# A DNA Polymerase V Homologue Encoded by TOL Plasmid pWW0 Confers Evolutionary Fitness on *Pseudomonas putida* under Conditions of Environmental Stress

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Plasmids in conjunction with other mobile elements such as transposons are major players in the genetic adaptation of bacteria in response to changes in environment. Here we show that a large catabolic TOL plasmid, pWW0, from *Pseudomonas putida* carries genes (*rulAB* genes) encoding an error-prone DNA polymerase Pol V homologue which increase the survival of bacteria under conditions of accumulation of DNA damage. A study of population dynamics in stationary phase revealed that the presence of pWW0-derived *rulAB* genes in the bacterial genome allows the expression of a strong growth advantage in stationary phase (GASP) phenotype of *P. putida*. When *rulAB*-carrying cells from an 8-day-old culture were mixed with Pol V-negative cells from a 1-day-old culture, cells derived from the aged culture out-competed cells from the nonaged culture and overtook the whole culture. At the same time, bacteria from an aged culture lacking the *rulAB* genes were only partially able to out-compete cells from a fresh overnight culture of the parental *P. putida* strain. Thus, in addition to conferring resistance to DNA damage, the plasmid-encoded Pol V genes significantly increase the evolutionary fitness of bacteria during prolonged nutritional starvation of a *P. putida* population. The results of our study indicate that RecA is involved in the control of expression of the pWW0-encoded Pol V.

In natural environments, bacteria are faced with many different types of stresses. Among them, nutritional stress is the most common for bacteria occupying various water and soil habitats (71). Additionally, environmental bacteria are frequently exposed to cycles of drying and rehydration. Microorganisms living in geographic areas where the temperature sometimes drops below zero are faced with series of freezing and melting. Many bacteria, especially those living in a phyllosphere, are exposed to UV irradiation. The UV wavelengths that reach the earth's surface can cause direct DNA damage by inducing the formation of DNA photoproducts whose accumulation can be lethal to cells through the blockage of DNA replication and RNA transcription (45). Some data published already more than 20 years ago indicate that freeze-thaw stress and drying cycles can also cause DNA damage which is mutagenic to a bacterium (3, 10, 65, 73). It has also been shown that oxidative DNA damage generated from endogenous metabolism in growth-arrested cells accumulates during stasis (7, 8).

In *Escherichia coli*, DNA polymerases Pol II, Pol IV, and Pol V are induced as part of the SOS regulon in response to DNA damage (23). The LexA repressor binds to a 20-bp consensus sequence in the operator region of the SOS regulon genes, suppressing their expression. RecA/single-stranded DNA nucleoprotein filament functions as a coprotease that stimulates LexA autoproteolysis (37). The timing, duration, and level of expression can vary for each LexA-regulated gene, depending on the location and binding affinity of the SOS boxes relative to

the strength of the promoter. Therefore, some genes may be partially induced in response to even mild levels of DNA damage of endogenous origin, while other genes are induced only if high-level or persistent DNA damage is present in a cell (13). One of the earliest induced genes (<1 min after SOS induction) is *polB*, encoding Pol II, but transcription from the promoter of the *umuD* and *umuC* genes encoding Pol V is up-regulated among the latest (reviewed in reference 68). The DNA polymerase Pol V can continue DNA replication when the replication fork is collapsed at a blocking lesion, but this DNA synthesis is error-prone (23, 62). Therefore, Pol V-catalyzed error-prone translesion synthesis takes place when all error-free processes for overcoming the replication block at a DNA lesion site have failed (63, 66). The regulation of expression of Pol V in *E. coli* is complex. In addition to control at the transcriptional level, RecA controls the expression of Pol V at the posttranslational level. Firstly, the UmuD protein must undergo RecA-mediated autocleavage to produce UmuD′. Only the (UmuD′)$_2$C complex functions as a DNA polymerase. Additionally, Pol V processivity requires interactions with both the β-clamp and the tip of the RecA nucleoprotein filament (21, 23, 46).

Many plasmids possess the ability to increase the survival and mutation rates of their UV-irradiated bacterial host (22, 25, 32, 33, 35, 38, 50, 53, 58–60, 79). These phenotypes are connected with the presence of genes exhibiting homology to *E. coli umuDC* genes. For example, the presence of *rulAB* genes encoding homologues of Pol V in plasmids is proposed to contribute significantly to virulence and ecological fitness in diverse *Pseudomonas syringae* pathosystems because of the ability of the *rulAB* genes to confer UV tolerance, enabling bacteria to survive on leaf surfaces that are exposed to DNA-

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damaging UV irradiation (58). It is interesting that the genes on the *Pseudomonas aeruginosa* pPL1 plasmid which are responsible for protection against UV light can also protect the cells from X-ray and freeze-thaw damage (73). Thus, the protective role of Pol V homologues against different types of environmental stresses may be more general than conferring UV tolerance on a bacterium.

Many environmental bacteria extend their nutritional diversity by harboring catabolic pathways for the degradation of aromatic compounds on large low-copy-number plasmids. The toluene degradation plasmid pWW0 (74) from *Pseudomonas putida* strain mt-2 has been one of the most studied catabolic plasmids. The vast majority of publications on this plasmid have concentrated on the two catabolic operons (*xyl* operons) and on the regulatory genes *xylR* and *xylS* responsible for their expression (48). The entire catabolic region of pWW0 is part of the two nested transposons Tn*4651* and Tn*4653* (70). The plasmid functions (replication, maintenance, and transfer functions) of pWW0 are all clustered within a 46-kb sector which has been termed the IncP-9 core of the plasmid (24). The complete sequence of pWW0 was published only recently (24). An analysis of the nucleotide sequence of pWW0 has revealed that the plasmid backbone carries genes (designated *ruvA* and *ruvB* in the pWW0 sequence annotation) whose deduced polypeptide products show high percentages of identity with the family of UV resistance proteins (24).

Here we assign a novel function to pWW0. We show that the *ruvAB* genes (which we renamed *rulAB*) identified in pWW0 encode the DNA polymerase Pol V function, and we demonstrate that the presence of these genes significantly enhances the fitness of bacteria under conditions of long-term starvation. Our results indicate that the Pol V genes from TOL plasmid pWW0 increase the probability of accumulation of beneficial mutations in *P. putida* cells, allowing the genetic adaptation of bacterial populations under conditions of environmental stress.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used for this study are described in Table 1. Complete medium was Luria-Bertani (LB) medium (42). M9 minimal medium (2) was supplemented with a solution of trace salts (4) at a final concentration of 2.5 ml/liter. Casamino Acids and glucose were added to the minimal medium at final concentrations of 0.2% and 10 mM, respectively. Solid medium contained 1.5% Difco agar. Antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; tetracycline, 80 μg/ml; carbenicillin, 1,000 to 3,000 μg/ml; and rifampin, 100 μg/ml. The final concentration of potassium tellurite was 25 μg/ml. *E. coli* was incubated at 37°C and *P. putida* was incubated at 30°C if not otherwise stated. *E. coli* was transformed with plasmid DNA as described by Hanahan (26). *P. putida* was electrotransformed as described by Sharma and Schimke (54).

