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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 ± 87 × 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 immunocompromised 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

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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 0.5×10^{-9} to 2.2×10^{-8}/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, Crispr/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 homogentisic acid, the substrate of 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 PAO1*ΔmutL,* 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^{9} 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.

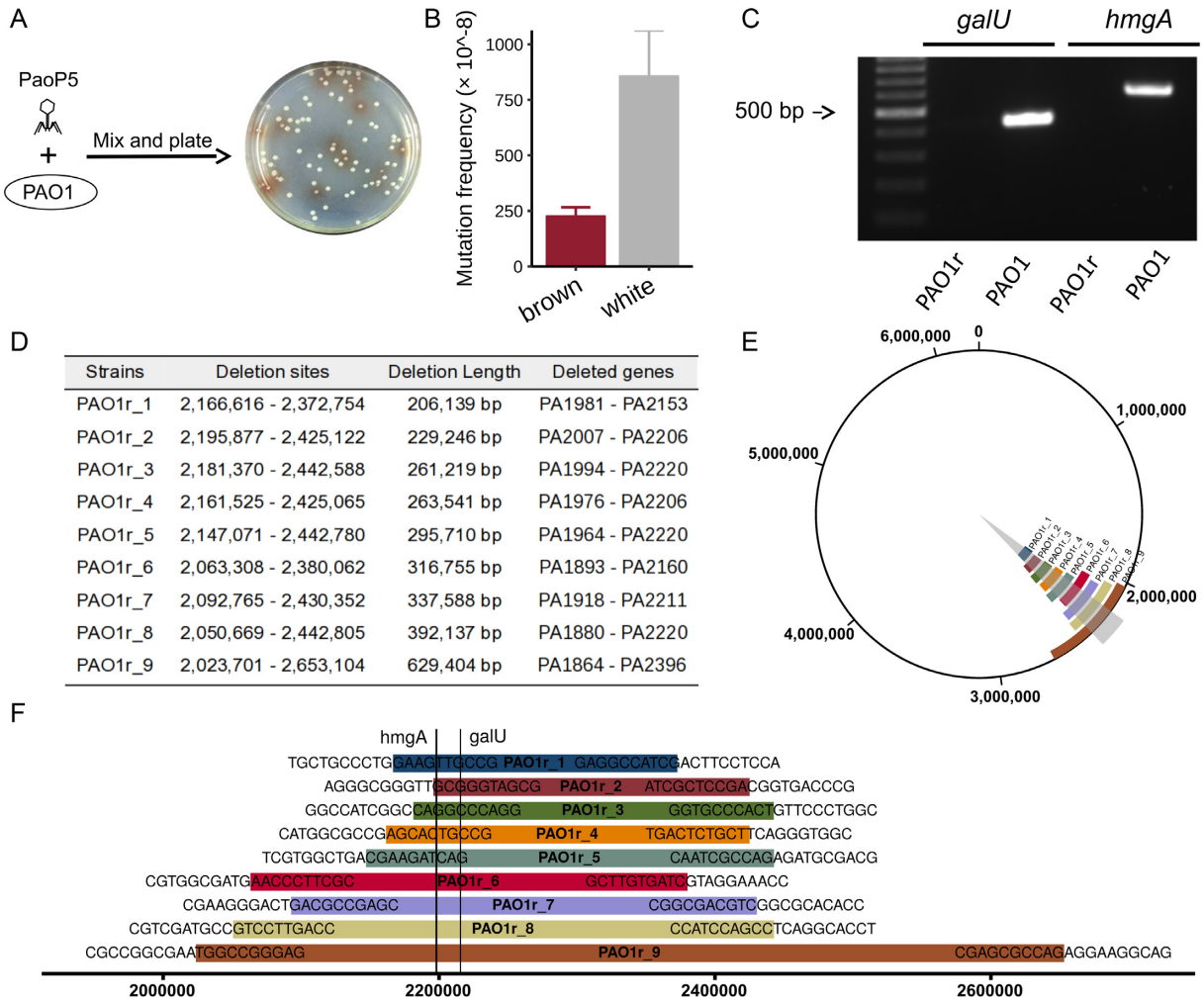
This method is robust in isolating phage resistant mutants and all the colonies grown on the plate tested were phage resistant.

