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RESEARCH ARTICLE

Pseudomonas Aeruginosa Colonising Patients with Cystic Fibrosis: Antibiotic Resistance and Growth Conditions Determined by *in vivo* and *in vitro* Gene Expression

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ABSTRACT

Background: *Pseudomonas aeruginosa* is the main pathogen responsible for lung destruction in cystic fibrosis, becoming difficult to eradicate in chronic infection.

Aims: To describe antibiotic resistance among strains of *P.aeruginosa* isolated from sputa of patients with cystic fibrosis. To investigate *in vivo* and *in vitro* expression of genes related to antibiotic resistance and anaerobic growth.

Methods: Sputa (*in vivo*) and strains (*in vitro*) from 26 patients were obtained during 17 months. Genotypes were compared by random polymorphic DNA amplification. Expression of *nirS* (anaerobic respiration) and *mexY* (MexXY efflux pump) were measured by quantitative real time polymerase chain reaction. Expression levels of *nirS* in aerobiosis and anaerobiosis were compared to estimate oxygenation status within lungs. Mutations in the regulator gene *mexZ* were investigated in sputa expressing *mexY* and were correlated with strains' antibiotic resistance.

Results: Nine patients and 56 sputa were finally analysed. Seven patients carried a single genotype. Gene *mexY* was detected in all the sputa; expression levels were higher in sputa with *mexZ* mutations. Multi-resistance was frequent. Resistance profiles not always correlated with *mexY* expression levels or *mexZ* mutations. Comparison of *in vivo* and *in vitro nirS* expression indicated mainly aerobic and microaerophilic environments within sputa.

Discussion: Mutations in e *mexZ* are frequent in strains of *P.aeruginosa* colonising patients with cystic fibrosis. Presence of these mutations correlates with increased expression of *mexY in vivo* and *in vitro,* but no with *in vivo* antibiotic resistance. Results of *nirS* expression suggest that the lungs represent heterogeneous environments regarding oxygenation status. This complexity explains that mechanisms of growth and antibiotic resistance within the lungs of these patients are still largely unknown.

Conclusions: After many years of research few studies, including the present, revealed different aspects of *in vivo* growth of *P. aeruginosa*. We determined a cut-off to discriminate between sputa containing *mexZ* wild type and mutated alleles and showed that comparison of *in vivo* and *in vitro nirS* expression allows to predict oxygenation status. So far, none of the studies can explain all the factors influencing the behaviour of *P.aeruginosa* colonising cystic fibrosis patients making it difficult to design new therapeutic strategies.

Introduction

Cystic fibrosis (CF) is a severe inherited disease due to mutations in the gene that codifies for the CF transmembrane conductance regulator (CFTR), a channel that mainly conducts chloride but that is also involved in maintaining transmembrane flow of bicarbonate, sodium and potassium. It is present in several human cells and different kind of mutations determine different CFTR defects. These defects are grouped in seven different classes that can be the target for different therapeutic strategies. Respiratory disease due to lung damage is the most severe and frequent manifestation, with chronic sino-pulmonary infections and acute exacerbations [1,2]. Dysfunction in electrolytic transport produces an increased viscosity of mucus and a decreased muco-ciliary clearance, making microbial colonisation easier [1,3].

Some microbial species preferentially colonises lungs of patients with CF since childhood. The microbiota has been changing over the years, probably due to the use of different therapeutic approaches. By the age of 5 years more than half patients are colonised with *Staphylococcus aureus* and less than 30% also carry other respiratory pathogens (*Haemophilus influenzae, Stenotrophomonas maltophilia, Achromobater spp., Burkholderia cepacia*). Later, *Pseudomonas aeruginosa* appears and becomes predominant [4,5]. From 2003, a change in the frequency of pathogens has been observed, with a marked increase of *S. aureus* and *S. maltophilia* and a concomitant increase in antimicrobial resistance (multi-drug resistant *P. aeruginosa*, methicillin-resistant *S. aureus*) [6] .

In CF, lungs constitute a heterogeneous environment where aerobic zones coexist with anaerobic zones generated by bronchial obstruction due to mucus plugs. *P. aeruginosa* initially colonises the mucosa producing intermittent infections, but promptly adapts to the environment by forming biofilms. At this stage infections become chronic and the bacterium is almost impossible to eradicate [7]. Biofilms are organised communities of microorganisms composed of one or several species covered with a polymeric hydrated matrix of polysaccharides, proteins and nucleic acids of their own synthesis. This matrix is called extracellular polymeric substances (EPS) or extracellular matrix (ECM) and is a key element in the development of complex, three-dimensional, attached communities [8,9]. Within biofilms bacteria become more resistant to the action of immune system and to chemical agents such as antibiotics [8,10]. Two phenomena related to antibiotics action can develop: resistance and tolerance [11]. Growth in the biofilms leads to phenotypic and genotypic diversity in a short time [3,7,12] and this can be in part related to exposure to antibiotics [13].

P. aeruginosa is a facultative aerobe that preferentially uses oxygen as final electron acceptor during aerobic respiration but can also grow and multiply in anaerobiosis if there are enough nitrite or nitrate concentrations to obtain energy from denitrification. Four reductases are involved in this process, one of which is NirS. Arginine deamination is an alternative way but is less effective than denitrification [14-17].

Hypoxemic conditions in CF lungs were demonstrated by direct measuring of oxygen in the mucosal surface [14] and by the identification of strict anaerobic bacteria [14,18]. *P. aeruginosa* adapt to these conditions by preferentially using anaerobic respiration [19].

