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Brief report

Study of involvement of ImuB and DnaE2 in stationary-phase mutagenesis in *Pseudomonas putida*

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ABSTRACT

Several bacterial species carry in their genomes a so-called “mutagenesis” gene cluster encoding ImuB which is similar to Y-family DNA polymerases, and DnaE2 related to the catalytic subunit DnaE of Pol III. Y-family DNA polymerases are known to be involved in stationary-phase mutagenesis and DnaE2 homologues characterized so far have expressed a mutator phenotype. In this study, we raised a question about the involvement of ImuB and DnaE2 in stationary-phase mutagenesis. Here, we show that *Pseudomonas putida* ImuB and DnaE2 have antagonistic effects on stationary-phase mutagenesis. ImuB facilitated accumulation of stationary-phase mutants up to two-fold. In contrast to that, DnaE2 had no significant effect on emergence of 1-bp deletion mutants and moreover, it acted as an anti-mutator in accumulation of base substitution mutants in starving bacteria. Similar antagonistic effects of DnaE2 and ImuB on mutagenesis appeared also in UV-mutagenesis study. This data distinguishes the DnaE2 of *P. putida* from its homologues studied in other organisms.

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1. Introduction

Mutagenesis occurring in resting cells is well documented both in bacterial and eukaryotic cells. In microbial world, this process is commonly called “adaptive mutation” or “stationary-phase mutation” [1,2]. Stress-induced activation of error-prone DNA polymerases is an important mechanism in stationary-phase mutagenesis (reviewed in Refs. [3–5]). *E. coli* has five DNA polymerases, three of which, Pol II, Pol IV, and Pol V, are induced as part of SOS regulon in response to DNA damage [6]. These polymerases can continue DNA replication if replication fork is collapsed at a blocking lesion, whereas the synthesis of DNA by Pol IV and Pol V is error-prone [6,7].

Error-prone DNA polymerases Pol IV and Pol V have been shown to participate in stationary-phase mutagenesis in *E. coli* [8–11]. Differently from enterobacteria, *umuDC* orthologs encoding Pol V are absent from genomes of many bacterial species studied.

A multiple gene cassette *lexA2-imuA-imuB-dnaE2* was recently described in *Pseudomonas putida* [12]. This operon encodes another LexA repressor (LexA2), which negatively regulates the operon, a protein annotated as Sula (ImuA), a protein exhibiting similarity to Y-family DNA polymerases (ImuB), and a second copy of an alpha subunit of the DNA polymerase Pol III (DnaE2) [12]. Similar gene cluster or part of it has been identified in many families of Proteobacteria

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[12,13]. Phylogenetic analyses revealed that the gene cluster, also known as “mutagenesis cassette”, has undergone several reorganizations since its inception in actinobacteria, and that it has dispersed across the bacterial domain through combination of vertical inheritance, lateral gene transfer and duplication [13]. Absence of *umuDC* orthologs encoding Pol V from genomes of a wide variety of bacterial species carrying the “mutagenesis cassette” has suggested that this gene cassette may replace the functions of *umuDC* system [13,14]. So far, biological role of that widespread gene cluster for bacteria has been addressed only in few studies. It is known that DnaE2 mediates SOS mutagenesis in *Mycobacterium tuberculosis* and contributes to the emergence of drug resistance *in vivo* [15]. Also, DNA polymerases encoded by this cluster are responsible for majority of DNA damage-induced mutations in *Caulobacter crescentus* [14]. This widespread operon was shown to be DNA damage-induced in *P. putida* [12], but its role in mutagenesis has not been studied so far.

