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Adaptation of *Stenotrophomonas maltophilia* in cystic fibrosis: molecular diversity, mutation frequency and antibiotic resistance

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ABSTRACT

Due to the continuous exposure to a challenging environment and repeated antibiotic treatment courses, bacterial populations in cystic fibrosis (CF) patients experience selective pressure causing the emergence of mutator phenotypes. In this study we investigated the genotypic diversity, mutation frequency and antibiotic resistance of *S. maltophilia* isolates chronically colonizing CF patients. *S. maltophilia* was isolated from a total of 90 sputum samples, collected sequentially from 19 CF patients admitted between January 2008 and March 2012 at the University Hospital Essen, Germany. DNA fingerprinting by repetitive-sequence-based PCR revealed that 68.4% (n=13) of CF patients harbored different *S. maltophilia* genotypes during the 4-year study course. Out of 90 *S. maltophilia* isolates obtained from chronically colonized CF patients, 17.8% (n=16) were hypomutators, 27.7% (n=25), normomutators, 23.3% (n=21), weak hypermutators and 31.2% (n=28) strong hypermutators. We also found that mutation rates of the most clonally related genotypes varied over time with the tendency to become less mutable. Mutator isolates were found to have no significant increase in resistance against eight different antibiotics versus nonmutators. Sequencing of genes *mut*L, *mut*S and *uvr*D genes demonstrated the occurrence of amino acid changes in their corresponding proteins. Here, we could demonstrate that several different *S. maltophilia* genotypes are present in CF patients and as a sign of adaption their mutation status switches over time to a less mutator phenotype without increasing resistance. These results suggest that *S. maltophilia* attempts to sustain its biological fitness as mechanism for long-term persistence in the CF lung.

Introduction

The pathophysiology of cystic fibrosis (CF) lung disease is characterized by dehydration of airway surfaces and impaired mucociliary transport. Consequently, under conditions of increased viscosity and osmolarity, effective eradication of pathogens in these patients does not occur, generally leading to chronic lung colonization/infection (Rowe et al., 2005). In children, *Staphylococcus aureus* and *Haemophilus influenzae* are the most frequently isolated pathogens, whereas adults are often colonized by *Pseudomonas aeruginosa* (Hauser et al., 2011).

Different worldwide CF centers have observed that *Stenotrophomonas maltophilia´*s prevalence has increased (de Vrankrijker et al., 2010). However, there is considerable debate about whether this pathogen is a real marker of disease severity or if it is causally related to disease progression (Waters et al., 2011; Waters et al., 2013).

Since the bacterial populations in CF patients are constantly exposed to a challenging environment and repeated antibiotic treatments, strong diversification over time will occur, inducing the emergence of mutator phenotypes (Tenaillon et al., 1999). In general, a mutator microorganism exhibits an increased spontaneous mutation rate due to defects present in DNA repair and error avoidance systems (Oliver, 2010; Oliver and Mena, 2010). Studies related to mutator prevalence in CF patients have been mainly focused on *P. aeruginosa* (Oliver et al., 2000; Ciofu et al., 2005; Henrichfreise et al., 2007; Ferroni et al., 2009), whereas data concerning other CF pathogens is still limited (Prunier et al., 2003; Román et al., 2004; Turrientes et al., 2010). Furthermore, hypermutation has been recognized as a key mechanism for increasing antimicrobial resistance in *P. aeruginosa* (Maciá et al., 2005; Oliver and Mena, 2010).

The significance of chronic/ or persistent colonization of *Stenotrophomonas maltophilia* in the CF airway (disease) has not been well clarified, which leads to widely disparate views regarding the relevance of this bacterium in these patients (Valdezate et al., 2001; Goss et al., 2004). To expand the knowledge on *S. maltophilia* adaptation to CF airways, we investigated the genotypic diversity, mutation frequency and antibiotic resistance of *S. maltophilia* isolates chronically colonizing CF patients.

