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Topoisomerase I mutants: The gene on pBR322 that encodes resistance to tetracycline affects plasmid DNA supercoiling

(transcription/pUC9)

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ABSTRACT **Plasmid pBR322 DNA isolated from topoisomerase I mutants of *Escherichia coli* and *Salmonella typhimurium* exhibits a distinctive supercoiling distribution characterized by an extremely heterogeneous distribution of linking numbers that contains highly negatively supercoiled topoisomers. Analysis of the supercoiling distributions of deletion and insertion derivatives of pBR322 shows that the presence of the gene on pBR322 encoding resistance to tetracycline is responsible for the unusual supercoiling distribution. Both an intact promoter and a portion of the remainder of the gene, but not the gene product, are required. However, no particular section of the gene outside the promoter appears to be necessary; only the size of the section remaining appears to be important. These observations suggest that transcription of this gene may be responsible for its effect on DNA supercoiling.**

Supercoiling greatly affects the substrate properties of DNA molecules (1, 2) and has been shown to be important in replication, transcription, recombination, repair, and transposition of DNA in bacteria (reviewed in refs. 3–5). It is important, therefore, to understand the interactions that regulate the level of supercoiling and to learn, in molecular terms, what occurs when the normal controls are perturbed or absent.

The level of supercoiling in bacteria depends on the opposing activities of at least two topoisomerases (6–10): DNA gyrase (11), which introduces negative superhelical turns, and topoisomerase I (12), which removes negative superhelical turns. Synthesis of these enzymes appears, in turn, to be regulated by DNA supercoiling (13–15). In addition, the product of the *himA* gene apparently regulates expression of one of the gyrase genes (*gyrA*) (13). The role of the third known bacterial topoisomerase, topoisomerase III (16), is not yet known.

DNA gyrase is an essential enzyme in bacteria: conditional lethal mutations have been obtained in the genes encoding gyrase subunits (17–19), and gyrase is the target of two classes of antibiotic (11, 20, 21). In contrast, topoisomerase I is not essential: deletion mutants are viable (22). However, topoisomerase I deletion mutants of *Escherichia coli* grow well only because they have acquired compensatory mutations (7, 23). Thus, *E. coli* apparently requires both DNA gyrase and topoisomerase I, but mutations can overcome the need for topoisomerase I, whereas no substitutes exist for gyrase.

A possible clue to the function of topoisomerase I comes from the observation that there are sequence-dependent differences in supercoiling among DNAs isolated from topoisomerase I mutants (10). Surprisingly, two of the DNAs found to have very different supercoiling were the closely related plasmids pUC9 and pBR322. This result meant that DNA sequences whose presence affects supercoiling in the absence of topoisomerase I could potentially be identified using a simple recombinant DNA approach. The present work reports the results of such an analysis of pBR322 DNA.

MATERIALS AND METHODS

Plasmid Construction. Restriction enzymes were purchased from New England Biolabs, International Biotechnologies (New Haven, CT), and Bethesda Research Laboratories. All plasmids constructed are derivatives of pBR322 (Fig. 1). pBR322 DNA was digested with one or two restriction enzymes, and single-stranded DNA ends were filled in using the Klenow fragment of *E. coli* DNA polymerase I (from International Biotechnologies). The DNA was then recircularized using T4 DNA ligase (from International Biotechnologies) and used to transform strain DM800. Published procedures (28, 29) and the recommendations of the suppliers were used. Plasmid DNA was isolated from transformants exhibiting the expected pattern of antibiotic resistance and subjected to restriction analysis. Transformants carrying plasmids with the expected structure were used for studies of supercoiling. These plasmids are shown schematically in Fig. 2.

