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Pseudomonas aeruginosa infections are prevented in cystic fibrosis patients by avian antibodies binding *Pseudomonas aeruginosa* flagellin

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Abstract

Pseudomonas aeruginosa (PA) is the main cause of morbidity and mortality in cystic fibrosis (CF) patients. CF patients with chronic PA infections have a more rapid deterioration of their lung function and the bacteria become impossible to eradicate from the lungs. Antibiotic resistance among PA strains in CF patients is steadily increasing. Specific chicken (IgY) antibodies against PA have been shown to have potential to prevent PA infections in CF. Anti-Pseudomonas IgY reduces PA adhesion to epithelia, but the mechanism has not been fully elucidated. To gain further insight into the prophylactic effect of these antibodies, the immunoreactivity was investigated by 2D electrophoresis of PA strains, immunoblotting and MALDI-TOF-MS. To confirm the identity of the proteins, the tryptic peptides were analyzed by MALDI-TOF-MS to accurately measure their monoisotopic masses as well as determine their amino acid sequences. In order to facilitate fragmentation of the peptides they were N-terminally or C-terminally labeled. Several strains were investigated and anti-Pseudomonas IgY was immunoreactive against all of these strains, which strengthens its potential as a prophylactic treatment against PA. Flagellin was identified as the major antigen. Flagellin is the main protein of the flagella and is crucial for establishing infections in hosts as well as being involved in PA chemotaxis, motility, adhesion and inflammation. Furthermore, secreted flagellin elicits an inflammatory response. In conclusion, anti-Pseudomonas IgY binds flagellin, which may prevent PA infections in CF patients by hindering host invasion.
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Keywords: 2D electrophoresis; Antigen; IgY; Mass spectrometry; Proteomics

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene coding for the CFTR protein [1,2]. This protein is a cAMP-regulated chloride channel. A defective CFTR protein function leads to impaired chloride, sodium and water balance. Manifestations from several organs are involved, but CF is primarily a pulmonary disease due to a predisposition for bacterial lung infections. Lung infections by the opportunistic bacteria *Pseudomonas aeruginosa* (PA) is one of the main causes of morbidity and mortality in CF patients. It is not known why CF patients are more affected by this bacterium than by other microbes. One theory is that PA has an increased ability to adhere to CF epithelial cells than normal epithelia [3]. Another theory is that CF patients may have a decreased ability to clear PA infections [4,5].

The first PA infections can usually be cured but after repetitive PA infections most patients become chronically infected and the bacteria form different phenotypes, including an alginate-producing, mucoid variant, which forms a biofilm. It is difficult for antibiotics to penetrate the biofilm and it is impossible to completely eradicate a chronic infection. The recommended strategy is to treat PA with early high dose antibiotics which have lowered the incidence of chronic infections [6]. Some drawbacks are an increased risk of antibiotic resistance [7] and antibiotic toxicity such as hearing loss and renal failure [8–10]. An alternative and complement treatment to

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antibiotics could be specific chicken antibodies (IgY) against PA.

IgY has potential as an oral immunotherapy against several types of microbes [11–14]. Specific antibodies are produced by immunization of hens and purification of antibodies from the yolk of their eggs. We have earlier shown that anti-Pseudomonas IgY may prevent PA infections in CF patients [15]. The number of PA infections was reduced in CF patients receiving IgY, and thereby the need for antibiotics was reduced while lung function was maintained.

Anti-Pseudomonas IgY prevents adhesion of PA to dermal epithelial cells *in vitro* [16], but the mechanism has not been fully elucidated. Therefore, we investigated the immunoreactivity of anti-Pseudomonas IgY to further characterize the prophylactic effect of these antibodies and gain increased understanding about the immunological mechanisms behind this immunotherapy and antigens important for adhesion.

2. Experimental

2.1. Anti-Pseudomonas IgY preparation

Hens were immunized as previously described with the following PA strains: PAO1, PAO3, PAO5, PAO6, PAO9 and PAO11 [15]. Antibodies were purified from egg yolk by Fresenius-Kabi (Uppsala, Sweden). To reduce batch to batch variations at least 300 eggs from 10 hens were pooled. The same preparations were given to a number of Swedish CF patients.

