

# A role for topoisomerase III in a recombination pathway alternative to RuvABC

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## Summary

**The physiological role of topoisomerase III is unclear for any organism. We show here that the removal of topoisomerase III in temperature sensitive topoisomerase IV mutants in *Escherichia coli* results in inviability at the permissive temperature. The removal of topoisomerase III has no effect on the accumulation of catenated intermediates of DNA replication, even when topoisomerase IV activity is removed. Either *recQ* or *recA* null mutations, but not *helD* null or *lexA3*, partially rescued the synthetic lethality of the double topoisomerase III/IV mutant, indicating a role for topoisomerase III in recombination. We find a bias against deleting the gene encoding topoisomerase III in *ruvC53* or  $\Delta$ *ruvABC* backgrounds compared with the isogenic wild-type strains. The topoisomerase III RuvC double mutants that can be constructed are five- to 10-fold more sensitive to UV irradiation and mitomycin C treatment and are twofold less efficient in transduction efficiency than *ruvC53* mutants. The overexpression of *ruvABC* allows the construction of the topoisomerase III/IV double**

**mutant. These data are consistent with a role for topoisomerase III in disentangling recombination intermediates as an alternative to RuvABC to maintain the stability of the genome.**

## Introduction

Topoisomerases are ubiquitous enzymes that are required for nearly all aspects of nucleic acid metabolism (Champoux, 2001; Wang, 2002; Corbett and Berger, 2004). Becoming covalently bound to the DNA, topoisomerases make transient breaks in DNA, pass DNA strands through the breaks, and then reseal the DNA. Topoisomerases allow cells to modulate three topological forms of DNA: linking number (Lk) (the number of times the two strands of DNA are intertwined), catenanes (intermolecular linking of multiple DNA molecules), and knots (intramolecular tangling of one DNA molecule). These enzymes are divided into two groups: those that alter Lk in steps of one (type-1) and those that alter Lk in steps of two (type-2).

*Escherichia coli* contains four topoisomerases: two type-1 topoisomerases (topoisomerases I and III) (Sharma and Mondragon, 1995) and two type-2 topoisomerases (DNA gyrase and topoisomerase IV) (Levine *et al.*, 1998). The cellular roles for three of these have been established. Topoisomerase I (encoded by the *topA* gene), and topoisomerase IV (encoded by *parC* and *parE*) counter gyrase (encoded by *gyrA* and *gyrB*) to regulate DNA supercoiling levels (Zechiedrich *et al.*, 2000). Topoisomerase IV is also responsible for untangling catenanes and knots (Adams *et al.*, 1992; Zechiedrich and Cozzarelli, 1995; Zechiedrich *et al.*, 1997; Deibler *et al.*, 2001).

Topoisomerase III is well conserved across evolution, yet the *in vivo* function of topoisomerase III remains elusive (reviewed in Wang, 2002). Biochemical studies have shown that *E. coli* topoisomerase III relaxes negative supercoils and unties knots that are single-stranded or that contain single-stranded regions (DiGate and Mariani, 1988; Du *et al.*, 1995). The enzyme is most efficient at decatenating DNA substrates containing a nick or a gap (DiGate and Mariani, 1988). Topoisomerase III is also capable of unlinking what has been termed 'precatenanes' (Ullsperger *et al.*, 1995) that may occur during DNA replication (Hiasa and Mariani, 1994; Peter *et al.*, 1998; Nurse *et al.*, 2003). It has been reported that puri-

fied *E. coli* topoisomerase III and RecQ helicase together can catenate intact double-stranded supercoiled DNA (Harmon *et al.*, 1999). This was the first evidence that a type-1 topoisomerase is capable of performing a type-2 topoisomerase function with covalently closed double-stranded DNA. In addition, a genetic study has suggested that topoisomerase III and topoisomerase I activity might be required for chromosomal segregation following homologous recombination in *E. coli* (Zhu *et al.*, 2001). It has also been reported that the overexpression of *topB* can rescue cells lacking *topA* by disentangling R-loops (Broccoli *et al.*, 2000).

Additional data imply that topoisomerase III plays an important cellular role. *E. coli* cells lacking topoisomerase III display an increase in recombination between regions of short homology and an increase in frameshift mutations (Schofield *et al.*, 1992; Uematsu *et al.*, 1997). The consequences of the loss of topoisomerase III activity have been studied more extensively in eukaryotic cells. Mice lacking topoisomerase III $\alpha$  die during the early stages of embryogenesis and are reabsorbed (Li and Wang, 1998). Removal of the  $\beta$  isoform of topoisomerase III is better tolerated. Top3 $\beta^{-/-}$  mice survive to term, but have reduced lifespans and reduced fertility, compared with their wild-type littermates (Kwan and Wang, 2001). Further examination of cells from these mice revealed aneuploidy (Kwan *et al.*, 2003). *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* topoisomerase III null mutants sporulate at a lower efficiency than wild-type strains (Goodwin *et al.*, 1999). This defect in meiosis can be suppressed by preventing genetic recombination (Gangloff *et al.*, 1999). *S. cerevisiae* cells lacking topoisomerase III display an increase in deletions of short repetitive sequences, an increase in recombination at regions of short homologies, and also exhibit a significant increase in homologous recombination-dependent rearrangements of rDNA arrays (Gangloff *et al.*, 1994).

The phenotypes of topoisomerase III mutant cells are similar to the phenotypes of cells with mutations in the RecQ family of helicases. This helicase family includes the human BLM and WRN helicases, which are implicated in the Bloom and Werner syndromes (Ellis *et al.*, 1995), Sgs1 from *S. cerevisiae*, and Rqh1 from *S. pombe* (Gangloff *et al.*, 1994). Mutations in these RecQ homologues result in an increase in chromosomal translocations and recombination between regions of homologous sequences (Ellis *et al.*, 1995; Yu *et al.*, 1996; Hanada *et al.*, 1997; Stewart *et al.*, 1997; Myung *et al.*, 2001). In both yeast and humans, a physical interaction between topoisomerase III and RecQ helicases has been shown (Gangloff *et al.*, 1994; Wu *et al.*, 2000; Johnson *et al.*, 2000; Bennett *et al.*, 2000; Fricke *et al.*, 2001). Deletion of the genes encoding the RecQ homologues suppresses partially some of the defects in both budding and fission

yeast lacking topoisomerase III (Gangloff *et al.*, 1994). Based upon these data, it has been suggested that defects in topoisomerase III may be important in the human Bloom and Werner syndromes (Wu *et al.*, 1999). Topoisomerase III malfunction has also been implicated in Ataxia-telangiectasia, although it is unclear how (Fritz *et al.*, 1997; Ju and Muller, 2003). A common feature of these diseases is genomic instability, which can contribute to a predisposition to cancer.

The data presented here support a model in which RecA and RecQ create Holliday junction intermediates that topoisomerase III resolves in an alternate pathway to the RuvABC pathway. This topoisomerase-mediated resolution would always result in non-crossover recombination thus preventing genomic rearrangements associated with crossover recombination.

## Results

### *Effect of topoisomerase III on DNA supercoiling, decatenating and unknotting in vivo*

One possible cellular role of topoisomerase III is that it unlinks catenated intermediates of DNA replication (Hiasa and Mariani, 1994; Harmon *et al.*, 1999; Wu *et al.*, 1999; Harmon *et al.*, 2003; Nurse *et al.*, 2003). We tested this directly by measuring the level of catenanes in a plasmid substrate, pJB3.5d (Bliska and Cozzarelli, 1987), in the absence of topoisomerase III. If topoisomerase III unlinks catenanes, then replication intermediates should accumulate in the cell. In a strain lacking the gene encoding topoisomerase III (*topB*), there were no catenanes (data not shown). At the same time, catenanes accumulated in the positive control, an isogenic topoisomerase IV temperature sensitive mutant at the non-permissive temperature. Steady-state plasmid DNA knotting levels were identical in the presence or absence of topoisomerase III (data not shown).

We reasoned that the strong decatenating activity of topoisomerase IV could mask a small, but potentially important, role of topoisomerase III. Therefore, to measure catenane accumulation when both topoisomerase III and topoisomerase IV activities were removed, we transduced the  $\Delta topB$  allele into a strain that contains a norfloxacin-resistant allele of gyrase, *gyrAL83*. The production of catenanes requires DNA replication and, thus, a functional gyrase (Adams *et al.*, 1992). Normally, norfloxacin targets both gyrase and topoisomerase IV in *E. coli*, but topoisomerase IV is specifically inhibited and catenanes accumulate when the drug is added to the *gyrAL83*-containing strain (Khodursky *et al.*, 1995). Non-synchronous plasmid replication takes place at a steady state level. It takes mere seconds to complete one round of plasmid replication; at any given time, ~10% of the

plasmids are undergoing DNA replication (Adams *et al.*, 1992). In addition, the inhibition of topoisomerase IV with norfloxacin is not absolute. The enzyme retains activity at a rate  $\sim 1/100$  the normal level (Zechiedrich and Cozzarelli, 1995; Zechiedrich *et al.*, 1997). Consequently, DNA replication intermediates represent a snapshot of the steady-state replication process. This snapshot shows that the accumulation of catenated plasmid DNA was the same in both the *gyrAL83* (Fig. 1, lanes 2–6) and  $\Delta topB *gyrAL83* (Fig. 1, lanes 8–12) cells. Therefore, topoisomerase III does not appear normally to assist topoisomerase IV in unlinking catenated intermediates of plasmid DNA replication.$

We also measured DNA supercoil relaxation following gyrase inhibition with norfloxacin (Zechiedrich *et al.*, 2000) in the presence or absence of topoisomerase III (data not shown). The rate or extent of DNA supercoil relaxation by topoisomerases I and IV was identical with and without topoisomerase III. The rate or extent of DNA supercoil relaxation by topoisomerase I plus or minus topoisomerase III was also identical following inhibition of gyrase and topoisomerase IV activity with norfloxacin in the *gyrA<sup>+</sup> parC<sup>-</sup>* strain. The removal of topoisomerase IV activity alone with norfloxacin in the *gyrAL83 parC<sup>+</sup>* strain causes a shift to more negatively supercoiled plasmid DNA (Zechiedrich *et al.*, 2000). We found that the shift in the presence or absence of topoisomerase III was identical. Thus, topoisomerase III seems to play no role in regulating DNA supercoiling in *E. coli*.

#### Synthetic lethality of a topoisomerase III null allele with topoisomerase IV temperature-sensitive alleles

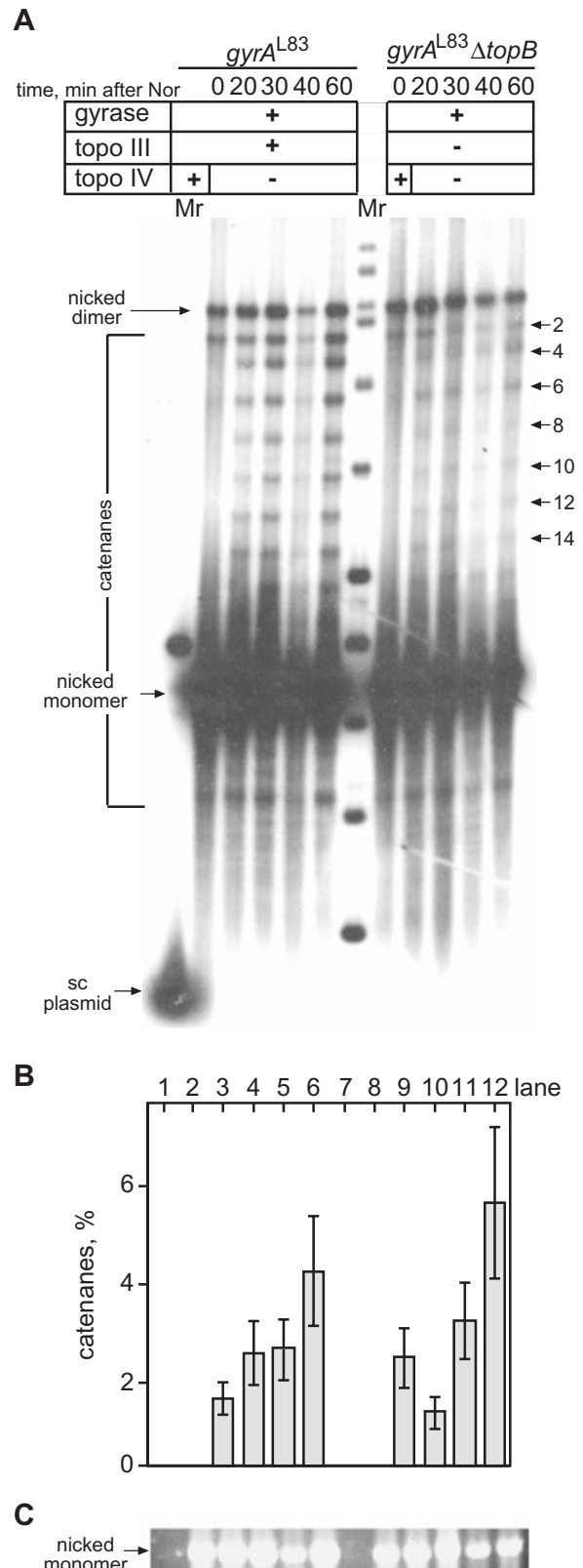
We attempted to transduce the *topB* deletion ( $\Delta topB::kan$ ) into a topoisomerase IV temperature-sensitive mutant, *parE<sup>ts</sup>*, at the permissive temperature, 30°C (Kato *et al.*, 1990). We obtained very few, slowly growing colonies that were not viable at 37°C (data not shown). This result indicates that the combination of the two mutant alleles

**Fig. 1.** Effect of *topB* on plasmid replication catenane accumulation.

A. Autoradiograph of the high resolution gel shown partly in (C). Strains (see Table 3) LZ5 (*gyrAL83*) and LZ2369 (*gyrAL83*  $\Delta topB$ ) were grown to mid-logarithmic phase. Before treating the cultures with norfloxacin, an aliquot of each culture was taken (0 min). After norfloxacin was added, aliquots were taken at the times indicated above each lane. The catenanes are indicated by arrows on the right of the gel.

