Investigation of the *algT* operon sequence in mucoid and non-mucoid *Pseudomonas aeruginosa* isolates from 115 Scandinavian patients with cystic fibrosis and in 88 *in vitro* non-mucoid revertants

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Pseudomonas aeruginosa is the dominant pathogen causing chronic lung infections in patients with cystic fibrosis (CF). After an initial phase characterized by intermittent colonizations, a chronic infection is established upon conversion of *P. aeruginosa* from the non-mucoid to the mucoid, alginate-overproducing phenotype. During the chronic infection the isolation of both mucoid and non-mucoid isolates in CF sputum samples is very common. The purpose of the present study was to establish, by sequence analysis, the types of mutations present in the *algTmucABD* operon in a large number of mucoid and non-mucoid *P. aeruginosa* isolates from Scandinavian CF patients and in *in vitro*-derived non-mucoid revertants. Mucoid (83) and non-mucoid isolates (103) from 91 Scandinavian patients with chronic *P. aeruginosa* infection and 24 non-mucoid isolates from intermittently colonized CF patients were investigated. In addition, 88 spontaneous non-mucoid revertants obtained *in vitro* from nine mucoid CF isolates were also included in the study. Mutations in *mucA* were found in 92 % of the mucoid and in up to 70 % of the non-mucoid isolates from chronically infected patients, indicating that the majority of non-mucoid isolates are revertants. None of the non-mucoid isolates from intermittently colonized CF patients harboured *mucA* mutations. Although *algT* has been considered an important gene for secondary-site mutations responsible for reversion to non-mucoidy, only 30 % of the *mucA*-mutated non-mucoid CF isolates had mutations in *algT*. In contrast, 83 % of the *in vitro*-derived spontaneous non-mucoid revertants had mutations in *algT*, showing that in the CF lung there is a selection for non-mucoid revertants with secondary-site mutations in genes other than *algT*. In addition, we report, to our knowledge for the first time, loss-of-function mutations in the negative regulators *mucB* and *mucD* in CF clinical isolates. In some of the CF isolates these mutations are associated with moderate alginate production. In conclusion, most non-mucoid isolates from chronically infected CF patients are revertants and the mechanism of reversion is *algT*-independent in the CF lung.

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INTRODUCTION

Establishment of the *Pseudomonas aeruginosa* chronic lung infection in the cystic fibrosis (CF) lung is preceded by a period of intermittent colonization with *P. aeruginosa*

strains typically resembling those found in the environment, i.e. non-mucoid and antibiotic sensitive (Doggett *et al.*, 1966; Høiby, 1977). This stage is temporary and represents a window of opportunity for the aggressive treatment of the infection (Frederiksen *et al.*, 1997; Høiby *et al.*, 2005). Untreated, the infection will eventually become chronic, characterized by the appearance of

Abbreviations: CF, cystic fibrosis; PMN, polymorphonuclear leukocytes.

mucoid *P. aeruginosa* isolates (Doggett *et al.*, 1966; Høiby, 1977; Pedersen, 1992) and an inflammatory response dominated by polymorphonuclear leukocytes (PMNs) (Høiby *et al.*, 1993). It has been shown that the reactive oxygen species liberated by PMNs can cause conversion of *P. aeruginosa* from non-mucoid to mucoid (Mathee *et al.*, 1999). Besides the oxygen radicals, various other stress conditions occur in the CF lung environment, such as high osmolarity, dehydration, nutrient limitation and antibiotics, all of which may affect alginate production (Bayer *et al.*, 1990; Berry *et al.*, 1989; DeVault *et al.*, 1990; Speert *et al.*, 1990; Terry *et al.*, 1991; Bagge *et al.*, 2004).

Once the infecting bacteria become mucoid, the infection is very difficult to eradicate due to the biofilm mode of growth (Høiby *et al.*, 2005). Clinically, the presence of mucoid variants is associated with poor prognosis, deterioration of the lung function and increased tissue damage (Pedersen, 1992). The main characteristic of the mucoid phenotype is the production of a thick mucopolysaccharide layer consisting of the exopolysaccharide alginate (Evans & Linker, 1973). Great phenotypic heterogeneity has been shown among mucoid and non-mucoid CF isolates, including colony morphology, biofilm formation properties, antibiotic resistance and media-dependent alginate production (Fyfe & Govan, 1980; Govan *et al.*, 1983; Pugashetti *et al.*, 1982; Drenkard & Ausubel, 2002; Haussler, 2004; Lee *et al.*, 2005).

The alginate biosynthesis operon (*algD*–*algA*, PA3540–PA3551) is under the control of the *algD* promoter. A key element in *algD* gene regulation is the alternative sigma factor AlgT (also termed AlgU or σ^{22}), which induces the expression of *algD* and increases the expression of regulatory proteins that enhance *algD* transcription (Govan & Deretic, 1996). The *algT* gene belongs to an operon with four other genes, *mucA*–*mucB*–*mucC*–*mucD*.

Mutations in either *mucA*, *mucB* or *mucD* can lead to conversion to mucoidy (Martin *et al.*, 1993a, b; Goldberg *et al.*, 1993; Wood & Ohman, 2006), suggesting that the products of these three genes have negative regulator effects on *algT*. While *mucA* mutants are highly mucoid, *mucB* mutants have a slightly mucoid phenotype (Martin *et al.*, 1993a; Mathee *et al.*, 1997), suggesting that MucA is the primary control element of AlgT activity. Mutations in *P. aeruginosa* *mucD* confer alginate production during growth on certain media (e.g. *Pseudomonas* isolation agar), suggesting that MucD can also act as a negative effector of AlgT activity (Boucher *et al.*, 1996).

MucA acts as an anti-sigma factor that binds and sequesters AlgT, thus affecting its ability to transcribe (Schurr *et al.*, 1996). MucB is also a negative regulator that binds to MucA and forms a signal transduction complex that normally keeps the AlgT transcriptional activity low (Schurr *et al.*, 1996; Mathee *et al.*, 1997; Rowen & Deretic, 2000). The function of MucC is generally unknown but it has been reported as being both a positive and a negative regulator (Boucher *et al.*, 1997a). MucD is a close

homologue of *Escherichia coli* HtrA (DegP), a periplasmic serine protease that is involved in the proteolysis of abnormal proteins and is required for resistance to oxidative and heat stress (Boucher *et al.*, 1997b). It has been shown that the proteolytic motif of MucD is important for its regulation of alginate production as well as temperature resistance (Wood & Ohman, 2006). The *mucD* gene also has a secondary promoter, independent of AlgT, within the *algT* operon (Wood & Ohman, 2006).

Activation from a distance is a unique feature of the *algD* promoter. Besides the control exerted by AlgT, MucA and MucB, the response regulators AlgR (Deretic *et al.*, 1989; Nikolskaya & Galperin, 2002), AlgB (Wozniak & Ohman, 1991; Goldberg & Dahnke, 1992) and the DNA-binding protein AlgZ (Baynham *et al.*, 1999) also exert control of *algD* transcription.

