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The genetic control of DNA supercoiling in *Salmonella typhimurium*

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We have elucidated the genetic control of DNA supercoiling in *Salmonella typhimurium*. The level of superhelix density is controlled by two classes of genes. The only member of the first class is *topA*, the structural gene for topoisomerase I. The second class, *tos*, (topoisomerase one suppressor) consists of at least two genes, one of which is linked to *gyrA*, the structural gene for the topoisomerase subunit of DNA gyrase. Deletions of *topA* result in oversupercoiling of plasmid DNA. These mutations do not require the acquisition of second-site compensatory mutations to allow cell growth, in contrast to the situation in *Escherichia coli*. However, *tos* mutations, unlinked to *topA*, have been isolated which reduce plasmid superhelix density. We conclude that the level of DNA supercoiling in *S. typhimurium* is a dynamic balance between the effects of the gene products of *topA* (relaxation) and *tos* (supercoiling) which act independently of each other. Using a variety of combinations of these mutations we have constructed a series of isogenic strains, each of which has a different but precisely defined level of plasmid supercoiling; the series as a whole provides a wide range of supercoiling both above and below the wild-type level.

Key words: DNA supercoiling/*S. typhimurium*/topoisomerase I/gyrase

Introduction

DNA isolated from prokaryotic sources is usually negatively supercoiled. The degree of supercoiling affects many cellular processes, including replication (Drlica and Snyder, 1978), integrative recombination (Mizuuchi *et al.*, 1980), transposition (Isberg and Syvanen, 1982) and transcription (Wahle and Mueller, 1980; Yang *et al.*, 1979). The maintenance of negative supercoiling *in vivo* is presumed to depend on a functional balance between the enzymes DNA gyrase and topoisomerase I. DNA gyrase introduces negative turns in a topologically constrained duplex molecule in a reaction requiring ATP (Gellert *et al.*, 1976). In contrast, topoisomerase I relaxes negatively supercoiled DNA without an energy requirement (Wang, 1971). The normal level of chromosomal superhelix density may therefore be changed by affecting the function of one or other of these enzymes.

The structural genes for both topoisomerase I and DNA gyrase have been identified in *Escherichia coli* and *Salmonella typhimurium* (Bachmann, 1983; Sanderson and Roth, 1983). In *E. coli* the two subunits of gyrase are encoded by *gyrA* and *gyrB*, located at 48 and 83 minutes, respectively, on the chromosomal map. The gene for topoisomerase I, *topA*, is located at 28 minutes between *trp* and *cysB*. Strains deleted for *topA* are non-viable unless an additional mutation is also present (DiNardo *et al.*, 1982; Pruss *et al.*, 1982). Such second-site mutations, some of which have been mapped to *gyrA* and *gyrB*, presumably compensate for the oversupercoiling which results when *topA* is deleted.

In *S. typhimurium* the *topA* gene has been shown to be identical to the previously characterised *supX* locus (Trucksis *et al.*, 1981). Mutations in *supX* were first isolated by their ability to suppress the auxotrophic requirement of strains bearing the promoter mutation *leu-500* (Mukai and Margolin, 1963). Subsequently Trucksis *et al.* (1981) showed that *supX* mutations, which like *topA* in *E. coli* map between *trp* and *cysB*, result in the loss of topoisomerase I antigenicity. Mutations in *topA* (*supX*) have a variety of phenotypic effects in addition to the suppression of the *leu-500* promoter mutation, including suppression of certain *lac* promoter mutations (Dubnau and Margolin, 1972), increased production of a number of enzymes (Dubnau and Margolin, 1972; Graf and Burns, 1973), increased u.v. light sensitivity and the loss of u.v. light-induced mutagenesis (Overbye and Margolin, 1981).

We have defined the genetic basis for the control of DNA supercoiling in *S. typhimurium*. Here we describe the isolation and characterization of several well defined *topA* mutations. The effects of these mutations on DNA supercoiling were followed by observing the superhelix density of a plasmid carried by these strains. Unlike the situation in *E. coli*, strains of *S. typhimurium* carrying deletions of the *topA* gene are viable, even in the absence of any mutations compensating for the oversupercoiling. However, second-site mutations which reduce the oversupercoiling can be selected. Thus the level of DNA supercoiling in *S. typhimurium* is controlled by two classes of genes with opposing effects. The superhelix density of DNA in wild-type cells results from a balance between the effects of the products of these two classes of genes. The genetic analysis is therefore in correspondence with known enzymology. We have used various combinations of these mutations to construct a series of strains in which the DNA is supercoiled to levels both above and below that of the wild-type.

Results

Isolation of topA mutations

Mutations in the *topA* gene were isolated in two ways. (1) The *topA* gene lies between *trp* and *cysB* (Dubnau and Margolin, 1972; Trucksis *et al.*, 1981). By selecting for deletions simultaneously removing both *trp* and *cysB*, it was possible to delete the *topA* gene entirely, in the absence of any selective pressure for *topA* function. This was achieved using fusaric acid to select for spontaneous tetracycline-sensitive derivatives of CH340, which carries a Tn*10* insertion in *trp*. Following Tn*10* excision, which generally results in deletion or inversion of adjacent chromosomal DNA, Tet^s colonies were screened for auxotrophic requirements. Any which had simultaneously become both Trp^- and Cys^- were analysed further. Four such Trp^- Cys^- derivatives were selected

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(strains CH582, CH598, CH599, CH600) and all were shown to be deleted for *topA*, taking advantage of the promoter mutation *leu-500*; the Leu$^-$ phenotype of strains with this mutation is suppressed by mutations at the *topA* locus. To demonstrate that this Leu$^+$ phenotype was not due to simultaneous reversion of the *leu-500* mutation, these strains were transduced to Trp$^+$ Cys$^+$ with phage P22 grown on wild-type *S. typhimurium* LT2. All such transductants became Leu$^-$.