B. Quantification of catenanes. The intensities of the four-noded catenanes were compared with the intensity of the nicked monomer in each lane using phosphorimager analysis. The same was done with the six-noded catenanes. The values (%) of these two comparisons were averaged and are shown. Error bars represent standard error of the mean. These results are typical of what was seen in two additional experiments.

C. Ethidium-bromide stained gel of nicked monomer to show relative DNA loading.



**Fig. 2.** Linkage efficiency.

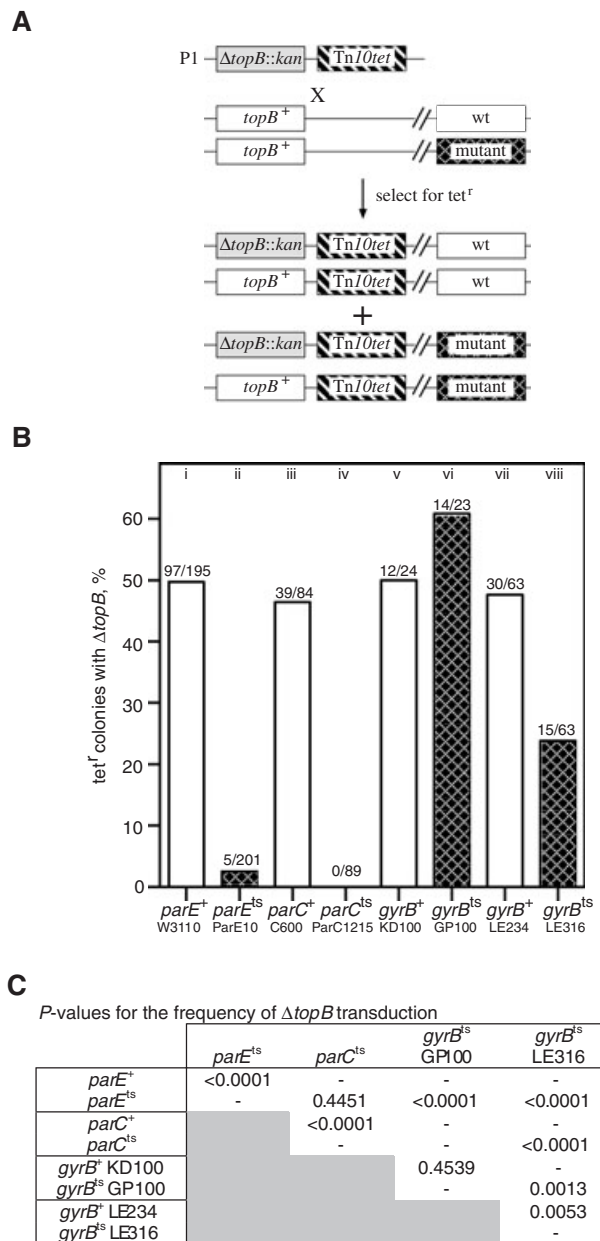
**A.** Schematic representation of the assay. A *Tn10tet* allele linked to  $\Delta topB::kan$  was transduced into various mutants and their isogenic wild-type strains. The transductants were selected for tetracycline resistance. These tetracycline-resistant colonies were then screened for kanamycin resistance ( $\Delta topB$ ). If there were a bias against combining the *topB* null allele with the mutant allele, a significant difference in linkage efficiency would be seen.

**B.** Efficiency of *topB* linkage in topoisomerase mutant strains. The experiment described in A was performed on topoisomerase IV (*parE<sup>ts</sup>* and *parC<sup>ts</sup>*) and two gyrase temperature-sensitive mutants (*gyrB<sup>ts</sup>*), as well as their isogenic wild-type strains (*parE<sup>+</sup>*, *parC<sup>+</sup>* and *gyrB<sup>+</sup>*). The strains tested are shown along the x-axis (and see Table 3), whereas the percentage of tetracycline-resistant colonies that were also kanamycin resistant is reported along the y-axis. The total number of tetracycline-resistant colonies screened and the number of those that contained the *topB* deletion is also shown above each bar. The general transduction frequency in *parE<sup>+</sup>* and *parE<sup>ts</sup>* strains was identical at 30°C (data not shown).

**C.** *P*-values for linkage efficiency results. To determine whether there was a significant difference between the number of mutant cells that were transduced with the  $\Delta topB::kan$  allele and the number of wild-type cells that contained the *topB* null allele, we performed a contingency chi-square test. We report the resultant *P*-values here in the form of a matrix. For example, the *P*-value for *parE<sup>+</sup>* and *parE<sup>ts</sup>* was <0.0001. Likewise, the *P*-value for *parC<sup>+</sup>* and *parC<sup>ts</sup>* was also <0.0001. *P*-values > 0.05 are considered insignificant.

was problematic to the cell. To test quantitatively whether the *topB* deletion could coexist with the topoisomerase IV mutation, we carried out genetic linkage experiments using bacteriophage P1-mediated transduction as outlined in Fig. 2A. In these experiments, an antibiotic resistance-encoding gene (*Tn10tet*) was transduced near  $\Delta topB::kan$  to make strain CRL6. We used CRL6 as the P1 donor to transduce *Tn10tet*  $\Delta topB::kan$  into cells containing either the *parE<sup>ts</sup>* allele or its isogenic wild type (*parE<sup>+</sup>*), selected for tetracycline resistance, and screened for kanamycin resistance. If the *topB* deletion can be tolerated by the *parE<sup>ts</sup>* strain, then the percentage of tetracycline-resistant colonies that are also kanamycin resistant will be equal in both the *parE<sup>ts</sup>* and *parE<sup>+</sup>* cells. Based upon their physical distance on the *E. coli* chromosome, these two markers should be transduced together ~50% of the time.

When selecting for tetracycline resistance, we found that the *parE<sup>+</sup>* recipients also acquired the kanamycin resistance gene with a frequency of about 50% (Fig. 2B, columns i, iii, v and vii) in four different strain backgrounds (W3110, C600, KD100 and LE234). In contrast, the *parE<sup>ts</sup>* cells that were resistant to tetracycline were rarely (5/201) also resistant to kanamycin (Fig. 2B, column ii). Chi-square statistical analysis showed a significant difference ( $P < 0.0001$ ) between  $\Delta topB$  transduction into the *parE<sup>ts</sup>* strain compared with the *parE<sup>+</sup>* strain at 30°C (Fig. 2C). The resultant 5/201  $\Delta topB$  *parE<sup>ts</sup>* cells were dead at 37°C, whereas  $\Delta topB$  and *parE<sup>ts</sup>* cells were fully viable at 37°C as measured by plating efficiency assays (data not shown). Therefore,  $\Delta topB$  and *parE<sup>ts</sup>* are synthetically



lethal. This was surprising because  $\Delta topB$  alone is viable and heretofore the robust *parE<sup>ts</sup>* mutant strain (Kato *et al.*, 1990) had appeared to retain wild-type topoisomerase IV activity at 30°C and 37°C (Adams *et al.*, 1992; Zechiedrich and Cozzarelli, 1995; Zechiedrich *et al.*, 2000; Deibler *et al.*, 2001). The synthetic lethal phenotype of  $\Delta topB$  is not restricted to the mutant *parE* subunit of topoisomerase IV, but is also seen with the *parC<sup>ts</sup>* allele (Fig. 2B, column iv). We were unable to combine  $\Delta topB$  with *parC<sup>ts</sup>* ( $P < 0.0001$ ) (Fig. 2C). Thus, the removal of *topB* in either topoisomerase IV temperature-sensitive mutant background is lethal.

Although we had not previously observed any defect in transduction in the *parE*<sup>ts</sup> mutant (Zechiedrich and Cozzarelli, 1995; and see below), it was possible that this particular set of double antibiotic resistance markers was affected by the mutant allele of *parE*. Therefore, we tested the cotransduction efficiency of a pair of transposon markers in approximately the same chromosomal position as *topB* by transducing Tn10kan Tn10tet from strain CRL47 into *parE*<sup>ts</sup> and its isogenic wild-type strain. The transduction and cotransduction frequencies were equal (data not shown).

It was shown previously that the overproduction of topoisomerase III rescues the temperature-sensitivity of topoisomerase IV mutant strains (Nurse *et al.*, 2003). We utilized this finding to explore potential genetic interactions with topoisomerase III. We used IPTG to express *topB* from plasmid pPH1243 (Broccoli *et al.*, 2000), which rescued both *parC*<sup>ts</sup> and *parE*<sup>ts</sup> cells at their respective non-permissive temperatures, 37°C and 42°C, and did not affect the viability of the *parC*<sup>+</sup>(C600), *parE*<sup>+</sup>(W3110) or the *topB* null strain (in W3110). *topB* overexpression from pPH1243 allowed construction of the  $\Delta$ *topB parE*<sup>ts</sup> strain, which was now viable at 37°C (used below) and even 42°C (data not shown) in the presence of IPTG. In the absence of the inducer, the double topoisomerase mutant cells were not viable at either temperature (Fig. 5). That the  $\Delta$ *topB parE*<sup>ts</sup> strain was viable at 30°C in the absence of IPTG likely results from the well known leaky expression from the *trc* promoter. A lower copy number plasmid, pPH1095, containing the *topB* gene under its natural promoter (Broccoli *et al.*, 2000), rescued 93 ± 8.5% of *parE*<sup>ts</sup> colonies at 42°C (data not shown).

Gyrase and topoisomerase IV are the type-2 topoisomerases in *E. coli*. These enzymes can perform many of the same reactions in a test tube (Steck and Drlica, 1984; Marians, 1987; Ullsperger and Cozzarelli, 1996), yet they perform distinct roles in the cell (Deibler *et al.*, 2001). We tested whether temperature-sensitive gyrase mutations could coexist with  $\Delta$ *topB* using the same genetic linkage experiment as above. We found no difference ( $P = 0.4539$ , Fig. 2C) in the frequency of transducing  $\Delta$ *topB* into *gyrB*<sup>+</sup> (KD100) or *gyrB225ts* (GP100) (Steck and Drlica, 1984) at the permissive temperature, 30°C (Fig. 2B, compare vi with v). There was also no difference in the viability or colony size of the  $\Delta$ *topB gyrB225ts* colonies compared with *gyrB225ts* colonies at 30°C, 37°C or 42°C (data not shown).

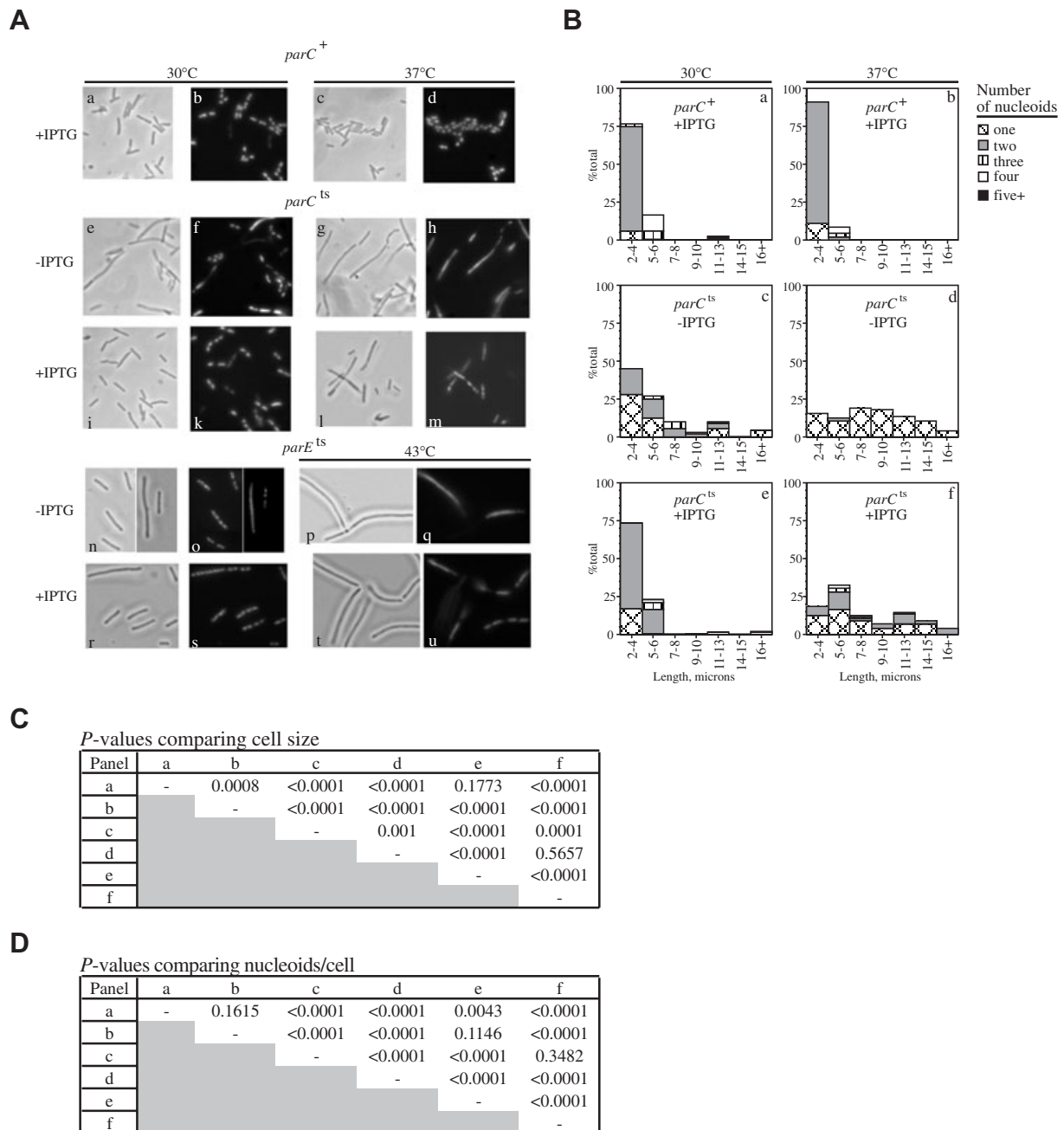
Recognizing that this particular gyrase mutant may not have impaired function at 30°C, we repeated the experiment with the *gyrB134ts* allele in strain LE316 (Orr *et al.*, 1979), which exhibits a supercoiling deficiency at 30°C (data not shown). There was a significant decrease ( $P = 0.0053$ ) in the percentage of *gyrB*<sup>ts</sup> cells that also

contained the *topB* null allele relative to *gyrB*<sup>ts</sup> (Fig. 2B, compare column viii with vii). However, unlike what was seen in topoisomerase IV mutants, the overproduction of topoisomerase III, with IPTG concentrations ranging from 0.5 to 5 mM, did not rescue the *gyrB134ts* strain. In addition, there was no difference in the viability of the  $\Delta$ *topB gyrB134ts* double mutant strain at 30°C or 37°C (data not shown). Using Tn10tet linked to Tn10kan from strain CRL47 as described above, and we found a similar twofold decrease in the likelihood that *gyrB134ts* cells contained both markers compared with *gyrB*<sup>+</sup> cells. We conclude that the *gyrB134ts* allele decreases general cotransduction and is not synthetically lethal with  $\Delta$ *topB*.

