**Pseudomonas aeruginosa** MutL promotes large chromosomal deletions through non-homologous end joining to prevent bacteriophage predation

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ABSTRACT

**Pseudomonas aeruginosa** is an opportunistic pathogen with a relatively large genome, and has been shown to routinely lose genomic fragments during environmental selection. However, the underlying molecular mechanisms that promote chromosomal deletion are still poorly understood. In a recent study, we showed that by deleting a large chromosomal fragment containing two closely situated genes, hmgA and galU, *P. aeruginosa* was able to form ‘brown mutants’, bacteriophage (phage) resistant mutants with a brown color phenotype. In this study, we show that the brown mutants occur at a frequency of $227 \pm 87 \times 10^{-8}$ and contain a deletion ranging from ~200 to ~620 kb. By screening *P. aeruginosa* transposon mutants, we identified *mutL* gene whose mutation constrained the emergence of phage-resistant brown mutants. Moreover, the *P. aeruginosa* MutL (PaMutL) nicking activity can result in DNA double strand break (DSB), which is then repaired by non-homologous end joining (NHEJ), leading to chromosomal deletions. Thus, we reported a noncanonical function of PaMutL that promotes chromosomal deletions through NHEJ to prevent phage predation.

INTRODUCTION

Large-scale genomic deletions have been observed in many bacterial species as a mechanism for adaptation to specialized environments or lifestyles, such as in obligate pathogens and symbionts (1,2). Genome reduction through genomic DNA deletion is a commonly observed successful strategy that allows pathogens to better adapt to new environments by removal of genes that are no longer necessary for survival (3–8).

*Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium and opportunistic pathogen that is capable of surviving in diverse freshwater and terrestrial environments, as well as infecting a wide-range of susceptible hosts (9). In humans, *P. aeruginosa* is able to colonize and infect various body sites including the lungs, urinary tract, and the eye, and cause severe infections in burn victims and immuno-compromised patients (10,11). In cystic fibrosis patients, *P. aeruginosa* establishes a chronic infection early in life that is extremely difficult to eradicate (12,13).

Genomes of *P. aeruginosa* are relatively large and can range in size from 5.5 to 7 Mb. Analyses of the genomes of diverse strains of *P. aeruginosa* have shown that the high adaptability of this organism can be partly explained by the remarkable plasticity of its genome (14). In addition to genome rearrangements and horizontal acquisition of genes, genome reduction has also been observed, particularly among clinical strains isolated from chronic infections (12,13,15). Hocquet et al. sequenced four clinical *P. aeruginosa* strains and revealed large chromosomal deletions (66–270 kb). Using *in vitro* assays, they demonstrated that these strains are highly resistant to two bacterial pyocin toxins, toxic proteins mediating *P. aeruginosa* inter-strain competition (16), which may enhance their survival in a mixed bacterial population during chronic infection in the host (5). Smith et al. collected *P. aeruginosa* strains from a patient over an 8-year span, and detected a large deletion of 188 kb that eliminated 139 genes (17). Dingemans et al. reported a clinical *P. aeruginosa* strain, whose genes encoding all type
III secretion system and several virulent factors were missing (18). However, the mechanisms responsible for large-scale genomic deletions in P. aeruginosa are poorly understood, and the genetic factors that promote such reductive evolution have not yet been determined.

In general, site-specific and spontaneous deletion are the two main causes of genomic fragment deletion. Site-specific deletion mediated by prophage or other mobile genetic elements are common and occurred at a high frequency (19). For example, an 89 kbp pathogenicity island in Streptococcus suis, which is flanked by a 15 bp direct repeat, was excised from the genome with a frequency of \( \sim 3.2 \times 10^{-4} \) (20). On the contrary, spontaneous deletion was considered as a result of the dysfunction of DNA replication and repair system (3,21). In Salmonella enterica, the DNA loss rate was estimated to be 0.05 bp per chromosome per generation (21). Sanna et al. estimated that the apparent deletion rate in S. enterica ranges from 0.5 \( \times 10^{-9} \) to 2.2 \( \times 10^{-9} \)/cell/generation (1). However, the molecular mechanisms for spontaneous deletions are not well studied.

Genomic fragment deletion is not only one of the strategies used by bacteria to facilitate chronic infections, but also a phage resistance mechanism (7). Phages outnumber bacteria in the natural environment, and play an important role in modulating bacterial communities across different environments (22). On the other hand, bacteria have evolved diverse mechanisms to resist phage infection, including preventing phage adsorption, preventing DNA injection, restriction and modification system, abortive infection, Crisper/Cas system (23). Deletion of the gene(s) involved in phage receptor synthesis could result in the loss of phage receptor, and therefore resist phage adsorption and infection (7).

Previously, we identified two types of phage-resistant mutants in P. aeruginosa based on white or brown colony pigmentation (Figure 1A) (7). Further study showed that while all the white phage-resistant mutants (named white mutants) contained single nucleotide variations conferring phage resistance (24), the brown phage-resistant mutants (named brown mutants), which occurred at a frequency of \( \sim 10^{-6} \), carried a large chromosomal deletion. The deleted fragment contained two closely situated genes, hmgA and galU. The galU mutation results in a lack of O-antigen (25), which is required for phage adsorption. Deletion of hmgA results in the accumulation of a red compound called hmgA (26). Therefore, these phage-resistant genomic deletion mutants can be easily identified based on color.