Besides controlling alginate production, AlgT is the *P. aeruginosa* equivalent of the extreme heat-shock sigma factor σ^E of *E. coli* (Yu *et al.*, 1995). Congruent with the functions played by the σ^E in *E. coli*, AlgT is involved in the regulation of a number of other systems in *P. aeruginosa*, such as heat shock, osmotic protection and protection against reactive oxygen species (Martin *et al.*, 1994; Yu *et al.*, 1995; Firoved *et al.*, 2002). It has also been shown that AlgT represses flagellum biosynthesis in *P. aeruginosa* (Tart *et al.*, 2006). Thus, the presence of uncontrolled AlgT in mucoid isolates interferes with the normal physiology of the cell and therefore the selective advantage of mucoid forms must be critical for the ability of this organism to persistently colonize and chronically infect CF patients (Schurr *et al.*, 1994). Besides, bacteria use a lot of energy to produce alginate and therefore it is not surprising that the mucoid phenotype is highly unstable in the absence of the selective pressure, e.g. once the strains are taken out from the CF lung (Govan *et al.*, 1979; Ohman, 1981).

Non-mucoid isolates are often isolated from the CF sputum samples with or without simultaneous isolation of the mucoid isolates (Doggett, 1969; Høiby, 1975). These non-mucoid isolates are either wild-type *P. aeruginosa* strains or revertants from the mucoid isolates (Schurr *et al.*, 1994). The revertants might occur by repair of the mutation in *mucA*, or by secondary-site mutations in *algT* or other alginate regulatory genes (DeVries & Ohman, 1994). Inactivation of *algT* is the most frequent mechanism that has been demonstrated for revertants obtained *in vitro* (DeVries & Ohman, 1994; Schurr *et al.*, 1994). However, this is not the only mechanism of conversion to the non-mucoid phenotype; several alternative pathways have been suggested for the reversion to the non-mucoid state (Schurr *et al.*, 1994). It has been shown that the non-mucoid revertants due to mutations in *algT* become motile through upregulation of the flagella and this was considered an adaptive mechanism allowing *P. aeruginosa* to swim towards areas of higher oxygen concentration (Wyckoff *et al.*, 2002; Tart *et al.*, 2006). However, the nature of the non-mucoid isolates has not previously been

investigated on a large number of *P. aeruginosa* isolates collected from the sputum of CF patients.

In a recent study of 37 sequential isolates from 10 CF patients attending the Hannover CF Clinic, Germany, *mucA* mutations were found in both mucoid and non-mucoid isolates but the occurrence of secondary-site mutations responsible for the reversion to the non-mucoid phenotype was not investigated (Bragonzi *et al.*, 2006).

The purpose of the present study was to analyse the mutations in the *algTmucABD* operon, which encodes the most important regulators of alginate production. This was performed in a large collection of mucoid and non-mucoid isolates from the sputum of Scandinavian CF patients with chronic or intermittent *P. aeruginosa* infection as well as in *in vitro*-obtained non-mucoid revertants. The correlation between the genotype of the alginate regulatory genes and the alginate morphotypes is discussed.

METHODS

CF patients, bacterial strains and growth conditions. One hundred and sixty-six paired mucoid and non-mucoid isolates from 83 Scandinavian CF patients with chronic *P. aeruginosa* lung infection were investigated (Denmark, 38 patients; Sweden, 26 patients; and Norway, 19 patients). These patients were randomly chosen from the CF patients attending six different CF clinics (total number of CF patients/number of chronically infected with *P. aeruginosa*): Copenhagen (240/112) and Århus (119/73) in Denmark; Uppsala (44/21) and Lund (109/27) in Sweden; and Oslo and Bergen (201/73) in Norway. Identification of the different *P. aeruginosa* clones in the six CF centres done by PFGE showed a number of different *P. aeruginosa* clones harboured by a number of patients as follows: Copenhagen, 21 different clones/twelve to two patients; Århus, nine different clones/four to two patients; Oslo, two different clones/ten to nine patients; Uppsala, one clone/three patients; and Lund, three clones/four to two patients. Since the Århus centre was established in 1991 and a number of CF patients transferred from Copenhagen, five clones were common, each harboured by four to two patients. The 83 pairs of mucoid and non-mucoid isolates included in this study had similar PFGE patterns and therefore were clonally related. Twenty non-mucoid isolates from eight Danish chronically infected CF patients from whom only non-mucoid colonies were routinely identified were also included in the study.

In addition, non-mucoid isolates from 24 early intermittently colonized Danish CF patients were included in this study.

The CF patients were considered chronically infected when *P. aeruginosa* was cultured in the sputum for six consecutive months or serum contained ≥ 2 precipitating antibodies to *P. aeruginosa* by crossed-immunoelectrophoresis (Høiby, 1977).

Sputum samples obtained by expectoration or endolaryngeal suction were Gram-stained and examined under the microscope to confirm the origin from the lower airways, with the exception of the samples from Norway. The sputum samples of the 83 Scandinavian CF patients were plated on Blue agar plates (a modified Conrad Drigalski medium selective for Gram-negative rods, Statens Serum Institute, Copenhagen, Denmark, containing: peptone 10 g, yeast extract 5 g, NaCl 5 g, agar 11 g, detergent 0.05 g, sodium thiosulphate 1 g, bromthymolblue 0.1 g, lactose 9 g and glucose 0.4 g).

One mucoid and one non-mucoid *P. aeruginosa* isolate, which were simultaneously present in the sputum samples of the 83 Scandinavian

patients, were collected from the Blue agar plate and considered a pair of isolates.

The sputum samples of the eight Danish CF patients who were registered in the records as presenting only non-mucoid isolates were plated on PIA (Pseudomonas isolation agar, Difco, containing peptone 20 g, magnesium chloride 1.4 g, potassium sulphate 10 g, Irgasan 25 mg and agar 13.6 mg), LB and Blue agar plates and incubated for at least 7 days. Colonies with different morphotypes were observed and a total of 20 colonies were isolated (one and up to four different colonies per sputum sample).

From the sputum samples of CF patients with intermittent colonization only non-mucoid isolates were cultured and one isolate from each patient was collected. All isolates were stored at -80°C in broth supplemented with 10 % glycerol.

Spontaneous non-mucoid revertants isolated *in vitro*. Nine different mucoid CF isolates and PAO1 *mucA22* (Mathee *et al.*, 1999) were grown for 4–7 days in continuous culture in flow-cell biofilms as previously described (Lee *et al.*, 2005). The bacteria recovered from biofilm reverted to the non-mucoid phenotype in a proportion of 80 %, and 17 different non-mucoid revertants were isolated.

The nine different mucoid CF isolates and PAO1 *mucA22* were also grown in shaking aerobic conditions for 4 or 7 days and 12 different spontaneous non-mucoid revertants were isolated.

In addition, 60 separate shaking cultures, 30 in aerobic and 30 in anaerobic conditions, of one CF mucoid isolate were performed. One non-mucoid revertant from each of the 60 cultures was isolated.

