Emergence of a \textit{mutL} Mutation Causing MLST-PFGE Discrepancy among \textit{Pseudomonas aeruginosa} Isolates from a Cystic Fibrosis Patient

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An MLST-type shift (from ST242 to ST996) was detected in *Pseudomonas aeruginosa* isolates with a uniform PFGE-pattern obtained from a chronically-colonized patient. MLST mutational change involved the *mutL* gene with the consequent emergence of a hyper-mutable phenotype. This observation challenges the required neutrality of *mutL* as an appropriate marker in MLST typing, and alerts to limitations of MLST-only based population studies in chronic infections under constant antibiotic selective pressure.

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Pulsed-field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) methods are molecular typing tools used to study the genetic relationship among bacterial isolates (7). PFGE technique has been widely used to identify particular epidemic clones causing nosocomial outbreaks and to follow changes in the identity of isolates along chronic infections, as in *Pseudomonas aeruginosa* cystic fibrosis (CF) lung infection (8,11). MLST provides useful information about the population biology of international genetic lineages (clones and clonal complexes) producing infections worldwide (4,13). We have recently documented a high MLST-based genetic diversity among clinical carbapenem non-susceptible *P. aeruginosa* in Spain (6). In this work we detected widespread ST175 and ST646 clones. Despite of these results, differences in their PFGE pattern were observed, probably due to local evolution (6).

In the present work we report a discrepancy between MLST and PFGE typing tools in a collection of 21 *P. aeruginosa* isolates obtained from a single CF-patient attended in our CF Unit from 2003 to 2009. PFGE typing was performed as follows: from an overnight culture of 5 ml of LB broth (Difco, Detroit, MI) of each isolate, an aliquot of 1 ml was centrifuged and the pellet was mixed with 200 µl of SE solution pH=7.5 in the same eppendorf. Optical density was adjusted between 0.6 and 2.5 using the Nanodrop System (Thermo Scientific, Wilmington, DE) at 590 nm. Subsequently, 200 µl of 2% agarose was added to the eppendorf, gently mixed, and deposited in appropriate plug molds. Overnight protein lysis was carried out at 56°C in 2 ml of lysis solution (EDTA 0.5 M pH=9; 10% sarkosyl, and 25 mg/ml of proteinase K). Plugs were then washed twice in 2 ml of TE during 30 min. A third part of each plug was digested
with 15 U of SpeI (Roche, Diagnostics, Indianapolis, IN) at 37°C during at least 2 hours. Electrophoresis was carried out in a 1.2% agarose gel in 0.5xTBE at 14°C with the following settings: 6 v/cm², 5-40s, and 22 hours. For MLST typing we followed the scheme developed by Keith Jolley at the University of Oxford (http://pubmlst.org/paeruginosa).

The study of the sequential isolates obtained from the CF-patient revealed a MLST shift during chronic infection. Along seven consecutive years nineteen isolates grouped in ST242 (allele code 28,5,5,11,3,15,44). However, in the last three years two isolates clustered in ST996 (allele code 28,5,5,44,3,15,44), which only differs from ST242 in the mutL allele sequence (indicated in the allele code in bold letters and underlined). On the contrary, all PFGE patterns were identical among isolates grouping both STs (Figure 1) and different to those obtained in STs from other patients that were included for comparison, suggesting the persistence of a single clonal lineage. The change from mutL allele 11 to allele 44 involved two nucleotidic changes: 554G→A and 929A→T. While the first represents a neutral (synonymous) change, probably fixed by genetic drift, the second presented an AAG-ATG change in the 310 codon giving rise to an amino acid substitution (K310M). This amino acidic sequence had previously been detected in our laboratory in a hyper-mutable CF-<i>P. aeruginosa</i> isolate (10). Interestingly, ST996 isolate with the MutL changes had a mutation frequency, measured in three independent experiments in Luria Bertani agar plates supplemented with 300 mg/L of rifampicin (10), with a median value of 4.11x10⁻⁶ (range: from 7x10⁻⁶ to 1.22x10⁻⁶) higher than that belonging to the ST242 clone, with a median value of 1.2x10⁻⁸ (range: 5x10⁻⁹ to 7.44x10⁻⁸). The lysine at position 310 of the deduced MutL sequence was aligned with the lysine residue at position 307 of the <i>Escherichia coli</i> MutL protein, crucial for the ATPase activity of the protein, and therefore required for
the DNA mismatch repair function (10, 1). Allele mutL-11 found in ST996 has been previously registered in the *P. aeruginosa* MLST database, and corresponds to a Single Locus Variant (SLV) of mutL-44 ST242 (9). The complete analysis of the database showed that both STs only presented genetic relation with ST456 (Allele code: 28, 68, 5, 11, 3, 15, 44) which is an SLV of ST242 and Double Locus Variant (DLV) of ST996.

It is tempting to suggest that K310M change, influencing the mismatch repair system of the ST996, might have been selected by hitch-hiking with advantageous adaptive mutations favored by hypermutation, including antibiotic resistance (2, 3, 5, 10). Clinical chart review of this patient shows that during the last three years he received more than 270 days per year of intravenous ceftazidime, tobramycin, ampicillin, and piperacillin/tazobactam in combination with oral doses of linezolid, trimethoprim, ciprofloxacin, levofloxacin, and azithromycin, and inhaled tobramycin and colistin. All antibiotics were used at the maximum doses due to frequent *P. aeruginosa* exacerbations. If we cannot completely rule out the possibility of a new ST996 acquisition, our data strongly suggest that mutL mutations occur *in vivo* as consequence of the adaptive benefit of a mutL hypermutable variant when confronted with strong selective pressures exerted by frequent antibiotic therapy.

The *in-vivo* modification of the MLST type in *P. aeruginosa* isolates with identical PFGE pattern has practical consequences for the *P. aeruginosa* typing strategies. We recommend the application of both typing systems to follow chronic colonization, as occurs in CF patients. Long-term persistent clones in a patient or in a group of patients might accumulate single nucleotide polymorphisms (SNPs) (12, 14). It should be noted that a hyper-mutable status increases the possibility of production of
novel single nucleotide variants eventually giving rise to novel MLST changes, particularly in case of chronic infections, such as CF.

In conclusion, the mutL based MLST modification of the P. aeruginosa isolates recovered from a single CF-patient is an alert to false “multiple colonization and/or the coexistence of multiple lineages” if only the MLST technique is applied, and the value of PFGE for a single-patient population analysis is not taken into consideration.

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Figure 1. Dendrogram based on Dice’s coefficient of the PFGE-SpI patterns of 8 isolates corresponding to ST242 and the 2 isolates matched with ST996.