#### *Effect of topB overexpression on the cell morphology of topoisomerase IV temperature-sensitive mutants*

Originally, the *parC*<sup>ts</sup> and *parE*<sup>ts</sup> mutant strains were identified by a defect in DNA nucleoid partitioning and inviability at high temperatures (Hirota *et al.*, 1968; Kato *et al.*, 1988; Kato *et al.*, 1990). These mutants also exhibit a septation defect, resulting in cells that are longer than normal. To try to understand how topoisomerase III rescues the conditional lethality of the topoisomerase IV mutants, we analysed the effect of *topB* overexpression on cell length and DNA partitioning in the *parC*<sup>ts</sup> and *parE*<sup>ts</sup> compared with isogenic wild-type strains. Shown in Fig. 3 are light (left) and fluorescence (right) micrographs of DAPI stained cells. IPTG-induced overexpression of *topB* did not affect the morphology of the wild-type strains (Fig. 3A a–d and data not shown), as expected from previous experiments with arabinose-induced *topB* overexpression (Zhu *et al.*, 2001).

The *parC*<sup>ts</sup> mutant exhibited significant DNA segregation and cell septation defects, even at the permissive temperature (30°C) (Fig. 3A e and f), with ~50% of the population abnormal (Fig. 3B, compare panel c with panel a). At 37°C, the non-permissive temperature, most cells were filamented and contained non-partitioned DNA (Fig. 3A g, h and Fig. 3B d). The overexpression of *topB* had a dramatic effect on the *parC*<sup>ts</sup> mutant at 30°C, returning its morphology nearly to that of wild type (Fig. 3A i, k and Fig. 3B, compare panel e with a) and rescuing nucleoid partitioning in ~75% of the cells. At 37°C (Fig. 3A l, m and 3B f), the overexpression of *topB* increased the amount of partitioned DNA from almost none (Fig. 3A e–h and Fig. 3B d) to ~45% (Fig. 3A i–m and Fig. 3B f); whereas, ~90% of the wild-type cells were partitioned at 37°C. However, *topB* overexpression did not rescue the filamentation defect (Fig. 3B compare d with f). The *parE*<sup>ts</sup> cells were longer than their isogenic wild type at 30°C and contained what appeared to be discrete, partitioned nucleoids (Fig. 3A n, o). Again, the overexpression of *topB*



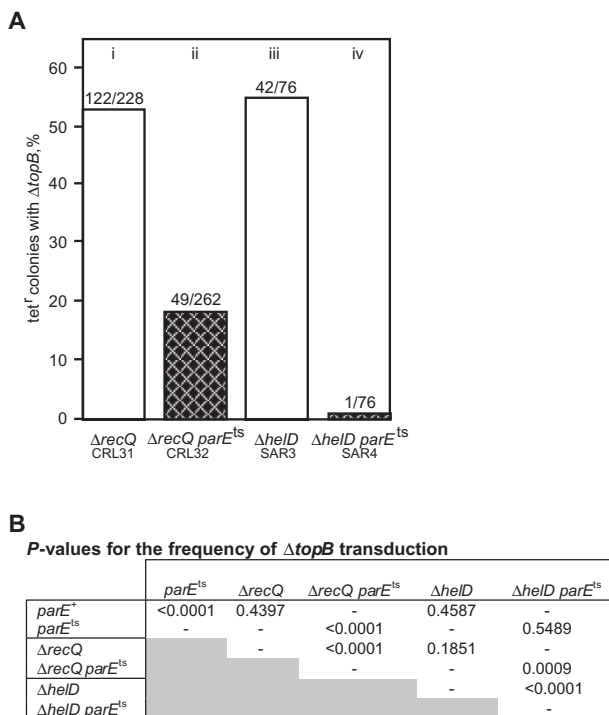
**Fig. 3.** Effect of *topB* overexpression on the morphology of topoisomerase IV mutant cells.

A. Topoisomerase IV temperature-sensitive mutant cells exhibit a DNA partition and filamentation defect that is partially rescued by topoisomerase III overproduction. C600 (*parC*<sup>+</sup>), ParC1215 (*parC*<sup>ts</sup>) and ParE10 (*parE*<sup>ts</sup>) cells containing plasmid pPH1243 (IPTG inducible *topB*) were grown in the presence or absence of 0.5 mM IPTG at the permissive temperature, 30°C, or at the non-permissive temperature 37°C (*parC*<sup>ts</sup>) or 43°C (*parE*<sup>ts</sup>) for 2 h. Panels a–d show *parC*<sup>+</sup> cells grown with IPTG at 30°C (a, b) or 37°C (c, d). Panels e–h show *parC*<sup>ts</sup> cells grown without IPTG and panels i–m with IPTG, at 30°C (e, f, i, k) or 37°C (g, h, l, m). Panels n–q show *parE*<sup>ts</sup> cells grown without IPTG and panels r–u with IPTG at 30°C (n, o, r, s) or 43°C (p, q, t, u). Light microscopy phase contrast images of cells (left) and the corresponding DAPI-stained DNA bright field images (right) are shown for each condition.

B. Quantification of the effect of overexpression of *topB* on the morphology of *parC*<sup>+</sup> and *parC*<sup>ts</sup> cells. Analysis of *parC*<sup>+</sup> cells are shown in panels a and b, and analysis of the *parC*<sup>ts</sup> mutant cells in panels c–f. Cell length and the number of DNA nucleoids in the cells were measured. Length is shown on the x-axis, and the percent total number of cells of a given length is shown on the y-axis. In addition, the number of nucleoids (1 to >6) is shown by the shading. The number of cells counted, *n*, was 115 for panel a; 125 for panel b; 88 for panel c; 94 for panel d; 121 for panel e; and 95 for panel f.

C. *P*-values concerning the cell length ± *topB* overexpression. *P* < 0.05 was considered significant. *P*-values were determined from chi-square analysis.

D. *P*-values concerning the number of nucleoids/cell ± *topB* overexpression. *P* < 0.05 was considered to be a significant difference. *P*-values were determined from chi-square analysis.



**Fig. 4.** The effect of helicase mutations on the synthetic lethality of  $\Delta topB$   $parE^{ts}$  strains.

A. The linkage assay described in Fig. 2 was performed in various mutants and their isogenic wild-type strain (W3110). As in Fig. 2, these experiments were conducted at 30°C. RecQ and helicase IV (encoded by the *helD* gene) are 3'→5' helicases. The results for the isogenic wild type of these strains,  $parE^+$ , are shown in Fig. 2B. As described in Fig. 2,  $\Delta topB::kan Tn10tet$  was transduced into the strains shown along the x-axis. The percentage of colonies with both markers is reported along the y-axis, and above each column is the number of colonies with both markers over the number of colonies tested.

B. P-values for linkage efficiency results (see *Experimental procedures*). The data for  $parE^+$  and  $parE^{ts}$  are shown in Fig. 2.  $P > 0.05$  is considered insignificant. P-values were determined from chi-square analysis.

did not rescue the filamentation phenotype of the  $parE^{ts}$  cells (Fig. 3A, compare panel n with r). At 42°C, the  $parE^{ts}$  cells exhibited a severe DNA segregation defect, similar to the  $parC^{ts}$  cells, which was partially rescued by the overexpression of *topB* (Fig. 3A t, u). Thus, over-production of topoisomerase III partially compensates for the DNA partitioning defect, but not for the filamentation defect, observed in the topoisomerase IV temperature-sensitive mutants. This is in agreement with the results reported by Nurse *et al.* (2003) for the  $parE^{ts}$  strain using an arabinose-inducible overexpression system.

#### *RecQ is required for the synthetic lethality of the ΔtopB parE<sup>ts</sup> double mutant*

In *S. cerevisiae* and *S. pombe*, deletion of the genes that encode the respective RecQ homologues rescued the

topoisomerase III null phenotypes (Gangloff *et al.*, 1994; Goodwin *et al.*, 1999; Maftahi *et al.*, 1999). Based upon these results and the finding that purified RecQ and topoisomerase III can catenate double-stranded DNA (Harmon *et al.*, 1999), it was hypothesized that RecQ helicases create a DNA substrate that topoisomerase III resolves (Gangloff *et al.*, 1994; Wu *et al.*, 1999). Therefore, we examined the effect of RecQ on the synthetic lethal phenotype of the  $\Delta topB$   $parE^{ts}$  double mutant.

We first constructed the  $\Delta recQ::cat$   $parE^{ts}$  and  $\Delta recQ::cat$   $parE^+$  strains, for which the transduction efficiency and growth were identical (data not shown). Then, using the linkage efficiency assay depicted in Fig. 2A, we asked whether the absence of RecQ would allow the *topB* deletion to be combined with the  $parE^{ts}$  allele at 30°C. Removal of the *recQ* gene increased the cotransduction efficiency of  $\Delta topB$  into the  $parE^{ts}$  strain ~7.5 times (compare Fig. 2B, column ii with Fig. 4A, column ii) ( $P \leq 0.0001$ ) (Fig. 4B). Thus, the removal of *recQ* partially rescues the synthetic lethal phenotype of the  $\Delta topB$   $parE^{ts}$  mutant cells.

Removing another 3'→5' helicase, helicase IV (Mendonca *et al.*, 1995), did not rescue the synthetic lethality of the  $\Delta topB$   $parE^{ts}$  strain (compare Fig. 4A iv with Fig. 2B ii) because the combination of the two topoisomerase mutations was still lethal. There was no detectable difference in transduction efficiency or strain growth between  $parE^{ts}$  and  $\Delta helD$   $parE^{ts}$  strains (data not shown).

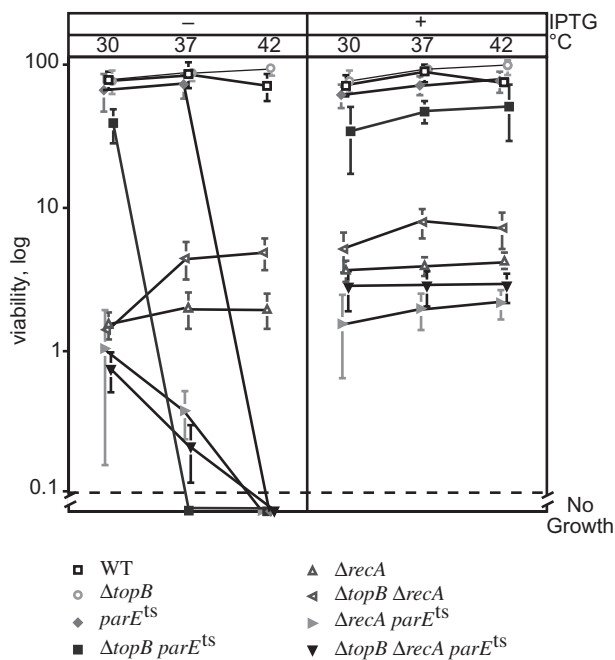
#### *Effect of recA on ΔtopB, parE<sup>ts</sup> and ΔtopB parE<sup>ts</sup> viability*

The filamentation and chromosome segregation defects of the  $\Delta topA$   $\Delta topB$  double mutant were suppressed by deleting the gene encoding the strand-invasion protein, RecA (Zhu *et al.*, 2001). Furthermore, the prevention of meiotic recombination in *S. cerevisiae* was able to rescue the sporulation defect seen in  $\Delta top3$  cells (Gangloff *et al.*, 1999). It has been suggested that topoisomerase III, in conjunction with Sgs1, suppresses the production of crossover recombinants during double strand break repair in *S. cerevisiae* (Ira *et al.*, 2003). Thus, it is possible that topoisomerase III could play a role in RecA-mediated recombination (Wang *et al.*, 1990; Bailis *et al.*, 1992; Wu *et al.*, 1999; Zhu *et al.*, 2001; Wang, 2002).

To determine whether RecA plays a role in the synthetic lethality of the  $\Delta topB$   $parE^{ts}$ , we measured the viability of  $\Delta topB$   $parE^{ts}$  in the absence *recA*. Because plasmid pPH1243 allowed the construction of the  $\Delta topB$   $parE^{ts}$  double mutant strain, we transduced the  $\Delta recA::Tn10$  allele into  $\Delta topB$   $parE^{ts}$ ,  $\Delta topB$ ,  $parE^{ts}$  and their isogenic wild-type strain in the presence of pPH1243 at 30°C. There was no difference in transduction frequency among these strains. The number of colonies formed on Luria–

Bertani (LB) agar plates containing ampicillin with (right) or without (left) 0.5 mM IPTG was divided by the number of cells spread as determined by cell counts using a Petroff-Hauser counter. The viability of the various otherwise isogenic strains is shown (Fig. 5). All strains lacking the *recA* gene were less viable compared with *recA*<sup>+</sup> strains (Fig. 5). Despite this overall reduction in viability, the  $\Delta topB \Delta recA parE^{ts}$  triple mutant was viable at 37°C in the absence of IPTG-induced *topB* expression, whereas the  $\Delta topB parE^{ts}$  strain did not grow under these conditions ( $P = 0.0095$ ). Thus the deletion of *recA* is able to partially rescue the  $\Delta topB parE^{ts}$  double mutant at 37°C.