In all *in vitro* experiments the growth medium was FB minimal medium [1 mM MgCl_2 , 0.1 mM CaCl_2 , non-chelated trace elements, 2 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$, 6 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} \text{ l}^{-1}$, 3 g $\text{KH}_2\text{PO}_4 \text{ l}^{-1}$, 3 g $\text{NaCl} \text{ l}^{-1}$] supplemented with 0.2 % Casamino acids and 0.2 % glucose. Anaerobic growth was performed in the presence of 0.2 % KNO_3 in an anaerobic chamber (85 % N_2 , 5 % CO_2 , 10 % H_2 ; Don Whitley Scientific).

Investigation of the alginate morphotypes on PIA and LB media. Different alginate morphotypes were classified according to Schurr *et al.* (1994) on the basis of their phenotypes on two media, PIA and LB (Fig. 1). Type I was mucoid on PIA and LB; type II was mucoid on PIA but lost its mucoid appearance on LB; type III was non-mucoid on both PIA and LB; and type IV displayed very slight but detectable mucoidy on both media after an incubation period of at least 4 days (Boucher *et al.*, 1996; Schurr *et al.*, 1994). In order to classify the 83 pairs of mucoid and non-mucoid isolates into the different phenotypes, the isolates were subsequently grown on Blue agar plates, PIA plates and LB plates and followed for 7 days. The 20 isolates of the eight CF patients with chronic non-mucoid *P. aeruginosa* were recovered after prolonged growth of the sputum samples on the three different media.

Genotyping by PFGE. All isolates were genotyped by PFGE as described previously using *SpeI* enzyme (Ojeniyi *et al.*, 1991; Römmling & Tümmler, 2000). After PFGE, the band patterns were visualized by ethidium bromide staining and then photographed (GelDoc imaging system, Bio-Rad). The patterns were analysed by Fingerprinting II software (Bio-Rad). The clonal relatedness of the individual pairs of mucoid and non-mucoid *P. aeruginosa* was confirmed according to Tenover *et al.* (1995). Isolates with PFGE patterns that differed from each other by two to three bands were considered clonally related, as this pattern is consistent with a single genetic event, a point mutation or an insertion or deletion of the DNA. Isolates with PFGE patterns that differed by more than three bands were considered to belong to different strains.

Fig. 1. Examples of the four alginate morphotypes on the basis of their phenotypes on two media, PIA (left) and LB (right), after 2 days incubation at 37 °C. Type I was mucoid on both PIA and LB; type II was mucoid on PIA but lost its mucoid appearance on LB; type III was non-mucoid on both PIA and LB. After 7 days incubation at 37 °C type IV showed increased alginate production on PIA and remained non-mucoid on LB.

Sequence analysis of *algTmucABD* operon. DNA extraction was carried out according to the manufacturer using a commercial DNA isolation kit (Puregene, Gentra Systems).

For PCR amplification and sequencing of *algT*, *mucA*, *mucB* and *mucD*, the primers shown in Table 1 were used. PCR amplification of the entire *algTmucABD* operon was carried out using DYNazyme EXT DNA polymerase (FINNZYMES).

The sequencing was done on a Macrogen automatic DNA sequencer ABI3700. The numbers of reads were between two and four for each gene of each strain. The sequence results were compared with the strain PAO1 sequence (www.pseudomonas.com) with DNASIS MAX version 2.0 (Hitachi Software Engineering), in order to determine the occurrence of sequence variants within the *algT* operon.

Complementation analysis. Triparental mating was used to mobilize the *algT*-containing recombinant plasmid pCD100 from *E. coli* JM109 to non-mucoid *P. aeruginosa* isolates showing a *mucA* *algT* mutant genotype with the conjugation helper plasmid pRK600 (Kessler *et al.*, 1992), as previously described (Hoffmann *et al.*, 2005). Transconjugants of *P. aeruginosa* were selected on PIA agar containing 80 µg tetracycline ml⁻¹, and complementation of *algT* mutations was scored by the mucoid phenotype observed on PIA plates after incubation at 37 °C to confirm that the mutations found in *algT* were responsible for the non-mucoid phenotype.

Alginate measurement. Alginate production was measured using a borate/carbazol method (Knutson & Jeanes, 1968) on overnight cultures in ox broth of the 83 pairs of mucoid and non-mucoid *P. aeruginosa* isolates from chronically infected CF patients. D-Mannuronate lactone (Sigma) was used to calibrate a standard curve, and PAO1 and PAO1 *mucA22* were used as control strains. The results are presented as medians (range) and the unpaired *t*-test (Statview 4.5 software, Abacus Concepts) was used to compare the different alginate morphotype groups. Our group has previously shown that this method is specific for alginate (Pedersen *et al.*, 1989).

Table 1. Primers used to amplify and sequence the genes in the *algTmucABD* operon

Primer	Sequence (5'–3')
<i>algT</i> -sek1	CCTGAGCCCCGATGCAATCCATTTTCG
<i>algT</i> -sek2	GGTATGCCTTGATGAAGGCTTCCTGC
<i>algT</i> -sek3	GCTGTATCGGATCGCCATCAACACC
<i>algT</i> -sek4	GGACAGAGTTTCCTGCAGGGCTTCAC
<i>mucA</i> -1	CTCTGCAGCCTTTGTTGCGAGAAG
<i>mucA</i> -1 rev	CTGCCAAGCAAAAGCAACAGGGAGG
<i>mucA</i> -2	GTGCGTCTGTACAACCAGAACGACG
<i>mucA</i> -2 rev	GTCGTTCTGGTTGTACAGACGCACG
<i>mucB</i> -1	ATCCGCCGTCAGTGGTACAG
<i>mucB</i> -1 rev	CGAGCAGGACGAGCAGGTAC
<i>mucB</i> -int1	CAGTGGTCCTTGCGGTGACT
<i>mucB</i> -int2	TTCAGCAGCAGCGACTTCAA
<i>mucD</i> -1	GTCCGATTTCGGCCTGAGTCT
<i>mucD</i> -1 rev	ACGCAGGTAACGGATTGACG
<i>mucD</i> -int1	GATCAACCCGGGTAACCTCCG
<i>mucD</i> -int2	AGATCTGCGAGTTGATGCCG
<i>mucD</i> -int3	CATCCTCACCAACAATCACGTCGTGG
<i>mucD</i> -int4	GCTCGCTACGGTCGGACAGG
<i>mucD</i> -int5	CAGCGCAAGTCCCTGAGCATGG
<i>mucD</i> -int6	GCTTCATGTTGCCACCAGGTGC

RESULTS

Clonal distribution of the mucoid and non-mucoid isolates

Among the 38 Danish patients with chronic *P. aeruginosa* infection, two dominant clones were found: Dk1 (16 patients) and Dk2 (9 patients). CF patients from both the Copenhagen and the Århus CF centres harboured these clones. The presence of these two dominant clones was published previously by our group (Ojeniyi *et al.*, 1993; Jelsbak *et al.*, 2007). Among the 26 Swedish patients, three different clones were found among patients attending the Lund CF centre: L1 (three patients), L2 and L3 (each infecting two patients). One patient from Lund and one patient from Århus harboured the same clone. Among the 19 Norwegian patients, one dominant clone was found, clone N (13 patients). The presence of this dominant clone among Norwegian CF patients was published previously (Fluge *et al.*, 2001). Unique clones among *P. aeruginosa* isolates from Denmark (Ud), Sweden (Us) and Norway (Un) were found in the rest of the patients.

