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Mutator Genes Giving Rise to Decreased Antibiotic Susceptibility in *Pseudomonas aeruginosa*[▽]

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Screening of the PA14 genomic transposon mutant library for resistance to ceftazidime, tobramycin, and ciprofloxacin led to the discovery of several mutants that appeared in more than one screen. Testing of the frequency of mutation to ciprofloxacin resistance revealed previously known mutator genes, including *mutS* and *mutL*, as well as mutators that have not yet been described for *P. aeruginosa*, including PA3958 and RadA (PA4609).

Pseudomonas aeruginosa is a metabolically versatile bacterium capable of living in multiple ecological niches (18), including the airways of cystic fibrosis (CF) patients. One of the challenges that it faces in the CF lung environment is intensive antibiotic therapy. Hypermutable *P. aeruginosa* isolates, with an increased chromosomal mutation frequency leading to multidrug resistance acquisition, are very frequently found in chronic CF lung infections (37% [1] and 54.5% [14], respectively). Weak hypermutators with modestly increased mutation frequencies are also observed in the early stages of infection (5%), but the genetic basis for these is unknown (8). For *Escherichia coli*, more than 30 different mutator genes with functions in DNA repair or error avoidance systems have been described to date (5), some of which have also been reported to lead to an increase in mutation frequency in *P. aeruginosa*.

We addressed the question of whether we would detect novel mutator genes in *P. aeruginosa* when screening the PA14 nonredundant transposon mutant library (10) for decreased susceptibility to different antibiotics, assuming that a hypermutator phenotype would lead to a decreased susceptibility to more than one class of antibiotics.

Overnight cultures were used to inoculate antibiotic-containing Mueller-Hinton agar plates with approximately 3×10^4 cells per spot, which still allowed the detection of resistant variants in a hypermutable population (15). The antibiotic concentrations used for screening were $2\times$ the MIC of the parent strain, PA14, for ceftazidime, ciprofloxacin, and tobramycin (i.e., 4, 0.2, and 1 μg/ml, respectively), with tobramycin also being screened at $1 \times$ the MIC (0.5 μg/ml). Low antibiotic concentrations were chosen to also detect mutations that lead to small decreases in antibiotic susceptibility, since these might be clinically significant if accumulated in a stepwise manner. Agar plates were checked after 24 h and 48 h of incubation at 37°C. The mutants corresponding to growth on the respective agar plates were confirmed to be less susceptible to the selective drug by determination of the MICs (22) after 24 h and 48 h. Mutants with a reproducible ≥2-fold increase in the MIC detected in more than one screen were studied further, as were transposon mutants with known mutator genes and genes that were annotated to be involved in DNA repair even if they appeared in only one screen.

Not all mutants that were detected in more than one antibiotic resistance screen showed an increase in mutation frequency (Table 1). For these (PA5001, PA1767, and PA1766), other, as yet undetermined mechanisms must have led to the observed reduced susceptibility.

As expected, several mutants with a mutator phenotype had disruptions in DNA repair genes. The mutators *mutS* and *mutL* were detected in all three screens (Table 1). The mechanism of the mismatch repair has been well characterized for *E. coli* and involves MutS, MutL, MutH, and UvrD. While MutS, MutL, and UvrD homologs are present in *P. aeruginosa* and mutations in the respective genes are found in clinical isolates with mutator phenotypes (12, 13), *P. aeruginosa* lacks a MutH endonuclease homolog (7). A transposon interruption in gene PA3958, belonging to the endonuclease/exonuclease/phosphatase family (2), led to a threefold increase in the mutation frequency. Reintroducing a functional copy of the gene PA3958 on a plasmid lowered the mutation frequency to the wild-type level. Similarly, an independently generated PA3958 mutant (6) showed a fourfold increase in mutation frequency. Thus, the PA3958 gene product may be involved in DNA repair. Although *uvrD* was not detected in any of the screens, we confirmed that the deletion of the *uvrD* gene in PA14 led to the expected increase in the mutation frequency (Table 1). Growth curves of the UvrD parent and selected ciprofloxacin-resistant mutants showed a decreased fitness compared to the PA14 wild-type and selected ciprofloxacin-resistant mutants, respectively (Fig. 1). This might explain why none of the resistance screens detected the *uvrD* transposon mutant and also the lower occurrence of *uvrD* mutants in the clinic (12, 13).