In this study, we generated a chromosomal deletion model in P. aeruginosa strain PAO1 by infecting with phage PaoP5 (27) and selecting for brown-colored colonies. By screening the PAO1 transposon mutants carrying mutation in genes that might play a role in DNA repair, we unexpectedly identified PAO1ΔmutL, which is defective in generating phage-resistant brown mutants containing the large DNA deletion. Further biochemistry studies demonstrated that the nicking function of P. aeruginosa MutL (PaMutL) could also result in DNA double strand break, which is then repaired by NHEJ proteins Ku and LigD, leading to chromosomal DNA deletion. Thus, our study revealed a non-canonical function of PaMutL in promoting chromosomal deletion through NHEJ to prevent phage predation.

### MATERIALS AND METHODS

#### Bacteria and bacteriophage growth conditions

Pseudomonas aeruginosa PAO1 wildtype and deletion mutants, as well as lytic phage PaoP5 (27) were stored in our laboratory at −80°C in 15% glycerol. P. aeruginosa was routinely grown in Luria–Bertani (LB) broth with shaking at 37°C. When required, the antibiotics carbenicillin, gentamicin or tetracycline were used to select the constructed mutant strains. The concentrations used in the different experiments are described below.

#### Selection of bacteriophage-resistant mutants

Pseudomonas aeruginosa was grown at 37°C in LB. When the bacterial culture reached OD600 of 0.2, it was diluted in ten-fold increments down to \( 10^{-3} \). 0.01-ml aliquots of the 10\(^0\)–10\(^3\) dilutions were mixed with 10\(^0\) PFU (plaque-forming units) of phage, plated on LB agar, and the plates were incubated at 37°C for 24 h.

The phage sensitivity of the isolated colonies was tested by double layer agar assay, as previously described (28). Briefly, 3 ml of 0.8% soft agar was mixed with 200 μl overnight culture of bacteria and was layered onto bottom agar (1.5%). Finally, 1 μl of phages were deposited onto the agar and incubated overnight at 37°C. The absence of plaque formation was indicative of phage resistance.

This method is robust in isolating phage resistant mutants and all the colonies grown on the plate tested were phage resistant.

#### Bacterial genome sequencing

Nine brown mutants from independent biological repeats were selected. Bacterial genomic DNA was extracted using UNIQ-10 Column Bacterial Genomic DNA Isolation Kit (sangon bitotec:SK1202), and then sent to Novogene Corporation for sequencing using the Illumina Hiseq2500 platform (paired-end 125 bp, ~1 Gbp/sample). After demultiplexing of raw reads, Trimmomatic (29) was used to remove adapter sequences and low quality bases. BWA (30) was used to map clean reads to the reference genome sequence of PAO1. Samtools (31) was then used to prepare the data for use with the Integrative Genomics Viewer (IGV) (32). DNA fragment deletion locations were manually checked with IGV and SeqKit (33) and validated by PCR. The sequence data is available in the NCBI Sequence Read Archive under SRA accession number SRP092581.

#### Calculating the frequency of bacteriophage-resistant mutants

To determine the frequency of bacteriophage-resistant mutants for each strain, a single colony was picked and inoculated into LB. Cultures were grown with agitation until reaching \( \sim 3 \times 10^8 \) CFU/ml and then diluted in ten-fold increments down to \( 10^{-6} \). 0.1-ml aliquots of \( 10^{-6} \) and \( 10^{-5} \) dilutions of each culture were plated on LB agar without phage (three plates for each dilution) to determine the
number of *P. aeruginosa* CFU. Then, 0.1-ml aliquots from the 10⁰–10⁻³ dilutions were mixed with 10⁹ PFU of phage, plated on LB agar, and incubated at 37°C for 24 h. To calculate frequencies of brown mutants and total (brown and white) mutants, mean numbers of brown and total mutants derived from 1 ml of culture were divided by mean total CFU in the same 1 ml of original culture. Five biological repeats were performed for each strain, and the final frequency was expressed as mean±standard deviation.

**Screening of transposon mutants defective in producing phage-resistant brown mutants**

A *P. aeruginosa* PAO1 transposon mutant library was acquired from the University of Washington Genome Center (34). We chose the following mutants to test due to their known or potential involvement in DNA repair or their known or putative nuclease activity: recA, recB, mutL, uvrA, uvrB, uvrC, uvrD, xthA, Nei1, Nth, TagA. The screening was performed by mixing log phase bacterial culture with phage, and plating on LB agar plates containing tetracycline (65 μg/ml). After overnight growth at 37°C, we identified any brown mutants that appeared.

**Complementation of mutL, ligD, ku, recA and recB**

The *mutL* gene was amplified by PCR (the sequences of primers MutL-F and MutL-R are listed in Supplementary Table S1), and the PCR product was purified and digested with SacI and BamHI. The digested PCR product was then ligated into SacI/BamHI-treated pUCP24 to generate pucp-*mutL*. The Δ*mutL* complementation strain was generated by electroporation (35) of pucp-*mutL* into strain Δ*mutL*, while the *mutL* overexpression strain was made by electroporation of pucp-*mutL* into wild-type PAO1 followed by selection on LB agar containing gentamicin (20 μg/ml).

Complementations of *ligD, ku, recA* and *recB* was performed using the same method, with the primers listed in Supplemental Table S1. The PaMutL N-terminal do-
main (NTD) was complemented into ΔmutL using the same method with the primers NTD-u and NTD-d.