(2) Mutations in *topA* were selected in CH340 by spreading cells onto minimal glucose plates supplemented only with tryptophan. Spontaneously arising leucine prototrophic colonies fell into two distinct size classes. Colonies of wild-type size (2 mm after 48 h at 37°C) arose at a low frequency and were shown to be due to mutations mapping within the leucine operon, presumably reversions of *leu-500*; these were not considered further. The second class of colonies arose at a higher frequency, were much smaller and were presumed to harbour spontaneous *topA* mutations suppressing the leucine auxotrophy. Four of these strains (CH594 to CH597) were shown to have a mutation in *topA* by demonstrating that they still contained the original *leu-500* mutation, and that the suppressing mutation was linked to *trp*. This was shown as follows. The presumed *topA leu-500* strains were transduced to Trp$^+$ with P22 grown on wild-type cells. Of the resulting Trp$^+$ Tet^S transductants, 46% (11/24) remained Leu$^+$ and retained the parental small colony type. However, 54% became Leu$^-$ and regained the wild-type (*topA*$^+$) colony morphology. This linkage between *trp* and the *leu-500*-suppressing mutation is in close agreement with previous estimates of the linkage between *topA* and *trp* (Dubnau and Margolin, 1972). The spontaneous mutations in *topA*, selected by suppression of *leu-500*, are hereafter referred to as point mutations although we have not discounted the possibility that some of them may be short deletions.

topA strains grow slowly

Strains carrying a *topA* mutation grow slowly compared with the parental strain CH340, even on LB agar. The rates of growth in LB liquid medium of strains carrying point (*topA2763, topA2764, topA2765, topA2766*) or deletion (*△topA2762, △topA2767, △topA2768, △topA2769*) mutations in *topA* were identical, with a doubling time of 64 min. This is to be compared with a doubling time of 29 min for the parental strain CH340 and for the wild-type LT2.

topA strains oversupercoil plasmid DNA

The identification of *topA* as the structural gene for topoisomerase I implies that mutations at this locus concur with changes in the superhelix density of the DNA within the cell. All of the strains used in this study carried pLk1, a small circular plasmid of 2233 bp. This plasmid would be expected to adopt the level of supercoiling imposed on it by the functional topoisomerases in the cell. To examine the level of supercoiling of pLk1, plasmid DNA was extracted from the various strains and electrophoresed in chloroquine-containing agarose gels. This is illustrated in Figure 1 where plasmid DNA isolated from the parental strain (CH340) is compared with that isolated from strains carrying a point or a deletion mutation of *topA*. It is clear that the *topA* mutations significantly alter the supercoiling of pLk1 DNA. The use of two different concentrations of intercalator confirmed that pLk1 extracted from *topA* strains is oversupercoiled relative to that of their *topA*$^+$ parent (not shown).

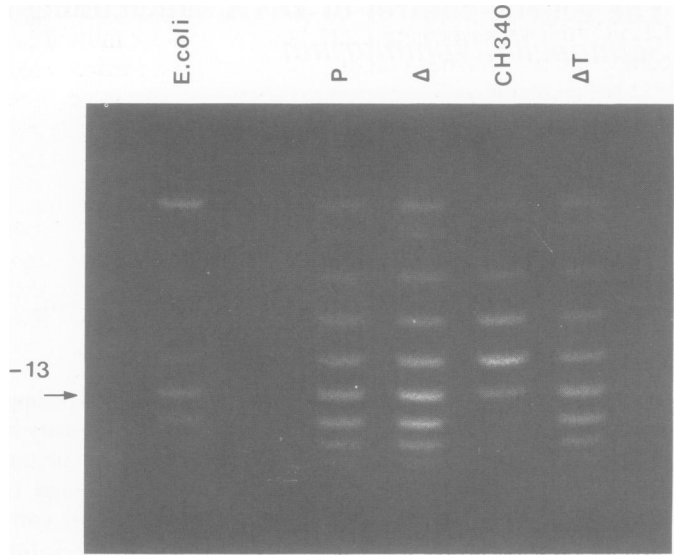

Fig. 1. Plasmid supercoiling in *topA* strains of *S. typhimurium*. pLk1 DNA was electrophoresed in 1.0% agarose containing 2.0 μg/ml chloroqine. Strain CH594 (**track P**) contains a point mutation in the *topA* gene. CH582 (**track** △) contains a *topA* deletion. CH340 is the parental *topA*$^+$ strain from which these were derived. CH583 (**track** △**T**) was derived by transduction of the deletion *△topA2762* into CH340. Absolute linking differences may be estimated by comparison with pLk1 prepared from *E. coli* strain HB101. The linking difference of its most intense topoisomer is indicated at the left.

Table I. Comparison of superhelix densities in four plasmids of different sizes in strain CH582

Plasmid	Size bp	Linking difference $\triangle Lk$	Superhelix density σ
pLk1	2233	−13.5	−0.0635
pLk4	2500	−15.2	−0.0638
pLk3	2907	−17.7	−0.0640
pAT153	3657	−22.3	−0.0640

The mean value is $\sigma = -0.0639 \pm 0.00025$ (SD).

Interestingly, the degree of pLk1 supercoiling was identical in all of the *topA* strains isolated, whether the lesion was a deletion or a point mutation. Therefore it seems that *topA* mutations isolated by suppression of the *leu-500* mutation, as well as the *trp-topA-cysB* deletions, result in the complete loss of topoisomerase I function.

The absolute linking differences of pLk1 DNA isolated from *S. typhimurium* strains were determined by comparison with pLk1 DNA extracted from the *E. coli* K-12 strain HB101. The linking difference of pLk1 from HB101 was measured accurately by the band counting method (Keller, 1975). Compared with the value of −13.2 observed in *E. coli*, the mean linking difference of pLk1 in the parental strain CH340 is −11.9. In the strains carrying *topA* mutations this value falls to a linking difference of −13.5. This corresponds to an increase in superhelix density from $\sigma = -0.056$ to -0.064.

