Different mutations in *muc*A gene of *Pseudomonas aeruginosa* mucoid strains in cystic fibrosis patients and their effect on *algU* gene expression

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SUMMARY

Alginate biosynthesis in *Pseudomonas aeruginosa* is a highly regulated process in which *algU* and *mucA* genes are key elements. Mutations in *mucA* gene determine alginate operon overexpression and exopolysaccharide overproduction. In our study, 119 strains of *P. aeruginosa* were isolated from sputa of 96 cystic fibrosis patients and 84/119 showed non-mucoid phenotype, while 35/119 showed mucoid phenotypes. *mucA* gene was amplified and sequenced in all strains revealing mutations in 29/35 mucoid strains (82%) and in one non-mucoid strain. 4/29 strains showed mutations never described that generated premature stop and much shorter MucA proteins. In all mutated strains, *algU* gene expression was analyzed to determine if mutations in *mucA*, resulting in a strong loss of its protein, could significantly influence its function and subsequently the biosynthetic pathways under *algU* control.
Analysis of *algU* expression disclosed that the length significantly affects the expression of genes involved in the production of alginate and in the motility and hence survival of *P. aeruginosa* strains in cystic fibrosis lungs.

Key words: *mucA* gene, Cystic fibrosis, Real-time PCR, Alginate.

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INTRODUCTION

Pseudomonas aeruginosa is commonly found in soil, water, wet surfaces of plants, vegetables and fruit, but it can cause illness in immunocompromised and hospitalized patients, especially in the presence of local favoring conditions (trauma, burns, surgical wounds, ulcers, etc.). The most important diseases caused by *P. aeruginosa* are lung infections in patients with predisposing local causes such as mechanical ventilation or cystic fibrosis (CF).
There is an age-specific pattern of bacteria responsible for pulmonary disease in CF, according to which, infections in adolescence subsequently recur as co-infections with other opportunistic aerobic and anaerobic Gram-negative bacilli (Armstrong *et al.*, 1997; Lyczak *et al.*, 2002; Lambiase *et al.*, 2010). The wide distribution of genotypes of *P. aeruginosa* suggests an environmental acquisition. The first isolates of *P. aeruginosa* from respiratory sputa of CF patients appear to have characteristics comparable to those of environmental strains such as non-mucoid phenotype and sensitivity to antibiotics, whereas the next isolates are resistant to antimicrobials and are frequently mucoid (Burns *et al.*, 2001). The mucous material released by the bacteria outside the cell and in which the bacteria are incorporated was indicated as alginate, a negatively charged copolymer composed of α-D-mannuronic acid and guluronic acid.
The CF lung environment, with its stress conditions (release of reactive oxygen intermediates from PMNs, high osmolarity, dehydration, nutrient limitation, presence of antibiotics) influ-

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ences the production of alginate resulting in the conversion of *P. aeruginosa* from the non-mucoid to mucoid phenotype (Mathee *et al.*, 1999; Bagge *et al.*, 2004; Roychoudhury *et al.*, 1991; Mahenthiralingam, *et al.*, 1994). There is an interval of years between the initial colonization by *P. aeruginosa* and its conversion to mucoid phenotype, preceded by a period of intermittent colonization by strains similar to environmental isolates with a non-mucoid phenotype, movement skills and sensitivity to antibacterial drugs (Hoiby *et al.*, 2005). Mucoid *P. aeruginosa* acquisition is then associated with a significant clinical deterioration (Rosenfeld *et al.*, 2001; Nixon *et al.*, 2001).

The synthesis of alginate is complex and involves 12 genes belonging to the *algA-algD* operon. The expression of the alginate operon is highly regulated and under the control of the *algD* promoter. A key element in *algD* gene regulation is the anti-sigma factor MucA. MucA is a transmembrane protein with its N-terminal domain in the cytoplasm and the C-terminus in the periplasm; it acts as an anti-sigma factor that can bind and sequester the alternative sigma factor AlgU through the N-terminal cytoplasmic domain with consequences on the activities of *algU* (Ramsey & Wozniak, 2005). MucA can also act as a regulator of *algU* transcription forming a complex with MucB and altering the signal transduction pathway that maintains the low transcriptional activity of *algU* (Rowen & Deretic, 2000). The alternative sigma factor AlgU is a positive regulator of alginate production and negative regulator of motility associated with flagella (Tart *et al.*, 2005).