**Mutagenesis assays.** *P. putida* overnight cultures grown on LB medium were diluted 1:100 in fresh LB medium to ensure the exponential growth of bacteria. After the cultures reached mid-exponential phase (optical density at 580 nm of 0.6 to 0.8), 10 ml of each culture was transferred to a 100-mm-diameter sterile petri dish for UV irradiation. 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 apparatus. Aliquots (100 μl) were removed from the petri dish and diluted in LB medium, and individual 2.5-ml cultures were grown in test tubes overnight with shaking. In order to study the effect of mitomycin C (MMC) on the frequency of mutation, mid-exponential-phase cultures of bacteria were diluted by adding 100 μl of cell culture to 2.5 ml of fresh LB broth supplemented with 2 μg MMC/ml and cultured overnight with aeration. The frequency of spontaneous Rif^r mutations was determined in parallel experiments with cells originated from the same initial cultures that were used for the inoculation of cultures subjected to treatment with exogenous DNA-damaging agents. In all cases (untreated cells or cells exposed to either UV irradiation or MMC), 0.5-ml samples of cell suspension from each overnight culture were pelleted by centrifugation, and the collected cells were resuspended in 50 μl LB broth and plated onto LB plates containing 100 μg/ml rifampin. Colonies were counted on rifampin-containing plates incubated for 24 h. The frequency of mutation to Rif^r was calculated per 10^9 plated cells by using the Lea-Coulson method of the median (34, 49). *P* values were calculated by using the Mann-Whitney test (57). The frequency of mutation was determined for at least 60 independent cultures.

**UV irradiation survival assays.** *P. putida* cells were grown to mid-exponential growth phase as described above, and after proper dilution, approximately 10^3 to 10^4 cells were plated onto LB plates. The plated cultures were subjected to UV-C irradiation at a dose of 50 J/m². Irradiated plates were incubated in the dark for 24 h before the enumeration of CFU. Survival was expressed as the number of CFU detected after irradiation as a percentage of those detected after no treatment. At least 60 independent measurements were performed for each strain studied.

**Construction of plasmid for insertion of *rulAB* genes into the *P. putida* chromosome.** The NruI- and MunI-cleaved DNA fragment originated from the TOL plasmid pWW0 carrying the *rulAB* operon was cloned into SmaI- and EcoRI-cleaved pBluescript SK(+) to obtain the plasmid pSKrulAB. Then, using the enzymes HindIII and Ecl136II, the *rulAB* genes were cloned into pUC18Not. The resulting plasmid, pUCNotrulAB, was used to subclone the *rulAB* genes as a NotI-cleaved DNA fragment into the mini-transposon mini-Tn5 Tel-carrying delivery plasmid pJMT6 (51) Plasmid pUTtelrulAB was selected in *E. coli* strain CC118 λ*pir* (27). Plasmid pUTtelrulAB, which was not able to replicate in hosts other than *E. coli* CC118 λ*pir*, was conjugatively transferred into *P. putida* PaW85 by using a helper plasmid, pRK2013 (16), and transconjugants carrying random insertions of the mini-transposon containing the *rulAB* genes in the chromosome of PaW85 were isolated.

**Stationary-phase competitions.** To study the expression of the growth advantage in stationary phase (GASP) phenotype in *P. putida* and the effect of the presence of *rulAB* genes on relative fitness in stationary phase, subpopulations of aged and nonaged cultures were mixed 1:1,000 (vol/vol) and cocultured in 50 ml LB medium at room temperature (22°C) for 30 days. Nonaged cultures were grown overnight in LB medium, and aged cells were derived from 8-day-old cultures grown in LB medium. Markers to distinguish the competitors in mixed populations were resistance to tetracycline (to mark cells of the aged subpopulation) and resistance to kanamycin (to mark cells of the nonaged subpopulation), obtained by inserting either the tetracycline resistance (*tet*) or kanamycin resistance (*kan*) gene into the same neutral position in the PaW85 chromosome (details for construction are described below). Additionally, in order to reduce the possible risk of contamination that could happen during periodic sampling of cells from stationary-phase cultures, plasmid pPR9TT (52), encoding carbenicillin resistance, was introduced into competitor cells, allowing the cultivation of bacteria in the presence of carbenicillin (1,500 μg/ml) in the growth medium. Individual subpopulation titers were determined after periodic sampling (5-day periods), serial dilution, and plating of cells onto selective medium containing appropriate antibiotics.

Marking the competitor cells with different antibiotic resistance genes was carried out as follows. The *tnpA* and *tnpC* genes of the chromosomally locating transposon Tn*4652* (29) were chosen as targets to mark the strains with antibiotic resistance genes. The kanamycin resistance gene was cloned as an EcoRI DNA fragment from pUC4K into the pBluescript KS(+) vector to obtain plasmid pKSkm. The *tnpA* and *tnpC* genes originated from Tn*4652* and cloned into plasmid pKS2.8Cla/Eco were cleaved with NruI and HindIII (HindIII ends were blunt ended) and ligated with the *kan* gene-containing Ecl136II-HincII DNA fragment originated from pKSkm. The resulting plasmid, in which internal sequences of the *tnpAC* genes were partially deleted and replaced with the *kan* gene, was named pKStnpAC::km. Another construct, pKStnpAC::tet, carrying the *tet* gene insertion within the *tnpAC* region, was created by cloning the *tet* gene from pBR322 as a Van91- and EcoRI-cleaved DNA fragment into the NruI- and HindIII-cleaved plasmid pKS2.8Cla/Eco. Subsequently, the *tnpAC* genes interrupted with different antibiotic resistance-encoding marker genes were cloned with EcoRI- and Acc65I-generated ends from pKStnpAC::km and pKStnpAC::tet into the EcoRI- and Acc65I-cleaved plasmid pGP704 L (44) to obtain plasmids pGP704tnpAC::km and pGP704tnpAC::tet, respectively. The plasmids pGP704tnpAC::km and pGP704tnpAC::tet, able to replicate in *E. coli* CC118 λ*pir* but not in other hosts, were conjugatively transferred into *P. putida* PaW85, and the chromosomally locating *tnpAC* genes were replaced by the interrupted genes by homologous recombination. The PaW85 derivative strains PaW85km and PaW85tet carrying either the *kan* or *tet* gene in the same chromosomal location were verified by PCR. The PaWrulAB cells were marked with the same antibiotic resistance genes, using the same strategy as that described for