**sacB insertion**

First, galU was amplified from PAO1 using primers galU-F and galU-R (Supplementary Table S1), the PCR product was digested with KpnI/XbaI, and the digested product was ligated into KpnI/XbaI-digested pEX18Gm to generate pEX-galU. Plasmid pEX18Gm contains a sacB gene for sucrose selection. To account for the high mutation frequency of sacB, we cloned another copy of sacB into pEX-galU. SacB was amplified from pEX18Gm (sacB-F and sacB-R), then ligated into EcoRI/KpnI-digested pEX-galU to generate pEX-galU/sacB. pEX-galU/sacB was then electroporated into wild-type PAO1 or ΔmutL, and selected on LB agar containing gentamicin (20 μg/ml). pEX-galU/sacB inserted into the bacterial genome by single cross over, which was confirmed by PCR. Then, brown mutants of PAO1::sacB and ΔmutL::sacB were selected by sucrose and phage.

**PaMutL and N-terminal domain (NTD) purification**

The mutL gene was amplified by PCR (MutL-P-F and MutL-P-R) and the PCR product was purified and digested with NdeI/NotI. The digested PCR product was then ligated into NdeI/NotI-treated PET-21a to generate pET-PaMutL, which was transferred into *Escherichia coli* BL21(DE3) and selected on LB agar containing ampicillin (100 μg/ml). pET-PaMutL was transformed into E. coli cells were grown at 37°C with shaking in LB medium supplemented with ampicillin (100 μg/ml) to an OD600 of 0.6 before 0.15 mM IPTG was added to induce protein production. Culture was further incubated for 16 h at 18°C with shaking. The His-tagged PaMutL was then purified as previously described (36). Purified PaMutL protein was >95% pure as determined by SDS/PAGE stained with Coomassie Brilliant Blue (Supplementary Figure S1). Protein concentrations are expressed as monomer equivalents when cited in molar terms.

A truncated protein (PaMutLΔ340–633 aa), which only contains the N-terminal domain (NTD) while missing endonuclease domain, was purified using the same method with the primers: NTD-P-u and NTD-P-d.

**Cleavage assay of PaMutL.**

Cleavage assay was performed in a reaction mix containing protein (500 nM), DNA substrate (100 ng plasmid) and 5 mM MgCl₂ in endonuclease buffer (20 mM Tris, pH 7.5; 0.1 mg/ml BSA; 100 mM NaCl) with a final volume of 20 μl. The cleavage reaction was incubated at 37°C for 30 min. The reaction was stopped by adding 40 mM EDTA and 10% glycerol, and the products were analyzed by gel electrophoresis after ethidium bromide staining.

**Construction of MutL mutants in plasmids**

The A20V, G97S, N37H (37) and R474A (36) mutants were constructed in plasmids as previously described, with primers listed in Supplementary Table S1.

ΔmutL::sacB is resistant to tetracycline and gentamicin. Thus, to complement MutL mutants in plasmid, we first cloned a carbenicillin resistant gene from plasmid pUCP-Nde (38) into pUCP24 at the multiple cloning site to generate pUCP24-cb. Then, the PaMutL mutant fragments were ligated into Sall/BamHI double digested pUCP24-cb and the resulting plasmid was transferred into ΔmutL::sacB to select for the colonies that were resistant to carbenicillin (300 μg/ml).

**Construction of ΔligDΔku double mutant**

ΔligDΔku double mutant was constructed by knocking out gene ku in ΔligD mutant background. Briefly, the left arm and right arm of ku was amplified from PAO1 genome using primers Ku-LAF/Ku-LAR and Ku-RAF/Ku-RAR. The gentamycin resistant gene was cloned with primers Ku-GmF/Ku-GmR. Then the three fragments were ligated by overlap PCR using primers Ku-LAF and Ku-RAR. The PCR fragment was purified and ligated into pEX18Tc. The resulting plasmid was electroporated into ΔligD to select for the gentamicin (20 μg/ml) resistance colonies. The deletion of ku was further confirmed by PCR.

**Statistical analyses**

Student’s t-test was used to compare two-group data, and one way ANOVA followed by Tukey’s HSD post hoc was used to compare three or more groups. A P value < 0.05 was considered as statistically significant.

**RESULTS**

**A chromosomal DNA deletion model in *P. aeruginosa strain PAO1***

PAO1 was infected by lytic phage PaoP5 and both brown and white mutants were selected for further study (Figure 1A). Brown mutants occurred at a frequency of 227 ± 87 × 10⁻⁸ (Figure 1B). As expected, all the brown mutants were missing both the *hmgA* and galU genes (7), as revealed by PCR (Figure 1C).

To characterize the deletion, nine brown mutants were picked from 9 independent biological replicates and sequenced. All nine mutants contained deletion of *hmgA* and *galU*-containing DNA fragments ranging from 206 to 629 Kb, which account for 3.16% to 9.67% of the PAO1 genome (Figure 1D and E). Intriguingly, the deletion sites of all nine mutants are unique. Furthermore, no homologous sequences are found flanking the deletion regions among the nine selected mutants (Figure 1F). Thus, chromosomal deletion in this selection model of PAO1 shows three clear features: high frequency (~10⁻⁶), non-site specificity, and large deletions (206–629 kb).

**MutL promotes chromosomal deletions in PAO1**

To investigate the deletion mechanism, we selected the PAO1 transposon mutants carrying mutation in genes that might be involved in DNA repair, and screened for mutants that were defective in generating phage-resistant brown colonies. The screening resulted in the identification of
Figure 2. MutL is required for large chromosomal deletions in *P. aeruginosa*. (A) Representative pictures of the phage-resistant mutants of PAO1, ΔmutL, ΔmutL::mutL and PAO1::mutL. Many more white mutants were generated in the ΔmutL background, and most mutants generated from the mutL overexpression strain were brown mutants. (B) The frequency of brown mutants and white mutants for each strain selected by phage. No brown mutants were detected from ΔmutL, while overexpression of mutL significantly increased the frequency of brown mutants to 1468 ± 427 × 10⁻⁸ (P < 0.05, calculated by one way ANOVA). (C) Genomic organization of PAO1::sacB. Two copies of sacB were inserted into the PAO1 genome immediately downstream from galU. Thus, PAO1::sacB is sensitive to both phage and 5% sucrose selection. (D) The frequency of brown mutants and white mutants for each strain selected by phage and 5% sucrose. Inserting two copies of the sacB gene significantly decreased the rate of white mutants after selection with both phage and 5% sucrose. More than 10⁸ ΔmutL::sacB cells were subjected to selection by phage and sucrose without any brown mutants being detected. Each experiment was repeated five times. The asterisks mark P-value of < 0.05 as calculated by Student’s t-test.