To show that this value is not a special property of the plasmid pLk1, these measurements were compared with the ability of a *topA* strain to oversupercoil three other plasmids: pAT153 (3.65 kb) and two derivatives of this plasmid, pLk3

(2.9 kb), pLk4 (2.5 kb). Each plasmid was used to transform CH582 to Tet^r, displacing pLk1 because of their mutual incompatibility. The mean linking differences were determined and their corresponding superhelix densities calculated. The results are summarised in Table I. The superhelix density remained constant within experimental error at $\sigma = -0.064$, irrespective of plasmid size.

topA mutations may be transduced with normal frequencies

Transducing lysates of phage P22 grown on all four strains carrying point mutations in *topA* were used to transfer the *topA* mutations into a *topA*$^+$ background. CH340 was used as the recipient, selecting for Leu$^+$ transductants (suppressing the *leu-500* mutation of CH340). The frequencies at which Leu$^+$ transductants arose (i.e., at which *topA* is transferred) were directly comparable with the frequency at which TT29 (His$^-$) was transduced to His$^+$ using the same transducing lysates. Similar transductions using the *trp-topA-cysB* deletions as donors resulted in tryptophan and cysteine auxotrophy in addition to the suppression of the *leu-500* mutation. However, the frequencies at which the *trp-topA-cysB* deletions could be transferred were an order of magnitude lower than that found for the point mutations. This difference is presumably due to the lower efficiency with which large deletions homologously recombine with a wild-type region of the chromosome (Chelala and Margolin, 1974).

The transduction of *topA* mutations into a *topA*$^+$ background is accompanied by the expected increase in the level of plasmid supercoiling. For example, in Figure 1 the topoisomer distribution of pLk1 extracted from CH583, produced by transducing *△topA2762* into CH340, is identical to that of strain CH582 from which the transducing lysate was made. The same result was obtained from all transductions of *topA* mutations into CH340.

Spontaneous second-site mutations affect growth and superhelix density

When *topA* strains were appropriately diluted and spread on LB agar, a small number of fast growing colonies arose spontaneously. In the case of point mutations in *topA*, some of these fast growers were presumably direct reversions to *topA*$^+$. However, in strains carrying *topA* deletions the mutations resulting in fast growth must be at a second site, suppressing *topA*. We name this locus *tos* (topoisomerase one suppressor). Four such suppressors of *△topA2762* were characterised in detail (see Table II). In all cases the doubling time of strains carrying the second site mutations is reduced to a value between that of the *topA*$^-$ parent (CH582; 64 min) and that of the *topA*$^+$ strain (CH340; 29 min).

It might be expected that the increased growth rates of *topA tos* strains would be accompanied by a reduction in DNA supercoiling to a level below that imposed by the *topA* mutation alone. Plasmid supercoiling in *topA tos* strains is compared with that in the *topA* strain CH582 in Figure 2. It is evident that strains carrying both *topA* and *tos* mutations supercoil pLk1 to a lesser extent than do the strains carrying the *topA* mutations alone. However, the degree to which the different *tos* mutations suppress the oversupercoiling caused by the *△topA2762* mutations varies. Furthermore, the reduction of plasmid supercoiling by progressively more stringent *tos* mutations results in values of superhelix density below that of the parental strain. Mean linking differences, superhelix densities and corresponding growth rates for each strain are listed in Table II.

We observed that the *tos* mutations fall into a limited number of distinct classes. Out of 48 fast growing colonies (six from each of eight separate cultures) only four types of *tos* mutations recurred which could be distinguished by their effects on plasmid supercoiling. These four classes are exemplified by *tos-1, tos-2, tos-3* and *tos-4*. Topoisomer distributions of pLk1 extracted from these strains are shown in Figure 2. All other *tos* mutations resulted in a level of DNA supercoiling indistinguishable from one or other of these four strains. We have calculated the frequency at which *tos* mutations arise to be 10^{-8}; lower than expected for random point mutations in a single gene. This, together with the small number of discrete classes of *tos* mutations, suggests that mutation at only a small number of sites in the gene or genes responsible can result in the Tos$^-$ phenotype; the majority of mutations are presumably lethal or neutral.

The *tos* mutations are not linked to *topA* by transduction. This was shown by transducing CH340 to leucine prototrophy using P22 grown on *topA tos* strains. In repeated transductions the resultant colony morphology, rate of growth in culture and plasmid superhelix densities were identical to those of the original *topA tos*$^+$ strains.

tos mutations in a topA$^+$ *background undersupercoil plasmid DNA*

The *tos* mutations were separated from the *topA* deletion (*△topA2762*) present in the strain in which they were originally isolated by replacing this deletion with wild-type DNA. This was achieved by selecting for Trp$^+$ Cys$^+$ transductants on leucine-supplemented minimal glucose plates using a P22 lysate of LT2. Without exception the growth rates of the resultant *topA*$^+$ *tos* strains were increased to levels closer to that of the wild-type than those of their *topA tos* parents (see Table II).

Having shown that *tos* mutations consistently reduce the oversupercoiling in *topA* strains we reasoned that, in isolation, these *tos* mutations might reduce plasmid supercoiling to levels below that of the wild-type. Figure 3 illustrates the range of plasmid supercoiling in *topA*$^+$ strains containing *tos* mutations compared with that of the wild-type. To take one example, the *tos-4* mutation in a *topA*$^+$ background reduces the mean linking difference of pLk1 to a value of -10.0, well below that of the wild-type (-11.9). The plasmid superhelix densities of these *topA*$^+$ *tos* strains have been determined and are presented in Table II. Note that when the *topA tos* strains are ranked in order of decreasing superhelix density, the *topA*$^+$ *tos* strains exhibit the same order. The ability to separate *topA* and *tos* mutations has enabled us to construct a series of strains with plasmid superhelix densities both above and below that of the wild-type. Within this range, however, there is a considerable degree of overlap in supercoiling between the *topA tos* and the *topA*$^+$ *tos* series of strains which may be seen in Figure 3. As anticipated, the *tos* mutations in isolation from *topA* without exception reduce the plasmid superhelix density below the wild-type level.