Among the 24 CF patients with intermittent colonization, three clones, each harboured by two patients, were found, while the rest of the patients harboured unique clones.

The genotyping analysis showed that with the exception of one patient from Sweden and one from Denmark who shared the same clone, no cross-infection occurred among the Danish, Swedish and Norwegian CF patients attending the six different clinics in Scandinavia. Patients who moved to a newly established clinic in Denmark harboured some of the dominant clones from the original centre.

Distribution of the different alginate morphotypes

Four alginate morphotypes could be distinguished, in agreement with Schurr *et al.* (1994): see Fig. 1.

The alginate phenotype of the mucoid and non-mucoid isolates from the 83 chronically infected CF patients was initially determined on Blue agar plates. Subsequent culture on PIA and LB plates showed that 87% of the non-mucoid and 89% of the mucoid maintained their phenotype on PIA and LB. This means that 87% of the non-mucoid isolates showed a type III alginate production and 89% of the mucoid isolates a type I alginate production.

However, six non-mucoid and eight mucoid isolates showed a phenotype of Alg⁺ on PIA and Alg⁻ on LB, corresponding to a type II alginate morphotype, and five non-mucoid isolates showed slightly increased alginate production on PIA after 7 days incubation, corresponding to type IV alginate production.

From the eight CF patients with chronic infection with non-mucoid *P. aeruginosa*, five isolates showed type IV alginate production, starting to produce increased amounts of alginate after prolonged incubation, while the rest of the

15 non-mucoid isolates maintained their phenotype and showed type III alginate production.

When the phenotypic characterization of alginate production (types I, II, III and IV) as judged by the colony morphology on PIA and LB plates was compared to the level of alginate production measured on overnight culture in ox broth, a good correlation was found. The median amount of alginate measured in isolates that showed a type I, II, III and IV morphotype was 180.69 (16.8–757.9), 79.6 (1.07–449), 0 (0–27.9) and 2 (0–496) mg l⁻¹, respectively. The differences between the different groups were statistically significant. This shows that colony morphology on PIA plates correlates with the amount of alginate produced by the isolates in a rich medium such as ox broth.

Mutations in *mucA*, *mucB* and *mucD* in non-mucoid and mucoid *P. aeruginosa* isolates from chronically infected patients

The DNA sequence analysis of *mucA* carried out on non-mucoid (103) and mucoid (83) *P. aeruginosa* isolates from chronically infected Scandinavian CF patients showed that 71 (69%) of the non-mucoid isolates and 77 (93%) of the mucoid isolates had mutations in *mucA*. No mutations in *mucA* were found in the non-mucoid isolates from 24 intermittently colonized patients. The types of *mucA* mutations in Scandinavian mucoid and non-mucoid isolates are presented in Table 2.

The most common mutation was a C/T transition at position 349 or 352 bp of the *mucA* gene (*mucA* consists of 585 bp), which resulted in a stop codon, followed by the *mucA22* mutation, a deletion of a G within the homopolymeric stretch of five G residues located between positions 429 and 433 of the *mucA* coding region.

An association between the dominant clones and the types of *mucA* mutation was observed: clone Dk1 and T349C, clone Dk2 and *mucA22*, and clone N and T352C. These associations provide an explanation for the high frequency with which these types of *mucA* mutations were found among the Scandinavian CF isolates. However, the same types of mutations were also found in unrelated clones from different geographical locations in Scandinavia, suggesting that these loci represent hot-spot regions for mutation in *mucA*. In addition, the *mucA22* mutation was reported previously in several mucoid isolates from the USA, Europe and Australia (Boucher *et al.*, 1997b; Bragonzi *et al.*, 2006; Anthony *et al.*, 2002). Isolates belonging to the Dk1, Dk2 and N clones showed several other types of mutations.

The sequence alterations found in *mucB* and *mucD* in mucoid and non-mucoid isolates of the chronically infected CF patients are presented in Table 3. For *mucB* we identified several insertions and deletions, leading to frameshifts and premature stop codons. We also identified a T deletion at position 41 of *mucD* leading to a premature stop codon. These types of gene alterations probably result

Table 2. Types of mutations and the respective changes determined in the *mucA* sequence of mucoid (M) and non-mucoid (NM) CF *P. aeruginosa* isolates

The number of isolates with the respective mutations and the clonal distribution of the isolates are also shown.

Mutation in <i>mucA</i>	Sequence change	M (83)	NM (103)	Clones
C137T	Ala/Val		5	Ud
ΔCC160	Frameshift, stop codon 474	1		Dk1
ΔTA162	Frameshift, stop codon 474		1	N
Insertion C at 222	Frameshift, stop codon 478	1		Ud
Δ14 bp at 223	Frameshift, stop codon 462	1	1	Ud
Insertion 5 bp at 253	Frameshift, stop codon 289	2	2	L3
Δ5 bp at 266	Frameshift, stop codon 471	1		Ud
Δ14 bp at 297	Frameshift, stop codon 567	1	1	Dk2
Insertion 4 bp at 300	Frameshift, stop codon 481	1	1	Ud
Δ105 bp at 307	Frameshift, stop codon 478	3	4	N
Insertion T at 326	Frameshift, stop codon 478	1	1	Dk1
Δ42 bp at 339	Frameshift, stop codon 541		2	N
C340T	Stop codon	1	3	Us
T349C	Stop codon	15	16	Dk1, Un, Ud
T352C	Stop codon	14	8	N, L2, Ud, Us
ΔG357	Frameshift, stop codon 385	1	1	Us
ΔG358	Frameshift, stop codon 478	1		Us
ΔC360	Frameshift, stop codon 385	1	1	Un
ΔC365	Frameshift, stop codon 385	2	1	Ud, Un
T367C	Stop codon	1	1	Us
Δ19 bp at 368	Frameshift, stop codon 420	1	1	Ud
Insertion C at 368	Frameshift, stop codon 478	1		Ud
C382T	Stop codon	1		L2
G421T	Stop codon	1		L1
C424T	Stop codon	2	1	Us
ΔG430	Frameshift, stop codon 439	21	18	Dk2, Us, Un, Ud
Insertion CG at 431	Frameshift, stop codon 441	1		Us
Insertion GCG at 431	Frameshift		1	Us
ΔG at 432	Frameshift, stop codon 439	1	1	Un

in loss of function of their gene products and have apparently not been reported before. Some isolates had several point mutations leading to multiple amino acid changes in MucB or MucD. However, in a large number of the CF isolates simultaneous mutations in *mucA* and *mucB* or *mucD* were identified (results not shown). Therefore, the alginate regulator roles played by *mucB* and *mucD* are difficult to assess from our data. We identified five isolates with sequence alterations in *mucB* or *mucD* and wild-type *mucA*. One isolate showed a mucoid phenotype only on PIA (type II) and four isolates showed a mucoid phenotype after prolonged incubation (type IV) (Table 3). The isolates with type II alginate production had a 4 bp insertion at position 808 in *mucB*, resulting in a frameshift and a premature stop codon at position 855 (*mucB* consists of 951 bp). Two of the isolates with type IV alginate production harboured a Δ162 bp at 235 and an 8 bp insertion at 466, resulting in premature stop codons in *mucB*. The other two isolates with type IV alginate production showed a ΔT41, resulting in a stop codon in *mucD* (*mucD* consists of 1425 bp), and several point mutations in *mucD* (A409G, C673G, G1321A), resulting in

amino acid changes (Table 3). These findings suggest that these sequence alterations might result in loss of function of the negative regulators MucB or MucD, leading to increased transcription of the alginate operon when growing on PIA plates or after prolonged incubation.

