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Cross-Sectional and Longitudinal Multilocus Sequence Typing of *Pseudomonas aeruginosa* in Cystic Fibrosis Sputum Samples▽

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Multilocus sequence typing (MLST) is a genetic typing tool designed to provide information about the relatedness of isolates at the core genome level. The utility of MLST in regard to cystic fibrosis (CF)-related infection with *Pseudomonas aeruginosa* is unknown. The molecular clock speed of the MLST genes was studied using 219 colonies isolated longitudinally from 49 patients with CF. A cross-sectional study examining 27 to 46 colonies per sputum sample for samples from 16 patients was also undertaken. The molecular clock speed was estimated to be 2.05×10^{-5} (upper 95% confidence limit) or 4.75×10^{-6} (50% confidence limit) point mutations per nucleotide per year. In the cross-sectional study, 50% of patients were infected with more than one sequence type. There was evidence of point mutations, recombination events, and coinfection with epidemic and unique strains. A clonal complex that was highly genetically distinct from the rest of the *P. aeruginosa* population was identified. The MLST scheme uses genes with an appropriate clock speed and provides useful information about the genetic variation of *P. aeruginosa* within and between patients with CF.

Multilocus sequence typing (MLST) is a typing tool based on the DNA sequences of several housekeeping genes. Housekeeping genes are chosen because they are assumed to be under low selection pressure and therefore to have a low molecular clock speed, i.e., to undergo mutations at a low rate. This allows MLST to provide data about the core evolutionary genome of a bacterium which allow deductions about population structure and the relatedness of different strains. Where populations are rapidly diversifying by recombination, as may occur in a clinical outbreak, MLST is able to identify closely related isolates (22) which may have undergone changes in one or two loci. MLST has recently been used to estimate the molecular clock speed of the housekeeping genes in cystic fibrosis (CF)-related *Burkholderia cepacia* complex infection and to identify presumed recombination events in infected patients (24). We hypothesized that the clock speed of the housekeeping genes of the *Pseudomonas aeruginosa* MLST scheme in patients with CF would be of the same order and that point mutations and recombination events would be identified. These hypotheses were tested by examining sequential isolates from chronically infected patients with CF (clock speed study) and by examining multiple isolates from single sputum samples (cross-sectional study).

MATERIALS AND METHODS

Bacterial isolates for the clock speed study. A set of 47 *P. aeruginosa* isolates cultured, at different time points ranging from 4 to 44 months apart, from 23 patients (H01 to H23) with CF was provided by the Health Protection Agency Laboratory, Colindale, United Kingdom. They had been sent from the CF centers of three London hospitals (Great Ormond Street, Kings College, and Chelsea and Westminster Hospitals) for strain genotyping, and one colony from each sputum sample had been stored in cryobeads at −80°C following identification as *P. aeruginosa* by formation of pyocyanin on Kings A agar and oxidase positivity, or by the API20NE test for non-pigment formers. Isolates were transported to Warwick on agar slopes, streaked to purity, and stored at −80°C in 90% heart infusion broth and 10% glycerol. No clinical information was available for these isolates, which were part of a genotype surveillance study.

Another set of 172 *P. aeruginosa* isolates were collected from sputum samples provided, at different time points ranging from 1 to 10 weeks apart, by 26 patients (W01 to W26) from the West Midlands Adult CF Unit who had been infected for over 12 months. Single colonies of each morphotype from each *P. aeruginosa* isolation plate were transported to the University of Warwick in charcoal agar transport swabs, streaked to purity, and stored in heart infusion broth and glycerol at −80°C.

DNA was extracted using the Promega Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions, and MLST was performed as previously described (1). The point mutation rate was estimated using the Poisson distribution as previously described (24), assuming that the isolation of the same sequence type (ST) from a patient over a period of time demonstrated genetic stability of the housekeeping genes over that period regardless of any additional STs isolated. Other data were compared with isolates in the pubMLST database (http://pubmlst.org/paeruginosa) using DnaSP v4.1 (16).