Materials and methods

Bacterial strains

S. maltophilia was isolated from a total of 90 sputum samples, sequentially collected from 19 chronically colonized CF patients admitted between January 2008 and March 2012 at the University Hospital Essen, Germany. Sputum samples from CF patients attending the University Hospital were collected from the patients as part of standard care. Chronic colonization/infection by *S. maltophilia* was defined as the persistent presence of this bacterium in 2 or more sputa within a period of 12 months (Goncalves Vidigal et al., 2013; Valdezate et al., 2001). Isolates that grew on selective agar medium (Goncalves Vidigal et al., 2011) were identified by oxidase test, MicroScan® WalkAway system (Siemens, Erlangen, Germany) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay (Vitek MS, bioMérieux, Marcy l´Etoile, France).

Isolation of genomic DNA from clinical specimens

Genomic DNA extraction was performed using the UltraCleanTM microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA), in accordance with the manufacturer´s instructions. The extracted DNA was stored at - 20^{0}C until amplification was conducted.

Molecular typing by repetitive-sequence-based-PCR (rep-PCR)

Rep-PCR was carried out using the DiversiLab® bacterial DNA fingerprinting kit (bioMérieux, Marcy l´Etoile, France), following the manufacturer´s recommendations. Briefly, each reaction required 35ng of genomic DNA, 2.5U of AmpliTaq DNA polymerase, and 1.5µL of 10X PCR buffer (QIAGEN, Hilden, Germany). Thermal cycling parameters were as follows: initial denaturation at 94^{0}C for 2min, 35 cycles of denaturation at 94^{0}C for 30s, annealing at 60^{0}C for 30s, extension at 70^{0}C for 90s,

and a final round of extension at 70^0C for 3min. Analysis of rep-PCR amplicons was

conducted by the DiversiLab system software, in which different sizes and intensities of amplified fragments were separated by a microfluidic Labchip and detected using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The degree of relatedness was defined by cluster analysis, using the Pearson correlation coefficient and UPGMA to create dendrograms. Isolates with a similarity of ≥95% were considered as a "genotype". We used ATCC 13637 reference strain as a quality control. Subculture stability and reproducibility of rep-PCR results were defined by the average similarity of >98% of three different colonies obtained at three time points (1, 5 and 10 days).

Mutation frequency assay

Mutation frequencies conferring rifampicin resistance were estimated as previously described (Turrientes et al., 2010). Independent triplicate Luria Bertani (LB) broth culture were grown overnight in an orbital incubator at 36°C with agitation (150rpm). Aliquots (100µL) of 10^{-6} dilutions of the overnight cultures were inoculated onto LB-rifampicin plates (250 mg/L), further 500µL aliquots were seeded onto LB plates. Colony counts of LB and LB-rifampicin plates were carried out after 24h and 48h incubation period, respectively. The mean number of mutants for each isolate was calculated. Isolates were classified into four categories based on their mutation frequencies: hypomutator ($f \leq 8 \times 10^{-9}$), normomutator ($8 \times 10^{-9} < f < 4 \times 10^{-8}$), weak hypermutator ($4 \times 10^{-8} < f < 4 \times 10^{-7}$) and strong hypermutator ($f \geq 4 \times 10^{-7}$) (Turrientes et al., 2010).

PCR amplification and sequencing of mismatch repair genes from *S. maltophilia* isolates

In attempt to uncover the underlying mechanisms involved in increased mutation frequency, five *S. maltophilia* strains (two hypermutators and three hypomutators) were randomly selected for investigation. After inducing mutation with rifampicin, genomic DNA extraction of isolates was performed as previously described. Three primer pairs were designed to span *mutL*, *mutS* and *uvrD* genes (Table 2). Amplification reactions were conducted in a 50 µL final volume containing of 1 ng of genomic DNA, 5mM dNTP mix, 1.5 $MgCl_2$, 1× PCR buffer, 50 µM of each primer, 2.5 Unit *Taq* DNA polymerase (Qiagen, Hilden, Germany). The PCR program was carried out as follows: denaturation step at 95 °C for 10 min, followed by 40 cycles consisting of a denaturation step at 95 °C for 30s, 1 min at the appropriate annealing temperature, and 72 °C for 1 min, with a final extension step of 72 °C for 10 min. Aliquots of PCR products were run alongside a 100 base pair DNA ladder (GE Healthcare, Germany) on a 0.6 % gel. PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and sequenced using both forward and reverse primers (LGC Genomics, Berlin, Germany).