Bacterial genome sequencing

Nine brown mutants from independent biological repeats were selected. Bacterial genomic DNA was extracted using UNlQ-10 Column Bacterial Genomic DNA Isolation Kit (sangon bitotec:SK1202), and then sent to Novogene Corporation for sequencing using the Illumina Hiseq 2500 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



Strains	Deletion sites	Deletion Length	Deleted genes
PAO1r_1	2,166,616 - 2,372,754	206,139 bp	PA1981 - PA2153
PAO1r_2	2,195,877 - 2,425,122	229,246 bp	PA2007 - PA2206
PAO1r_3	2,181,370 - 2,442,588	261,219 bp	PA1994 - PA2220
PAO1r_4	2,161,525 - 2,425,065	263,541 bp	PA1976 - PA2206
PAO1r_5	2,147,071 - 2,442,780	295,710 bp	PA1964 - PA2220
PAO1r_6	2,063,308 - 2,380,062	316,755 bp	PA1893 - PA2160
PAO1r_7	2,092,765 - 2,430,352	337,588 bp	PA1918 - PA2211
PAO1r_8	2,050,669 - 2,442,805	392,137 bp	PA1880 - PA2220
PAO1r_9	2,023,701 - 2,653,104	629,404 bp	PA1864 - PA2396

Figure 1. Characteristics of deletion mutants selected by phage infection. (**A**) Brown mutants are selected after phage PaoP5 infection. (**B**) Phage-resistant mutant frequencies in total occur at $1083 \pm 524 \times 10^{-8}$. The brown mutants were detected with a frequency of $227 \pm 87 \times 10^{-8}$. The data was calculated from five biological replicates. (**C**) Both the *hmgA* and *galU* genes were deleted in the brown mutants, as was detected by PCR. (**D**) Deletion sites and deleted genes in all the brown mutants. (**E**) Locations of deletions in the chromosomes of nine brown mutants compared with wild-type PAO1. (**F**) Close-up of E, indicating the deleted chromosomal regions of the nine brown mutants. The locations of the *hmgA* and *galU* genes are indicated.

number of *P. aeruginosa* CFU. Then, 0.1-ml aliquots from the 10^0–10^{-3} dilutions were mixed with 10^9 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, mutS, 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 *ΔmutL* complementation strain was generated by electroporation (35) of pucp-*mutL* into strain *ΔmutL*, 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).

Transformed *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 $MgCl_2$ in endonuclease buffer (20 mM Tris, pH7.5; 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 SalI/BamHI double digested pUCP24-cb and the resulting plasmid was transfered 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 $227 \pm 87 \times 10^{-8}$ (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 ($\sim 10^{-6}$), 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