Components of the oxidized guanine residue repair system, MutT, MutY, and MutM, have strong, moderate, and weak

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TABLE 1. Mutation frequencies of wild-type PA14 and transposon mutants of the nonredundant PA14 mutant library

PA14 locus	PA01 homolog	Name of mutated gene	Polarity[a]	Description of gene product	Detection of mutant in resistance screen[b]			Mutation frequency[c]	Fold increase
					CAZ	TOB	CIP		
Wild type								1.2×10^{-7}	
PA14_66120	PA5001		No	Conserved hypothetical protein	+	+	−	1.8×10^{-7}	1.5
PA14_41710	PA1767		Yes	Putative membrane protein	+	+	−	5.5×10^{-8}	0.5
PA14_41730	PA1766		No	Conserved hypothetical protein	+	+	−	6.7×10^{-8}	0.6
PA14_17500	PA3620	*mutS*	No	DNA mismatch repair protein MutS	+	+	+	8.5×10^{-6}	71.8
PA14_65350	PA4946	*mutL*	No	DNA mismatch repair protein MutL	+	+	+	8.5×10^{-6}	71.6
PA14_71870	PA5443	*uvrD*	No	DNA helicase II	−	−	−	3.7×10^{-6}	31.0
PA14_12670	PA3958		No	Endonuclease/exonuclease/phosphatase family	−	−	+	4.0×10^{-7}	3.4
PA14_57190	PA4400	*mutT*	No	Probable pyrophosphohydrolase	−	−	+	2.8×10^{-6}	23.6
PA14_67990	PA5147	*mutY*	No	A/G-specific adenine glycosylase	−	+	−	1.2×10^{-6}	10.1
PA14_04670	PA0357	*mutM*	No	Formamidopyrimidine-DNA glycosylase	−	+	−	6.5×10^{-7}	5.5
PA14_01720	PA0140	*ahpF*	No	Alkyl hydroperoxide reductase subunit F	−	−	+	8.9×10^{-8}	0.8
PA14_60990	PA4609	*radA*	No	DNA repair protein RadA	+	+	−	1.8×10^{-6}	15.0
PA14_25230	PA3002	*mfd*	Yes	Transcription repair coupling factor	−	+	−	4.6×10^{-7}	3.9
PA14_25220	PA3003		No	Conserved hypothetical protein	−	+	+	1.4×10^{-7}	1.1
PA14_54590	PA0750	*ung*	No	Uracil-DNA glycosylase	−	−	−	4.7×10^{-7}	4.0

[a] Potential polarity was assessed based on the chromosomal context of the mutated gene.
[b] CAZ, ceftazidime; TOB, tobramycin; CIP, ciprofloxacin.
[c] To determine the rate of mutation to ciprofloxacin resistance, appropriate dilutions of a concentrated overnight cell culture grown from a single colony were plated on agar containing 0.5 μg/ml ciprofloxacin or on antibiotic-free medium. Cell counts were determined after visible colonies had formed after incubation at 37°C (36 h for mutants).

mutator phenotypes, respectively, in *E. coli* (3). The same tendencies occurred in *P. aeruginosa* PA14 homologs, since *mutT*, *mutY*, and *mutM* deletions lead to 24-, 11-, and 6-fold increases in mutation frequencies, respectively. To date, clinical isolates *of P. aeruginosa* with *mutT* or *mutM* mutations have not been described (16), although the lack of a *mutY* PCR band for clinical mutator strains from two different patients was reported (14).