The action of aminoglycosides, an important class of antibiotics used to treat acute exacerbations, is compromised within biofilms because their transport through the cytoplasmatic membrane is oxygen-dependant [20]. *P. aeruginosa* can also acquire resistance to aminoglycosides by two main mechanisms: active elimination of the molecule by efflux pumps and enzymatic hydrolysis [21]. Overexpression of the inducible efflux pump MexXY-OprM is considered the most frequent mechanisms of acquired aminoglycoside resistance in CF patients [22] and is due to mutations in different genes involved in the regulation of expression, notably *mexZ* repressor, *fusA1* that encodes for an elongation factor G and *amgRS* that encodes for a two-component regulatory system [23].

We aimed to investigate the growth conditions of *P. aeruginosa* and the expression of some determinants of resistance by measuring genetic expression directly into the sputa (*in vivo*) and in strains isolated from these sputa (*in vitro*) in patients with CF.

METHODS

Patients and strains

A convenience sample of 26 patients who attended the Department of Respiratory Diseases of the University Hospital Centre (CHU) at Besançon (France) and who agreed to participate was studied. Sequential sputa were collected between February 2006 and June 2007 under respiratory physiotherapy both during chronic colonisation and during acute exacerbations. Part of each sputum sample was used for semi-quantitative culture, isolation of *P. aeruginosa* strains and antibiotic susceptibility at the Bacteriology Laboratory at CHU. The remaining sample was frozen at -80°C immediately after collected and was used for genetic analysis at the Department of Microbiology and Molecular Medicine of the University Medical Centre (CMU) of Geneva School

of Medicine.
The study was accepted by the Research Ethics Committee and all patients signed informed consent to participate.

Genotyping
To compare genotypes between strains isolated from each patient, random amplification of polymorphic DNA (RAPD) with primer 207 [24] was used. PCR conditions were as follows: 94°C for 5 minutes; 45 cycles at 94 °C for 1 minute; 36°C for 1 minute; 72°C for 2 minutes and final extension at 72°C for 10 minutes. PCR products underwent electrophoresis on 1.5% Tris-Borate-EDTA (TBE) agarose gel and the banding patterns were visually analysed. Two strains were considered to belong to the same genotype if its RAPD profile was not different in more than 2 bands.

DNA and RNA extraction from sputa
Two samples per each sputum were extracted and analysed in parallel. The sputum was solubilised in 4 ml of Trizol per gram of sputum. Dithiothreitol (DTT) was added to obtain a final concentration of 100 µg/ml (0.64 mM). After homogenization, 2 aliquots of 5 ml were transferred into 2 Falcon tubes of 14 ml. The extraction was performed with 1 ml of chloroform. Samples were centrifuged at 10,000 rpm for 15 minutes at 4°C. The upper phase was removed for RNA extraction; the inter- and lower- phases were kept at 4°C for DNA extraction. Four ml of isopropanol were added to the upper phase, then mixed and incubated for 10 minutes and finally centrifuged at 4000 rpm for 45 minutes. The supernatant was removed and the RNA pellet was suspended in 1 ml of 75% ethanol. After centrifugation, the supernatant was removed and the RNA pellet was dried for 10 minutes. RNA was suspended in 75 µl of RNAse-free water, incubated at 65°C for 10 minutes, then treated with DNase (Promega RQ1 DNAse™) for 50 minutes. Samples were purified using RNeasy columns (Qiagen™) and RNA was eluted with 30 µl of RNase-free water. RNA concentration was measured at 260 nm and then stored at -80°C.
DNA was extracted from the interphase by adding back extraction buffer and then mixing for 3 minutes. The tubes were then centrifuged at 10,000 rpm for 15 minutes at 4°C. Isopropanol (0.6 ml) was added to the supernatant and mixed for 5 minutes at room temperature. After centrifugation at 10,000 rpm at 4°C for 15 minutes, the pellet containing the DNA was recovered and suspended in 1 ml of 75% ethanol, then centrifuged again. The DNA pellet was dried, suspended in 10 mM Tris buffer pH 8.0 and stored at -20°C.

RNA extraction from bacterial strains
Strains were grown in Luria-Bertani broth (LB) to reach mid-exponential growth phase (DO_{600} between 1.5-2.0). Aliquots of approximately $1x10^9$ bacteria were treated with 2 volumes of RNA stabiliser (RNAprotect bacteria, Qiagen™). Strains were also grown in solid media (LB + 2% agar -LBA) in aerobiosis (37°C and room atmosphere during 18-22 hs) and in anaerobiosis (LBA supplemented with 50mM of KNO3 at 37°C and atmosphere generated with GeneBag™ (Biomérieux) to obtain less than 0.1% of oxygen) at the same time. After incubation, bacterial suspensions corresponding to $5x10^8$ colony forming units were prepared in sterile water by measuring the DO_{600}. These suspensions were mixed with 2 volumes of RNAprotect bacteria reagent (Qiagen™) and supplier's instructions were followed. Each strain was studied in duplicate both in aerobiosis and anaerobiosis. Bacterial pellets were suspended in 100 µl of lysozyme (3 mg/ml) and RNA was extracted according to the supplier's protocol. Extracted RNA was eluted in 45 µl of RNAse-free water and treated immediately with DNAse RQ1 (Promega™) in the presence of 2 µl of RNAsin (Promega™). The reaction was incubated at 37°C for 60 minutes. RNA concentration was measured at 260 nm and frozen at -80°C.

Reverse transcription
One µg of RNA was used for reverse transcription in a mix containing 8 µl of 2.5 mM dNTPs, 0.5 µl of random hexanucleotides at 500 µg/ml and RNAse-free water (total volume 24 µl). The reaction was incubated at 65°C for 5 minutes and then chilled on ice. Eight µl of first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT), 4 µl of $MgCl_2$ and 2 µl of RNAsin (Promega™) were added and incubated during 10 minutes at room temperature, then 2 minutes at 42°C. Half of the sample (19 µl) was transferred to a new tube and 1 µl of reverse transcriptase ImProm-II (Promega™) was added. The remaining sample was used as control. Complementary DNA (cDNA) synthesis was carried out at 42°C for 50 minutes. The enzyme was then inactivated at 70°C for 15 minutes and the samples were diluted in 45 µl of RNAse-free water.

