J. Microbiol. Biotechnol. (2005), **15**(6), 1214–1220

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Inactivation of *mutS* Leads to a Multiple-Drug Resistance in *Pseudomonas putida* ATCC12633

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Received: January 5, 2005
Accepted: April 7, 2005

Abstract Decreased porin-mediated outer membrane penetration of hydrophilic antibiotics is a common mechanism of antibiotic resistance in Gram-negative bacteria. This study was undertaken to determine whether a null mutation in *Pseudomonas putida* would suppress porin synthesis, and therefore reduce the susceptibility of the organism to streptomycin, norfloxacin, and tetracycline. Inverse PCR amplification and double-stranded DNA sequencing were used to identify chromosomal genes carrying Tn*phoA'*-1 inserts. Genome database available was used to identify putative homologue genes, one of which encodes protein with homology to domains of the MutS of *P. putida*, suggesting a crucial role in the multidrug resistance. Increased resistance to streptomycin, norfloxacin, and tetracycline might be due to accumulation of compensatory mutations. Either no growth or slow growth was observed in *P. putida* KH1027 when grown in minimal medium containing gluconate, glucose, or citrate; however, it is not clear whether the growth patterns contributed to the multidrug resistance.

Key words: *Pseudomonas putida*, inverse PCR, DNA sequencing, multidrug resistance

Multidrug resistance presents a serious problem in the treatment of bacterial infections. Low membrane permeability and active efflux are generally associated with multidrug resistance [8, 17, 28]. Porins are major outer membrane proteins, which form transmembrane pores that permit diffusion into bacterial cells of hydrophilic compounds, including sugars, amino acids, and antibiotics. Loss or alteration of the porins may result in an increased resistance to a variety of drugs. Special transport proteins that translocate specific antibiotics can be inactivated by mutations. Synthesis of the OmpF porin of *Escherichia coli* is highly and reversibly inhibited by 5 mM salicylate, which results in resistance to β-lactam antibiotics, chloramphenicol, and tetracycline [27, 35]. Burns and Clark [4] have also demonstrated effects of sodium salicylate on the outer membrane permeability of *Pseudomonas cepacia* and found that there was apparent decrease in the amount of a 40 kDa minor outer membrane protein, OpcS, in the outer membrane of bacteria grown in medium containing salicylate. However, the susceptibility of the organism to chloramphenicol, trimethoprim, and ciprofloxacin was decreased in *P. cepacia*, whereas the susceptibility to a β-lactam and ceftazidime was unchanged [4]. These findings suggest that OpcS might function as a selective antibiotic-permeable porin, which can be suppressed by growth in the presence of salicylate. Multidrug resistance can also occur by decreasing the internal concentration of the antibiotics through drug efflux transporters. The multidrug resistance is often associated with the overexpression of the transporters that recognize and efficiently expel a broad range of structurally unrelated compounds from the cells [16]. In Gram-negative bacteria, the majority of multidrug transporters share a common three-component organization: a transporter located in the inner cytoplasmic membrane functions with an outer membrane channel and a periplasmic accessory protein. In this arrangement, efflux complexes traverse both the inner and the outer membranes and thus facilitate direct passage of the substrate from the cytoplasm or the cytoplasmic membrane into the external medium. Direct efflux of drugs into the external medium is advantageous for Gram-negative bacteria because, in order to come in again, the expelled drug molecules must cross the outer membrane with low permeability. Hence, drug efflux works synergistically with the low permeability of outer membrane. The synergy explains the observation that Gram-negative bacteria become hypersusceptible to various drugs either by the inactivation of efflux pumps or by the permeabilization of the outer membrane. Four different efflux complexes, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-

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OprM, have so far been characterized and encoded in the same gene cluster on chromosome from *P. aeruginosa* [16]. The MexAB-OprM pump system exhibits the broadest substrate specificity known among multidrug transporters, since it accommodates β-lactams [21], chloramphenicol, tetracycline, quinolones [20], trimethoprim [16], novobiocin, and organic solvents [22].