PAO1ΔmutL, which was not able to produce any brown-colored mutants, suggesting that mutL may be necessary for the observed chromosomal deletions (Figure 2A).

Not surprisingly, due to the known role of MutL in the mismatch repair system (39–41), deletion of mutL significantly increased the point mutation rate. PAO1ΔmutL generated white phage-resistant mutants with a 16-fold higher frequency (17 104 ± 9631 × 10⁻⁸) than wild type PAO1 (Figure 2B).

The predominance of the white-colored mutant made it difficult to identify brown-colored mutants when plated on an agar plate. Therefore, we inserted two copies of the sacB gene after the galU gene in both PAO1 and PAO1ΔmutL (Figure 2C) for counter-selection. This allowed us to screen for mutants that were resistant to both phage and 5% sucrose. Due to the close proximity of the inserted sacB to galU, it is unlikely to affect the frequency of brown mutants observed in either the PAO1 or PAO1ΔmutL backgrounds. However, the frequency of white mutants should decrease dramatically, since white mutants would have to carry point mutations in both inserted sacB genes in addition to another gene conferring phage resistance in order to survive the phage and 5% sucrose selection. As expected, ΔmutL::sacB generated much fewer white mutants (201 ± 188 × 10⁻⁸), and no brown mutants were detected from ΔmutL::sacB after selection by phage and 5% sucrose. PAO1::sacB generated brown mutants with a frequency of 84 ± 54 × 10⁻⁸, which is several fold lower than that observed in wild-type PAO1 (Figure 2D). We reason that the reduced frequency may be due to the presence of SacB protein that is generated before the deletion event, leading to decreased viability when plated on sucrose-containing LB agar plates. (The detailed mutation frequencies of each strain are listed in Supplemental Information Table S2.)

Complementation of mutL not only allowed PAO1ΔmutL to regain the ability to generate brown...
Nicking activity of PaMutL results in DSB and is essential for chromosomal deletions

The bacterial mismatch repair system has been extensively studied for four decades, with PaMutL being a known mismatch repair endonuclease with single stranded DNA incising function (36,42). However, our data clearly show that deletion of mutL abolishes the chromosomal DNA deletions in PAO1, suggesting that PaMutL may also possess the ability to cut double-stranded DNA, resulting in double-stranded breaks, which has been suggested in vitro by Correa et al previously (36).

To test whether PaMutL can make DSB, PaMutL with a His-tag at the C-terminus was purified from E. coli cells bearing an IPTG-inducible expression plasmid pET-PaMutL. PaMutL endonuclease activity was tested using a supercoiled plasmid, pUCP24. Recovery of linear plasmid product was observed (Figure 3A). To further confirm that the cleavage activity was not due to contaminating nucleases, a truncated protein (PaMutL340–633 aa, named NTD), with deleted endonuclease domain, was purified in an identical fashion to the wild-type protein (Supplementary Figure S1). Since the PaMutL C terminal (CTD) dimerization domain possesses metal ion-dependent endonuclease activity, the deletion of CTD completely blocked the endonuclease activity in vitro (Figure 3A).

To determine if the double-stranded cleavage activity is sequence specific, a supercoiled plasmid was incubated with PaMutL in endonuclease buffer and then linearized with EcoRI. Supercoiled plasmid digested with EcoRI, or digested with NTD and EcoRI, were used as controls. As shown in Figure 3B, PaMutL and EcoRI generated a wide range of DNA fragment sizes, as indicated by the production of DNA smear below the linearized plasmid, while EcoRI alone or NTD and EcoRI double-digested plasmid formed a specific band due to the presence of one single EcoRI cutting site in plasmid pUCP24. These results suggest that the double-stranded cleavage activity of PaMutL does not possess sequence specificity.

The key residues for MutL nicking functions have been investigated previously in vitro (36,37) (Figure 3C). However, whether these residues are essential for generating chromosomal deletions in vivo are unknown. Thus, we constructed A20V, G97S, N37H, R474A mutants, as well as NTD, in plasmids and transferred into mutL::sacB background. None of these mutants can restore the brown mutant phenotype when selected with sucrose and bacteriophage. To test whether these residues are essential for mismatch repair, the transformed strains were tested with PaoP5, and none of them could restore the mismatch repair function of PaMutL, as indicated by the high frequency of white mutants similar to that of mutL. The in vivo genetic studies suggest that ATP binding domain, ATP hydrolysis domain, endonuclease motif are all essential for mismatch repair, as well as making DSB and subsequent chromosomal deletions in vivo (Figure 3C and D).

NHEJ promotes chromosomal deletion while Homologous Recombination (HR) inhibits it

DSB in bacteria is mainly repaired by NHEJ (43–45) or HR (46–48). In brown mutants, the deletion sites are not site-specific and no homologous sequence was found around the deletion sites. Thus, we infer that the DSB made by PaMutL is repaired by NHEJ through Ku and LigD. Ku is a DNA end-binding protein that binds to the DSBs and then the DSBs are sealed by a specialized DNA ligase LigD.