TABLE 1. Bacterial strains and plasmids used for this study

| Strain or plasmid | Genotype or construction | Source or reference |
|---|---|---|
| **Strains** | | |
| *E. coli* | | |
|   TG1 | *supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ (*traD36 proAB⁺ lac1�q lacZ*ΔM15) | 12 |
|   DH5α | *supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1* | Invitrogen |
|   CC118 λpir | Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λpir phage lysogen | 27 |
| *P. putida* | | |
|   PaW85 | Wild type, carries Tn*4652* in chromosome | 5 |
|   PaWrulAB | PaW85 carrying the *rulAB* genes from pWW0 in chromosome | This study |
|   PaW85km | PaW85 carrying Kmʳ gene from pUC4K inserted into Tn*4652* | This study |
|   PaW85tet | PaW85 carrying Tetʳ gene from pBR322 inserted into Tn*4652* | This study |
|   PaWrulABkm | PaWrulAB carrying Kmʳ gene from pUC4K inserted into Tn*4652* | This study |
|   PaWrulABtet | PaWrulAB carrying Tetʳ gene from pBR322 inserted into Tn*4652* | This study |
|   PaW85 *recA*::*tet* | PaW85 *recA*-defective derivative | 67 |
|   PaWrulAB *recA*::*tet* | PaWrulAB *recA*-defective derivative | This study |
| **Plasmids** | | |
|   pWW0 | Toluene catabolic plasmid, carries *rulAB* (originally named *ruv*AB) genes | 24, 74 |
|   pBluescript KS(+) | Cloning vector (Apʳ) | Stratagene |
|   pBluescript SK(+) | Cloning vector (Apʳ) | Stratagene |
|   pUC18Not | Cloning vector (Apʳ) | 27 |
|   pUC4K | Cloning vector (Kmʳ) | Amersham |
|   pBR322 | Cloning vector (Apʳ Tetʳ) | 6 |
|   pSKrulAB | pBluescript SK(+) containing *rulAB* genes cloned from pWW0 as NruI-MunI fragment into SmaI- and EcoRI-cleaved vector | This study |
|   pUCNotrulAB | pUC18Not containing *rulAB* genes cloned from pSKrulAB as HindII-Ecl136II fragment into same restrictase-cleaved vector | This study |
|   pJMT6 | Delivery plasmid for mini-Tn5 Tel (Apʳ Telʳ) | 51 |
|   pUTtelrulAB | pJMT6 containing *rulAB* genes cloned as NotI fragment from pUCNotrulAB into NotI-cleaved vector | This study |
|   pKS2.8Cla/Eco | Partial *tnpAC* sequence from Tn*4652* cloned as ClaI-EcoRI fragment into pBluescript KS (+) | This study |
|   pKSkm | Kmʳ gene from pUC4K cloned as EcoRI fragment into pBluescript KS (+) | This study |
|   pKStnpAC::km | Kmʳ gene from pKSkm cloned as Ec1136II-HincII fragment into NruI-HindIII-cleaved pKS2.8Cla/Eco | This study |
|   pKStnpAC::tet | Tetʳ gene from pBR322 cloned as Van91-EcoRI fragment from pBR322 into NruI-HindII-cleaved pKS2.8Cla/Eco | This study |
|   pGP704 L | Delivery plasmid for homologous recombination | 44 |
|   pGP704tnpAC::km | *tnpAC*::*km* sequence-containing EcoRI-Acc65I fragment from pKStnpAC::km in pGP704 L | This study |
|   pGP704tnpAC::tet | *tnpAC*::*tet* sequence-containing EcoRI-Acc65I fragment from pKStnpAC::tet in pGP704 L | This study |
|   pRK2013 | Helper plasmid for conjugal transfer of pGP704 L | 16 |
|   pPR9TT | Broad-host-range vector (Cmʳ Apʳ) | 52 |
|   pKSPrulAB | pBluescript KS(+) containing PCR-amplified *rulAB* promoter region cloned into EcoRI-cleaved vector | This study |
|   pKTluxAB | pKT240-derived promoter-probe vector | 67 |
|   pKTPrulABluxAB | *rulAB* promoter region cloned from pKSPrulAB as Acc65I-SmaI fragment into Acc65I-SmaI-cleaved pKTluxAB | This study |
|   pUCNotPrulABluxAB | *rulAB* promoter-*luxAB* transcriptional fusion from pKTPrulABluxAB as BamHI fragment in pUC18Not | This study |
|   pPR9TTPrulABluxAB | *rulAB* promoter-*luxAB* transcriptional fusion from pUCNotPrulABluxAB as NotI fragment in pPR9TT | This study |
|   pKTlacZ | pKT240-derived promoter-probe vector (Apʳ) | 28 |
|   pKTPrulABlacZ | *rulAB* promoter region cloned from pKSPrulAB as HincII-SmaI fragment into SmaI-cleaved pKTlacZ | This study |

marking PaW85. The resulting strains were named PaWrulABkm and PaWrul-ABtet.

**Studies of transcription from the *rulAB* promoter.** A DNA fragment containing a putative *rulAB* gene promoter region was amplified by PCR, using pWW0 DNA as a template with the oligonucleotides BamHIrulAB (5′-CCTTGGATCCCATAATGCACGCGCCTCAG-3′) and XhoIrulAB (5′-CCTTCTCGAGTCCGCAGCGGGGCTTGG-3′), complementary to the nucleotides at positions −121 to −140 and 81 to 98, respectively, relative to the ATG initiator codon of the *rulAB* genes. The amplified DNA fragment was cloned into EcoRV-cleaved pBluescript KS(+) to obtain pKSPrulAB. The *rulAB* promoter region was then subcloned as an Acc65I- and SmaI-generated DNA fragment upstream of the *luxAB* reporter genes present in plasmid pKTluxAB. The resulting plasmid was named pKTPrulABluxAB. To obtain the *rulAB* promoter construct in a single-copy plasmid, the *rulAB* promoter-*luxAB* gene expression cassette was subcloned with BamHI ends from pKTPrulABluxAB into the BamHI site in pUC18Not (resulting in the plasmid pUCNotPrulABluxAB) and finally as a NotI DNA fragment into pPR9TT to obtain the plasmid pPR9TTPrulABluxAB. The effect of the presence of the DNA-damaging agent mitomycin C (2 μg/ml) in the growth medium of bacteria on transcription from the *rulAB* promoter was studied by measuring the luciferase activity as specified previously (67). Bacteria were grown exponentially in M9 glucose medium supplemented with Casamino Acids (CAA) solution.
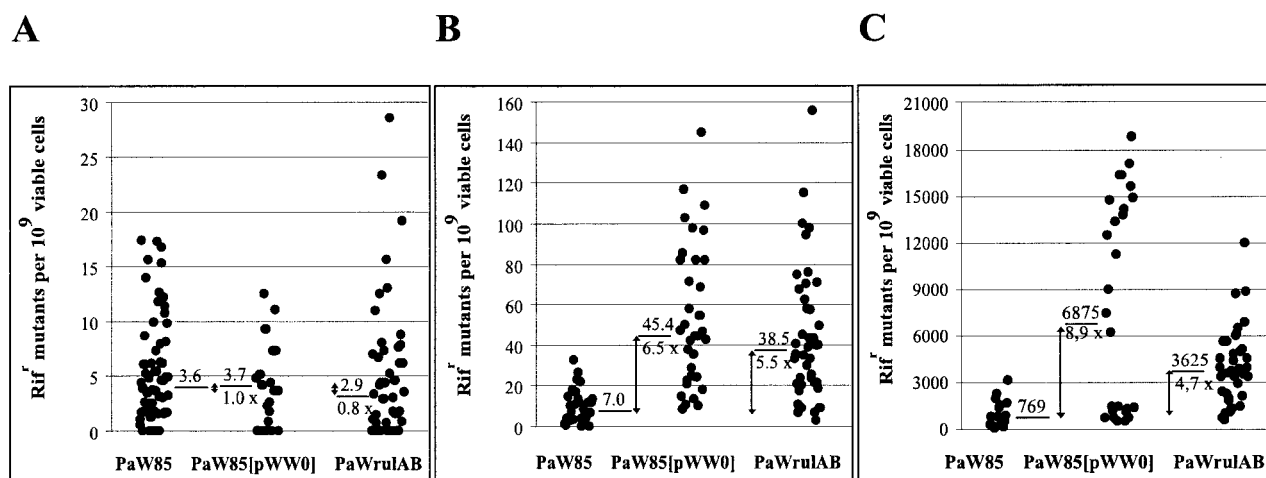
**A**    **B**    **C**