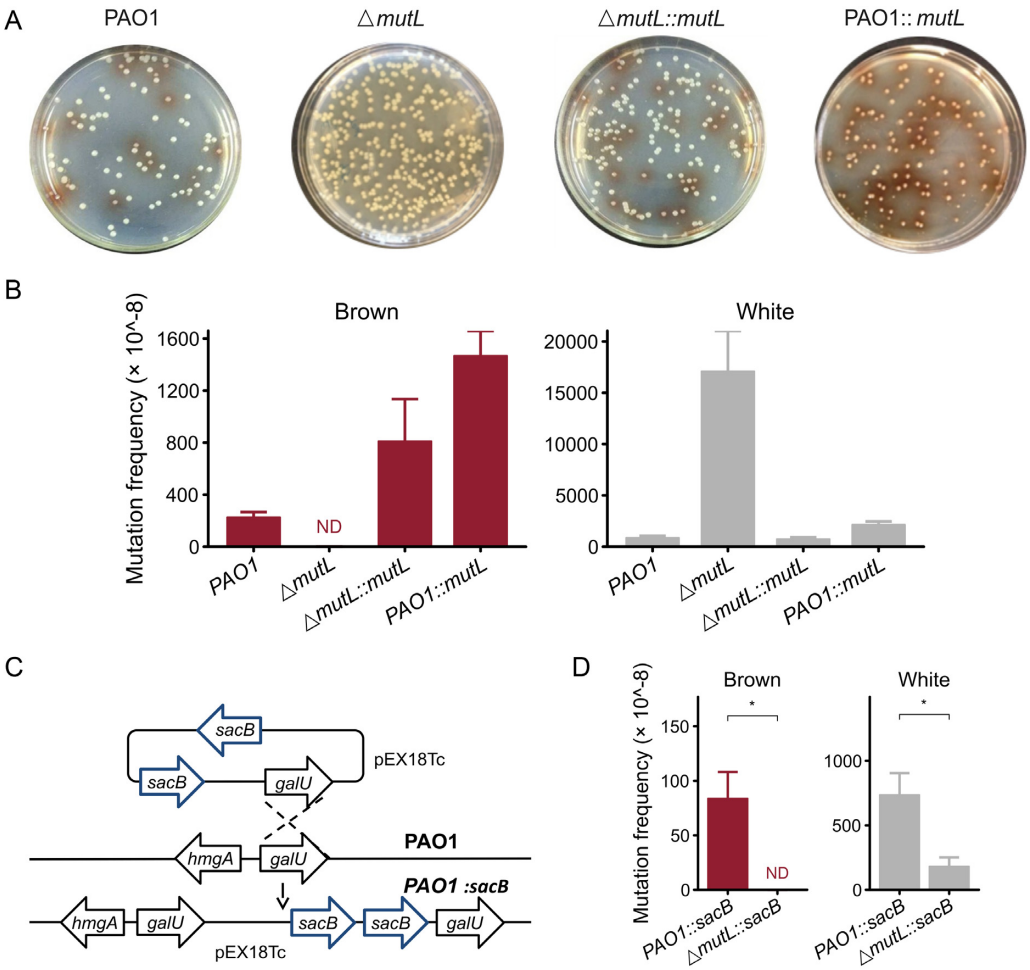


Figure 2. MutL is required for large chromosomal deletions in *P. aeruginosa*. (**A**) Representative pictures of the phage-resistant mutants of PAO1, *ΔmutL, ΔmutL::mutL* and PAO1*::mutL*. Many more white mutants were generated in the *ΔmutL* background, and most mutants generated from the *mutL* overexpression 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 $1468 \pm 427 \times 10^{-8}$. ($P < 0.05$, 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 10^8 *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.

PAO1*ΔmutL*, 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. PAO1*ΔmutL* generated white phage-resistant mutants with a 16-fold higher frequency ($17\ 104 \pm 9631 \times 10^{-8}$) 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 PAO1*ΔmutL* (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 PAO*1ΔmutL* 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 ($201 \pm 188 \times 10^{-8}$), 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 $84 \pm 54 \times 10^{-8}$, which is several folds 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 PAO1*ΔmutL* to regain the ability to generate brown

mutants, but also increased the frequency of brown mutants to $811 \pm 725 \times 10^{-8}$, which is almost a 4-fold increase compared to PAO1 wild-type. Overexpression of MutL in wild-type PAO1 significantly increased the frequency of brown mutants from $227 \pm 87 \times 10^{-8}$ to $1468 \pm 427 \times 10^{-8}$ (Figure 2B). These genetic studies indicate that MutL is essential for large chromosomal deletions in *P. aeruginosa*.

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 (PaMutLΔ340–633 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 infected 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