In vivo and in vitro gene expression

The following genes were selected for study over sputum samples and strains: *nirS* (coding for a nitrite reductase necessary for anaerobic growth using denitrification); *mexY* (inner membrane antiporter component of the MexXY-OprM efflux pump); and

mexZ (*mexY* repressor). The selection was made on the basis of previous experiments on 70 sputa and 39 strains from 20 patients where the following genes involved in denitrification were tested: *nirS*, *norB* and *narG*. We found that *nirS* and *norB* were always induced in anaerobiosis, but *nirS* gave higher values. On the other hand, *narG* was not always induced (data not shown). The MexXY-OprM efflux pump was chosen because its over-expression is the most common mechanism of aminoglycoside resistance in CF isolates [23] and it can also contribute to ß-lactam and fluroquinolone resistance, three main antibiotics in the treatment of CF patients, although these resistances may be isolated from patients without CF [25,26].

Real time PCR (Rotor-Gene 3000 – Corbett™, Australia) was used to quantify cDNA with 3 µl of cDNA as template and the following reaction components: 7.5 µl of QuantiTect SYBR Green™ (Qiagen), 0.9 µl of each template and 2.7 µl of water.

The housekeeping gene *rpsL* was always amplified along with the three genes under investigation to normalise the results; expression levels were calculated as number of copies per gram of sputum and normalised by the number of copies of *rpsL*. Primers' sequences (5'-3'):

rpsL-F: GCAAGCGCATGGTCGACAAGA;
rpsL-R, CGCTGTGCTCTTGCAGGTTGTGA;
mexY1, TGGTCAACGTCAGCGCCAGCTAT;
mexY2, TCGACGATCTTCAGGCGGTTCTG;
mexZ-F1, CGGCGCGACAGTAGCATATAAT;
mexZ-R1, TCGAAATCGATTCGGAACAAG;
nirS-1: CCATCCGAAGTCCTCGCACCTCT;
nirS-2: TTCATCGCCGCGCTTGTTGTACT;

Two samples per sputum were extracted and analysed in parallel; the mean value was then used. *In vitro nirS* induction was calculated as the ratio anaerobic expression / aerobic expression (*nirS*/*rspL* in anaerobiosis / *nirS*/*rspL* in aerobiosis). Growth conditions were classified as aerobic or anaerobic by comparing expression rates *in vivo* and *in vitro* (in aerobiosis and anaerobiosis) for each patient. Due to the variability observed in *nirS* induction between different strains from the same patient, we used the mean ratio of expression of all the strains isolated from a same sputum sample. *nirS* induction rates were compared to those of the reference strain PAO1 (www.pseudomonas.com). To establish if variations of *mexY* expression were caused by mutations in *mexZ*, a region 1Kbp of genomic DNA (gDNA) extracted from sputa that includes this gene was amplified. The sequences of the obtained amplicons were compared to the sequence of *mexZ* from strain PAO1.

Antibiotic resistance

Antibiotic resistance was determined at CHU Besançon by Kirby-Bauer disk diffusion method to the following classes: aminoglycosides (gentamycin, amikacin, tobramycin), fluroquinolones (ciprofloxacin) and beta-lactams (tazobactam-piperacillin, ceftazidime, imipenem, meropenem). Results were interpreted according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2023 [27], except for gentamycin for which no breakpoints are available from 2020 for this committee, so it was interpreted according to EUCAST 2019 [28]. Antibiotic profiles were correlated with the level of expression of *mexY* and mutations in *mexZ*. Strains were classified as multi-resistant when they presented resistance to 3 or more classes of antibiotics [29]. For the purposes of this paper and to simplify the interpretation, we denominate strains with no resistance to the 3 tested classes as "fully susceptible" although they can present resistance to no tested antibiotics.

Statistical analysis

One-tway ANOVA test with a significance level of 0.5 was used to compare mean *mexY*/*rpsL* values between "susceptible/susceptible, increased exposure", "resistant" to only one antibiotic class and multi-resistant strains, and to compare differences in the expression of in vivo *mexY* expression between wild type and *mexZ* mutant strains.

RESULTS

Bacterial load in sputa and genotyping of strains

One hundred twelve sputa were obtained from 26 patients. Fourteen were excluded because one or more of the following reasons: intermittent or no colonisation with *P. aeruginosa*; less than 2 sputa per patient; absence of strain/s isolated from the sputa. Twelve patients were retained for further study. Eleven samples from 3 patients had low bacterial load (less than $2x10^6$ *rpsL* copies/gram and less than 10^5 CFU/ml of sputum in cultures) and were excluded from the study. The final study population consisted in the remaining 9 patients (identified with letters from A to I) from whom 56 sputa were included because they contained more than 10^6 copies/gram of *rpsL* and more than 10^6 CFU/ml of sputum. Bacterial load determined by qRT-PCR of gDNA from sputa correlated with semi-quantitative cultures.

Analysis by RAPD demonstrated that 7 patients carried a single genotype while the other 2 carried two different genotypes each one (data not shown).

The sample collection time period for each patient was as follows: C 17 months; A, F, G and H 16 months; E 14 months; I 9 months; D 8 months; B 7 months.

Antibiotic resistance and expression of MexXY efflux pump

Resistance percentages to the tested antibiotics were as follows: gentamycin 46, amikacin 38, tobramycin 51, ciprofloxacin 61, tazobactam-piperacillin 56, ceftazidime 56, imipenem 61 and meropenem 39. Nine strains from 3 patients were susceptible to all tested antibiotics while 4 strains from 3 patients were resistant to all. These two were the most frequent resistance profiles, followed by other multi-resistant profiles. In total, 33 different profiles were found. Most of them included resistance to ß-lactams (28/33) and to aminoglycosides (24/33), while ciprofloxacin was present in 20/33. Profiles are shown in table 1 along with distribution by patient. Regarding resistance to aminoglycosides: 11 strains were resistant to 1, 10 were resistant to 2 and 10 were resistant to the 3 tested antibiotics of this class. No statistical differences were found between "resistant" vs. "susceptible/susceptible, increased exposure" strains for any antibiotic.