The frequency of brown mutants for Δku and ΔLigD were 45 ± 25 × 10⁻⁸ and 64 ± 48 × 10⁻⁸ respectively, while the ΔkuΔLigD double mutant generated much fewer brown mutants (30 ± 18 × 10⁻⁸), which is almost 7-fold less than that of PAO1 wild-type. Complementation of ku and LigD significantly increased the frequency to 1947 ± 420 × 10⁻⁸ and 558 ± 205 × 10⁻⁸, respectively (Figure 4A). These ge-
MutL is a well-characterized component of the DNA mismatch repair (MMR) system, which corrects errors that occur during DNA replication (49). In *E. coli*, this DNA repair pathway has been well-studied (50). Mismatch sensing protein MutS first binds to a mismatch site, then recruits MutL (51). The MutS-MutL complex activates the endonuclease MutH, which uses the absence of methylation as a strand discrimination signal to nick the unmethylated GATC site in the newly synthesized strand. The resulting DNA segment is excised by an exonuclease and repaired by DNA polymerase and DNA ligase (52). However, this methyl-directed MMR is only found in *E. coli* and a small group of closely related Gamma-proteobacteria. MutL homologues in other bacteria as well as in eukaryotes possess endonuclease activity that generates the strand cleavage and directs the repair system. In *Bacillus subtilis*, upon mismatch detection, MutS is loaded at the mismatch site and MutL is recruited by MutS to nick the nascent DNA strand (52). Then, the mismatch-containing strand is degraded by exonuclease WalJ and a polymerase and ligase perform resynthesis to complete the correction (41).

In *P. aeruginosa*, MutL has been shown to be part of the MMR system *in vivo* (53), and it also possesses endonuclease activity that can cleave single-stranded DNA (36, 42). More recently, double-stranded DNA cleavage activity has been suggested for MutL *in vitro*. Correa *et al.* demonstrated that, in the presence of ATP, Mg²⁺ and Mn²⁺, both *Bacillus subtilis* MutL (BsMutL) and *P. aeruginosa* MutL (PaMutL) were able to initiate a second cut on nicked plasmid DNA to yield a linear product (36). However, the biological significance and the double-stranded cleaving specificity of PaMutL have not been investigated.

The double-stranded DNA cleaving activity of PaMutL on plasmid pUCP24 (38) was confirmed in this study (Figure 3A, B), and the cleaving activity of PaMutL was shown to be unspecific. The key residues for MutL in mismatch repair and nicking have been well characterized *in vivo* and *in vitro*, however, their functions in generating deletion mutants are unknown. We used *in vivo* genetic model to demonstrate that ATP binding domain, ATP hydrolysis domain, and endonuclease motif are all essential for generating DSBs and subsequent deletions.

Previous studies have shown that *P. aeruginosa* MutS recognizes point mutations and recruits MutL to the mismatched site (54). In our deletion model, ΔmutS had a much higher point mutation rate, as reflected by the high frequency (21.521±8.542×10⁻⁸) of white mutants; while brown mutants in ΔmutS::sacB were detected with a frequency of 171±82×10⁻⁸ (data not shown), which is similar to wild-type PAO1. These data suggest that MutS is required for mismatch repair; however, the double-stranded DNA cleaving function of MutL might be independent of MutS. Further studies are needed to better understand the cleavage and regulation of MutL *in vivo*.

**NHEJ contributes to chromosomal deletion while HR inhibits it**

PaMutL makes DNA double strand break *in vivo*, which is deleterious to bacteria. Bacteria mainly employ two basic mechanisms to repair DSB: HR (46, 47) and NHEJ (43–
Figure 5. Model of the mechanism by which MutL makes DSB and results in a DNA fragment deletion through NHEJ that protects P. aeruginosa from phage infection. PaMutL generates DSB occasionally. The end points of DSB are then joined by Ku and LigD. Brown mutants with a deleted galU/hmgA region are identified after phage infection.

HR utilizes a homologous template to repair the lost genetic information, and maintain chromosome integrity. Our genetic studies confirmed that both HR and NHEJ are involved in DSB repair. While HR maintains the genome integrity, DSB repaired by NHEJ is error-prone and results in chromosomal deletion.

NHEJ has been extensively studied over the last decade as a key approach to repair DSB, but its physiological and ecological importance in bacteria remains to be elucidated. In this study, we showed that NHEJ ligates the DSBs in P. aeruginosa and results in chromosomal deletion, thus increasing genetic diversity to allow P. aeruginosa to better survive the adverse environmental selections, such as resisting phage infection and establishing chronic infection in the host.

Based on our results, we propose a model to explain the chromosomal DNA deletion event in P. aeruginosa (Figure 5). The nicking activity of PaMutL makes double-stranded breaks occasionally. Then, the broken DNA ends were bound and bridged by Ku and sealed by LigD ligase. Since the cleavage by MutL is non-site-specific, and NHEJ ligation is error prone, the deletion site in each mutant may be different (Figure 1F). Among the spontaneous deletion mutants observed in our study, those brown mutants with deletions of the galU/hmgA-containing regions were resistant to phage adsorption and selected after phage infection.

Ecological perspectives on P. aeruginosa MutL

Genetic heterogeneity allows bacteria to better adapt to environmental stresses and promote survival in novel and changing environments (55). The plasticity of the P. aeruginosa genome allows for adaptation in the clinical setting, where adaptation to the host or resistance to antibiotics is essential for bacterial survival (5), as well as in environmental settings, where evading phage predation is of most importance (7). Our study revealed the dual role of MutL in generating both point mutation and large deletion mutation, thus contributing to the genetic heterogeneity in P. aeruginosa.

