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Depletion of RNase HI activity in *Escherichia coli* lacking DNA topoisomerase I leads to defects in DNA supercoiling and segregation

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Abstract

Gyrase-mediated hypernegative supercoiling is one manifestation of R-loop formation, a phenomenon that is normally suppressed by topoisomerase I (*topA*) in *Escherichia coli*. Overproduction of RNase HI (*rnhA*), an enzyme that removes the RNA moiety of R-loops, prevents hypernegative supercoiling and allows growth of *topA* null mutants. We previously showed that *topA* and *rnhA* null mutations are incompatible. We now report that such mutants were viable when RNase HI or topoisomerase III was expressed from a plasmid-borne gene. Surprisingly, DNA of *topA* null mutants became relaxed rather than hypernegatively supercoiled following depletion of RNase HI activity. This result failed to correlate with the cellular concentration of gyrase or topoisomerase IV (the other relaxing enzyme in the cell) or with transcription-induced supercoiling. Rather, intracellular DNA relaxation in the absence of RNase HI was related to inhibition of gyrase activity both *in vivo* and in extracts. Cells lacking *topA* and *rnhA* also exhibited properties consistent with segregation defects. Overproduction of topoisomerase III, an enzyme that can carry out DNA decatenation, corrected the segregation defects without restoring supercoiling activity. Collectively these data reveal 1) the existence of a cellular response to loss of RNase HI that counters the supercoiling activity of gyrase and 2) supercoiling-independent segregation defects due to loss of RNase HI from *topA* null mutants. Thus RNase HI plays a more central role in DNA topology than previously thought.

Keywords

topA; topoisomerase I; gyrase; RNase HI; segregation

Introduction

Negative DNA supercoiling is a regulated feature of bacterial chromosomes (Menzel and Gellert, 1983; DiNardo *et al.*, 1982; Pruss *et al.*, 1982; Drlica, 1992) that is important for most processes involving DNA strand separation. In *E. coli*, supercoiling regulation is achieved largely by DNA topoisomerases with opposing enzymatic activities. DNA gyrase, encoded by *gyrA* and *gyrB*, introduces negative supercoils, while two other enzymes, DNA topoisomerase I (*topA*) and DNA topoisomerase IV (*parC* and *parE*), remove excess negative supercoils (Pruss *et al.*, 1982; Zechiedrich *et al.*, 2000). Although the ability to create *topA* null mutants is a clear indication that topoisomerase I is not required for survival

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(Sternglanz *et al.*, 1981; Stupina and Wang, 2005), growth of such mutants often requires compensatory mutations that reduce negative supercoiling (DiNardo *et al.*, 1982; Pruss *et al.*, 1982). Indeed, very high levels of supercoiling (hypernegative supercoiling, detected as plasmid topoisomers that cannot be resolved by electrophoresis in agarose gels (Pruss, 1985)) can accumulate in *topA* null mutants (Pruss, 1985) and correlate with growth inhibition (Massé and Drolet, 1999a,b).

One major pathway for generating hypernegative supercoiling in *topA* mutants involves R-loop formation coupled to gyrase activity (Massé and Drolet, 1999c). Hypernegative supercoiling is initiated by the accumulation of negative supercoils behind moving RNA polymerase when topoisomerase I is absent (Massé and Drolet, 1999c). These negative supercoils promote re-hybridization of nascent transcripts to the template DNA strand, which leaves the non-template strand unpaired and the DNA relaxed. Relaxed DNA is a substrate for gyrase, which then introduces more supercoils that lead to increased R-loop formation, more DNA relaxation, and increased gyrase action (Drolet *et al.*, 1994; Phoenix *et al.*, 1997; Drolet, 2006). Removal of RNA-DNA hybrids by overproduction of RNase HI suppresses the accumulation of hypernegative supercoils and the associated growth defect (Drolet *et al.*, 1995; Massé and Drolet, 1999c). Growth inhibition and hypernegative supercoiling also correlate with major gene expression defects, as illustrated by the accumulation of truncated RNAs (Baaklini *et al.*, 2004; Baaklini *et al.*, manuscript submitted). Within this context, our inability to construct double *topA rnhA* null mutants did not come as a surprise (Massé and Drolet, 1999b; Drolet *et al.*, 1995). Indeed, it was predicted that such double mutants would die from toxic levels of hypernegative supercoiling.

In the present work, we constructed *topA rnhA* double mutants that grew when *rnhA* was expressed from a plasmid-borne gene. Unexpectedly, we found that under non-permissive conditions the DNA of such mutants was relaxed, not hypernegatively supercoiled. Depletion of RNase HI activity in *topA* null mutants triggered a cellular response that inhibited supercoiling by gyrase. Moreover, the growth inhibition of *topA rnhA* null double mutants correlated with phenotypes reminiscent of segregation defects: extensive cell filamentation with abnormal nucleoid structures and accumulation of anucleate cells. Overproduction of topoisomerase III (*topB*), a type IA enzyme like topoisomerase I, corrected the segregation defects and allowed *topA rnhA* double mutants to grow without restoring supercoiling. Such activity was consistent with topoisomerase III being able to decatenate daughter DNA molecules (Hiasa *et al.*, 1994) and use R-loops as a substrate (Broccoli *et al.*, 2000). Thus high-level expression of a plasmid-borne *topB* gene provided a second way to allow conditional growth of *topA rnhA* double mutants. Below we describe measurements of bacterial DNA supercoiling and cell morphology that reveal an important role for RNase HI in preventing transcription from perturbing DNA topology beyond viable bounds.