Bacterial isolates for the cross-sectional study. Sixteen patients at the West Midlands Adult CF Unit who had been infected with *P. aeruginosa* for over 12 months provided sputum samples as part of their routine clinical care. The samples were vortexed at 37°C in an equal volume of Sputasol, and then an aliquot of 0.5 to 1.0 ml was transported to the University of Warwick in 7-ml universal containers. Serial dilutions of 10^{-1} to 10^{-6} were made with phosphate-buffered saline, and 50-μl portions of the 10^{-2}, 10^{-4}, and 10^{-6} dilutions were spread onto the following agar plates before overnight incubation at 37°C: brain heart infusion, *P. aeruginosa* isolation agar (PIA), PIA containing 2 mg/liter ciprofloxacin, PIA containing 8 mg/liter ceftazidime, and PIA containing 8 mg/liter tobramycin. Forty-seven colonies were picked, making sure colonies from all plates and of all morphologies were represented, using a 1-μl sterile loop and then streaked to purity on brain heart infusion agar. Alkaline lysates were made as previously described (21) in 96-well plates for use as PCR templates for MLST as described above.

Ethical approval for the studies was granted by the local research ethics committee.

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RESULTS

Molecular clock speed of *P. aeruginosa* housekeeping genes. Twenty STs were identified among the 47 Health Protection Agency isolates from 23 patients, and no changes of ST were seen over time (a total of 41.2 isolate-years). Data from the West Midlands Adult CF Unit are represented in Table 1. In all 49 patients there were STs which were present unchanged over a period of time. In total, these stable STs represented 50.73 isolate-years. Thus, the observed number of point mutations in 2,882 base pairs (the total size of the seven housekeeping genes) in 49 patients over 50.73 years was zero. Assuming that point mutations follow a Poisson distribution, calculations based on the upper 95% confidence limit (i.e., the probability of a higher mutation rate is 5%) revealed an estimated rate of 2.05×10^{-5} point mutations per nucleotide per year. Using the 50% confidence limit, the rate was estimated to be 4.75×10^{-6} point mutations per nucleotide per year.

According to previous studies, an estimate of the evolutionary time since the existence of a putative common ancestor for a species can be calculated by dividing the average distance between alleles at synonymous locations by the estimated clock speed (9). Using DnaSP, the average distance between alleles at synonymous locations for all isolates in the pubMLST database was 0.0328, which gives an estimated evolutionary time of 6,910 years (50% confidence limit) for *P. aeruginosa*.

In patient W26, a clonal complex of three STs (ST-366, ST-368, and ST-372, which are single-locus variants of each other) was found, which forms a very separate group, or clade, in the phylogenetic tree of *P. aeruginosa*. They all contain *trpE1*, an old allele, and ST-372 contains *guaA29*, an old allele, but their other five alleles were novel. BLAST searches for these novel alleles and the 16S ribosomal subunit genes of these isolates all returned *P. aeruginosa* isolates as the closest matches. Using DnaSP, the average nucleotide difference between this novel clonal complex and the other STs in the MLST database was 4.5%, whereas the average difference across all the other STs was just 0.85%.

Diversity of *P. aeruginosa* in sputum samples. Sixteen patients provided sputum samples for this study. The mean age was 28.3 years (standard deviation [SD], 7.1), 56.3% were male, the mean body mass index was 21.6 (SD, 3.4), and the mean forced expiratory volume in 1 sec (FEV_1) was 49.8% predicted (SD, 21.8). Forty-seven isolates per sputum sample were tested, and full MLST data were obtained for 27 to 46 of the 47 isolates (mean, 34.8) per patient. Eight patients (50%) had only one ST, and eight patients (50%) had more than one ST; six (37.5%) had two STs, one (6.3%) had three STs, and one (6.3%) had four STs (Table 2).

Data were analyzed (chi-square test or Mann-Whitney U test) according to whether patients had one ST or multiple STs in their sputum sample. No statistical differences were found for sex, age, FEV_1, mean body mass index, use of inhaled antimicrobials, use of azithromcyin, or number of courses of intravenous antimicrobials in a year. In five of the eight patients with multiple STs, the use of antimicrobial agar helped to select out particular STs. Patients with multiple STs were more likely to have been previously found (data not shown) to harbor hypermutator strains of *P. aeruginosa* than patients with one ST (62.5% versus 14.3%), but the association did not reach statistical significance (chi-square test, $P = 0.057$).