Growth rate is affected by variations in DNA supercoiling. Table II shows that deviation in the plasmid supercoiling either above or below the level of the wild-type results in a lower growth rate. Clearly the wild-type level of supercoiling represents the optimum for most rapid growth.

tos-1 is linked to the gyrA locus

Since mutations in *tos* cause a decrease in DNA supercoiling, the function of the *tos* gene(s) must be the introduction of

supercoiling into DNA. Because the only known enzyme capable of performing this function is gyrase, it seemed probable that *tos* was in fact *gyr*. To test whether any of the *tos* mutations were at or close to *gyrA* we made use of strain TT5345 which has the tetracycline resistance transposon Tn*10* 55% linked by co-transduction to *gyrA*. If a *tos* mutation is similarly linked to this transposon this would be consistent with *tos* and *gyrA* being identical. The four *topA tos* strains were transduced to Tet^r using a P22 lysate of TT5345 and their plasmid superhelix density measured. The consequence of replacing a *tos* mutation with the wild-type allele would be to increase the plasmid superhelix density to the level found in a *topA* strain. By this criterion the *tos-2, tos-3* and *tos-4* mutations were found to be unlinked to the Tn*10* by P22 transduction. However, five out of 12 Tet^r transductants of CH584 (△*topA2762 tos-1*) exhibited an increased level of plasmid supercoiling. These results are consistent with the *tos-1* mutation being a mutation in *gyrA*.

Discussion

S. typhimurium strains carrying mutations in the *topA* gene show changes in the level of DNA supercoiling. Strains carry-

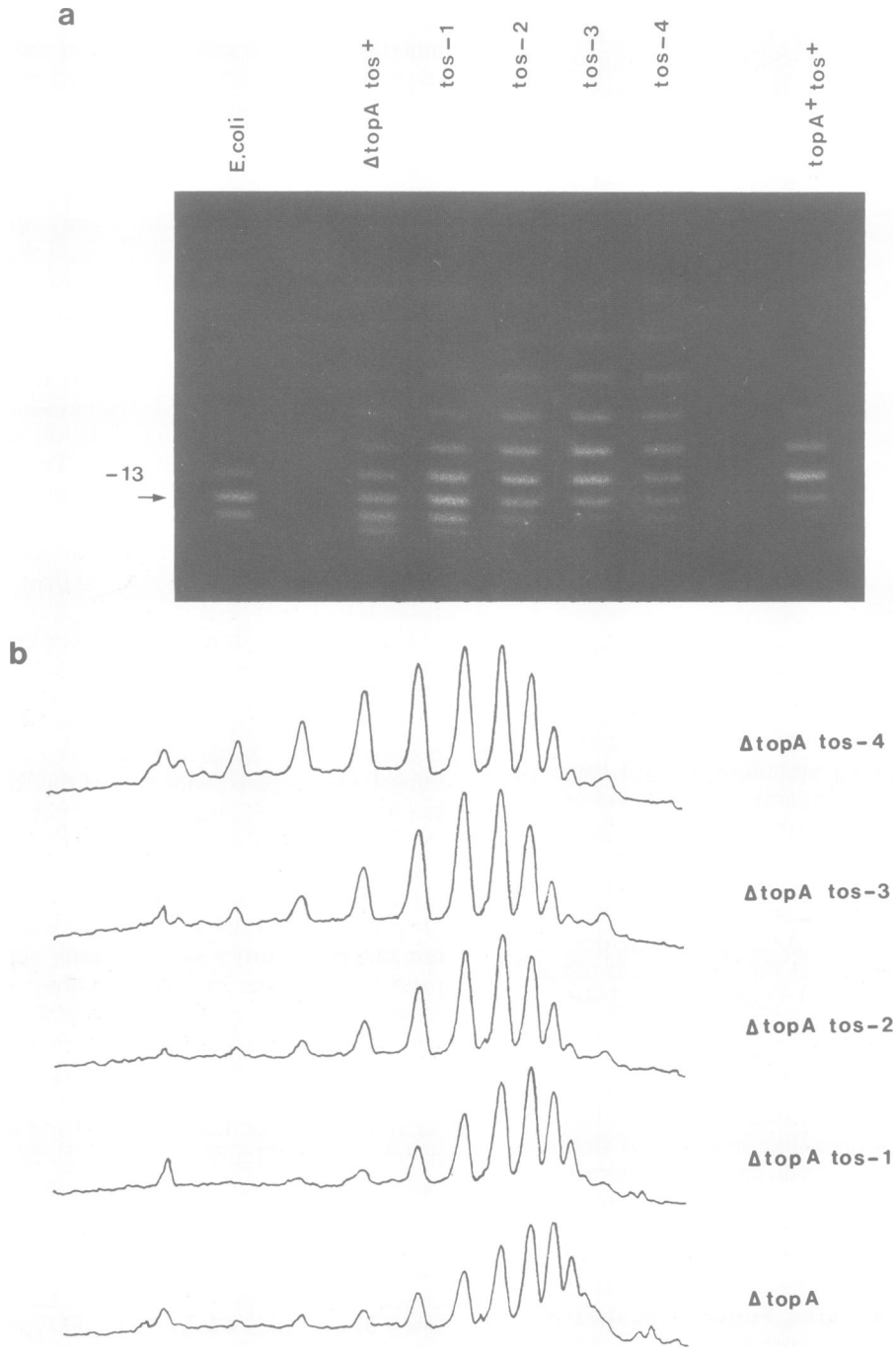

Fig. 2. *tos* mutations reduce plasmid supercoiling in a *topA* strain. The four *tos* mutations (of strains CH584, CH585, CH586 and CH588 containing *tos-1* to *tos-4* mutations respectively) were isolated in the △*topA2762* strain CH582. **(a)** pLk1 DNA extracted from these strains was electrophoresed in 1.0% agarose in the presence of 1.5 μg/ml chloroquine; pLk1 from *E. coli* provides calibration. **(b)** Densitometer tracings of a photographic negative of an equivalent agarose-chloroquine gel; strain genotypes are as indicated.

