Clonal analysis of *Inquilinus limosus* isolates from six cystic fibrosis patients and specific serum antibody response

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Inquilinus limosus is a novel Gram-negative bacterium of the subdivision α -Proteobacteria recently found in the airways of patients with cystic fibrosis (CF). Here, the authors report on the clinical courses of six CF patients colonized with *I. limosus*. Five patients suffered from either an acute respiratory exacerbation or a progressive loss of pulmonary function, whereas one patient was in a stable clinical situation. This study focused on two aims: (i) the clonal analysis of *I. limosus* isolates by random amplified polymorphic DNA (RAPD)-PCR, and (ii) the clarification of whether the presence of *I. limosus* in the respiratory tract is associated with a specific serum antibody response. Serum IgG was detected by immunoblotting using *I. limosus* whole-cell-lysate proteins as antigens. Sera from healthy blood donors ($n = 10$) and from CF patients colonized with *Pseudomonas aeruginosa* ($n = 10$) were found to be immunoblot negative. All six *Inquilinus*-positive patients raised serum IgG antibodies against various *I. limosus* antigens. Surprisingly, in one patient, a specific *I. limosus* serum antibody response was already detected 1 year prior to *Inquilinus*-positive sputum cultures. Two prominent antigens were characterized by MALDI-MS: a 23 kDa protein revealed homology to the outer membrane lipoprotein OmlA of *Actinobacillus pleuropneumoniae*, and an 18 kDa protein to a protein-tyrosine phosphatase of *Burkholderia cepacia*. In conclusion, detection of *I. limosus* is accompanied by a specific serum antibody response and may reflect the infectious/pathogenic potential of *I. limosus*. Moreover, IgG immunoblotting may be useful to detect early infection with *I. limosus* and may support the selective cultivation of this novel emerging pathogen.

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INTRODUCTION

In the airways of patients with cystic fibrosis (CF), *Pseudomonas aeruginosa* is the predominant respiratory pathogen, and contributes considerably to morbidity and mortality. Other commonly isolated bacteria are *Staphylococcus aureus*, *Haemophilus influenzae* and inherently multi-resistant organisms, such as members of the *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*. In addition, unusual non-fermenting Gram-negative rods, such as *Ralstonia* spp. and *Pandoraea* spp., have been increasingly recognized (Berlinger & Appleman, 2000; Coenye *et al.*, 2002b; Jorgensen *et al.*, 2003; Wallet *et al.*, 2002), and, as a

consequence of difficulties in cultivation and identification, their true prevalence might even be higher (Burns *et al.*, 1998; Gilligan, 1991; Rajan & Saiman, 2002). The genus *Inquilinus* was defined in 2002, when 51 unknown bacterial CF isolates were characterized by molecular methods. Seven isolates were classified into the novel genus *Inquilinus* and belonged to the species *I. limosus* (Coenye *et al.*, 2002a). Already in 1999, an alpha-proteobacterium with no specific relative was isolated from a lung-transplanted CF patient during respiratory exacerbation and subsequently recognized as *I. limosus* (Coenye *et al.*, 2002b; Pitulle *et al.*, 1999). Recently, two groups have reported on a few cases of *I. limosus* among German and French CF patients (Chiron *et al.*, 2005; Wellinghausen *et al.*, 2005). From these eight published cases, four CF patients alone showed signs of acute respiratory exacerbation and/or spirometric deterioration. In six patients, *I. limosus* was detected in at least

Abbreviations: CF, cystic fibrosis; CRP, C-reactive protein; RAPD, random amplified polymorphic DNA; WCL, whole-cell lysate.

two follow-up specimens. Except for one French CF patient, all of them were chronically colonized with *P. aeruginosa*. Nevertheless, the pathogenic potential, the impact on respiratory function and the risk of patient-to-patient transmission of *I. limosus* are still unclear, and the environmental habitat of *I. limosus* is unknown.