This study was undertaken to determine whether a null mutation in *P. putida* would suppress porin synthesis, and therefore reduce the susceptibility of the organism to streptomycin, norfloxacin, and tetracycline. Inverse PCR amplification and double-stranded DNA sequencing were used to identify chromosomal genes carrying Tn*phoA'* inserts. Genome sequences of *Pseudomonas* spp. available were used to identify putative homologue genes, one of which encodes a protein homologous to domains of the MutS of *P. putida*, suggesting a crucial role in the multidrug resistance.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely cultured with shaking (200 rpm) at 37°C (*E. coli*) or 30°C (*P. putida*) in rich Luria-Bertani (LB) medium and on LB agar [14]. The following antibiotics were used at the indicated concentrations for *P. putida*: 50 μg/ml kanamycin or 100 μg/ml rifampin. *E. coli* strains carrying plasmids were grown in the presence of antibiotics: 100 μg/ml ampicillin or 50 μg/ml kanamycin. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Quantum), when required, was used to detect β-galactosidase activity of recombinants on plates [15, 41].

P. putida MK1 and mutant strains were cultured with a density of 5×10^8 cells/ml, and were diluted 100- to 1,000-fold at 30°C in M9 medium [12, 43]. The minimal medium used for growth was carbon-free M9 minimal medium supplemented with appropriate carbon sources: 5% glucose, 5% lactose, 5% fructose, 5% (wt/vol) sucrose, 5% galactose, 10 mM acetate, 10 mM citrate, 10 mM gluconate, 10 mM pyruvate, or 10 mM succinate, respectively.

Transposon Mutagenesis

This mutagenesis was performed as previously described [14, 39]. *P. putida* MK1 was a rifampin-resistant strain of *P. putida* ATCC12633 (purchased from the American Type Culture Collection) and isolated by successive culture on LB plates containing 100 μg/ml rifampin. Rifampin-resistance of *P. putida* was utilized for counterselection in trasposon mutagenesis. The mutant strains were constructed by insertion of the Tn*phoA'*-1 element of *E. coli* BW16948. In addition, the promoterless *lacZ* of Tn*phoA'*-1 could be reacted as a reporter gene for the individual promoter [41]. Suicide vector pBW30 was transferred by exoconjugation using a nitrocellulose membrane (Millipore membrane, 25 mm, 0.45 μm pore size). *E. coli* BW16948 carrying pBW30 was cultivated at 37°C in 5 ml of LB broth containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. *P. putida* was grown at 30°C in 5 ml of LB broth without antibiotics. *P. putida* MK1 (a recipient strain) and *E. coli* (a

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
Escherichia coli		
Top10F'	F'{*lacI*^{q}, Tn*10*(Tet^R)} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15Δ*lacX*74 *deoR recA*1 *araD*139 Δ(*ara-leu*)7697 *galU galK rpsL*(Str^R)*endA*1 *nupG*	INVITROGEN
BW16948	pBW30:: Tn*phoA'*-λ pir/RP4::MuKan *recA thr*-1 *leuB*6 *lacY*1 *tonA*21 *supE*44	[37]
Pseudomonas putida		
ATCC12633	Prototroph	American Type Culture Collection
MK1	*P. putida* ATCC12633 Rif^r	This study
KH1001	Tn*phoA'*-1 mutant of *P. putida* MK1; Km^r, Rif^r	This study
KH1010	Tn*phoA'*-1 mutant of *P. putida* MK1; Km^r, Rif^r	This study
KH1015	Tn*phoA'*-1 mutant of *P. putida* MK1; Km^r, Rif^r	This study
KH1027	Tn*phoA'*-1 mutant of *P. putida* MK1; Km^r, Rif^r	This study
Plasmids		
pCR2.1	Topoisomerase (TOPO) activated TA cloning vector with T-A cloning site of *lacZ*; Ap^r, Km^r	INVITROGEN
pIPCR27	pCR2.1 with 1.96 kb fragment from *Inverse PCR* fragment cloned into the *Eco*Rsite	This study
pRS551	*lacZ* probe vector for southern hybridization; Ap^r, Km^r	[41]