Other Methods. *E. coli* strains DM800 [Δ(*topA,cysB*)*204 gyrB225 acrA13*] (7) and DM4100 [*topA*$^+$ *gyrB*$^+$ *cysB242*] (22) were used. Plasmid DNA was isolated from cells grown in M9 medium (29) containing 1 mM $MgSO_4$, 0.04 mM $CaCl_2$, 0.4% glucose, 0.5% Casamino acids, L-cysteine (60 μg/ml), and ampicillin (35 μg/ml). Tetracycline (20 μg/ml) was also present in three instances (see Fig. 4, lanes a, b, and f). Published conditions (10) of cell growth, plasmid DNA isolation, and gel electrophoresis were used.

RESULTS

Sequences in pBR322 Cause High Levels of Plasmid DNA Supercoiling in a Topoisomerase I Mutant. Plasmid pBR322 DNA isolated from topoisomerase I mutants of *E. coli* and *Salmonella typhimurium* exhibits a supercoiling distribution that contrasts strikingly with that of plasmid DNA isolated from wild-type cells (10). The distribution from the mutants is characterized by an extreme heterogeneity in linking number, which is primarily due to the presence of topoisomers having above normal levels of supercoiling (10). The most negatively supercoiled topoisomers have a superhelix density more than twice the average superhelix density of pBR322 DNA isolated from a wild-type control. Such a supercoiling distribution is obtained even when pBR322 DNA is isolated from the topoisomerase I deletion mutant DM800 (10), which contains a compensatory mutation that reduces gyrase activity (13). [Bacterial chromosomes isolated from DM800 are

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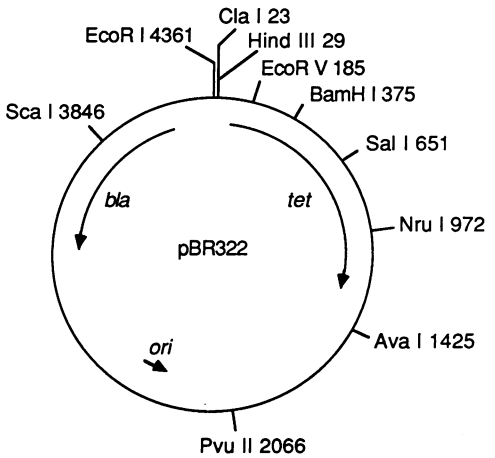

FIG. 1. Restriction map of pBR322 (24, 25). Transcription of *tet* is initiated at nucleotide 45 (26); translation probably starts at position 86 and ends at 1273 (25). The *tet* gene comes from pSC101 (24), and its expression on that plasmid is induced by tetracycline (27). On pBR322, however, *tet* is expressed constitutively because the plasmid lacks the gene encoding the *tet* repressor (24, 27).

less negatively supercoiled than normal (8).] The unusual supercoiling distribution is most clearly resolved by two-dimensional gel electrophoresis in the presence of chloroquine or ethidium bromide (10); examples of this distribution, obtained using plasmids constructed in the present study, are shown (Fig. 3). The unusual distribution can also be detected by electrophoresis in one-dimensional gels containing chloroquine (Fig. 4; ref. 10). At a low concentration of the drug, the constituent topoisomers of pBR322 DNA isolated from a wild-type strain are resolved (lane b), whereas most of the pBR322 DNA isolated from a topoisomerase I deletion mutant (lane a) migrates as unresolved, negatively supercoiled DNA. Comparison of Fig. 3 with the corresponding one-dimensional gels (Fig. 4, lanes i and k; Fig. 5, lane j) shows that one can predict the presence of the unusual supercoiling distribution from the distribution seen on a one-dimensional gel.