Homology searches and sequence alignments

Sequences of *S. maltophilia mutL*, *mutS* and *uvrD* genes were retrieved from NIH GenBank database (www.ncbi.nlm.nih.gov) to design the primers and to verify similarities. Alignments were performed using the Clustal Omega program (Sievers et al., 2011). Sequences of all samples were examined manually to verify for local

misalignment errors relative to the reference genome (*S. maltophilia* D457), mobile element insertions, and large insertions and/or deletions.

Antimicrobial susceptibility testing

The *in vitro* activities of ceftazidime, colistin, co-trimoxazole, fosfomycin, levofloxacin, moxifloxacin, tigecycline and tobramycin, against all 90 *S. maltophilia* isolates were tested. Susceptibility testing was performed using MIC test strips (Liofilchem, Roseto degli Abruzzi, Italy) and resistance breakpoints were those of Clinical and Laboratory Standards Institute (CLSI) guidelines (M07-A8). For colistin, we used the breakpoints established for *P. aeruginosa*. *S. maltophilia* ATCC 16637 was tested for quality assurance purposes. A suspension with adjusted density of 0.5 McFarland standard was plated on Mueller-Hinton agar. MIC was read after incubation at 35 °C for 24 h. According to the manufacturer's guidelines, the zone of growth inhibition that intersected the E-test strip was interpreted as the MIC.

Statistical analysis

Fisher´s test was used for comparison of resistance between nonmutator (hypomutator and normomutator) and mutator (weak hypermutator and strong hypermutator) groups. The analyses were performed with the Prism 5.0 software package (Graph Pad Software, San Diego, CA, USA). A *P* value of <0.05 was considered statistically significant.

Results

A total of 90 *S. maltophilia* isolates were collected from the sputum of 19 CF patients considered chronically colonized. The mean age of CF patients for the onset of chronic colonization with *S. maltophilia* was 25±12.8 years (range 11-65 years), of which 52.6% of subjects were female. The mean number of sputa collected samples per patient was 4.8 (±3.3). Mutation in the cystic fibrosis transmembrane conductance regulator did not correlate with number of isolates obtained per sputa or *S. maltophilia* genotype.

To better understand the genetic structure (i.e. degree of relatedness) of *S. maltophilia* population in chronically colonized CF patients, these bacterial isolates were fingerprinted using the semi-automated, rep-PCR technique (DiversiLab® system). Table 1 summarizes demographic and genotypic characteristics of the CF patients, as well as the genotype characteristics of *S. maltophilia* isolates obtained from these patients. Genotypic analysis of the consecutive clinical isolates demonstrated the presence of 38 different genotypes. A total of 13 (68.4%) CF patients harbored different *S. maltophilia* genotypes during the course of the study. For example, patient 3 (Figure 1) had the highest number of diverse genotypes (n=8) within 34 months. Patients 7, 10 and 11 showed the presence of three genotypes, whereas patients 8, 9, 13, 14, 15 and 17 had only two. Our data also show that 31.6% (n=6) of these patients were colonised with unique clones (Table 1). Interestingly, we also observed that 10 patients (1, 3, 4, 7, 10, 11, 13, 15, 16 and 17) shared *S. maltophilia* genotypes.

To exclude confounding factors that might alter rep-PCR banding patterns of *S. maltophilia* due to increasing culture age and/or clonal expansion during growth of multiple subcultures, stability of rep-PCR patterns were investigated. For that, three distinguished colonies from a clinical CF isolates were collected at three different

time points (1, 5 and 10 days), and rep-PCR was performed. We were able to demonstrate that the *S. maltophilia* genotype was subculture stable and that rep-PCR gave reproducible results (Figure 2).

