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Polyphosphate kinase regulates error-prone replication by DNA polymerase IV in *Escherichia coli*

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Summary

The *ppk* gene encodes polyphosphate kinase (Ppk), an enzyme that catalyses the polymerization of inorganic phosphate into long chains of polyphosphate (polyP). An insertion mutation in *ppk* causes a decrease in adaptive mutation in *Escherichia coli* strain FC40. Adaptive mutation in FC40 mostly results from error-prone DNA polymerase IV (Pol IV), encoded by *dinB*; most of the antimutagenic phenotype of the *ppk* mutant disappears in a *dinB* mutant strain. In addition, the *ppk* mutant causes a decrease in growth-dependent mutations produced by overexpressing Pol IV. However, the amount of Pol IV protein is unchanged in the *ppk* mutant strain, indicating that the activity or fidelity of Pol IV is altered. Adaptive mutation is inhibited both by the absence of Ppk, which results in low amounts of polyP, and by overproduction of Ppk, which results in high amounts of polyP, suggesting that an optimal level of polyP is necessary. Taken together, these results suggest a novel mechanism involving polyP that directly or indirectly regulates DNA polymerase activity or fidelity.

Introduction

Escherichia coli DNA polymerase IV (Pol IV) is a member of the Y-family of DNA polymerases that is characterized by their ability to synthesize past DNA lesions. However, Y-family DNA polymerases insert incorrect nucleotides at higher frequencies than normal replicative polymerases. Therefore, when DNA lesions are present (i.e. after UV irradiation) Y-family polymerases cause increased mutation rates (reviewed by Goodman, 2002). Error-prone polymerases, as they have been called, are found in all three domains of life. A deficiency in the human homologue, Pol η, causes a variant of the skin disease Xero-derma pigmentosum that results in increased sensitivity to UV light and a predisposition to skin cancer (Yamada *et al*., 2000).

Both of *E. coli*'s Y-family polymerases, Pol IV and Pol V, are induced as part of the LexA-controlled SOS regulon that responds to DNA damage (Kenyon and Walker, 1980;Bagg *et al*., 1981;Courcelle *et al*., 2001). A current model for *trans*-lesion DNA synthesis is as follows (Goodman, 2002). The normal replicative polymerase, Pol III, stalls at a DNA lesion, which results in induction of the SOS response; one of the Y-family polymerases (Pol IV or Pol V) is recruited to the replication fork, synthesizes past the lesion and continues replicating for a short period. The Y-family polymerase then is replaced by Pol III, allowing normal DNA replication to continue. Because of the high rate at which incorrect nucleotides are incorporated, DNA synthesis by Pol IV or Pol V results in mutations.

When bacteria are held under non-lethal selection, the non-growing cells accumulate mutations that relieve the selective pressure and allow the bacteria to grow. This phenomenon is known as adaptive mutation (Cairns and Foster, 1991). Pol IV is responsible for 50–80% of the adaptive mutations produced in the highly studied *E. coli* strain FC40 (Foster, 2000;McKenzie

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et al., 2001). FC40 is deleted for *lac* on the chromosome and the *lac* region is carried on the episome, F′128. *lacI* and *lacZ* are fused, eliminating the last four residues of *lacI*, all of *lacP* and *lacO*, and the first 23 residues of *lacZ* (Mueller-Hill *et al*., 1964). The *lacI–Z* gene fusion has a +1 frameshift in *lacI*, adding a G:C base pair to a run of three G:C base pairs, resulting in a Lac^- phenotype (Calos and Miller, 1981).

During non-selective growth, Lac^+ mutants of FC40 arise at a rate of about 10^{-9} per cell per generation. When incubating on minimal lactose media, Lac^+ mutants arise for about a week at a rate of 10^{-7} per cell per day (Cairns and Foster, 1991). Adaptive mutation in FC40 differs from growth-dependent mutation in several ways. Adaptive Lac^+ mutations result almost exclusively from −1 frame-shifts in runs of consecutive bases (Foster and Trimarchi, 1994;Rosenberg *et al*., 1994). In contrast, the mutations that occur during growth include deletions and duplications as well as simple frameshifts. The rate of adaptive mutation in FC40 is 100 times higher when the *lac* allele is on the episome than when it is on the chromosome (Foster and Trimarchi, 1995;Radicella *et al*., 1995). Furthermore, unlike growth-dependent mutation, adaptive mutation depends on the double-strand break repair activities of RecABCD (Cairns and Foster, 1991;Foster, 1993;Harris *et al*., 1994) and is enhanced by the expression of conjugal functions (Foster and Trimarchi, 1995;Galitski and Roth, 1995;Radicella *et al*., 1995). Evidence suggests that the conjugal function that is required for adaptive mutation is nicking the DNA at the conjugal origin (Rodriguez *et al*., 2002). Although the mechanism by which adaptive mutation occurs is controversial (Foster, 2004;Rosenberg and Hastings, 2004;Roth and Andersson, 2004), it is widely accepted that Pol IV causes most of the frameshifts that result in adaptive mutations.

Adaptive mutation is increased 100-fold in a strain defective for the RecG helicase (Foster *et al*., 1996;Harris *et al*., 1996). This increase is eliminated when Pol IV is also defective, indicating that the high rate of adaptive mutation in *recG* mutants is almost exclusively due to Pol IV (Layton and Foster, 2003). Because this 100-fold increase in adaptive mutation is easy to assay, we used a *recG* mutant strain to screen for mutations that decrease the amount or activity of Pol IV. A transposon was used to generate random insertions, and the resulting mutants were screened for low levels of adaptive mutation. One gene found during this screen was *rpoS*, which encodes the stationary-phase sigma factor, σ^{38}, that is required for transcription of genes induced upon entry into stationary phase (reviewed by Hengge-Aronis, 2002). Further analysis showed that Pol IV is induced about threefold during late stationary phase, and this induction is RpoS dependent (Layton and Foster, 2003).