Results

Absence of RNase HI prevents accumulation of hypernegative supercoiling associated with a *topA* deficiency

We previously described the effects of topoisomerase I defects in living cells using a set of *E. coli* strains in which a temperature-sensitive gyrase mutation allows the growth of *topA* null cells at 37°C but not at 28°C owing to reactivation of gyrase (Drolet *et al.*, 1995). In such strains hypernegatively supercoiled DNA accumulates following the temperature downshift unless RNase HI is overproduced (Massé and Drolet, 1999c). Overproduction of RNase HI at 28°C also restores growth. To better understand relationships among hypernegative supercoiling, R-loops, and growth inhibition, we constructed a conditional

topA rnhA double mutant (transduction was used to introduce an *rnhA::cam* allele into a *topA20::Tn10 gyrB(Ts)* strain carrying a plasmid expressing *rnhA* under control of the arabinose-inducible P_{BAD} promoter). Transductants were obtained by plating at 37°C in the presence but not in the absence of arabinose (not shown). When these transductants were transferred to liquid medium lacking arabinose, cells grew slowly, cell filamentation occurred, and culture growth stopped prematurely, irrespective of temperature (not shown). Thus, when RNase HI is absent, the growth defects of *topA* null *gyrB(Ts)* mutants were seen even at 37°C. Since colonies arose after plating on solid medium containing arabinose, we conclude that deleting *rnhA* in *topA* mutants is bacteriostatic, not bactericidal (not shown).

Supercoiling was measured with plasmid pGB2*nusB::kan*, a low copy-number derivative of pSC101 that is prone to R-loop-dependent hypernegative supercoiling in *topA* null mutants (Broccoli *et al.*, 2004). Plasmid topoisomers were resolved using agarose gel electrophoresis in the presence of 7.5 µg/ml of chloroquine, as previously described (Massé and Drolet, 1999c). Under this condition the more relaxed topoisomers migrated faster; hypernegatively supercoiled DNA also migrated rapidly. With a *gyrB(Ts)* control strain lacking only *topA* (strain PS152), hypernegatively supercoiled DNA accumulated after transfer of cells from 37 to 28°C (Fig. 1a, lane 2, indicated by [--]). When an *rnhA* null mutation was added (strain PS151, which also carried an inducible *rnhA* gene), and mutant cells from arabinose-containing agar were incubated overnight at 37°C in the absence of arabinose, followed by 1:1000 dilution into medium lacking arabinose, cell growth was sufficient for examination of plasmid supercoiling. A portion of the DNA was more relaxed at 37°C than in *topA* single mutant cells (Fig. 1a, compare lanes 1 and 3), and hypernegatively supercoiled DNA failed to accumulate following a transfer to 28°C (Fig. 1a, lane 4). The relaxed portion of topoisomers remained extensively relaxed following the temperature downshift (Fig. 1a, lanes 3 and 4, indicated by [rel]). These extensively relaxed topoisomers were not observed in wild-type (lanes 5 and 6) or in *gyrB(Ts)* (lanes 7 and 8) cells. Thus, removal of *rnhA* from a *topA*-deficient strain caused DNA relaxation and loss of hypernegative supercoiling.

When arabinose was added to the diluted (1:1000) overnight culture of the *topA rnhA* double mutant (strain PS151) to produce RNase HI from a plasmid-borne gene, supercoiling was restored at 37°C (Fig. 1a, compare lane 3, no arabinose, with lane 9, 0.05% arabinose) and accumulation of hypernegatively supercoiled DNA occurred following a temperature downshift (Fig. 1a, lane 10). Since RNase HI was overproduced in this experiment, *topA* null cells accumulated lower amounts of hypernegatively supercoiled DNA than observed with *topA* null cells having only wild-type levels of RNase HI (Fig. 1a, compare lanes 2, *topA* null cells, and 10, *topA rnhA* double mutant with 0.05% arabinose).

Two-dimensional gel electrophoresis confirmed the presence of hypernegatively supercoiled and extensively relaxed DNA, respectively, in the presence and absence of RNase HI (Fig. 1b; hypernegatively supercoiled DNA is seen at bottom part of the curves at high chloroquine concentration ((right panels, indicated by [--]); extensively relaxed DNA is at the far right part of the curves at lower chloroquine concentration (left and middle panels; lines were traced over the more relaxed topoisomers)). Additional two-dimensional gel analyses showed that the more relaxed topoisomers were not positively supercoiled (data not shown). Thus, removal of RNase HI relaxed DNA of *topA* null cells and prevented the accumulation of hypernegatively supercoiled DNA rather than increasing it.

Supercoiling deficit in a *topA rnhA* null double mutant after transcription inhibition

Excess negative supercoiling in *topA* mutants originates largely from transcription. To determine whether the reduced supercoiling in the *topA rnhA* double mutant is specific to transcription-induced supercoiling, mutant cells (strain PS151) were treated with rifampicin, an inhibitor of RNA polymerase. When rifampicin was added to a culture of *topA* null cells

(strain PS152, containing wild-type RNase HI) before the temperature downshift, almost all of the hypernegatively supercoiled topoisomers were lost (Fig. 2, top panels). When RNase HI was absent and most of the DNA was seen as extensively relaxed topoisomers, rifampicin had little effect (Fig. 2, bottom panels; a line was traced over the more relaxed topoisomers). Thus the extensive relaxation of supercoiling seen in *topA rnhA* double mutants was related to global supercoiling rather than being specifically linked to transcription. However, our results also show that the effect of the *rnhA* deletion on supercoiling following a temperature downshift was much stronger when transcription was allowed (Fig. 2, compare $-rif$ with $+rif$, $+$ and $-$ RNase HI).