2.2. PA strains for protein analysis

The following strains were used: PAO1, PAO503 (*met-9011*, derived from PAO1), PAO503-K2 (chloramphenicol resistant mutant due to hyperproduction of the MexAB-OprM efflux pump), HABS1 (serotype O1 reference strain), PAO-D (mutant with an insertion in the flagellum gene *fliD* of PAO1), PAO-NP (mutant with an insertion in the *pilA* gene of PAO1), PA-NED995 and PA-NED1033 (clinical strains from patients with external otitis). PAO1 and PAO503 were from Bruce Holloway, Monash University, Australia, and PAO-D and PAO-NP from Reuben Ramphal, University of Florida, Gainesville, FL, USA.

2.3. Cultivation of PA strains

PA strains were grown overnight in 100 ml Luria broth (LB) and the cultures were transferred to 2 L flasks with indentations, each containing 1 L LB. The flasks were incubated on a rotary shaker at 37 °C and the bacteria were harvested in the logarithmic phase OD (650 nm) approx. 0.7 after 3–4 h.

2.4. Protein purification

Proteins from individual PA strains were purified in two steps with ReadyPrep Sequential Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The protein composition was determined by 1D SDS-PAGE performed with Invitrogen's (Carlsbad, CA, USA) system and 4–12% polyacrylamide gels (Tris–HCl). The result was very similar in both steps and the supernatants were therefore pooled together. The total protein concentration was measured using the Bradford protein assay (Bio-Rad). Protein preparations were stored at −20 °C until further analysis.

2.5. 2D electrophoresis

Protein preparations were diluted in ReadyPrep 2-D starter kit rehydration/sample buffer (Bio-Rad) to a final concentration of 2 mg/ml. Seventeen centimetres long isoelectric focusing strips, pH 4–7 (Bio-Rad) were passively rehydrated overnight with 300 μl (600 μg) protein preparation under mineral oil at RT. Isoelectric focusing was carried out the next day for a total of 40 kV h using an IPG-phor unit (Bio-Rad) (20 °C). Focused strips were stored at −70 °C. Before the second electrophoresis step, strips were equilibrated 10 min in ReadyPrep 2-D starter kit equilibration buffers I and II (Bio-Rad). Thereafter the strips were attached to 8–16% SDS gels (Tris–HCl, 193 mm × 183 mm × 1.0 mm) Protean II Ready Gel, Precast gel (Bio-Rad). Electrophoresis was performed in a Protean II XI cell (Bio-Rad) and run for 30 min 16 mA/gel, to allow proteins to enter the gel, and then the resistance was increased to 24 mA/gel. Electrophoresis continued for nearly 5 h. Protein spots were visualized with Colloidal Blue Kit (Invitrogen) according to the manufacturer's instructions.

2.6. Immunoblotting

Proteins in the 2D gels were blotted onto nitrocellulose membranes overnight at 30 V in a tank transfer system (Trans-blot cell, Bio-Rad). Thereafter, the membranes were washed in PBS containing 0.5% Tween (PBS-T) for 30 min followed by blocking of unbound sites with 1% BSA in PBS-T for 1 h. After washing the membranes three times for 5 min, the primary antibody, anti-Pseudomonas IgY, diluted 1/1000 in PBS-T (or unspecific IgY as negative control), was added and incubated for 1 h. This was followed by three washes performed as above and then the membranes were incubated with a secondary antibody, alkaline phosphatase conjugated Rabbit anti-Chicken/Turkey IgY (Zymed, San Francisco, CA, USA) diluted 1/1000 in PBS-T. After three additional washes, 10 min each, the membranes were developed in NBT/BCIP substrate (Roche Diagnostics, Mannheim, Germany) until spots appeared after about 1 min. The membranes were rinsed in MQ-H_2O and air dried in the dark.

Western blot and colloidal blue staining was performed in duplicates for most strains tested.

2.7. In-gel tryptic digestion of protein spots

Colloidal blue-stained 2D gels and immunoblots were visually matched. Spots on gels corresponding to spots on immunoblots were picked and an in-gel tryptic digestion was performed. First, each gel spot was destained in 50 mM NH_4HCO_3, 50% methanol and thereafter the spots were dried in a SpeedVac. One microlitre (0.1 μg/μl) porcine trypsin

(Promega, Madison, WI, USA) was added and allowed to draw into each gel piece on ice for 30 min before incubating at 37 °C overnight. Tryptic digestion was stopped by addition of 20 μl 0.1% trifluoroacetic acid (TFA). After vortexing, the supernatant, containing peptide fragments, was transferred to a new tube.