Another gene that was found in the screen was *ppk*, which encodes polyphosphate kinase (Ppk). Ppk polymerizes the gamma phosphate of ATP into long chains of inorganic polyphosphate (polyP) (reviewed by Kornberg *et al*., 1999). *ppk* is in an operon with *ppx* that encodes an exopolyphosphatase (Ppx) responsible for degrading polyP (Akiyama *et al*., 1993). PolyP is found in all studied organisms in all domains of life (reviewed by Kornberg *et al*., 1999), and many functions have been proposed for it. PolyP activates Lon protease by binding to its DNA-binding domain; this binding allows Lon to degrade ribo-somal proteins in response to amino acid starvation (Kuroda *et al*., 2001;Nomura *et al*., 2004). *recA* and *rpoS* mRNA levels are decreased when polyP levels are undetectable (Shiba *et al*., 1997;Tsutsumi *et al*., 2000). Ppk is one of several proteins that form the RNA degradosome that regulates RNA turnover (Blum *et al*., 1997;Carpousis, 2002;Bernstein *et al*., 2004). PolyP that was purified with DNA from the fungus *Colletotrichum* inhibited DNA replication by *Taq* polymerase *in vitro* (Rodriguez, 1993). Finally, polyP forms a complex with RNA polymerase; *in vitro* studies have shown that polyP inhibits transcription from σ^{70} promoters but allows transcription from RpoS-dependent promoters at high salt concentrations (Kusano and Ishihama, 1997).

Here we report that Ppk is necessary for normal levels of adaptive mutation. Furthermore, Ppk is required for the increased rate of growth-dependent mutations produced by Pol IV overexpression. Our results suggest that Ppk is affecting the activity of Pol IV in a manner that is independent of any previously known factors that regulate this polymerase.

Results

Mutations in Ppk reduce adaptive mutation in FC40

In *E. coli* strain FC40 adaptive mutation is increased up to 100-fold by mutations in *recG* (Foster *et al*., 1996;Harris *et al*., 1996), and this increase is dependent on Pol IV (encoded by *dinB*) (Layton and Foster, 2003). To search for genetic factors that control Pol IV, we made random insertions of a transposon encoding chloramphenicol resistance (Cm^R), miniTn*10*Cm (Kleckner *et al*., 1991), into a *recG* mutant strain, and then screened the Cm^R isolates for reduced levels of adaptive mutation. One mutant found in this screen has an insertion in the *ppk* gene at nucleotide 345 in its coding sequence. The *ppk*::miniTn*10*Cm (hereafter referred to as *ppk*::Cm) mutation decreased adaptive mutation by approximately 80% in the *recG* mutant strain (Fig. 1) and up to 65% in the wild-type strain (see Fig. 2A). To determine whether another *ppk* mutant allele has a similar phenotype, we tested an allele that has the C-terminal end of *ppk* and the N-terminal end of *ppx* (the second gene in the operon) replaced by a kanamycin-resistance (Kn^R) cassette (Δ*ppk*::Kn); a strain carrying this allele was shown to have 1.5% of the Ppk and 22% of the Ppx activities of the wild-type strain (Kuroda *et al*., 1997). When transduced into FC40, the Δ*ppk*::Kn allele conferred a phenotype similar to that conferred by *ppk*::Cm (Fig. 1). To test whether the phenotype conferred by *ppk*::Cm is due solely to loss of *ppk* instead of to a polar effect on *ppx*, we deleted *ppx* from codon 94 to the end of its coding sequence and inserted a Kn^R gene to create Δ*ppx*::Kn. The Δ*ppx*::Kn allele did not affect adaptive mutation (Fig. 1). Thus, wild-type *ppk*, but not *ppx*, is required for normal levels of adaptive mutation.

To determine whether *ppk* and *dinB* are in the same pathway for adaptive mutation, we performed an epistasis test comparing adaptive mutation in the *ppk*, *dinB* and *ppk dinB* double mutant strains. Figure 2A shows that the double mutant strain had approximately half of the level of adaptive mutation as did the *dinB* mutant strain, indicating that most but not all of the Ppk antimutator effect can be attributed to decreased Pol IV activity. None of these mutant backgrounds affected cell viability on lactose plates (Fig. 2B).

Intermediate levels of polyP appear to be required for optimal levels of adaptive mutation

Polyphosphate kinase produces long chains of inorganic polyP and other studies have shown that *ppk* mutant strains have decreased amounts of polyP (Shiba *et al*., 1997). To test whether the observed decrease in adaptive mutation resulted from a lack of polyP, we attempted to complement the *ppk*::Cm defect with a multicopy plasmid (pBR322) carrying *ppk* under control of its own promoter. Presumably expressing *ppk* on a multicopy plasmid would increase polyP levels, especially in a strain without functional *ppx*. Surprisingly, the presence of this plasmid did not complement *ppk*::Cm for adaptive mutation (Fig. 3). When the same plasmid was transformed into the wild-type strain, the adaptive mutation levels were not affected (Fig. 3). Cell viability did not change over the course of the experiment (data not shown), indicating that overexpressing *ppk* is not toxic. In addition, the amount of Pol IV measured by Western blot analysis was not affected by overexpression of *ppk* (data not shown). As the *ppk*::Cm mutation is polar on *ppx*, these results suggest that when *ppk* is overexpressed, Ppx activity is required for normal levels of adaptive mutation. This conclusion was further supported by the fact that overexpressing *ppk* in the Δ*ppx*::Kn mutant strain also inhibited adaptive mutation (Fig. 3). As Ppx degrades polyP, overexpressing *ppk* in the Δ*ppx*::Kn mutant background

should result in relatively high levels of polyP. These results suggest that adaptive mutation is inhibited both when polyP levels are high and when they are low.