In contrast to pBR322 DNA, pUC9 DNA isolated from topoisomerase I mutants exhibits a supercoiling distribution similar to that of plasmid DNA isolated from wild-type cells (10). The marked difference in supercoiling between pUC9 and pBR322 DNA isolated from these mutants was surprising, since most of pUC9 (30) comes from pBR322 (Fig. 1). Plasmids pUC9 and pBR322 have the same origin of replication, but pUC9 is only about half as large as pBR322 and lacks the gene (*tet*) encoding resistance to tetracycline. In addition, pUC9 carries a short portion of the *lacZ* gene. Neither the difference in size between pUC9 and pBR322 nor the presence of tetracycline during selection or growth of strains transformed with pBR322 is responsible for the difference in supercoiling between the two plasmids. Dimers of pUC9 isolated from a topoisomerase I mutant exhibit a normal supercoiling distribution (10), and pBR322 DNA isolated from a transformant of the topoisomerase I deletion mutant DM800 that had never been exposed to tetracycline (Fig. 4, lane c) exhibits the same supercoiling distribution as pBR322 DNA isolated from a transformant of DM800 selected and grown in the presence of tetracycline (Fig. 4, lane a; ref. 10). Moreover, pUC9 DNA isolated from a derivative of DM800 that is resistant to tetracycline (due to an insertion of Tn*10*) and that was grown in medium containing tetracycline exhibits the same supercoiling distribution as pUC9 DNA isolated from strain DM800 (data not shown). Highly enriched growth medium, however, appears to increase slightly the heterogeneity of supercoiling of pUC9 DNA isolated from topoisomerase I mutants (data not shown), although growth medium does not detectably affect supercoiling of pBR322