Table II. Characterisation of the principal strains used in this study

Strain	Relevant genotype	Doubling time min	Linking difference $\triangle Lk$	Superhelix density σ	Free energy of plasmid supercoiling kcal/mol
CH340	*topA*$^+$ *tos*$^+$	29	−11.9	−0.056	35.7
CH582	△*topA2762*	64	−13.5	−0.064	47.1
CH584	△*topA2762 tos-1*	53	−12.9	−0.061	42.6
CH585	△*topA2762 tos-2*	49	−12.1	−0.057	37.1
CH586	△*topA2762 tos-3*	48.5	−11.6	−0.055	33.8
CH588	△*topA2762 tos-4*	49	−11.5	−0.054	33.2
CH589	*topA*$^+$ *tos-1*	29	−11.8	−0.055	35.1
CH590	*topA*$^+$ *tos-2*	36	−11.5	−0.054	33.8
CH591	*topA*$^+$ *tos-3*	34.5	−10.7	−0.050	28.3
CH593	*topA*$^+$ *tos-4*	36	−10.0	−0.047	24.3
HB101 (*E. coli*)	*topA*$^+$ *tos*$^+$		−13.2	−0.062	

All *S. typhimurium* strains carry *leu-500* and *ara-9* mutations.

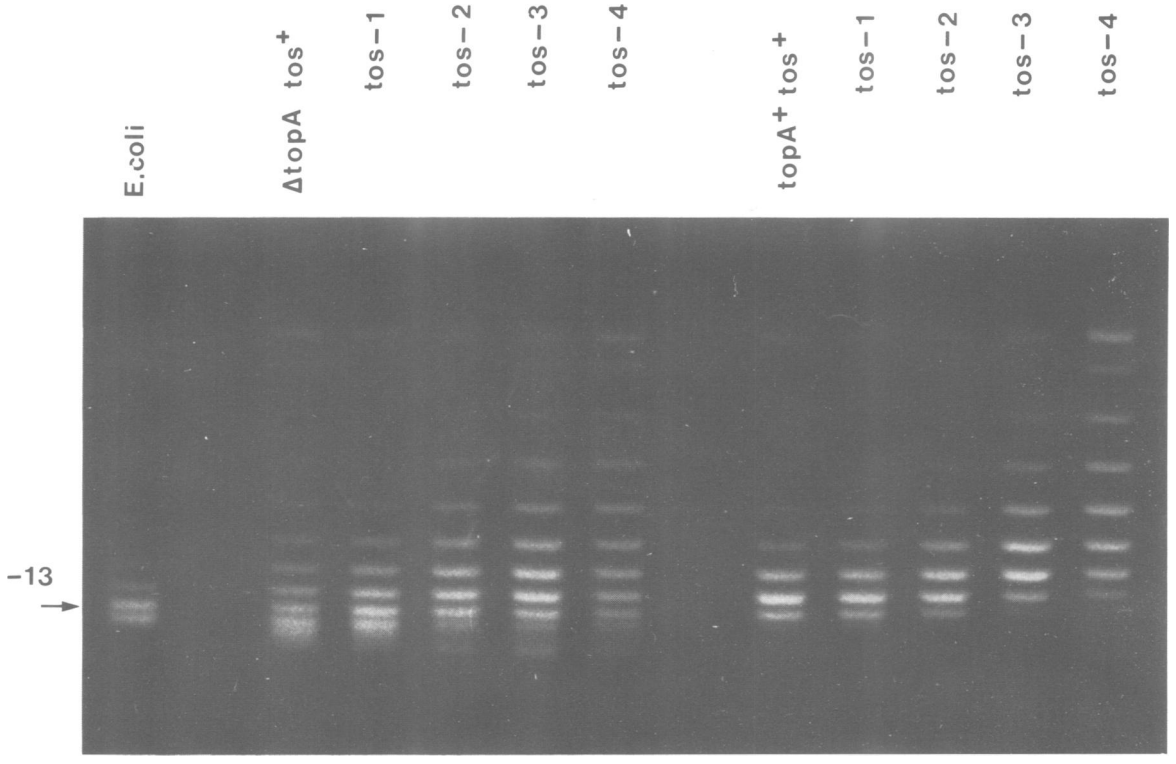

Fig. 3. Plasmid supercoiling in a variety of combinations of *topA* and *tos* mutations. 1% agarose gel containing 1.25 μg/ml chloroquine optimises resolution of the less supercoiled topoisomers of *topA*$^+$ *tos* strains at the expense of that of more highly supercoiled topoisomers isolated from the *topA* strain. Note the wide range of plasmid DNA supercoiling levels available in this series of strains.

ing *topA* deletions are viable and the deletions can be transduced into wild-type backgrounds at normal frequencies. Second site mutations, which reduce the high level of supercoiling in *topA* strains, are at a locus (or loci) we name *tos*; they are unlinked to the *topA* gene, but one *tos* mutation is linked to the *gyrA* locus. Different *tos* mutations reduce supercoiling to different extents and, when isolated from *topA* mutations (i.e., in a *topA*$^+$ background), result in plasmid supercoiling below that of the wild-type cells. We have thus constructed a series of strains bearing various *topA* and *tos* mutations which show a wide variation in levels of DNA supercoiling.

Total deletions of the *topA* gene, obtained by a site-directed procedure in the absence of any selection for or against *topA* function, resulted in an identical degree of over-supercoiling of plasmid DNA. While the presence or absence of topoisomerase I in *topA* strains has been shown by its antigenicity (Trucksis and Depew, 1981; Trucksis *et al.*, 1981)

and its activity (DiNardo *et al.*, 1982; Sternglanz *et al.*, 1981) in cell lysates, in this study we have made direct observations of the *in vivo* consequences for plasmid topology. We have shown that these *topA* deletions simultaneously suppress the auxotrophic requirement of strains bearing the chromosomal promoter mutation *leu-500*. Spontaneous mutations which suppress *leu-500* were originally mapped to a locus *supX* (Mukai and Margolin, 1963), which has recently been equated with *topA* (Trucksis *et al.*, 1981). We have also isolated a number of spontaneous mutations which suppress *leu-500*. The increased level of DNA supercoiling in these strains shows directly that a cellular topoisomerase is affected and clearly confirms the identity of *supX* as *topA*.

The level of DNA oversupercoiling in all strains containing *topA* mutations isolated by suppression of *leu-500* was identical to that in strains deleted for the entire *trp-topA-cysB* region. This implies that mutations selected for suppression of *leu-500* result in the total loss of topoisomerase I activity, and are not the consequence of a partial or a specific alteration in *topA* function. These results provide the first direct evidence that the suppression of the *leu-500* promoter mutation is associated with the oversupercoiling of DNA.