$\Delta recA$  cells are deficient in both the SOS response and in homologous recombination. Thus, we utilized a *lexA* mutant allele, *lexA3*, that produces a non-cleavable LexA protein to distinguish which RecA function was involved. Cells that contain *lexA3* are unable to derepress the LexA/SOS regulon in response to DNA damage (Little *et al.*, 1980). Employing P1 phage, we moved *lexA3* into the  $\Delta topB parE^{ts}$  strain (containing pPH1243) in the presence



**Fig. 5.** Effect of *recA* deletion on  $\Delta topB parE^{ts}$  viability. All strains tested contain plasmid pPH1243 and are isogenic.  $\Delta topB parE^{ts}$  strains are not viable at 37°C unless topoisomerase III is provided from pPH1243. *topB* expression was induced by adding 0.5 mM IPTG to the medium. All cells were grown to mid-logarithmic phase, serially diluted and spread onto two sets of plates (with or without IPTG) that were then incubated up to 72 h at the indicated temperature. The viability of wild-type,  $\Delta topB$ ,  $parE^{ts}$ ,  $\Delta topB parE^{ts}$ ,  $\Delta recA$ ,  $\Delta topB \Delta recA$ ,  $\Delta recA parE^{ts}$  and  $\Delta topB \Delta recA parE^{ts}$  strains was measured and is shown here on a log scale. The percentage of viable cells is reported on the y-axis. The x-axis displays the conditions under which the cells were grown. Cells containing the *parE*<sup>ts</sup> allele are shaded, whereas cells lacking the *recA* gene are represented by a triangle. Error bars represent standard deviations from at least three trials.

of 0.5 mM IPTG and measured the number of colonies/viable cells. Unlike the  $\Delta recA \Delta topB parE^{ts}$  strain, the *lexA3*  $\Delta topB parE^{ts}$  strain was not viable at 37°C without IPTG (data not shown). Therefore, *lexA3* does not rescue the synthetic lethality of the  $\Delta topB parE^{ts}$  mutant. We conclude that the synthetic lethality of the  $\Delta topB parE^{ts}$  strain does not involve LexA regulon induction. These results support the idea that the recombination function of RecA is required. Based upon these data, it appears likely that preventing the formation of recombination intermediates allows the survival of topoisomerase III/IV double mutant cells.

There was no significant difference in viability between any of the  $\Delta recA$ -containing strains at 30°C. At this temperature, the addition of IPTG to the medium increased the viability of the  $\Delta recA$ ,  $\Delta topB \Delta recA$  and  $\Delta topB \Delta recA parE^{ts}$  strains. In general, at all temperatures tested, the overexpression of topoisomerase III had a positive influence on cell viability in cells lacking *recA*, indicating that the increase in topoisomerase III levels was somehow advantageous.

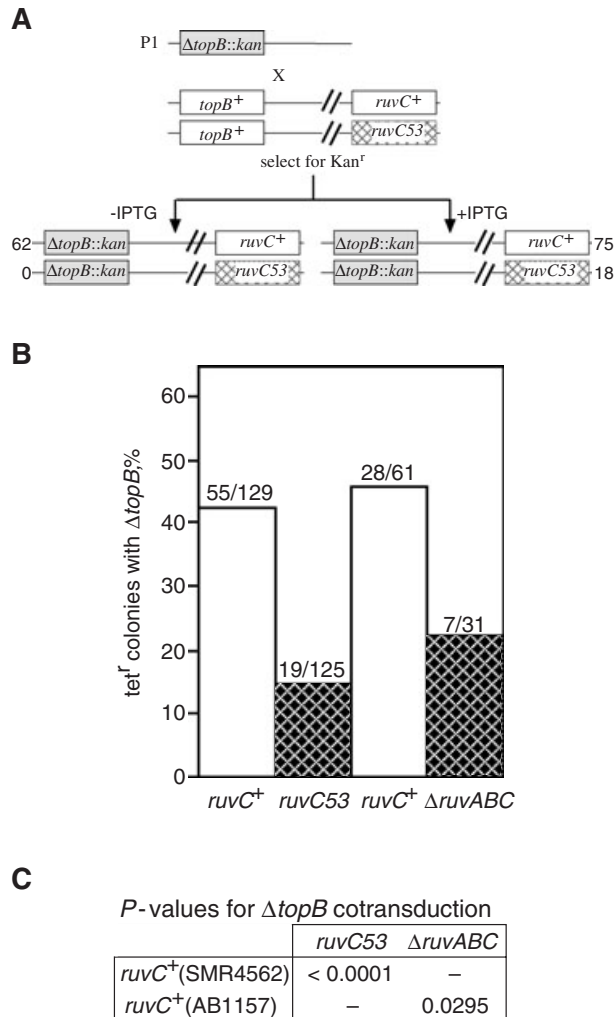
At 37°C, in the absence of IPTG, wild-type,  $\Delta topB$ , *parE*<sup>ts</sup> and  $\Delta recA$  strains were as viable as at 30°C. The  $\Delta topB parE^{ts}$  double mutant was not viable, and the  $\Delta topB \Delta recA$  strain was more viable compared with 30°C. This strain is also more viable than the  $\Delta recA$  strain ( $P = 0.041$ ). The viability of the  $\Delta recA parE^{ts}$  and  $\Delta topB \Delta recA parE^{ts}$  strains was decreased; however, these strains were viable in the absence of IPTG induced *topB* expression. This suggests that the deletion of *recA*, although somewhat deleterious to the *parE*<sup>ts</sup> strain (as suggested by the decreased viability of the  $\Delta recA parE^{ts}$  strain compared with the  $\Delta recA$  strain), obviates the need for *topB*.

At 42°C, in the absence of IPTG, none of the strains containing the *parE*<sup>ts</sup> allele grew. The rest of the strains showed no change in viability compared with 37°C. In addition, *topB* overexpression rescued the temperature-sensitivity of all the strains with the *parE*<sup>ts</sup> allele. These strains were just as viable as they were at 37°C + IPTG. These results were unexpected in the  $\Delta topB \Delta recA parE^{ts}$  and  $\Delta recA parE^{ts}$  strains. Because deleting *recA* allowed  $\Delta topB parE^{ts}$  cells to live at 37°C, it appeared that *topB* was acting down-stream of *recA* in the *parE*<sup>ts</sup> mutants. Based upon this, we expected that the overexpression of *topB* should have no effect on cells lacking *recA*.

#### Transduction of $\Delta topB$ into *ruvC53* or $\Delta ruvABC$

Human topoisomerase III $\alpha$  has been shown to cleave a synthetic double Holliday junction with the help of BLM helicase (Wu and Hickson, 2003). If topoisomerase III unlinks such recombination intermediates *in vivo*, then the cell should not tolerate the removal of both the major

Holliday junction resolving enzyme, RuvC (Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991; reviewed in West, 1997), and topoisomerase III. *ruvC*<sup>+</sup> and *ruvC53* (a loss of function mutant, Sharples and Lloyd, 1993) strains (in the 594 genetic background) were transformed with pPH1243.  $\Delta topB$  was transduced into these cells in either the absence or presence of IPTG.  $\Delta topB::kan$  could be transduced into *ruvC*<sup>+</sup>, but not *ruvC53* cells, in the absence of IPTG at 30°C (Fig. 6A). Next, we employed the linkage



**Fig. 6.** Transduction of  $\Delta topB$  into *ruvC* mutants. **A.** *ruvC53* (SMR635) and its isogenic wild type (SMR632) were transformed with pPH1243. The  $\Delta topB::kan$  allele was then transduced into these strains in either the absence or presence of IPTG. The transductants were then screened for the appropriate phenotypes, and the resulting number of colonies with the correct phenotype is reported. **B.** The linkage efficiency assay depicted in Fig. 2 was performed on *ruvC53* (SMR6047),  $\Delta ruvABC$  (JJC754) and their isogenic wild-type strains. The percentage of tetracycline-resistant cells that contained the *topB* deletion is shown. **C.** *P*-values for the linkage efficiency results as determined from chi-square analysis.

**Table 1.** Cotransduction efficiency<sup>a</sup> in *ruvC*<sup>+</sup> and *ruvC53* strains.

Donor strain	Position <sup>b</sup>	<i>ruvC</i> allele		
		<i>ruvC</i> <sup>+</sup>	<i>ruvC53</i>	<i>P</i> -value
CRL6 ( $\Delta topB$ )	40'	43% (129) <sup>c</sup>	15% (125)	<0.0001
CRL47	40'	13% (60)	11% (36)	0.7494
CRL45	54'	13% (60)	18% (55)	0.4747
CRL46	86'	52% (58)	58% (31)	0.5674

**a.** SMR4562 (*ruvC*<sup>+</sup>) and SMR6047 (*ruvC53*) were transduced with P1 lysates produced from the indicated donor strains.

**b.** Approximate chromosomal location (in minutes) of antibiotic-resistance markers.

**c.** Total number of colonies screened.

assay schematized in Fig. 2A.  $\Delta topB::kan$  Tn10tet was transduced into the *ruvC53* mutant strain and its isogenic (SMR4562) wild-type strain, selecting for tetracycline resistance. The percentage of *ruvC*<sup>+</sup> cells that contained both kanamycin and tetracycline resistance markers was 50% (Fig. 6B). This percentage was reduced by approximately threefold in the strain containing the *ruvC53* allele and approximately twofold in the  $\Delta ruvABC$  mutant (Fig. 6B; *P* = 0.0001 and *P* = 0.0295 respectively). These data suggest that the removal of both topoisomerase III and RuvC is detrimental to cells.

Throughout our experiments, we found a reduced number of transductants in cells containing the *ruvC53* allele compared with *ruvC*<sup>+</sup> cells, which agrees with previous results (Lloyd, 1991). It was possible that the role of RuvC in resolving the recombination intermediates that arise during transduction, or the chromosomal location of *ruvC* (41.9') could account for the reduced efficiency of  $\Delta topB::kan$  cotransduction with Tn10tet. To test both of these possibilities, we performed linkage efficiency assays using three different pairs of linked transposons (Table 1). We tested the cotransduction frequencies of transposon pairs from strains CRL45 (with the transposons located at 53.7' and 54.1'), CRL46 (86.4' and 86.8') and CRL47 (39.5' and 40.3') (*topB* is located at 39.7') and found no difference between *ruvC*<sup>+</sup> and *ruvC53* containing strains (Table 1). Because the probability of transducing both markers was the same, neither the chromosomal position of *ruvC* nor its role in transduction was responsible for the bias against  $\Delta topB::kan$  cotransduction into *ruvC53* mutant strains.

#### Characterization of $\Delta topB$ *ruvC53* cells

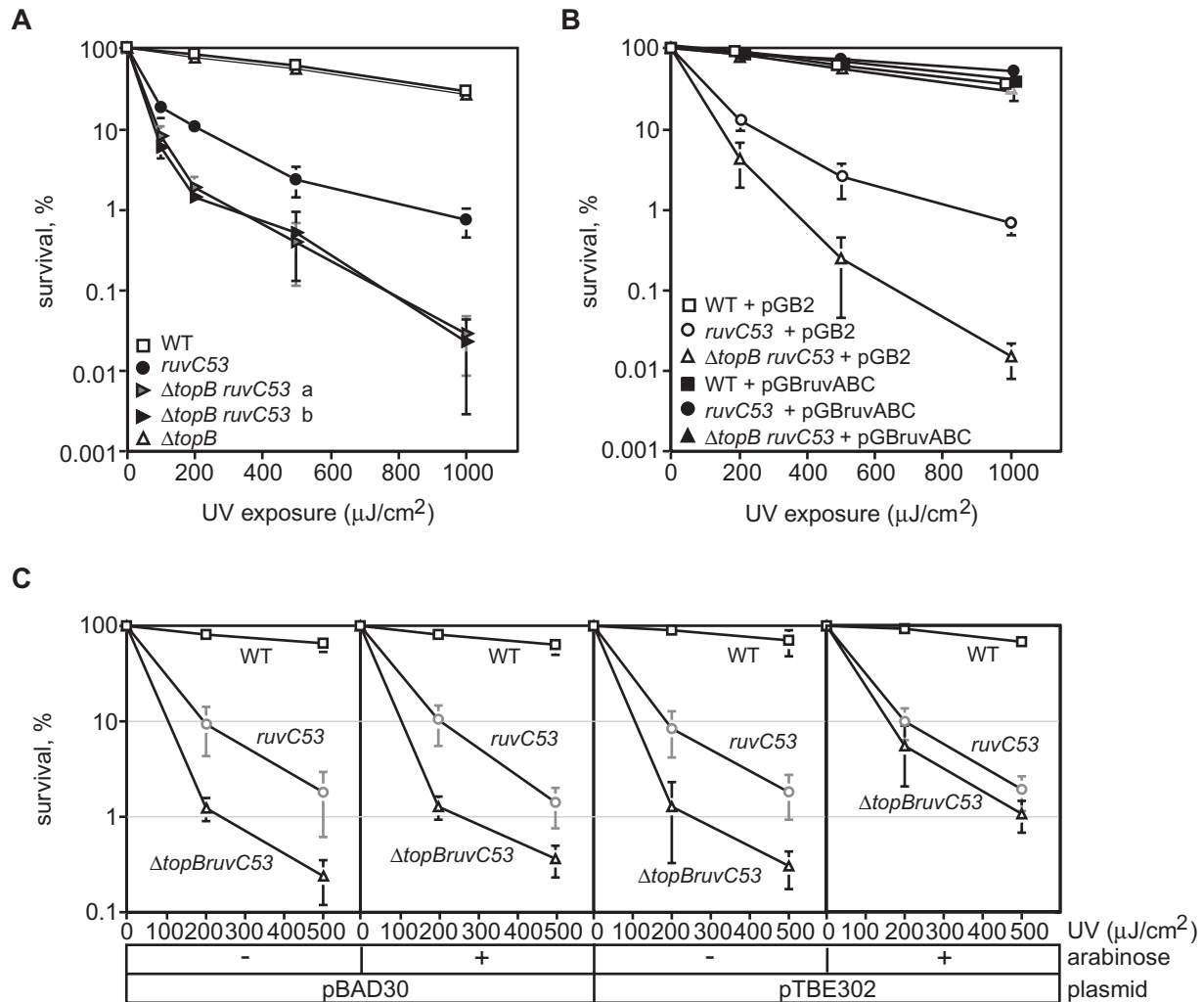
*ruvC53* mutants are characterized by an increased sensitivity to DNA damaging agents, as well as a decrease in transduction efficiency (Lloyd, 1991). If topoisomerase III acts in a pathway that provides an alternative route of Holliday junction disentangling to the RuvABC pathway, then the  $\Delta topB$  *ruvC53* double mutant strain should be

even more sensitive to DNA damage and further reduced in transduction efficiency than the *ruvC53* mutant.