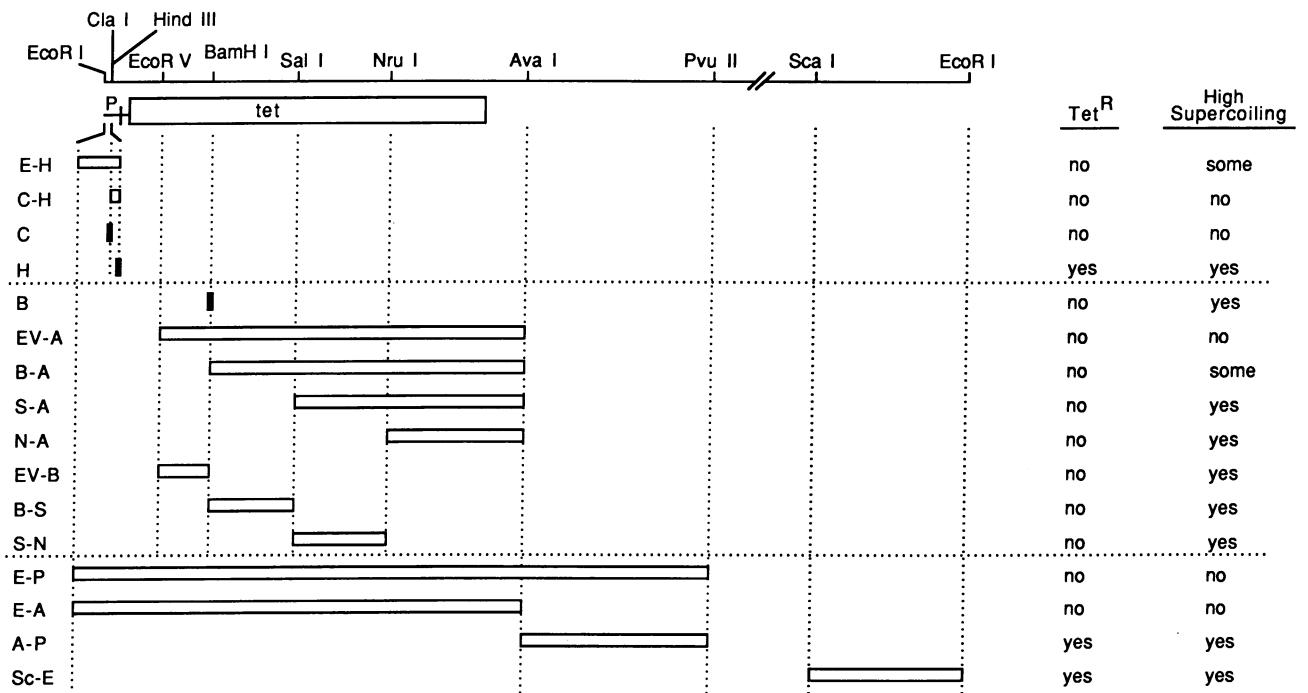

FIG. 2. Insertion and deletion derivatives of pBR322 constructed in this study. At the top of the figure is a restriction map of pBR322 linearized at the *EcoRI* site, showing the location of the *tet* promoter (P) and coding region (box). The distance from the *Eco*RI to the *Hin*dIII site has been expanded slightly in the rest of the figure. Open bars represent deletions of pBR322, and closed bars represent sites of insertion into pBR322. Insertions were created by cleaving pBR322 DNA with one restriction enzyme, filling in the single-stranded ends, and religating. Plasmid designations are shown on the left and correspond to the first letters of the restriction enzymes used in the construction. A summary of the results of this study is given on the right.

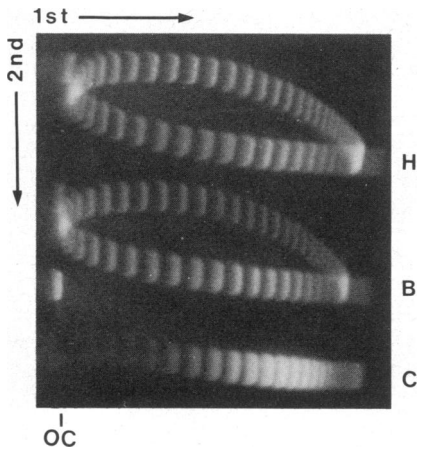

FIG. 3. Two-dimensional gel electrophoresis of pBR322 derivatives isolated from strain DM800 (Δ*topA*). DNA of the three insertion derivatives H, B, and C was analyzed on one gel; for all three plasmids, DNA from similar amounts of cells was loaded onto the gel. Chloroquine was present at 120 μg/ml in the first (horizontal) dimension and at 600 μg/ml in the second (vertical) dimension. The top two distributions are qualitatively the same as that of pBR322 DNA isolated from topoisomerase I mutants (10). During electrophoresis in the first dimension, topoisomers in the upper arcs of the top two distributions were negatively supercoiled while topoisomers in the lower arcs were positively supercoiled, causing the two arcs of each distribution to be superimposed. Electrophoresis in the second dimension resolves the superimposed topoisomers. The position of open circular (OC) DNA is indicated.

DNA isolated from these mutants (10). The supercoiling distribution of pUC9 DNA isolated from a topoisomerase I mutant grown in such medium is still very different from that of pBR322: most of the covalently circular pUC9 DNA (Fig. 4, lane g) appears as a ladder of topoisomers at the low

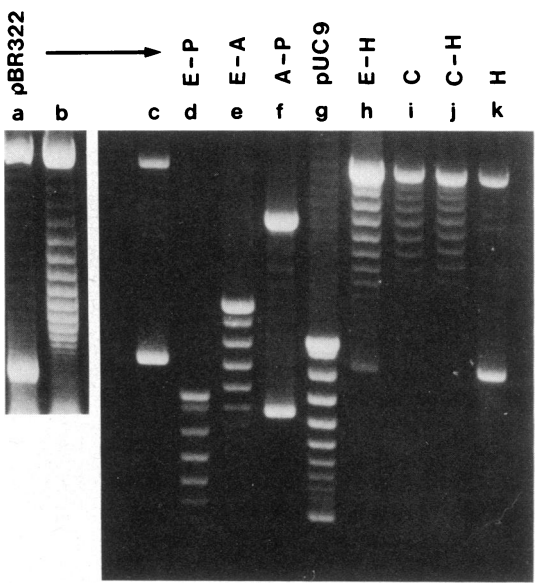

FIG. 4. Electrophoresis of plasmid DNA in one-dimensional gels containing chloroquine. DNA in lane b was isolated from strain DM4100 (*topA*$^+$); DNA in all other lanes was isolated from strain DM800 (Δ*topA*). Lanes c–k contain DNA from similar amounts of cells; lanes a and b contain DNA from more cells, but they are comparable to each other in amount of cells. Derivatives of pBR322 are designated as in Fig. 2. The DNA is negatively supercoiled at these concentrations of chloroquine (15 μg/ml in lanes a and b, 12 μg/ml in the rest); the bright band at the top of each lane is open circular DNA.