[a]Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Rif^r, rifampin resistance; Str^r, streptomycin resistance; Tet^r, tetracycline resistance.

donor strain) were mixed at the ratio of 5:1 in 5 ml of 10 mM $MgSO_4$ and filtered through nitrocellulose membrane. The filters were incubated on LB agar plate at 30°C for 24 h, and suspended in 5 ml of 10 mM $MgSO_4$. The mating cells were placed on the selective medium (containing 50 μg/ml kanamycin and 100 μg/ml rifampin) for the isolating of exoconjugants. The growing cells on such plate were only *P. putida* exoconjugants that have a transposon element inserted in their chromosomal DNA [41].

Test of Minimal Inhibitory Concentration (MIC)

The MICs of each antibiotic were determined by the broth dilution technique with an inoculum of 5×10^4 organisms/ml, as previously described [20]. The following antibiotics were used at the MICs for *P. putida*: streptomycin, norfloxacin, and tetracycline.

Southern Hybridization

DNA probes were obtained by PCR amplification of pRS551 [36] with primers *LacZ*-F (5'-ATGACCATGATT-ACGGATTCA-3') and *LacZ*-R (5'-CACTTCAACATCA-ACGGTAAT-3'). Amplified PCR products were labeled with the Random Prime Labelling system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Genomic DNA of *P. putida* MK1 and mutant strains was prepared by GENOME DNA kit (Bio101) and cleaved with *Hin*dIII [7, 13]. The digested DNA was visualized by ethidium bromide staining after resolution of 50 μl samples on 1% agarose gels. The gel was treated with 250 mM depurination solution (250 mM HCl), denaturation buffer (1.5 M NaCl and 0.5 M NaOH), and neutralizing buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5). Then, single-stranded DNA was transferred to 0.45 μm nitrocelluose membrane (Schleicher and Schuell, GmbH, Dassel, Germany) in a pyrex dish containing 20× SSC (0.3 M Na_3 citrate, 3 M NaCl, and pH 7.0). After DNA was transferred, the nitrocellulose membrane was exposed to UV in a UV cross-linker. Probes (*lacZ* of pRS551) were hybridized in 40 ml of Gold Hydridization Buffer (Amersham Pharmacia Biotech.) for 16 h at 42°C. The hybridized blot was washed in 5× SSC, primary wash buffer (0.4% SDS and 0.5× SSC), and secondary wash buffer (2× SSC), and then exposed to autoradiography film (Kodak, Rochester, NY, U.S.A.).

Inverse PCR (IPCR) and Sequencing

To amplify chromosomal genes from each mutant of *P. putida*, the following oligonucleotide primers were synthesized and used for inverse PCR [29, 42]: *phoA* primer (5'-TTCGGCATAATTACGTGC-3'), and *lacZ* primer (5'-ACTCGCTTTAATGATGAT-3'), which corresponded to nucleotides 316 to 333 bp of the *Tnp* gene and nucleotides 688 to 705 of the *lacZ* gene. Genomic DNA was digested with *Cla*I in 100 μl of reaction mixture containing 10 μl of 10× reaction buffer, and 10 μg of DNA, 50 U of enzyme (Takara). The reaction mix was incubated for 8 h at 37°C. The digested DNA was purified with a Gene Clean kit (Bio101) and then ligated in 20 μl of reaction mixture containing 2 μl of 10× reaction buffer, and 5 U of T4 DNA ligase (Takara). The reaction mixture was incubated for 16 h at 14°C. The self-ligates of digested DNA were added as templates to 100 μl of reaction mixture of inverse PCR, containing 10 μl of 10× reaction buffer, 1.5 mM $MgCl_2$, deoxynucleotide triphosphates (dNTPs), 2.5 U of *Thermus aquaticus* DNA polymerase (Puretech), and 0.6 M each of primers. Reaction mixture was heated for 10 min at 95°C to denature the DNA, and then cycled for 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C for 30 cycles. The source for the sequencing analysis was plasmid pIPCR27 derived from cloning a 1.96 kb IPCR fragment of KH1027 into the pCR2.1-TOPO (TOPO TA cloning system, Invitrogen). Nucleotide sequencing was carried out by Macrogen Biotechnologies Company (Seoul, Korea).