Table 1. Distribution of resistance profiles by patient

PROFILE	A	B	C	D	E	F	G	H	I	Total
No resistance (fully susceptible)			5	3				1		9
AN			1					1		2
CIP		1								1
IPM			1							1
AN-CIP		2								2
CAZ-CIP						1				1
CAZ-TZP								1		1
TM-CIP		1						2		3
CAZ-IPM-CIP						1				1
IPM-AN-CIP									1	1
IPM-GN-AN						1				1
CAZ-TZP-AN-CIP		1								1
CAZ-GN-AN-TM						1				1
CAZ-IPM-GN-TM-CIP							1			1
CAZ-TZP-GN-AN-TM								1		1
CAZ-TZP-IPM-MER									1	1
IPM-GN-TM-CIP							3			3
TZP-IPM-MER-CIP								1		1
CAZ-TZP-IPM-MER-AN	2									2
CAZ-TZP-IPM-MER-CIP	2								1	3
TZP-IPM-GN-TM-CIP							3			3
CAZ-TZP-IPM-MER-GN-TM					3					3
CAZ-TZP-GN-AN-TM-CIP								1		1
CAZ-TZP-IPM-AN-TM-CIP	1									1
CAZ-TZP-IPM-GN-TM-CIP							2			2
CAZ-TZP-IPM-MER-AN-CIP						1				1
TZP-IPM-MEM-GN-AK-CIP	1									1
TZP-IPM-MER-GN-TM-CIP							1			1
CAZ-TZP-IPM-MER-AN-TM-CIP								1		1
CAZ-TZP-IPM-MER-GN-AN-TM	2									2
CAZ-TZP-IPM-MER-GN-TM-CIP					2		1			3
CAZ-TZP-MER-GN-AN-TM-CIP						1				1
CAZ-TZP-IPM-MER-GN-AN-TM-CIP	**2**					**1**	**1**			**4**
Total	10	5	7	3	5	7	12	9	3	61

AN: amikacin; CAZ: ceftazidime; CIP: ciprofloxacin; GN: gentamycin; IPM: imipenem; MER: meropenem; TM:tobramycin; TZP: tazobactam-piperacillin.

In bold: profile resistant to all tested antibiotics.

Table 2. Distribution of number of strains according to the number of classes of antibiotics to which the strains are resistance and mean *mexY/rpsL* expression.

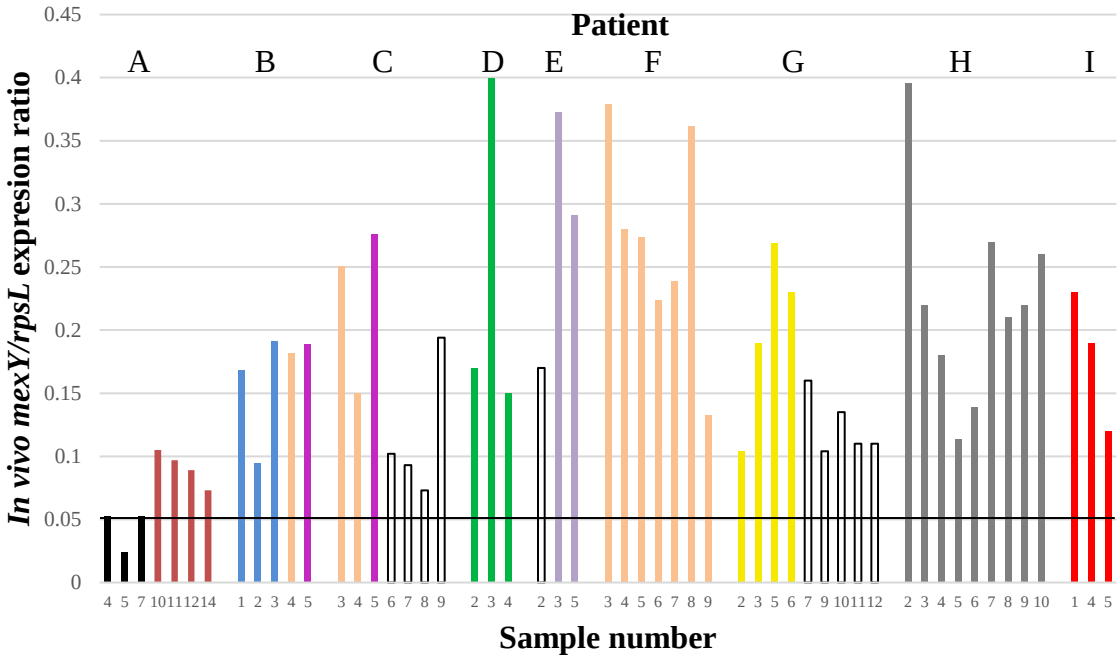
Profile	Mean *mexY in vivo*	Mean *mexY in vitro**
No resistance (fully susceptible)	0,198	16
Resistance to 1 class	0,155	20
Resistance to 2 classes	0,213	20
Multi-resistance	0,157	30

*Fold-change from PAO1

Expression of *mexY* was detectable in all the 53 analysed sputa at more than 5 x 10^4 copies/gram. The expression ratios of *mexY/rpsL* ranged from 0.02 (patient A) to 0.4 (patients D and H). No statistically significant differences were found in mean expressions of *mexY in vivo* ($p = 0.57$) or *in vitro* ($p = 0.13$) between fully susceptible, resistant to less than 3 antibiotic classes and multi-resistant strains. In fact, the lowest mean expression level was observed for multi-resistant strains (Table 2). Statistical differences were neither found between *in vivo* ($p = 0.70$) or *in vitro mexY* expression ($p = 0.13$) among strains resistant to 1, 2 or the 3 tested aminoglycosides.