In this study, we estimated the pathogenic potential of *I. limosus*, taking into account the clinical aspects of *Inquiliinus*-positive CF patients. Since 2002, we have identified *I. limosus* from respiratory secretions of six different CF patients. *I. limosus* isolates from these six patients were genotyped using random amplified polymorphic DNA (RAPD)-PCR, and their protein profiles were compared by SDS-PAGE. For *P. aeruginosa* and *B. cepacia*, it had already been shown that high serum antibody titres correlate with signs of respiratory exacerbation (Fomsgaard *et al.*, 1988; Hendry *et al.*, 2000). Therefore, we investigated the host serum IgG response against *I. limosus* by immunoblotting. Here we report for the first time on a specific serum antibody response against *I. limosus* antigens.

METHODS

Bacterial strains and subjects. *I. limosus* and *P. aeruginosa* isolates were recovered from sputa of four CF patients at the Ludwig-Maximilians-University Hospital, Munich, Germany, from one CF patient at the Heinrich Heine University Hospital, Dueseldorf, Germany, and from one CF patient at the Friedrich-Schiller-University Hospital, Jena, Germany. Antimicrobial susceptibility tests were performed for ceftazidime, ciprofloxacin, tobramycin, meropenem, fosfomycin and polymyxin using the reference agar dilution method according to the recommendations of the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) (National Committee for Clinical Laboratory Standards, 2000), and for cefepime, piperacillin-tazobactam, aztreonam, amikacin and gentamicin using the E-test method according to the manufacturer's recommendations (AB Biodisk).

RAPD-PCR and sequencing of 16S rDNA. Isolates were cultured in tryptone soy broth (TSB) at 37 °C to stationary phase. Total genomic DNA was extracted from *P. aeruginosa* and *I. limosus* CF isolates and typed by RAPD-PCR analysis as described previously for *P. aeruginosa* (Mahenthiralingam *et al.*, 1996) using primer 208 (5'-ACGGCCGACC-3'). Amplification of 489 bp 16S rDNAs was performed using universal primers FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 800r (5'-GAGTACCAGGTATCTAATCC-3'). Subsequently, the amplicons were sequenced using primer 800r.

Protein preparation. Isolates were cultured in TSB at 37 °C to stationary phase. Bacterial cells were harvested by centrifugation at 6800 g for 10 min at 4 °C. For preparation of bacterial whole-cell lysate (WCL) proteins, pellets were resuspended in 200 µl denaturing buffer containing 10% (v/v) glycerol, 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.003% (w/v) bromophenol blue and 50 mM Tris/HCl, pH 6.8, boiled for 5 min, and cleared by centrifugation (5 min at 21 000 g). Supernatant of 4 ml bacterial culture obtained by centrifugation (10 min at 6800 g) was precipitated by the addition of 10% trichloroacetic acid. After incubation on ice for 2 h, precipitated proteins were collected by centrifugation at 21 000 g for 30 min, washed with acetone, and resuspended in 20 µl denaturing buffer. Samples were heated at 100 °C for 10 min and centrifuged at 21 000 g for 10 min at 4 °C. The protein content of the samples was measured using the Bradford protein assay.

Pre-adsorption of sera. Patient serum samples were pre-adsorbed with boiled whole cells of either *P. aeruginosa* or *I. limosus* (Rogol *et al.*, 1983). Bacteria were grown at 37 °C overnight in TSB, adjusted to a density of 1×10^{10} cells ml⁻¹ in PBS and boiled for 15 min. Patient sera were diluted 1:20 in PBS containing antigens from 10^9 cells ml⁻¹ heat-inactivated *P. aeruginosa* or *I. limosus*, and incubated for 45 min at 37 °C with gentle shaking. Cells and formed immune complexes were separated from serum by centrifugation at 1700 g for 20 min.

Immunoblotting. SDS-PAGE was performed using a 12% SDS-polyacrylamide gel (Caballero *et al.*, 2001). Bacterial WCL proteins (10 µg) were loaded, and after electrophoresis, the separated proteins were stained with Coomassie blue or electroblotted to nitrocellulose filters, as described by Towbin *et al.* (1992). The membrane was blocked for 1 h with 1% BSA and washed three times for 5 min with PBS/Tween (0.5%). Blotting membranes were incubated for 3 h with either patients or control sera, using dilutions of 1:200, 1:500 or 1:2000, washed three times, and incubated for 1 h with alkaline phosphatase-conjugated anti-human IgG, diluted 1:5000. After washing, the antibody-reacting bands were visualized using Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (Sigma). The development was stopped after 1 min by washing the membranes in several changes of distilled water.