RESULTS

Isolation of Multidrug-Resistant Strains of *P. putida* MK1

Given that outer membrane porins permit hydrophilic antibiotics to diffuse into bacterial cells, it was of interest to create a null mutant of *P. putida* to investigate if a protein loss may result in an increased resistance to a variety of drugs. Therefore, a plasmid pBW30 carrying the Tn*phoA'*-1 transposon was used for transposon mutagenesis of *P. putida*. The plasmid having R6K origin requires the R6K-specified replication protein π and can be maintained only in host strains that produce this protein, such as the λ *pir* phage-lysogenized *E. coli* strains [14, 41]. Strain *P. putida* receiving pBW30 but lacking the π protein is non-permissive and does not maintain the transferred plasmid. Since Tn*phoA'*-1 and its cognate transposase were constructed as inserts in pBW30, exoconjugants stably expressing the marker of Tn*phoA'*-1 were selected for clones in which it has been transferred to a replicon of *P. putida*. Selection for the *P. putida* MK1 exoconjugants was carried out on LB plate containing kanamycin (selective marker of Tn*phoA'*-1) and rifampin.

To determine whether null mutations result in multidrug resistance, exoconjugants were tested on LB plates containing antibiotics streptomycin, norfloxacin, and tetracycline. As shown in Table 1, about 2,000 exoconjugants were screened, and four isolates reproducibly exhibited resistance against all three antibiotics tested.

To examine whether the transposon had been once inserted randomly into the chromosome of each mutant, Southern hybridization analysis was performed with a probe consisting of *lacZ*. Thus, DNA was isolated from *P.*

Table 2. Susceptibility of *P. putida* MK1 and exoconjugants to antibiotics.

Strain	MIC (mg/ml)[a]			
	Str	Nor	Tet	Reference
P. putida MK1	>15	2	3	This study
KH1001	>100	10	>3	This study
KH1010	>75	10	>3	This study
KH1015	>100	10	25	This study
KH1027	>100	20	50	This study

[a]MICs of each antibiotic were determined by the broth dilution technique: Str, streptomycin; Nor, norfloxacin; Tet, tetracycline.

putida MK1 and five exoconjugants, digested with restriction enzyme, and Southern blotted after agarose gel electrophoresis. Hybridization of this probe with DNA of four mutants resulted in a single hybridizing fragment, indicating that the mutations were due to single insertion, whereas one mutant showed more than one insertion or partial DNA digest (data not shown). No hybridization signals were observed with *P. putida* MK1, which does not include *lacZ* on the chromosome. Southern hybridization analysis also demonstrated that there were at least three different locations of Tn*phoA'*-1 in the chromosomes of *P. putida* exoconjugants (data not shown).

Susceptibility of Mutants to Streptomycin, Norfloxacin, and Tetracycline

To determine the susceptibility pattern of mutants, the susceptibilities of four mutants with single in sertion and *P. putida* MK1 were examined (Table 2). Mutants were 5 to 7 times more resistant to streptomycin, 5 to 10 times more resistant to norfloxacin, and 2 to 17 times more resistant to tetracycline than parent *P. putida* MK1. Interestingly, the KH1027 mutant exhibited higher levels of resistance to tetracycline than other strains. The MICs value of mutant strains, compared with that of *P. putida* MK1, indicated that the mutagenesis by transposable element in chromosomal DNA somehow influenced change of their phenotype and emergence of the antibiotic resistance.