Interestingly, the more common single-point mutations C338T and A631G found in *mucB* or A409G, C673G and G1321A in *mucD* that led to amino acid changes in the respective encoded proteins were reported previously in German isolates (Bragonzi *et al.*, 2006) and might suggest that these are hot-spot regions for mutations in *mucB* and *mucD*.

Mutations in the *algT* gene in non-mucoid *P. aeruginosa* isolates

Of the 71 non-mucoid isolates with a *mucA* mutant genotype, only 37 (52 %) had sequence alterations in *algT*. Moreover, some *algT* mutations, such as A55G resulting in a change of Lys to Glu (13 non-mucoid isolates), C115T resulting in a change of Arg to Trp (one non-mucoid

Table 3. Types of mutations and the changes determined in the sequence of *mucB* and *mucD* in mucoid (M) and non-mucoid (NM) isolates

The number of isolates with the respective mutation is shown. Isolates with reduced alginate production (such as type II or IV) are indicated. Some isolates had several mutations in *mucB* and *mucD*; these are shown by superscript numbers (¹⁻⁶) for each mutation type.

Mutation	Sequence change	M (83)	NM (103)
Mutations in <i>mucB</i>			
A77G	Asp/Gly	2	2
C157T	Pro/Ser	1	1
G179T ¹	Arg/Leu		1
C208T	Arg/Cys		1
Insertion GC at 235	Frameshift, stop codon 418	1	
Δ162 bp at 235	Frameshift, stop codon 787		4 (IV)
T245C ²	Val/Ala		1
G331T ³	Asp/Tyr		1
C338T* ⁴	Ser/Phe	6	6
A356G	Tyr/Cys	1	1
T420C* ⁵	Glu/Asp	1	
Insertion 8 bp at 466	Frameshift		1 (IV)
G468A ⁵	Glu/Asp		
Insertion A at 545	Frameshift, stop codon 853		1
ΔGGC at 550 ²	Frameshift, stop codon 945		
Insertion A at 570 ³	Frameshift		
Insertion T at 576 ³	Frameshift		
Insertion A at 594 ³	Frameshift		
Insertion 44 bp at 610	Frameshift, stop codon 717		1
G611A ⁶	Ala/Gly	3	3
A631G* ^{5,6}	Thr/Ala	3	3
Insertion 4 bp at 808 ⁴	Frameshift, stop codon 855		(II)
A847G ¹	Thr/Ala		
ΔT 858	Frameshift, stop codon 867	1	1
Insertion A at 906	Frameshift		1 (IV)
Mutations in <i>mucD</i>			
ΔT 41	Stop codon		1 (IV)
C47T	Ala/Val		1
A163G	Met/Val	1	1
A245G	Gln/Arg		1
T274C ¹	Cys/Arg	1	1
G361A ²	Glu/Lys		1
A409G* ^{1,2,3,4}	Ile/Val	42	39
C467T ³	Pro/Leu		1
T515G	Val/Gly		1
C673G* ⁴	Gln/Glu	1	1
A1004T	Val/Ala		1
A1040T	Asp/Val	2	2
G1321A* ⁴	Val/Ile	1	(IV)

*Mutations previously reported by Bragonzi *et al.* (2006).

isolate), C389T resulting in a change from Ala to Val (one non-mucoid isolate) and C430T resulting in a change of Arg to Cys (one non-mucoid isolate), were also found in the *algT* sequence of the paired mucoid isolates, suggesting that these mutations do not affect the functionality of AlgT.

Thus, *algT* mutations present only in isolates with a non-mucoid phenotype were found in 21 isolates representing 30 % of the *mucA* mutant non-mucoid revertants (Table 4). With the exception of C58T (one isolate), C496T (one isolate), C499T (seven isolates) and a T deletion at position 97 (one isolate), all the other mutations could be complemented by pCD100, encoding the wild-type *algT* (Table 4). Although we could not confirm by complementation the effect of the T deletion at position 97, this mutation very probably causes a loss of function of the gene product, as it leads to a frameshift and a stop codon at position 106 bp in *algT* (*algT* consists of 582 bp). Even though we did not succeed in the complementation of all point mutations, we cannot exclude that the functionality of the *algT* gene product is affected by these point mutations (Table 4). In contrast to the low percentage of CF non-mucoid revertants that had changes in *algT*, up to 83 % (73 out of 88) of the spontaneous non-mucoid revertants obtained *in vitro* showed mutations in *algT* (Table 5).

Fifteen of the 88 *in vitro* revertants had a wild-type *algT* allele and, interestingly, 14 of them were recovered after anaerobic or biofilm growth. This might suggest that under stress conditions such as low oxygen tension, a selection of isolates with impaired AlgT function might occur.

The types of *algT* mutations found in the 21 clinical non-mucoid revertants were different from the types of mutations found in the 88 *in vitro*-obtained isolates. However, a 9 bp insertion found in 3 out of 21 CF isolates was frequently observed in the *in vitro* isolates, especially

Table 4. Types of mutations and the determined changes in the sequence of *algT* in non-mucoid clinical isolates with a *mucA* mutant genotype

The number of isolates showing the respective mutation is shown. Complementation by wild-type *algT* is also indicated.

Mutation in <i>algT</i> in clinical isolates	Complementation	Sequence changes	No. of isolates (21)
C58T		Arg/Trp	1
ΔT97		Frameshift, stop codon 106	1
Insertion 9 bp at 138	pCD100	Frameshift, stop codon 589	3
C142T	pCD100	Stop codon	2
A143G	pCD100	Gln/Arg	1
G164T	pCD100	Cys/Phe	1
A212T	pCD100	Tyr/Phe	1
T224C	pCD100	Leu/Pro	1
G344A	pCD100	Gly/Asp	1
G496T		Gly/Cys	1
C499T		Pro/Ser	7
Insertion T at 525	pCD100	Frameshift, stop codon 536	1

after aerobic growth. Interestingly, a mutation at codon 29 leading to a change of Tyr to Cys previously found by DeVries & Ohman (1994) in non-mucoid revertants was also present among the *in vitro* revertants obtained in this study.