We found that all the patients were colonised with strains carrying mutations in *mexZ*. Wild type *mexZ* allele was only found in the first 3 sputum samples obtained from patient A; these sputa expressed the lowest levels of *mexY*. On this basis, we have established an arbitrary threshold to classify sputum samples containing wild type and mutated *mexZ*; it corresponded to the mean expression of *mexY/rpsL* of these 3 sputa, being the value of 0.06 (Figure 1).

Figure 1. Expression level of *mexY in vivo* and corresponding status of *mexZ*.



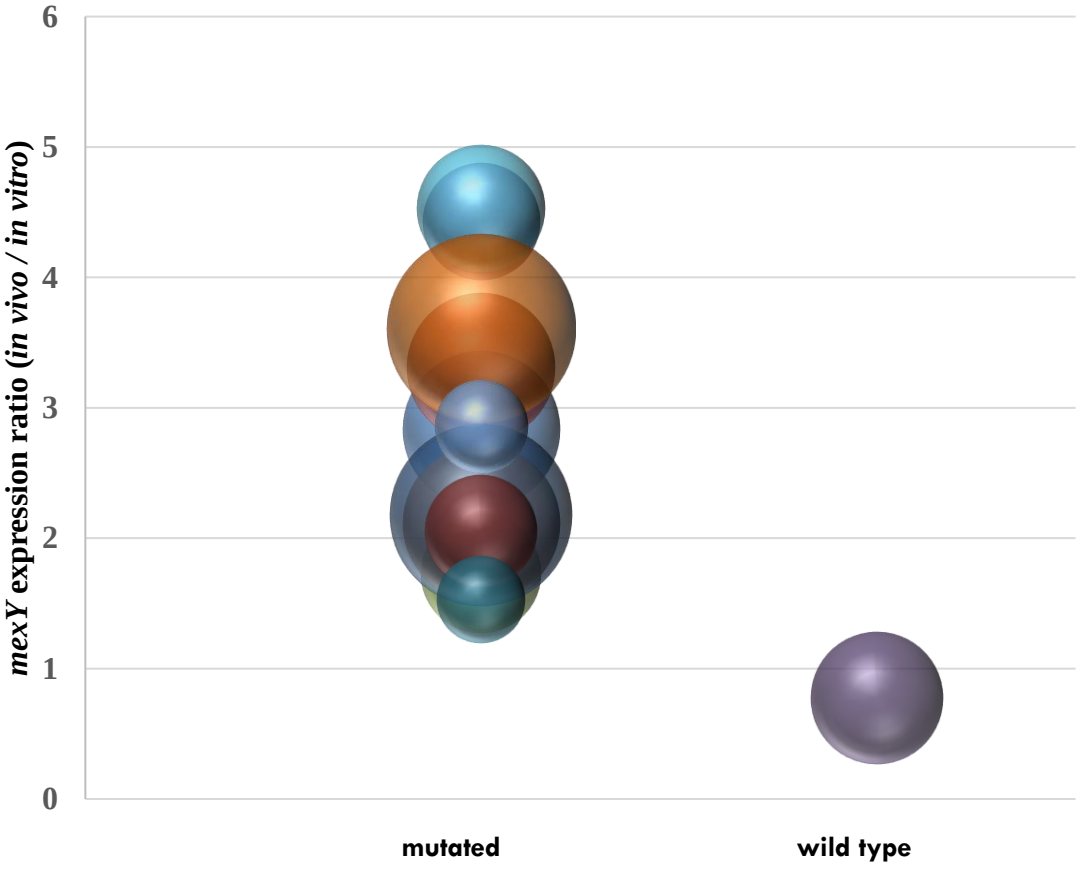
Wild type	Detected *mexZ* mutations									Not sequenced
	T12A	Δ1bp	Δ	D155G	G137D + L138R	Δ8bp	G195E	IS4	Δ21bp	

Black line : threshold to differentiate *mezZ* wild type from mutants (0,06)

Expression of *mexY in vitro* could be determined for 33 strains. Results for each patient ranged from: 8 to 19 (patient A); 12 to 25 (patient B); 3 to 30 (patient C); 14 (both strains from patient D); 24 (one strain from patient E); 25 to 33 (patient F); 26 to 81 (patient G); 11 to 42 (patient H); 9 to 21 (patient I). Expression levels of *mexY in vivo* were compared to that *in vitro* by dividing *mexY/rpsL* ratio from each sputum sample by the mean ratio of *mexY/rpsL* of all strains corresponding to each patient. Figure 2 shows in vivo / in vitro ratios according to *mexZ* allele status. It demonstrates that *in vivo mexY* expression in sputa containing *mexZ* mutated populations was 1 to 5 times higher than the expression of the 3 sputa from patient A ($p = 0.0035$). Among 43 sequenced strains, mean *mexY in vitro* expression ranged from 0.043 (wild type) to 0.231 (Δ mutation) but, again, no statistically significant differences were found in the expression level when comparing strains carrying each mutation.

Regarding correlation between *mexZ* mutations and antibiotic resistance profile, we found that the 3 wild type strains were resistant to 2 or 3 antibiotic classes; the 3 strains with the double mutation G137D + L138R were susceptible to all tested antibiotics; and the 3 strains with Δ1bp were only resistant to ciprofloxacin +/- amikacin. No other correlations were evident between type of *mexZ* mutation and resistance profile.

Figure 2. Expression level of *mexY* (*in vivo / in vitro* ratio) in sputum samples containing wild type and mutated *mexZ*.