FIG. 1. Study of the effect of the presence of *rulAB* genes from TOL plasmid pWW0 on frequency of mutation in growing cells of *P. putida*. Mutation frequencies were compared for PaW85, PaW85(pWW0), and the PaW85 derivative PaWrulAB carrying the *rulAB* genes in the chromosome. The frequency of mutation to Rif^r per $10^9$ cells was calculated using the Lea-Coulson method of the median (34, 49). Every spot on the figure stands for the result of one independent experiment. The horizontal lines shows the median values of the total results. The double arrows show the differences between the median values for PaW85 and PaW85(pWW0) or for PaW85 and PaWrulAB. (A) Spontaneous mutation frequency in growing cells of *P. putida* in the absence of an exogenous DNA-damaging agent. (B) Effect of exposure of bacteria to UV irradiation (UV-C dose of 100 J/m²) on mutation frequency. (C) Effect of the presence of mitomycin C (2 μg/ml) in the growth medium of bacteria on mutation frequency.

In order to study the effect of the growth phase of bacteria on transcription from the *rulAB* promoter, the promoter region was subcloned from pKSPrulAB as a HincII- and SmaI-generated DNA fragment into the SmaI site of the promoter-probe vector pKTlacZ (28) to obtain the plasmid pKTPrulABlacZ. β-Galactosidase (β-Gal) activities in *P. putida* cells carrying pKTPrulABlacZ were measured by a modification of the standard protocol of Miller (42), as specified previously (69). Bacteria were grown in M9 glucose medium supplemented with CAA solution.

## RESULTS

**TOL plasmid pWW0 confers UV mutability and tolerance to UV irradiation on *P. putida*.** Various plasmids in *Pseudomonas* have been shown to express the UV mutagenesis phenotype, i.e., an increase in mutation frequency of up to 100-fold above spontaneous levels (35, 36, 40, 56, 60). We found that the transfer of TOL plasmid pWW0 into the plasmid-free strain *P. putida* PaW85 also resulted in an increased frequency of mutations if bacteria were irradiated with UV-C light. The frequencies of appearance of spontaneous Rif^r mutants were similar for plasmid-free *P. putida* PaW85 and for PaW85 harboring pWW0 (Fig. 1A). At the same time, the exposure of pWW0-carrying bacteria to UV-C irradiation (254 nm) at a dose of 100 J/m² resulted in a 10-fold increase in the number of Rif^r mutants above spontaneously induced levels (Fig. 1B). This was about five times higher than that observed in the case of plasmid-free cells ($P < 0.0001$). Next, we studied whether the exposure of pWW0-harboring *P. putida* to other DNA damage-inducing agents could also induce a higher level of mutability. The addition of the mutagen mitomycin C (2 μg/ ml) to the growth medium of the bacteria led to an increase of 2 to 3 orders of magnitude in the frequency of mutation compared to the spontaneous levels (Fig. 1A and C). The median number of Rif^r mutants per $10^9$ cells was about nine times higher in the case of the pWW0-carrying strain than for the plasmid-free strain ($P < 0.0001$). Thus, in both cases of UV

irradiation of bacteria and exposure of cells to DNA damage-inducing chemicals, the presence of the TOL plasmid pWW0 in *P. putida* significantly increased the frequency of mutations.

Plasmids expressing UV mutability usually confer tolerance to UV irradiation. Our results clearly demonstrate that pWW0 confers UV tolerance on *P. putida* (Fig. 2). The percentage of survival for pWW0-harboring *P. putida* increased >10-fold in comparison with that for plasmid-free *P. putida* ($P < 0.0001$) when bacterial cultures were exposed to UV-C irradiation at a dose of 50 J/m².

**TOL plasmid pWW0 encodes a DNA polymerase Pol V homologue.** An analysis of the complete sequence of the TOL
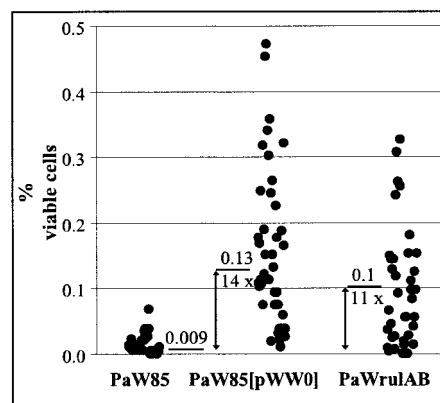


FIG. 2. Study of the effect of the presence of the *rulAB* genes on UV tolerance of *P. putida*. The percentage of survival of bacteria after UV-C irradiation at dose of 50 J/m² is shown. Every spot on the figure stands for the result of one independent experiment. The horizontal lines show the median values of the total results. The double arrows show the differences between the median values for PaW85 and PaW85(pWW0) or for PaW85 and PaWrulAB.

plasmid pWW0 by Greated et al. (24) revealed the genes *ruvA* (Q8VMP5) and *ruvB* (Q8VPM6), related to *P. syringae rulA* (Q52416) and *rulB* (Q52417) (61), respectively. The *rulA* and *rulB* genes confer UV irradiation tolerance and mutability on *P. syringae* (30, 58). These genes are distantly related to *umuDC* (encoding *E. coli* Pol V) and are able to complement an *E. coli umuDC* mutant (30), which indicates that the *rulAB* genes encode a Pol V homologue. The designation of the *rulAB* homologues carried on pWW0 as *ruvAB* is misleading because genes with the same name are located chromosomally in many studied bacterial species, including *P. putida* (www.tigr .org). The chromosomal *ruvAB* genes encode a Holliday junction helicase complex necessary for branch migration along DNA. Therefore, to avoid confusion, we decided to rename the plasmid-carried *ruvAB* genes with the designation *rulAB*, similar to that of other *umuDC* homologues identified in environmental bacteria.