DISCUSSION

Sequence analysis of the *algT* operon in a large number of mucoid and non-mucoid *P. aeruginosa* isolates from Scandinavian CF patients showed that the majority of the non-mucoid isolates are revertants from the mucoid phenotype as they have mutations in *mucA*. Although *algT* has been considered one of the main genes for secondary-site mutations resulting in reversion to non-mucoidy, inactivation of *algT* was found in only 30 % of the CF revertants. However, a much higher percentage (86 %) of the *in vitro* spontaneous revertants showed inactivation of *algT*. Previous studies showed that 9 out of 28 (DeVries & Ohman, 1994) and two out of three (Schurr *et al.*, 1994) spontaneous revertants had mutations in *algT*.

These data show that although *algT* mutants can grow and survive in laboratory conditions, they are less proficient in the CF lung environment due to the role played by AlgT in adaptation to stress. The large variety of the *algT* mutations found in the present and previous studies might suggest

that *algT* contains no hot-spot regions for mutations as is the case for its negative regulator, *mucA*.

The role played by AlgT in stress adaptations has been established by several studies.

Transcriptomic analysis revealed differential AlgT-dependent expression of the *osmC* and *osmE* genes, which potentially play a role in stress defence (Palma *et al.*, 2004). Therefore, *algT* mutants would be less proficient in adapting to osmotic stress. It has also been shown that *algT* mutants of *P. aeruginosa* showed increased susceptibility to reactive oxygen intermediates (Martin *et al.*, 1994; Yu *et al.*, 1996), indicating that even though *algT* mutants occur frequently *in vitro* (DeVries & Ohman, 1994; Schurr *et al.*, 1994), they are probably destroyed by the host response *in vivo*. Finally, in a recent paper (Wood *et al.*, 2006) *algT* was identified together with *algB* and *algR* as an important regulator of the alginate induction by environmental factors such as cell-wall-inhibitory antibiotics. If *algT* activation is involved in the response to cell-wall stress, then an *algT* mutant could be more susceptible to cell-wall antibiotics and therefore easily destroyed in the lung by the antibiotic treatment of the chronic lung infection. If this is true, we can not exclude a selection bias towards non-mucoid revertants with wild-type *algT*, which might be more resistant to antibiotics. Another possible explanation could be that mutations that arise during the strong

Table 5. Types of mutations and the determined sequence changes in *algT* found in non-mucoid revertants obtained *in vitro*

The conditions under which these revertants occurred (biofilm, aerobic and anaerobic growth) are shown. The numbers of isolates with the respective mutations are indicated.

Mutation in <i>algT</i>	Sequence alteration	Growth condition	No. of isolates (88)
Δ16 bp at 21	Frameshift, stop codon 64	Biofilm	1
C37T	Trp/Arg	Biofilm 1; anaerobic 11	12
Insertion 11 bp at 50	Frameshift, stop codon 90	Anaerobic	1
A86G	Tyr/Cys	Biofilm	1
Insertion T at 97	Frameshift, stop codon 205	Anaerobic	1
C122T	Ala/Val	Biofilm	1
Insertion 9 bp at 138	Frameshift, stop codon 589	Aerobic 22; biofilm 4	26
G145A	Asp/Asn	Aerobic	1
Insertion 9 bp at 147	Frameshift, no stop codon	Aerobic 9; anaerobic 3	12
ΔT167	Frameshift, stop codon 294	Aerobic	1
C182T	Ala/Val	Aerobic	1
Insertion C at 192	Frameshift, stop codon 204	Aerobic	1
ΔT204	Frameshift, stop codon 295	Aerobic	1
ΔT214	Frameshift, stop codon 294	Biofilm	1
G230T	Arg/Leu	Biofilm	1
Insertion 7 bp at 342	Frameshift, stop codon 385	Aerobic	1
C361T	Arg/Trp	Anaerobic	1
C412T	Stop codon	Biofilm	1
ΔT476	Frameshift, stop codon 487	Aerobic	1
ΔG507	Frameshift, no stop codon	Aerobic	1
A508G	Thr/Ala	Biofilm	1
ΔG511	Frameshift, no stop codon	Anaerobic	5

selection pressure in the CF lung are different from the mutations that arise during non-selective growth in the laboratory. Interestingly, *in vitro* spontaneous non-mucoid isolates with wild-type *algT* were recovered after anaerobic or biofilm growth conditions, suggesting that the non-mucoid CF isolates might occur in the anaerobic conditions encountered in the mucus plugs present in the conductive airways of the CF lungs.

Although the existence of alternative pathways that cause revertance to the non-mucoid phenotype has been postulated, the site of these mutations is not yet known. However, the investigations of the alginate morphotypes on LB and PIA agar might suggest that mutations in the regulatory genes *algB* and *algR* could be candidates for the secondary mutations responsible for *in vivo* revertance. PIA medium contains Irgasan, the trade name for triclosan, which has been shown to be a strong alginate inducer by a mechanism dependent on *algT*, *algB* and *algR* (Wood *et al.*, 2006). Thus, lack of inducibility of alginate production on PIA agar (type III alginate production) should characterize mutants in one of the three genes. Our study shows that the majority of the CF non-mucoid revertants showing a type III morphotype had no mutations in *algT* and therefore mutations in *algB* and *algR* could explain this morphotype.

The *mucA* mutations identified in non-mucoid CF isolates were also found in the paired mucoid isolates. In agreement with previous studies (Boucher *et al.*, 1997b; Yoon *et al.*, 2006; Bragonzi *et al.*, 2006), mutations in *mucA* were found to be the main mechanism of conversion to mucoidy. However, we were also able to identify loss-of-function mutations in *mucB* or *mucD* in some non-mucoid isolates with increased alginate production on PIA plates (type II) or after prolonged incubation (type IV). As these non-mucoid isolates with attenuated alginate production *in vitro* have mutations in the alginate regulatory genes, they might have the potential for increased alginate production *in vivo* in the CF lung. In some CF patients with chronic *P. aeruginosa* infections no mucoid isolates are cultured. We suggest that these *P. aeruginosa* isolates have *mucB* or *mucD* mutations and might be misinterpreted as non-mucoid in the clinical microbiological laboratory.

Mucoid isolates with a wild-type *mucA* allele were found in 7 % of the isolates, showing, in agreement with previous studies (Martin *et al.*, 1993b), that other loci are involved in the conversion to mucoidy. However, this seems to be a rare event among Scandinavian CF isolates compared to mucoid isolates from CF clinics in the USA and Canada, where, according to Yoon *et al.* (2006), approximately 13 % of the mucoid isolates had a wild-type *mucA*.

As expected, 100 % of the non-mucoid strains isolated from intermittently colonized patients showed wild-type *mucA*, confirming that the initial colonization of the CF patients occurs with environmental wild-type isolates that convert to alginate overproducers under the selection pressure in the CF lung. The demographic data showed

that the intermittently colonized patients were much younger than the chronically colonized patients. These intermittently colonized patients are in the window of opportunity for the treatment of the infection before mutations in *mucA*, responsible for conversion to mucoidy, occur.

Non-mucoid isolates from chronically colonized CF patients with wild-type *mucA* allele were found in 30 % of the isolates, suggesting that these isolates might be the original wild-type isolates that infected the patient. However, some of these isolates showed *algT* mutations (data not shown), raising the possibility that acquisition of a wild-type *mucA* allele had occurred by horizontal gene transfer.