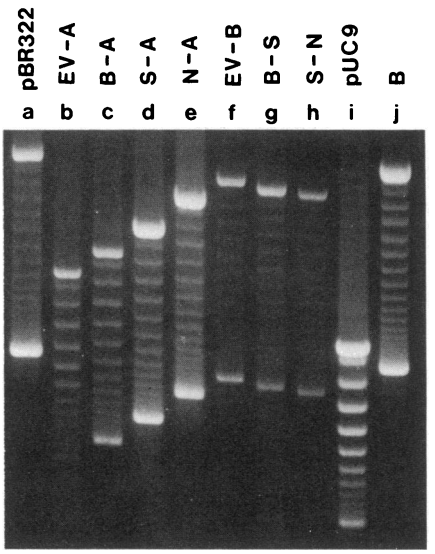

FIG. 5. Effect of alterations in the *tet* coding region on plasmid DNA supercoiling. DNA was isolated from strain DM800 (Δ*topA*) and subjected to electrophoresis in a one-dimensional gel containing chloroquine (12 μg/ml); all lanes contain DNA from similar amounts of cells. Derivatives of pBR322 are designated as in Fig. 2.

concentration of chloroquine at which most of the covalently circular pBR322 DNA (Fig. 4, lane c) migrates as a single, negatively supercoiled band. The difference in migration caused by the size difference of the plasmids is not relevant to the supercoiling distributions.

It seemed likely that the DNA segments deleted from or inserted into pBR322 in constructing pUC9 contain sequences responsible for the difference in supercoiling between the two plasmids. An initial experiment confirmed this expectation and showed that the region deleted from pBR322 (Fig. 1: the *Eco*RI–*Pvu* II fragment containing the *tet* gene) is required for the unusual supercoiling distribution: when a plasmid constructed by deleting this region from pBR322 is isolated from the topoisomerase I deletion mutant DM800, highly negatively supercoiled topoisomers are absent (Fig. 4, compare lanes c and d). As a control, DNA outside of this region was deleted from pBR322. The resulting plasmid, which carries a deletion from the *Eco*RI site to the *Sca* I site, exhibits pBR322-like supercoiling when isolated from strain DM800 (data not shown). All subsequent constructions, therefore, were designed to identify the sequences within the *Eco*RI–*Pvu* II fragment that are required for high levels of supercoiling.

The approach used was to take advantage of unique restriction sites within this region of pBR322 to make deletions or small insertions of defined extent (Fig. 2). To determine the effect of the alterations on supercoiling, plasmid DNA was isolated from the *E. coli* topoisomerase I deletion mutant DM800 (Δ*topA gyrB225*), grown to late exponential phase. The growth phase at which the cells are harvested is important: pBR322 DNA isolated from strain DM800 grown to late stationary phase (aeration overnight in the presence or absence of chloramphenicol) does not exhibit high levels of supercoiling (data not shown).

The sequences in pBR322 required for high levels of supercoiling were further localized to the *Eco*RI–*Ava* I fragment: a plasmid constructed by deleting that region from pBR322 displays a distribution lacking high levels of supercoiling when isolated from strain DM800 (Fig. 4, lane e), whereas a plasmid constructed by deleting from the *Ava* I to the *Pvu* II site displays pBR322-like supercoiling (Fig. 4, lane f). This latter result also suggests that high levels of plasmid DNA supercoiling are not related to low copy number, since

deleting the *Ava* I–*Pvu* II fragment should eliminate a protein that reduces pBR322 copy number (31). The *Eco*RI–*Ava* I fragment is almost entirely made up of the *tet* gene (Fig. 2), suggesting that transcription of that gene (rather than the presence of preferred sites of gyrase activity or of other protein binding), might be responsible for the region's effect on supercoiling. The subsequent plasmid constructions were designed to test this idea.