In *E. coli topA* deletions are lethal and cannot exist in the absence of a compensatory mutation (DiNardo *et al.*, 1982; Pruss *et al.*, 1982). As a result *topA* mutations cannot be transferred into a wild-type genetic background by transduction. We show here that the situation in *S. typhimurium* is clearly different. Three lines of evidence indicate that *S. typhimurium* strains bearing *topA* mutations are viable in the absence of compensating mutations. Firstly, *topA* mutations can be transduced into a wild-type background at the same frequency at which other genes can be transduced. This frequency is three orders of magnitude higher than the frequency at which spontaneous compensatory mutations arise. Secondly, although *topA* transductants grow more slowly than wild-type cells, a 36 h incubation is sufficient for such transductants to grow to small colonies. In contrast *topA* transductants of *E. coli* are unable to grow although after 4–6 days incubation spontaneous second site mutations arise which allow growth (DiNardo *et al.*, 1982). Finally, the level of plasmid supercoiling is identical in all of the *topA* deletion strains we have isolated, an observation which would not be expected were each deletion to be accompanied by a compensatory mutation. Moreover, the level of supercoiling is unchanged upon transduction of these deletions to wild-type backgrounds. These observations clearly preclude the existence of compensatory second site mutations in these *topA* strains and demonstrate the viability of *S. typhimurium* strains totally lacking topoisomerase I function.

S. typhimurium strains carrying *topA* mutations grow slowly. However, spontaneous second site mutations arise at low frequency resulting in faster growth. These *tos* mutations compensate for the oversupercoiling caused by the *topA* lesion, reducing the level of superhelix density. The compensating *tos* mutations reduce the plasmid supercoiling of *topA* strains towards and beyond that of the wild-type as their stringency increases. One of the *tos* mutations is linked to *gyrA*. The remaining *tos* mutations are unlinked to *gyrA* and at least one additional locus must be involved. We think it probable that this locus is *gyrB*, but we have been unable to test this to date. In *E. coli* some compensatory mutations have been mapped to *gyrA* and *gyrB*. In addition, the *tos* mutations are probably equivalent to the *supX* 'modifier' class of mutations identified by Overbye and Margolin (1981) in response to u.v. light induced mutagenesis.

We have separated by transduction the *tos* mutations from the *topA* deletion required for their selection. In all cases the plasmid supercoiling in *topA*$^+$ *tos* strains is lower than that of the wild-type strain. As a result we have been able to isolate a series of strains in which the plasmid superhelix density varies over a wide range. Within this range there is an appreciable overlap of supercoiling between the *topA tos* and the *topA*$^+$ *tos* strains. Compared with the superhelix density of plasmid isolated from the wild-type strain ($\sigma = -0.056$), this value may be increased by up to 13.4% (in uncompensated *topA* strains $\sigma = -0.064$), or reduced by up to 16.0% (in the most undersupercoiled *tos* strain $\sigma = -0.047$). This provides a range of superhelix densities in which the most supercoiled level is 36% higher than the least. It is useful to consider the implications for the free energy of DNA supercoiling, which can be used to couple to structural transitions such as promoter unwinding. Plasmid free energy is quadratically related to linking difference. Plasmid extracted from *topA* strains therefore has almost twice the free energy of that isolated from the most undersupercoiled strain, a very significant difference.

Using four different sizes of plasmid we have shown that the value of superhelix density is independent of plasmid size. This suggests that the functional topoisomerase enzymes in the cell respond directly to the levels of torsional stress in the DNA; the only alternative explanation, that the enzyme recognises directly the linking number, is highly implausible.

In summary the level of DNA supercoiling in *S. typhimurium* is controlled genetically by *topA* and *tos* genes. The mutations we have isolated demonstrate the opposing effects of these genes, illutrated diagrammatically in Figure 4. The free energy of supercoiling is raised by a functional *tos* gene product and lowered by *topA* gene product, such that the superhelix density of wild-type *S. typhimurium* DNA is the result of the balance between the two activities. Thus the genetic description of supercoiling control is straightforward, and in complete accord with enzymological considerations.

The existence of this series of isogenic strains, each with a different level of DNA supercoiling will be of considerable advantage in the study of topologically mediated control of gene expression. Previous studies of supercoiling-dependent gene expression in living cells have usually employed gyrase inhibitors such as nalidixic acid, coumermycin A_1, novobiocin and oxolinic acid (Sanzey, 1979; Smith *et al.*, 1978; Yang *et al.*, 1979). While these studies have been invaluable in identifying genes which are likely to be sensitive to decreased DNA supercoiling, the variations in supercoiling effected by these drugs, and their possible pleiotropic influences, have not been determined. Gyrase inhibitors only allow the investigation of the effects of superhelix densities below the wild-type level. The use of the strains decribed here, which have genetically determined and well defined superhelix densities, will allow systematic study of the effects of the variation of DNA supercoiling *in vivo* on a wide range of cellular processes without the above uncertainties.

Materials and methods

Genetic techniques

All strains used in this study except *E. coli* K-12 HB101, are derivatives of *S. typhimurium* LT2. Their genotypes are shown in Table III. All bacteria were grown at 37°C with aeration in LB medium. Screening and selection for