In order to control whether the *rulAB* genes from pWW0 could confer UV irradiation-induced mutability on *P. putida*, we cloned the *rulAB* genes from the TOL plasmid and inserted them within mini-Tn5Tel[r] into the chromosome of *P. putida* PaW85. Potassium tellurite was added to the growth medium of bacteria only for the initial selection of clones carrying mini-Tn insertions in the chromosome but not in later experiments. Ten different locations of the mini-Tn were examined for the ability of the *rulAB* genes to express the UV mutagenesis phenotype. We found that 9 *rulAB* gene insertions of the 10 investigated insertions expressed the UV mutagenesis phenotype at levels similar to that determined for *P. putida* carrying the TOL plasmid pWW0. We selected one of these *rulAB* gene-carrying clones for further studies and named it PaWrulAB. The growth rates of PaW85 and PaWrulAB were compared in LB liquid cultures. We did not observe any differences in the growth rates of these strains during the exponential growth of bacteria. Also, the numbers of CFU per ml during the first 3 days of bacterial cultivation were similar between the two strains compared (data not shown).

The frequencies of appearance of Rif[r] mutants were similar for both UV-irradiated pWW0-harboring *P. putida* and *P. putida* carrying the *rulAB* genes on the chromosome (Fig. 1B). The effect of mitomycin C on mutagenesis was approximately twofold larger in PaW85(pWW0) than in PaWrulAB carrying the chromosomally located *rulAB* genes (Fig. 1C). Similar to the case for pWW0-harboring *P. putida*, about 0.1% of PaWrulAB cells survived after exposure to UV radiation (Fig. 2). These data indicate that the DNA damage-induced mutability and UV resistance phenotype conferred by pWW0 are determined by the *rulAB* genes and that the expression of these genes is not significantly influenced by other pWW0-carried genes.

In order to rule out the possibility that other genes in pWW0 might be involved in UV mutagenesis and UV tolerance, an additional control experiment was performed. We constructed a pWW0 derivative lacking the functional *rulAB* operon. The original *rulAB* gene cluster in pWW0 was replaced with a DNA sequence in which the internal sequence of the *rulAB* operon (nucleotides 401 to 1361, starting from ATG of the *rulA* gene) was deleted and contained an insertion of the kanamycin resistance gene. We found that if bacteria were exposed to DNA-damaging agents, the frequency of appearance of Rif[r] mutants

was the same both in the case of the plasmid-free PaW85 and in the case of PaW85 carrying the TOL plasmid derivative with inactivated *rulAB* genes (data not shown).

**The *rulAB* genes from pWW0 confer the GASP phenotype on *P. putida*.** The presence of plasmids is advantageous for bacteria under harsh environmental conditions. However, even if plasmid-harboring cells can grow on a wider spectrum of substrates than those lacking a catabolic plasmid, they quickly consume the available nutrients and enter stationary phase. Thus, in natural environments, bacteria spend the majority of their existence under conditions of starvation. Upon the onset of starvation, there is intense selective pressure for any mutation that confers a selective advantage. Populations of stationary-phase cells have been shown to be highly dynamic: waves of fitter mutants constantly arise and take over previous populations (17, 18). The growth advantage in stationary phase (GASP) phenotype was initially observed in starving *E. coli* populations (78). Since then, the GASP phenotype has been observed in many different bacterial species (references 18 and 81 and references therein). In the case of *P. putida*, the GASP phenotype has been reported only for conditions of phosphate starvation (reviewed in reference 81).

Using this information, we decided to study whether other starvation conditions could also allow detection of the GASP phenotype of *P. putida* and whether the pWW0-derived *rulAB* genes could influence the occurrence of GASP mutations. The appearance of GASP mutants is detectable in competition experiments. Cells containing advantageous alleles permitting growth during stationary phase (GASP mutants) will increase in number relative to the rest of the population as the culture ages. We marked the *P. putida* strains used for GASP competition experiments with different antibiotic resistance markers (resistance to kanamycin or to tetracycline). The antibiotic resistance genes were inserted into the chromosomally locating transposon Tn4652 (for details, see Materials and Methods). Cells from an 8-day-old LB culture were inoculated as a numerical minority (1:1,000 [vol/vol]) into a fresh (1-day-old) LB culture. Both subpopulations were monitored for the next 30 days, and their relative proportions in the population were determined by plating cells onto selective media containing appropriate antibiotics.

We monitored competitions between pairs of aged and young subpopulations of PaW85 cells themselves, aged and young subpopulations of PaWrulAB cells themselves, an aged subpopulation of PaWrulAB cells and a young subpopulation of PaW85 cells, and vice versa. Nine parallel competition experiments were carried out with each pair. Representative examples of GASP competition phenotypes which appeared during the cocultivation of different subpopulations are shown in Fig. 3, and data about the coevolution of the mixed populations are summarized in Table 2. In all cases studied, at the beginning of the experiment the titer of aged cells increased, becoming equal to the number of cells of the young subpopulation, but later the patterns of GASP takeovers diverged, being remarkably affected by the presence or absence of the *rulAB* genes in bacteria. The cocultivation of aged and young subpopulations of PaW85 cells revealed a mild expression of the GASP phenotype: the relative proportion of cells derived from aged cultures was increased about 5 to 10 times, with a concomitant decrease in the number of cells of the young
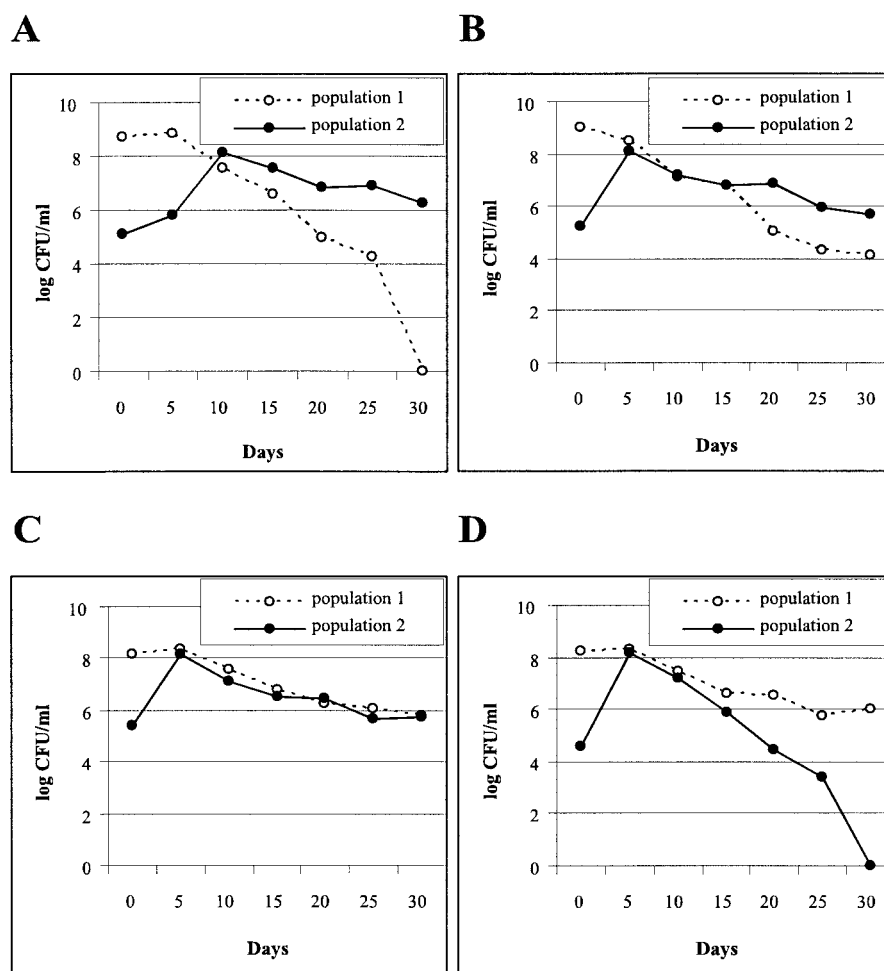
**A**