DISCUSSION

The molecular clock speed of 4.75×10^{-6} point mutations per nucleotide per year for *P. aeruginosa* estimated in this study was similar to that (2.36×10^{-6}) found for the *Burkholderia cepacia* complex (24) and *Helicobacter pylori* (2) using the same methodology. The slow clock speed implies that the genes chosen for the MLST scheme were appropriate and should give robust information about the organism's core genome. The estimated evolutionary time of *P. aeruginosa* is much shorter than those of the *B. cepacia* complex and of *Salmonella enterica* serovar Typhi, but these estimates depend upon the assumption that polymorphisms occur predominantly by mutation, whereas the *B. cepacia* complex and *P. aeruginosa* also evolve by recombination, making the estimates less robust. Furthermore, the MLST database probably contains more isolates from clinical sources than from environmental sources, and the clock speed was estimated from clinical isolates, which may bias any estimation of evolutionary time. Indeed, clinical isolates may represent, or contain, a more recent evolutionary group within *P. aeruginosa*. In keeping with that possibility, a small study of marine *P. aeruginosa* strains around Japan (8) suggests that different STs may predominate in particular ecologies, though one of their freshwater isolates had the same ST (ST-179) as the isolates from patient W27. It would be interesting to compare clinical and environmental isolates from the MLST database in the future when it will contain data from more diverse isolates.

The clock speed estimate has limitations which need to be borne in mind. MLST analysis is possible for only a limited proportion of the bacterial load in a sputum sample, which itself represents a limited proportion of the bacterial load of the lung. Therefore, as for any study of CF respiratory microbiology, sampling limits the conclusions that can be drawn. In particular, it is impossible to tell whether any genetic differences between isolates are due to genetic change over time or merely to sampling different coinfecting strains over time. To avoid this problem, we followed previous methodology (2) in focusing on strains which had not changed over time in order to estimate the clock speed. Nevertheless, interesting genetic differences were found and are discussed below, though the overall methodology and its limitations must be borne in mind.

There were five patients in whom the possibility of point mutation or recombination was demonstrated (Table 1). Patients W12, W20, W25, and W26 all harbored isolates with STs which differed by only one nucleotide. In each case the difference resulted in a novel allele (i.e., one which was not previously represented in the MLST database). The presence of single novel nucleotide differences is more in favor of point mutation than of recombination (4). Patient W14 harbored isolates which were identical apart from a 4-nucleotide insertion in the *ppsA* allele. Patient W26 harbored isolates which were identical apart from a 5-nucleotide difference in *guaA*. Since this difference did not result in a novel allele, a recombination event is very likely. In all of these examples the changes may have occurred in vivo or, in view of how common infection with multiple strains is, may represent diversity in the

TABLE 1. Allelic profiles of isolates from the clock speed study (West Midlands patients only)