RESULTS

Case reports

All suspected *I. limosus* isolates in respiratory secretions from six CF patients (two male and four female) have been confirmed by 16S rDNA sequencing. Four of the patients were followed at the University Hospital, Munich, and two of them were treated at two other German CF centres. At the first isolation of *I. limosus*, their age ranged from 15 to 32 years (Table 1). All six patients have been chronically colonized with *P. aeruginosa* for more than 2 years, and *I. limosus* has been detected in all patients in at least one follow-up specimen. Further data on the clinical status, antibiotic treatment and microbiological results are summarized in Table 1. In patients 1 and 6, *I. limosus* was initially misidentified as *P. aeruginosa* because of the very mucoid morphotype and a positive cytochrome oxidase reaction. Retrospectively, the stored isolates were rechecked by 16S rDNA sequencing and identified as *I. limosus*. Patients 1–4 showed signs of an acute respiratory exacerbation following the first *I. limosus* detection, whereas patient 6 showed a respiratory decline without signs of acute exacerbation. Patient 5 was in a stable clinical situation. In patient 3, retrospectively, we realized that *I. limosus* had been detected 1 year earlier by a different laboratory, but in only one of seven respiratory specimens and during a stable clinical situation (Wellinghausen *et al.*, 2005). The greatest clinical deterioration was observed in patient 1 (Fig. 1). This 15-year-old girl, chronically colonized with two *P. aeruginosa* morphotypes (isolates PA1-1 and PA1-2) had been admitted to hospital due to an acute pulmonary exacerbation associated with new lower-lobe infiltrates of the left lung. A white blood cell count of $39\,400\ \mu\text{l}^{-1}$ as well as a C-reactive protein (CRP) level of $26.6\ \text{mg dl}^{-1}$ indicated a

Table 1. Clinical data of CF patients colonized with *I. limosus*

Abbreviations: p.o., oral; by inh., by inhalation; i.v., intravenous; IL, *I. limosus*; PA, *P. aeruginosa*; STM, *S. maltophilia*; CT, *Comamonas testosteroni*; SM, *Serratia marcescens*; SA, *Staph. aureus*; CA, *Candida albicans*; CFA, *Candida famata*; AF, *Aspergillus fumigatus*; AMK, amikacin; AZT, azithromycin; CAZ, ceftazidime; CXM, cefuroxime; LEX, cefalexine; CIP, ciprofloxacin; COL, colistin; FOF, fosfomycin; LVX, levofloxacin; MEM, meropenem; TEC, teicoplanin; TOB, tobramycin; VAN, vancomycin.

Patient data	Patient no.					
	1	2	3	4	5	6
<i>I. limosus</i> isolate no.	IL1-1, IL1-2	IL2	IL3	IL4	IL5	IL6
Sex	Female	Male	Female	Female	Female	Male
Age (years)	16*	19	17	20	17	35
Age (years) at first PA detection	3	12	Childhood	17	6	25
Age (years) at first IL detection	15	18	15	19	16	32
Microbiological results						
Pathogens before first IL detection	PA	PA, STM, CA, AF	PA	PA, SA	PA, SA, CT	PA, STM, CFA
Months IL was the only pathogen/no. of sputa	3/9	—†	—†	—†	—†	—†
Pathogens associated with IL	PA	PA	PA, CA‡, AF‡	PA, SA, CA, AF	PA, STM, SA, CA, AF	PA, STM, SM
Antibiotic therapy						
Continuous regimen before IL detection						
p.o.	CIP	LEX	AZT, CIP	AZT	AZT, CXM	LEX
by inh.	TOB	TOB	COL/TOB			TOB
Last antibiotic regimen before IL detection						
p.o.	None	CIP		None	None	None
by inh.	None	None		COL	None	None
i.v.	CAZ, TOB	None	MEM, COL	None	AMK, CAZ, VAN	TOB
Regimen after/at first IL detection						
p.o.	None	None	None	None	None	None
by inh.	None	None	None	None	None	None
i.v.	MEM, TOB, CIP	(MEM, CIP, TOB)§	MEM, COL	MEM, VAN, CIP	TOB	None
Regimen was changed to i.v.	TEC, TOB, FOF/VAN, CAZ, LVX	—	—	TEC, TOB	—	—
Clinical status at first IL detection	Severe exacerbation	Stable, serious decline 8 months later	Exacerbation (when <i>I. limosus</i> was detected a second time)	Exacerbation	Stable	Respiratory decline, no exacerbation
FEV ₁ (% predicted) before/after first IL detection	30/24	104/95/90§	77‡/57	41/26	35/45	122/105
IgG immunoblot	+	+	+	+	+	+
Patient group	1	2	1	2	1	1