In *P. aeruginosa* environmental strains, the formation of mucoid colonies is extremely rare and the production of alginate is very low. This means that in the environmental strains of *P. aeruginosa* as well as those isolated initially from sputa of CF patients, the *algD* operon is usually repressed.

The conversion to the mucoid phenotype may occur as a result of chromosomal mutations. *mucA* mutants show a highly mucoid phenotype, and *mucA* gene mutations have been found in a range between 44% and 84% of clinical isolates of mucoid CF patients (Anthony *et al.*, 2002; Boucher *et al.*, 1997; Ciofu *et al.*, 2008).

Our work is an analysis of the influence of *mucA* gene mutations in mucoid strains of *P. aeruginosa* on *algU* expression levels designed to establish if different mutations in the *mucA* gene resulting in length and/or sequence changes of its protein, influence its function as a controller of alginate production and swimming ability.

MATERIALS AND METHODS

Study population and bacterial strains

The study was performed on 119 strains of *P. aeruginosa* isolated in the Functional Area of Microbiology of the "Federico II" University Hospital of Naples from respiratory specimens of cystic fibrosis patients. Microbiological samples (sputa) were obtained during the period January 2007 to December 2009 from 300 CF patients (145 males and 155 females; mean age 16.21 years; range 0.5–50 years) who were regularly attending the Regional Referral CF Center of Naples. CF diagnosis was confirmed by standard methods (sweat chloride concentration above 60 mmol/L) and by genetic analysis. Sputum samples obtained from all patients during the study period were mixed with an equal volume of 1% dithiothreitol before incubation at 37°C for 30 min. All specimens were examined microscopically and the identification of isolates was obtained by evaluating the appearance of colonies on selective McConkey agar, oxidase test and, finally, on the basis of biochemical tests carried out by the Vitek automated system (bioMérieux, Marcy l'Etoile; France). Strains were stored at -80°C as stock culture in Brain Heart Infusion Broth (Difco, Detroit, USA) containing 20% glycerol. *P. aeruginosa* ATCC 27853 was used as reference strain.

Genomic DNA extraction

For each strain of *P. aeruginosa* 2-3 colonies were picked from a pure culture, diluted in 8 ml of Brain Heart Infusion Broth and allowed to grow for 18 h with shaking at 37°C. Then 3 mL of bacterial culture were centrifuged at 8000 rpm for 5 min, resuspended in GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris HCl) and incubated in the presence of 0.1 mg/ml lysozyme (Sigma, CA, USA) and 0.6 mg/ml proteinase K (Sigma) for 90 min at 55°C. The cell lysate was then subjected to a phenol and then chloroform-isoamyl alcohol

extractions. Na-acetate 2.33 M and 3v ethanol were added to the aqueous phase, centrifuged and precipitated, left to air dry and resuspended in TE (10 mM Tris, 25 mM EDTA, pH 8) 10 mM. Genomic DNA was quantitatively evaluated by spectrophotometry and then checked by electrophoresis on 1% agarose gel.

PCR and sequencing

To identify mutations in the *mucA* gene, PCR was performed using the primer pair AFOR-AREV (AFOR: 5'-GCGGATGAACTCGAGTTG; AREV: 5'-CACTGACGGCGGATTGTT) (Edwards and Saunders, 2001). The reaction mixture contained 10 pmol of each primer, 200 μM each dNTP, 3 mM $MgCl_2$, and 0.5 U Taq polymerase (Sigma). The amplification program consisted of the following phases: initial denaturation at 94°C for 10 min; 30 cycles of denaturation at 94°C for 2 min, annealing at 57°C for 1 min and elongation at 72°C for 2 min; final elongation at 72°C for 10 min.

Subsequently, the amplification products were sequenced at the Center for Genetic Engineering (CEINGE) of Naples using the same PCR primers.

RNA extraction

For each isolate, 2-3 colonies were picked from a pure culture, diluted in 8 mL of Brain Heart Infusion broth and incubated with shaking for about 2 h at 37°C until the exponential phase of bacterial growth curve. 1 mL of culture, corresponding to approximately 2-5x10^7 cfu/ml, was centrifuged for 5 min at 8000 rpm and the bacterial pellet was resuspended in 200 μl of GTE with the addition of 100 μg lysozyme, to allow the enzymatic lysis of the bacterial cell wall. For the subsequent extraction an RNeasy Mini kit (Qiagen, Germantown, USA) was used. The RNA extract was eluted in 30 μl of Rnase free water and then incubated at 37°C for 20 min in the presence of 4 U DNase-RNase free (Bio-Rad, CA, USA). The DNase activity was blocked by the addition of 25 mM EDTA and heat denaturated for 10 min at 75°C.

