International Journal of Medical Microbiology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

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

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ARTICLE INFO

Article history:
Received 14 June 2013
Received in revised form 26 March 2014
Accepted 19 April 2014

Keywords:
Cystic fibrosis
Stenotrophomonas maltophilia
Rep-PCR
Mutation frequency
Antibiotic resistance

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 the mismatch repair genes *mutL*, *mutS* and *uvrD* revealed alterations that resulted in 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; Brooke, 2012).

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, 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 are 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 *S. 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,

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http://dx.doi.org/10.1016/j.ijmm.2014.04.002
1438-4221/

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 two or more sputa within a period of 12 months (Gonçalves 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 °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 35 ng of genomic DNA, 2.5 U of AmpliTaq DNA polymerase, and 1.5 μL of 10× PCR buffer (QIAGEN, Hilden, Germany). Thermal cycling parameters were as follows: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 70 °C for 90 s, and a final round of extension at 70 °C for 3 min. 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 (150 rpm). Aliquots (100 μL) of 10^{-6} dilutions of the overnight cultures were inoculated onto LB-rifampicin plates (250 mg/L), further 500 μL aliquots were seeded onto LB plates. Colony counts of LB and LB-rifampicin plates were carried out after 24 h and 48 h 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).

Table 1
Primers designed to amplify MMR genes of *S. maltophilia* and PCR conditions.

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

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

In attempt to uncover the underlying mechanisms involved in different mutation frequencies, 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 1). Amplification reactions were conducted in a 50 μL final volume containing of 1 ng of genomic DNA, 5 mM 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 30 s, 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.

Please cite this article in press as: Vidigal, P.G., et al., Adaptation of *Stenotrophomonas maltophilia* in cystic fibrosis: Molecular diversity, mutation frequency and antibiotic resistance. Int. J. Med. Microbiol. (2014), http://dx.doi.org/10.1016/j.ijmm.2014.04.002