AhpF is another component of defense against oxidative damage caused by reactive oxygen intermediates during aerobic growth. The *ahpF* transposon mutant of PA14 was detected in the ciprofloxacin screen. An *ahpF* deletion in *E. coli* leads to an approximately fourfold increase in the rifampin resistance mutation frequency (4), while *Salmonella enterica* serovar Typhimurium *ahp* deletion mutants do not show an elevated mutation frequency (5). We did not observe an increased mutation frequency of the *aphF* mutant compared to the wild type.

The above-mentioned mechanisms of prevention of damage due to reactive oxygen intermediates are relevant only during aerobic growth. For *E. coli*, it was shown that even the strong mutator phenotype of *mutT* deletion mutants was completely suppressed under anaerobic conditions (19). Since *P. aeruginosa* is thought to grow under reduced oxygen tension during chronic lung infections (17), the lung environment might not select for mutations in *ahpF*, *mutT*, *mutM*, or *mutY*.

A transposon mutant interrupted in the gene PA4609, the product of which is 78% similar to the *E. coli* RadA/Sms protein, was detected in two screens. It was shown to have a 15-fold-increased mutation frequency compared to the wild type, PA14 (Table 1), which was complemented to the wild-type level by reintroducing the gene PA4609 on a plasmid. RadA plays a role in the repair of collapsed replication forks by recombinational events. However, effects of *radA* deletions in *E. coli* are generally observed only when they are combined with *recG* or *ruvABC* mutations due to redundancy in recombinational functions (11). The increase in mutation frequency in *Pseudomonas* due to a transposon insertion in *radA* opens interesting questions about recombinational repair mechanisms in this species.

Mutants with disruptions of the two genes of the operon, PA3002 and PA3003, were detected in the tobramycin screen and in the tobramycin and ciprofloxacin screen, respectively. While we could not detect an increase in the mutation frequency for the PA3003 mutant, we observed a fourfold increase for the PA3002 deletion mutant (where PA3002 is the first gene in the operon). The annotated gene product Mfd shows 73% similarity to Mfd from *E. coli*. The latter displaces the RNA polymerase stalled at transcriptional blockages due to DNA damage and stimulates the recruitment of repair proteins to DNA lesions on the transcribed strand. The modestly increased mutation frequency of the PA14 *mfd* transposon mutant can thus most likely be attributed to the fact that a stalled RNA polymerase inhibits the repair of DNA lesions (20). An *mfd* transposon mutant was also detected in an independent gentamicin resistance screen of the PA01 *lux* fusion library (9) performed previously (data not shown). This previous screen also detected a mutant with a disruption of PA0750, coding for the uracil glycosylase Ung. The respective PA14 *ung* mutant showed a fourfold increase in mutation frequency. This result is in accordance with a sevenfold increase in the rifampin mutation frequency caused by inhibition of Ung via an inhibitory protein in *P. aeruginosa* (21).

This study demonstrates that broad screening of genomic libraries for resistance to multiple antibiotics can be utilized to identify mutator genes. Apart from the expected high-level hypermutators, we also detected mutators with modest increases in mutation frequencies. Disruption in those genes might be the genetic basis for the weak hypermutator pheno-