Levels of topoisomerase IV and gyrase proteins are not altered by deletion of *rnhA* from *topA* null cells

Changes in topoisomerase IV and gyrase concentrations were measured to address the possibility that levels of these proteins account for the extensive DNA relaxation associated with depletion of RNase HI activity in *topA* null cells. ParC and ParE were not overproduced in the *topA20::Tn10 gyrB* (Ts) mutant when RNase HI was depleted, as assessed by western blot experiments (Fig. 3, compare lanes 5 and 6 with lanes 7 and 8) and northern blot experiments (data not shown). GyrA and GyrB levels also exhibited no significant difference between the presence and absence of RNase HI (Fig. 3, compare lanes 5 and 6 with lanes 7 and 8).

Lack of significant change in ParC, ParE, GyrA and GyrB level was also observed when the *rnhA* gene was inactivated in the $\Delta topA gyrB$ (Ts) strain carrying pPH1243 (Fig. 3, lanes 1 to 4). This plasmid, which expressed *topB* (topoisomerase III) from an IPTG-inducible promoter, allowed the recovery of *rnhA::cam* transductants in a $\Delta topA$ recipient strain, but only when IPTG was present (Fig. 4a and data not shown). Western blot experiments with a transductant (strain SB383) confirmed that overproduction of topoisomerase III occurred (Fig. 4b). Plasmid pPH1243 also allowed *topA rnhA* double mutants to grow unless cultures were diluted into IPTG-deficient medium, in which case growth eventually stopped during log phase. Thus, introduction of two restrictive conditions, depletion of RNase HI or removal of topoisomerase III overexpression failed to reveal changes in gyrase and topoisomerase IV concentrations that could explain the extensive DNA relaxation observed with *topA rnhA* double mutants.

Supercoiling in a *topA rnhA* double mutant is not significantly affected by quinolone-mediated inhibition of topoisomerase IV

We also considered the possibility that supercoiling by gyrase was reduced. To test this hypothesis, we used the *gyrA*^{L83} allele to render gyrase resistant to norfloxacin, thereby allowing selective quinolone-mediated inhibition of topoisomerase IV, the only remaining relaxing activity in *topA* mutants (Zechiedrich *et al.*, 2000). The *gyrA*^{L83} allele was introduced into isogenic $\Delta topA gyrB$ (Ts) and $\Delta topA gyrB$ (Ts) *rnhA::cam* strains carrying pPH1243 to obtain, respectively, strains VU21 and PS160. Dilution into medium lacking IPTG gradually removed suppression of growth defect by topoisomerase III, but allowed enough growth for examination of plasmid supercoiling (as pointed out in a subsequent section, topoisomerase III overexpression has no effect on supercoiling).

Negative supercoiling increased at 37°C after inhibition of topoisomerase IV by norfloxacin in both the *topA rnhA* and the *topA* mutants following dilution (Fig. 5, compare *topA rnhA*, strain PS160, lanes 1 and 2 with *topA*, strain VU21, lanes 5 and 6). However, a significant proportion of the topoisomers remained relaxed in the *topA rnhA* mutant (Fig. 5, lane 2, indicated by [rel]). Following a shift to 28°C, the addition of norfloxacin to inhibit topoisomerase IV strongly stimulated the accumulation of hypernegatively supercoiled DNA

in the *topA* mutant (Fig. 5, compare lanes 7 and 8, indicated by [--]), whereas it did not appreciably change the supercoiling level in the *topA rnhA* mutant (Fig. 5, compare lanes 3 and 4). Collectively these results show that topoisomerase IV is not responsible for the strong supercoiling deficit in *topA rnhA* double mutants. It is likely that supercoiling by gyrase is impaired when both RNase HI and topoisomerase I are absent.

GyrI, a gyrase inhibitor, is not involved in supercoiling inhibition found in a *topA rnhA* double mutant

GyrI is encoded by *sbmC*, a well-characterized inhibitor of gyrase (Nakanishi *et al.*, 1998; Chatterji and Nagaraja, 2002). Since GyrI is part of the SOS regulon (Baquero *et al.*, 1995), which is chronically expressed in *rnhA* mutants (Kogoma *et al.*, 1993; McCool *et al.*, 2004), it is expected to be at elevated levels in *topA rnhA* double mutants. To examine involvement of GyrI in the supercoiling inhibition in *topA rnhA* double mutants, an *sbmC::kan* allele was introduced into isogenic $\Delta topA$ *gyrB*(Ts) and $\Delta topA$ *gyrB*(Ts) *rnhA::cam* strains carrying pPH1243, to obtain, respectively, strains VU64 and VU70. Both extensive DNA relaxation and lack of hypernegative supercoiling, which are characteristic of the *topA rnhA* double mutant, were seen whether or not *sbmC* was present (Fig. 6, compare lanes 1 and 4 with lanes 2 and 5, respectively). The lack of a significant supercoiling effect from the absence of GyrI was also illustrated by comparing supercoiling in strains VU64 (Fig. 6, *rnhA*⁺, lanes 3 and 6) and VU70 (Fig. 6, *rnhA*⁻, lanes 2 and 5). Thus, GyrI is not responsible for the DNA relaxation seen in *topA rnhA* double mutants. We also examined a *lexA3* mutant in which the SOS regulon is non-inducible. DNA was relaxed, and hypernegative supercoiling was absent in a *lexA3 topA rnhA* mutant (not shown).