We measured the UV sensitivity of two different  $\Delta topB$  *ruvC53* mutants from the cotransduction assay shown in Fig. 6B compared with an isogenic set of strains in an FC40 genetic background. Both double mutants were up to ~20 times more sensitive to UV irradiation than the cells containing the *ruvC53* allele alone (Fig. 7A).  $\Delta topB$  cells displayed the same sensitivity to UV light as wild-type cells (Fig. 7A). We repeated these experiments in an

MG1655 background and found identical results (data not shown).

As shown previously, a plasmid encoding the *ruvABC* genes is able to complement a *ruvABC* null strain (Seigneur *et al.*, 1998; Flores *et al.*, 2001; Grompone *et al.*, 2002). This plasmid, pGBruvABC, also complemented the UV sensitivity of the *ruvC53* strain (Fig. 7B). The overexpression of *ruvABC* rescued the UV sensitive phenotype of  $\Delta topB$  *ruvC53* cells to the wild-type level. The vector, pGB2, had no effect.



**Fig. 7.** UV sensitivity of  $\Delta topB$  *ruvC53*.

A. An isogenic set of strains, including two different  $\Delta topB$  *ruvC53* mutants [CRL43 ('a') and CRL51 ('b')] were exposed to increasing amounts (0–1000  $\mu\text{J cm}^{-2}$ ) of UV irradiation (x-axis). The number of colonies surviving each UV dose was counted and divided by the number of colonies present on an unexposed plate; this percentage is shown on the y-axis. Symbols representing strains containing the *ruvC53* allele are filled, while strains containing  $\Delta topB$  are represented by triangles. Error bars represent standard deviation from three experiments with duplicates of each strain; where no error bar is seen, the error is smaller than the symbol.

B. Wild-type (SMR4562), *ruvC53* (SMR6047) and  $\Delta topB$  *ruvC53* (CRL51) strains were transformed with a plasmid containing the *ruvABC* genes, pGBruvABC (shaded symbols), or with empty vector, pGB2 (open symbols).

C. Wild-type (SMR4562), *ruvC53* (SMR6047) and  $\Delta topB$  *ruvC53* (CRL51) strains were transformed with a plasmid containing the *topB* gene under the control of an arabinose-inducible promoter, pTBE302, or with empty vector, pBAD30. The matrix along the x-axis also indicates which plasmid was present, as well as whether 0.2% arabinose was in the media.

We measured the effect of overexpressing *topB* on the UV hypersensitive phenotype of the  $\Delta topB$  *ruvC53* mutants. Plasmid pTBE302 contains *topB* under the control of an arabinose-inducible promoter (Zhu *et al.*, 2001). *topB* overexpression had no effect on the UV sensitivity of the *ruvC53* mutant in either the presence or absence of 0.2% arabinose (Fig. 7C). However, when *topB* was induced in the  $\Delta topB$  *ruvC53* mutant, the tolerance to UV irradiation was increased to that of the *ruvC53* mutant. This was not seen in the absence of arabinose, nor with vector (pBAD30) alone (Fig. 7C, first three panels).

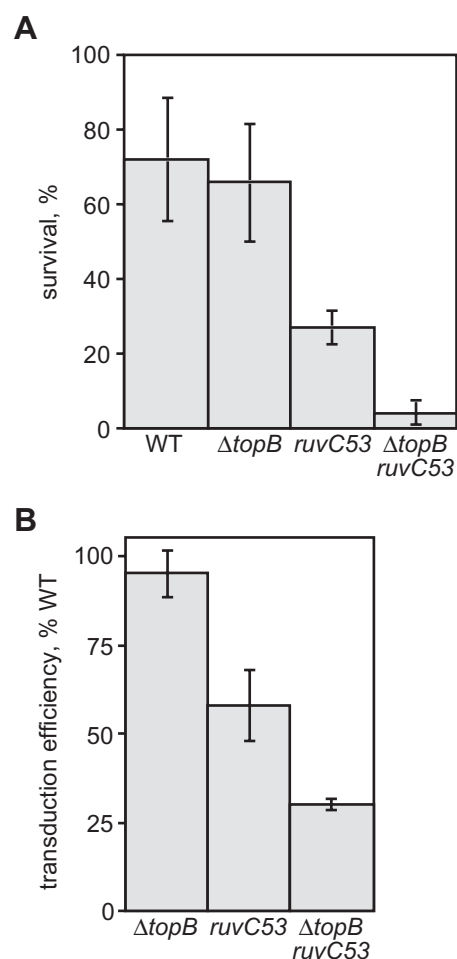
In addition, the  $\Delta topB$  *ruvC53* mutant was approximately five times more sensitive to mitomycin C ( $0.5 \mu\text{g ml}^{-1}$ ) than the *ruvC53* mutant (Fig. 8A). The  $\Delta topB$  strain was the same as wild type. The transduction efficiency of the  $\Delta topB$  *ruvC53* (CRL76) was also approximately twofold decreased relative to the *ruvC53* (CRL75) strain (Fig. 8B); a Poisson statistical analysis showed these values to be distinct with a confidence limit of 95%.

The hypersensitivity to DNA damaging agents, as well as the decreased transduction efficiency of the  $\Delta topB$  *ruvC53* double mutant strain compared with the *ruvC53* strain suggest that topoisomerase III is involved in processing recombination intermediates in an alternate pathway to RuvC.

#### Effect of *ruvABC* overexpression on topoisomerase double mutants

RuvABC might be expected to prevent the synthetic lethality of the topoisomerase III topoisomerase IV double mutant, but this rescue would require increased cellular levels of *ruvABC*. We tested whether an increase in RuvABC levels would allow the construction of the  $\Delta topB$  *parE*<sup>ts</sup> or  $\Delta topB$  *parC*<sup>ts</sup> mutants. The cotransduction efficiency assay described in Fig. 2A was carried out in *parE*<sup>+</sup>, *parE*<sup>ts</sup>, *parC*<sup>+</sup> and *parC*<sup>ts</sup> containing strains that had been transformed with either pGBruvABC or pGB2 (Fig. 9). In general, the frequency of  $\Delta topB::kan$  cotransduction into wild-type strains was reduced in the presence of pGBruvABC, compared with pGB2 alone or no plasmid (Fig. 2B). Perhaps *ruvABC* overexpression increases RuvC-mediated DNA cleavage to yield smaller chromosomal replacements by P1 transduction.

Despite the resulting decrease in the transduction of both markers, the overexpression of *ruvABC* from pGBruvABC allowed the cotransduction of  $\Delta topB$  into the *parE*<sup>ts</sup> strain at the same efficiency as the *parE*<sup>+</sup> strain (Fig. 9A). *ruvABC* overexpression also allowed the construction of the  $\Delta topB$  *parC*<sup>ts</sup> strain (Fig. 9A), and the frequency of  $\Delta topB$  cotransduction was similar

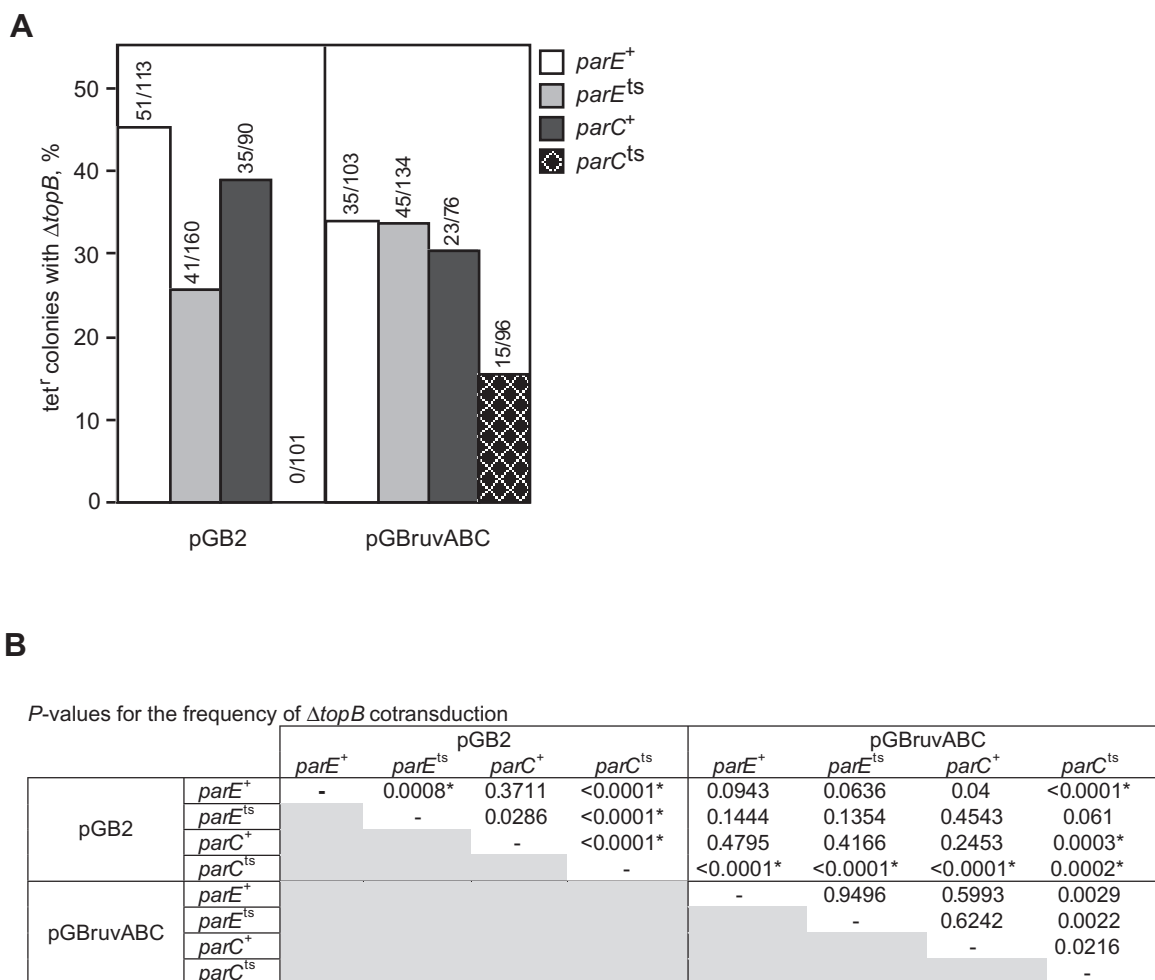


**Fig. 8.** Mitomycin C sensitivity and transduction efficiency of  $\Delta topB$  *ruvC53*.

A. An isogenic set of strains [SMR4562 (WT), CRL48 ( $\Delta topB$ ), SMR6047 (*ruvC53*) and CRL51 ( $\Delta topB$  *ruvC53*)] was tested for sensitivity to  $0.5 \mu\text{g ml}^{-1}$  of mitomycin C. The number of colonies that grew over 72 h on the mitomycin C plate was compared with the number of colonies that grew on LB over the same time period. This percentage of survival is shown along the y-axis. Error bars represent standard deviation from three trials done in duplicate.

B. WT (MG1655),  $\Delta topB$  (QZ103), *ruvC53* (CRL74) and  $\Delta topB$  *ruvC53* (CRL76) strains were transduced with an MOI of 0.1 of Tn10 at 51.75' (CAG18468). The transduction efficiency was calculated for these strains, and shown as a fraction of WT along the y-axis. WT transduction efficiency was  $2.04 \times 10^{-7}$  ( $\pm 8.39 \times 10^{-8}$ ). Error bars represent standard deviation from four trials done in duplicate.

to the *parC*<sup>+</sup> strain. The presence of pGB2 (in the presence or absence of spectinomycin) itself partially relieved the synthetic lethality of  $\Delta topB$  *parE*<sup>ts</sup>; however, there was still a statistically significant difference in cotransduction efficiency between *parE*<sup>ts</sup> and *parE*<sup>+</sup> cells. pGB2 had no effect on the cotransduction of  $\Delta topB$  into the *parC*<sup>ts</sup> strain. No other plasmid tested (pBR322 or pACYC184) produced such an effect. The number of colony forming units in the  $\Delta topB$  *parE*<sup>ts</sup>



**Fig. 9.** Effect of *ruvABC* overexpression on  $\Delta topB$  cotransduction efficiency.

A. *parE*<sup>+</sup>, *parE*<sup>ts</sup>, *parC*<sup>+</sup> and *parC*<sup>ts</sup> cells were transformed with pGBruvABC or the vector control, pGB2. We then performed the cotransduction efficiency assay described in Fig. 2A. The percentage of tetracycline-resistant colonies that were also kanamycin resistant is reported along the y-axis. The total number of tetracycline-resistant colonies screened and the number of those that contained the *topB* deletion is also shown above each bar.

B. *P*-values for cotransduction efficiency results. A contingency chi-square test was utilized to determine *P*-values. Asterisks denote significant values at a 95% confidence limit. Because of the multiple comparisons, we applied the Bonferroni method to correct for a type 1 error in determining which *P*-values were significant. The *P*-values that are significant are denoted with an asterisk only if it is lower than the limit ( $\alpha = 0.05$ ) divided by the number of samples compared (28). This same correction was performed in all previous statistical analyses; however, the correction had no effect because the *P*-values were already quite low. For  $\alpha = 0.05$ , the adjusted value is 0.0018; values above these levels were not considered significant.

strain harbouring pGBruvABC was twofold greater than in the  $\Delta topB$  *parE*<sup>ts</sup> pGB2 strain at both 30°C and 37°C (data not shown).