Gene Identification by Inverse PCR

To locate the insert of Tn*phoA'*-1 in the chromosomes of mutants, inverse PCR (IPCR) amplification of the upstream region of a Tn*phoA'*-1 insertion was performed and followed by sequencing of the PCR product [28]. Since there is only one *Cla*I site in the Tn*phoA'*-1 and one in flanking DNA near enough, the resulting fragment could be ligated and amplified. By using primers, *phoA* (5'-TTCGGCATAATTACGTGC-3') and *lacZ* (5'-ACTCGCT-TTAATGATGAT-3'), IPCR was able to obtain a single PCR band that could be cloned and identified (Fig. 1).

The IPCR-generated fragments from mutants of *P. putida* were cloned into the pCR2.1-TOPO vector using

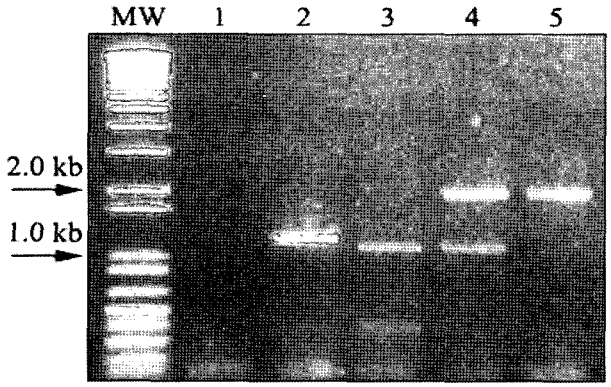

Fig. 1. Results of Inverse PCR (IPCR).
Total genomic DNA was digested to completion with *Cla*I and ligated. The ligated mixture was then amplified with *phoA* and *lacZ* primers. Lane 1, *Pseudomonas putida* MK1 (negative control); lane 2, KH1001; lane 3, KH1010; lane 4, KH1015; lane 5, KH1027; lane MW, molecular weight maker.

the TOPO T-A cloning system (Invitrogen). The cloned PCR products consisted of DNA sequences adjacent to one end of the transposon insertion, which was selected for DNA sequence analysis to search for location of insertion sites. Sequencing of the 2.0-kb PCR fragment from strain *P. putida* KH1027 (Fig. 1, lane 5) with the M13 forward and reverse primers showed that the cloned fragment carried 1,298 bp adjacent to the insert. BlastN and BlastX searches of existing gene and protein database were performed using the DNA sequence of this fragment, and significant homology of amino acid sequence was found; with 96% identity to MutS of *P. putida* (GenBank accession no. AB039965), 83% to MutS of *Pseudomonas aeruginosa* (accession no. AF220055), 61% to MutS of *Salmonella typhimurium* (accession no. U16303), and 60% to Fdv of *Escherichia coli* (accession no. M64730) (Fig. 2). However, sequencing of the PCR fragments of KH1001, KH1010, and KH1015 (Fig. 1, lanes 2, 3, and 4) did not allow us to identify sequences adjacent to the Tn*pho'*-1 insert.

Growth of MutS Mutant on Various Carbon Sources

P. putida KH 1027 mutant exhibited a pattern of multidrug resistance that was different from the patterns shown by the parental strain and other exoconjugant strains. To investigate if increased resistance resulted from loss of porins that permit diffusion of hydrophilic compounds into bacterial cells, the growth of mutant and parental strains in minimal medium containing various carbon sources was compared. The *P. putida* KH 1027 strain grew as fast as the parental strain, when provided with acetate, pyruvate, or succinate (Table 3). However, the mutant grew slowly on glucose or citrate, compared with its parental strain, whereas no growth of the mutant was seen in the presence of gluconate.