Here, we have investigated involvement of ImuB and DnaE2 in stationary-phase mutagenesis in *P. putida*. The results presented here demonstrate that these proteins have opposite roles in stationary-phase mutagenesis. The DnaE2 reduces the frequency of base substitutions but does not affect 1-bp deletions, whereas the ImuB increases the rate of occurrence of both types of mutations. Similar antagonistic effect of these two proteins on mutation frequency was also observed in UV-mutagenesis assay. Thus, biological effects of ImuB and DnaE2 on mutagenesis in *P. putida* differ from those described for ImuB and DnaE2 homologues from other organisms.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are described in table (Supplementary data S1). Complete medium was Luria–Bertani (LB) medium [16], and minimal medium was M9 [17]. Casamino acids (CAA) and glucose were added to the minimal medium at final concentrations 0.4% and 0.2%, respectively. Phenol minimal plates contained 2.5 mM phenol as a sole carbon and energy source. Antibiotics were added at following concentrations: ampicillin at 100 µg/ml, kanamycin at 50 µg/ml, tetracycline at 80 µg/ml, carbenicillin at 1000–3000 µg/ml and rifampin at 100 µg/ml. *E. coli* was incubated at 37 °C and *P. putida* at 30 °C. *E. coli* and *P. putida* were electrotransformed as described by Sharma and Schimke [18]. *E. coli* strains DH5α (Invitrogen), and CC118 λpir [19] were used for DNA cloning procedures and HB101 [20] as a host for helper plasmid pRK2013 [21] necessary for mobilization of non-conjugative plasmids.

2.2. Construction of *P. putida* *dnaE2* and *imuB* knockout strains

The *imuB* (PP3118) and *dnaE2* (PP3119) sequences of *P. putida* KT2440 were obtained from The Institute for Genomic Research website (<http://www.tigr.org>). The sequences of

these genes were amplified by PCR from genomic DNA of *P. putida* strain PaW85 [22] (this strain is isogenic to KT2440). Internal sequences of *imuB* and *dnaE2* genes were replaced with antibiotic resistance marker genes *Km^r* from pKSKm [23] and *Tet^r* from pBR322 [24]. DNA fragments carrying sequences from 5'- and 3'-ends of particular gene and antibiotic resistance determinant between these sequences were inserted into plasmid pGP704 L [25] not able to replicate in hosts other than *E. coli* CC118 λpir. The wild-type alleles of *imuB* and *dnaE2* present in the chromosome of *P. putida* strain PaW85 were replaced with the modified ones by homologous recombination. Derivatives of the plasmid pGP704 L carrying replacement cassettes were conjugatively transferred into *P. putida* PaW85 using a helper plasmid pRK2013. Integration of the whole delivery plasmid into a target site was excluded by testing transconjugants for the resistance to carbenicillin (only those unable to grow in the presence of 1500 µg/ml carbenicillin were considered to be true recombinants, generated as a result of double recombination events). PaW85 derivatives with desired gene knockouts were verified by PCR analysis. *dnaE2* knockout strain PaWDnaE2 contains 144 nucleotides from 5'-end and 467 nucleotides from 3'-end of the *dnaE2* gene (this strain lacks 2489 nt-long internal sequence from the *dnaE2* gene). *imuB* knockout strain PaWImuB contains 418 nucleotides from 5'-end and 616 nucleotides from 3'-end of the *imuB* gene (198 internal nucleotides are replaced with tetracycline resistance gene from pBR322 [25]). *imuB* and *dnaE2* double knockout strain PaWImuBDnaE2 was constructed by using PaWImuB as a recipient for replacement of original *dnaE2* sequence with the Δ *dnaE2::Km* sequence as described above. *imuB* and *dinB* double knockout strain PaWDinBImuB was generated by replacing the original *imuB* sequence in the strain PaWDinB [26] with the Δ *imuB::Km* sequence by homologous recombination using a procedure similar to that described above. Details of the constructions of these strains are presented as Supplementary data S2.