Inactivating the pBR322-*tet* Promoter Eliminates High Levels of Supercoiling. If transcription of the pBR322-*tet* gene is responsible for high levels of supercoiling, alterations in the *tet* promoter should affect supercoiling. Unique sites for three restriction enzymes lie within or just outside of the pBR322-*tet* promoter: the *Eco*RI, *Cla* I, and *Hin*dIII sites (32). These were used to construct four plasmids carrying different promoter mutations (Fig. 2): a deletion from the *Eco*RI to the *Hin*dIII site (27 base pairs), a deletion from the *Cla* I to the *Hin*dIII site (3 base pairs), an insertion at the *Cla* I site (2 base pairs), or an insertion at the *Hin*dIII site (4 base pairs). All of the mutations except the insertion at the *Hin*dIII site eliminated ability of the plasmid to confer resistance to tetracycline. The plasmid carrying the insertion at the *Hin*dIII site confers resistance to tetracycline, as expected (32). All three mutations that eliminate resistance to tetracycline also eliminate most or all of the highly negatively supercoiled DNA from the supercoiling distribution (Fig. 4, lanes h–j; Fig. 3, sample C). In contrast, the plasmid carrying the insertion at the *Hin*dIII site exhibits pBR322-like supercoiling (Fig. 4, lane k; Fig. 3, sample H). These results are consistent with the idea that transcription of the pBR322-*tet* gene may be responsible for the supercoiling distribution of pBR322 DNA isolated from topoisomerase I mutants. However, they are also consistent with a preferred site for gyrase activity at the *tet* promoter or with an involvement of the *tet* gene product. The next set of plasmid constructs was designed to help distinguish among these three possibilities.

Part of the Coding Region of the pBR322-*tet* Gene Is Required for High Levels of Supercoiling. If transcription of the pBR322-*tet* gene is responsible for high levels of supercoiling, the coding region should be required, in addition to the promoter. Thus, one would predict that leaving the promoter intact but removing most of the remainder of the gene should eliminate high levels of supercoiling. Moreover, the length of the coding region remaining might be important, but an active *tet* gene product should not be required. These predictions were tested by constructing a set of plasmids carrying deletions of decreasing size in the *tet* coding region. The promoter distal end of these deletions is at the *Ava* I site; the promoter proximal ends are at the *Eco*RV, *Bam*HI, *Sal* I or *Nru* I sites (Fig. 2), creating plasmids having the first 143, 335, 611, or 930 transcribed nucleotides, respectively, of the *tet* gene. Since all of these plasmids have an intact *tet* promoter, all should exhibit pBR322-like supercoiling when isolated from strain DM800 if that supercoiling distribution is due only to a preferred site of gyrase activity at the *tet* promoter. On the other hand, none should exhibit high levels of supercoiling if an active *tet* gene product is required, since none confers resistance to tetracycline. The result of the experiment is that the plasmid having the longest deletion does not exhibit high levels of supercoiling (Fig. 5, lane b), but the two plasmids having the shortest deletions do (Fig. 5, lanes d and e). Thus, the occurrence of high levels of supercoiling requires the *tet* coding region, depends on the length of coding region present, and does not require an active *tet* gene product, as expected if transcription of the *tet* gene were responsible.

No Particular Portion of the pBR322-*tet* Coding Region Is Required for High Levels of Supercoiling. The plasmid having the second longest deletion in the set of plasmids constructed above exhibits an intermediate supercoiling distribution (Fig. 5, lane c): unresolved negatively supercoiled topoisomers are present, but in relatively lower amounts than in pBR322 DNA (Fig. 5, lane a). This observation suggests that supercoiling is a continuous function of the length of the *tet* gene and argues against the possibility that special sites in the coding region are required for high levels of supercoiling. To investigate this question further, a set of plasmids carrying different, but relatively short, internal deletions of the *tet* gene was constructed. The plasmids in this set all have about the same length of *tet* gene remaining, but they lack different consecutive portions of the gene (Fig. 2). All of these plasmids exhibit pBR322-like supercoiling when isolated from strain DM800 (Fig. 5, lanes f–h), supporting the idea that there are no special sites within the coding region required for high levels of supercoiling.