Cell extracts of a *topA rnhA* double mutant exhibit no supercoiling activity and significantly inhibit supercoiling of wild-type cell extracts

The restoration of negative supercoiling is slow and requires a significant increase in the population cell density of strain PS151 (*topA rnhA* double mutant carrying pBAD*rnhA*) following the addition of arabinose to produce RNase HI (not shown). This result suggested involvement of one or more stable factors in inhibiting supercoiling in *topA rnhA* double mutants. To test this idea, we prepared extracts of wild-type (strain AQ634), *gyrB*(Ts) (strain MA249), *gyrB*(Ts) *topA* (strain PS152) and *gyrB*(Ts) *topA rnhA* (strain PS151) cells. Extracts from both wild-type and *gyrB*(Ts) strains exhibited supercoiling activity (Fig. 7a), although with the *gyrB*(Ts) strain an endonuclease activity was also detected (lane 6). Maximum supercoiling activity was seen at 3.5 μ g total protein in the reaction mixture for the *gyrB*(Ts) strain (lane 9); the same amount of protein from wild-type cells exhibited more supercoiling activity (compare wild-type, lane 4 with *gyrB*(Ts), lane 9), and a higher supercoiling level was achieved (not shown). Although supercoiling activity was also detected in crude extracts of *topA* null *gyrB*(Ts) cells (Fig. 7b, lanes 7 to 12, top panel), it was significantly lower than in extracts of isogenic *topA*⁺ *gyrB*(Ts) cells (Fig. 7a, lanes 6 to 10) and was better seen when the gel was probed with a [³²P]-labelled DNA fragment (Fig. 7b, lanes 7 to 12, bottom panel). Even with radioactive probing, supercoiling activity was undetectable in extracts from the *topA rnhA* double mutant grown in the absence of arabinose to deplete RNase HI activity (Fig. 7b, lanes 1 to 6, bottom panel). These results are consistent with crude extracts of *topA rnhA* null cells containing factor(s) that significantly reduce supercoiling activity.

As a further test for factor(s) inhibiting supercoiling, aliquots of extracts from both *topA* and *topA rnhA* double mutant cells were mixed with extracts from wild-type cells. As shown in Fig. 7c, extracts from the *topA rnhA* double mutant significantly inhibited supercoiling activity of wild-type extracts (compare lanes 1 and 5, which used 2.5 μ g protein from the wild-type cell extract with, respectively, 5 and 0 μ g protein from the *topA rnhA* double

mutant extract). In contrast, inhibition of supercoiling activity by the extract from *topA* null cells was weak (Fig. 7c, compare lanes 1 and 6, using 2.5 µg protein from the wild-type cell extract with 5 µg protein from the *topA rnhA* or the *topA* null extracts). Thus, a strong inhibitory activity present in extracts of *topA rnhA* double mutant explains the failure to detect supercoiling activity and may explain, at least in part, the impairment of supercoiling by gyrase in *topA rnhA* double mutants.

Topoisomerase III overproduction complements the growth defect of a *topA rnhA* double mutant without restoring supercoiling

The data described above failed to relate the growth defect of *topA rnhA* double mutants to excess negative supercoiling. Since in *E. coli* topoisomerase III is not involved in the regulation of supercoiling (Zechiedrich *et al.*, 2000; Lopez *et al.*, 2005), we expected DNA of *topA rnhA* double mutants to remain relaxed during suppression of growth defect by overexpression of topoisomerase III. To test this hypothesis we made use of the finding that in a *topA* null mutant hypernegative supercoiling of pPH1243 is stimulated both by transcription from the strong *P_{trc}* promoter, which is activated by the addition of IPTG, and by translation inhibitors (Broccoli *et al.*, 2004; Broccoli and Drolet, unpublished results; spectinomycin was added 15 min before shifting from 37 to 28°C). In one experimental set, IPTG was added throughout growth at 37°C to overexpress topoisomerase III; in the second set, IPTG was added after spectinomycin so that the transcription effect of IPTG on pPH1243 supercoiling could be observed in the absence of excess topoisomerase III. Fig. 8 shows that pPH1243 exhibited hypernegative supercoiling upon adding IPTG, either before (lane 2) or after (lane 6) the addition of spectinomycin to the *topA* null mutant. In contrast, relaxation of pPH1243 was observed whether or not topoisomerase III was overproduced and irrespective of the presence of spectinomycin in the *topA rnhA* double mutant (Fig. 8, lanes 3, 4, 7 and, 8). Two-dimensional gel electrophoresis confirmed relaxation of pPH1243 (data not shown). As expected, supercoiling was restored in this *topA rnhA* double mutant following the introduction of a plasmid carrying the *rnhA* gene (data not shown), indicating that relaxation is reversible and due to the absence of RNase HI. Collectively, these data support the assertion that overexpression of topoisomerase III suppresses the growth defect of *topA rnhA* double mutants without restoring supercoiling.

Cells of a *topA rnhA* double mutant form long filaments packed with unevenly distributed DNA

Despite the presence of a mechanism to prevent hypernegative supercoiling, *topA rnhA* double mutants do not grow without suppression. This result suggests the presence of supercoiling-independent mechanism(s) by which the absence of RNase HI dramatically perturbs cell physiology. As shown above, the growth defect of a *topA rnhA* double mutant can be rescued by overproducing topoisomerase III (Fig. 4), which can allow chromosome segregation by removing precatenanes during replication (Nurse *et al.*, 2003). We therefore examined the possibility that the growth inhibition of *topA rnhA* double mutants might be related to segregation defects.