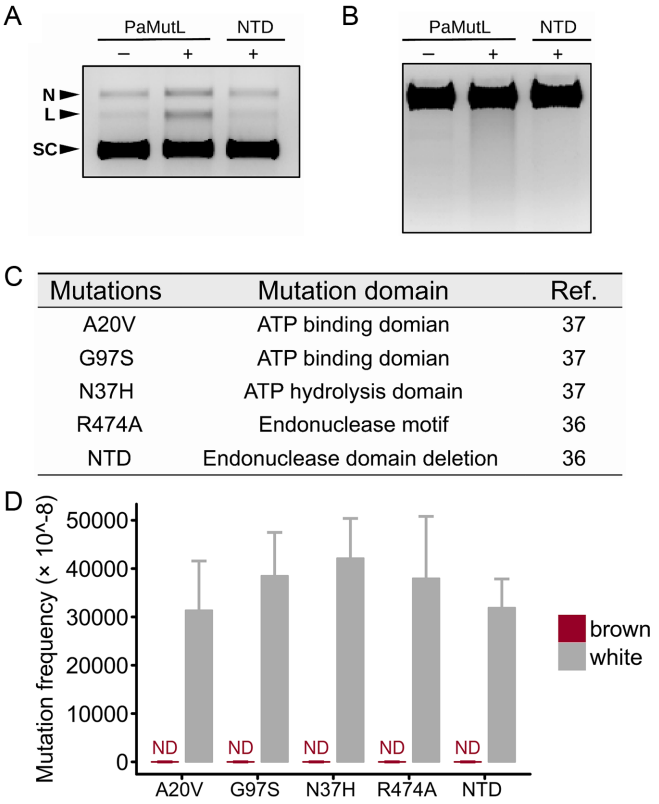


Figure 3. Nicking activity of PaMutL results in DSB and is essential for chromosomal deletions. (**A**) Confirmation that the nicking function of PaMutL can results in DSB *in vitro*. Linear DNA was observed from PaMutL (500nM) digested plasmid DNA, but NTD (500 nM) did not cleave dsDNA. SC, supercoiled; L, linearized; N, nicked. (**B**) DSB made by PaMutL is not site-specific. After incubation of plasmid pUCP24 with PaMutL (200 nM) or NTD (200 nM), the DNA was linearized with EcoRI. The products were analyzed by gel electrophoresis after ethidium bromide staining. (**C**) Key residues for PaMutL nicking function. (**D**) A20V,G97S, N37H, R474A and NTD mutants cannot restore the brown mutants, nor could decrease the frequency of white mutants in *mutL::sacB*.

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 $45 \pm 25 \times 10^{-8}$ and $64 \pm 48 \times 10^{-8}$ respectively, while the *ΔkuΔLigD* double mutant generated much fewer brown mutants ($30 \pm 18 \times 10^{-8}$), which is almost 7-fold less than that of PAO1 wild-type. Complementation of *ku* and *LigD* significantly increased the frequency to $1947 \pm 420 \times 10^{-8}$ and $558 \pm 205 \times 10^{-8}$, respectively (Figure 4A). These ge-

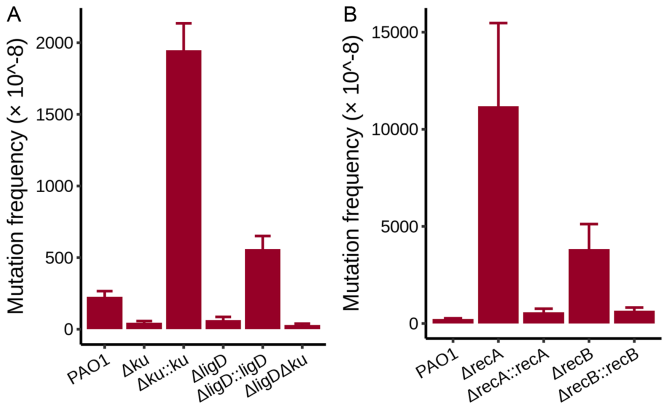


Figure 4. NHEJ promotes chromosomal deletions while HR inhibits it. (**A**) Knockout of *LigD* and *Ku* significantly decreased the frequency of brown mutants, while complementation of *LigD* or *Ku* increased the deletion frequency to $1947 \pm 420 \times 10^{-8}$ and $558 \pm 205 \times 10^{-8}$, respectively ($P < 0.05$, calculated by one way ANOVA). (**B**) Knockout of *recA* and *recB* significantly increased the frequency of brown mutants to $11192 \pm 9569 \times 10^{-8}$ and $3823 \pm 2898 \times 10^{-8}$, respectively ($P < 0.05$, calculated by one way ANOVA).

netic studies indicate that Non-Homologous End Joining can repair DSBs and results in chromosomal deletions.