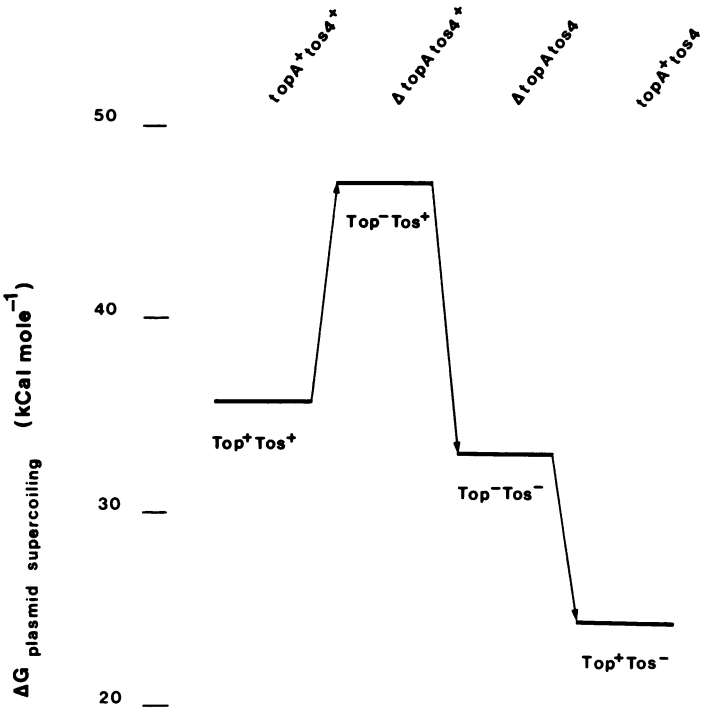

Fig. 4. Schematic representation of the relationship between the free energy of plasmid supercoiling and the genotypes of wild-type and mutant strains of *S. typhimuirum*. *topA* mutations increase free energy of supercoiling while *tos* mutations reduce it such that a 2-fold range of free energy is encompassed. Coincident presence of *topA* and *tos* mutations leads to an approximate cancellation of effects and results in levels of supercoiling around that of the wild-type strain.

amino acid requirements were performed using minimal glucose agar plates on which amino acid supplements (10 μmol) were spread immediately before use. Amino acid auxotrophy was determined by radial streaking on minimal glucose plates around filter paper disks impregnated with the appropriate amino acid (1 μmol). Tn*10*-induced deletions were isolated as Tet^s derivatives by the fusaric acid selection procedure of Bochner *et al.* (1980). Transductions were carried out using a high frequency transducing derivative of phage P22 *int-4* (Schmeiger, 1971).

Measurement of growth rates

Growth rates were measured as doubling times of culture densities in LB broth. The $A_{550\ nm}$ of 10 ml cultures at 37°C was measured at 10 min intervals in a Bausch and Lomb Spectronic 20 spectrophotometer and plotted as *A versus* time. The gradient of the linear portion was used to calculate the doubling time.

Enzymes

Restriction enzymes were purchased from Bethesda Research Laboratories and used as directed. DNA polymerase I Klenow fragment and T4 DNA ligase (BRL) were used as described previously (Lilley, 1981). Chicken reticulocyte topoisomerase I was the gift of T. Kimura; the reactions were performed in 200 mM NaCl, 10 mM Tris pH 8, 10 mM EDTA at 25°C.

Plasmids

All plasmids in this study are derivatives of pAT153 (Twigg and Sherratt, 1980). pLk1 is the product of *Eco*RI and *Ava*I cleavage, *Pol*I filling of the resultant termini and blunt end ligation. This 2233-bp plasmid was used to transform *E. coli* strain HB101 to Amp^r. pLk3, a 2907-bp plasmid conferring Tet^r was made by *Eco*RI and *Pst*I cleavage of pAT153, S1 nuclease trimming of the overhanging single-stranded ends followed by blunt end ligation. A smaller 2500-bp Tet^r plasmid pLk4, arose fortuitously on transformation of HB101 to Tet^r after construction of pLk3. pLk1 DNA purified from *E. coli* was transformed into *S. typhimurium* strain CH601, thereby creating CH340 from which all other strains except LT2, TT5345 and TT29 were derived. The size of this plasmid was chosen to provide optimum gel electrophoretic resolution of topoisomers while remaining in the range in which the free energy of supercoiling is a linear function of size (Horowitz and Wang, 1984).

Extraction of plasmid

pLk1 was purified from *E. coli* following chloramphenicol amplification of a

Table III. Strain list

Strain	Genotype	Construction
CH582	Δ*topA2762 leu-500 ara-9*	Tn*10* excision from CH340
CH583	Δ*topA2762 leu-500 ara-9*	Transduction of topA2762 from CH582 into CH340
CH584	Δ*topA2762 tos-1 leu-500 ara-9*	Spontaneous mutation in CH582
CH585	Δ*topA2762 tos-2 leu-500 ara-9*	Spontaneous mutation in CH582
CH586	Δ*topA2762 tos-3 leu-500 ara-9*	Spontaneous mutation in CH582
CH588	Δ*topA2762 tos-4 leu-500 ara-9*	Spontaneous mutation in CH582
CH589	*tos-1 leu-500 ara-9*	Transduction of CH584 to Top$^+$
CH590	*tos-2 leu-500 ara-9*	Transduction of CH585 to Top$^+$
CH591	*tos-3 leu-500 ara-9*	Transduction of CH586 to Top$^+$
CH593	*tos-4 leu-500 ara-9*	Transduction of CH588 to Top$^+$
CH594	*topA2763 leu-500 ara-9 trp-1016*::Tn*10*	Spontaneous mutation of CH340 to Leu$^+$
CH595	*topA2764 leu-500 ara-9 trp-1016*::Tn*10*	Spontaneous mutation of CH340 to Leu$^+$
CH596	*topA2765 leu-500 ara-9 trp-1016*::Tn*10*	Spontaneous mutation of CH340 to Leu$^+$
CH597	*topA2766 leu-500 ara-9 trp-1016*::Tn*10*	Spontaneous mutation of CH340 to Leu$^+$
CH598	Δ*topA2767 leu-500 ara-9*	Tn*10* excision from CH340
CH599	Δ*topA2768 leu-500 ara-9*	Tn*10* excision from CH340
CH600	Δ*topA2769 leu-500 ara-9*	Tn*10* excision from CH340
PM596	*leu-500 ara-9*	From P. Margolin
CH601	*leu-500 ara-9 trp-1016*::Tn*10*	Transduction of *trp1016*::Tn*10* from TT101 into PM596
CH340	*leu-500 ara-9 trp-1016*::Tn*10*	Transformation of pLk1 into CH601
TT5345	*his-203 zeh-775*::Tn*10*	From J. Roth

All strains except PM596, CH601 and TT5345 carry plasmid pLk1.

logarithmic phase culture. Cells were lysed by treatment with lysozyme, EDTA, Triton X-100 and the cleared lysate was centrifuged to equilibrium in an isopycnic gradient containing caesium chloride and ethidium bromide (Clewell and Helinski, 1969). Plasmid DNA used for linking difference measurements was isolated from *S. typhimurium* strains grown to an optical density at 550 nm of 1.0 by alkaline lysis procedure of Birnboim and Doly (1979) followed by isopycnic gradient centrifugation as above. We note that the use of saturated cultures gave a somewhat broader distribution of topoisomers.