## Discussion

### *A role for topoisomerase III in decatenation?*

It has been suggested that a role for topoisomerase III is to unlink DNA during replication (Wang *et al.*, 1990; Wu *et al.*, 1999; Wang, 2002; Nurse *et al.*, 2003). This is reasonable considering that: (i) purified topoisomerase III can

unlink catenanes containing a nick or a gap (DiGate and Marians, 1988; Hiasa and Marians, 1994) as well as catenate intact double strand DNA in the presence of excess RecQ (Harmon *et al.*, 1999); (ii) purified topoisomerase III unlinks precatenanes in a reconstituted replication assay (Hiasa and Marians, 1994; Nurse *et al.*, 2003); and (iii) the overexpression of *topB* rescues the lethality of topoisomerase IV temperature-sensitive strains (Nurse *et al.*, 2003).

Additional results are difficult to resolve with a model for topoisomerase III in decatenation. First, if at normal cellular levels, topoisomerase III unlinks precatenanes, it

does so poorly because it leaves 90% of replicated plasmid DNA as closed circular catenane links that only topoisomerase IV can resolve (Zechiedrich and Cozzarelli, 1995). Indeed, the overproduction of topoisomerase III at any level cannot rescue fully a *parE* null mutant strain (Nurse *et al.*, 2003). On the cellular level, the overproduction of topoisomerase III rescued the partition defect in only ~45% of the *parE*<sup>ts</sup> and *parC*<sup>ts</sup> cells and did not rescue the filamentation phenotype at the non-permissive temperature (Fig. 3B and Nurse *et al.*, 2003). These results suggest that topoisomerase III cannot efficiently perform the essential function of topoisomerase IV, which is either a subtle, but important, role in DNA supercoiling maintenance (Zechiedrich *et al.*, 2000), the unlinking of daughter chromosomes at the end of replication (Adams *et al.*, 1992; Zechiedrich and Cozzarelli, 1995) or unknotting DNA (Deibler *et al.*, 2001). Second, we find that the removal of topoisomerase III does not cause catenated plasmid replication intermediates to accumulate (data not shown). Even when the activity of the main cellular decatenating enzyme, topoisomerase IV, is absent, the removal of topoisomerase III activity does not cause an additional accumulation of catenanes when compared with the removal of only topoisomerase IV (Fig. 1). Third, topoisomerase III does not have the ability to preferential unlink rather than link DNA that the type-2 topoisomerases have (Rybenkov *et al.*, 1997). Therefore, topoisomerase III is as likely to link as to unlink DNA. Fourth, unlinking of replicated chromosomal DNA must occur in all living cells, yet topoisomerase III in a *parE*<sup>ts</sup> background, is dispensable at the permissive temperature when *recA* or *recQ* is eliminated (Figs 4 and 5). Finally, it is difficult to see how a role in decatenation can result in the phenotypes of increased mutation and genome rearrangement observed for topoisomerase III mutant cells.

The evidence indicates that topoisomerase III does not unlink catenated replication intermediates as a normal cellular role. When overproduced, this enzyme may remove 'precatenanes' (Hiasa and Marians, 1994; Ullsperger *et al.*, 1995; Peter *et al.*, 1998; Nurse *et al.*, 2003), resulting in fewer links for topoisomerase IV to remove, but topoisomerase IV is absolutely required to perform this role normally.

In general, topoisomerases are in high abundance. In *E. coli*, topoisomerase I, gyrase and topoisomerase IV are thought to range from 500 to 10 000 mol cell<sup>-1</sup>. Topoisomerase III is much less abundant (1–10 mol cell<sup>-1</sup>; DiGate and Marians, 1989). The *topB* gene encodes rare codons; this might explain the low protein abundance because the charged tRNAs for these codons are rate limiting (DiGate and Marians, 1989). The codon usage of the other topoisomerases is skewed to the abundant tRNAs (Table 2). The mean codon bias index (CBI) (Benetzen and Hall, 1982) for the five known topoisomerase

**Table 2.** Codon bias index.

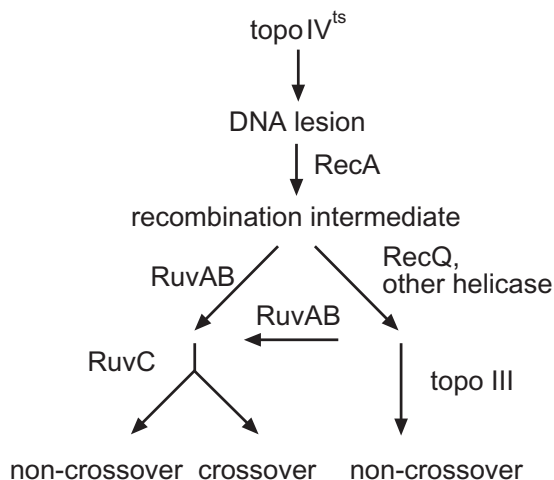
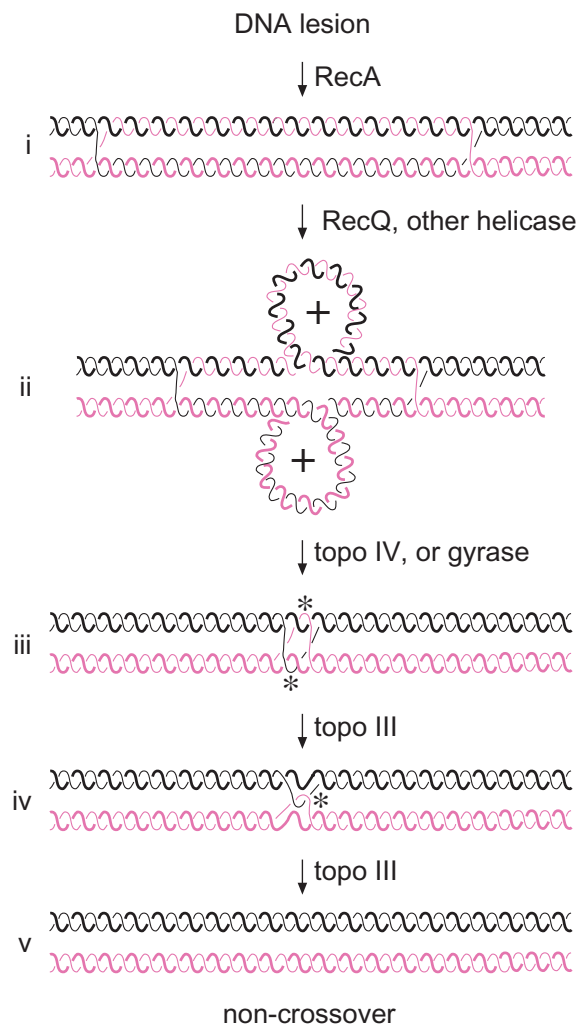
Gene	Codon bias index <sup>a</sup>
Topoisomerases	
<i>topA</i>	0.332
<i>gyrA</i>	0.496
<i>gyrB</i>	0.514
<i>parC</i>	0.351
<i>parE</i>	0.323
<i>topB</i>	0.192
DNA repair and recombination	
<i>ruvA</i>	0.208
<i>ruvB</i>	0.278
<i>ruvC</i>	0.207
<i>recG</i>	0.262
<i>recQ</i>	0.029
<i>helD</i>	0.150
<i>uvrD</i>	0.251

a. As formulated in Benetzen and Hall (1982).

genes was  $0.403 \pm 0.094$  (a value of zero would indicate random codon choice; a value of one would indicate preferred codon choice). Proteins involved in DNA repair are often in low abundance (discussed in Friedberg *et al.*, 1995). Excluding *recA* (0.558), the mean CBI for *E. coli* DNA repair and recombination genes (see complete list in *Experimental procedures*) was  $0.175 \pm 0.079$ . The values for topoisomerases and repair genes were distinct ( $P < 0.001$ ) by the student's *t*-test. Topoisomerase III, with a CBI = 0.192, is as likely to encode rare codons as preferred codons, and more closely resembles the DNA repair and recombination genes (Table 2).

#### *A model for the maintenance of genomic stability*

Homologous recombination is important in repairing DNA lesions caused by DNA replication errors and arrest (Kuzminov, 2001), exposure to chemicals and ionizing radiation (Cromie *et al.*, 2001), and the DNA breaks involved in proper chromosome segregation during meiosis (Kleckner, 1996). These types of repair often proceed through a homologous DNA strand invasion event, which is mediated in bacteria by RecA (reviewed in Kowalczykowski and Eggleston, 1994; Camerini-Otero and Hsieh, 1995). However, RecA-independent processes exist (Bierne *et al.*, 1997; Morag *et al.*, 1999; Lovett *et al.*, 2002; Ikeda *et al.*, 2004). Recombination intermediates are formed, such as D-loops, Holliday junctions and double Holliday junctions. In *E. coli*, one way to resolve these intermediates is through the combined activities of RuvABC (West, 1997). RuvAB (Fig. 10A, left branch) promotes branch migration and targets the endonuclease, RuvC, to the Holliday junction (West, 1997). RuvC cleaves the Holliday junction to generate non-crossovers and crossovers, although non-crossover products are preferred (West, 1997; Cromie and Leach, 2000).

**A****B**

We hypothesize that topoisomerase III acts in a separate pathway involved in processing recombination intermediates (Fig. 10A). We cannot envision a role for topoisomerase III in resolving single Holliday junctions. Indeed, synthetic single Holliday junctions are not cleaved by human topoisomerase III $\alpha$  (Wu and Hickson, 2003). Instead, we show a model in which RecQ helicase migrates two Holliday junctions toward each other, resulting in a build up of positive DNA supercoils (Fig. 10B ii). Based upon previous biochemical studies, topoisomerase IV could remove the positive supercoils (Crisona *et al.*, 2000) (or DNA gyrase could introduce negative DNA supercoils to prevent the formation) to allow additional branch migration of the Holliday junctions towards each other until they meet (Fig. 10B ii–iii). The Holliday junctions would most likely converge in a parallel conformation (Schwacha and Kleckner, 1995). This would result in the formation of a structure that has three single-stranded links, indicated by asterisks, two initial (Fig. 10B iii) and one that would remain when the first two were unlinked (Fig. 10B iv). Topoisomerase III might unlink this substrate, which is similar to its preferred *in vitro* substrate (DiGate and Marians, 1988). In support of this, human topoisomerase III $\alpha$  was shown to cleave a synthetic double Holliday junction in the presence of BLM helicase (Wu and Hickson, 2003). Although not perhaps its normal reaction, when overexpressed (Wallis *et al.*, 1989) or when topoisomerase III is removed (Broccoli *et al.*, 2000; Zhu *et al.*, 2001), topoisomerase I may also unlink the merged Holliday junctions through a separate pathway.

**Fig. 10.** Model for a role of topoisomerase III.

A. Topoisomerase III acts in a recombination pathway that is separate from RuvC. Topoisomerase IV temperature-sensitive mutant strains (ParE10 and ParC1215) lead to some type of DNA damage, perhaps through hindering replication fork progression. This leads to a DNA lesion. Recombinational repair is initiated by RecA. The recombination intermediate can then be processed by two separate pathways. One involves RuvABC. The end result would be either a crossover or non-crossover. The other pathway involves RecQ, and probably another helicase/branch migration protein that creates a substrate for topoisomerase III to unlink. Topoisomerase III-mediated resolution would always result in a non-crossover product. RuvAB can act upon the recombination intermediate in the topoisomerase pathway to channel the intermediate for resolution by RuvC. These two pathways might compete with one another for various types of DNA damage. B. A possible substrate for topoisomerase III – mediated resolution. A DNA lesion, followed by RecA-mediated strand invasion leads to the formation of two Holliday junctions (i). A helicase or branch migration enzyme moves the Holliday junctions towards each other and leads to the build-up of (+) supercoils (ii). Topoisomerase IV or gyrase removes the supercoils to allow branch migration to continue until the two Holliday junctions are juxtaposed (iii). Topoisomerase III resolves this through three single-stranded DNA passage events. The first two strand passages leave a singly linked hemi-catenane, which is readily unlinked by topoisomerase III *in vitro* (DiGate and Marians, 1988). The topoisomerase pathway always yields a non-crossover product (v). The asterisks denote where topoisomerase III would act upon the DNA.

Topoisomerase-mediated resolution of Holliday junctions would always result in non-crossover products (Fig. 10B v). A reasonable prediction of this model is that the removal of *topB* from any organism would result in an increase in chromosomal rearrangements. Over generations, this could lead to the shuffling of the genome.

That a purified topoisomerase can cleave Holliday junctions in a test tube has been shown (Sekiguchi *et al.*, 1996; Palaniyar *et al.*, 1999; Wu and Hickson, 2003). Topoisomerase-mediated cleavage in all of these studies was measured by incubating a synthetic Holliday junction with topoisomerase and a divalent cation. Cleavage is trapped by adding SDS to denature the topoisomerase. Proteinase K is added to digest the covalently bound topoisomerase. It is not clear in any of these cases whether a topoisomerase would actually finish the resolution reaction: break a DNA strand, pass a DNA strand through the break, and religate the break. This is required for Holliday junction resolution *in vivo*. Merged Holliday junctions form a hemi-catenane. Hemi-catenanes have been shown previously to be excellent substrates for topoisomerase III-mediated DNA strand passage.

Branch migration of Holliday junctions by helicases has been seen in T4 and T7 bacteriophages (Kong *et al.*, 1997). In addition to RuvAB, the *E. coli* helicases DnaB, RecG and RecQ have also shown branch migration activity *in vitro* (Harmon and Kowalczykowski, 1998; Bolt and Lloyd, 2002; Kaplan and O'Donnell, 2002; McGlynn and Lloyd, 2002). The actions of a helicase could disrupt D-loops to prevent the formation of Holliday junctions; however, it is unclear how branch migration by a helicase could process an existing Holliday junction to unlink the DNA strands. Alternately, RecQ might unwind various DNA structures (reviewed in Bennett and Keck, 2004) into a substrate that topoisomerase III recognizes and can disentangle, or RecQ-mediated branch migration of two Holliday junctions can lead to the formation of a DNA structure similar to a hemi-catenane that topoisomerase III unlinks as shown in Fig. 10B.