*Patient died in May 2004 at the age of 16 years.

†*I. limosus* was always associated with other pathogens.

‡Data from Wellinghausen *et al.* (2005).

§Eight months later when a serious decline occurred, *I. limosus* was still detectable.

||No change in the regimen was necessary.

Fig. 1. Clinical data of patient 1. Lung function measured as FEV₁ (percentage predicted, ●, solid line) and CRP (mg dl⁻¹, ○, dotted line) at different time-points are shown. Because of severe pulmonary deterioration, spirometry could not be performed between September and December 2002. The solid arrow indicates isolation of *P. aeruginosa*, the dotted arrow indicates isolation of *I. limosus*. The dashed arrow shows the time period when sera were available and *I. limosus* antibodies were detected.

significant systemic inflammatory response. Over 2 months, patient 1 was treated intravenously with different antibiotic regimens (see Table 1). *I. limosus* (isolate IL1-1) was first detected in a re-evaluation of her microbiological status 3 weeks after admission to hospital, due to lack of clinical response to anti-pseudomonal therapy with two different regimens and increasing CRP levels. The third antibiotic regimen finally led to clinical improvement and her discharge after 2 months of hospitalization. Subsequently, *I. limosus* was the only pathogen detected in her respiratory secretions over a period of 3 months with nine consecutive sputum samples. During antibiotic therapy with meropenem, tobramycin and ciprofloxacin, a meropenem-resistant *I. limosus* variant (isolate IL1-2) emerged. When, besides *I. limosus*, *P. aeruginosa* reoccurred in the patient's sputum 3 months later (isolate PA1-3), its RAPD-PCR banding pattern showed no differences to earlier *P. aeruginosa* isolates (PA1-1 and PA1-2), indicating their clonality (PA RAPD-PCR patterns are not shown). Despite ongoing intensive therapy, the girl died in May 2004 due to respiratory failure.

Microbiological results

The microbiological characteristics of *I. limosus* isolated from patient 1 (IL1-1, IL1-2), patient 2 (IL2), patient 3 (IL3), patient 4 (IL4), patient 5 (IL5) and patient 6 (IL6) were concordant with earlier-described *I. limosus* isolates, except for negative catalase production (Chiron *et al.*, 2005; Coenye *et al.*, 2002b; Pitulle *et al.*, 1999; Wellinghausen *et al.*, 2005). On routine culture media, all collected *I. limosus* isolates grew very slowly with non-pigmented colonies, and showed an extremely mucoid morphotype, which was also stable after 10 passages. 16S rDNA sequence analysis (400 bp) revealed 100% homology with *I. limosus* (accession no. AY043373) (Coenye *et al.*, 2002b). All *I. limosus* isolates were resistant to polymyxin B, fosfomycin and the

tested β -lactams, except for carbapenems. Only isolates IL1-2, IL2 and IL4 were additionally resistant to meropenem. In contrast to *P. aeruginosa*, all *I. limosus* isolates were resistant to tobramycin, but isolates IL1-1, IL1-2 and IL5 were susceptible to amikacin, and isolates IL1-1 and IL1-2 were susceptible to gentamicin.