2.8. MALDI-TOF-MS

MALDI-TOF-MS experiments were performed in an Autoflex (Bruker Daltonics, Bremen, Germany) of reflector type time-of-flight mass spectrometer, equipped with a pulsed nitrogen laser working at 337 nm. The instrument was operated in the positive ion mode with delayed extraction at an accelerating voltage of 20 kV and a variable voltage reflectron. The parameter settings were optimized to analyze peptides in both reflector and Post Source Decay (PSD) mode. Before analyses, the instrument was externally calibrated with Bruker standard peptide mixture consisting of seven peptides ranging from *m*/*z* 1046.51 to 3147.

One microlitre tryptic digest was mixed with 1 μl matrix, α-cyano-4-hydroxy-*trans*-cinnamic acid (Agilent Technologies, Palo Alto, CA, USA). Next, 1 μl of the mixture was applied on the MALDI sample plate (Bruker Scout 384/400 AnchorChip) and allowed to air dry (dried-droplet method) before being placed in the mass spectrometer.

For identification of proteins, peptide masses as well as their partial amino acid sequence in given mass-spectra were matched to proteins in ProFound sequence database (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe).

2.9. Peptide sequencing

To confirm the identity of the proteins, peptide fragments were sequenced by MALDI-TOF-MS working in the PSD mode. All samples used for PSD analysis were analyzed in the reflector mode. The ion selector was set at a chosen *m*/*z* value and the presence of neighboring ions was considered by setting a window around the target mass (±10%). The instrument was set for PSD and ion selector was set to *m*/*z* of precursor ions one at a time. The laser intensity was adjusted to obtain unit mass resolution. For each of the voltage segments 200 laser shots were collected. Spectra were finally pasted together after calibration of each segment to yield a single PSD data set, which was interpreted manually. In order to improve fragmentation of the peptides as well as to simplify interpretation of the PSD-spectra, the tryptic peptides were modified at the N-terminal with chemical-assisted fragmentation (CAF). In some cases the lysine containing peptides were also modified at lysine residues at the C-terminal with Lys-tag.

2.9.1. CAF modification

CAF modification was performed with an Ettan CAF-MALDI Sequencing Kit (GE Healthcare Amersham Biosciences AB, Uppsala, Sweden) which sulfonates the N-terminal and adds 136 Da to the primary amines of the peptide fragment [17]. A μZipTip C_{18} (Millipore, Bedford, MA, USA) was washed with 80% acetonitrile containing 0.1% TFA followed by equilibration with 0.1% TFA in water. Thereafter peptide sample was drawn up and down about 15 times to allow desalting and concentration of the peptides on the stationary phase of the tip. After that the tip was washed with 0.1% TFA to remove salts. CAF reagent was dissolved in CAF buffer (10 μg/μl) and drawn slowly up and down 15 times. After at least 3 min incubation, the reaction was stopped by adding stop buffer to remaining CAF reagent. The tip was washed in 0.1% TFA and the sample eluted in 5 μl 80% acetonitrile containing 0.1% TFA.

2.9.2. Lys-tag modification

Peptide samples were lyophilized. 0.8 mg Lys-tag (2-methoxy-4.5-dihydro-1H-imidazole) from QMX laboratories Ltd., Thaxted Essex, UK, was dissolved in 10 μl H_2O and used to dissolve one lyophilized sample. The reaction was carried out at 55 °C for 3 h and then stopped by the addition of 1 μl 100% TFA and 20 μl 0.1% TFA. Lys-tag modifies the ε-amine group of the lysine residues at the C-terminal and adds 68 Da to the molecular mass of the peptides [18]. This converts the lysine residue to an arginine homolog, which facilitates the ionization and desorption of the peptide.

3. Results

PA proteins antigenic for anti-Pseudomonas IgY were identified with 2D gels, immunoblotting and MALDI-TOF-MS.

3.1. 2D electrophoresis and immunoblotting of P. aeruginosa

Protein patterns of the individual PA strains (PAO1, HABS1, PA503, PA503-K2, PAO-D *fliD*, PAO-NP *pilA*, PA-NED995 and PA-NED1033) were very similar, both in gel-to-gel reproducibility and between strains, on colloidal blue-stained 1D and 2D gels (data not shown). Almost all proteins were in the range of pH 4–7, which is why this range was chosen for further analysis using 2D SDS-PAGE. Although colloidal blue-stained 2D gels were similar (Fig. 1A and B), immunoblots of the gels could be divided into two groups, groups A and B, which displayed two different patterns (Fig. 1C and D). No protein spots appeared on control blots incubated with unspecific IgY. There was no difference in immunoblot pattern whether the strains had been used for immunization or not.

