa under anaerobic conditions. (A, B) Cytotoxic effects of culture supernatant fluids (black bars) and diluted culture supernatant fluids (1:10, grey bars and 1:100, white bars) of P intermedia (PI) grown anaerobically ($-O_2$) and Ps aeruginosa (PA) grown anaerobically ($-O_2$) or aerobically ($+O_2$) on the respiratory epithelial cell line A549 determined using propidium iodide (dead cells: red) and syto13 (all cells: green). The 1:100 diluted culture supernatant fluid of Ps aeruginosa grown aerobically corresponds to a cell number of $8 \times 10^8$ CFU/ml and is comparable to the undiluted culture supernatant fluids of anaerobically grown Ps aeruginosa and P intermedia ($7.8 \times 10^8$ CFU/ml each). Data were obtained from 4 or 5 independent experiments which were performed in triplicate. (C) Cytotoxic effects of culture supernatant fluids of PI or PA on neutrophils (PMN) from five healthy human subjects expressed as the percentage of live neutrophils. Data were obtained from three independent experiments performed in triplicate. (D,E) Neutrophil and macrophage numbers in lung tissue of six C57Bl/6 mice and the representative pictures of lung tissue sections from C57Bl/6 mice intratracheally challenged with culture supernatant fluids of PI or PA. Lung tissue sections were stained with specific antibodies against mouse neutrophils (D) or macrophages (E). Arrows depict positive staining. Original magnification $\times 100$. *p<0.025.
These data suggest that *P. intermedia* contributes to lung pathology if appropriate growth conditions are present which allow multiplication to high cell numbers (>10⁸ CFU/lung). This suggestion needs to be validated in further studies.

*P. intermedia* is recognised by specific serum antibodies in patients with CF

To investigate whether the *P. intermedia* in CF sputum samples is recognised by the adaptive immune system of patients, as has been investigated for *P. aeruginosa*, we determined antibody titres against *P. intermedia* in serum samples from patients with CF and healthy individuals using a newly developed ELISA (figure 3A). Cohorts comprised 17 patients with CF (median age 15 years) and 30 healthy individuals (median age 25 years). A cut-off at a reciprocal titre of 510 in the ELISA was determined as three times the unspecific binding value of a group of healthy human individuals. Patients with CF harboured positive antibody titres against *P. intermedia* antigens in 94.1% of serum samples while none of the healthy control serum samples was positive. Thus, IgG antibody titres were significantly increased in patients with CF compared with healthy individuals (p<0.001).

Western blotting and MALDI-TOF showed that serum antibodies from patients with CF but not antibodies from healthy individuals bind to a 64 kD protein (PIN A0573) with hypothetical function and to a 42 kD immunoreactive protein (PIN A0102) (figure 3B). In order to prevent unspecific Fc-mediated binding of the antibodies to PIN A0573 and PIN A0102, we blocked the membrane with 17 µg/ml of the Fc fragment of human IgG. The western blot results were corroborated when crossed immunoelectrophoresis was used to determine the number of different antibodies in serum samples from our CF population. In two high-titre serum samples only two antibody arcs against single *P. intermedia* cell antigens were visible (figure 3C); in contrast, *P. aeruginosa* provoked the production of up to 64 different antibodies during chronic infection in patients with CF (figure 3D). A weak non-correlation (r=−0.253, p>0.001) was obtained between antibody titres against *P. intermedia* cell proteins and lung function in patients with CF.

The data suggest that *P. intermedia* is recognised by the humoral immune response in patients with CF.

**Numbers of strict anaerobic bacteria in CF bronchopulmonary samples**

Tunney and colleagues have determined the numbers of anaerobes in sputum specimens from patients with CF. They reported total viable counts of *Prevotella* species of >10⁴–10⁷ CFU/g.
sputum. To exclude the possibility that sputum specimens are contaminated by Prevotella species from oral flora, we quantified these micro-organisms in bronchopulmonary samples collected by fibreoptic bronchoscopy. When bronchopulmonary samples from four patients with CF (median age 11.5 years) were cultured anaerobically on media we found different Prevotella species in numbers of $>10^3$–$>10^7$ CFU, corroborating the results by Tunney et al$^{17}$ and suggesting that bacterial growth had occurred within the lower airways rather than contamination of airway samples with the oral anaerobic flora during recovery (table 2).