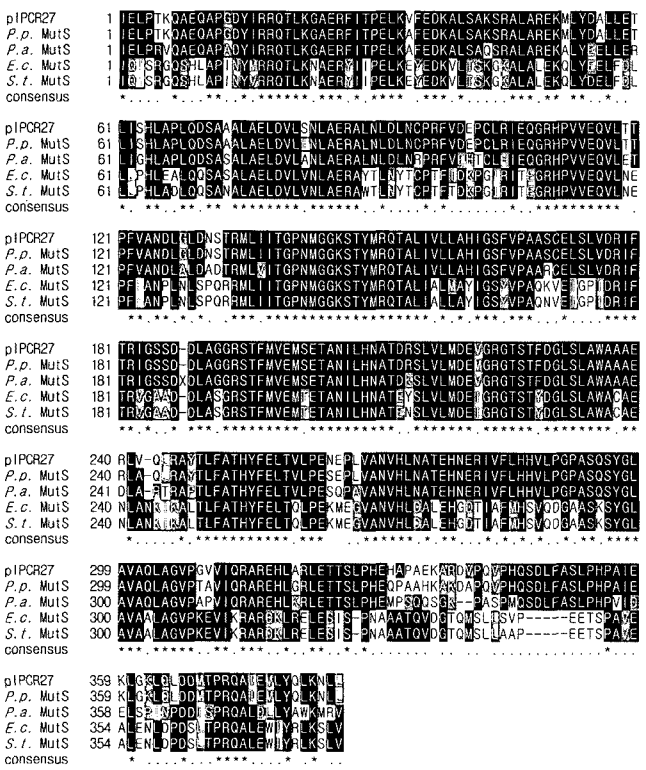

Fig. 2. Alignments of the IPCR sequence (pIPCR27) from *P. putida* KH1027 mutant to the MutS proteins of *P. putida* (*P. p.*), *P. aeruginosa* (*P. a.*), *E. coli* (*E. c.*), and *S. typhimurium* (*S. t.*). Amino acids that are conserved in five sequences are highlighted. The dashes indicate gaps that were included to improve alignment. MutS sequences for alignment were searched by the blastX and blastN of NCBI data bases.

DISCUSSION

In the present study, we have performed *in vivo* transposition of the *P. putida* genome with a Tn*phoA'*-1 transposable element and constructed mutant libraries consisting of thousands of individual random mutants. These libraries were used to screen *P. putida* with a random transposon-generated mutant to identify genes required for multidrug resistance. DNA fragment of the upstream region of a Tn*phoA'*-1 from *P. putida* KH1027 could be amplified and the amplified products consisted of DNA sequences at the adjacent end of the transposon insertion and were cloned for sequence analysis. Homologous searches of already known genes were able to identify the gene-carrying insert in *P. putida* KH1027, which was known to be *mutS*. Therefore, the genome sequence of *P. putida* KH1027 indicates that this organism appears to be deficient in MutS.

The mutation frequency of bacteria is essentially controlled by the DNA repair systems. The *dam*-directed mismatch repair (methyl-directed mismatch repair: MMR) system, which is encoded by *mutH*, *mutL*, *mutS*, and *uvrD* in *E. coli* [10], is the one of the most important systems. It plays a crucial role in repairing base mismatch insertions and deletions that arise during replication, as well as in controlling uptake of foreign DNA [26]. Another phenotypic property of *mutH*, *mutL*, and *mutS* is to increase recombination between homologous sequences and between related but nonhomologous sequences [6]. MutS binds specifically to mismatched base repair and insertion/deletion mispairs up to four nucleotides as well as adducted base mismatches [32, 37]. The DNA-bound MutS protein binds ATP and can form α-looped structures with concomitant hydrolysis of ATP in association with dimeric MutL, suggesting movement of the protein complex on DNA away from the mismatch [1]. After addition of MutH, the MutSLH complex cleaves DNA 5' to hemimethylated -GATC- sequences in the unmethylated strand. Subsequent excision repair from this nick occurs in either direction by one of several endonucleases, and directionality is imparted through interaction of the MutLS complex with helicase II, the *uvrD* gene product [40]. Resynthesis of the gapped DNA is accompanied by DNA polymerase III holoenzyme and subsequent ligation by DNA ligase. The mutator phenotype of strain with an inactivated *mutS* gene displays a high mutation frequency owing to its inability to repair mismatches. Strains exhibiting elevated mutation frequencies have been recently reported amongst populations of pathogenic *E. coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Helicobacter pylori* [2, 19, 30, 43]. In some cases, mutation frequencies to antibiotic resistance are 1,000-fold higher than normal strain. The majority of hypermutator populations harbor defects in *mutS*, which is the most frequent mutator allele [30].