Phages exist in diverse environments, and play an important role in structuring natural bacterial communities (22). Thus, the phage-host interactions occur extensively and a wide range of phages and their hosts have been used in experimental evolution studies to reveal general principles of phage biology. Phage-host co-evolution can accelerate molecular evolution on both sides (56). And coevolution with phages may promote the emergence of hypermutators, which are defective at the mismatch repair system (57). The presence of hypermutating bacterial strains increases genetic diversity within the population and enhances the ability to cope with phage. However, our study strongly indicates that phage resistance not only evolves via mutations, but also through chromosomal deletions. The brown mutants carry a large chromosomal fragment deletion including galU, a key gene involved in the biosynthesis of LPS, which is the receptor for phage PaoP5. Thus, the ecological significance of chromosomal deletion in phage-host co-evolution should be studied in the near future.

Genome reduction is a common strategy that allows bacteria to better adapt to new environments (3–7). Comparative genomics from symbiotic bacteria have shown that genome reduction occurs when this symbiotic lifestyle is stably established (58,59). For example, a recently identified strain of candidate bacterial phyla TM7 (TM7x) isolated from the oral cavity was shown to be an obligate epibiont of Actinomyces odontolyticus strain XH001 (8). TM7x displays
a highly reduced genome of only 705 kb, which strongly suggests that it has undergone large-scale genomic deletions, especially in comparison to the much larger genome of environmental TM7 S. aalbogensis (1,013 kb). However, the genetic mechanism for genome reduction is not well studied. In our study, we identified PaMutL as a key protein that promotes large chromosomal deletion through NHEJ. Thus, whether MutL and NHEJ can promote genome reduction in other bacteria is another interesting question.