The RNA subjected to DNase was purified by extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and quantified spectrophotometrically at 260 nm and checked for the integrity on a denaturing agarose gel 1%.

Synthesis of cDNA and RealTime PCR

For cDNA synthesis 250 μg of total RNA were used with 1 U of Enhancer avian AMV-RT (Sigma). For subsequent real-time PCR, 5 μl of cDNA mixture were used for each reaction. *algU* expression level was analyzed using the constitutively expressed 16S RNA gene. The primer pairs used in the amplification reaction were as follows: 16S FW: 5'-CGTCCGGAAACGGCCGCT-3'; 16S RW: 5'-CTCTCAGACCAGTTACGG-3'; algU FW: 5'-GCGACCTGGACCTGGGCT-3'; algU RW: 5'-TCCTCGATCAGCGGGATC-3' (Edwards & Saunders, 2001).

The amplification program consisted of the following phases: initial denaturation at 94°C for 10 min; 40 cycles of denaturation at 94°C for 20 s, annealing at 57°C for 20 s; melting curve 75 to 95°C.

The reactions were performed in a MiniOpticon (Biorad) and the curve analysis was performed with the program Gene Expression Macro Version 1.1.

Study of motility

Flagellum related motility (swimming) was evaluated measuring the diameter of swimming on LB-agar 0.3% plates. When swimming exceeded 6 mm it was considered indicative of the presence and function of the flagellum. Individual colonies obtained by growth on TSA plates were tooth-picked on LB-agar 0.3% plates. After incubation at 37°C for 18-20 h, the swimming diameter was measured. Experiments were performed in triplicate.

Genotyping by PFGE

DNA fingerprinting was carried out by the method described by Grothues *et al.* (1988). Briefly, isolates were grown overnight and suspended in SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5). The cell suspensions (4 McFarland units) were mixed with an equal volume of 2 % low-melting point agarose, molded into plugs at 4°C, and lysed with lysis buffer (1% N-lauryl sarcosine, 0.5 M EDTA, pH 8) to which Proteinase K (500 g/mL) had been added. The DNA contained in each mold was digested by 20U of SpeI restriction enzyme (New England Biolabs), in accordance with the manufacturer's instructions. Macrorestriction fragments were separated using the CHEF-DR III PFGE system (Bio-

Rad) at 10°C for 20 h, with an initial switch time of 5 s and a final switch time of 35 s, at a field strength of 6 V/cm. A ladder of lambda phage DNA concatemers was used as a size marker. Fragment patterns were compared according to the criteria described by Tenover (Tenover *et al.*, 1995). Based on these criteria, we considered isolates to be possibly related if their restriction patterns differed by 4-6 bands and closely related if their restriction patterns differed by no more than 2-3 bands. Isolates were considered different if their restriction patterns differed by 7 or more bands. The banding pattern produced from PFGE was analyzed using GelCompar II software and a dendrogram (Dice/unweighted pair-group method using average linkage [UPGMA]) produced to visualize the relatedness of the isolates.

Statistical analysis

The statistical differences were determined using ANOVA test. Differences in swimming motility and *algU* gene expression were considered significantly different at $P \leq 0.01$.

RESULTS

During the period January 2007 to December 2009 we collected 119 clinical isolates obtained from sputa of 96 patients chronically colonized by *P. aeruginosa* and admitted to the Cystic Fibrosis Centre of the University Hospital Federico II. 12/96 patients were infected by *P. aeruginosa* mucoid strains, 61/96 patients were infected by *P. aeruginosa* non-mucoid ones and 23/96 patients were co-infected by both mucoid and non-mucoid *P. aeruginosa* strains. We performed PFGE on all *P. aeruginosa* strains to share isolates with a different fingerprint. Mucoid and non-mucoid strains isolated from same patient always showed identical genotypes (Figure 1). Macrorestriction analysis on *P. aeruginosa* strains obtained from different patients showed different fingerprints with the possibility to be grouped into six major clusters. Strains grouped in each cluster showed a homology level of 80%. The clusters C and E grouped the major number of strains with diversity of less than 10% (Figure 2).

The *mucA* gene was amplified in all strains and sequenced to detect any mutations as a possible cause of the mucoid phenotype. Sequences obtained were compared with ones available online at the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLASTX) program to directly visualize changes or interruptions in the amino acid sequence.