Polyphosphate kinase does not affect the amount of Pol IV protein

Adaptive mutation could be low in *ppk* mutant strains because either the amount or the activity of Pol IV is decreased. For example, polyP levels have been shown to be important in transcription of *rpoS*, which encodes the RNA polymerase sigma factor that regulates the stationary-phase response (Shiba *et al*., 1997). Pol IV protein is induced about threefold during stationary phase, and this induction is dependent on *rpoS* (Layton and Foster, 2003). Therefore, it was possible that in *ppk* mutant strains, RpoS transcription is low and the RpoS-dependent induction of *dinB* is attenuated. To test this hypothesis, we incubated Δ*recG*, Δ*recG ppk*::Cm and Δ*recG rpoS*::Cm strains in minimal glycerol medium for 3 days. Samples were taken on days 2 and 3, and the amounts of Pol IV measured by Western blot analysis. Unlike *rpoS*::Cm mutant cells, *ppk*::Cm mutant cells had the same amount of Pol IV as wild-type cells after 3 days of continuous incubation (Fig. 4). This result indicates that the *ppk* antimutator phenotype is not due to a reduction in the amount of Pol IV or an increase in its degradation. Furthermore, the *ppk* antimutator phenotype is not exerted via *rpoS*.

Loss of Ppk inhibits growth-dependent mutations due to overexpression of Pol IV

Because there are other factors in addition to Pol IV that affect adaptive mutation (see *Introduction*), we used another assay that is independent of adaptive mutation to test the effect of Ppk on Pol IV activity. The rate of mutation in growing cells can increase up to 800-fold when Pol IV is overexpressed (Kim *et al*., 1997). Growth-dependent mutations giving resistance to tetracycline (Tc^R) can be measured easily in strain FC722, which has a +1 frameshift mutation in the *tetA* gene carried on its episome (Foster, 1997). Overexpression of Pol IV under control of an exogenous promoter on a multicopy plasmid resulted in an eightfold increase in the mutation rate to Tc^R (Fig. 5). The same plasmid carried by the *ppk*::Cm mutant strain resulted in only a threefold increase in the mutation rate to Tc^R (Fig. 5). The amount of Pol IV in these strains as determined by Western blot analysis was not affected by the *ppk*::Cm mutation (data not shown). These results indicate that Ppk is necessary for optimally high mutation rates due to Pol IV whether these mutations occur during growth or during stationary phase. Furthermore, as the *ppk*::Cam mutation affects Pol IV activity even when *dinB* is being transcribed from an exogenous promoter, Ppk is not exerting its effects by regulating *dinB* transcription.

Polyphosphate kinase does not affect the β-galactosidase levels of Lac^+ revertants

Both adaptive mutation to Lac^+ and growth-dependent mutation to Tc^R are dependent on the cell's ability to replicate the episome, transcribe the genes encoding the mutational targets and translate the corresponding proteins. To test the possibility that *ppk* mutants are defective in one of these processes, we mated a Lac^+ episome into the *ppk*::Cm mutant and wild-type strains and compared the β-galactosidase activities. The small (approximately 10%) difference in β-galactosidase activity between these strains is not statistically significant ($t = 1.49$, $P = 0.20$) (Table 1). As the amount of β-galactosidase (which is expressed solely from the episome) is nearly the same regardless of *ppk* activity, we conclude that *ppk* does not affect the copy number of the *lacZ* gene or the episome.

Overexpressing RecA does not suppress the ppk antimutator phenotype

Adaptive mutation is dependent on the recombination functions of RecA. Previous studies have shown that strains with no detectable polyP have decreased levels of the *recA* transcript (Tsutsumi *et al*., 2000). As expected, the levels of RecA protein were slightly lower in *ppk*::Cm mutant cells than in wild-type cells (Fig. 6A). To test whether this reduction was

responsible for the *ppk* antimutator phenotype, a multicopy plasmid encoding RecA was transformed into FC40 and its *ppk*::Cm derivative; the resulting strains were tested for adaptive mutation. As shown in Fig. 6B, overexpressing RecA did not suppress the *ppk*::Cm antimutator phenotype for adaptive mutation, indicating that the antimutator phenotype does not result from low levels of RecA.

Polyphosphate kinase also affects Pol V-dependent mutagenesis

In *E. coli*, Pol V (encoded by the *umuDC* genes) is necessary for mutagenesis by DNA-damaging agents such as UV light (Tang *et al*., 2000). To test whether Ppk affects Pol V activity, we assayed UV-induced mutagenesis to nalidixic acid resistance (Nal^R), which occurs by base substitutions in the genes that encode DNA gyrase (Yoshida *et al*., 1988). The *ppk*::Cm mutant strain had a 40% decrease in UV mutagenesis to Nal^R compared with the wild-type strain, but the *ppk*::Cm mutation had no effect in a *umuDC* mutant strain, which is missing Pol V (Fig. 7). These results indicate that Ppk not only affects Pol IV, but also affects the mutagenic activity of Pol V.

Discussion

Escherichia coli DNA Pol IV belongs to a family of error-prone polymerases some of whose members perform translesion DNA synthesis. A second important function for these polymerases may be to restart stalled replication forks (Goodman, 2002). It is hypothesized that error-prone polymerases gain access to replication forks by interacting with the β-clamp of DNA polymerase III (Wagner *et al*., 2000). Purified Pol IV can replicate only a few nucleotides by itself, but when interacting with the β-clamp it can replicate as many as 400 nucleotides (Tang *et al*., 2000;Wagner *et al*., 2000). Because many proteins (polymerase and others) bind to the β-clamp (Lopez de Saro *et al*., 2003), the polymerase that is most available may be the one targeted to the replication fork when normal replication stalls (Goodman, 2002). Polymerases II, IV and V are all induced in response to DNA damage (Goodman, 2002). Pol IV is also induced late in stationary phase independently of the SOS response (Layton and Foster, 2003). As there are about 250–1000 molecules of Pol IV in a non-induced cell and at least 2500 molecules in SOS-induced cells (Kim *et al*., 2001), there would appear to be enough molecules of Pol IV under most conditions to compete with other polymerases for the replication fork. The results presented here suggest that the mutagenic activity of Pol IV is modulated by Ppk and the amount of cellular polyP.