Several studies have confirmed the presence of mutators in CF patients with chronic respiratory infection caused by different bacteria (Oliver et al., 2000; Prunier et al., 2003; Román et al., 2004). For this reason, we carried out mutation frequency assay to verify the mutation status of *S. maltophilia* isolates obtained from chronically colonized CF patients. Of the 90 *S. maltophilia* isolates obtained from chronically colonized CF patients, mutation frequency results revealed that 17.8% (n=16) were hypomutators, 27.7% (n=25) normomutators, 23.3% (n=21) weak hypermutators and 31.2% (n=28) strong hypermutators. Eleven CF patients (57.9%) harbored at least one strong hypermutator isolate, whereas 7 (36.9%) of them harbored at least one hypomutator isolate. Interestingly, among the 10 patients that shared similar genotypes, 44.4% (n=4) were colonized by strong hypermutable, 33.3% (n=3) by weak hypermutable, and 22.2% (n=2) by normomutable and hypomutable isolates. We further examined if these genotypes due to the selection pressure of CF airways tended to become hypermutator. Remarkably, we noted that the mutation rates of most clonally related genotypes varied over time (Figure 3) with tendency to become less hypermutable, except for genotype 1.

In attempt to ascertain which modifications and/or mutations in *mutS*, *mutL* and *uvrD* genes might be responsible for the mutator phenotype, the entire sequence of these genes from five rifampicin-treated *S. maltophilia* isolates (three hypomutators and two hypermutators) were fully sequenced, and deduced amino acid sequences were compared. No insertions or deletions that might produce aberrant proteins in any of the sequenced genes compared with the reference

genome were detected. Notably, the presence of single nucleotide and double or triple nucleotide polymorphisms in all abovementioned genes, in comparison to reference strain, was detected in the rifampicin-treated *S. maltophilia* isolates. In Table 3 are listed the double and/or triple nucleotide changes detected. Based on the deduced amino sequences, our data demonstrated the following changes: A71P and T213A in MutL, T575A and A576E in MutS, P139A and A411V in UvrD, which were found in considerably highly conserved regions (Table 4). Only the amino acid modification A411V in the UvrD protein was unique to hypermutator isolates.

Conversely, there is a lack of study regarding the correlation of CF *S. maltophilia* mutator isolates with antibiotic resistance. To evaluate the impact of the high proportion of mutator isolates found in this study, susceptibility testing for eight widely used antimicrobial agents was performed (Figure 4). Tigecycline and co-trimoxazole had the best activity against *S. maltophilia* isolates. Interestingly, mutator isolates were found not significantly more resistant against the antibiotics than nonmutators (Table 5). In addition, the mean MIC of each antibiotic was not found to be statistically different between the nonmutator and mutator isolates.

Discussion

S. maltophilia is considered an emerging pathogen in the CF community, as a consequence of the repeated and long-term antipseudomonal therapies (Hauser et al., 201). Its ability to chronically colonize CF airways has been recently associated with lower levels of lung function (Waters et al., 2011), but the importance of this pathogen in CF patients is still debated. Not surprisingly, there is a growing interest in investigating host-pathogen interactions responsible for progressive CF lung

diseases (Denton et al., 1998; Valdezate et al., 2001; Turrientes et al., 2010) in order to gain a better understanding of the conditions required for bacterial colonisation/persistence.

Our data demonstrated a high degree of diversity in *S. maltophilia* consecutively isolated from chronically colonized CF patients. These results are in agreement with previous studies which used other typing methods (Denton et al., 1998; Valdezate et al., 2001). In a study from the UK, 45 *S. maltophilia* CF isolates (1 single isolate from each of 40 patients and 5 isolates from a single patient) collected during a period of 28 months were analyzed. ERIC-PCR analysis revealed the presence of 41 different genotypes, of these, four pairs of patients shared the same type. The five isolates that were colonizing the same patient were distinct from one another (Denton et al., 1998). A further prospective, observational investigation at the Hospital Ramóny Cajal CF Unit, Spain, evaluated 76 *S. maltophilia* isolates from 25 CF patients (Valdezate et al., 2001). A total of 62 *S. maltophilia* samples from 11 chronically colonised patients (44%) were recovered. Their data revealed that each patient had one to five strains with different PFGE profiles.