Of all the strains tested, 29/35 mucoid strains (82%) and one non-mucoid strain showed mutations of which 4 never described (Table 1). Of these strains, 16/30 (50%) showed deletion of a G in position 440 (ΔG440). This deletion changed the reading frame of the *mucA* gene, with the formation of a premature stop codon. Another 9/30 strains showed transitions that determined premature stop. PA12, PA74, PA86, PA91 non-mucoid and PA91 mucoid showed mutations never described (Table 1).

Strain PA12 showed a substitution of T in position 239 with the formation of a stop codon. Strain PA74 showed a deletion of 60 bases in the central region and the resulting shift of the reading frame gave an alteration of the amino acid sequence. Strain PA86 showed a deletion of a guanine in position 376 that produced a shift in the reading frame, and then in the amino acid sequence, with the formation of a premature stop

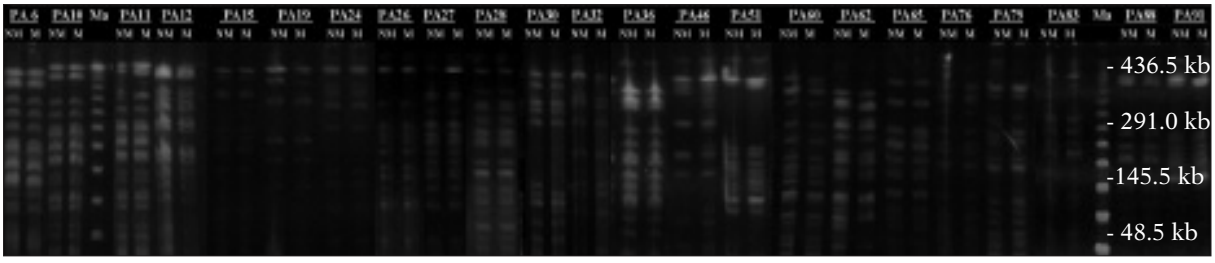

FIGURE 1 - *PFGE analysis of non-mucoid (NM) and mucoid (M) strains of P. aeruginosa isolated from the same patient. Ma: Molecular size marker Pulse Marker 50-1000 kb (Sigma).*