The *ppk* mutant derivative of the wild-type strain, FC40, exhibited a threefold decrease in adaptive mutation with no loss in cell viability (Fig. 2). Pol IV is responsible for up to 80% of the adaptive mutations in the wild-type strain (Foster, 2000;McKenzie *et al*., 2001); in *recG* mutants, adaptive mutation is increased 10- to 100-fold (Foster *et al*., 1996;Harris *et al*., 1996), and all these extra mutations result from Pol IV (Layton and Foster, 2003). As expected, defects in *ppk* had a relatively larger effect (fivefold decrease) in *recG* mutant strains because more of the mutations result from Pol IV. An epistasis test in $recG^+$ strains showed that the *ppk dinB* double mutant had a greater reduction in adaptive mutation than the *dinB* single mutant (Fig. 2A), suggesting that Ppk has yet an additional effect on adaptive mutation. However, the increase in growth-dependent mutations caused by overexpression of Pol IV was also reduced by the *ppk* mutation, independently indicating that Ppk affects Pol IV mutagenic activity. Finally, we demonstrated that the activity of β-galactosidase expressed from the episome is not affected by loss of Ppk, eliminating the trivial possibility that Ppk affects the number or expression of the mutational targets (episomal *lacZ* or *tetA*). All of these results indicate that Ppk and/or its product, polyP, directly or indirectly affects the ability of Pol IV to cause mutations. Ppk also affects Pol V-dependent UV mutagenesis, suggesting that mutagenesis of all Y-family polymerases may be regulated by Ppk. However, we do not know

as yet whether it is the polymerase activity or the fidelity of polymerization that is affected. In addition, it is possible that loss of Ppk reduces the amount of Pol V after UV damage.

The fact that loss of Ppk inhibited Pol IV-dependent mutation during growth is especially intriguing. Ppk is known to affect cells under stress (UV irradiation, nutritional downshift, amino acid starvation), but our results were obtained while the cells were growing in rich medium. The target for mutations during growth was reversion of a frameshift mutation in the *tetA* gene carried on the episome. Preliminary results show that when the same target for mutations is on the chromosome, the *ppk* mutant allele did not reduce growth-dependent mutations caused by overexpression of Pol IV (data not shown). One explanation for this difference is that Pol IV is more likely to be involved in DNA synthesis on the episome than on the chromosome. For example, Pol IV may participate in recombination-dependent DNA synthesis, which may occur more often on the episome because of the episome's high frequency of recombination (Seifert and Porter, 1984;Carter *et al*., 1992). PolyP could facilitate the recruitment of Pol IV for DNA synthesis primed by recombination intermediates. If this hypothesis is true, *ppk* and *recA* should be epistatic for reduction of growth-dependent mutations produced by Pol IV overexpression, a hypothesis which we are currently testing.

The only known function for Ppk is the production of chains of inorganic polyP. In *ppk* mutants, the cellular levels of long-chained polyP molecules (about 600 inorganic phosphate residues) are reduced, but detectable levels of short-chained polyP molecules (about 60–70 inorganic phosphate residues) remain (Castuma *et al*., 1995). The source of these short-chained polyP molecules in *ppk* mutant strains is unknown (Castuma *et al*., 1995). When Ppx is overexpressed, all classes of polyP are undetectable (Shiba *et al*., 1997). Strains that overexpress Ppx have lower levels of RecA during SOS induction than do wild-type cells (Tsutsumi *et al*., 2000). We found that *ppk* mutant cells also have less RecA protein even when not induced for SOS (Fig. 6A). Adaptive mutation does not occur in the absence of RecA (Foster, 1993); however, as overexpressing RecA from a multicopy plasmid did not suppress the *ppk* mutant phenotype (Fig. 6B), we conclude that the *ppk* antimutator effect does not result from low levels of RecA. Thus, although RecA levels are decreased in a *ppk* mutant strain, they appear to be sufficient for adaptive mutation. Note that having more RecA did not increase mutation in the wild-type strain, suggesting that RecA is normally not limiting for adaptive mutation.

Bacterial strains that overproduce Ppx also have decreased levels of RpoS (Shiba *et al*., 1997). As the induction of Pol IV in late stationary phase is dependent on RpoS (Layton and Foster, 2003), it was possible that RpoS levels were low in *ppk* mutants, resulting in less Pol IV. However, the results shown in Fig. 4 indicate that in late stationary phase *ppk* mutant cells have the same amount of Pol IV protein as do wild-type cells. Thus, the amount of RpoS in *ppk* mutant strains is sufficient to keep the levels of Pol IV normal. RpoS also affects adaptive mutation independently of Pol IV (Layton and Foster, 2003,Lombardo *et al*., 2004); therefore, it is possible that Ppk affects these other RpoS-dependent functions in adaptive mutation, which would explain why the *ppk*::Cm mutant has an antimutator phenotype even in the absence of Pol IV (Fig. 2A). However, it is clear that the majority of the effect of Ppk on adaptive mutation is exerted on Pol IV independently of RpoS.

The simplest explanation for our results is that polyP modulates the mutagenic activity of Pol IV by affecting its ability to synthesize DNA, the accuracy at which it does so, or both. While overexpressing Ppk had no effect on adaptive mutation in a strain with functional Ppx, it strongly inhibited adaptive mutation in a strain with no Ppx (Fig. 3). As Ppx degrades polyP, these results, together with the antimutagenic phenotype of *ppk* mutants, suggest that there is an optimal level of polyP for maximum expression of Pol IV's mutagenic activity. At this optimal level, polyP may be itself a positive activator of Pol IV's mutagenic activity, or it may act indirectly by activating an activator or inhibiting an inhibitor of Pol IV's mutagenic activity.

It is also possible that the activity of Pol IV is affected by the loss of Ppk protein itself (although the antimutagenic effect of overproducing Ppk must have some other cause). For example, Ppk is one of several proteins that bind to the C-terminus of RNase E to form the RNA degradosome that regulates mRNA turnover (Blum *et al*., 1997;Carpousis, 2002;Bernstein *et al*., 2004). Thus, Ppk could indirectly regulate Pol IV by changing the mRNA levels of a direct regulator of Pol IV. However, the function of Ppk in the RNA degradosome has not been clearly defined (Blum *et al*., 1997). In addition, a C-terminus deletion of *rne*, the gene that encodes RNase E, does not inhibit adaptive mutation (J.D. Stumpf and P.L. Foster, unpubl. data).