RAPD-PCR analysis and protein profiles of *P. aeruginosa* and *I. limosus* isolates

RAPD-profiles of sequential *I. limosus* isolates IL1-1 and IL1-2 were identical. *I. limosus* isolates IL2, IL3, IL4, IL5 and IL6 showed different RAPD profiles, demonstrating that each patient was infected by a distinct strain (Fig. 2). Additionally, the *I. limosus* WCL protein patterns were compared by SDS-PAGE showing that all seven isolates can be distinguished reproducibly by their protein profile (Fig. 3a). Strikingly, the isogenic pair IL1-1 and IL1-2 showed slightly distinct protein profiles, suggesting two expression variants of one clone. The most prominent differences were found for the 23 kDa protein named P23 and the 18 kDa protein named P18.

P. aeruginosa and *I. limosus* IgG-immunoblotting reactivity of sera from patient 1, with and without pre-adsorption

In a first approach, we analysed the IgG reactivity of sera from patient 1 against antigens of its autologous *P. aeruginosa* and *I. limosus* isolates. In Fig. 3(a), the Coomassie-stained protein patterns of WCL proteins of three sequential *P. aeruginosa* isolates and two sequential *I. limosus* isolates obtained from patient 1 are shown. Fig. 3(b–e) shows the corresponding IgG immunoblots developed with an early (serum 1) and a late serum (serum 2, 1 year later). Serum 2 reacted strongly with *P. aeruginosa* and *I. limosus* antigens. The strongest IgG-reactive antigens for all three *P. aeruginosa* isolates were located at ~20 kDa. For IL1-1, but

Fig. 2. RAPD-PCR analysis of *I. limosus* isolates IL1-1, IL1-2, IL2, IL3, IL4, IL5 and IL6. Numbers to the left show relative positions (in bp).

not for IL1-2, the strongest reactive protein bands were found for the proteins P23 and P18. Additionally, there were numerous IgG-reactive bands in the range 36–90 kDa. We examined 10 sera from CF patients continuously infected/colonized with *P. aeruginosa* for more than

2 years, but free of *I. limosus* in sputum cultures, and 10 from healthy/adult volunteers (blood donors) as controls. These 20 sera showed no reactivity with Western blots of *I. limosus* isolates IL1-1 and IL1-2 (serum dilution 1 : 200). As expected, pooled sera of *Pseudomonas*-positive, *Inquilinus*-negative CF patients were reactive for *P. aeruginosa* IgG immunoblotting (Fig. 3f; serum dilution 1 : 200). To assess the specificity of immunoblots, the two sequential sera from patient 1 were cross-adsorbed with boiled *P. aeruginosa* or *I. limosus* antigens (see Methods). *P. aeruginosa* cross-adsorption of sera resulted in significant reduction of the reactivity of *Pseudomonas* immunoblots, whereas the *Inquilinus* immunoblot patterns remained unchanged. Cross-adsorption of sera with *I. limosus* removed *I. limosus*-specific antibody reactivity quantitatively, except for those recognized as P18 and two proteins of about 36 and 85 kDa in size. Possibly, the strongly reactive P18 was not accessible to serum antibodies when boiled *Inquilinus* cells were used for cross-adsorption. Interestingly, serum 1 obtained 1 year before the initial isolation of *I. limosus* from sputum of patient 1 was already found to be immunoblot positive (Fig. 3e), although with weaker reactivity than serum 2.

Fig. 3. IgG immunoblots of WCL proteins of *P. aeruginosa* and *I. limosus* isolates from patient 1 incubated with sera from patient 1 and from negative controls. Lane 1, *P. aeruginosa* PA1-1, mucoid; lane 2, PA1-2, non-mucoid; lane 3, PA1-3, non-mucoid. PA1-3 was isolated after *I. limosus* infection. Lanes 4 and 5, *I. limosus* isolates with the different phenotypes IL1-1 and IL1-2, respectively. (a) Coomassie blue-stained SDS-PAGE. (b–d) Patient 1 serum 2 (from 12 December 2002; dilution 1 : 2000); (b) non-absorbed; (c) pre-absorbed with *I. limosus* (absIL); (d) pre-absorbed with *P. aeruginosa* (absPA). (e) Patient 1 serum 1 (from 22 August 2001; dilution 1 : 2000), pre-absorbed with *P. aeruginosa* (absPA). (f) Pooled control sera of *Inquilinus*-negative, *Pseudomonas*-positive patients ($n=10$; dilution 1 : 200). Numbers to the right show relative positions (in kDa). PA, *P. aeruginosa*; IL, *I. limosus*.