The adaptation of bacterial population to new or challenging environments normally results in spontaneous generation of mutators (Tenaillon et al.,1999; Giraud et al., 2001). Over the last decade, it has been extensively shown that there is a high prevalence of *P. aeruginosa* mutators in chronically colonised CF patients in Spain (37%) and Denmark (54%) (Oliver et al., 2000; Ciofu et al., 2005). In the current study, the presence of high proportion of mutable isolates of *S. maltophilia* (approximately 31%) was detected, suggesting rapid adaptation of this bacterium population in order to survive in the CF lung airway. Previously, mutation frequency regarding CF patients chronically colonized by *S. maltophilia* isolates had only

partially been addressed by a Spanish group (Turrientes et al., 2010). A total of 48 clinical isolates obtained from 13 CF patients (3 of them had only a single isolate) suffering from chronic infection were analyzed. The authors observed that 16.7% of the CF *S. maltophilia* isolates were strong hypermutators.

Additionally, when investigating the genotypes shared by different CF patients over time, we noticed that after adaptation to CF lung environment, these *S. maltophilia* isolates tended to revert to the original nonmutator state. This can be explained by the fact that accumulation of deleterious mutations may reduce bacterial population fitness (Taddei et al., 1997).

Increased mutation rates are commonly associated with defects in the mismatch repair (MMR) system, especially in the gene *mut*S (Oliver et al., 2002; Chopra et al., 2003; Turrientes et al., 2010). However, it is still unclear, based on the available sequence annotation, whether the modifications mentioned are actually relevant or responsible mutations for increasing mutation frequency in hypermutator isolates, as we could not confirm the findings described by Turrientes et al. (2010) in MutS. In contrast, amino acid positions 575 and 576 (usually T and A respectively) are located in available in MutS sequences from a wide range of bacterial genera, which is considerably highly conserved. Therefore, the sequence around these amino acid changes is involved in ATP-binding cassette domain and it is highly conserved among other different available sequences, it suggests that the structure of this region is relevant for MutS activity. Therefore, the abovementioned changes might be responsible for increasing mutation frequency, by modifying the activity of MutS protein.

In an additional experiment, we observed that rep-PCR did not detect differences between the genotype profiles of normomutator and induced-strong

hypermutator isolates (data not shown), as it has been shown for *P. aeruginosa* by pulsed-field gel electrophoresis (PFGE) (García-Castillo et al., 2012). Thus, there is no clear correlation between changes in mutation and genotype, which might provide information whether the strains persist or if they are newly acquired from the environment.

Nowadays, development of antibiotic resistance is recognized as a rising problem resulting in limited antibiotic treatment options. Hypermutation plays a role in the evolution of bacterial resistance (Chopra et al., 2003). Studies have demonstrated that *P. aeruginosa* mutators were more frequently resistant to different antibiotics than nonmutators isolates (Oliver et al., 2000; Ciofu et al., 2005; Henrichfreise et al., 2007). To the best of our knowledge, this study investigated for the first time the presence of an association between mutation frequencies in CF *S. maltophilia* isolates and antibiotic resistance. However, we did not find a significantly correlation between *S. maltophilia* mutators and increasing antibiotic resistance as shown for *Pseudomonas*. The reason for that is mainly unclear.

CF and non-CF patients are mainly colonized by a dominant *P. aeruginosa* descendant clone (adaptive radiation), which it is recovered over the years (Oliver et al., 2000; Maciá et al., 2005; Ciofu et al., 2005; Ferroni et al., 2009; Hogardt and Heeseman, 2010; Oliver, 2010). Because of that, *P. aeruginosa* is aggressively treated by long-term and repeated courses of antibiotics, and therefore, the selective pressure against this pathogen is extremely high (Hauser et al., 2011). Contrary, there is still a lack of guidelines to eradicate *S. maltophilia* (therapy duration and antimicrobial agents are not well established), consequently, the selective pressure against this pathogen might be lower (Döring et al., 2012).

Some limitations of this study should be noted. Firstly, as a typing method we have used rep-PCR instead of PFGE. Rep-PCR has been reported in previous

studies as a useful tool in the fingerprinting of *S. maltophilia* hospital outbreak isolates because it showed a high adjusted Rand`s coefficient (0.697) (Fluit et al., 2010; Overdevest et al., 2011). Secondly, we did not test the hypothesis whether naturally hypermutable strains are associated with a biological benefit or cost for the colonization in a host. However, bearing these facts in mind, our findings reveal important insights that may need to be considered in future studies, especially those involving potential hosts.