PolyP has been shown to inhibit *Thermus aquaticus* DNA polymerase activity *in vitro* (Rodriguez, 1993), so another possibility is that polyP directly interacts with Pol IV and either inhibits its ability to synthesize DNA or reduces the accuracy at which it does so. As polyP is a long, negatively charged molecule like DNA, it may interact with many DNA-binding proteins. As mentioned above, polyP affects RpoS, which binds to DNA, and the RNA degradosome, which binds to RNA. Recently, polyP and DNA have been shown to compete for the same binding region of the Lon protease; polyP binding is hypothesized to activate Lon by freeing it from the DNA (Nomura *et al*., 2004). If polyP competes with DNA or RNA for binding sites to other proteins, this may explain how polyP levels influence many different cellular processes, including DNA replication. Future experiments are needed to determine whether polyP binds to DNA polymerases, or to factors that affect them, and whether this binding regulates the amount or accuracy of DNA synthesis.

Experimental procedures

Bacterial strains

All bacterial strains are *E. coli* K12 derivatives and are listed in Table 2. Genetic manipulations were as described previously (Miller, 1992). The *ppk*::Cm insertion was isolated in a screen for antimutators that was previously described (Layton and Foster, 2003). The Δ*ppx*::Kn allele was created using the techniques described by Datsenko and Wanner (2001). Mutant alleles were transferred by P1 transduction, selecting for appropriate markers. PFG282 was constructed by mating a Lac^+ episome into PFG281 (FC36 *ppk*::Cm) and selecting for Pro^+.

Plasmids

The plasmids used are listed in Table 2. Standard molecular biology techniques were as described (Ausubel *et al*., 1988). To make the *ppk*-overexpressing plasmid, *ppk* was amplified with primers whose sequences were CAGCCGGATCCCTG TAAATCGCAAGCTCC and GCCGAAAGCTTTTTGAAC CAAGATCGACC; the resulting polymerase chain reaction (PCR) fragment contained the whole *ppk* gene plus 121 nucleotides before the start codon and 69 nucleotides after the stop codon. The fragment was digested and ligated into BamHI- and HindIII-digested pBR322. To make pPFG96, *dinB* was amplified with primers whose sequences were GCCGATATAGAATTCATGCGTAAAATCATTCATGTGGATA and GCAGCCAAGCTTTCATCATAATCCCAGCACCAGTT GTC; the resulting PCR fragment contained the entire *dinB* gene and two nucleotides past the stop codon. The fragment was digested and ligated into BamHI- and EcoRI-digested pBAD24. That *dinB* was under control of the *araBAD* promoter was confirmed by Western blot analysis (data not shown), which showed that Pol IV was expressed from the plasmid at much higher levels than from the exogenous genes even in the absence of arabinose.

β-Galactosidase assays

Four independent cultures of each strain were grown to saturation in M9 glycerol minimal medium at 37°C. β-Galactosi-dase assays were performed as described (Miller, 1992).

Mutation experiments

Media and protocols were as described previously (Cairns and Foster, 1991;Miller, 1992;Foster, 1994;Foster *et al.*, 1996). When required, antibiotics were added to rich media at the following concentrations: carbinicillin, 100 μg ml^{-1}; kanamycin, 30 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; nalidixic acid, 40 μg ml^{-1}; rifampicin, 100 μg ml^{-1}; when added to minimal media, antibiotics were added at half of the above concentrations except for chloramphenicol. For small-scale adaptive mutation experiments (Figs 1,3 and 5), approximately 10^7 cells of each strain were spread on each quadrant of a lactose minimal plate, which was then incubated for 5 days at 37°C (Foster *et al.*, 1996). Lac^+ colonies that arose each day were counted, and the results are given as the total number of Lac^+ colonies appearing from days 3–5. For the large-scale adaptive mutation experiment (Fig. 2A), lactose minimal plates were spread with about 10^8 cells of strains FC40 and PFG74 plus 10^9 cells of FC29 scavenger cells or 10^9 cells of strains PFB243 and PFG274 and no scavenger cells. Lac^+ colonies that arose each day were counted. To determine the viability of the Lac^- cells while incubating on lactose, plugs were removed from between Lac^+ colonies and the number of viable cells was determined by spreading appropriate dilutions on LB plus rifampicin plates (which selects against FC29). To calculate the number of Lac^+ revertants per cell, the number of Lac^+ colonies was divided by the mean number of Lac^- cells plated (Cairns and Foster, 1991). Statistical calculations were as given in Zar (1984).

To determine growth-dependent mutation rates (Fig. 5), overnight cultures of each strain were diluted 10^5-fold into LB broth plus appropriate antibiotics and 20 independent 1 ml aliquots were grown at 37°C overnight. A sample of 0.1 ml (approximately 10^8 cells) of each culture was spread on an LB plus tetracycline (Tc) plate and incubated at 37°C; the colonies arising after 24 h were counted. Mutation rates and confidence limits were calculated using the Lea–Coulson method of the median as described in Rosche and Foster (2000).

For UV mutagenesis (Fig. 7), a saturated culture of each strain was diluted 10^5-fold into LB broth plus appropriate drugs, and eight independent 1 ml aliquots were distributed and grown overnight at 37°C. An aliquot of 0.5 ml of each culture was added to 4.5 ml of 0.1 M $MgCl_2$ in a glass Petri plate and irradiated with agitation for 25 s with UV light at 1.6 J m^{-2} s^{-1}. Samples of the cultures were taken before and after irradiation, diluted, and plated on LB plates to determine the fraction of survivors, which ranged from 54% to 68% (data not shown). Before and after irradiation, 0.5 ml aliquots of each culture were diluted into 4.5 ml of LB broth and incubated at 37°C with aeration for at least 4 h. After this outgrowth, a sample of 0.1 ml (approximately 10^8 cells) was plated on LB plus nalidixic acid plate and incubated at 37°C for 24 h. Samples of the outgrown cultures were also diluted and plated on LB medium to determine total cell numbers. Results are given as the mean number of Nal^R mutants per 10^8 cells.