2.3. Isolation of *Phe⁺* revertants and UV mutagenesis assay

Assay system for recording the frequency of different types of point mutations in starving *P. putida* was based on activation of phenol monooxygenase gene *pheA* enabling bacteria to utilize phenol as growth substrate and form colonies on selective plates. The reporter gene *pheA* was altered in RSF1010-derived tester plasmids either by +1 frameshift mutation or by introducing TAG translational stop codon into the *pheA* [26]. Conditions for isolation of phenol-degrading *Phe⁺* revertants were the same as in our previous study [27]. Several independently isolated clones of the same gene knockouts were used in mutagenesis assay to verify reproducibility of the results. In order to find out whether bacteria will die during starvation on the selective medium, we determined the number of colony forming units (CFU) for at least five independent starving cultures of a particular strain studied. Viability of bacteria was determined on the same plates that were used for isolation of *Phe⁺* revertants. No differences in viability were found between the wild-type strain PaW85 and its derivatives PaWDnaE2, PaWImuB, PaWImuBDnaE2 and PaWDinBImuB during incubation of bacteria under starva-

tion conditions. Additionally, we compared the number of plasmid copies in wild-type strain and in its derivatives as performed in our previous studies [26,27], but no differences were found.

UV mutagenesis assay was performed as previously described [23]. Irradiation with UV-C light was performed at a 254-nm wavelength at a dose of 100 J/m² using a Stratagene UV-Stratalinker 1800. Colonies were counted on rifampin-supplemented plates incubated for 24 h. The frequency of mutation to Rif^R was calculated per 10⁹ plated cells using the Lea–Coulson method of the median [28,29]. *P*-values were calculated using the Mann–Whitney test [30]. The frequency of mutation was determined at least in four separate experiments, each including 40 independent cultures.

3. Results and discussion

3.1. *ImuB* and *DnaE2* have different effects in stationary-phase mutagenesis in *P. putida*

Translesion DNA synthesis provided by Y-family DNA polymerases Pol IV and Pol V is an important mechanism in stationary-phase mutagenesis. Many bacteria lack Pol V genes *umuD* and *umuC* and carry instead a multiple gene cassette containing *dnaE2*, which encodes a second copy of alpha subunit of DNA polymerase III and *imuB* which is related to genes for Y-family DNA polymerases [13]. *DnaE2* orthologs from *M. tuberculosis* and *C. crescentus* are error-prone and responsible for DNA-damage induced mutations [14,15]. *ImuB* is required for *DnaE2*-dependent damage-inducible mutagenesis in *C. crescentus* [14]. Here, we asked whether *ImuB* and *DnaE2* encoded by *P. putida* *lexA2-imuA-imuB-dnaE2* “mutagenesis cassette” could contribute to stationary-phase mutagenesis.

In order to examine involvement of *ImuB* and *DnaE2* in stationary-phase mutagenesis in *P. putida*, we constructed the

strains PaWImuB and PaWDnaE2 carrying internal deletion in *imuB* (PP3118) and *dnaE2* (PP3119), respectively. The assay system to measure frequency of different types of point mutations was as described previously [26]. The reporter gene *pheA* encoding phenol monooxygenase was altered in RSF1010-derived tester plasmids either by +1 frameshift mutation or by introducing TAG translational stop codon into the *pheA* gene. Point mutations restoring functional *pheA* sequence enabled bacteria to utilize phenol as a growth substrate. –1 deletions restoring the reading frame of the *pheA* reporter gene occurred mostly in CCC repeat flanking the inserted A nucleotide [26].

The results presented in Fig. 1 revealed that the absence of functional *dnaE2* gene enhanced appearance of base substitution mutations in starving *P. putida*. Compared to the wild-type strain, the frequency of accumulation of Phe⁺ revertants was 2.5–3 times elevated in PaWDnaE2 during the 10-days starvation period studied. Thus, in contrast to our expectations, *DnaE2* did not elevate mutation frequency but rather acted as an antimutator in stationary-phase mutagenesis of *P. putida*. Notably, the lack of *DnaE2* increased the frequency of appearance of Phe⁺ revertants occurred due to base substitutions over five times also in growing populations of *P. putida*. The median value of the mutation frequency was 0.8 per 1 × 10⁹ cells in case of wild-type and 4.5 per 1 × 10⁹ cells in case of *DnaE2*-defective *P. putida* (data not shown). Differently from base substitution mutations, *DnaE2* had no influence upon appearance of 1-bp deletions in starving *P. putida* (Fig. 1). The lack of *ImuB* resulted in up to two-fold lower frequency of emergence of both base substitution mutants and 1-bp deletions in starving *P. putida* (Fig. 1). In conclusion, *ImuB* and *DnaE2* had different effects on stationary-phase mutagenesis: the presence of *DnaE2* reduced the frequency of base substitutions having no significant effect on appearance of frameshift mutations, whereas *ImuB* increased the frequency of both types of mutations.