In conclusion, our results show that *S. maltophilia* isolates chronically colonizing CF patients have a high degree of diversity and are often characterized as mutator strains with the tendency to become less mutable status. The presence of mutator *S. maltophilia* strains was not associated with increase of antibiotic resistance. Our data indicate that *S. maltophilia* tries to preserve its biological fitness as mechanism for long-term persistence in the CF lung. Future studies investigating the adaption of *S. maltophilia* in the host are warranted.

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Conflicts of interest

None of the authors have any conflicts of interest to declare.

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Legends

Figure 1. **Molecular epidemiology of *S. maltophilia* samples isolated from patient 3**. Dendrogram, virtual gel and the similarity matrix generated by the DL software. The gel-like image and similarity matrix exhibit the presence of 8 different genotypes.

Figure 2. Assessment of subculture stability and reproducibility of rep-PCR. *S. maltophilia* was cultivated for 1, 5 and 10 days (indicated by the first number) and colonies from three sites (indicated by the second number) were investigated by rep-PCR. The horizontal bar at the bottom left shows the percent similarity coefficient of these samples. Subculture stability and reproducibility of *S. maltophilia* are shown by a high index of similarity (>98%).

Figure 3. **Mutation status of the genotypes clonally related and shared among different CF patients over time**. Each black dot represents a single isolate obtained from a patient. The black line links the isolates that share the same genotype pattern according to rep-PCR. The similarity among isolates was established as ≥95% according to Pearson correlation index.

Figure 4. Distribution of resistant hypomutator, normomutator, weak hypermutator and strong hypermutator CF *S. maltophilia* isolates. Antibiotic resistance was determined against ceftazidime, colistin, co-trimoxazole, fosfomycin, levofloxacin, moxifloxacin, tigecycline and tobramycin using E-test strips. Abbreviations: CEF_{res} - ceftazidime resistant; LEV_{res} - levofloxacin resistant; COL_{res} - colistin resistant; SXT_{res} - co-trimoxazole resistant; TGC_{res} - tigecycline resistant; TOB_{res} - tobramycin resistant; MXF_{res} – moxifloxacin resistant: FOS_{res} - fosfomycin resistant.

Table 1. Demographic and genotypic characteristics of 90 *S. maltophilia* isolates obtained from 19 CF patients.

Patient	Sex	Age (years)	CF mutation	No. of sputa	Genotypes
Patient 1	F	17	dF508/R553X	9	**1**,8,9,22,32
Patient 2	M	12	dF508/dF508	2	30
Patient 3	F	11	dF508/dF508	15	**11**,12,**13**,**14**,**16**,21,23,37
Patient 4	F	28	dF508/327-26-A>G	10	10,**13**,**14**,**16**
Patient 5	M	27	dF508/dF508	5	34
Patient 6	M	24	dF508/dF508	2	6
Patient 7	F	21	not identified	7	**1**,2,28
Patient 8	F	36	not identified	3	24,25
Patient 9	F	27	R347P/G542X	3	5,38
Patient 10	M	21	dF508/R31C	4	**13**,**14**,15
Patient 11	M	18	dF508/R553X	3	3,20,**33**
Patient 12	F	12	dF508/dF508	2	29
Patient 13	M	27	G1069R/3007delG	3	**18**,19
Patient 14	M	20	dF508/dF508	6	26,36
Patient 15	M	19	dF508/dF508	2	**11**,**27**
Patient 16	F	14	not identified	6	17,**18**,**27**,31,**33**
Patient 17	M	47	dF508/2789+5G>A	4	**18**,28
Patient 18	F	65	dF508/-	3	35
Patient 19	F	31	dF508/dF508	2	4

The numbers in bold refer to the genotypes which are shared by different patients.