Molecular biology techniques

Molecular biology techniques were used as previously described (Ausubel *et al.*, 1988). For immunoblots, cultures were grown in minimal glycerol medium plus appropriate drugs at 37° C and samples were taken 2 and 3 days after inoculation. Samples were centrifuged, and the cells were resuspended in SDS-PAGE sample loading buffer without dye and boiled for 15 min. Total protein was determined in each sample by Bradford assays (Bio-Rad Laboratories). Aliquots containing 40 μg of protein were diluted into sample loading buffer with dye and loaded onto an SDS 12% polyacrylamide gel. Proteins were separated by electrophoresis and then electrotransferred to Immobilon-P membranes (pore size, 0.45 μm; Millipore). The membranes were probed with rabbit anti-Pol IV polyclonal antiserum (obtained from H. Ohmori) or rabbit anti-RecA polyclonal antiserum (obtained from R. Woodgate). The reactions were visualized using alkaline phosphatase-conjugated goat anti-rabbit secondary antibody and developed using the Western-light chemiluminescence reagent (Applied Biosystems).

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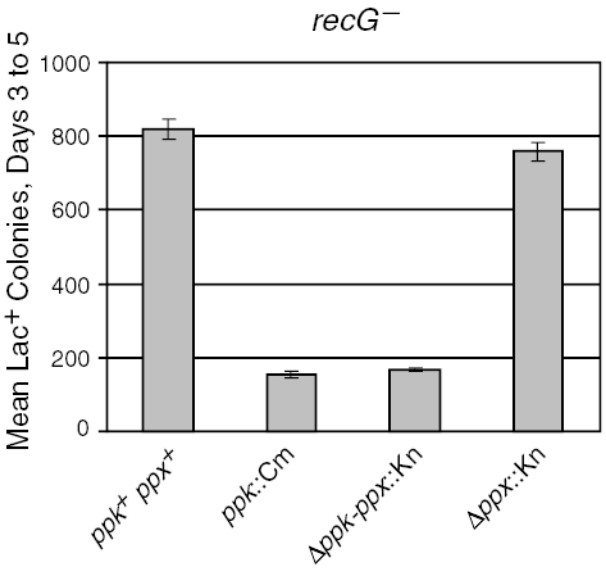

Fig 1.

Disrupting the *ppk* gene reduces Lac$^+$ adaptive mutation. Approximately 10^7 cells from four independent cultures were spread on each quadrant of a lactose minimal plate in a small-scale experiment (see *Experimental procedures*). The data are the mean of the total number (±SEM) of Lac$^+$ colonies appearing on days 3–5. All strains are *recG* mutants. *ppk*$^+$*ppx*$^+$ = FC526, *ppk*::Cm = PFG39, Δ*ppk-ppx*::Kn = PFG203, Δ*ppx*::Kn = PFG162.

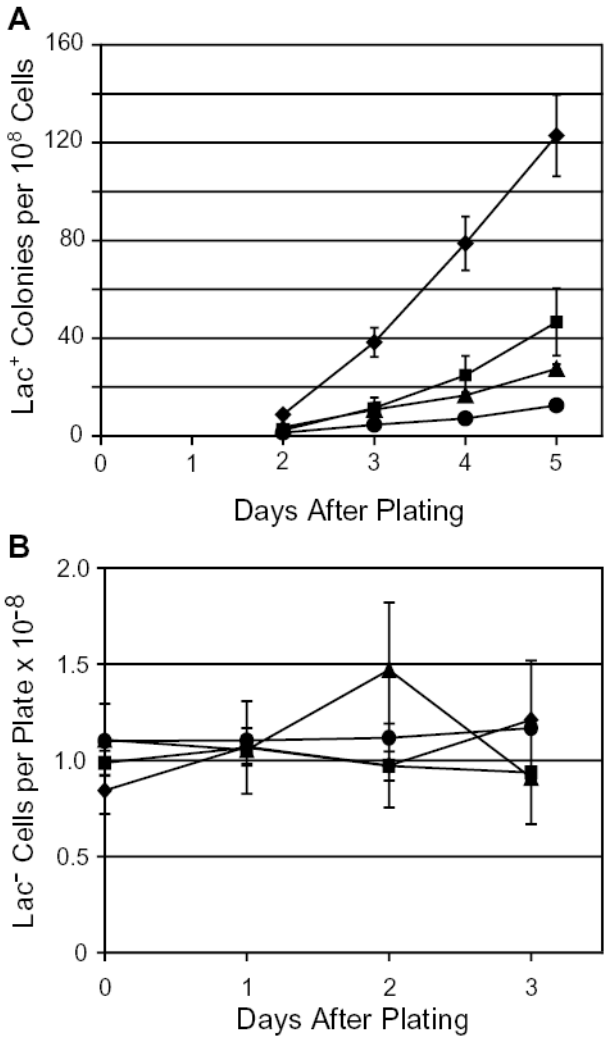

Fig 2.

Most of the effect of *ppk* on adaptive mutation results from decreased Pol IV activity.

A. The cumulative number of Lac^+ revertants per 10^8 Lac^- cells. About 10^8 cells from six independent cultures of FC40 and PFG74 (*ppk*), and 10^9 cells from six independent cultures of PFB243 (*dinB*) and PFG274 (*ppk dinB*) were plated on lactose minimal plates. Data points are the means (±SEM). ♦, wild type = FC40; ▪, *ppk*::Cm = PFG74; ▲, Δ*dinB*::Zeo = PFB243; •, *ppk*::Cm Δ*dinB*::Zeo = PFG274.

B. The viability of the strains during incubation on lactose plates. Each day, three plates per strain were used to determine the number of viable Lac^- cells (see *Experimental procedures*). Data points are the means ± SEM. Symbols are the same as in (A).