In addition, we tried to isolate secreted proteins from culture supernatants of *I. limosus*. Neither from stationary TSB, nor from Ca^{2+} -depleted culture supernatants (known to induce type III secretion in *P. aeruginosa*), could we detect supernatant proteins by SDS-PAGE (Coomassie staining) or by immunoblotting (data not shown).

In summary, we demonstrated that the presence of *I. limosus* in the respiratory tract of patient 1 was associated with a specific IgG serum antibody response against distinct *I. limosus* antigens. Moreover, a serum antibody response to *I. limosus* antigens may be detectable before *I. limosus* can be cultivated.

***I. limosus* IgG-immunoblotting analysis of the reactivity of patient sera against diverse *I. limosus* WCL proteins**

In a second approach, we analysed the serum IgG reactivity of the six patients against autologous and heterologous *I. limosus* isolates to demonstrate antibody response, directed to dominant common antigens and strain-specific antigens. As expected, pooled control sera of *Pseudomonas*-positive, *Inquilinus*-negative CF patients showed no reactivity with Western blots of all *I. limosus* isolates (Fig. 4b, serum dilution 1:200). To obtain sufficient visible bands in immunoblots incubated with sera from patients 2 to 6, we had to use serum dilutions of 1:500, in contrast to 1:2000 with sera from patient 1.

Sera from patient 1 showed cross-reactivity to all five heterologous *I. limosus* isolates (Fig. 4c). The IgG immunoblot incubated with sera from patient 2 showed weak reactivity with heterologous *Inquilinus* antigens, but strong reactivity to WCL antigens of the autologous IL2 isolate, in particular in the range 36–47 kDa (Fig. 4d). Serum IgG from patient 3 was highly reactive for numerous *Inquilinus* protein bands likely present in all seven *I. limosus* isolates (Fig. 4e), even when using serum dilutions of 1:2000 (results not shown). Moreover, in the range 20–30 kDa, additional reactive bands appeared with the autologous and heterologous *I. limosus* isolates that were not recognized by sera from patients 1 and 2. The IgG immunoblot incubated with serum from patient 4 showed strong antibody reactivity to antigens of the autologous IL4 WCL as well as to IL2 WCL (in the range 36–47 kDa), but only weak reactivity against WCLs of IL1-1, IL1-2, IL3, IL5 and IL6 (Fig. 4f). Serum from patient 5 showed high cross-reactivity for diverse antigens of all seven *I. limosus* isolates (Fig. 4g), especially for IL2 and IL4. Serum from patient 6 showed, equivalent to sera from patients 3 and 5, cross-reactivity to WCLs of all seven *I. limosus* isolates (Fig. 4h). Among patients 2–6, only sera from patients 2 and 3 showed reactivity with P23 and weak reactivity with P18 from isolate IL1-1.

In summary, sera from all six *I. limosus*-infected patients showed IgG reactivity against numerous proteins of WCLs of *I. limosus* with varying intensity. Except for patients 3 and

6, all patients developed the strongest reactivity against antigens of autologous *I. limosus* isolates. Only sera from patients 1–3 recognized the proteins P18 and P23 of isolate IL1-1. Thus, the patients were separated into two serum-reactivity groups. Sera from the first group (patients 1, 3, 5 and 6) showed a strong cross-reactivity against autologous and heterologous *I. limosus* antigens, whereas sera from the second group (patients 2 and 4) showed strong reactivity predominantly against the autologous *I. limosus* antigens and only weak reactivity against heterologous antigens.