Perhaps, in the absence of RecQ, the topoisomerase-mediated disentangling mode of Holliday junction resolution is either not used or is inefficient. This could explain why the deletion of the *recQ* gene can relieve partly the synthetic lethality of the  $\Delta topB parE^{ts}$  double mutant (Fig. 4) and may explain why the deletion of the RecQ homologues, *rqh1* or *sgs1*, in yeast relieves the defects of topoisomerase III mutations (Gangloff *et al.*, 1994; Goodwin *et al.*, 1999; Maftahi *et al.*, 1999; Oh *et al.*, 2002). The BLM helicase has been shown to stimulate the activity of human topoisomerase III $\alpha$  (Wu and Hickson, 2002).

Our data show that RuvABC can act on recombination intermediates normally metabolized by the topoisomerase pathway, as evidenced by the rescue of  $\Delta topB ruvC53$  to wild-type levels by *ruvABC* overexpression

(Fig. 7B). Whether RuvAB can act after RecQ, as denoted as an arrow in Fig. 10A or competes more readily with RecQ for a recombination intermediate is unknown. In contrast, topoisomerase III cannot act upon the DNA substrate once processed by RuvAB for RuvC because topoisomerase III overproduction did not have any effect on *ruvC53* mutant cells (Fig. 7C). What decides the fate of the Holliday junction is unknown; relative binding affinities, enzyme abundance, and preferred sites of enzyme activity may all come into play. It is also possible that normally the topoisomerase pathway might be used exclusively for resolving double Holliday junctions whereas RuvC might be preferred for the resolution of single Holliday junctions.

Because of RuvC, topoisomerase I, RecG, and sometimes RusA, the absence of topoisomerase III is normally tolerated by *E. coli*. However, when topoisomerase IV is mutated and RecA and RecQ are present, the removal of topoisomerase III may lead to cell death because the chromosomes become intertwined and trapped in the recombination intermediate structures. Failure to unlink the DNA would prevent chromosomal segregation, thus impeding cell division and resulting in a partition defective phenotype. A similar partition defective phenotype was seen in *S. pombe* and *S. cerevisiae* cells that lack topoisomerase III (Gangloff *et al.*, 1999; Maftahi *et al.*, 1999). Interestingly, similar cell morphology also resulted from the overproduction of the RecQ homologue from *S. pombe*, Rqh1 (Oh *et al.*, 2002); more Rqh1 might lead to more intermediates funnelled into the topoisomerase pathway than those enzymes can process.

Our data suggest that the *parE<sup>ts</sup>* and *parC<sup>ts</sup>* alleles cause an increase in DNA lesions that must be processed by topoisomerase III or RuvC. The topoisomerase IV mutants at their non-permissive temperatures result in: (i) increased negative supercoiling of the DNA (Zechiedrich *et al.*, 2000), which might explain; (ii) increase in cell growth and DNA replication rates; (iii) increased catenation (Adams *et al.*, 1992; Zechiedrich and Cozzarelli, 1995; Zechiedrich *et al.*, 1997) and knotting (Deibler *et al.*, 2001). Any of these could lead to an increase in DNA damage, which in turn might overwhelm topoisomerase III and RuvC, both of which, as discussed above, are not abundant in the cell. Indeed, even at 30°C, the SOS response is activated in *parE<sup>ts</sup>* strains (data not shown), indicating the presence of DNA damage. In addition, preliminary results show that *ruvC53 parE10* cells are more sensitive to UV light than *ruvC53* single mutants and that *parE10* cells are also more sensitive to UV irradiation than wild-type cells at higher (but still permissive) temperatures (C. R. Lopez, J. Galloway and E. L. Zechiedrich, unpubl. results). We show here that *recA parE10* cells are less viable than *recA* cells (Fig. 5B). This has been reported previously (Grompone *et al.*, 2004).

Grompone *et al.* also found that a *priA parE10* double mutant displays a synthetic lethal phenotype. This suggests that the *parE10* allele causes a hindrance to DNA replication. These data all point to *parE10* causing DNA damage.

There also appears to be a *recA* independent repair pathway that is involved in dealing with the problems caused by the *parE10* allele. Although the *recA parE10* double mutant has reduced viability, it is still viable. If the tolerance of the damage caused by the *parE10* allele involved only *recA*, then the *recA parE10* would not be viable. The stochastic nature as to which repair pathway is chosen (McCool *et al.*, 2004) would explain why the deletion of *recA* rescues some of the *topB parE10* mutants, but not all of them. We think that it is also possible that *topA* mutants could lead to DNA damage that requires *recA*, *topB* and possibly *ruvC* for repair.

The functional complexity of the roles of proteins involved in DNA segregation, DNA repair, DNA recombination, and the modulation of DNA topology exemplifies the adaptive nature of bacteria. Rather than these enzymes having redundant functions, each has a specific role. Multiple enzymes functioning in multiple alternative pathways probably allow cells to adapt to a variety of conditions.

## Experimental procedures

### Chemicals and reagents

All antibiotics, DNaseI, RNaseA and DAPI were from Sigma. Supercoiled DNA and 1KB plus DNA ladders were from Invitrogen. Proteinase K was from Boeringer Mannheim. Nylon Zeta-Probe Blotting Membranes were from Bio-Rad. IPTG and formaldehyde were from Fisher Scientific. [ $\alpha$ - $^{32}$ P] dCTP and Megaprime DNA Labelling System were purchased from Amersham Pharmacia.

### Bacterial allele construction and plasmids

$\Delta$ *recQ::cat* contains the chloramphenicol acetyltransferase (*cat*) gene flanked by FRT sites replacing codon 32–567 of the *recQ* gene. This allele was constructed by linear replacement (Datsenko and Wanner, 2000). The primers used to generate the linear fragment were *recQ1*: 5'-TAAA CAGGTTTTACAAGAAACCTTTGGCTACCAACAGTTTCGC CCGGCCGTTAGGCTGGAGCTGCTTC and *recQ2*: 5'-CATGCGCACGAATCAGCGCCATAAACGGTTTGCCAAAGC GTTCCAGCTTGCGTATGAATATCCTCCTTAGT where the underlined sequence corresponds to *recQ* coding sequence and the bold sequence corresponds to plasmid pKD3 (Datsenko and Wanner, 2000). Plasmid pPH1243 (Broccoli *et al.*, 2000) contains the *topB* gene with 200 bp of 5' upstream sequence under the control of the *trc* promoter and is derived from the pTRC99a vector (provided by Marc Drölet, University of Montreal, Canada). pTBE302 (Zhu *et al.*, 2001) contains the *topB* gene under the control of an arabinose-inducible promoter. It is based on the pBAD30 vector. pGBruvABC (Flores *et al.*, 2001) contains the *ruvAB* and

*ruvC* genes cloned into the low copy number vector pGB2 (generously provided by Benedicte Michel, Genetique Microbienne, Institute National de la Recherche Agronomique, Jouy en Josas, France). Plasmid pJB3.5d has been described previously (Bliska and Cozzarelli, 1987).

### Bacterial strains

All bacterial strains used are listed in Table 3. P1 transductions were performed with P1<sub>virA</sub> according to Miller (Miller, 1972). To make strain CRL6, first the Tn10*tet* allele from strain CAG18465 was transduced into strain QZ103 ( $\Delta$ *topB::kan*). The resulting  $\Delta$ *topB::kan* Tn10*tet* alleles from this strain were transduced (selecting for tetracycline resistance) into the following strains: W3110, C600, ParE10, ParC1215, LE234, LE316, CRL31, CRL32, SAR3, SAR4, SMR6047 and SMR4562. These transductants were then screened for kanamycin resistance at 30°C to determine whether the  $\Delta$ *topB* allele was cotransduced (all subsequent experiments with these strains were performed at 30°C), and we verified the presence of the original mutation in these strains by measuring temperature sensitivity at 42°C (ParE10, ParC1215 and LE316) or UV sensitivity (SMR6047).

To construct CRL22 and CRL36, the  $\Delta$ *topB::kan* Tn10*tet* alleles from CRL6 and the  $\Delta$ *topB::kan* allele from QZ103 were transduced into ParE10 [pPH1243] *parE10*+ [pPH1243] in the presence of 0.5 mM IPTG and 100  $\mu$ g ml<sup>-1</sup> of ampicillin in addition to the selection agent (tetracycline or kanamycin respectively). This allowed the  $\Delta$ *topB* allele to coexist with the *parE10* allele at 30°C. Tetracycline and kanamycin resistance, and temperature sensitivity were verified.

Strain LZ2369 was made by transducing the  $\Delta$ *topB::kan* allele from QZ103 into a strain LZ5 (*gyrA*L83). To construct strains CRL38 and CRL40, the  $\Delta$ *recA* and *lexA3* alleles were transduced into strains LZ2549 (W3110 + pPH1243) and CRL36 ( $\Delta$ *topB::kan parE10*+ pPH1243). CRL45, CRL46 and CRL47 were constructed by transducing Tn10 from strains CAG18468, CAG18491 and CAG 18465 into strains CAG18522, CAG18557 and CAG18518. Kanamycin and tetracycline resistance were verified. CRL2 [P1(QZ103) X ParE10] and CRL35 [P1(CRL6) X ParE10] were constructed as above. Throughout the text we refer to these strains by their relevant phenotype.

### Linkage efficiency assay

Tn10 was linked to  $\Delta$ *topB::kan*, as described above.  $\Delta$ *topB::kan* Tn10 from donor strain CRL6 was then transduced into an isogenic set of mutants, selecting for tetracycline resistance. Transductants were given up to 160 h to appear at 30°C. The subsequent transductants were then screened for kanamycin resistance. A contingency chi-square statistical analysis was performed to determine whether the difference in linkage values was significant. *P*-values are given. A-value  $\leq$  0.05 was considered significant, with a 95% confidence level. Transduction frequency was identical in *parE*<sup>+</sup> and *parE*<sup>ts</sup> strains when selecting for tetracycline resistance (CRL6) or chloramphenicol resistance (SMR6201 and SWM1001).

Table 3. Bacterial strains.

Strain	Genotype	Construction/reference
AB1157	F <sup>-</sup> <i>thr-1 leuB6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 Δgpl-proA</i> )62 <i>hisG4 argE3 rpsL31Sm<sup>R</sup></i> ) <i>tsx-33 supE44 kdgK51rfbD1 mgl-51 qsr<sup>-</sup> rac<sup>-</sup> λ<sup>-</sup></i>	Howard-Flanders <i>et al.</i> (1964)
CAG18465	MG1655 <i>zdj-225::Tn10</i>	Singer <i>et al.</i> (1989)
CAG18468	MG1655 <i>nupC510::Tn10</i>	Singer <i>et al.</i> (1989)
CAG18491	MG1655 <i>metE3079::Tn10</i>	Singer <i>et al.</i> (1989)
CAG18518	MG1655 <i>zdi-3122::Tn10kan</i>	Singer <i>et al.</i> (1989)
CAG18522	MG1655 <i>zfb-3135::Tn10kan</i>	Singer <i>et al.</i> (1989)
CAG18557	MG1655 <i>fadAB3165::Tn10kan</i>	Singer <i>et al.</i> (1989)
CRL2	W3110 <i>ΔtopB::kan parE10</i>	P1(QZ103) × ParE10
CRL3	W3110 <i>ΔtopB::kan</i>	P1(QZ103) × W3110
CRL6	QZ103 <i>zdj-225::Tn10</i>	P1(CAG18465) × QZ103
CRL19	W3110 <i>ΔtopB::kan zdj-225::Tn10</i>	P1(CRL6) × W3110
CRL22	W3110 <i>ΔtopB::kan zdj-225::Tn10 parE10</i> [pPH1243]	P1(CRL6) × ParE10 [pPH1243]
CRL31	W3110 <i>ΔrecQ::cat</i>	P1(SMR6201) × W3110
CRL32	W3110 <i>ΔrecQ::cat parE10</i>	P1(SMR6201) × ParE10
CRL35	W3110 <i>ΔtopB::kan zdj-225::Tn10 parE10</i>	P1(CRL6) × ParE10
CRL36	W3110 <i>ΔtopB::kan parE10</i> [pPH1243]	P1(QZ103) × ParE10 [pPH1243]
CRL37	W3110 <i>Δ(srIR-recA)306::Tn10 parE10</i> [pPH1243]	P1(GY8322) × ParE10 [pPH1243]
CRL38	W3110 <i>Δ(srIR-recA)306::Tn10 ΔtopB::kan parE10</i> [pPH1243]	P1(GY8322) × CRL36
CRL39	W3110 <i>Δ(srIR-recA)306::Tn10</i> [pPH1243]	P1(GY8322) × W3110 [pPH1243]
CRL40	W3110 <i>lexA3 malB::Tn9 ΔtopB::kan parE10</i> [pPH1243]	P1(SMR841) × CRL36
CRL41	W3110 <i>lexA3 malB::Tn9</i> [pPH1243]	P1(SMR841) × W3110 [pPH1243]
CRL43	FC40 <i>ruvC53 upp::Tn10dtet + 1 eda::Tn10cat ΔtopB::kan zdj-225::Tn10</i>	P1(CRL6) × SMR6047
CRL45	CAG18522 <i>nupC510::Tn10</i>	P1(CAG18468) × CAG18522
CRL46	CAG18557 <i>metE3079::Tn10</i>	P1(CAG18491) × CAG18557
CRL47	CAG18518 <i>zdj-225::Tn10</i>	P1(CAG18465) × CAG18518
CRL48	FC40 <i>ΔtopB::kan zdj-225::Tn10</i>	P1(CRL6) × SMR4562
CRL51	FC40 <i>ruvC53 upp::Tn10dtet + 1 eda::Tn10cat ΔtopB::kan zdj-225::Tn10</i>	P1(CRL6) × SMR6047
CRL74	MG1655 <i>ruvC53 upp::Tn10dtet + 1 eda::Tn10cat</i>	P1(SMR6047) × MG1655
CRL76	MG1655 <i>ruvC53 upp::Tn10dtet + 1 eda::Tn10cat ΔtopB::kan</i>	P1(SMR6047) × QZ103
CRL79	W3110 <i>Δ(srIR-recA)306::Tn10 ΔtopB::kan</i> [pPH1243]	P1(GY8322) × CRL3 [pPH1243]
CS85	<i>ruvC53 eda-51::Tn10</i>	R.G. Lloyd (Nottingham) via R.D. Kolodner
C600	F <sup>-</sup> <i>λ<sup>-</sup> thr-1 leuB6 thi-1 lacY1 supE44 tonA21 fhuA21</i>	Appleyard (1954)
DG75	F <sup>-</sup> <i>thy leu</i>	Wolf <i>et al.</i> (1968)
FC40	<i>Δ(lac-proAB)<sub>xiii</sub> thi ara Rif<sup>R</sup> [F<sup>-</sup> proAB<sup>+</sup> lacl<sub>q</sub> lacl33 ΩlacZ]</i>	Cairns and Foster (1991)
GP100	KD100 <i>gyrB225ts</i>	Steck and Drlica (1984)
GY8322	<i>Δ(srIR-recA)306::Tn10</i> [mini-F <i>recA</i> <sup>+</sup> ]	A. Bailone
JJC754	AB1157 <i>ΔruvABC::cat</i>	Seigneur <i>et al.</i> (1998)
KD100	Streptomycin sensitive derivative of DG75	Steck and Drlica (1984)
LE234	F <sup>-</sup> <i>metB argE ilv tna</i>	Orr <i>et al.</i> (1979)
LE316	LE234 <i>gyrB134 coufs</i>	Orr <i>et al.</i> (1979)
LZ5	C600 <i>gyrA<sup>83</sup> zei-723::Tn10</i>	Khodursky <i>et al.</i> (1995)
LZ6	C600 <i>zei-723::Tn10</i>	Khodursky <i>et al.</i> (1995)
LZ2369	LZ5 <i>ΔtopB::kan</i>	P1 (QZ103) × LZ5
LZ2371	LZ6 <i>ΔtopB::kan</i>	P1 (QZ103) × LZ6
MG1655	F <sup>-</sup> <i>λ<sup>-</sup> rph-1</i>	Guyer <i>et al.</i> (1981)
ParC1215	C600 <i>parC1215ts</i>	Kato <i>et al.</i> (1988)
ParE10	W3110 <i>parE10ts</i>	Kato <i>et al.</i> (1990)
QZ103	MG1655 <i>ΔtopB::kan</i>	Zhu <i>et al.</i> (2001)
SAR3	W3110 <i>ΔhelD::cat</i>	P1(SWM1001) × W3110
SAR4	W3110 <i>ΔhelD::cat parE10</i>	P1(SWM1001) × ParE10
SMR632	594 <i>hsdR<sub>K</sub><sup>-</sup> M<sub>K</sub><sup>+</sup></i>	Motamedi <i>et al.</i> (1999)
SMR635	594 <i>hsdR<sub>K</sub><sup>-</sup> M<sub>K</sub><sup>+</sup> ruvC53 eda-51::Tn10</i>	P1 (CS85) × SMR632
SMR841	<i>lexA3 malB::Tn9</i>	McKenzie <i>et al.</i> (2000)
SMR4562	genotype identical to FC40, independent construction	McKenzie <i>et al.</i> (2000)
SMR6047	SMR4562 <i>ruvC53 upp::Tn10dtet + 1 eda::Tn10cat</i>	Bull <i>et al.</i> (2001)
SMR6201	594 <i>ΔrecQ::cat</i>	This study
SWM1001	AB1157 <i>ΔhelD::cat</i>	Mendonca <i>et al.</i> (1993)
W3110	F <sup>-</sup> <i>λ<sup>-</sup> IN(rrnD-rrnE) 1 rph-1</i>	Kato <i>et al.</i> (1990)
594	F <sup>-</sup> <i>λ<sup>-</sup> lac-3350 galK2 galT22 rpsL179 rpsL179 IN(rrnD-rrnE)1</i>	Weigle (1966)