Patient	Time (wk)	ST	Allele[a]						
			acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*
W01	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	12.3	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	0	170	*36*	*5*	*29*	*7*	*4*	*10*	*7*
	19.9	170	*36*	*5*	*29*	*7*	*4*	*10*	*7*
W02	0	347	*40*	*5*	*17*	*5*	*4*	*15*	*7*
	0	348	*22*	*20*	*11*	*3*	*3*	*3*	*7*
	20.1	348	*22*	*20*	*11*	*3*	*3*	*3*	*7*
	0	353	*88*	*27*	*28*	*3*	*4*	*13*	*7*
	4.1	353	*88*	*27*	*28*	*3*	*4*	*13*	*7*
W03	0	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	12.1	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
W04	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	18.3	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	0	170	*36*	*5*	*29*	*7*	*4*	*10*	*7*
	18.1	170	*36*	*5*	*29*	*7*	*4*	*10*	*7*
W05	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	5.7	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
W06	0	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	10.9	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	0	236	*28*	*5*	*1*	*5*	*4*	*32*	*10*
	0	349	*89*	*30*	*64*	*78*	*48*	*24*	*32*
	13.9	349	*89*	*30*	*64*	*78*	*48*	*24*	*32*
W07	0	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	1	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	1.4	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	0	236	*28*	*5*	*1*	*5*	*4*	*32*	*10*
	17.9	236	*28*	*5*	*1*	*5*	*4*	*32*	*10*
W08	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	11.3	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
W09	0	349	*89*	*30*	*64*	*78*	*48*	*24*	*32*
	2	349	*89*	*30*	*64*	*78*	*48*	*24*	*32*
W10	0	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	7	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
W11	0	350	*16*	*5*	*5*	*74*	*2*	*10*	*10*
	7.7	350	*16*	*5*	*5*	*74*	*2*	*10*	*10*
W12	0	237	*85* (1)	*1*	*59*	*6*	*1*	*33*	*42*
	0	238	*5*	*1*	*59*	*6*	*1*	*33*	*42*
	9.1	238	*5*	*1*	*59*	*6*	*1*	*33*	*42*
W13	0	354	*16*	*5*	*5*	*79*	*2*	*10*	*10*
	7.3	354	*16*	*5*	*5*	*79*	*2*	*10*	*10*
W14	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	0	148-ins	*17*	*5*	*1*	*3*	*13*	*6-ins*[b] (4)	*7*
	8.1	148-ins	*17*	*5*	*1*	*3*	*13*	*6-ins* (4)	*7*
	0	352	*107*	*3*	*20*	*62*	*4*	*7*	*3*
	3.9	352	*107*	*3*	*20*	*62*	*4*	*7*	*3*
W15	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	0	367	*36*	*53*	*29*	*7*	*4*	*10*	*7*
	13.9	367	*36*	*53*	*29*	*7*	*4*	*10*	*7*
W16	0	351	*106*	*66*	*11*	*3*	*29*	*4*	*9*
	6.9	351	*106*	*66*	*11*	*3*	*29*	*4*	*9*
W17	0	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	5.6	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
W18	0	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	12	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	12	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
W19	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	7.3	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
W20	0	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	2.9	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	0	374	*6*	*5*	*11*	*81* (1)	*4*	*23*	*1*
	2.9	374	*6*	*5*	*11*	*81* (1)	*4*	*23*	*1*
W21	0	17	*11*	*5*	*1*	*7*	*9*	*4*	*7*
	18.7	17	*11*	*5*	*1*	*7*	*9*	*4*	*7*
	0	370	*6*	*5*	*36*	*63*	*4*	*49*	*2*
	9.3	370	*6*	*5*	*36*	*63*	*4*	*49*	*2*
W22	0	170-del	*36*	*5*	*29*	*7-del*[c]	*4*	*10*	*7*
	7.7	170-del	*36*	*5*	*29*	*7-del*	*4*	*10*	*7*
W23	0	146	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	0	231	*6*	*5*	*36*	*63*	*4*	*30*	*2*
W24	0	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	11.3	148	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	0	231	*6*	*5*	*36*	*63*	*4*	*30*	*2*
	9.1	231	*6*	*5*	*36*	*63*	*4*	*30*	*2*
W25	0	231	*6*	*5*	*36*	*63*	*4*	*30*	*2*
	14.1	231	*6*	*5*	*36*	*63*	*4*	*30*	*2*
	0	376	*6*	*5*	*36*	*63*	*4*	*30*	*59* (1)
W26	0	366	*87*	*70*	*79*	*80*	*53*	*48*	*1*
	0	368	*90* (1)	*70*	*79*	*80*	*53*	*48*	*1*
	9.6	368	*90* (1)	*70*	*79*	*80*	*53*	*48*	*1*
	0	372	*90*	*70*	*29* (5)	*80*	*53*	*48*	*1*

[a] The number of nucleotide differences between alleles of one ST and another ST is indicated in parentheses (e.g., in patient W12 *acsA85* differs from *acsA5* by one nucleotide).
[b] 6-ins is the same as *ppsA6* but with a 4-bp insertion.
[c] *mutL7*-del is the same as *mutL7* but with a 52-bp deletion from bp 26 to 77.

TABLE 2. Allelic profiles of the STs found in the cross-sectional study of patient sputum samples

Patient	ST[a]	Proportion of ST	Allele[b]						
			acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*
W03	146	35/35	*6*	*5*	*11*	*3*	*4*	*23*	*1*
W04	148	34/34	*17*	*5*	*1*	*3*	*13*	*6*	*7*
W10	146	39/39	*6*	*5*	*11*	*3*	*4*	*23*	*1*
W27	179	31/31	*36*	*27*	*28*	*3*	*4*	*13*	*7*
W28	232	36/36	*17*	*5*	*11*	*64*	*4*	*4*	*2*
W29	148	30/30	*17*	*5*	*1*	*3*	*13*	*6*	*7*
W30	148	27/27	*17*	*5*	*1*	*3*	*13*	*6*	*7*
W31	146	32/32	*6*	*5*	*11*	*3*	*4*	*23*	*1*
W01	148 (c)	8/43	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	170	35/43	*36* (5)	*5*	*29* (3)	*7* (4)	*4* (2)	*10* (4)	*7*
W07	148	11/46	*17* (1)	*5*	*1*	*3* (5)	*13* (2)	*6* (5)	*7* (2)
	236 (c)	31/46	*28*	*5*	*1*	*5*	*4*	*32*	*10*
	239	1/46	*28*	*5*	*1*	*5*	*43* (1)	*32*	*10*
	240	3/46	*28*	*5*	*1*	*5*	*4*	*32*	*43* (1)
W12	238 (c)	1/32	*5*	*1*	*59*	*6*	*1*	*33*	*42*
	237	30/32	*85* (1)	*1*	*59*	*6*	*1*	*33*	*42*
	243	1/32	*86* (2)	*1*	*59*	*6*	*1*	*33*	*42*
W18	146 (c)	18/42	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	231	24/42	*6*	*5*	*36* (1)	*63* (1)	*4*	*30* (4)	*2* (2)
W22	146 (c)	4/28	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	170-del	24/28	*36* (2)	*5*	*29* (1)	*7-del*[c]	*4*	*10* (3)	*7* (1)
W23	146 (c)	30/35	*6*	*5*	*11*	*3*	*4*	*23*	*1*
	231	5/35	*6*	*5*	*36* (1)	*63* (1)	*4*	*30* (4)	*2* (2)
W24	148 (c)	19/30	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	231	11/30	*6* (7)	*5*	*36* (1)	*63* (1)	*4* (2)	*30* (5)	*2* (1)
W32	148 (c)	30/37	*17*	*5*	*1*	*3*	*13*	*6*	*7*
	233	7/37	*16* (5)	*5*	*30* (1)	*11* (3)	*13*	*31* (5)	*41* (1)