Measurement of linking difference

The absolute linking difference ($\triangle Lk$) of each plasmid extracted and purified from *E. coli* was measured by the band counting method of Keller (1975). Eight sets of overlapping distributions of topoisomers were prepared by incubating pLk1 with topoisomerase I in the presence of appropriate concentrations of ethidium bromide. These were electrophoresed in three separate 1% agarose gels containing 0, 3 and 9 ng/ml ethidium bromide respectively and correspondence between gels was established by identification of the centre of the distribution of a particular set of topoisomers. To determine the absolute linking difference, the number of topoisomers between the fully relaxed and the native supercoiling states were counted. The linking differences of pLk1, pLk3, pLk4 and pAT153 extracted from strains of *S. typhimurium* were established by direct comparison of topoisomer distributions in chloroquine-containing agarose gels with that of equivalent plasmid DNA extracted from *E. coli*. Electrophoresis was performed in 1.0% agarose (Sigma), 90 mM Tris pH 8.3, 90 mM borate, 10 mM EDTA at 3 V/cm for 15 h. Topoisomer distributions were resolved by the inclusion of chloroquine phosphate (Sigma) at the concentrations specified in the figure legends. After electrophoresis gels were stained in 1 μg/ml ethidium bromide for 1 h and chloroquine removed by soaking in distilled water for at least 4 h before photographing under u.v. illumination. Topoisomer distributions were quantified by densitometry of photographic negatives using a Joyce Loebl recording microdensitometer. Mean linking differences were calculated by mathematically interpolating to the centres of the Gaussian distribution sampled at the three most abundant topoisomers by the method of Kolb and Buc (1982). Superhelix densities (σ) of pLk1 were calculated from the resultant linking differences by the following equation:

$$\sigma = \frac{10.5 \triangle Lk}{2233}$$

Energy calculations

The free energy of supercoiling of pLk1 at 37°C was calculated from the equation:

$$\triangle G_L = \frac{1100RT \triangle Lk^2}{N}$$

where $\triangle G_L$ is free energy as a function of linking difference (corrected for the temperature dependence of the winding angle), N is 2233 bp, R is the gas constant and T is 310°K (Depew and Wang, 1975; Pulleyblank *et al.*, 1975; Wang *et al.*, 1983).

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References

Bachmann,B.J. (1983) *Microbiol. Rev.*, **47**, 180-230.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513-1523.
Bochner,B.R., Huang,H.-C., Schieven,G.L. and Ames,B.N. (1980) *J. Bacteriol.*, **143**, 926-933.
Chelala,C.A. and Margolin,P. (1974) *Mol. Gen. Genet.*, **131**, 97-112.
Clewell,D.B. and Helinski,D.R. (1969) *Proc. Natl. Acad. Sci. USA*, **62**, 1159-1166.
Depew,R.E. and Wang,J.C. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4275-4279.
DiNardo,S., Voelkel,K.A., Sternglanz,R., Reynolds,A.E. and Wright,A. (1982) *Cell*, **31**, 43-51.
Drlica,K. and Snyder,M. (1978) *J. Mol. Biol.*, **120**, 145-154.
Dubnau,E. and Margolin,P. (1972) *Mol. Gen. Genet.*, **117**, 91-112.
Gellert,M., Mizuuchi,K., O'Dea,M.H. and Nash,H.A. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 3872-3876.
Graf,L.H. and Burns,R.O. (1973) *Mol. Gen. Genet.*, **126**, 281-301.
Horowitz,D.S. and Wang,J.C. (1984) *J. Mol. Biol.*, **173**, 75-91.
Isberg,R.R. and Syvanen,M. (1982) *Cell*, **30**, 9-18.
Keller,W. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4876-4880.
Kolb,A. and Buc,H. (1982) *Nucleic Acids Res.*, **10**, 473-485.
Lilley,D.M.J. (1981) *Nature*, **292**, 380-382.
Mizuuchi,K., Gellert,M., Weisberg,R.A. and Nash,H.A. (1980) *J. Mol. Biol.*, **141**, 485-494.
Mukai,F.H. and Margolin,P. (1963) *Proc. Natl. Acad. Sci. USA*, **50**, 140-148.
Overbye,K.M. and Margolin,P. (1981) *J. Bacteriol.*, **146**, 170-178.
Pruss,G.J., Manes,S.H. and Drlica,K. (1982) *Cell*, **31**, 35-42.
Pulleyblank,D.E., Shure,M., Tang,D., Vinograd,J. and Vosberg,H.-P. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4280-4284.
Sanderson,K.E. and Roth,J.R. (1983) *Microbiol. Rev.*, **47**, 410-453.
Sanzey,B. (1979) *J. Bacteriol.*, **138**, 40-47.
Schmeiger,H. (1971) *Mol. Gen. Genet.*, **110**, 378-381.
Smith,C.L., Kubo,M. and Imamoto,F. (1978) *Nature*, **275**, 420-423.
Sternglanz,R., DiNardo,S., Voelkel,K., Nishimura,Y., Hirota,Y., Becherer, K. and Wang,J.C. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2747-2751.
Trucksis,M. and Depew,R.E. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2164-2168.
Trucksis,M., Golub,E.I., Zabel,D.J. and Depew,R.E. (1981) *J. Bacteriol.*, **147**, 679-681.
Twigg,A.J. and Sherratt,D. (1980) *Nature*, **283**, 216-218.
Wahle,E. and Mueller,K. (1980) *Mol. Gen. Genet.*, **179**, 661-667.
Wang,J.C. (1971) *J. Mol. Biol.*, **55**, 523-533.
Wang,J.C., Peck,L.J. and Becherer,K. (1983) *Cold Spring Harbor Symp. Quant. Biol.*, **47**, 85-91.
Yang,H.-L., Heller,K., Gellert,M. and Zubay,G. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3304-3308.

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