Expression of nirS in vitro and in vivo

All strains except 3 grew in anaerobiosis. These strains corresponded to 3 different patients and all of them exhibited a small colony phenotype. As expected, the expression level of *nirS* was higher in anaerobiosis than in aerobiosis, meaning that its expression was induced under anaerobic conditions. Nevertheless, induction rates were highly variable, with a range between 2 and 1494. Moreover, 2 strains isolated from the same sputum could have very different induction rates, sometimes close to a 20-fold (ex.: strains 5a and 5b from patient G) (Table 3). The median induction rate was 38, which is slightly higher than the value measured for PAO1 reference strain (between 20 and 30).

Table 3. Induction of *nirS*

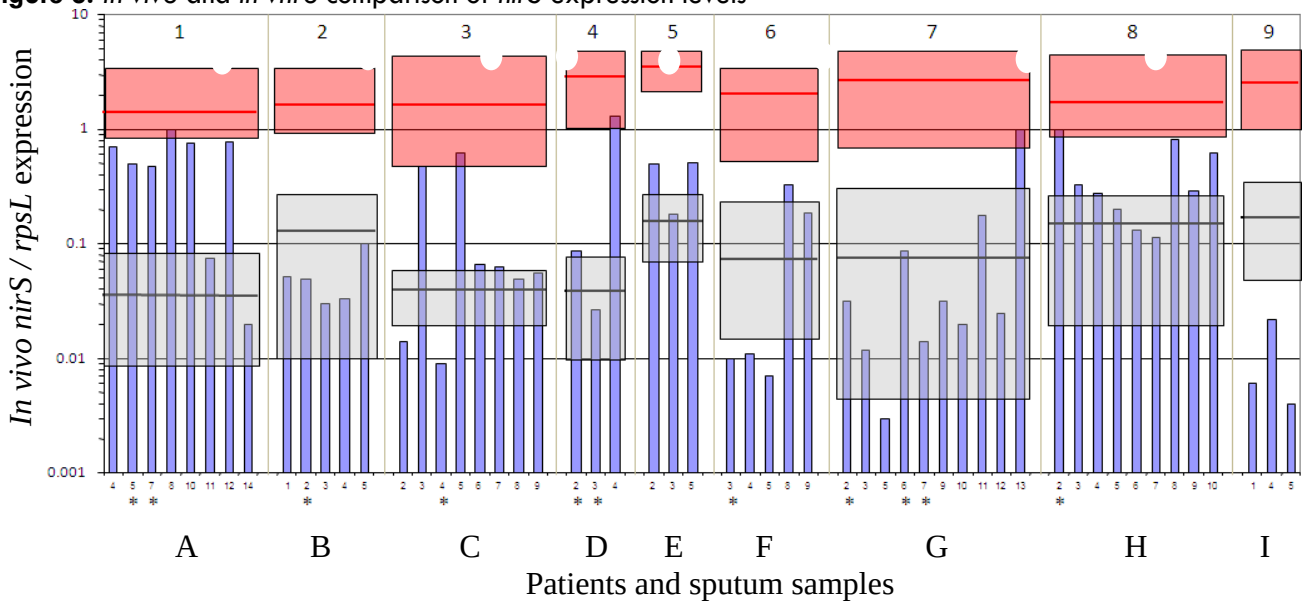
Patient	Strain	*nirS*/*rpsL* aerobiosis	*nirS*/*rpsL* anaerobiosis	*nirS* induction
A	4	0.003	3.278	1261
	5a	**0.141**	**0.308**	**2**
	5b	**0.011**	**0.237**	**21**
	6	0.008	1.408	183
	10	0.230	0.870	4
	12	0.013	2.830	216
	14	0.017	0.027	2
B	**1a**	**0.401**	**3.352**	**8**
	1b	**0.010**	**0.988**	**99**
	2	0.002	0.124	77
	3	0.041	1.302	32
	4	0.160	2.320	15
C	3	0.054	4.850	90
	4	0.020	1.040	52
	5a	**0.001**	**0.011**	**8**
	5b	**0.059**	**3.630**	**62**
	8	0.058	0.480	8
D	2	0.048	3.088	64
	3a	**0.002**	**0.147**	**74**
	3b	**0.007**	**5.016**	**760**
	4	0.090	3.380	38
E	3	0.280	2.169	8
	5	0.070	5.170	74
F	**3a**	**0.028**	**3.400**	**121**
	3b	**0.015**	**2.780**	**185**
	4a	**0.039**	**0.700**	**18**
	4b	**0.057**	**2.820**	**49**
	5	0.040	0.760	19
	8	0.257	3.390	13
	9	0.110	0.520	5
	2	0.036	0.700	19
	3	0.005	4.344	965
	4a	**0.324**	**1.480**	**5**
	4b	**0.022**	**3.160**	**147**
	5a	**0.023**	**1.880**	**82**
	5b	**0.002**	**2.540**	**1494**
	6	0.031	5.050	163

G	**7a**	**0.160**	**3.440**	**22**
	7b	**0.026**	**2.350**	**90**
	9	0.095	4.250	45
	10	0.019	2.550	134
	11	0.370	18.600	50
	12	0.739	21.000	28
	13	0.220	0.427	2
H	**2a**	**0.079**	**2.094**	**27**
	2b	**0.022**	**1.394**	**63**
	3	0.005	0.137	29
	4	0.093	0.990	11
	6	0.291	1.838	6
	7	0.110	1.330	12
	8	0.107	1.296	12
	9	0.346	29.910	86
	10	0.369	4.550	12
I	1	0.054	5.110	95
	4	0.085	1.557	18
	5	0.389	1.081	3

In bold: strains isolated from the same sputum sample.

By measuring cDNA levels for the gene *nirS* directly into sputum samples and after normalisation with *rpsL*, we determined *nirS* expression *in vivo*. These values were compared with those obtained *in vitro* for each patient (Figure 3).