3.2. Identification of antigenic proteins

MALDI-TOF-MS analysis produced excellent spectra (Fig. 2A and B) and most spots matched well to proteins in the database. In group A, the major antigenic protein spots were all identified as the same protein, flagellin type a. In group B four large spots were also identified as flagellin, but of b-type. Some spots could not be identified by MALDI-TOF MS.

One modified peptide from each type of flagellin was subjected to PSD MALDI sequencing in order to confirm the identity of the proteins (Fig. 2C and D). The obtained PSD spectra were not of optimal quality due to the low analyte concentration and thus low peak resolution and poor mass accuracy.

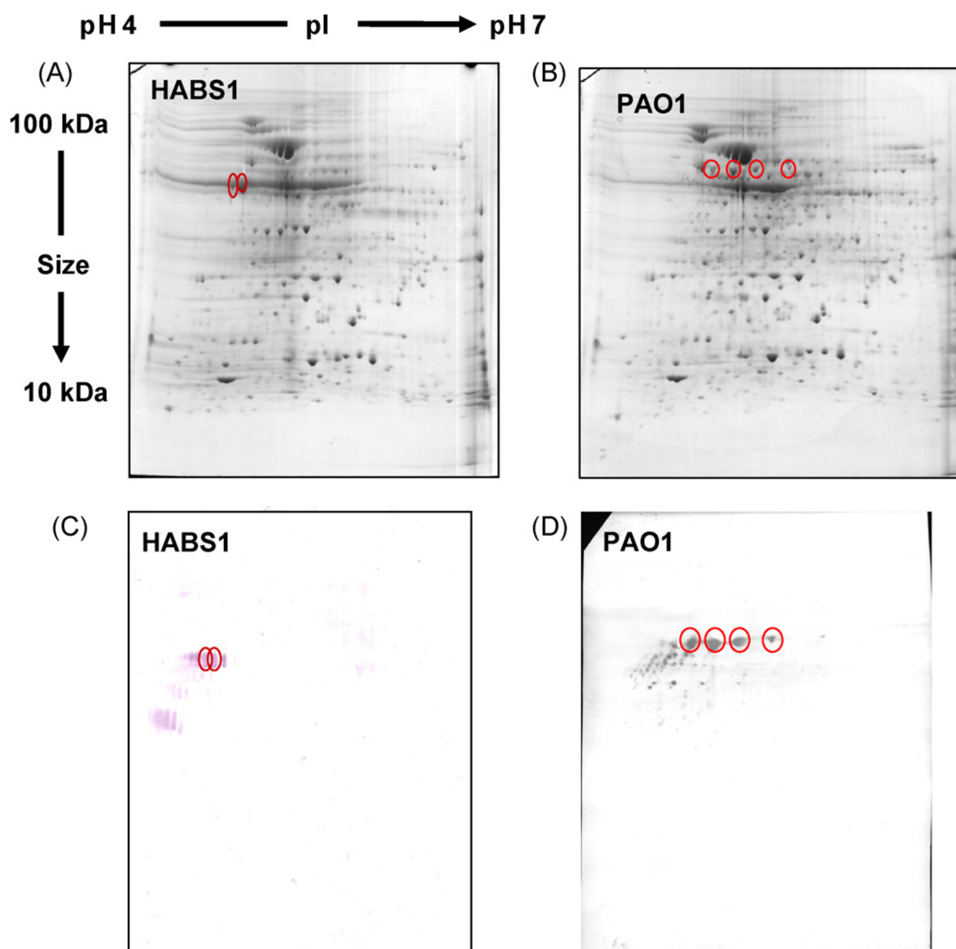

Fig. 1. (A and B) Colloidal blue-stained 2D SDS-PAGE gels of *Pseudomonas aeruginosa* (PA) showed similar patterns between strains, (C and D) but immunoblots of PA proteins incubated with anti-pseudomonas IgY could be divided into two groups depending on the spot pattern. Immunogenic proteins (circled) were identified with MALDI-TOF-MS and database searches as flagellin types a and b.