Tn10 and Tn10kan were transduced into *ruvC*<sup>+</sup> (SMR4562) and *ruvC53* (SMR6047) strains. The cotransduction frequency was ~32% from CRL45, 67% from CRL46 and 17% from CRL47.

More *ΔtopB parE*<sup>ts</sup> colonies do arise if given more recovery time to appear after transduction; however, greater incubation time (~150 h) does not result in a greater proportion of *ΔtopB parE*<sup>ts</sup> colonies (data not shown). Even given more time after

the transduction to grow, none of the late arising tetracycline-resistant colonies were also kanamycin resistant in the *parC<sup>ts</sup>* background.

ParE10 and ParC1215 strains (and their isogenic wild-type strains) were transformed with pGBruvABC or empty vector (pGB2).  $\Delta topB::kan$  Tn10 from CRL6 was then transduced into these strains as above.

#### Plating efficiency assays

Cultures were grown to mid-logarithmic phase ( $A_{600} = 0.4$ ) in LB medium containing  $100 \mu\text{g ml}^{-1}$  ampicillin and  $0.5 \text{ mM}$  IPTG. A  $50 \mu\text{l}$  aliquot of a  $10^{-4}$  dilution of the culture was spread onto two sets of LB agar plates containing  $100 \mu\text{g ml}^{-1}$  ampicillin with and without  $0.5 \text{ mM}$  IPTG. The plates were incubated up to 72 h at  $30^\circ\text{C}$ ,  $37^\circ\text{C}$  or  $42^\circ\text{C}$ . The number of colonies on each plate was counted after each overnight incubation.

#### Microscopy

WT (C600 and W3110), *parC<sup>ts</sup>* and *parE<sup>ts</sup>* mutant strains containing plasmid pPH1243 were grown in LB medium with  $100 \mu\text{g ml}^{-1}$  ampicillin and either  $0.5 \text{ mM}$  or no IPTG at  $30^\circ\text{C}$ . Mid-logarithmic phase cells ( $A_{600} = 0.4$ ) were diluted to  $A_{600} = 0.01$  and shifted to  $37^\circ\text{C}$  (*parC<sup>ts</sup>* and C600) or  $43^\circ\text{C}$  (*parE<sup>ts</sup>* and W3110) and allowed to grow for two additional hours. Cells were prepared for microscopy essentially as described in (Pringle *et al.*, 1991). Briefly, cells were fixed by the addition of formaldehyde ( $37\% \text{ w/v}$  stock) to  $5\% \text{ w/v}$  and incubated for at least 1 h at  $25^\circ\text{C}$ . Cells were then washed with  $0.1 \text{ M KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (pH 7.0), placed on poly-L-lysine treated glass slides and overlaid with mounting medium that contained  $225 \text{ ng ml}^{-1}$  4',6-diamidino-2-phenylindole dihydrochloride (DAPI). A Zeiss Axioplan fluorescence microscope was used. Images were captured using a Photometrics Cool-Snap HQ camera with MetaVue v.6r6 software.

#### Analysis of catenane accumulation by high-resolution gel electrophoresis

Strains LZ5 and LZ2369, containing plasmid pJB3.5d, were grown at  $37^\circ\text{C}$  in LB medium containing  $100 \mu\text{g ml}^{-1}$  ampicillin. At  $A_{600} = 0.4$ , a  $5.7 \text{ ml}$  aliquot was taken (0 min). Norfloxacin was added to a final concentration of  $60 \mu\text{M}$  to inhibit topoisomerase IV activity (Khodursky *et al.*, 1995). Additional  $5.7 \text{ ml}$  aliquots were taken 20, 30, 40 and 60 min after addition of drug. Plasmid DNA was isolated by alkaline lysis immediately after taking the aliquots. The DNA was nicked with DNaseI, subjected to high-resolution gel electrophoresis (Sundin and Varshavsky, 1981), transferred to nylon membranes, and probed with [ $\alpha$ - $^{32}\text{P}$ ] dCTP labelled linearized pJB3.5d DNA.

Strains W3110, ParE10 and CRL3, containing pBR322, were grown at  $30^\circ\text{C}$  in LB medium containing ampicillin ( $100 \mu\text{g ml}^{-1}$ ). Upon reaching  $A_{600} = 0.4$ , a  $5.7 \text{ ml}$  aliquot was taken (0 min) and the cultures were shifted to  $42^\circ\text{C}$ . Aliquots of  $5.7 \text{ ml}$  were then taken at 15 and 30' after this temperature shift. Plasmid DNA was isolated by alkaline lysis immediately after taking the aliquots. The DNA was nicked with DNaseI, subjected to high-resolution gel electrophoresis

(Sundin and Varshavsky, 1981), transferred to nylon membranes, and probed with [ $\alpha$ - $^{32}\text{P}$ ] dCTP-labelled linearized pBR322 DNA.

#### Analysis of plasmid supercoiling levels

Strains LZ5, LZ6, LZ2369 and LZ2371, containing plasmid pJB3.5d, were grown at  $37^\circ\text{C}$  as described above and previously (Zechiedrich *et al.*, 2000). At  $A_{600} = 0.4$ , a  $5.7 \text{ ml}$  aliquot was taken (0 min). Norfloxacin was added to a final concentration of  $60 \mu\text{M}$  to inhibit topoisomerase IV activity (Khodursky *et al.*, 1995). Additional  $5.7 \text{ ml}$  aliquots were taken 20, 30, 40 and 60 min after addition of drug. Plasmid DNA was isolated by alkaline lysis immediately after taking the aliquots. The DNA was subjected to gel electrophoresis on a  $1.2\%$  agarose gel containing either  $2 \mu\text{g ml}^{-1}$  (for relaxed DNA) or  $4 \mu\text{g ml}^{-1}$  (for supercoiled DNA) chloroquine (Zechiedrich *et al.*, 2000). The DNA was then transferred to nylon membranes, and probed with [ $\alpha$ - $^{32}\text{P}$ ] dCTP labelled linearized pJB3.5d DNA.

#### UV sensitivity assay

Cells were grown to  $A_{600} = 0.4$  in the LB broth at  $30^\circ\text{C}$ . After normalizing the amount of cells to  $A_{600} = 0.4$ , serial dilutions were performed. Aliquots ( $50 \mu\text{l}$ ) of  $10^{-4}$  dilutions were spread onto LB plates (unless otherwise noted). For cells containing the *ruvC53* allele, a  $50 \mu\text{l}$  aliquot of a  $10^{-3}$  dilution was also plated. Cells were allowed to grow on the plates for 30 min at  $24^\circ\text{C}$ . The plates were then exposed to the indicated dosage of UV light using a CL-1000 ultraviolet crosslinker and incubated overnight at  $30^\circ\text{C}$ . Colonies were allowed to grow for 72 h at  $30^\circ\text{C}$ , followed by another 72 h at  $24^\circ\text{C}$ . Colonies were counted after each night of incubation. Each strain was tested a minimum of three times.

#### Mitomycin C sensitivity

Cells were grown to mid-logarithmic phase ( $A_{600} = 0.4$ ). After normalization to 0.4, the cultures were serially diluted. Fifty microlitres of both a  $10^{-3}$  and  $10^{-4}$  dilution (for strains with the *ruvC53* allele) or  $50 \mu\text{l}$  of the  $10^{-4}$  dilution were spread onto LB plates containing  $0.5 \mu\text{g ml}^{-1}$  of mitomycin C, as well as an LB plate without drug. The plates were incubated for up to 72 h at  $30^\circ\text{C}$ .

#### Transduction efficiency

A P1 lysate of CAG18468 was titered, and an MOI of 0.1 was used to infect the strains tested. There was no significant difference in cell density at  $A_{600} = 0.4$  for WT, *ruvC53*,  $\Delta topB$  and  $\Delta topB$  *ruvC53* strains. After normalizing 1 ml of culture to 0.4, the cells were pelleted and resuspended in  $0.5 \text{ ml}$  of  $0.03 \text{ M MgSO}_4$   $0.015 \text{ M CaCl}_2$ . In addition to cells incubated with phage, a tube of each strain was also incubated without phage. After 20 min at  $30^\circ\text{C}$ , the cells were pelleted and resuspended in  $150 \mu\text{l}$  of LB + sodium citrate. The cells were then allowed to recover for 45 min at  $30^\circ\text{C}$ . All  $150 \mu\text{l}$  were spread onto LB-Tet plates. In addition to this,  $25 \mu\text{l}$  of non-transduced cells were also spread onto a plain LB plate. After plating, cells were incubated overnight at  $30^\circ\text{C}$ . After recording the number of transductants, the plates were incubated an additional 72 h at the same temperature. Each strain was

tested in duplicate. There appeared to be no significant difference in viability among the strains tested. The number of possible transductants = number of colonies present without selection agent  $\times$  dilution used ( $10^{-5}$ ). Efficiency = number of transductants/number of possible transductants.

### Cell viability

The viability of the indicated strains was measured by diluting an overnight culture in fresh LB broth and growing to  $A_{600} = 0.4$ . After normalization to 0.4, the culture was serially diluted in 1% NaCl. Fifty microlitres of a  $10^{-3}$  (for *recA* strains) or  $10^{-4}$  dilution were spread onto plates with or without 0.5 mM IPTG and incubated for up to 72 h at 30°C, 37°C or 42°C. The number of colonies was counted after each overnight incubation period. After plating the diluted cells, the 5  $\mu$ l of the normalized culture were placed onto a Petroff-Hauser slide to determine the number of cells  $\text{ml}^{-1}$ . Viability = colonies/cells plated.

### Codon bias index calculation

Codon bias index was calculated according to Bennetzen and Hall (1982). The sequence of each gene in question was retrieved from the NCBI website and inserted into DNA Strider (version 1.01). DNA Strider then determined the frequency of each codon. From this information, we calculated: (i) number of preferred codons used (as defined by Bennetzen and Hall), (ii) number of preferred codons by random chance (sum of the total number of codons encoding each particular amino acid divided by the number of possible codons for each amino acid. For example, if there were 20 phenylalanines encoded, then  $20 \div 2$  (because there are two codons that encode phenylalanine) and so forth for each amino acid and (iii) the total number of codons (not counting Met, Asp or Trp).

CBI = (number of preferred codons – number of preferred codons by random chance)  $\div$  (total number of codons – number of codons encoding Met, Asp and Trp) – number of preferred codons by random chance).

In addition to the genes shown in Table 2, CBI was calculated for *mutH* (0.181), *mutL* (0.194), *mutS* (0.254), *phrB* (0.066), *recB* (0.210), *recC* (0.211), *recD* (0.112), *recF* (0.026), *recJ* (0.204), *recO* (0.081), and *recR* (0.224).

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