Figure 3. *In vivo* and *in vitro* comparison of *nirS* expression levels



*Samples collected during acute exacerbations.
*Samples collected during acute exacerbations.
Mean levels (red and grey lines) and the range (boxes) of *in vitro* expression in aerobiosis and anaerobiosis with respect to the expression levels *in vivo* (Y axis). Grey zones represent *nirS*/*rpsL* rates measured in

aerobiosis (20% of oxygen) while red zones represent ratios measured in anaerobiosis (<0.1% of oxygen). Zones localized between grey and red boxes define a microaerophilic zone.

Anaerobic *in vitro* expression is comparable between the 9 patients (rates 1.3 to 3.6); in contrast, aerobic *in vitro* expression shows great variability (0.04 to 0.18). These results are due to a very high aerobic *nirS* expression level for few strains from patients B, E, H and I. According to clinical information, no relationship exists between *nirS* expression during acute exacerbations (asterisks in Figure 3) compared to chronic disease.

Discussion and conclusions

The objective of this study was to further understand the behaviour and the growth conditions of *P. aeruginosa* when colonising the complex environment of the lungs of patients with CF. We adapted a previous published method [30] for the *in vivo* analysis of genetic expression in tracheal aspirates from intubated patients and applied it to analyse sputum samples of 9 CF patients. In this way we measured the expression of the *mexY* gene, coding for the protein MexY (the inner membrane antiporter of the three-component MexXY-OprM efflux pump [22]) and a gene involved in anaerobic growth by denitrification (*nirS* nitrite-reductase).

Two out of the nine patients (22%) carried a single genotype identified by RAPD. This finding is in agreement with previous analysis of *P. aeruginosa* from chronic stage of CF patients showing that in a period of up to 20 months cloning-derived mutants exhibited up to 20% divergence in genomic macrorestriction patterns [31]. Nevertheless, it has been demonstrated that mutator strains are present in less than 10% of *P. aeruginosa* strains in early stages of CF but this figure reaches up to 60% in chronic infection [32]. In fact, it is known that over longer periods of time (up to 25 years) hypermutation leading to a loss of virulence, adaptation to biofilms and acquisition of higher antibiotic resistance levels, among others, is necessary to establish chronic infection [12,33].

Even though our study period was relatively short we observed high rates of antibiotic resistance, being the lowest for amikacin (38%), and 41% of strains exhibited multi-resistance. As we do not have enough clinical data, these findings may indicate that most patients were already at a chronic stage of the disease.

It is worth to point out that susceptibility testing guidelines, such as EUCAST, have undergone deep changes over the last years. For *P. aeruginosa* these changes included the elimination of breakpoints for gentamycin because of its low efficacy and more recently the recommendation to use amikacin only for urinary tract infections. Tobramycin remains as the only aminoglycoside for use in infections originating in other body sites. However, the mechanism that we investigated is the most frequent for intrinsic and acquired aminoglycoside resistance in CF patients and we were able to demonstrate that its overexpression is directly linked to the presence of *mexZ* mutant populations.

All strains from patient D were fully susceptible and, for a global *in vitro mexY* expression level of 3 to 81-fold-change in reference to PAO1, the *in vitro* level for this patient was low (14-fold-change); nevertheless *in vivo mexY* expression were among the highest. On the opposite, all strains from patient G were multi-resistant while *in vitro mexY* expression was the highest (26 to 81-fold-change) but *in vivo mexY* expression were from average to low. It seems clear that other *in vivo* factors that we did not account for play an important role in gene expression.

We found that only 1 out of the 9 studied patients was colonised by wild type *mexZ* repressor gene. Among the remaining 8 patients, all the identified mutations in *mexZ* were predicted as non-tolerated by the SIFT algorithm (https://sift.bii.a-star.edu.sg), indicating a complete loss of the function of this protein. These mutant populations were probably selected during aminoglycoside treatment. Of note, all the involved patients received amikacin or tobramycin at a certain point during the study period. The regulator *mexZ* was previously reported as the most frequently mutated gene among 29 CF patients [33,34]. The fact that no statistically significant differences were observed in *mexY* expression between strains fully susceptible or resistant to one, two or three antibiotic classes, could indicate that other factors influence the expression of antibiotic resistance and/or of MexXY-OprM. Actually, it has recently been demonstrated that mutations in genes other than *mexZ* (*fusA1* and *amgRS*) have a higher impact on reducing aminoglycoside susceptibility and that combination of mutations in the three genes have a stronger effect [23].

For three patients the type of *mexZ* mutation correlated with the resistance profile, although the association was inverse to what was expected: strains harbouring wild type *mexZ* were resistant to 2 classes (not including aminoglycosides) or multi-resistant (including aminoglycosides) while double mutants were fully susceptible. It is expected that the resistant strains express other mechanisms.

In comparison with *in vitro* measurements, *mexY* expression was 2 to 5 times higher than the expression in sputum samples containing mutant populations. This also suggests that in the absence of repression mediated by *mexZ* it could be an

additional increase in the expression of *mexY* when bacteria develop *in vivo*.

The gene *mexY* was not induced in anaerobiosis (data not shown). This finding indicates that environmental factors other than the atmosphere have an incidence in the increased expression of this gene. One of such factors could be the presence of reactive oxygen species (ROS) generated during the chronic inflammation phase of CF. In fact, it was previously suggested that oxidation of bacterial DNA by ROS is responsible for an increased risk of hypermutation [32] and that hydrogen peroxide is able to induce *mexY in vitro* and to increase the frequency of strains resistant to aminoglycosides [35].