However, the obtained partial amino acid sequence information for these peptides was useful for conformation of the results and not for de novo sequencing.

Some protein spots appeared on immunoblots, but not on colloidal blue-stained gels. These proteins are probably in low abundance, but very immunogenic, or may be difficult to stain. Alternatively, they could be degraded flagellin.

4. Discussion

The main cause of mortality and morbidity in CF patients is PA lung infections leading to a decline in lung function. Avian antibodies against PA, anti-Pseudomonas IgY, could be used as a complement to antibiotics to fight these infections [15]. In this study, patients gargled with an antibody preparation every night after tooth brushing. The number of positive PA cultures and the need for antibiotics decreased considerably during IgY treatment, while good lung function and nutritional status was maintained. The hypothesis is that IgY binding PA prevents adhesion of the bacteria to oropharynx, which is the route by which PA enters the lower airways [19]. Anti-Pseudomonas IgY reduces adhesion to dermal epithelial cells *in vitro* [16].

Here, we show that anti-Pseudomonas IgY binds the PA protein flagellin. Flagellin is the major component of the flagellum which is required for PA motility and chemotaxis [20,21]. The flagellum is also involved in invasion and the establishment of infection in the host [22]. Mutant strains lacking the gene coding for flagellin, *fliC*, are less capable of inducing infection and mortality [23], and monoclonal antibodies against flagellin reduce mortality and morbidity in murine models [24,25].

Flagellin is highly conserved among bacterial species and triggers innate and adaptive immunity [21,22]. Many bacteria secrete flagellin which elicits a strong inflammatory response in epithelial cells. It is recognized by the host TLR 5 receptor [26], which initiates inflammation. Excessive, persistent inflammation in response to bacterial infections contributes to lung damage in CF patients [27]. It leads to lung destruction and decline in pulmonary function.

Flagellin is an important virulence factor, but at the same time flagellated strains are more frequently phagocytosed than non-flagellated ones [28]. When PA infections progress in CF patients, the bacteria become non-flagellated and non-motile [22], probably to evade the immune system and persist in the lungs. Mucoid PA produces alginate and the alternative sigma factor which induces expression of alginate also inhibits flagellin