Cells were stained with DAPI and prepared for fluorescence microscopy such that both cell morphology and DNA content could be examined. In the absence of topoisomerase III overproduction, cells of the *topA rnhA* double mutant (strain SB383) formed long filaments full of DNA, and nucleoid structures were altered (Fig. 9a, panel 2). Anucleate cells also accumulated (Fig. 9b, yellow arrows), as did cells with very low amounts of chromosomal DNA (Fig. 9b, green arrows). Formation of such cells could result from septum closure on nucleoids, a typical manifestation of partition defects (guillotine effect; Niki *et al.*, 1991). Clearly, the *topA rnhA* double mutant fails to produce a significant number of normal cells. However, more than half of the cells containing aberrant nucleoid structures likely remain

viable, as cells of strain SB383 incubated in liquid medium lacking IPTG form colonies when plated on solid media containing IPTG to overproduce topoisomerase III (data not shown). Overproduction of topoisomerase III reduced both cell filamentation and DNA content, and it caused DNA to be more regularly distributed within the cells (Fig. 9a, panel 1). Topoisomerase III overproduction also reduced the number of anucleate cells by more than half. Segregation defects were also apparent in the *topA20::Tn10 rnhA::cam gyrB* (Ts) mutant carrying pBAD*rnhA* (strain PS151; Fig. 9a, panel 4, -ara); they were corrected by the addition of arabinose to produce RNase HI (panel 3, +ara). Moreover, cells of single *rnhA* and *topA* null mutants failed to show major morphological or nucleoid defects (Fig. 9c, panels 2 and 3 respectively). Thus, combinations of *topA* and *rnhA* null mutations lead to phenotypes reminiscent of segregation defects, thereby explaining the growth inhibition phenotype of *topA rnhA* double mutants.

Discussion

The work described above, which focused on the properties of *topA rnhA* double mutants, revealed new features of how DNA topology is regulated in bacterial cells. One level concerns DNA supercoiling. Basal supercoiling, which is defined as supercoiling in the absence of transcription (Drlica *et al.*, 1988), is set largely by the supercoiling activity of gyrase opposed by the relaxing activities of topoisomerase I and topoisomerase IV. Transcription, through the generation of R-loops coupled with gyrase activity, produces hypernegative supercoiling that is countered by topoisomerase I and RNase HI. The growth defect associated with a deficiency of topoisomerase I is suppressed by partially defective gyrase, overexpression of topoisomerase IV, or overexpression of RNase HI. Surprisingly, *topA rnhA* null double mutants not only lacked hypernegative supercoiling but also contained DNA that was relaxed (Fig. 1 and Fig. 8). Evidence was found for the interesting possibility that RNase HI normally regulates factor(s) impairing supercoiling by gyrase. A second level of topology control concerns decatenation of replicated chromosomes. Decatenation is largely a function of topoisomerase IV (Zechiedrich and Cozzarelli, 1995). In our work, a *topA rnhA* double mutant exhibited a defect in chromosome segregation that was corrected by overexpression of topoisomerase III (Fig. 9), an enzyme that has no effect on supercoiling and is capable of decatenation both *in vitro* and *in vivo* (Hiasa *et al.*, 1994; Nurse *et al.*, 2003). Thus RNase HI, either directly or indirectly, influences both basal levels of supercoiling and chromosome segregation as discussed in the following sections.

Supercoiling in *topA rnhA* double mutants

Previous work showed that gyrase-mediated hypernegative supercoiling occurs during transcription *in vitro* in the absence of RNase HI (Drolet *et al.*, 1994; Phoenix *et al.*, 1997) and in *topA* null mutants when RNase HI is not overproduced (Massé and Drolet, 1999c). We expected removal of *rnhA* from *topA* mutants to lead to extensive hypernegative supercoiling, since cells lacking both topoisomerase I and RNase HI cannot be constructed (Massé and Drolet, 1999c; Drolet *et al.*, 1995). Unexpectedly, hypernegative supercoiling failed to accumulate in *topA rnhA* double mutants that had been constructed by conditional expression of *rnhA* or *topB*. However, DNA of such mutants was more relaxed than usual. Relaxation was not an indirect consequence of cell death, because deleting *rnhA* in *topA* null mutants was bacteriostatic, not bactericidal. When arabinose was added to non-growing *topA rnhA* null cells carrying pBAD*rnhA* to induce the synthesis of RNase HI, both growth and supercoiling were restored. More importantly, DNA remained extensively relaxed in a *topA rnhA* double mutant when cell growth was restored by overproducing topoisomerase III. Only when a plasmid carrying *rnhA* was introduced into this mutant that supercoiling was also restored. Therefore, DNA relaxation correlated with depletion of RNase HI activity in *topA* mutants.

Since supercoiling level is set primarily by opposing topoisomerase activities, we considered the possibility that the supercoiling deficit in *topA rnhA* null double mutants was due either to an excess of DNA relaxation activity by topoisomerases or to a loss of gyrase supercoiling activity. One well established mechanism by which excess negative supercoiling is prevented in *topA* mutants is the overproduction of topoisomerase IV (Kato *et al.*, 1990; Free and Dorman, 1994). However, western blot experiments showed that topoisomerase IV is not overproduced in *topA* null cells lacking RNase HI activity. Moreover, inhibition of topoisomerase IV by norfloxacin failed to significantly raise supercoiling in a *topA rnhA* double mutant carrying the *gyrA*^{L83} quinolone-resistance allele. Thus topoisomerase IV was not responsible for the supercoiling deficit in *topA rnhA* double mutants. In agreement with the results of previous experiments showing that topoisomerase III is not involved in supercoiling regulation in *E. coli* (Zechiedrich *et al.*, 2000; Lopez *et al.*, 2005), overproducing this enzyme had no effect on supercoiling in either *topA* single or *topA rnhA* double mutants. Therefore, relaxation activity of topoisomerases is unlikely to explain the supercoiling deficit in *topA* null cells depleted of RNase HI activity.