[a] c, comparator ST.
[b] The difference between alleles of one ST and its comparator ST is indicated in parentheses (e.g., in patient W07 *nuoD43* in ST-239 differs from *nuoD4* in ST-236 by one nucleotide).
[c] *mutL7*-del is the same as *mutL7* but with a 52-bp deletion from bp 26 to 77.

environment from which the patients acquired their infections, leading to transient or permanent infection with similar STs, with different ones being isolated at different time points. Interestingly, in a separate study, patients W12, W14, and W26 were found to harbor hypermutator strains, a phenotype which increases the rate of point mutation up to 1,000-fold (6, 14) and also increases recombination (11).

Previous studies examining 1 to 10 colonies from sputum samples have demonstrated at least transient coinfection with different strains of *P. aeruginosa* in 33 to 48% of patients (17, 20, 23), and longitudinal studies examining different morphotypes from sputum samples have given values of 13 to 75% (3, 5, 10, 13, 15, 18). In the first study to examine more than 20 colonies per sample and to use MLST, we found that 50% of our patients had coinfection. We expected coinfection to be associated with younger age (since infection usually stabilizes later in the course of infection) and also, therefore, with better lung function, but these associations were not found. An association with hypermutator presence was found, but seven of the eight coinfected patients harbored very genetically different STs (Table 2), making coinfection with different strains the likely explanation for the genetic diversity rather than hypermutator presence increasing genetic drift. However, patient W12 had STs differing by only one or two nucleotides and patient W07 had STs differing by only one nucleotide, suggesting point mutation in these two patients, both of whom harbored hypermutators. Thus, genetic drift, which has been identified previously by longitudinal studies of the genome (19), can be identified by MLST.

Several epidemic strains were identified: ST-146 (the Liverpool epidemic strain) was found in the sputa of six patients, ST-148 (the Midlands 1 epidemic strain) was found in the sputa of seven patients, and ST-231 was found in the sputa of three patients. The other 11 STs were found only in the sputa of individual patients. Previous studies (7, 12) demonstrated that epidemic strains are capable of replacing unique strains in the CF lung, but seven of the eight patients in this study were infected with other STs as well as epidemic STs, suggesting that replacement with epidemic strains is not the rule.

In patient W26, who harbored hypermutator strains, an unusual clonal complex of three STs was found, which had an average distance of 4.5% from the rest of the STs in the MLST database. For the *B. cepacia* complex the variation within species, which had been defined by DNA-DNA hybridization and phenotypic tests, is less than 3%, and the variation between species within the complex is 3% to 6% (A. Baldwin, personal communication, 2007). By analogy with these values, this clonal complex may represent a new subspecies or genomovar of *P. aeruginosa*.

Conclusions. The housekeeping genes for the *P. aeruginosa* MLST scheme had a molecular clock speed in keeping with that of the *B. cepacia complex* in CF-related infection, so their inclusion in the MLST scheme was appropriate. MLST identified possible point mutations and recombination events, with

hypermutators possibly playing a role in point mutation, as well as identifying the presence of a clonal complex of isolates highly genetically distinct from the rest of the population. MLST analysis of more than 20 isolates per sputum sample identified coinfection with different strains in 50% of the patients, in keeping with previous data, and frequent coinfection of unique strains with epidemic strains. Thus, MLST provides useful information about the genetic variation of *P. aeruginosa* within and between patients with CF.

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