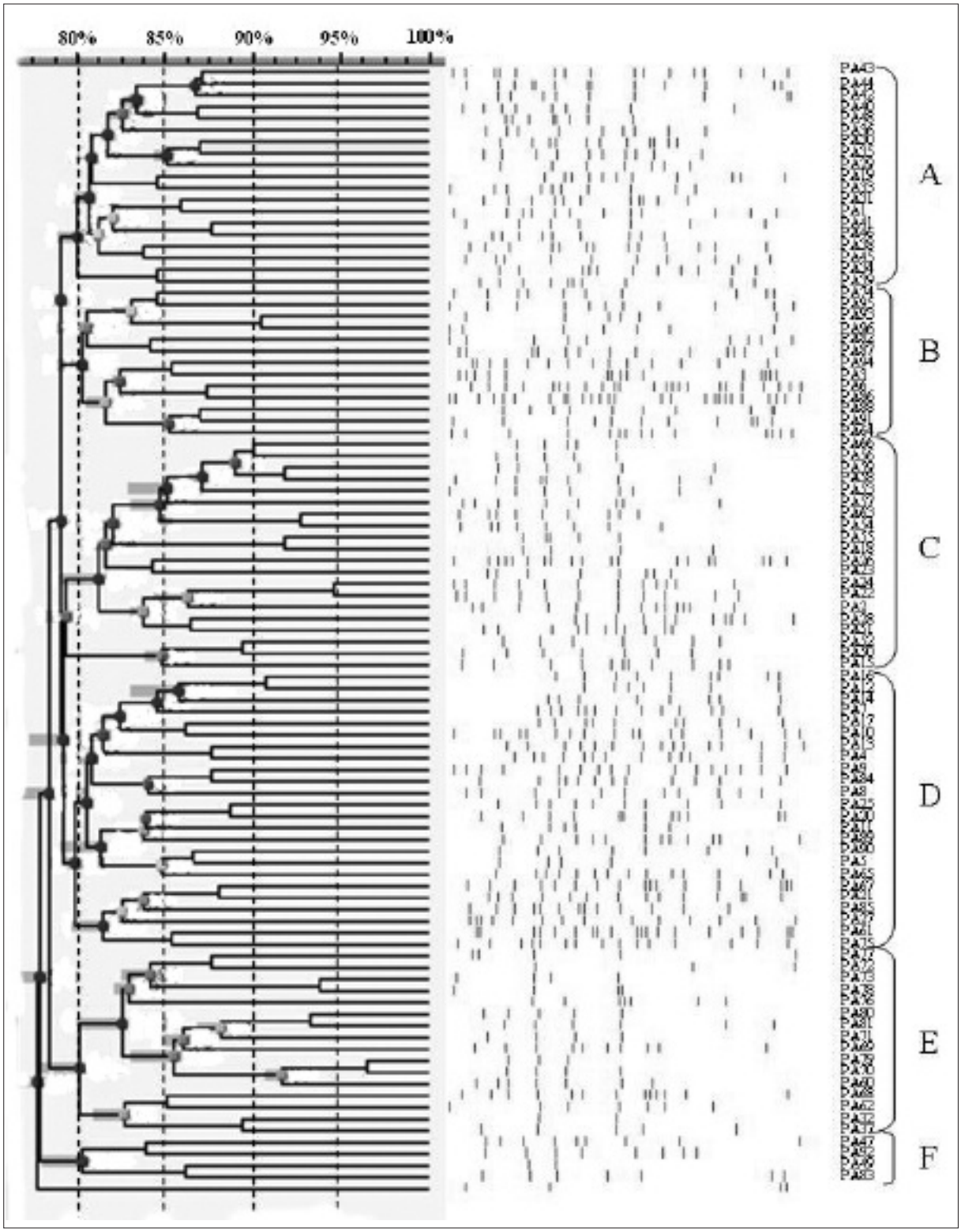

FIGURE 2 - *Phylogenetic analysis of digitized PFGE SpeI profile of the 96 P. aeruginosa. Cluster analysis is based on the percentage distance index. The numbers indicate the strains and the letters indicate the clusters.*

TABLE 1 - *mucA mutations in P. aeruginosa strains. Bold letters indicate new mutations described.*

Isolate	*Colony morphotype*	*mucA mutation*	*Stop codon*
PA1, PA2, PA3, PA4, PA5, PA7, PA8, PA9, PA13, PA14, PA16, PA17, PA18, PA20, PA21, PA22, PA23, PA25, PA29, PA31, PA33, PA34, PA35, PA37, PA38, PA39, PA40, PA41, PA42, PA43, PA44, PA45, PA47, PA48, PA49, PA50, PA52, PA53, PA54, PA55, PA56, PA57, PA58, PA59, PA63, PA64, PA66, PA67, PA68, PA69, PA71, PA72, PA73, PA75, PA78, PA85, PA87, PA89, PA92, PA93, PA96	PANM	-	TGA at 592
PA6	PAM/PANM	△G440/-	TGA at 450
PA10	PAM/PANM	-/-	TGA at 592
PA11	PAM/PANM	△G440/-	TGA at 450
PA12	**PAM/PANM**	**A→T_{239}/-**	**TAA at 239**
PA15	PAM/PANM	C→T_{446}/-	TAG at 446
PA19	PAM/PANM	C→T_{359}/-	TAA at 359
PA24	PAM/PANM	-/-	TGA at 592
PA26	PAM/PANM	-/-	TGA at 592
PA27	PAM/PANM	△G440/-	TGA at 450
PA28	PAM/PANM	△G440/-	TGA at 450
PA30	PAM/PANM	-/-	TGA at 592
PA32	PAM/PANM	-/-	TGA at 592
PA36	PAM/PANM	△G440/-	TGA at 450
PA46	PAM/PANM	△G440/-	TGA at 450
PA51	PAM/PANM	-/-	TGA at 592
PA60	PAM/PANM	C→T_{446}/-	TAG at 446
PA61	PAM	C→T_{359}/-	TAA at 359
PA62	PAM/PANM	C→T_{446}/-	TAG at 446
PA65	PAM/PANM	△G440/-	TGA at 450
PA70	PAM	G440/-	TGA at 450
PA74	**PAM**	**$△_{359-418}$/-**	**TGA at 487**
PA76	PAM/PANM	△G440/-	TGA at 450
PA77	PAM	△G440/-	TGA at 450
PA79	PAM/PANM	C→T_{446}/-	TAG at 446
PA80	PAM	△G440/-	TGA at 450
PA81	PAM	C→T_{377}/-	TAG at 377
PA82	PAM	△G440/-	TGA at 450
PA83	PAM/PANM	C T_{446}/-	TAG at 446
PA84	PAM	△G440/-	TGA at 450
PA86	**PAM**	**△G376/-**	**TGA at 396**
PA88	PAM/PANM	C→T_{446}/-	TAG at 446
PA90	PAM	△G440/-	TGA at 450
PA91	**PAM/PANM**	**$△_{264-411}$/$△_{264-411}$**	**TGA at 450**
PA94	PAM	△G440/-	TGA at 450
PA95	PAM	△G440/-	TGA at 450

Colony morphotype: mucoid (M) and non-mucoid (NM).