Table 2. Primers designed to amplify MMR genes of *S. maltophilia* and PCR conditions

Gene	Primer sequence (5→3)	Annealing temperature (°C)
mutL	F1: *CAGTTCTTCGCAGCCAAGTC* R1: *GTCAGGATGCGTTCCAGAGT* F2: *ATGCGGTGGAAACCCTGAT* R2: *ACCGGTGATGACCAGCAT* F3: *CCGTGCCTGAAGATCGAG* R3: *TCAGTCGGTACAGCGCTTC*	55°C 50°C 50°C
mutS	F1:*AGGAACCAGCGGTCGATCT* R1:*GTGGTGGTGGCGGATTCT* F2:*GACCGGCAGTCCGTTCTC* R2:*GCGAGCTGTTCTACAACGTG* F3:*CGTGTATCGGAATACAGATCG* R3:*CGGAGATCCTCATCAACCAG*	55°C 55°C 55°C
uvrD	F1: *GTAGGCCATCACCAGCCACT* R1: *GAGCACTGGAGCAAGGAAAG* F2: *ACCAGCTCGTCCATGTCCT* R2: *CGCTGCTGTCGCACTACC* F3: *CCTGCACGTTCTCGACCT* R3: *ATGTCTCCCACCTGCTTGAT*	50°C 52°C 52°C

Table 3. Nucleotide polymorphisms in the *mut*L, *mut*S and *uvr*D gene of S. maltophilia isolates

Strain	Mutator phenotype	***mut*L nucleotide position**	***mut*S nucleotide position**	***uvr*D nucleotide position**
		1377918-1377920	**1377831-1377832**	**4633207-4633208**
reference	normomutator	ACG	CG	GC
Smal1	hypomutator	CAA	GC	GC
Smal 2	hypermutator	CAA	GC	GC
Smal 3	hypomutator	GCG	GC	CT
Smal 4	hypermutator	GCG	GC	CT
Smal 5	hypomutator	GCG	GC	CT

Table 4. Amino acid polymorphisms in the MutL, MutS and UvrD genes of *S. maltophilia* isolates

Strain	Mutator phenotype	MutL amino acid in position						MutS amino acid in position			UvrD amino acid in position	
		71	**166**	**213**	**348**	**349**	**350**	**538**	**575**	**576**	**139**	**411**
reference	normomutator	A	L	T	A	A	Q	E	T	A	P	A
Smal1	hypomutator	P	L	A	P	V	H	G	A	E	A	A
Smal 2	hypermutator	P	R	A	P	V	H	G	A	E	A	V
Smal 3	hypomutator	P	L	A	P	V	H	G	A	E	A	A
Smal 4	hypermutator	P	L	A	P	V	H	D	A	E	A	V
Smal 5	hypomutator	P	L	A	P	V	H	D	A	E	A	V

Table 5. Comparison of MICs for different antimicrobial agents tested against *S. maltophilia* isolates with nonmutator and mutator phenotypes[a]

	Nonmutator isolates (n=41)		Mutator isolates (n=49)		
Antibiotic	No. (%) resistant	Mean MIC[b]	No. (%) resistant	Mean MIC[b]	Statistical significance (*P*)[c]
Ceftazidime	63.4	26.5	46.9	18.6	0.1402
Colistin	56.0	6.8	59.1	7.3	0.8319
Co-trimoxazole	14.6	0.3	18.3	0.3	0.5634
Fosfomycin	60.9	37.2	67.3	51	0.6589
Levofloxacin	24.3	1.1	32.5	1.3	0.4855
Moxifloxacin	26.8	0.6	32.5	1	0.6463
Tigecycline	2.4	1	4.0	1.5	1.0000
Tobramycin	82.9	82.8	87.7	78.3	0.5593

[a] Nonmutator isolates (hypomutator and normomutator) included the isolates with frequencies of $\leq 8 \times 10^{-9}$ and $8 \times 10^{-9} < f < 4 \times 10^{-8}$; whereas rmutator isolates (weak hypermutator and strong hypermutator) had a frequency of $4 \times 10^{-8} < f < 4 \times 10^{-7}$ and $\geq 4 \times 10^{-7}$. [b] Geometric mean of the MIC in mg/L. [c] *P* values (Fisher´s test) resulting from comparison of the MICs among nonmutator and mutator isolates.