Western blot experiments also demonstrated that levels of gyrase protein were not altered following the depletion of RNase HI activity in *topA* mutants. Two experiments indicated that the supercoiling deficit is related to impairment of supercoiling by gyrase. First, while inhibiting topoisomerase IV by norfloxacin in a single *topA* null mutant strongly promoted hypernegative supercoiling, it did not significantly stimulate supercoiling in a double *topA rnhA* null mutant. Second, supercoiling activity could not be detected in cell extracts of a *topA rnhA* null double mutant (strain PS151), and these extracts significantly inhibited the supercoiling activity when mixed with extracts from wild-type cells. Thus, the supercoiling deficit in *topA* null cells depleted of RNase HI activity is likely related to a cellular response that leads to the inhibition of gyrase.

The cellular response leading to the impairment of gyrase activity in double *topA rnhA* null mutants is currently unknown. The response is related neither to the SOS regulon, which is constitutively induced in the absence of RNase HI (Kogoma *et al.*, 1993; McCool *et al.*, 2004), nor to the presence of the gyrase inhibitor, GyrI, a member of the SOS regulon (Baquero *et al.*, 1995). Previous results also failed to demonstrate an effect of GyrI on supercoiling *in vivo* (Chatterji *et al.*, 2003). While supercoiling by gyrase can be prevented by a direct interaction between the enzyme and a specific inhibitor acting like GyrI, it may also be inhibited by low [ATP]/[ADP] (Westerhoff *et al.*, 1998; Drlica, 1992) and indirectly by proteins that interact with DNA, such as the abundant nucleoid proteins Fis, H-NS and HU (Travers and Muskhelishvili, 2005ab). Additional experiments are required to work through the many possible ways in which gyrase activity can be lowered.

Segregation defect and growth inhibition suppressed by topoisomerase III

The growth inhibition associated with *topA rnhA* double mutants correlated with phenotypes seen previously with chromosomal segregation defects: extensive cell filamentation, abnormal nucleoid structures, and accumulation of anucleate cells. Thus, the simultaneous absence of topoisomerase I and RNase HI leads to segregation defects not observed with cells lacking only one of the enzymes. R-loops may be involved, since a relationship between topoisomerase I and RNase HI is well established. If R-loops persist in *topA rnhA* double mutants, particularly at *oriK* sites where constitutive, stable DNA replication is initiated (Kogoma, 1997), excess replication could occur. Over-replication may saturate the segregation capacity of the cell, thus requiring more decatenation activity than can be provided by excess topoisomerase III.

Unregulated over-replication is also known to lead to collapse of replication forks, DNA double-strand breaks (Kouziminova *et al.*, 2004; Simmons *et al.*, 2004; Michel *et al.*, 2007),

and ultimately hyper-recombination, which can cause segregation defects (Lopez *et al.*, 2005; Zhu *et al.*, 2001; Zahradka *et al.*, 1999; Magner *et al.*, 2007). Accumulation of unresolved recombination intermediates interferes with chromosome segregation; resolution of these structures can be performed by topoisomerase III (Lopez *et al.*, 2005; Zhu *et al.*, 2001), even when it is present at normal or very low levels (less than 10 copies per cell; Digate and Mariani, 1989).

The extensive DNA relaxation in *topA rnhA* double mutants could also contribute to segregation defects, since the inhibitory effect of temperature-sensitive gyrase mutations on segregation (Steck and Drlica, 1984) is thought to be due to DNA relaxation that then suppresses decatenation by topoisomerase IV (Zechiedrich *et al.*, 1997; Holmes and Cozzarelli, 2000). Overproduction of topoisomerase III allows chromosome segregation when topoisomerase IV is inactive (Nurse *et al.*, 2003). However, in the context of extensive DNA relaxation, the ability of topoisomerase III to perform decatenation might be reduced because single-stranded DNA regions, the substrate for topoisomerase III, are expected to be infrequent. Such regions can also be provided by R-loops (Broccoli *et al.*, 2000).

Biological function of RNase HI

The first function attributed to RNase HI in *E. coli* was a role in the removal of RNA primers of Okazaki fragments (Funnell *et al.*, 1986; Ogawa and Okazaki, 1984). However, this enzyme cannot remove the last ribonucleotides at the RNA-DNA junctions. In fact, 5'-3' exonuclease activity (e.g. polymerase I) plays the major role in the removal of RNA primers. Interestingly, RNase H activity in various bacterial species was recently shown to be dispensable for complete RNA primer removal (Fukushima *et al.*, 2007). Only 5'-3' exonuclease activity was shown to be indispensable for this process. Based on the results presented in the present work we propose that a major function of RNase HI, and possibly other bacterial RNase H molecules, involves the control of DNA topology via R-loops.

Experimental procedures

E. coli strains

Escherichia coli strains used in this work are described in Table 1. Strains were constructed by transduction with phage P1_{vir} as previously described (Miller, 1992). When needed, tetracycline (10 µg/ml), chloramphenicol (15 µg/ml) or kanamycin (50 µg/ml) was added to the medium. PCR was used to confirm the presence of only the *rnhA::cam* allele and the *sbmC::kan* allele, respectively, on the chromosome of the *rnhA* null and the *sbmC* null transductants.