**B**



**C**



**D**



FIG. 3. Representative examples of GASP competition phenotypes which appeared during the cocultivation of aged and nonaged subpopulations of *P. putida*. For simplification, the competing subpopulations are marked as population 1 and population 2. Different expression levels of the GASP phenotype are shown for population 1, which consists of cells derived from a 1-day-old (nonaged) culture, and for population 2, derived from an 8-day-old (aged) culture. Population 2 was able to out-compete population 1 either completely (strong GASP phenotype, panel A) or partially (mild GASP phenotype, panel B) or displayed an equal fitness to that of population 1 (equal GASP phenotype, panel C). In some cases, population 2 was either partially or completely out-competed by population 1 (no GASP phenotype, panel D).

subpopulation. Aged PaWrulAB cells expressed the strong GASP phenotype in seven competition experiments, completely out-competing the young PaW85 subpopulation, and in two experiments the milder expression of the GASP phenotype became apparent. Interestingly, in most cases young PaWrulAB cells were also able to completely or partially out-compete aged PaW85 subpopulations. Only in two cases did both subpopulations display equal fitness. We also studied the competition between aged and fresh subpopulations of PaWrulAB cells. Compared with the population dynamics observed in the case of the coevolution of aged and young PaW85 subpopulations, the *rulAB*-carrying cells expressed a stronger GASP phenotype. Taken together, the results of our experiments indicate that the *rulAB* genes from TOL plasmid pWW0 increase the probability of *P. putida* cells accumulating beneficial mutations allowing the appearance of the GASP phenotype.

**Regulation of expression of the pWW0-encoded Pol V homologue in *P. putida*.** The finding that the presence of *rulAB*

genes in the bacterial genome enhances the fitness of *P. putida* during prolonged starvation indicates that the *rulAB* genes are expressed in late-stationary-phase cells. As a next step, we decided to study the expression of the *rulAB* genes in *P. putida* in the presence or absence of exogenous DNA-damaging agents in the growth environment. The effects of growth phase of bacteria and the duration of starvation in stationary-phase cultures were also studied.

A putative promoter region of the *rulAB* genes is similar to the promoter region of the *umuDC* genes characterized in *E. coli* (31) (Fig. 4A). A putative SOS box, which may function as a binding site for the LexA repressor at the *rulAB* promoter, overlaps the −10 sequence. In many bacteria studied, DNA damage stimulates a RecA-mediated cleavage of the LexA repressor, resulting in an increased level of transcription of the DNA damage-induced genes. The genome of *P. putida* KT2440 contains two *lexA* homologues—*lexA1*, whose product binds the *E. coli*-like LexA-binding sequence (11), and *lexA2*, encoding a repressor protein which binds to a site closely related to

TABLE 2. Study of the effect of the presence of pWW0-derived *rulAB* genes on expression of GASP phenotype in *P. putida*

| Competing subpopulations[a] | No. of cases with GASP phenotype[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cells from 8-day-old culture | | | | Cells from 1-day-old culture | | | |
| | Strong | Mild | Equal | None | Strong | Mild | Equal | None |
| PaW85 (8) + PaW85 (1) | 0 | 8 | 1 | 0 | 0 | 0 | 1 | 8 |
| PaWrulAB (8) + PaW85 (1) | 7 | 2 | 0 | 0 | 0 | 0 | 0 | 9 |
| PaW85 (8) + PaWrulAB (1) | 0 | 0 | 2 | 7 | 5 | 2 | 2 | 0 |
| PaWrulAB (8) + PaWrulAB (1) | 2 | 5 | 0 | 2 | 2 | 0 | 0 | 7 |

[a] Numbers 8 and 1 in parentheses indicate how many days the two subpopulations were grown separately in LB before starting competition experiments with mixed populations. Cells from 8-day-old cultures were mixed with 1-day-old cultures at a ratio of 1:1,000 (vol/vol).
[b] Number of cases when the particular subpopulations expressed the GASP phenotype in nine parallel competition experiments if cells derived from 8-day-old and 1-day-old cultures were allowed to coevolve for 30 days in stationary-phase cultures. The expression of the GASP phenotype in nine parallel competition experiments is shown separately for cells derived from both aged (8-day-old) and nonaged (1-day-old) cultures. Representative examples of expression of the strong, mild, and equal GASP phenotypes or the lack of expression of the GASP phenotype are illustrated in Fig. 3.

a LexA-binding motif found in cyanobacteria (1). The *rulAB* promoter region contains a DNA sequence, CTGTATATG CAAACAG, which perfectly matches the *E. coli* LexA repressor-binding consensus CTGTN$_8$ACAG. In order to study whether transcription from the *rulAB* promoter could be inducible by DNA damage, we cloned the *rulAB* promoter upstream of the *luxAB* reporter in plasmid pPR9TT to obtain plasmid pPR9TTPrulABluxAB. The luciferase activities measured in exponentially growing *P. putida* cells carrying pPR9TTPrulABluxAB indicated that the promoter of the *rulAB* genes is already active, even in the absence of the DNA damage-inducing chemical MMC (Fig. 5A). However, bacteria exposed to MMC for 3 h expressed a threefold higher level of luciferase activity than bacteria grown in the absence of this mutagen (Fig. 5A). This indicated that DNA damage can induce transcription of the *rulAB* genes. Proceeding from these data, we investigated whether the induction of transcription of the *rulAB* genes is achieved by a classical SOS response requiring the RecA-mediated inactivation of the LexA repressor or if some other mechanisms are behind the regulation of transcription from the *rulAB* promoter. A comparison of the transcription levels from the *rulAB* promoter in a *P. putida recA*-defective strain grown in the presence or absence of MMC demonstrated that the RecA protein is needed for the induction of transcription from this promoter in the presence of DNA damage. No effect of DNA damage on transcription from this promoter could be observed in the *recA*-defective *P. putida* strain PaW85 *recA*::*tet* (Fig. 5B). The RecA activity was also needed for *rulAB*-mediated UV mutagenesis in *P. putida*. As shown in Fig. 6, the effect of the presence of the *rulAB* genes in the bacterial chromosome on the frequency of occurrence of UV irradiation-stimulated rifampin-resistant mutations completely disappeared in the RecA-negative background.

