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Sequence variability and functional analysis of MutS of hypermutable *Pseudomonas aeruginosa* cystic fibrosis isolates

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Abstract

In this study, we investigated the variability of MutS among *Pseudomonas aeruginosa* recovered from cystic fibrosis (CF) patients. Sequencing of the *mutS* gene of 15 hypermutable *P. aeruginosa* isolates obtained from different patients revealed high rates of nucleotide substitutions as compared to that of strain PAO1. Significantly more synonymous than non-synonymous nucleotide substitutions have been found, indicating that generally MutS is highly conserved. The functional analysis of MutS variants by complementation of a PAO1 *mutS* mutant revealed 5 isolates with a defective MutS due to frameshift mutations or amino acid substitutions. This work supports the hypothesis that the respiratory tract of CF patients represents an environment that favors the selection of highly adaptive mutator phenotypes.

Keywords: Cystic fibrosis; *Pseudomonas aeruginosa*; Mutator strains; MutS; TGGE

Introduction

Cystic fibrosis (CF) is a hereditary human disorder characterised by chronic pulmonary infection, most frequently caused by *Pseudomonas aeruginosa* (Rajan and Saiman, 2002). Despite advances in therapy and a continuous increase in life expectancy, *P. aeruginosa* airway infection remains the major cause of morbidity and mortality among this population. During chronic infection the pseudomonades exhibit a remarkable adaptability to the CF lung that becomes obvious by the emergence of diverse *P. aeruginosa* phenotypes related to one clone (Mahenthiralingam et al., 1996). Interestingly, this diversification has been linked to alterations in the bacterial DNA methyl-directed mismatch repair (MMR) system (Oliver et al., 2000, 2002). Bacterial MMR mutants with high mutation rates (termed mutators) exhibit not only spontaneous mutability but are also hypersensitive to base substitutions by mutagenic agents or oxidative stress, the latter in particular being associated with the excessive inflammation in the lungs of CF patients. However, the emergence of *P. aeruginosa* isolates may substantially contribute to the rapid selection of lung-adapted, e.g. highly antibiotic-resistant variants (Ciofu et al., 2005).

To explore how defects of MutS contribute to the emergence of *P. aeruginosa* mutator strains of diverse

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phenotypes during CF lung disease we analysed 15 hypermutable *P. aeruginosa* isolates from different CF patients for *mutS* sequence variability. Moreover, we verified the functionality of predicted MutS variants by complementation of a PAO1 *mutS* mutant. The characterisation of CF mutator isolates might be useful to better understand adaptive strategies of *P. aeruginosa* during chronic CF lung disease and to define adequate anti-pseudomonal therapies. For the assessment of hypermutability, phenotypic tests such as determination of mutation frequencies and routine susceptibility tests appear to be applicable (Macia et al., 2004). To identify underlying defects leading to high mutation frequencies, molecular methods are indispensable. For the genetic analyses of mutators, sequencing of MMR genes represents the currently accepted ''gold standard''. Furthermore, the temperature gradient gel electrophoresis (TGGE) is an inexpensive method, that has been applied for the screening of DNA fragments for small sequence changes up to single point mutations (Wartell et al., 1998; Yoshino et al., 1991). Therefore, we supposed that TGGE may be a promising alternative to whole gene sequencing for the screening of *P. aeruginosa* isolates for mutated MMR alleles.

In this work, we evaluated the applicability of TGGE in comparison to gene sequencing to identify *mutS* mutators among 15 hypermutable *P. aeruginosa* CF isolates. Moreover, by *mutS* sequence analysis and complementation of a PAO1 *mutS* mutant we identified 5 loss-of-function *mutS* strains with varying *mutS* mutations. By PFGE we showed that almost all hypermutable *P. aeruginosa* isolates investigated were of different clonal origin. These findings suggest that during CF, *mutS* mutator strains evolve more frequently by independent mutational events and subsequent selection of the mutator phenotype than by the transmission of preselected *mutS* strains.

Materials and methods

Hypermutable *P. aeruginosa* CF isolates

To recover hypermutable (HM) *P. aeruginosa* isolates, 100 sputum samples obtained from different CF patients (58 male and 42 female patients, 8–37 years) with a well-documented history of chronic *P. aeruginosa* lung infection were processed as follows: after liquefaction with dithiothreitol, 200 µl of sputum samples were plated on selective agar containing 300 µg/ml rifampicin. Then, colonies grown after incubation for 48 h at 37 °C were subcultured on rifampicin agar to confirm the resistotype. Streptomycin mutation frequencies (MFSM) were measured in triplicate to determine the hypermutable phenotype as described previously (Oliver et al., 2000). Prior to MFSM determination, susceptibility was verified by estimating the streptomycin minimal inhibitory concentrations (MICs) using the Etest method (VIVA Diagnostika, Cologne, Germany). Initial *P. aeruginosa* CF isolates randomly selected from our strain collection were used as a control group. For the primarily streptomycin-resistant isolates HM14 and HM15, chloramphenicol mutation frequencies were determined similarly using 200 µg/ml chloramphenicol to assess hypermutability. MICs of strains HM1 to HM15 were determined for ceftazidime, meropenem, tobramycin, and ciprofloxacin by agar dilution according to CLSI standards and breakpoints (CLSI, 2005). Multiple antimicrobial resistance was defined as resistance against at least two of the tested agents.

Molecular methods

For TGGE-PCR reactions, 5 µl crude bacterial lysates of *P. aeruginosa* were used as a template. PCR primers generating 4 slightly overlapping *mutS* subfragments (S1–S4) representing MutS structural domains I and II, III, IV, and V were designed to analyse the complete *mutS* gene of 2568 bp. The following forward (f) and reverse (r) primers were used to generate amplicons S1–S4: fS1 (5′-GCC CGT ATG ACC GAC CTC T-3′), rS1 (5′-CCG AGT CGC GAT CGA AGT-3′), 611 bp; fS2 (5′-CCG CGC GCC ATG GGA CTT CGA T-3′), rS2 (5′-TTC GGC GAG TTC GGG ATA-3′), 606 bp; fS3 (5′-CAC CAC CAT CGG CAC CTA T-3′), rS3 (5′-GTT GGC CAC GAA CGG TGT-3′), 597 bp; and fS4 (5′-TGG TCG AGC AGG TGC TGG-3′), rS4 (5′-ATT CTA GCA GCT TGT GCG G-3′), 792 bp. For TGGE-PCR, a 40-bp G/C-rich sequence (GC-clamp) was added to the 5′-end of each reverse primer. This GC-clamp (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) ensures that the target sequence is in the lower melting domain and therefore improves the detection of mutated DNA fragments (Myers et al., 1985). The DCode Universal Mutation Detection System (BioRad Laboratories, Munich, Germany) was used for sequence-specific separation of TGGE-amplicons on a denaturing 6% (w/v) polyacrylamide–bisacrylamide gel (6 M urea, 1.25 × TAE, pH 8.3) gel. A constant voltage of 130 V and a temperature ramp of 1 °C/h were applied. To determine the temperature range, a melting profile of TGGE amplicons S1–S4 was calculated by means of MacMelt software (BioRad Laboratories, Munich, Germany). For *mutS* gene sequencing, subfragments S1–S4 were generated using the same primers as described for TGGE, but without the GC-clamp. Sequences of both strands of the PCR products were determined in duplicate to ensure proper sequencing results.

Hypermutable *P. aeruginosa* isolates were typed by pulse field gel electrophoresis (PFGE) analysis using