In conclusion, most of the non-mucoid CF isolates are revertants from mucoid isolates and the mechanism of revertance is *algT*-independent; this is in contrast to the *in vitro*-obtained isolates, which frequently show mutations in *algT*. Investigations are continuing in our laboratory to find out which genes might be responsible for the conversion to non-mucoidy *in vivo*.

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REFERENCES

- Anthony, M., Rose, B., Pegler, M. B., Elkins, M., Service, H., Thamotharampillai, K., Watson, J., Robinson, M., Bye, P. & other authors (2002). Genetic analysis of *Pseudomonas aeruginosa* isolates from the sputa of Australian adult cystic fibrosis patients. *J Clin Microbiol* **40**, 2772–2778.
- Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E. P. & Høiby, N. (2004). *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. *Antimicrob Agents Chemother* **48**, 1175–1187.
- Bayer, A. S., Eftekhari, F., Tu, J., Nast, C. C. & Speert, D. P. (1990). Oxygen-dependent up-regulation of mucoid exopolysaccharide (alginate) production in *Pseudomonas aeruginosa*. *Infect Immun* **58**, 1344–1349.
- Baynham, P. J., Brown, A. L., Hall, L. L. & Wozniak, D. J. (1999). *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and *algD* transcriptional activation. *Mol Microbiol* **33**, 1069–1080.
- Berry, A., DeVault, J. D. & Chakrabarty, A. M. (1989). High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. *J Bacteriol* **171**, 2312–2317.
- Boucher, J. C., Martinez-Salazar, J., Schurr, M. J., Mudd, M. H., Yu, H. & Deretic, V. (1996). Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. *J Bacteriol* **178**, 511–523.

- Boucher, J. C., Schurr, M. J., Yu, H., Rowen, D. W. & Deretic, V. (1997a). *Pseudomonas aeruginosa* in cystic fibrosis: role of *mucC* in the regulation of alginate production and stress sensitivity. *Microbiology* **143**, 3473–3480.
- Boucher, J. C., Yu, H., Mudd, M. H. & Deretic, V. (1997b). Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of *muc* mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect Immun* **65**, 3838–3846.
- Bragonzi, A., Wiehlmann, L., Klockgether, J., Cramer, N., Worlitzsch, D., Doring, G. & Tümmler, B. (2006). Sequence diversity of the *mucABD* locus in *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Microbiology* **152**, 3261–3269.
- Deretic, V., Dikshit, R., Konyecsni, W. M., Chakrabarty, A. M. & Misra, T. K. (1989). The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. *J Bacteriol* **171**, 1278–1283.
- DeVault, J. D., Kimbara, K. & Chakrabarty, A. M. (1990). Pulmonary dehydration and infection in cystic fibrosis: evidence that ethanol activates alginate gene expression and induction of mucoidy in *Pseudomonas aeruginosa*. *Mol Microbiol* **4**, 737–745.
- DeVries, C. A. & Ohman, D. E. (1994). Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. *J Bacteriol* **176**, 6677–6687.
- Doggett, R. G. (1969). Incidence of mucoid *Pseudomonas aeruginosa* from clinical sources. *Appl Microbiol* **18**, 936–937.
- Doggett, R. G., Harrison, G. M., Stillwell, R. N. & Wallis, E. S. (1966). An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis of the pancreas. *J Pediatr* **68**, 215–221.
- Drenkard, E. & Ausubel, F. M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**, 740–743.
- Evans, L. R. & Linker, A. (1973). Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. *J Bacteriol* **116**, 915–924.
- Firoved, A. M., Boucher, J. C. & Deretic, V. (2002). Global genomic analysis of AlgU (σ^E)-dependent promoters (sigmulon) in *Pseudomonas aeruginosa* and implications for inflammatory processes in cystic fibrosis. *J Bacteriol* **184**, 1057–1064.
- Fluge, G., Ojieniyi, B., Høiby, N., Digraanes, A., Ciofu, O., Hunstad, E., Haanaes, O. C. & Storrosten, O. T. (2001). Typing of *Pseudomonas aeruginosa* strains in Norwegian cystic fibrosis patients. *Clin Microbiol Infect* **7**, 238–243.
- Frederiksen, B., Koch, C. & Høiby, N. (1997). Antibiotic treatment of initial colonization with *Pseudomonas aeruginosa* postpones chronic infection and prevents deterioration of pulmonary function in cystic fibrosis. *Pediatr Pulmonol* **23**, 330–335.
- Fyfe, J. A. & Govan, J. R. (1980). Alginate synthesis in mucoid *Pseudomonas aeruginosa*: a chromosomal locus involved in control. *J Gen Microbiol* **119**, 443–450.
- Goldberg, J. B. & Dahnke, T. (1992). *Pseudomonas aeruginosa* AlgB, which modulates the expression of alginate, is a member of the NtrC subclass of prokaryotic regulators. *Mol Microbiol* **6**, 59–66.
- Goldberg, J. B., Gorman, W. L., Flynn, J. L. & Ohman, D. E. (1993). A mutation in *algN* permits trans activation of alginate production by *algT* in *Pseudomonas* species. *J Bacteriol* **175**, 1303–1308.
- Govan, J. R. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60**, 539–574.
- Govan, J. R., Fyfe, J. A. & McMillan, C. (1979). The instability of mucoid *Pseudomonas aeruginosa*: fluctuation test and improved stability of the mucoid form in shaken culture. *J Gen Microbiol* **110**, 229–232.
- Govan, J. R., Fyfe, J. A. & Baker, N. R. (1983). Heterogeneity and reduction in pulmonary clearance of mucoid *Pseudomonas aeruginosa*. *Rev Infect Dis* **5** (Suppl. 5), S874–S879.
- Haussler, S. (2004). Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa*. *Environ Microbiol* **6**, 546–551.
- Hoffmann, N., Rasmussen, T. B., Jensen, P. O., Stub, C., Hentzer, M., Molin, S., Ciofu, O., Givskov, M., Johansen, H. K. & Høiby, N. (2005). Novel mouse model of chronic *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis. *Infect Immun* **73**, 2504–2514.
- Høiby, N. (1975). Prevalence of mucoid strains of *Pseudomonas aeruginosa* in bacteriological specimens from patients with cystic fibrosis and patients with other diseases. *Acta Pathol Microbiol Scand Suppl* **83**, 549–552.
- Høiby, N. (1977). *Pseudomonas aeruginosa* infection in cystic fibrosis. Diagnostic and prognostic significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. A survey. *Acta Pathol Microbiol Scand Suppl* **262**, 1–96.
- Høiby, N., Giwercman, B., Jensen, E. T., Johansen, H. K., Kronborg, G., Pressler, T. & Kharazmi, A. (1993). Immune response in cystic fibrosis – helpful or harmful? In *18th European CF Conference*, pp. 133–139. Edited by H. Escobar, F. Baquero & L. Suarez. Madrid: Elsevier.
- Høiby, N., Frederiksen, B. & Pressler, T. (2005). Eradication of early *Pseudomonas aeruginosa* infection. *J Cyst Fibros* **4** (Suppl. 2), 49–54.
- Jelsbak, L., Johansen, H. K., Frost, A. L., Thøgersen, R., Thomsen, L. E., Ciofu, O., Yang, L., Haagensen, J. A., Høiby, N. & Molin, S. (2007). Molecular epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of cystic fibrosis patients. *Infect Immun* **75**, 2214–2224.
- Kessler, B., de Lorenzo, V. & Timmis, K. N. (1992). A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. *Mol Gen Genet* **233**, 293–301.
- Knutson, C. A. & Jeanes, A. (1968). A new modification of the carbazole analysis: application to heteropolysaccharides. *Anal Biochem* **24**, 470–481.
- Lee, B., Haagensen, J. A., Ciofu, O., Andersen, J. B., Høiby, N. & Molin, S. (2005). Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *J Clin Microbiol* **43**, 5247–5255.
- Martin, D. W., Schurr, M. J., Mudd, M. H. & Deretic, V. (1993a). Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of *mucB* causes conversion to mucoidy. *Mol Microbiol* **9**, 497–506.
- Martin, D. W., Schurr, M. J., Mudd, M. H., Govan, J. R., Holloway, B. W. & Deretic, V. (1993b). Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proc Natl Acad Sci U S A* **90**, 8377–8381.
- Martin, D. W., Schurr, M. J., Yu, H. & Deretic, V. (1994). Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to σ^E and stress response. *J Bacteriol* **176**, 6688–6696.
- Mathee, K., McPherson, C. J. & Ohman, D. E. (1997). Posttranslational control of the *algT* (*algU*)-encoded σ^{22} for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). *J Bacteriol* **179**, 3711–3720.