Plasmids

pBAD18*rnhA* was constructed by placing an EcoRI-HindIII fragment carrying the *rnhA* gene under the control of the P_{BAD} promoter of pBAD18 (Guzman *et al.*, 1995). The *rnhA* gene was obtained by PCR from pSK760 (Drolet *et al.*, 1995) by using d(GTCAGAATTCCAGGAAGTCTACCAGA) and d(GTCAAAGCTTGGCAATGTCGTAAACC) oligonucleotides. pGB2*nusB::kan* is a pSC101 derivative that was constructed by inserting the pUC4K EcoRI fragment carrying a kanamycin-resistance cassette into the ScaI site of pGB2*nusB* (Friedman *et al.*, 1990). pPH1243 is a pTrc99a derivative carrying the *topB* gene under the control of the IPTG-inducible P_{trc} promoter (Broccoli *et al.*, 2000).

Plasmid extraction for supercoiling analysis

Cells were grown overnight at 37°C in LB medium supplemented with cysteine (50 µg/ml, for the RFM475 derivatives), thymine (10 µg/ml, for the MA251 derivatives) or tryptophan

(50 µg/ml, for VU35 and VU95 strains). When required, ampicillin (50 µg/ml), spectinomycin (30 µg/ml), arabinose (0.05%) or IPTG (1 mM) were added. Overnight cultures diluted in pre-warmed medium (37°C), were grown to an OD₆₀₀ of ~0.5 at which time an aliquot of cells was recovered for plasmid extraction while the remaining culture was transferred to 28°C. Aliquots of cells were recovered for plasmid extraction at the indicated times. Growth was arrested by transferring cells into a tube filled with ice, thus immediately lowering the temperature of the culture to 0°C. Plasmid DNAs were extracted by alkaline lysis as previously described (24). For experiments using derivatives of MA251, overnight cultures were diluted 1:1000; they were diluted to an OD₆₀₀ of 0.03 for the experiments using SB383, VU21, PS160, VU64, and VU70.

Plasmid topoisomer analysis

One-dimensional and two-dimensional agarose gel electrophoresis in the presence of chloroquine was performed in 0.5 × TBE as described (Massé *et al.*, 1997). After electrophoresis, the gels were dried and prepared for *in situ* hybridization with random prime-labelled probes as described (Massé *et al.*, 1997). Images were obtained by using a Phosphorimager Typhoon 9400 (Amersham Biosciences).

Western blot analysis

The equivalent of 200 µl of cell culture at an OD₆₀₀ of 0.7 was used for Western blot analysis. The cell pellets were lysed by boiling in sodium dodecyl sulphate (SDS). The proteins were separated by SDS-polyacrylamide (7.5 %) gel electrophoresis. Western blots were performed as described previously (Sambrook *et al.*, 1989) by using nitrocellulose membranes (Hybond-ECL, GE Healthcare). After transfer, the membranes were stained with Ponceau S (Fisher Scientific) to confirm that similar amounts of protein were loaded in each lane. ParC and ParE antibodies were obtained from Dr Kenneth J. Marians (Memorial Sloan-Kettering Cancer Center, New York, New York). GyrA and GyrB antibodies were purchased from John Innes Enterprises Ltd (John Innes Centre, Norwich Research Park Colney, Norwich, UK). TopB antibodies were obtained from Dr Russell DiGate (Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, Philadelphia, PA). The ECL Plus detection kit (GE Healthcare) was used to reveal the specific proteins.

Gyrase assays in crude cell extracts

Cells were grown in LB medium at 37°C to an OD₆₀₀ of 0.7 and transferred to 28°C for 30 minutes. Cells were recovered and prepared for gyrase assays in crude extracts as previously described (DiNardo *et al.*, 1982). 0.2 µg of relaxed pBR322 DNA (prepared by using wheat germ topoisomerase I from Sigma-Aldrich) was used in the assays.

Microscopy

RFM443, RFM475, PH379 and SB383 cells were grown overnight on LB plates supplemented, when required, with cysteine (50 µg/ml), ampicillin (50 µg/ml), chloramphenicol (10 µg/ml) and/or tetracycline (10 µg/ml). When needed, IPTG (1 mM) was added to the plates of SB383 cells. PS151 cells were grown overnight on LB plates supplemented with thymine (50 µg/ml) and, when needed, arabinose (0.05%). The plates were incubated at 37°C. After overnight growth, cells were resuspended in pre-warmed (37°C) liquid LB medium (supplemented as requested) to obtain a starting OD₆₀₀ of about 0.01. Cells were grown at 37°C to an OD₆₀₀ of 0.8. 150 µl of cells were harvested, centrifuged, and resuspended in 77% ethanol (fixing solution). The cells were washed with 500 µl of 0.9% NaCl, centrifuged and resuspended in 100 µl of 0.9% NaCl. 3 µl of the fixed samples were spread on slides pre-treated with a Poly-L-Lysine solution (Sigma) and allowed to air dry at room temperature. 5 µl of slow fade gold antifade reagent with DAPI (4',6-

diamidino-2-phenylindole; from Invitrogen) was deposited on the slides and sealed with a cover glass. Fluorescence pictures were obtained with a Nikon E600 equipped with a 100-W mercury lamp and standard DAPI filters using the X100 oil immersion objective. The images were captured on the computer using the Nikon ACT-1 software. Exposure time was 1/50s at maximum sensitivity. For phase contrast microscopy, the microscope was adjusted to the phase contrast optical system and pictures were taken at X100 oil immersion objective with the Ph3 annulus. Exposure time was 1/120s at normal sensitivity. The images were processed with Adobe Photoshop.