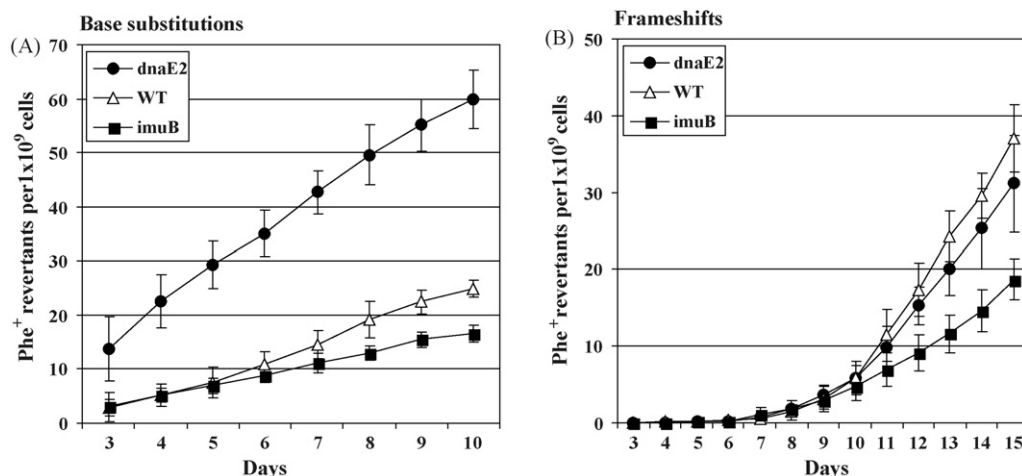


Fig. 1 – Accumulation of Phe⁺ revertants on phenol minimal plates in *P. putida* wild-type strain PaW85 (WT), and in its *DnaE2*- or *ImuB*-defective derivatives PaWDnaE2 (*dnaE2*) and PaWImuB (*imuB*). Effect of *DnaE2* and *ImuB* on accumulation of base substitution and 1-bp deletion mutants. About 5 × 10⁸ *P. putida* cells were plated onto phenol minimal plates from overnight liquid M9 medium-grown independent cultures. Data for at least five parallel experiments are presented. In all cases, means ± S.D. (error bars) for at least 10 plates calculated per 1 × 10⁹ cells are shown.

3.2. Accumulation of stationary-phase mutants in *ImuB* and *DnaE2* double-knockout strains differs from that observed in strains lacking only one of these enzymes

The frequency of accumulation of base substitution mutations in double mutant PaWImuBDnaE2 and wild-type strain was comparable (Fig. 2A) indicating that the mutator phenotype of PaWDnaE2 (Fig. 1A) is dependent on ImuB. Interestingly, although the absence of DnaE2 alone did not affect significantly the frequency of frameshift mutations (Fig. 1B), the frequency of appearance of this type of mutations was approximately two-fold reduced in PaWImuBDnaE2 compared to PaWImuB (compare Figs. 1 and 2). PaWImuBDnaE2 is the only strain in which we have observed decline in mutation frequency as a result of *dnaE2* knockout.

ImuB from *C. crescentus* possibly cooperates with DnaE2 in DNA damage-inducible mutagenesis as no phenotypic effect of DnaE2 was demonstrated in this organism in the absence of ImuB [14]. The opposite effects of ImuB and DnaE2 in occurrence of base substitution mutants in *P. putida* indicated that in this organism DnaE2 does not require the activity of ImuB for its functioning. The decline in mutation frequency in ImuB-defective but not in DnaE2-defective bacteria demonstrated that *imuB* functions as a mutator allele in stationary-phase mutagenesis. At the same time, the fact that the frequency of 1-bp deletions was reduced in starving PaWImuB was surprising because our previous studies [26] revealed that PP1203-encoded Pol IV was responsible for the generation of majority of 1-bp deletions in long-term-starved bacteria. The results obtained using double-knockout strain PaWDinBImuB make the picture even more complicated. The frequency of accumulation of 1-bp deletion mutants in *P. putida* strain PaWDinBImuB was similar to that measured in PaWImuB (compare Figs. 1 and 2), being about 10-fold higher compared to the rate of accumulation of revertants in a Pol IV-deficient strain PaWDinB (Fig. 2). Hence, one may speculate that ImuB somehow influences DNA polymerase traffic. It is possible that