Several mechanisms of antibiotic resistance have been unraveled in *P. aeruginosa*, which warrants closer examination of *P. putida* KH1027. Mutational resistance to norfloxacin in *P. aeruginosa* has been associated with modification of the intracellular target of the drug (DNA gyrase) or alteration

Table 3. Growth in M9 medium containing various carbon sources.

Strains	Carbon source[a,b]									
	Glu	Lac	Fruc	Su	Gal	Ace	Cit	Gluc	Pyr	Suc
P. putida MK1	+++	-	-	-	-	+	++	++	++	++
KH1027	+	-	-	-	-	+	+	-	++	++

[a]Glu, glucose; Lac, lactose; Fruc, fructose; Su, sucrose; Gal, galactose; Ace, acetate; Cit. citrate; Gluc, gluconate; Pyr, pyruvate; Suc, succinate.
[b]+, indicates positive degree of growth; -, indicates negative results of growth.

of outer membrane permeability [9, 11, 34]. Since only one type of gyrase mutation has been identified in *P. aeruginosa*, alteration of outer membrane permeability is associated with norfloxacin resistance. By analogy, resistance to norfloxacin of *P. putida* KH1027 may be due to an alteration or modification of porins and lipopolysaccharide in the outer membrane. Active efflux of tetracycline is a resistance mechanism found in *P. aeruginosa*, which express efflux systems, MexAB-OprM, MexCD-OprJ, and MexXY-OprM, with broad substrate specificity [16]. Although tetracycline can diffuse readily through the inner membrane bilayer, the lipid bilayer of the outer membrane is relatively impermeable to lipophilic tetracycline, and tetracycline can penetrate the outer membrane by the porin OmpF. In mutants with decreased OmpF expression, there is an increase in the MIC for tetracycline [5, 25, 33]. By analogy, a similar mutation in the porin for the tetracycline would lead to high levels of resistance. Streptomycin, as an aminoglycoside, appears to use a specific transporter to enter the cell [16]. The active efflux system of streptomycin, MexXY-OprM, is a main mechanism of resistance found in *P. aeruginosa*. No active efflux systems have been found in *P. putida*; therefore, the decreased porin-mediated outer membrane penetration appears to limit target access to streptomycin. Although the action of the mutator is accumulative in the presence of three antibiotics, its primary effect has been considered to be the reduction of the porin(s) in the outer membrane [18]. However, since MutS was completely lost by the inserted inactivation of *mutS*, several mutations that modify outer membrane structural components can lead to multidrug resistance, which would occur in *P. putida* KH1027 rather than other mutants. A mutator could also facilitate modification of the active sites of detoxification enzymes to shift the resistance from resistance to a low dose to a high dose and extend its resistance spectra [31, 38]. Thus, these studies suggest that increased mutation and homologous recombination rates in the *mutS* mutant would facilitate the appearance of *P. putida* phenotypes able to adapt to the changing environment [30]. Similar observations were also made by previous studies that describe significantly higher frequency of antibiotic resistant cells in mutator *P. aeruginosa* pathogenic isolates and *E. coli* uropathogenic isolates [23, 24].

No growth and slow growth were observed in KH1027, when grown in minimal medium containing gluconate, glucose, or citrate; however, it is not clear whether the growth patterns contributed to the multidrug resistance. MutS mutant could accumulate compensatory mutations that limit costs of antibiotic resistance [3], which may lead to loss of functions that were dispensable in the condition where rich medium (LB) was used for selection of resistants. Thus, since a high mutation frequency allows faster adaptation, a mutator strain would be initially beneficial, but deleterious in secondary environment [3].

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