TABLE 2 - *Amino acid sequences obtained by translation of mucA gene with novel mutations. Stop codon and alternative amino acid sequences are reported in grey.*

Strain	*Amino acid sequence of MucA protein*
Wild-type	MSREALQETLSAVMDNEADELELRRVLAACGEDAELRSTWSRYQLARSVMHREPTLPKLDIAAAVSAALA DEAAPPKAEKGPWRMVGRLAVAASVTLAVLAGVRLYNQNDALPQMAQQGTTPQIALPQVKGPAVLAGYSE EQGAPQVITNSSSSDTRWHEQRLPIYLRQHVQQSAVSGTESALPYARAASLENR*
PA12	MSREALQETLSAVMDNEADELELRRVLAACGEDAELRSTWSRYQLARSVMHREPTLPKLDIAAAVSAALA DEAAPP*
PA74	MSREALQETLSAVMDNEADELELRRVLAACGEDAELRSTWSRYQLARSVMHREPTLPKLDIAAAVSAALA DEAAPPKAEKGPWRMVGRLAVAASVTLAVLAGVRLYNQNDALPQMAGRLXRXAXGAXGDHQLLVQRYPLA*
PA86	MSREALQETLSAVMDNEADELELRRVLAACGEDAELRSTWSRYQLARSVMHREPTLPKLDIAAAVSAALA DEAAPPKAEKGPWRMVGRLAVAASVTLAVLAGVRLYNQNDALPQMAQQGTTPRSPCLR*
PA91	MSREALQETLSAVMDNEADELELRRVLAACGEDAELRSTWSRYQLARSVMHREPTLPKLDIAAAVSAALA DEAAPPKAEKGPWRSWPATAKSRGRRR*

codon. Strain PA91 showed a deletion of 147 bases in the central region of the gene with a modification of the reading frame and formation of a premature stop. Same mutation was observed in PA91 non-mucoid strain. Amino acid sequences of the 4 truncated proteins are reported in table 2 and mutation in PA12 proved to give rise to the shortest protein. The remaining strains had wild-type *mucA* sequences.

30/119 strains were included into three groups depending on MucA protein length and colony morphology and subjected to *algU* gene expression analysis by real-time PCR to verify the influence of *mucA* mutations on expression levels. The first group included eight non-mucoid strains that showed no mutation in the *mucA* gene and the unique non-mucoid strain with mutation (PA91). The second group included the six mucoid