DISCUSSION

Fig. 2 contains a summary of the results presented above. This work shows that DNA sequences in the promoter and in the coding region of the *tet* gene on pBR322 are required for the high levels of supercoiling found in pBR322 DNA isolated from a topoisomerase I deletion mutant. Resistance to tetracycline, however, is not required. Neither the *tet* promoter nor the coding region alone are sufficient for the high levels of supercoiling; both must be present. Moreover, no particular sequence in the *tet* gene outside of the promoter appears to be necessary; instead, the length of the gene seems to be important. The simplest interpretation of these results is that the high levels of supercoiling are a consequence of transcription of the *tet* gene.

Possible mechanisms whereby transcription of a gene could have the observed effect on supercoiling are R-loop formation and/or the binding of RNA polymerase. Each process would reduce apparent superhelicity by unwinding the DNA double helix and could thereby make the molecule a good substrate for gyrase. About 400 base pairs of DNA would have to be unwound to account for the highest linking difference observed (10). If this unwinding is caused by the binding of RNA polymerase, 24 polymerase molecules would be required (33).

It seems unlikely that preferred sites of gyrase activity, in the absence of other factors, are responsible for the high levels of supercoiling. Although preferred sites of gyrase binding *in vivo* have been identified in the pBR322-*tet* gene (34, 35) and may be relatively uniformly distributed throughout the gene (35), no prominent sites have been reported in the *tet* promoter. Moreover, two different classes of preferred gyrase site would be needed to account for the present results: a specific site at the *tet* promoter and a set of interchangeable sites distributed throughout the rest of the gene. It also seems unlikely that the high levels of supercoiling are related to plasmid copy number: a plasmid lacking the element of pBR322 copy control that is missing from pUC9 shows pBR322-like supercoiling (Fig. 4, lane f), and the relative copy numbers of the plasmids (qualitative estimates based on Figs. 3–5 and data not shown) exhibit no obvious correlation with plasmid DNA supercoiling.

Although no particular sequences within the *tet* coding region appear to be required for high levels of supercoiling, the 5′ end of the gene (between the *Hin*dIII and *Eco*RV sites) was not deleted in this study. Specific sites required for high levels of supercoiling might, therefore, be present in the leader or at the beginning of the coding region of the *tet* gene. Such sites would have to be necessary, but not sufficient: some plasmids containing the 5′ end of the *tet* gene do not show high levels of supercoiling (Fig. 2, plasmids C-H, C, and EV-A).

Why transcription of *tet*, but apparently not of the other genes on pBR322, might have such a profound effect on

supercoiling has not been addressed in this work. Obvious possibilities are that the effect is specific for the *tet* promoter or that the position or orientation of the *tet* gene on pBR322 is responsible. Whether sequences in pUC9 affect plasmid DNA supercoiling has also not been addressed. Since pUC9 DNA (Fig. 4, lane g) isolated from strain DM800 has relatively more highly negatively supercoiled topoisomers than the plasmid (Fig. 4, lane d) constructed by deleting the *Eco*RI–*Pvu* II fragment from pBR322, it seems likely that the short portion of the *lacZ* gene on pUC9 causes an increase in plasmid DNA supercoiling in topoisomerase I mutants. A variety of genes in bacteria, therefore, may have the potential to increase the supercoiling of the DNA molecule on which they reside; one role of topoisomerase I may be to prevent or correct increases from such sources. Two other groups have reported that differences in plasmid primary structure cause differences in supercoiling (36, 37).

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