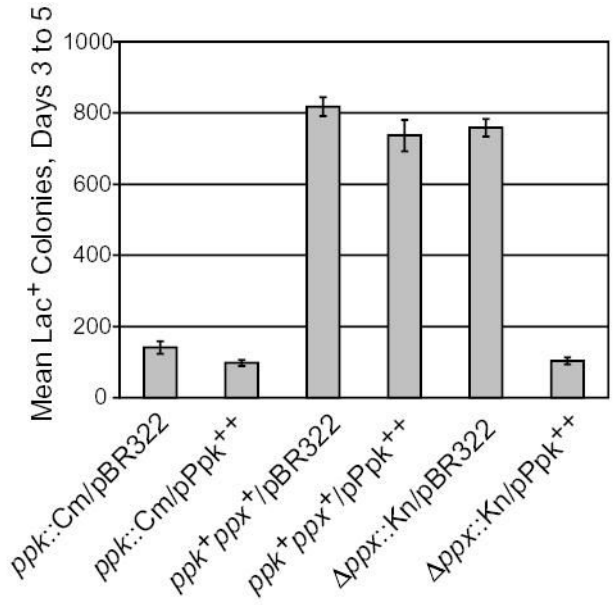

Fig 3.

Overexpressing Ppk is antimutagenic in *ppx* mutant cells. Approximately 10^7 cells from four independent cultures were spread on each quadrant of a lactose minimal plate in a small-scale adaptive mutation experiment (see *Experimental procedures*). The data are the means of the total number (±SEM) of Lac^+ colonies appearing on days 3–5. All strains are *recG* mutants. *ppk*::Cm/pBR322 = PFG87; *ppk*::Cm/pPpk^{++} = PFG176; *ppk*$^+$*ppx*$^+$/pBR322 = PFG89; *ppk*$^+$*ppx*$^+$/pPpk^{++} = PFG175; Δ*ppx*::Kn/pBR322 = PFG323; Δ*ppx*::Kn/pPpk^{++} = PFG322.

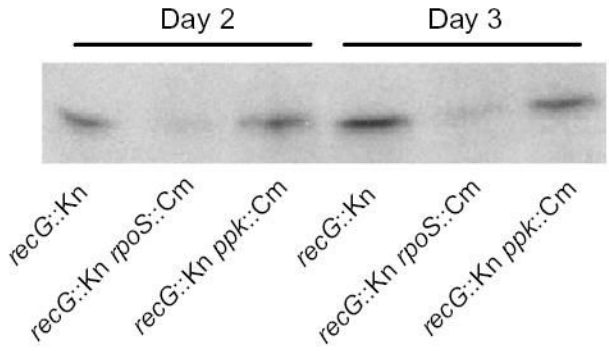

Fig 4.

The amount of Pol IV declines in an *rpoS* mutant strain but not in wild type or a *ppk* mutant strain during prolonged incubation in minimal medium. Samples were taken 2 and 3 days after inoculation and equal amounts of total protein from each strain were loaded in each lane. Pol IV was detected by immunoblotting. Δ*recG*::Kn = FC526; Δ*recG*::Kn *rpoS*::Cm = PFG36; Δ*recG*::Kn *ppk*::Cm = PFG39.

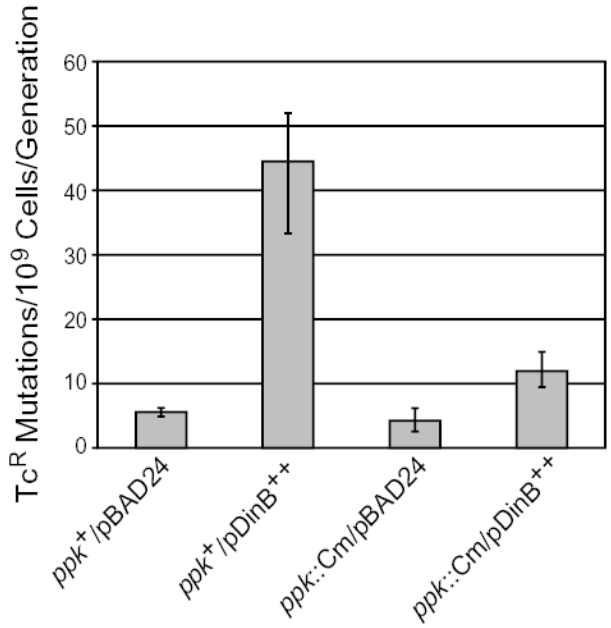

Fig 5.

The *ppk* mutant allele reduces the growth-dependent mutation rate due to overexpression of Pol IV. Values are mutation rates to tetracycline resistance; error bars are 95% confidence levels. *ppk*$^{+}$/pBAD24 = PFG265; *ppk*$^{+}$/pDinB^{++} = PFG266; *ppk*::Cm/pBAD24 = PFG267; *ppk*::Cm/pDinB^{++} = PFG268.

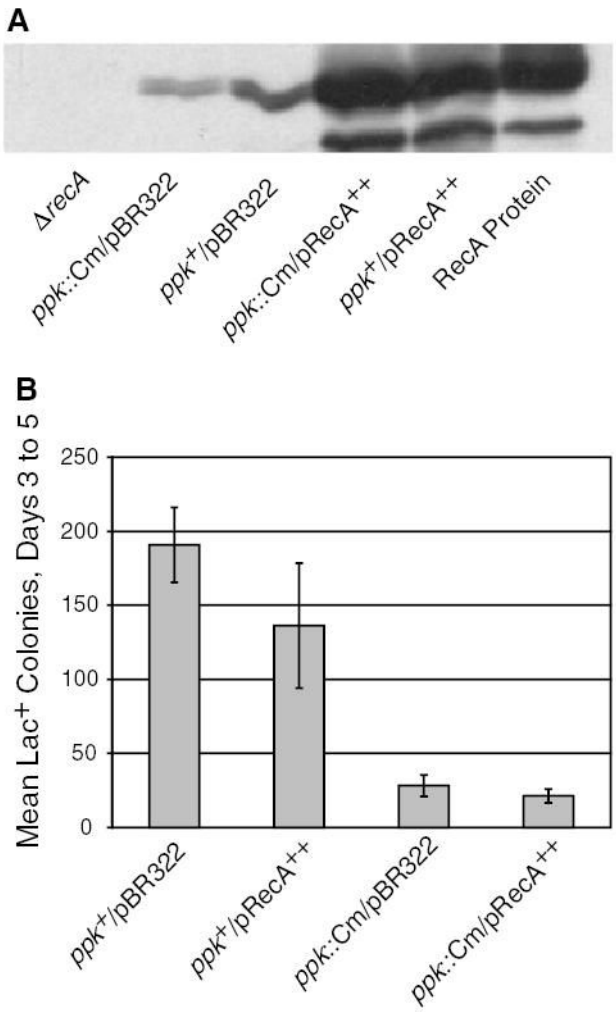

Fig 6.