Culture-independent diagnosis yielded a higher prevalence of Prevotella species in sputum specimens from patients with CF. Not all patients with antibodies against P. intermedia had P. intermedia recovered from sputum taken at the time the serum was obtained. While this may be due to the persistence of specific antibodies even after eradication of the pathogen, it may also result from a lack of growth of the anaerobes on culture plates (ie, a false negative diagnosis). We therefore tested a culture-independent novel FNA probe for the identification of Prevotella spp. According to the sequence information of the distributor, P. intermedia cannot be distinguished from P. nigrescens by this technique. The specificity of the probe for P. intermedia and P. nigrescens was confirmed by applying the probe to a large number of other Prevotella strains (table 1). Four other bacterial strains were also used (table 1). Only negative staining results were obtained (data not shown). The probe was applied to 17 sputum samples from patients with CF and identified Prevotella spp. in 47% of the samples (figure 4A, B).

In contrast, growth of Prevotella spp. from sputum specimens on culture plates was only achieved in four cases (23%). To specify to which species grown colonies belong, we applied MALDI-TOF-MS. As gold standards for detection of Prevotella species by MALDI-TOF-MS, 27 different sequenced Prevotella strains stored in our large strain collection at the Institute of Medical Microbiology and Hygiene were used (table 1). As examples, distinct MALDI-TOF-MS patterns for P. intermedia, P. nigrescens and P. melaninogenica are depicted in figure 4C–E. Using these references, two of the four Prevotella sputum isolates were identified as P. intermedia and the other two as P. nigrescens and P. melaninogenica, respectively. These results were corroborated by the sequence analysis of the four Prevotella CF isolates (accession numbers: HM998855, HM998854, HM998855, HM998856).

Collectively, these data show that Prevotella species and possibly other obligate anaerobic species present in sputum specimens from patients with CF may be missed by culture-dependent methods.

### DISCUSSION

**Contribution of P. intermedia to the pathogenicity of lung disease in CF**

In this study we show that P. intermedia may contribute to the complex pathophysiology in the chronically infected CF lung. Several lines of evidence support this notion. First, Prevotella species were detected in numbers $>10^7$ CFU, showing that bacterial growth had occurred within the lower airways rather than contamination of airway samples with the oral anaerobic flora during recovery (table 2). This result corroborates data from other investigators.$^{17}$

Second, in contrast to healthy individuals, P. intermedia antigens are recognised by the adaptive immune system of the vast majority of patients with CF resulting in specific antibody titres, which again argues against contamination of airway samples with the oral anaerobic flora during recovery (figure 3A). The fact that, in some patients, positive serum antibody titres against P. intermedia antigens were found in the absence of the microorganism may be explained by either a false negative detection of P. intermedia (see below) or the possibility that patients who cleared the microorganism still had circulating antibodies. Similar phenomena occur with P. aeruginosa serum antibody titres and detection of the microorganism in CF airway specimens.$^{30, 31}$

Third, extracellular toxins of P. intermedia are cytotoxic for human alveolar type II cells and neutrophils (figure 1A, B,C), and they induce the influx of macrophages and neutrophils in the airway lumen thereby increasing lung inflammation (figure 1D, E). Using assays for gelatin, elastin and casein cleavage, we were unable to identify proteases with these activities in culture supernatants of P. intermedia strain ATCC 25611 which had pronounced cytotoxicity to A549 cells.

Last, P. intermedia possesses a surface protein$^{32}$ which, in analogy to S. aureus,$^{33}$ may avoid opsonophagocytosis by binding the Fc portion of human IgG, rendering specific antibodies inefficiently protective (figure 3B).