DATA AVAILABILITY

The bacterial genome sequence data is available in the NCBI Sequence Read Archive under SRA accession number SRP092581.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Koskineni,S., Sun,S., Berg,O.G. and Anderson,D.J.I. (2012) Selection-driven gene loss in bacteria. PLoS Genet., 8, e1002787.
2. Lee,M.C. and Marx,C. (2012) Repeated, selection-driven genome reduction of accessory genes in experimental populations. PLoS Genet., 8, e1002651.
3. Moran,N.A. (2002) Microbial minimalism: genome reduction in bacterial pathogens. Cell, 108, 583–586.
4. Tani, Y., Hattori,K., Suzuki,K. and Miyawaki, K. (2008) Spontaneous deletion of a 209-kilobase-pair fragment from the Escherichia coli genome occurs with acquisition of resistance to an assortment of infectious phages. Appl. Environ. Microbiol., 74, 4256–4263.
5. Hoquet,D., Petitjean,M., Rohmer,L., Valot,B., Kulasekara,H.D., Bedel,E., Bertrand,X., Pleulet,P., Kohler,T., Pantelet,A. et al. (2016) Pyomelanin-producing Pseudomonas aeruginosa selected during chronic infections have a large chromosomal deletion which confers resistance to pyocyanin. Environ. Microbiol., 18, 3482–3493.
6. Birch,A., Hauser,A., Ruttner,C. and Hutter,R. (1991) Chromosomal deletion and rearrangement in Streptomyces glaucescens. J. Bacteriol., 173, 3531–3538.
7. Le,S., Yao,X.Y., Lu,S.G., Tan,Y.L., Rao,X.C., Li,M., Jin,X.L., Wang,J., Zhao,Y., Wu,N.C. et al. (2014) Chromosomal DNA deletion confers phage resistance to Pseudomonas aeruginosa. Sci. Rep., 4, 4738.
8. He,X., McLean,J.S., Edlund,A., Yooseph,S., Hall,A.P., Liu,S.Y., Dorsstein,P.C., Esquenazi,E., Hunter,R.C., Cheng,G. et al. (2015) Cultivation of a human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. Proc. Natl. Acad. Sci. U.S.A., 112, 244–249.
9. Stover,C.K., Pham,X.Q., Erwin,A.L., Mizoguchi,S.D., Warrener,P., Hickey,M.J., Brinkman,F.S., Hufnagle,W.O., Kowalki,D.J., Lagrou,M. et al. (2000) Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature, 406, 959–964.
10. Khaledi,A., Schniederjans,M., Pohl,S., Rainer,R., Bodenhofer,U., Xia,B.Y., Klawonn,F., Bruchmann,S., Preusse,M., Eckweiler,D. et al. (2016) Transcriptome Profiling of Antimicrobial Resistance in Pseudomonas aeruginosa. Antonie. Agents Chemother., 60, 4722–4733.
11. Qin,X., Zerr,D.M., McNutt,M.A., Berry,J.E., Burns,J.L. and Kapur,R.P. (2012) Pseudomonas aeruginosa syntrophy in chronically colonized airways of cystic fibrosis patients. Antoniob. Agents Chemother., 58, 5971–5981.
12. Marvig,R.L., Dolce,D., Sommer,L.M., Petersen,B., Ciofu,O., Campana,S., Molin,S., Taccetti,G. and Johansen,H.K. (2015) Within-host microevolution of Pseudomonas aeruginosa in Italian cystic fibrosis patients. BMC Microbiol., 15, 218.
13. Marvig,R.L., Sommer,L.M., Molin,S. and Johansen,H.K. (2015) Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis. Nat. Genet., 47, 57–64.
14. Winsor,G.L., Griffiths,E.J., Lo,R., Dhillon,B.K., Shay,J.A. and Brinkman,F.S. (2016) Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. Nucleic Acids Res., 44, D646–D653.
15. Didelot,X., Walker,A.S., Peto,T.E., Crook,D.W. and Wilson,D.J. (2016) Within-host evolution of bacterial pathogens. Nat. Rev. Microbiol., 14, 150–162.
16. Ghequire,M.G. and De Mot,R. (2014) Ribosomally encoded antibacterial proteins and peptides from Pseudomonas. FEMS Microbiol. Rev., 38, 523–568.
17. Smith,E.E., Buckley,D.G., Wu,Z.N., Saenphimmachak,C., Hoffman,I.R., D’Argenio,D.A., Miller,S.J., Ramsey,B.W., Speert,D.P., Moskovitz,S.M. et al. (2006) Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc. Natl. Acad. Sci. U.S.A., 103, 8487–8492.
18. Dingemans,J., Ye,L., Hildebrand,F., Tontodonati,F., Craggs,M., Biloqc,F., De Vos,D., Crabbe,A., Van Houdt,R., Malfroot,A. et al. (2014) The deletion of TonB-dependent receptor genes is part of the genome reduction process that occurs during adaptation of Pseudomonas aeruginosa to the cystic fibrosis lung. Pathogens Dis., 71, 26–38.
19. Grindley,N.D.F., Whiteson,K.L. and Rice,P.A. (2006) Mechanisms of site-specific recombination. Annu. Rev. Biochem., 75, 567–605.
20. Li,M., Shen,X., Yan,J., Han,H., Zheng,B., Liu,D., Cheng,H., Zhao,Y., Rao,X., Wang,C. et al. (2011) GI-type T4SS-mediated horizontal transfer of the 89K pathogenicity island in epidemic Streptococcus suis serotype 2. Mol. Microbiol., 79, 1670–1683.
21. Nilsson,A.I., Koskineni,S., Eriksson,S., Kugelberg,E., Hinton,J.C. and Andersson,D.I. (2005) Bacterial genome size reduction by experimental evolution. Proc. Natl. Acad. Sci. U.S.A., 102, 12112–12116.
22. Salmond,G.P. and Fineran,P.C. (2010) A century of the phage: past, present and future. Nat. Rev. Microbiol., 13, 777–786.
23. Labrie,S.J., Samson,J.E. and Moineau,S. (2010) Bacteriophage resistance mechanisms. Nat. Rev. Microbiol., 8, 317–327.
24. Lim,W.S., Phang,K.K., Tan,A.H., Li,S.F. and Ow,D.S. (2016) Small colony variants and single nucleotide variations in P1 region of PBI Phage-Resistant Pseudomonas aeruginosa. Front. Microbiol., 7, 282.
25. Priebe,G.P., Dean,C.R., Zaidi,T., Meluleni,G.J., Coleman,F.T., Coutinho,Y.S., Noto,M.J., Urban,T.A., Pier,G.B. and Goldberg,J.B. (2004) The galU Gene of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. In Infect. Immun., 72, 4224–4232.
26. Rodriguez-Rojas,A., Menah,G., Borrell,N., Oliver,A. and Blazquez,J. (2009) Inactivation of the hmgA gene of Pseudomonas aeruginosa leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. Microbiology (UK), 155, 1050–1057.
27. Shen,M., Le,S., Jin,X., Li,G., Tan,Y., Li,M., Zhao,X., Shen,W., Yang,Y., Wang,J. et al. (2016) Characterization and comparative genomic analyses of Pseudomonas aeruginosa phage PaP5: New members assigned to PAK, Pl-like viruses. Sci. Rep., 6, 34067.
28. Le,S., He,X., Tan,Y., Huang,G., Zhang,L., Lux,R., Shi,W. and Hu,F. (2013) Mapping the tail fiber as the receptor binding protein responsible for differential host specificity of pseudomonas aeruginosa bacteriophages PaPi and JG004. *PLoS One*, 8, e68562.