Using the same set of samples, we measured the *in vivo* and *in vitro nirS* expression to estimate growth conditions of *P. aeruginosa* in the lungs of these patients. All the 56 studied strains demonstrated *nirS* induction under anaerobic conditions, although expression levels were highly variable. PAO1 induction rates were similar to what is described by elsewhere (ratio 20-30) [15]. Induction of *nirS* is regulated in anaerobiosis by the transcriptional activator Dnr which is, in turn, under control of the regulator of nitrate reduction in anaerobiosis Anr [36,37] and of the NarL/NarX nitrate detection system[36]. *nirS* operon is directly or indirectly repressed by the quorum-sensing regulator RhlR [38]. Anr and Dnr are necessary for anaerobic but not for microaerophilic growth [15]. We assume that both Anr and Dnr regulators were functional in all the strains that were able to grow in anaerobiosis (all but three). The differences in induction rates could be the result of a peripheric regulation via de quorum-sensing system or of *nirS* expression under microaerophilic conditions[39].

If we extrapolate *in vitro* values to sputum samples we can establish the following classification: 32 out of 54 (59%) sputa give values compatible with aerobic conditions, 6/54 (11%) give values in the lower limit of anaerobic zone and the remaining 16 (30%) show microaerophilic conditions. Sputa from patients B and I are all classified as aerobic while most samples from patient A show levels closer to anaerobiosis. Samples from patient G exhibit a temporal tendence towards anaerobiosis but no such tendence is observed for the other patients. In accordance with our findings, a previous article of *in vitro* experiments mimicking CF lungs conditions shows that *P. aeruginosa* grows preferentially under aerobic or microaerobic atmosphere [15]. *In vivo* data obtained in the present study is in favour of an heterogeneous environment where different bacterial sub-populations develop using aerobic respiration (with oxygen as final electron acceptor) in the mucous surface and under microaerophilic/anaerobic conditions deeper within the mucus, as it was previously published [14,15]. Bacteria developing within zones of aerobic conditions are probably planktonic, as opposed to those developing in zones of low oxygen concentration, situation that promotes biofilm production.

We analysed more than 50 sputa allowing us to compare *in vivo* expression for a same patient and to follow its evolution. Our study period was 17 months so, even if it is not a neglectable follow-up time, it limited our possibility to observe trends in the behaviour of *P. aeruginosa* during chronic infections. Nevertheless, we were able to obtain some useful conclusions.

The present study allowed us to predict different aminoglycoside efficacy according to *mexY in vivo* expression and growth conditions. For instance, patient A exhibited the lowest *mexY in vivo* expression levels. Nevertheless, most of the strains isolated from their sputa indicates growth under microaerophilic/anaerobic atmosphere, conditions under which aminoglycosides do not have activity. In contrast, all sputum samples from patient F indicate aerobic conditions but *mexY* expression was among the highest. According to these two parameters and to the persistence of *mexZ* mutated populations over a 17-month period, it is reasonable to predict that aminoglycoside treatment will only allow to eradicate a small proportion of *P. aeruginosa* population. Inhaled tobramycin, the only recommended aminoglycoside for respiratory infections [27,40], reaches intraluminal concentration well over the minimal inhibitory concentration for *P. aeruginosa* [41]. In consequence, mutant *mexZ* population could be eradicated during tobramycin inhaled treatment under aerobic conditions. Sputum samples analysed in the present study were obtained under respiratory physiotherapy and should come from the lower respiratory tract. However, we cannot exclude the possibility that sputum quality was no homogeneous, i.e., there could be samples coming from upper respiratory tract with exposition to higher oxygen concentrations.

Antibiotics such as aminoglycosides, quinolones and ß-lactams could eliminate planktonic bacteria which account for the biggest bacterial mass accumulated during acute exacerbations, but they would not have any effect on bacteria growing within biofilms. This is the concerning group of bacteria that acts as a reservoir during chronic infections.

Although expression levels were variable and did not indicate a particular trend, we were able to establish a threshold to differentiate basal levels of *mexY* expression, corresponding to wild type *mexZ* gene, and overexpression corresponding to a mutated population. Our results show that

heterogeneous populations (wild type and mutated) of *P. aeruginosa* colonise CF lungs. In our study population, *mexZ* mutated populations were by far the most frequent (3/54 sputa and 1/9 patients). Even though we do not have complete clinical data we know that all patients were under aminoglycoside prophylaxis or treatment at some point during the study period. It would be interesting to investigate if variations in the genetic expression in a single patient is a result of relative proportions of wild type and mutated populations or if they are rather due to a modulation of *mexY* expression within a single homogeneous population. The occurrence of different *mexZ* mutations, even among strains from a same patient, suggests a repeated selection and might be independent from mutant *mexZ* alleles.

We did not find a correlation between *mexY* expression and aminoglycoside's susceptibility profile. This observation was already made twenty years ago but in that case no mutations in *mexZ* regulator were found [26], although the authors conclude that MexXY overexpression in aminoglycoside resistant strains occurs via mutation in one or more genes that were not identified at the time of the study. We now know that mutations in *fusA1* and *amgRS* have a greater impact than *mexZ* in reducing aminoglycoside susceptibility [23].

Few studies have so far analysed gene expression of P. aeruginosa *in vivo* in patients with CF, either by measuring gene expression directly into clinical samples [42-44] or under laboratory conditions mimicking human conditions or animal models [45,46]. It is worth noting that molecular analysis for the present study was performed more than ten years ago. Great advances had been made in the field of genomics since then, with transcriptomics and proteomics widely used now-a-days. Yet, new treatment strategies focusing on the complex environment of CF lungs are lacking.

Conclusions

Measures of *in vivo* and *in vitro* gene expression showed that sputum samples harbouring wild type *mexZ* alleles expressed the lowest levels of *mexY*, allowing us to establish cut-off of to differentiate sputum samples carrying mutant from wild type *mexZ*. We also estimated that the isolated strains grew preferentially under aerobic or microaerophilic conditions in the lungs. As well as other few studies investigating *in vivo* gene expression of *P. aeruginosa*, our results shed some light about the behaviour of this bacterium when colonising patients with CF. However, they cannot entirely explain all factors influencing the persistent infection and the progression of antibiotic resistance, making it difficult to design new therapeutic approaches.

Conflict of interests

None

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