MALDI-MS sequencing of P23 and P18

P23 and P18 of IL1-1 were excised from SDS-PAGE and submitted for MALDI-MS analysis. Compared to the Mascot database, P23 revealed closest homology to the outer membrane lipoprotein OmlA of *Actinobacillus pleuropneumoniae* (Gram & Ahrens, 1998), with a protein score of 79 ($P < 0.05$) (accession no. gi29469957). The result for P18 with a protein score of 74 ($P < 0.05$) pointed to a homologous protein-tyrosine phosphatase of *B. cepacia* (accession no. gi46321582). *I. limosus* isolates IL1-2, IL2, IL3, IL4, IL5 and IL6 were also checked for the presence of P23 and P18. Therefore, Coomassie-stained protein bands of SDS-PAGE runs with the same electrophoretic mobility as P18 and P23 were analysed by MALDI-MS. We did not obtain sequences which indicated similarity to P18 or P23.

DISCUSSION

I. limosus is a novel species of the subdivision α -*Proteobacteria*. At present, only a small number of isolates have been reported, exclusively among CF patients (Chiron *et al.*, 2005; Coenye *et al.*, 2002a; Pitulle *et al.*, 1999; Wellinghausen *et al.*, 2005). Nevertheless, in agreement with published cases, the *I. limosus* isolates detected in respiratory secretions of six patients in our laboratory represent an increasing local prevalence in CF. As in the cases of patients 1 and 6, retarded growth and the similarity to mucoid *P. aeruginosa* may easily lead to overlooking and misidentification of *I. limosus*. In earlier studies, *Inquilinus*-positive patients were colonized with individual strains showing no clonality in PFGE analysis (Chiron *et al.*, 2005; Wellinghausen *et al.*, 2005). To characterize the *I. limosus* isolates from our six patients, we compared them using both RAPD-PCR and SDS-PAGE of bacterial WCLs. The different banding patterns in RAPD-PCR indicated that this could be a suitable method to discriminate *I. limosus* isolates [results were confirmed by PFGE analysis, as described by Wellinghausen *et al.* (2005); data not shown]. The individual RAPD-PCR and WCL protein profiles demonstrated that each patient was infected/colonized by an individual *I. limosus* clone. The fact that patients 1–3 were treated in the same outpatient CF clinic during overlapping time periods suggests that there was no transmission of *I. limosus* among these patients. As expected, comparison of sequential *I. limosus* isolates of patient 1 revealed identical RAPD profiles, but slightly different

Fig. 4. (a) Coomassie blue-stained SDS-PAGE of WCL proteins from *I. limosus* isolates of patients 1–6 (IL1-1, IL1-2, IL2, IL3, IL4, IL5 and IL6). (b–h) IgG immunoblots incubated with pooled control sera of *Inquilinus*-negative, *Pseudomonas*-positive patients (b) ($n = 10$; dilution 1 : 200), and with sera from patients 1 (c), 2 (d), 3 (e), 4 (f), 5 (g) and 6 (h) (dilution 1 : 500). IL, *I. limosus*; numbers at the top show *I. limosus* isolate numbers. Numbers on the right show relative positions (in kDa).

protein patterns. In contrast to IL1-1, IL1-2 showed no detectable expression of antigens P18 and P23, suggesting an adaptation of *I. limosus* during the course of infection. Interestingly, these two *I. limosus* variants co-existed in the respiratory tract. We demonstrated by immunoblotting that

patient 1 showed a strong antibody response, especially against P18 and P23. As a putative lipoprotein, P23 might be localized in the cell envelope of *I. limosus*, and its access to antibodies is not surprising, possibly explaining the loss of P23 reactivity after cross-adsorption with *I. limosus*. P18 is

probably localized cytoplasmically and thus might not be accessible for cross-adsorption. *I. limosus* isolates of the remaining five patients did not express P18 and P23 in detectable amounts, although patients 2 and 3 showed a weak reactivity in this size range and probably against these proteins. During the course of infection, the expression of P18 and P23 might have been counter-selected, and then might have disappeared in the WCLs of isolates IL2 and IL3, as occurred for isolate IL1-2.