29. Bolger,A.M., Lohse,M. and Usadel,B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30, 2114–2120.
30. Li,H. and Durbin,R. (2010) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, 25, 1754–1760.
31. Li,H., Handsaker,B., Wysoker,A., Fennel,T., Ruan,J., Homer,N., Marth,G., Abecasis,G., Durbin,R. and Genome Project Data Processing, S. (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, 25, 2078–2079.
32. Robinson,J.T., Thorvaldsdottir,H.,Winckler,W.,Guttman,M.,Lander,E.S.,Getz,G. and Mesirov,J.P. (2011) Integrative genomics viewer. *Nat. Biotechnol.*, 29, 24–26.
33. Shen,W., Le,S., Li,Y. and Hu,F. (2016) SeqKit: A Cross-Platform and ultrafast toolkit for FASTA/Q file manipulation. *PLoS One*, 11, e0163962.
34. Jacobs,M.A., Ahwood,A., Thaipisuttikul,I., Spencer,D., Haugen,E., Ernst,S., Will,O., Kaul,R., Raymond,C., Levy,R. et al. (2003) Comprehensive transposon mutant database of Pseudomonas aeruginosa. *Proc. Natl. Acad. Sci. U.S.A.*, 100, 14339–14344.
35. Choi,K.H., Kumar,A. and Schweizer,H.P. (2006) A 10-min method for preparation of highly electro competent Pseudomonas aeruginosa cells: application for DNA fragment transfer between plasmid and chromosome transformation. *J. Microbiol. Methods*, 64, 391–397.
36. Correa,E.M.E.,Martina,M.A., De Tullio,L.,Argarana,C.E. and Barra,J.L. (2011) Some amino acids of the Pseudomonas aeruginosa MutL D(D/Q)HAI(X)2E(X)4E conserved motif are essential for the 3D structure of the protein but not for the 3D in vitro endonuclease activity. *DNA Repair*, 10, 1106–1113.
37. Boltz,N.J., Lenhart,J.S., Weindorf,S.C. and Simmons,L.A. (2012) Residues in the N-terminal domain of MutL required for mismatch repair in Bacillus subtilis. *J. Bacteriol.*, 194, 5361–5367.
38. Cronin,C.N. and McIntire,W.S. (1999) pUCP-Nco and pUCP-Nde: Escherichia-Pseudomonas shuttle vectors for recombinant protein expression in Pseudomonas. *Anal. Biochem.*, 272, 112–115.
39. Polosina,Y.Y. and Cupples,C.G. (2010) MutL: conducting the cell’s response to mismatched and misaligned DNA. *Bioessays*, 32, 51–59.
40. Groothuizen,F.S., Winkler,I., Cristovao,M., Fish,A., Winterwerp,H.H.K., Reumer,A., Marx,A.D., Hermans,N., Nicholls,R.A., Murshudov,G.N. et al. (2015) MutS/MutL crystal structure reveals that the MutS sliding clamp loads MutL onto DNA. *eLife*, 4, e06744.
41. Guarna,A. and Charbonnier,J.B. (2015) Insights from a decade of biophysical studies on MutL: Roles in strand discrimination and mismatch removal. *Prog. Biophys. Mol. Biol.*, 117, 149–156.
42. Correa,E.M., De Tullio,L., Velez,P.S., Martina,M.A., Argarana,C.E. and Barra,J.L. (2013) Analysis of DNA structure and sequence requirements for Pseudomonas aeruginosa MutL endonuclease activity. *J. Bacteriol.*, 154, 505–511.
43. Stephanou,N.C., Gao,F., Bongiorno,P., Ehr,R., Schnappinger,D., Shuman,S. and Glickman,M.S. (2007) Mycobacterial nonhomologous end joining mediates mutagenic repair of chromosomal double-strand DNA breaks. *J. Bacteriol.*, 189, 5237–5246.
44. Deriano,L. and Roth,D.B. (2013) Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. *Annu. Rev. Genet.*, 47, 433–455.
45. Lieber,M.R. (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu. Rev. Biochem.*, 79, 181–211.
46. Courcelle,J., Wendel,B.M., Livingstone,D.D. and Courcelle,C.T. (2015) RecBCD is required to complete chromosomal replication: implications for double-strand break frequencies and repair mechanisms. *DNA Repair*, 32, 86–95.
47. Jasin,M. and Haber,J.E. (2016) The democratization of gene editing: Insights from site-specific cleavage and double-strand break repair. *DNA Repair*, 44, 6–16.
48. Shen,L., Wang,Q., Liu,R., Chen,Z., Zhang,X., Zhou,P. and Wang,Z. (2018) LncRNA Inc-R1 regulates homologous recombination repair of DNA double-strand breaks by stabilizing RAD51 mRNA as a competitive endogenous RNA. *Nucleic Acids Res.*, 46, 717–729.
49. Kadyrova,L.Y. and Kadyrova,F.A. (2016) Endonuclease activities of MutL’s alpha and its homologs in DNA mismatch repair. *DNA Repair*, 38, 42–49.
50. Bhagwat,A.S. and Lieb,M. (2002) Cooperation and competition in mismatch repair: very short-patch repair and methyl-directed mismatch repair in Escherichia coli. *Mol. Microbiol.*, 44, 1421–1428.
51. Qiu,R.Y., Sakato,M., Saco,E.J., Wilkins,H., Zhang,X.D., Modrich,P., Hingorani,M.M., Erie,D.A. and Weninger,K.R. (2015) MutL traps MutS at a DNA mismatch. *Proc. Natl. Acad. Sci. U.S.A.*, 112, 10914–10919.
52. Lenhart,J.S., Pillon,M.C., Guarna,A., Bitech,J.S. and Simmons,L.A. (2016) Mismatch repair in Gram-positive bacteria. *Res. Microbiol.*, 167, 4–12.
53. Jacquelin,D.K., Filiperti,A., Argarana,C.E. and Barra,J.L. (2005) Pseudomonas aeruginosa MutL protein functions in Escherichia coli. *Biochem. J.*, 388, 879–887.
54. Oliver,A., Baquero,F. and Blazquez,J. (2002) The mismatch repair system (mutS, mutL and uvrD genes) in Pseudomonas aeruginosa: molecular characterization of naturally occurring mutants. *Mol. Microbiol.*, 43, 1641–1650.
55. Magdanova,L.A. and Golyansnaya,N.V. (2013) Heterogeneity as an adaptive trait of microbial populations. *Microbiology*, 82, 1–10.
56. Paterson,S., Vogwill,T., Buckling,A., Benmayor,R., Spiers,A.J., Thomson,N.R., Quail,M., Smith,F., Walker,D., Libberton,B. et al. (2010) Antagonistic coevolution accelerates molecular evolution. *Nature*, 464, 275–278.
57. Pul,C., Macia,M.D., Oliver,A., Schachar,J. and Buckling,A. (2007) Coevolution with viruses drives the evolution of bacterial mutation rates. *Nature*, 450, 1079–1081.
58. Boscaro,V., Koliko,M., Felletti,M., Vannini,C., Lynn,D.H. and Keeling,P.I. (2017) Parallel genome reduction in symbionts descended from closely related free-living bacteria. *Nat. Ecol. Evol.*, 1, 1160–1167.
59. Baumgartner,M., Roßler,S., Wicker,T. and Pernthaler,J. (2017) Letting go: bacterial genome reduction solves the dilemma of adapting to predation mortality in a substrate-restricted environment. *ISME J.*, 11, 2258–2266.