We studied the effect of the growth phase of bacteria on the level of transcription from the *rulAB* promoter. The results shown in Fig. 5C demonstrate that transcription from the promoter of the *rulAB* genes increases five to six times in late-stationary-phase cells. The level of β-Gal expression was only slightly increased if the activities of exponentially growing and stationary-phase cells sampled at hours 12 and 24 were compared, but the activity was significantly increased during prolonged incubation (cells sampled at hours 36, 48, and 60). Thus, the increased level of transcription of the *rulAB* genes in

late stationary phase might be one of the mechanisms allowing a higher level of expression of the pWW0-encoded Pol V in bacteria suffering prolonged nutritional starvation.

## DISCUSSION

In all living cells, DNA continuously incurs damage by endogenous and exogenous agents. To cope with DNA damage, bacteria have evolved several defense mechanisms. DNA repair pathways remove lesions in an essentially error-free way (20). Additionally, cells contain specialized DNA polymerases that are able to continue DNA replication when the replication fork is collapsed at a blocking lesion, with an associated risk of generating mutations (19). Here we have characterized an error-prone DNA polymerase Pol V homologue encoded by the toluene catabolic plasmid pWW0 in *P. putida* and studied its role on the fitness of bacteria under different environmental conditions.

The results presented in Fig. 1 and 2 demonstrate that the *rulAB* genes identified in pWW0 confer increased resistance to
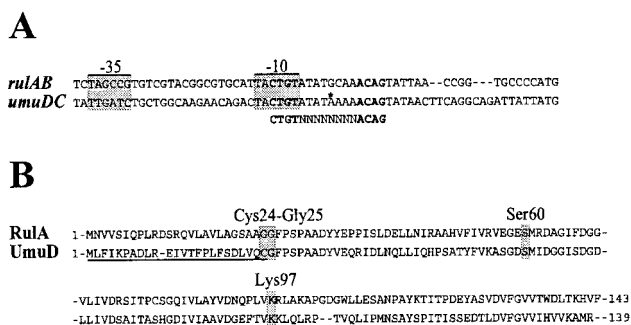


FIG. 4. (A) DNA sequence alignment of the putative promoter region of the *rulAB* genes and the promoter of the *E. coli umuDC* genes. The −35 and −10 hexamers of the promoters are shaded and marked by lines. The LexA-binding consensus nucleotides (15, 72) are shown below the DNA sequence alignment and are indicated in bold. The transcriptional start site mapped for the *umuDC* promoter (31) is indicated by an asterisk. (B) Amino acid sequence alignment of the N termini of the UmuD and RulA proteins. The N-terminal sequence of UmuD removed after the RecA-stimulated processing of UmuD to UmuD′ (9, 43, 55) is underlined. The cleavage site between Cys24 and Gly25 and amino acids important in the cleavage reaction (Ser60 and Lys97) of UmuD (41) are shaded.
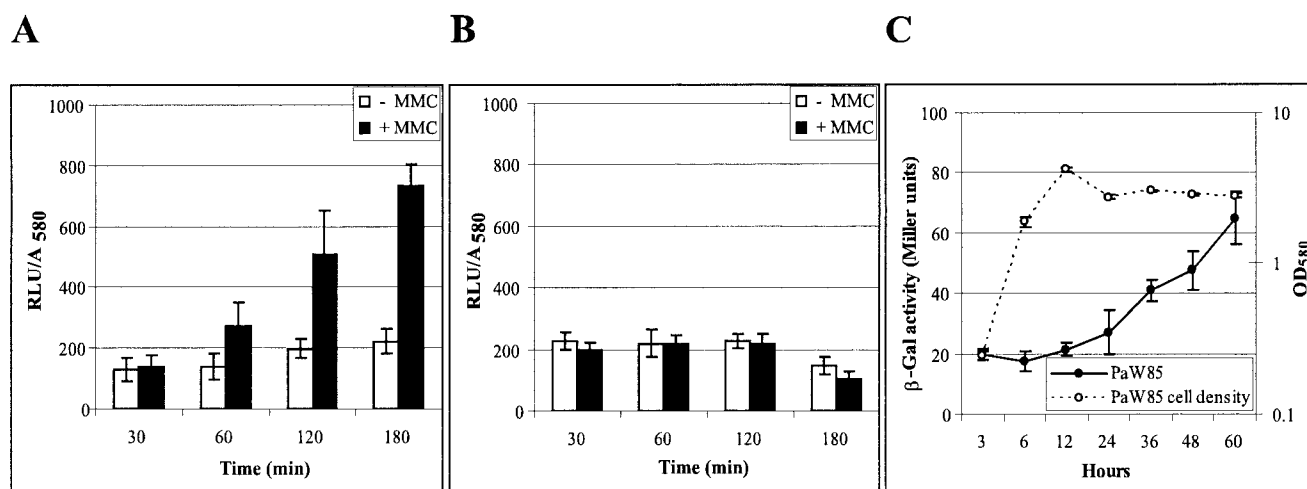
**A** **B** **C**



FIG. 5. Study of the effect of DNA damage on transcription from the *rulAB* promoter in *P. putida* wild-type strain PaW85 (A) and in its RecA-defective derivative strain PaW85 *recA*::*tet* (B). The promoter was cloned upstream of the reporter genes *luxAB* encoding luciferase, and the expression of the transcriptional fusion was measured with a single-copy pPR9TT-derived broad-host-range plasmid, pPR9TTPrulABluxAB. Transcription from the *rulAB* promoter was assayed by measuring the luciferase activity (relative luciferase units/optical density units at 580 nm) in cells grown in M9 medium supplemented with glucose and CAA in the presence or absence of the DNA-damaging agent MMC (2 μg/ml). (C) Effect of growth phase of bacteria on transcription from the *rulAB* promoter. β-Gal activity was measured in the *P. putida* wild-type strain PaW85 carrying the *rulAB* promoter-*lacZ* fusion in plasmid pKTPrulABlacZ. Bacteria were grown in M9 medium supplemented with glucose and CAA. The growth curve of the bacteria is indicated by a dashed line. The results of four independent experiments are presented. The standard deviations are shown in the figure (error bars). OD$_{580}$, optical density at 580 nm.