in the absence of ImuB and Pol IV some other DNA polymerase has favored access to replication apparatus and can thereby facilitate occurrence of frameshift mutations in starving bacteria. Further studies are in progress to test this possibility.

According to its sequence similarity, the *P. putida* ImuB and its orthologs form a branch in the UmuC superfamily of proteins that is distinct from *E. coli*-like DinB proteins (Pol IV), which are also present in majority of bacterial species studied [14]. At the same time, protein structure prediction using ModBase database [31] generated a most highly scored model for *P. putida* ImuB if DinB homologues Dbh and Dpo4 [32–35] were used as a template. DNA polymerases from different families have a similar overall shape resembling right hand composed of palm, finger and thumb domains [36]. The palm domain is structurally most conserved and contains three invariant carboxylates forming a catalytic center: D7, D105, and E106 of Dpo4, which are located on adjacent β strands 1 and 6, are in identical positions to the catalytic residues found in other Y-family polymerases [33]. Amino acid substitutions at any of these three conserved residues severely impair the polymerase activity [37,38]. Notably, none of these residues was present in the predicted palm domain of ImuB (Supplementary data S3). This raises a question whether ImuB has a DNA polymerase activity at all. Further work is necessary to elucidate biochemical activities of *P. putida* ImuB.

3.3. The effects of *ImuB* and *DnaE2* in UV-mutagenesis are similar to those observed in stationary-phase mutagenesis

Genetic data of DnaE2 homologues of *M. tuberculosis* and *C. crescentus* indicated that DnaE2 is an error-prone DNA polymerase [14,15]. DnaE2 from these organisms conferred a mutator phenotype after exposure of bacteria to UV irradiation. Therefore, it is possible that experimental conditions used by us to study stationary-phase mutagenesis did not

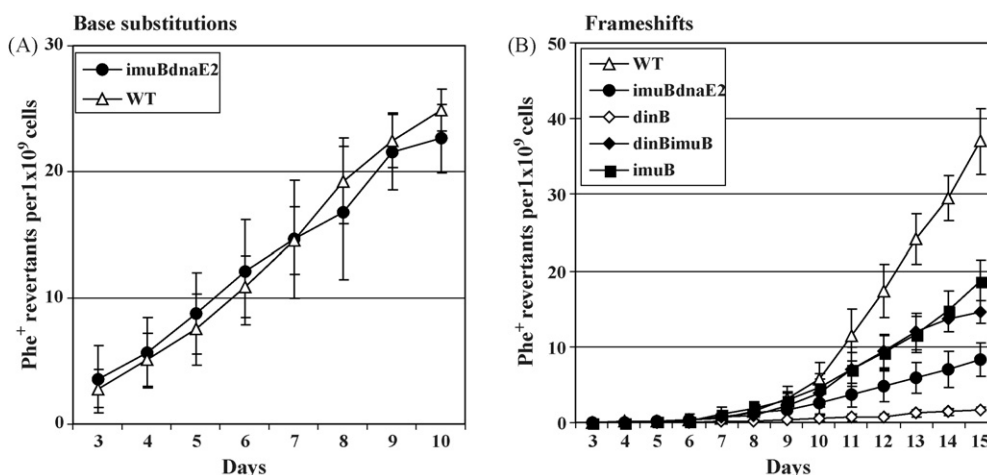


Fig. 2 – Accumulation of Phe⁺ revertants on phenol minimal plates in *P. putida* wild-type strain PaW85, and in its derivatives. Accumulation of base substitution mutants in PaW85 (WT) and PaWImuBDnaE2 (*imuBdnaE2*) and 1-bp deletion mutants in PaW85 (WT), PaWImuB (*imuB*), PaWImuBDnaE2 (*imuBdnaE2*), PaWDinB (*dinB*) and PaWDinBImuB (*dinBimuB*). About 5×10^8 *P. putida* cells were plated onto phenol minimal plates from overnight liquid M9 medium-grown independent cultures. Data for at least five parallel experiments are presented. In all cases, means \pm S.D. (error bars) for at least 10 plates calculated per 1×10^9 cells are shown.