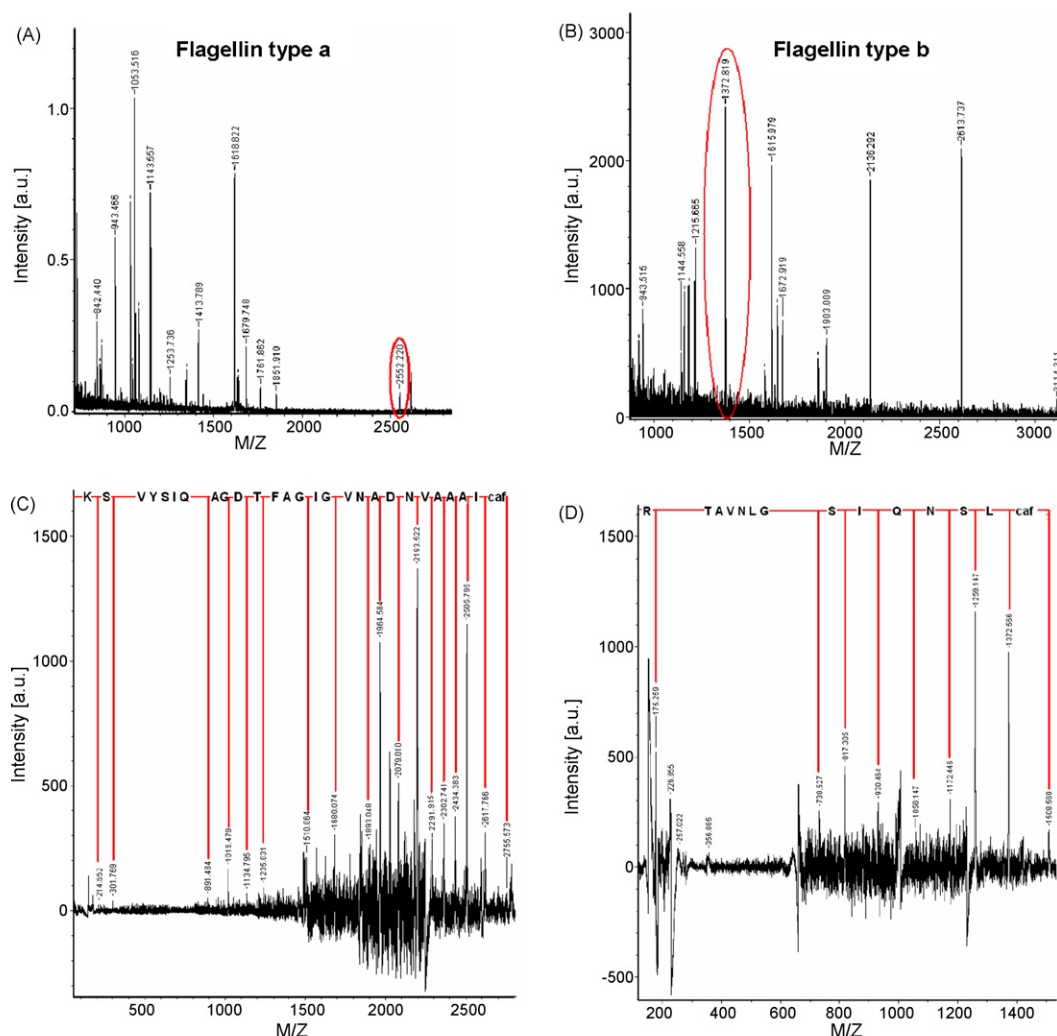

Fig. 2. MALDI-TOF spectra of flagellin (A) type a and (B) type b. Marked peaks were subjected to PSD analysis. Prior to the PSD analysis the peptides were Lys-tag and/or CAF modified. Amino acid sequences were determined by search of signals separated by the mass of an amino acid as shown in panels C and D.

gene expression [29]. PA grown in the presence of airway fluids from chronically infected CF patients represses *fliC* expression [30]. Therefore, anti-Pseudomonas IgY should be most effective at an early stage before infection is established. This should also be true for flagella based vaccines, which have been investigated [31,32]. Today there is no effective vaccine against PA on the market despite several studies in the field [33,34]. IgY might also be effective in the beginning of a PA infection in CF patients due to the low number of bacteria at the point in time. The same is true for vaccines.

PA flagellin can be divided into two types, a and b, depending on the size and antigenicity [35]. Anti-Pseudomonas IgY has affinity for both types. Type b has a molecular weight of 53 kDa, while type a flagellins are more heterologous, both in amino acid sequence and size (45–52 kDa), explaining the small variation in positions of the spots on immunoblots. This is probably also due to posttranslational modifications. For example, flagellin of both types have phosphorylated tyrosines [36] and undergo glycosylation [37,38]. Glycosylation is however less in type b [38].

Several strains were investigated, including some of the ones used for immunization. Anti-Pseudomonas IgY was immunoreactive against all of them, which strengthen its potential as a prophylactic treatment against PA.

In conclusion, anti-Pseudomonas IgY binds flagellin, which may prevent PA infections in CF patients by hindering host invasion due to reduced adherence. IgY may directly affect adherence or indirectly via reduced motility. Since flagellin also is involved in inflammation, the antibodies may also reduce the inflammatory response to PA in CF patients.

References

[1] J.M. Rommens, M.C. Iannuzzi, B. Kerem, M.L. Drumm, G. Melmer, M. Dean, R. Rozmahel, J.L. Cole, D. Kennedy, N. Hidaka, et al., Science 245 (1989) 1059.

[2] M.J. Welsh, A.E. Smith, Cell 73 (1993) 1251.

[3] L. Saiman, A. Prince, J. Clin. Invest. 92 (1993) 1875.

[4] G.B. Pier, M. Grout, T.S. Zaidi, J.B. Goldberg, Am. J. Respir. Crit. Care Med. 154 (1996) S175.

[5] G.B. Pier, M. Grout, T.S. Zaidi, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 12088.