So far, it is still unclear whether *I. limosus* contributes to the respiratory deterioration of CF patients or accompanies *P. aeruginosa* as a commensal. We addressed this issue by detecting a possible serum antibody response against *I. limosus* WCL antigens in sera from all six *Inquilinus*-positive patients using immunoblotting. All CF patients presented here had positive *I. limosus* sputum cultures, and five of them showed clinical signs of exacerbation or spirometric deterioration. Patient 1 showed a strong, specific IgG response against *I. limosus* detected by immunoblotting with *I. limosus* WCLs. Extensive pre-adsorption with *P. aeruginosa* did not diminish the IgG reactivity to *I. limosus* proteins, but cross-reactivity to *P. aeruginosa* could be substantially removed by adsorption with *I. limosus*. Thus, immunoblotting appears to be a specific serological technique for diagnosis of *I. limosus* pulmonary infections.

The detection of a positive *I. limosus* immunoblot in patients even before the culture-based detection of *I. limosus* indicates pre-existing colonization/infection. *I. limosus* was not detected at that stage by culture, probably due to a very low bacterial load, a slow growth rate and lack of experience with *I. limosus*. As described for *B. cepacia* infection, a specific serum antibody response may be detectable years prior to cultivation of the pathogen (Aronoff *et al.*, 1991). The other *Inquilinus*-positive patients also showed an immune response to *Inquilinus* antigens, albeit with weaker reactivity. The time-point of infection with *I. limosus* could not be exactly defined, and sera obtained before the first cultural detection of the organism were not available. Follow-up sera were available from patients 2 and 3, 6 months after the first isolation of *I. limosus*, but showed no increase in reaction against *I. limosus* antigens (data not shown). In contrast, sera of 10 CF patients with positive *P. aeruginosa* sputum cultures, but negative *I. limosus* cultures, showed IgG reactivity against *P. aeruginosa*, but not against *I. limosus*. As expected, healthy blood donors were also free of anti-*I. limosus* antibodies. In summary, we conclude that *I. limosus* may have the potential for invasion and eliciting an inflammatory response, which leads to a specific serum antibody response. Probably, *I. limosus* affects the respiratory function of CF patients in a similar manner to *P. aeruginosa* and *B. cepacia* (Hendry *et al.*, 2000; Johansen *et al.*, 2004; Lacy *et al.*, 1995).

With respect to immunoblot results, we may separate *Inquilinus*-positive patients into two groups. Group 1 showed an IgG reaction against both autologous and

heterologous *I. limosus* antigens. Group 2 showed only a weak reaction against heterologous antigens, but a strong reaction against autologous *I. limosus* antigens. However, the classification into these groups reflects the clinical situation poorly. For example, patient 5, classified into group 1 with various reactive bands against heterologous WCLs, had a stable lung function, while patient 1 (also from group 1) suffered from a severe clinical deterioration during *I. limosus* infection. These preliminary data indicate that the number of reactive bands in Western blot is not of prognostic value for the clinical course of *I. limosus* infection. It has been reported that intensive treatment regimes at the early stage of *P. aeruginosa* infection can have an effect on the pseudomonal antibody response (Johansen *et al.*, 2004). It can be speculated that the antibody production against *I. limosus* in the patients of this study diminished due to the recurrent antibiotic treatment that all of them had received in the past.

In general, the prevalence of *I. limosus* might be underestimated. Therefore, a screening method for *I. limosus* is required in order to identify *I. limosus* during the early and latent state of infection. This might help to initiate appropriate antibiotic treatment, taking into account the multi-resistant phenotype of the organism. As an IgG-antibody reaction was detectable in all six *I. limosus* sputum-culture-positive patients, we conclude that WCL immunoblots are a sensitive method to detect class-specific serum antibodies against *I. limosus* antigens and to predict colonization or infection of CF patients with *I. limosus*. Subsequently, a positive *Inquilinus* immunoblot might require the application of specific microbiological procedures to improve the specific detection of this novel pathogen in sputum cultures (e.g. prolonged incubation because of the slow growth rate). Extended serologic studies of *I. limosus* should be performed to identify dominant antigens common to all *I. limosus* isolates. In conjunction with serological diagnosis the screening method for *I. limosus* in respiratory secretions should be improved, either by fluorescent *in situ* hybridization (Hogardt *et al.*, 2000) and/or by PCR, which has proven to be very useful to control *B. cepacia* infection (Moore *et al.*, 2002).

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