UV irradiation on *P. putida*. Based on published data (76), pWW0 has been demonstrated to confer resistance to the reactive singlet oxygen species. Oxidative DNA damage accumulation within bacteria is a major contributor to the generation of stationary-phase mutations in bacteria (7), and the SOS response is induced in stationary-phase cells, even in the absence of exposure to exogenous DNA-damaging agents (64). It is possible that the pWW0-provided resistance to reactive oxygen species, observed already more than 20 years ago, is also conferred by the *rulAB* genes and that this resistance increases
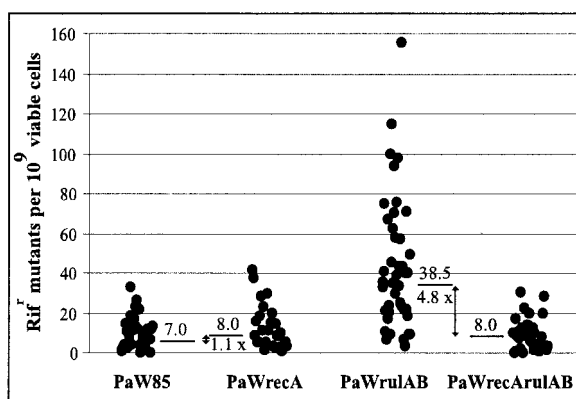


FIG. 6. Study of the role of RecA on *rulAB* gene-induced UV mutagenesis in *P. putida*. Every spot on the figure stands for the result of one independent experiment. The horizontal lines show the median values of the total results. The double arrows show the differences between the median values for PaW85 and PaW85 *recA*::*tet* (PaWrecA in the figure) or PaWrulAB and PaWrulAB *recA*::*tet* (PaWrecArulAB in the figure).

the survival rate of bacteria during prolonged starvation when oxidative DNA damage accumulates.

In *E. coli*, LexA binds specifically to a DNA motif known as the SOS box (72). A 16-bp consensus sequence, CTGN$_{10}$CAG, is the target for LexA in *E. coli* but also in many other members of the gamma subclass of *Proteobacteria*, including pseudomonads (reference 15 and references therein). Although the promoter region of the *rulAB* genes on pWW0 contains a DNA sequence showing a perfect match to the *E. coli* LexA-binding consensus sequence (Fig. 4A) and *P. putida* LexA1 binds an *E. coli*-like SOS box (11), the extent of increase of transcription from this promoter in *P. putida* was only threefold after the exposure of bacteria to the DNA-damaging chemical MMC. At the same time, we observed that MMC stimulates transcription from the *rulAB* promoter >20 times when similar expression studies were carried out using *E. coli* (data not shown). This indicates that although the binding sites for the LexA repressor are similar for both organisms, the *P. putida* LexA homologue LexA1 may bind the *E. coli*-like SOS box with a lower affinity. This possibility is also supported by our finding that the promoter of the *dinB* gene from *P. putida*, which also carries an *E. coli*-like SOS box, is only induced up to twofold by MMC in *P. putida* (67) but that a more-than-eightfold induction became apparent in *E. coli* (not shown). Thus, one may speculate that compared to *E. coli*, *P. putida* has evolved a regulatory system allowing a considerably high basal level of transcription of particular SOS regulon genes already under "normal" growth conditions of bacteria due to a reduced affinity of binding sites for the LexA repressor.

Published data indicate that the expression of some putative Pol V homologues does not require RecA activity (e.g., see reference 39). Our results indicate that the expression of the

pWW0-encoded RulAB protein is RecA dependent. The expression of *E. coli* Pol V is controlled by RecA at both the transcriptional and posttranslational levels. Posttranslational RecA-mediated proteolytic cleavage of UmuD to UmuD′ is required for the Pol V activity (23). We propose that the RulA protein is probably also activated by RecA-stimulated proteolysis for its role in DNA polymerase activity. This hypothesis is based on the findings that *rulAB*-dependent mutagenesis was completely abolished in the RecA-negative background of *P. putida* (Fig. 6) and that the *rulA* gene product shares the putative cleavage site as well as the amino acids involved in mechanism of the cleavage reaction conserved in UmuD-like proteins (Fig. 4B).

It has been argued that induced mutagenesis could help cells to survive periods of extreme environmental stress by acting as a mechanism of genetic adaptation of microbial populations (47). Thus, in addition to the protection of cells against DNA damage, the presence of plasmid-encoded Pol V may have an important role in the evolution of microbes under environmental stress. Data supporting this idea have been obtained from experiments with *E. coli*: in competition with the wild type, mutants lacking one or more SOS polymerases showed reduced long-term survival and evolutionary fitness (77). Under conditions of long-term starvation, cells with the growth advantage in stationary phase (GASP) phenotype arise by mutation, and because evolution is a continuous process, stationary-phase populations are repeatedly taken over by mutants with increased fitness (18, 78). So far, the appearance of the GASP phenotype in *P. putida* has been observed only for conditions of phosphate starvation (reviewed in reference 81). Here we show that *P. putida* was able to express the GASP phenotype under classical experimental conditions when competitions between young and aged subpopulations of *P. putida* PaW85 were carried out with bacteria grown aerobically in LB broth (Table 2). However, the GASP phenotype observed by us was weak and allowed only a partial displacement of the subpopulation that was derived from a nonaged culture by cells evolved in an aged culture. At the same time, the presence of the pWW0-derived *rulAB* genes in *P. putida* conferred in most cases a very strong GASP phenotype, completely out-competing the subpopulation of cells lacking the *rulAB* genes. Moreover, in several mixed cultures, the *rulAB*-expressing young subpopulation completely out-competed the aged population of cells lacking the *rulAB* genes. It seems very unlikely that the 1-day-old culture already contained GASP mutants. Rather, because the young PaWrulAB subpopulation was 3 orders of magnitude larger than the aged subpopulation of cells lacking the *rulAB* genes, during cocultivation the *rulAB*-carrying cells had a higher probability for the occurrence of mutations leading to the strong GASP phenotype.

The pattern of GASP takeovers diverged during the coevolution of subpopulations in the mixed cultures, not only between different combinations of subpopulations but also in the case of parallel experiments when we cocultivated subpopulations derived from the same pairs of cultures (Table 2). Monitoring the population dynamics within parallel experiments using the same pairs of competitors revealed that even if we started the competition experiments by mixing cells from the same cultures, the mixed populations evolved in different ways. This indicates that the GASP takeover populations that occur

in parallel mixed populations may carry different GASP mutations or that some may contain additional mutations having an opposite effect on fitness.

DNA polymerase Pol V homologues are frequently encoded by naturally occurring conjugative plasmids. These plasmids often carry multiple antibiotic resistance genes (75). The *rulAB* locus, which increases the survival of epiphytic *P. syringae* strains on leaf surfaces, is widely distributed among *P. syringae* virulence plasmids (59, 60, 80). The results presented in this study demonstrate that the catabolic TOL plasmid pWW0 carries the *rulAB* genes encoding a Pol V homologue. The association of Pol V genes with catabolic plasmids may be more general. For example, a complete sequence analysis of the naphthalene degradation plasmid pDTG1 (14) revealed the presence of genes that are very closely related to the *rulAB* genes from pWW0. The *Pseudomonas* plasmid CAM-OCT, which encodes catabolic pathways for *n*-alkanes and camphor utilization, also carries UV response genes (38). The UV response genes cloned from the CAM-OCT plasmid increased the ability to isolate mutant bacterial strains with novel metabolic properties (40). Additionally, McBeth and Hauer have mentioned in their article (40) that the phenol-degradative plasmid pVI150 carries UV resistance genes as well. Thus, it is possible that the presence of genes encoding Pol V homologues in catabolic plasmids may contribute to metabolic diversity in microbial populations via the highly mutagenic DNA synthesis provided by these enzymes if they are induced in stressed bacteria (e.g., under conditions of long-term nutrient starvation).

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