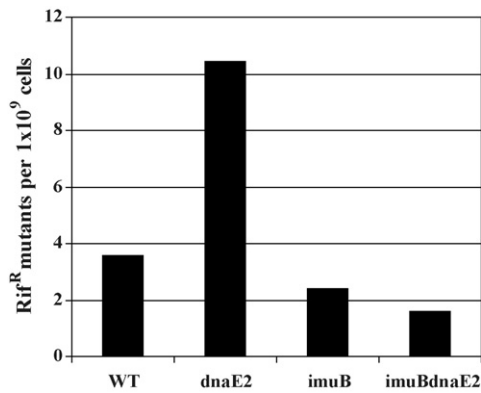


Fig. 3 – Contribution of ImuB and DnaE2 to mutagenic processes in UV-induced mutagenesis assay. The median value for frequency of Rif^R mutants per 10⁹ UV-C-irradiated cells of PaW85 (WT), PaWDnaE2 (dnaE2), PaWImuB (imuB), and PaWImuBDnaE2 (imuBdnaE2) is shown. Differences between frequencies of Rif^R mutations of PaWImuB, PaWDnaE2 and PaWImuBDnaE2 relative to the wild-type strain were statistically significant (*P*-value less than 0.05) at the 95% confidence level based on the Mann–Whitney test [30]. We have previously shown that *P. putida* expresses only weak UV-mutagenesis phenotype: not more than two-fold difference appeared in mutation rate between non-induced and UV-C-irradiated *P. putida* [23].

allow expression of the mutator phenotype of DnaE2. In order to clarify the role of *P. putida* DnaE2 and ImuB in mutagenesis, we carried out UV-mutagenesis study of *P. putida* wild-type strain and its DnaE2 or ImuB-defective derivatives using the same Rif^R assay as was used in the other studies to examine UV-induced mutagenesis. The lack of DnaE2 resulted in two-fold higher frequency of mutation compared to the wild-type bacteria (Fig. 3). Thus, the anti-mutator effect of DnaE2 with regard to appearance of base substitution mutations was not restricted to a particular test system employed but appeared to be more general. In accordance with data of stationary-phase mutagenesis assay the absence of ImuB reduced the mutation frequency up to two-fold also in UV-mutagenesis assay (Fig. 3). The double mutant PaWImuBDnaE2 exhibited mutation frequency comparable to that of PaWImuB (Fig. 3). The finding that DnaE2-defective strain had higher and ImuB-defective strain lower mutation frequency than the wild-type strain in the UV-mutagenesis assays fits well with our other data indicating that ImuB does not cooperate with DnaE2 in mutagenesis in *P. putida*.

Most recently, Sanders et al. [39] demonstrated that UV-induced mutagenesis in *P. aeruginosa* is dependent upon Pol I, and on second Pol III, DnaE2 (PolC). *P. aeruginosa* DnaE2 is the closest homologue to *P. putida* DnaE2. They share 73% identity, while *P. putida* DnaE and DnaE2 have only 30% identity. As the DnaE2 from *P. aeruginosa* displays phenotypic effects similar to those observed in *M. tuberculosis* and *C. crescentus* while *P. putida* DnaE2 does not, the DnaE2 from *P. putida* would provide a good model to study which distinguishes this DNA polymerase from error-prone DnaE2 homologues. In other words, study of *P. putida* DnaE2 would be useful to elucidate

mechanisms standing behind mutator phenotype of certain DnaE2 homologues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.dnarep.2007.01.010](https://doi.org/10.1016/j.dnarep.2007.01.010).

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