DSB in *P. aeruginosa* can also be repaired faithfully by HR, without loss of genomic information. Thus, HR should inhibit chromosomal deletion and the formation of brown mutants. As expected, the frequency of brown mutants for *ΔrecA* and *ΔrecB* increased to $11\ 192 \pm 9569 \times 10^{-8}$ and $3823 \pm 2898 \times 10^{-8}$, respectively (Figure 4B), which are much higher than that of PAO1. The complementation with *recA* and *recB* in a *ΔrecA* and *ΔrecB* background, respectively, decreased the frequency to $574 \pm 417 \times 10^{-8}$ and $663 \pm 352 \times 10^{-8}$. Thus, these genetic studies imply that HR system can contribute to the repair of MutL-generated DSBs without losing large chromosomal fragments; while disruption of HR will render more DSBs being repaired by NHEJ, which is error-prone and results in chromosomal deletion.

DISCUSSION

MutL promotes chromosomal DNA deletion that confers phage resistance to *P. aeruginosa*

In the current study, we identified *mutL* as a genetic determinant that promotes chromosomal DNA deletion in *P. aeruginosa.* Using a phage-selection model to detect chromosomal DNA deletion mutants (brown mutants), we found that *P. aeruginosa mutL* mutant displayed significantly increased point mutation frequency (white mutants), but lost the ability to generate brown mutants. In addition, PAO1*::sacB* generates brown mutants, while no brown mutant was detected in *ΔmutL*::*sacB* background. Since we did not screen $>10^8$ cells in each experiment, we cannot exclude the possibility that *ΔmutL*::sacB can still generate deletion mutants with a frequency of less than 10^{-8}. Nevertheless, our genetic studies strongly imply that MutL is required for chromosomal DNA deletion.

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, Mg^{2+} and Mn^{2+}, 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 ($21{,}521 \pm 8{,}542 \times 10^{-8}$) of white mutants; while brown mutants in *ΔmutS::sacB* were detected with a frequency of $171 \pm 82 \times 10^{-8}$ (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–

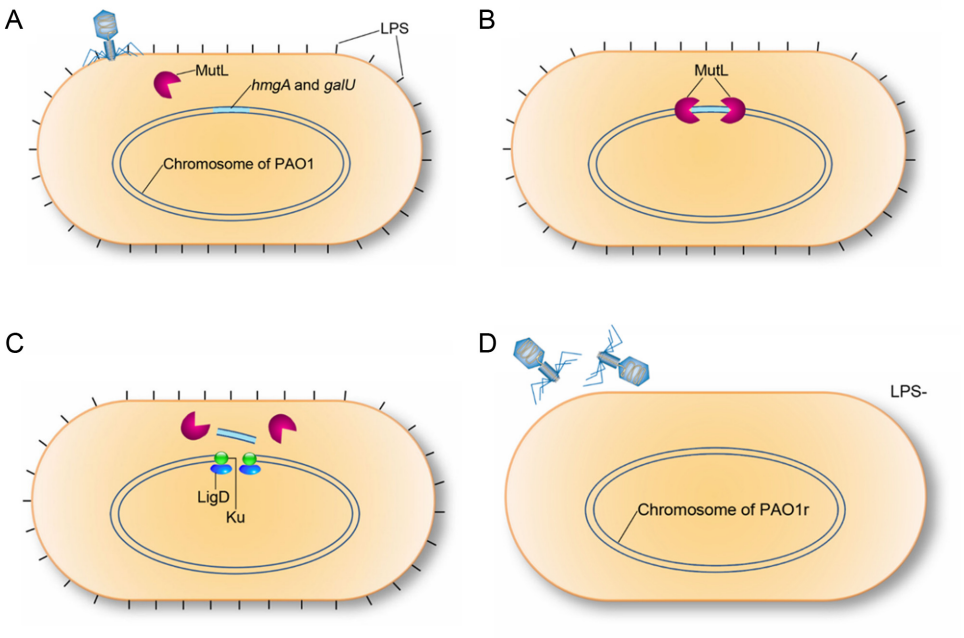


Figure 5. Model of the mechanism by which MutL makes DSB and results in a DNA fragment deletion through NHEJ that protects *P. aeruginosa* from phage infection. PaMutL generates DSB occasionally. The end points of DSB are then joined by Ku and LigD. Brown mutants with a deleted a *galU*/*hmgA* region are identified after phage infection.

45). 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. aalborgensis* (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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