[6] G. Doring, N. Hoiby, J. Cyst. Fibros. 3 (2004) 67.
[7] D.M. Livermore, Clin. Infect. Dis. 34 (2002) 634.
[8] M. Al-Aloul, H. Miller, S. Alapati, P.A. Stockton, M.J. Ledson, M.J. Walshaw, Pediatr. Pulmonol. 39 (2005) 15.
[9] M. Mulheran, C. Degg, S. Burr, D.W. Morgan, D.E. Stableforth, Antimicrob. Agents Chemother. 45 (2001) 2502.
[10] M. Sone, P.A. Schachern, M.M. Paparella, Hear. Res. 115 (1998) 217.
[11] D. Carlander, H. Kollberg, P.E. Wejaker, A. Larsson, Immunol Res. 21 (2000) 1.
[12] M. Kuroki, M. Ohta, Y. Ikemori, R.C. Peralta, H. Yokoyama, Y. Kodama, Arch. Virol. 138 (1994) 143.
[13] J.H. Shin, M. Yang, S.W. Nam, J.T. Kim, N.H. Myung, W.G. Bang, I.H. Roe, Clin. Diagn. Lab Immunol. 9 (2002) 1061.
[14] C. Kobayashi, H. Yokoyama, S.V. Nguyen, Y. Kodama, T. Kimata, M. Izeki, Vaccine 23 (2004) 232.
[15] H. Kollberg, D. Carlander, H. Olesen, P.E. Wejaker, M. Johannesson, A. Larsson, Pediatr. Pulmonol. 35 (2003) 433.
[16] D. Carlander, J. Sundstrom, A. Berglund, A. Larsson, B. Wretlind, H. Kollberg, Pediatr. Pulmonol. 240 (Suppl. 19) (1999).
[17] U. Hellman, R. Bhikhabhai, Rapid Commun. Mass Spectrom. 16 (2002) 1851.
[18] E.C. Peters, D.M. Horn, D.C. Tully, A. Brock, Rapid Commun. Mass Spectrom. 15 (2001) 2387.
[19] G. Doring, S.P. Conway, H.G. Heijerman, M.E. Hodson, N. Hoiby, A. Smyth, D.J. Touw, Eur. Respir. J. 16 (2000) 749.
[20] R.M. Macnab, J. Bacteriol. 181 (1999) 7149.
[21] A.N. Honko, S.B. Mizel, Immunol. Res. 33 (2005) 83.
[22] A. Prince, Am. J. Respir. Cell Mol. Biol. 34 (2006) 548.
[23] M. Feldman, R. Bryan, S. Rajan, L. Scheffler, S. Brunnert, H. Tang, A. Prince, Infect. Immun. 66 (1998) 43.
[24] W.J. Landsperger, K.D. Kelly-Wintenberg, T.C. Montie, L.S. Knight, M.B. Hansen, C.C. Huntenburg, M.J. Schneidkraut, Infect. Immun. 62 (1994) 4825.
[25] Y. Barnea, Y. Carmeli, E. Gur, B. Kuzmenko, A. Gat, L.F. Neville, R. Eren, S. Dagan, S. Navon-Venezia, Plast. Reconstr. Surg. 117 (2006) 2284.
[26] F. Hayashi, K.D. Smith, A. Ozinsky, T.R. Hawn, E.C. Yi, D.R. Goodlett, J.K. Eng, S. Akira, D.M. Underhill, A. Aderem, Nature 410 (2001) 1099.
[27] H. Heijerman, J. Cyst. Fibros. 4 (Suppl. 2) (2005) 3.
[28] E. Mahenthiralingam, D.P. Speert, Infect. Immun. 63 (1995) 4519.
[29] E.S. Garrett, D. Perlegas, D.J. Wozniak, J. Bacteriol. 181 (1999) 7401.
[30] M.C. Wolfgang, J. Jyot, A.L. Goodman, R. Ramphal, S. Lory, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 6664.
[31] G. Doring, F. Dorner, Behring Inst. Mitt. (1997) 338.
[32] G. Doring, C. Pfeiffer, U. Weber, A. Mohr-Pennert, F. Dorner, Am. J. Respir. Crit. Care Med. 151 (1995) 983.
[33] A.B. Lang, A. Rudeberg, M.H. Schoni, J.U. Que, E. Furer, U.B. Schaad, Pediatr. Infect. Dis. J. 23 (2004) 504.
[34] I.A. Holder, Vaccine 22 (2004) 831.
[35] J.S. Allison, M. Dawson, D. Drake, T.C. Montie, Infect. Immun. 49 (1985) 770.
[36] K. Kelly-Wintenberg, S.L. South, T.C. Montie, J. Bacteriol. 175 (1993) 2458.
[37] C.D. Brimer, T.C. Montie, J. Bacteriol. 180 (1998) 3209.
[38] A. Verma, M. Schirm, S.K. Arora, P. Thibault, S.M. Logan, R. Ramphal, J. Bacteriol. 188 (2006) 4395.