- Mathee, K., Ciofu, O., Sternberg, C., Lindum, P. W., Campbell, J. L., Jensen, P., Johnsen, A. H., Givskov, M., Ohman, D. E. & other authors (1999). Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology* **145**, 1349–1357.
- Nikolskaya, A. N. & Galperin, M. Y. (2002). A novel type of conserved DNA-binding domain in the transcriptional regulators of the AlgR/AgrA/LytR family. *Nucleic Acids Res* **30**, 2453–2459.
- Ohman, D. E. (1981). Genetic mapping of chromosomal determinants for the production of exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. *Infect Immun* **33**, 142–148.
- Ojienyi, B., Høiby, N. & Rosdahl, V. T. (1991). Genome fingerprinting as a typing method used on polyagglutinable *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *APMIS* **99**, 492–498.
- Ojienyi, B., Petersen, U. S. & Høiby, N. (1993). Comparison of genome fingerprinting with conventional typing methods used on *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *APMIS* **101**, 168–175.
- Palma, M., DeLuca, D., Worgall, S. & Quadri, L. E. (2004). Transcriptome analysis of the response of *Pseudomonas aeruginosa* to hydrogen peroxide. *J Bacteriol* **186**, 248–252.
- Pedersen, S. S. (1992). Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *APMIS Suppl* **28**, 1–79.
- Pedersen, S. S., Espersen, F., Høiby, N. & Shand, G. H. (1989). Purification, characterization, and immunological cross-reactivity of alginates produced by mucoid *Pseudomonas aeruginosa* from patients with cystic fibrosis. *J Clin Microbiol* **27**, 691–699.
- Pugashetti, B. K., Metzger, H. M., Jr, Vadas, L. & Feingold, D. S. (1982). Phenotypic differences among clinically isolated mucoid *Pseudomonas aeruginosa* strains. *J Clin Microbiol* **16**, 686–691.
- Römling, U. & Tümmler, B. (2000). Achieving 100 % typeability of *Pseudomonas aeruginosa* by pulsed-field gel electrophoresis. *J Clin Microbiol* **38**, 464–465.
- Rowen, D. W. & Deretic, V. (2000). Membrane-to-cytosol redistribution of ECF sigma factor AlgU and conversion to mucoidy in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Mol Microbiol* **36**, 314–327.
- Schurr, M. J., Martin, D. W., Mudd, M. H. & Deretic, V. (1994). Gene cluster controlling conversion to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: functional analysis in a heterologous host and role in the instability of mucoidy. *J Bacteriol* **176**, 3375–3382.
- Schurr, M. J., Yu, H., Martinez-Salazar, J. M., Boucher, J. C. & Deretic, V. (1996). Control of AlgU, a member of the σ^E -like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *J Bacteriol* **178**, 4997–5004.
- Speert, D. P., Farmer, S. W., Campbell, M. E., Musser, J. M., Selander, R. K. & Kuo, S. (1990). Conversion of *Pseudomonas aeruginosa* to the phenotype characteristic of strains from patients with cystic fibrosis. *J Clin Microbiol* **28**, 188–194.
- Tart, A. H., Blanks, M. J. & Wozniak, D. J. (2006). The AlgT-dependent transcriptional regulator AmrZ (AlgZ) inhibits flagellum biosynthesis in mucoid, nonmotile *Pseudomonas aeruginosa* cystic fibrosis isolates. *J Bacteriol* **188**, 6483–6489.
- Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.
- Terry, J. M., Pina, S. E. & Mattingly, S. J. (1991). Environmental conditions which influence mucoid conversion *Pseudomonas aeruginosa* PAO1. *Infect Immun* **59**, 471–477.
- Wood, L. F. & Ohman, D. E. (2006). Independent regulation of MucD, an HtrA-like protease in *Pseudomonas aeruginosa*, and the role of its proteolytic motif in alginate gene regulation. *J Bacteriol* **188**, 3134–3137.
- Wood, L. F., Leech, A. J. & Ohman, D. E. (2006). Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of sigma (AlgT) and the AlgW and Prc proteases. *Mol Microbiol* **62**, 412–426.
- Wozniak, D. J. & Ohman, D. E. (1991). *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for *algD* transcription. *J Bacteriol* **173**, 1406–1413.
- Wyckoff, T. J., Thomas, B., Hassett, D. J. & Wozniak, D. J. (2002). Static growth of mucoid *Pseudomonas aeruginosa* selects for non-mucoid variants that have acquired flagellum-dependent motility. *Microbiology* **148**, 3423–3430.
- Yoon, S. S., Coakley, R., Lau, G. W., Lyman, S. V., Gaston, B., Karabulut, A. C., Hennigan, R. F., Hwang, S. H., Buettner, G. & other authors (2006). Anaerobic killing of mucoid *Pseudomonas aeruginosa* by acidified nitrite derivatives under cystic fibrosis airway conditions. *J Clin Invest* **116**, 436–446.
- Yu, H., Schurr, M. J. & Deretic, V. (1995). Functional equivalence of *Escherichia coli* sigma E and *Pseudomonas aeruginosa* AlgU: *E. coli* *rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *P. aeruginosa*. *J Bacteriol* **177**, 3259–3268.
- Yu, H., Boucher, J. C., Hibler, N. S. & Deretic, V. (1996). Virulence properties of *Pseudomonas aeruginosa* lacking the extreme-stress sigma factor AlgU (sigma E). *Infect Immun* **64**, 2774–2781.

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