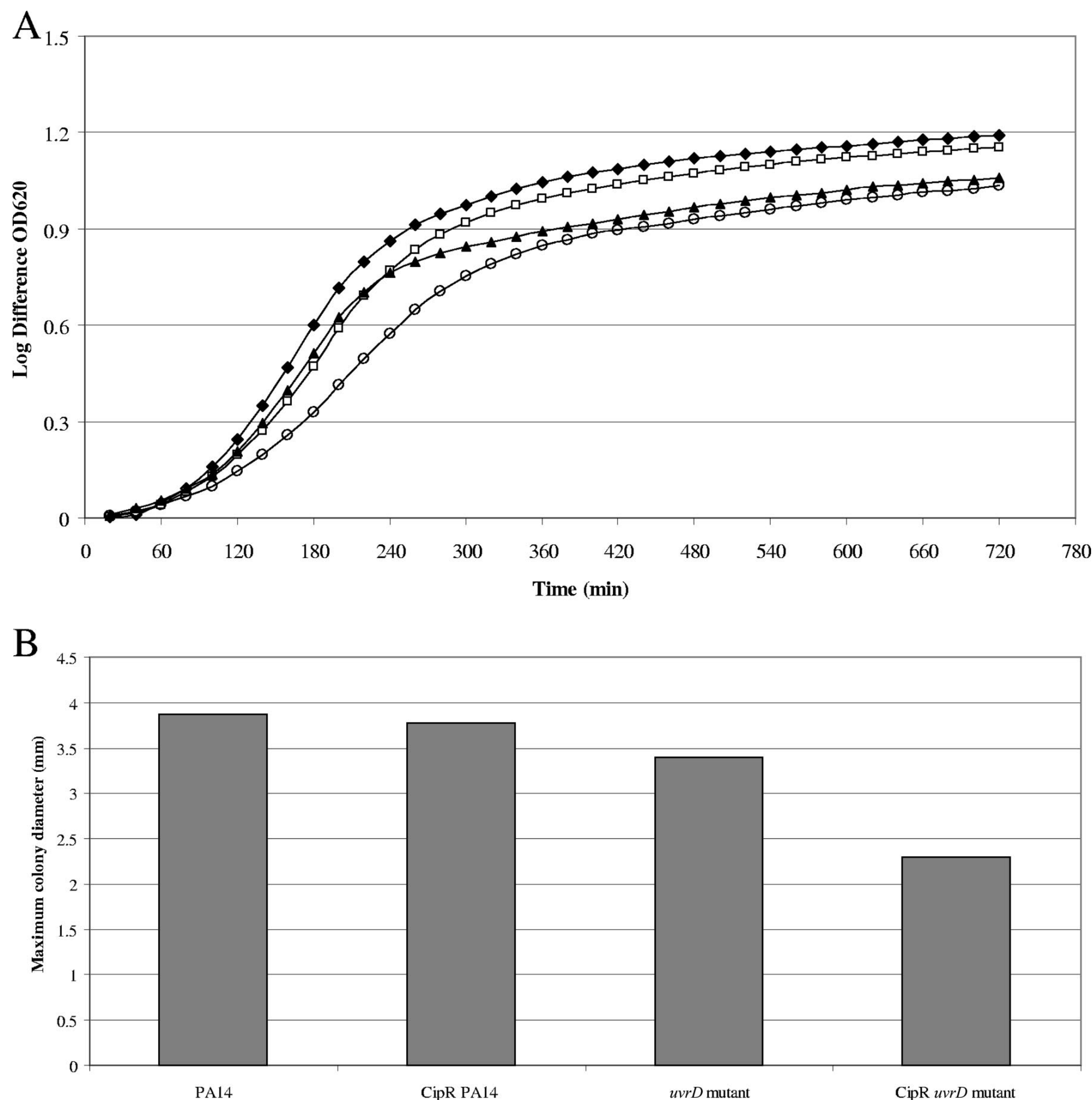

FIG. 1. Growth defects of the *uvrD* PA14 transposon mutant. (A) Growth curves (optical density at 620 nm [OD620]) of *P. aeruginosa* PA14 (closed diamonds), ciprofloxacin-resistant mutants of PA14 (open squares), the *uvrD* PA14 transposon mutant (closed triangles), and ciprofloxacin-resistant mutants of the *uvrD* PA14 transposon mutant (open circles). (B) Maximum colony diameter of single colonies of *P. aeruginosa* PA14 and the *uvrD* PA14 transposon mutant grown on Mueller-Hinton agar plates without antibiotic and on selection plates with 0.5 μg/ml ciprofloxacin (CipR) after 36 h of incubation at 37°C.

type seen in early CF isolates. Further work is needed to confirm the clinical relevance of these observations.

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