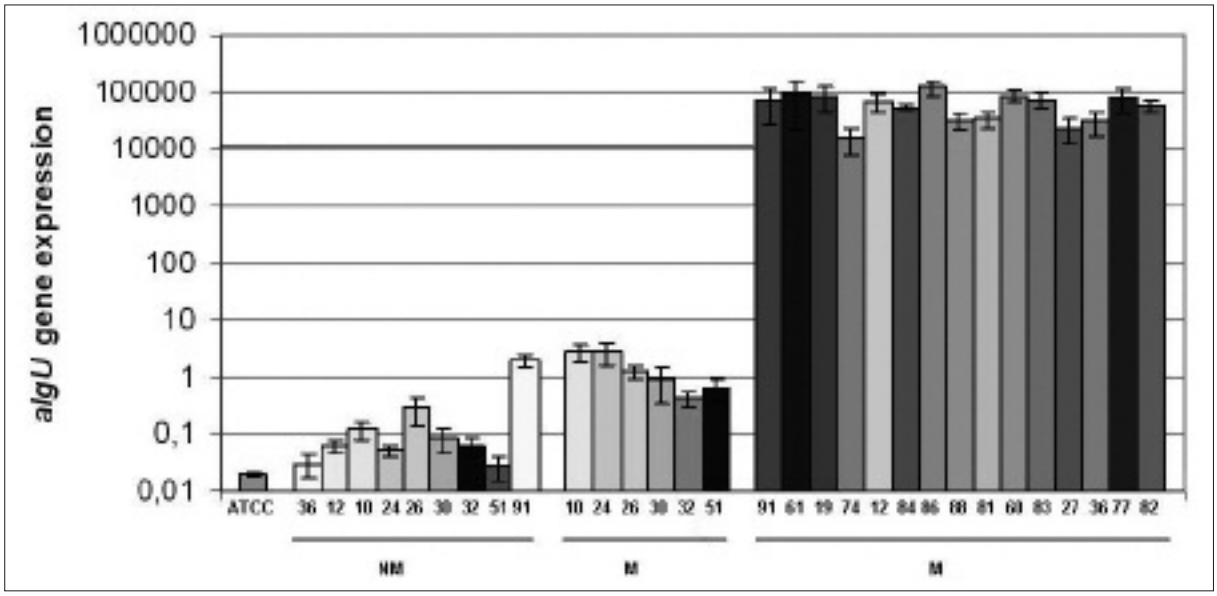

FIGURE 3 - *algU gene expression levels of 30 strains in comparison to P. aeruginosa ATCC 27853. Expression levels are significantly different between group III and other groups with P value of < 0.005. Numbers on X-axis refer to P. aeruginosa isolates (NM: non-mucoid isolates; M: mucoid isolates).*

TABLE 3 - *Swimming motility for non-mucoid (NM) and mucoid (M) strains. Swimming motility is significantly different between group III and the other two groups with a P value of <0.01.*

Strain	*Group*	*Swimming motility diameter (mm)*
ATCC		25±3
PA10 (NM)	I	27±3
PA12 (NM)		32±2
PA24 (NM)		35±2
PA26 (NM)		28±3
PA30 (NM)		29±3
PA32 (NM)		31±3
PA36 (NM)		34±1
PA51 (NM)		35±2
PA91 (NM)		13±1
PA10 (M)	II	10±2
PA24 (M)		10±1
PA26 (M)		17±1
PA30 (M)		12±2
PA32 (M)		13±1
PA51 (M)		13±1
PA91 (M)	III	3±1
PA61 (M)		16±2
PA74 (M)		22±2
PA84 (M)		15±1
PA86 (M)		5±1
PA36 (M)		12±1
PA12 (M)		5±1
PA19 (M)		7±1
PA27 (M)		8±2
PA60 (M)		13±1
PA77 (M)		9±1
PA81 (M)		15±2
PA82 (M)		7±2
PA83 (M)		13±1
PA88 (M)		15±1

strains without mutations in *mucA* and the same length as the wild-type protein. The third group included the PA91 mucoid strain and other fourteen mucoid strains, with ΔG440 mutations or mutations in nearby locations, producing a C-terminal domain truncated protein or the loss of the most central and terminal regions of the protein. *P. aeruginosa* ATCC 27853 was used as reference strain and the histogram in figure 3 shows *algU* gene expression levels of 30 strains in comparison to *P. aeruginosa* ATCC.

The group I strains did not have *algU* expression levels significantly higher than those of ATCC strain according to their non-mucoid phenotype (P >0.01). Also the strains belonging to group II did not have *algU* expression levels significantly higher than those of ATCC strain although they had a mucoid phenotype (P >0.01), revealing that the length of the protein MucA appeared to have a decisive role in influencing these values but it did not seem to be the only factor that influences the alginate production. Instead, the strains belonging to group III showed *algU* expression levels significantly higher than non-mucoid strains and than mucoid strains without mutation (P <0.005). The non-mucoid strain PA91, deleted in position 254-411, showed *algU* expression levels similar to *algU* expression levels of group II mucoid strains, although it presented non-mucoid phenotype; while his mucoid counterpart showed *algU* expression typical of mutated strains.

The ability to move in a solid medium (swimming) was analyzed and, as expected, reflects levels of *algU* expression. Infact the loss of motility of the groups II strains was not significantly different from the motility of group I strains with the wild type MucA protein (P >0.01).

As shown in table 3, instead, group III strains presented a reduced ability to move in comparison with strains with wild-type MucA protein. Finally, strain CF91, characterized by shorter MucA protein, showed reduced swimming motility in non-mucoid strain and total loss of swimming motility in mucoid ones (P <0.01).

DISCUSSION

The pathogenic action of *P. aeruginosa* is supported by many different virulence factors: pro-

teases, adhesins, flagella, biofilm formation and mucoid phenotype. The main characteristic of mucoid strains of *P. aeruginosa* is the production of the mucopolysaccharide alginate.