Importantly, the cytotoxic and inflammatory potential of culture supernatant fluids of P. aeruginosa grown anaerobically

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A, German Collection of Microorganisms and Cell Cultures (DSMZ); B, Institute for Medical Microbiology and Hygiene, Tübingen, Germany; identified by API.
to cell numbers comparable to \( \textit{P} \text{ intermedia} \) was much lower (figure 1A, D, E). Provided that comparable \( \textit{P} \text{ intermedia} \) cell numbers are reached in CF airways to those used in our in vitro assays (>10\(^5\) CFU), \( \textit{P} \text{ intermedia} \) would be more pathogenic than anaerobically grown \( \textit{P} \text{ aeruginosa} \). However, although growth under anaerobic environmental conditions is the dominant lifestyle for \( \textit{P} \text{ aeruginosa} \) in the CF lung\(^3\) and the its virulence decreases during chronic CF lung infection due to adaptive radiation,\(^3\) \( \textit{P} \text{ aeruginosa} \) can also multiply in aerobic compartments of the CF lung, in contrast to \( \textit{P} \text{ intermedia} \), and contribute to the pathophysiology of CF lung disease. Our animal experiments support this notion.

**Detection of \textit{Prevotella} species in bronchopulmonary samples of patients with CF**

In general, reports on anaerobic bacterial species in CF sputum samples are rare,\(^{14,20}\) although growth of obligate anaerobic species in CF sputum specimens may be facilitated by the high viscosity of the sputum\(^2\) and the presence of facultative anaerobic bacteria which rapidly consume oxygen, thus lowering the oxygen tension to degrees which allow obligate anaerobic bacteria to thrive.

In this study we show that the fluoreszenz in situ hybridisierung (FISH) method for detection of \( \textit{P} \text{ intermedia} \) and \( \textit{P} \text{ nigrescens} \) yields better results in this context than the classical API system. By using the novel PNA probe, 47% of the sputum samples were found positive for \( \textit{P} \text{ intermedia} \) and/or \( \textit{P} \text{ nigrescens} \) while only in 23% of the sputum samples were \( \textit{Prevotella} \) spp. identified by culture and API. One explanation is that we may have missed \( \textit{P} \text{ intermedia} \) during sputum solubilisation. Our finding may also explain in part why we detected \( \textit{P} \text{ intermedia} \)-specific serum antibody titres in several \( \textit{P} \text{ intermedia} \) culture-negative CF patients. Compared with the API system—which is known to have various disadvantages such as interpretation of biochemical reactions, long incubation time periods and costs—the novel PNA probe is much more rapid. Furthermore, FISH is more sensitive than API due to different sample processing. While the probe cannot be applied to detect these \( \textit{Prevotella} \) spp. in unprocessed CF sputum, solubilisation with 1,4-Dithiothreitol (DTT) followed by centrifugation and fluorescence microscopy is sufficient to obtain a result within 4 h. The PNA probe may allow rapid screening of \( \textit{P} \text{ intermedia} \) in the sputum from patients with CF which, in positive cases, may be followed

### Table 2 \textit{Prevotella} species in bronchopulmonary samples of patients with cystic fibrosis

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<th>Patient</th>
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<th>CFU/ml</th>
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<td>1</td>
<td>\textit{Prevotella denticola}</td>
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<td>4</td>
<td>\textit{Prevotella melaninogenica}</td>
<td>$5\times10^6$</td>
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by a more elaborate and time-consuming quantitative assessment of *P. intermedia*. These data can be used for the control of treatment directed against strict anaerobes.

Taken together, the results of this study suggest that *P. intermedia* plays a critical role in the complex pathophysiology of lung disease in patients with CF when cell numbers of >10⁶ CFU are reached in the anaerobic sputum plagues.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the ethic committees of the University of Tübingen, the Hospices Civils de Lyon and the Universität Claude Bernard, Lyon, France.

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