Overexpression of RecA does not suppress the *ppk* antimutator phenotype.

A. Western blot showing that a *ppk* mutant strain has less RecA than a *ppk*$^+$ strain, but not if RecA is overexpressed from a plasmid. Equal amounts of total protein from each strain were loaded in each lane; 10 μg of purified RecA protein (New England Biolabs) was loaded in the right lane. (The lower band is probably a degradation product).

B. Overexpression of RecA does not suppress the *ppk* mutant phenotype for adaptive mutation. Approximately 10^7 cells were spread on each quadrant of a lactose minimal plate. The data are the means of the total number (±SEM) of Lac$^+$ colonies appearing on days 3–5. Δ*recA* = FC348; *ppk*::Cm/pBR322 = PFG88; *ppk*$^+$/pBR322 = PFG90; *ppk*::Cm/pRecA^{++} = PFG92; *ppk*$^+$/pRecA^{++} = PFG94.

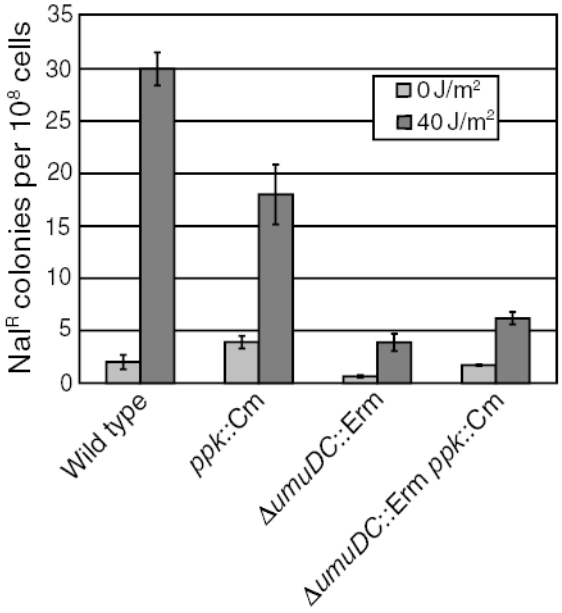

Fig 7.

The *ppk* mutant allele reduces UV mutagenesis when Pol V is present. Data points are the mean (±SEM) of Nal^R mutants per 10^8 cells. Wild type = FC40; *ppk*::Cm = PFG74; Δ*umuDC*::Erm = PFB286; Δ*umuDC*::Erm *ppk*::Cm = PFG414.

Table 1

β-Galactosidase activity of wild-type and *ppk* mutant strains.

Strain[a]	Relevant genotype	β-Galactosidase activity (Miller units)[b]	SEM
FC420	*ppk*$^+$	341	19.8
PFG282	*ppk*::Cm	306	11.7

[a] Lac$^+$ derivatives of the wild-type and *ppk* mutant strains.

[b] Four independent cultures for each strain were assayed. Data are the mean and SEM.

Table 2

Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant genotype	Source/reference
E. coli strains		
FC29	*ara* Δ(*lac-proB*)$_{XIII}$ *thi*/F′ Δ (*lacIZ*) Pro$^+$	Cairns and Foster (1991)
FC36	F$^-$ *ara* Δ (*lac-proB*)$_{XIII}$ *thi* Rif^R	Cairns and Foster (1991)
FC40	FC36/F′ Φ(*lacI33-lacZ*) Pro$^+$	Cairns and Foster (1991)
FC348	FC40 Δ*recA srl*::Tn*10*	Berardini *et al*. (1999)
FC420	FC36/F′ Lac$^+$ Pro$^+$	This study
FC438	FC40 *recG162 zib*636::Tn*10*	Foster *et al*. (1996)
FC526	FC40 Δ*recG*263::Kn	Foster *et al*. (1996)
FC722	FC40 with a Tc^S allele on the episome	Foster (1997)
PFB243	FC40 *dinB*::Zeo on chromosome and episome	Layton and Foster (2003)
PFB286	FC40 Δ*umuDC*::Erm	Frank *et al*. (1996)
PFG36	FC526 *rpoS*::miniTn*10* Cm	Layton and Foster (2003)
PFG39	FC526 *ppk*::miniTn*10* Cm	This study
PFG74	FC40 *ppk*::miniTn*10* Cm	This study
PFG87	PFG39/pBR322	This study
PFG88	PFG74/pBR322	This study
PFG89	FC526/pBR322	This study
PFG90	FC40/pBR322	This study
PFG92	PFG74/pPF2041	This study
PFG94	FC40/pPF2041	This study
PFG152	FC40 Δ*ppk*::Kn	This study
PFG162	FC438 Δ*ppx*::Kn	This study
PFG175	FC526/pPFG95	This study
PFG176	PFG39/pPFG95	This study
PFG202	FC722 *ppk*::miniTn*10* Cm	This study
PFG203	FC438 Δ*ppk-ppx35*::Kn	Kuroda *et al*. (1997); this study
PFG265	FC722/pBAD24	This study
PFG266	FC722/pPFG96	This study
PFG267	PFG202/pBAD24	This study
PFG268	PFG202/pPFG96	This study
PFG274	PFB243 *ppk*::miniTn*10* Cm	This study
PFG281	FC36 *ppk*::miniTn*10* Cm	This study
PFG282	PFG281/F′ Lac$^+$ Pro$^+$	This study
PFG322	PFG162/pPFG95	This study
PFG323	PFG162/pBR322	This study
PFG414	PFB286 *ppk*::miniTn*10* Cm	This study
Plasmids		
pBR322	None	Bolivar *et al*. (1977)
pPFG95	*ppk*$^+$ on pBR322	This study
pPF2041	*recA*$^+$ on pBR322	R. Brent (pers. comm.)
pBAD24	None	Guzman *et al*. (1995)
pPFG96	*dinB* $^+$ on pBAD24	This study