A key element of alginate operon regulation is the alternative sigma factor AlgU that interacts with MucA and acts as a negative regulator of alginate biosynthesis: in fact, the occurrence of mutations in *mucA* was found to be the most frequent mechanism of conversion to the mucoid form (Wood & Ohman, 2006).

This study analyzed 119 *P. aeruginosa* strains, isolated from a cohort of patients with cystic fibrosis. PFGE analysis revealed that all patients were chronically infected by strains with a unique macrorestriction profile, although 23 patients showed co-infection with two strains of *P. aeruginosa* with different morphology but a genetically related profile. We analyzed the sequence of the *mucA* gene in all strains showing that in 30 strains (40%) the *mucA* gene was mutated.

The most frequent mutation (16/30 strains) was found ΔG440, in agreement with known data. This mutation was found to cause a frame-shift mutation at the end of the gene encoding the periplasmic domain of MucA, resulting in the loss of the last 50 amino acid of wild type protein.

Of the remaining strains in which *mucA* was mutated, 5 had mutations never before described in the literature: in two cases a deletion of the central region resulted in a frame-shift and then in a premature stop; in other cases, the transition of a cytosine in thymine caused a premature stop codon.

Comparing the results of PFGE profiles and *mucA* mutations we found that isolates with the same mutation were not included in the same phylogenetic cluster, e.g. the isolates with DG440 were distributed in five different clusters. In particular PA91 and PA12, the isolates with major deletion in *mucA* gene, were included in two distinct branch of dendrogram highlighting that the high genetic variability of *P. aeruginosa* specie is not easily correlated to a single factor as *mucA* mutation but is useful to disclose co-infection due to identical strains with different morphotype.

The strains were classified into three groups according to the morphology of colonies and the length of the MucA protein: in this way group I comprised non-mucoid strains with the wild-type MucA protein, group II mucoid strains with the wild-type MucA protein, group III strains with a protein without the C-terminal domain or without the most part of protein. Group III included all strains with new mutations in *mucA* gene, that produced shorter proteins lacking of most part of the protein included the domain fundamental for interaction with AlgU.

The results of real-time PCR showed that the expression of the *algU* gene varied significantly in the three groups. In group II, *algU* gene expression levels were similar to those of reference strains. It can be assumed that in this group, in which *algU* expression levels are low but there is also production of alginate, gene regulation occurs at different levels as reported by Boucher (Boucher *et al.*, 2000). In the third group, however, there is a higher level of *algU* transcription, suggesting that the loss of central and terminal amino acids of the MucA protein adversely affects the production of AlgU. The non-mucoid strains PA91, that presented *mucA* gene mutated but non-mucoid phenotype, showed *algU* expression level typical of group II, although it can be included in group I because of its morphology.

algU was reported to affect not only the amount of alginate production, but also the expression of genes that control flagella functionality. The *fleQ* gene, involved in the regulation of the synthesis of the flagellum, was inhibited by the expression of the *algU* gene, thus in each of the strains analyzed a loss of mobility inversely proportional to alginate production was expected (Tart *et al.*, 2005).

Analysis of motility showed that strains with *mucA* mutations seemed to have a lower motility compared to the non-mucoid strains and mucoid strains without mutations in the *mucA* gene. The complete inactivation of the flagellum with consequent loss of motility, however, was only observed in the two strains which had *mucA* novel mutations. In addition, PA91 non-mucoid strain grouped with group II instead of group I in the motility analysis.

Tart *et al.* claimed that the flagella are essential for the pathogenesis and non-mobile strains are dramatically attenuated in their virulence. However, flagellin is an excellent immunizing agent and the loss of the flagellum reduces the release of mediators of the host's immune system giving to the organism a selective advantage. It can be assumed that the interaction between these two vir-

ulence factors may play a crucial role in chronic lung infection in CF patients.

In agreement with the data already known, which show the key role of the protein MucA in the regulation of the *algU* gene that is crucial in the regulation of virulence factors, the results obtained in our study show that the length and therefore the structure of the MucA protein are fundamental for the correct expression of genes involved in the production of alginate and motility, although we have also demonstrated that strains without *mucA* mutation show *algU* expression levels, mucoid phenotype and motility similar to mucoid.

Since the production of alginate is considered one of the most important virulence factors of this opportunistic pathogen in patients with CF, it is clear that the exopolysaccharide production is regulated at many different levels and mutation in *mucA* gene could also remain silent to influence the motility and then colonization and survival of strains of *P. aeruginosa* in cystic fibrosis lung.

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