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AQ634 (wild-type), MA249 (*gyrB*(Ts)), PS152 (*topA*⁻, *rnhA*⁺/pGB2nusB::kan) and PS151 (*topA*⁻, *rnhA*⁻/pGB2nusB::kan and pBAD18*rnhA*) cells were grown overnight at 37°C in the absence of arabinose. Overnight cultures were diluted 1:1000 in LB medium with or without arabinose (0.05%) as indicated and grown at 37°C. DNA was extracted just before the temperature downshift and 60 minutes after the downshift (28°C). DNA samples were loaded on agarose gels for 1-D (with 7.5 µg/ml of chloroquine in a) or 2-D (chloroquine concentrations in µg/ml are indicated by arrows in b) electrophoresis. The gels were hybridized with a probe to detect pGB2nusB::kan. [--] points to hypernegatively supercoiled DNA. [rel] indicates extensively relaxed DNA. In b), lines were traced over the more relaxed topoisomers.

Figure 2. Supercoiling in a *topA* *rnhA* null double mutant after transcription inhibition
 PS152 (*topA*⁻, *rnhA*⁺/ pGB2nusB::kan) and PS151 (*topA*⁻, *rnhA*⁻/ pGB2nusB::kan and pBAD18*rnhA*) cells were grown at 37°C in the absence of arabinose as indicated in the legend to Fig. 1. Rifampicin (250 µg/ml) was added or not as indicated, 15 minutes before the temperature downshift and the DNA was extracted 60 min after the downshift (28°C). DNA samples were loaded on an agarose gel for 2-D electrophoresis (chloroquine concentrations in µg/ml are indicated by arrows). The gel was hybridized with a probe to detect pGB2nusB::kan. Lines were traced over the more relaxed topoisomers (bottom panels).

Figure 3. Western blot analysis to measure topoisomerase IV and gyrase levels in *topA rnhA* mutants

RFM475 (*gyrB(Ts) ΔtopA*), SB383 (*gyrB(Ts) ΔtopA rnhA::cam/pPH1243*), PS147 (*gyrB(Ts) topA20::Tn10rnhA::cam/pBAD18rnhA*) and MA251 (*gyrB(Ts) topA20::Tn10*) cells were grown at 37°C in LB medium (no IPTG, no arabinose). Aliquots of cells were used for western blots as described in Experimental procedures. Lanes 1, 3, 5 and 7, aliquots of cells grown at 37°C; lanes 2, 4, 6 and 8, aliquots of cells exposed to 28°C for one hour.

IPTG

+

-

a)

IPTG

+

-

Temp °C

37

28

37

28

b)

←TopB

Figure 4. Complementation of a *topA rnhA* mutant by topoisomerase III overproduction
 In (a) SB383 (*gyrB*(Ts) Δ *topA rnhA::cam*/pPH1243) cells were streaked on solid LB medium with 1 mM or no IPTG as indicated. The plates were incubated overnight at 37°C. In (b) SB383 cells were grown in LB at 37°C with or without 1 mM IPTG as indicated and aliquots were used for western blots with anti-topoisomerase III antibodies. Aliquots were also obtained from cells 60 min after a temperature downshift to 28°C.

Figure 5. Topoisomerase IV inhibition does not promote hypernegative supercoiling in a double *topA rnhA* mutant

PS160 (*topA*⁻, *rnhA*⁻ *gyrA*^{L83}/pPH1243) and VU21 (*topA*⁻, *rnhA*⁺ *gyrA*^{L83}/pPH1243) cells were grown at 37°C in LB without IPTG to log phase and norfloxacin (60 µM final) was added or not as indicated. Aliquots of cells were taken for DNA extraction 30 min later and the remaining cells were transferred to 28°C. 30 min later aliquots of cells were taken for DNA extraction. DNA was analyzed in an agarose gel with 7.5 µg/ml chloroquine. The gel was hybridized with a probe to detect pPH1243 topoisomers. [--] point to hypernegatively supercoiled DNA. [rel] indicates extensively relaxed DNA.

Figure 6. GyrI, a gyrase inhibitor, plays no role in supercoiling inhibition in a *topA rnhA* null double mutant

SB383 (*topA*⁻, *rnhA*⁻, *sbmC*⁺/pPH1243), VU70 (*topA*⁻, *rnhA*⁻, *sbmC*⁻/pPH1243) and VU64 (*topA*⁻, *rnhA*⁺, *sbmC*⁻/pPH1243) cells were grown at 37°C in the presence of 1 mM ITPG. Spectinomycin was added to 400 µg/ml 15 min before the temperature downshift to 28°C. DNA was extracted 60 min after the downshift and samples were loaded on an agarose gel (with 7.5 µg/ml of chloroquine) for 1-D electrophoresis. The gel was hybridized with a probe to detect pPH1243 topoisomers. [---] point to hypernegatively supercoiled DNA.

Figure 7. Supercoiling assays in crude cell extracts

Crude cell extracts were prepared and used for supercoiling assays as described in Experimental procedures. In (a), the amounts of protein used in the assays were 28 μ g (lanes 1 and 6), 14 μ g (lanes 2 and 7), 7 μ g (lanes 3 and 8), 3.5 μ g (lanes 4 and 9) and 1.75 μ g (lanes 5 and 10). The reactions were incubated at 30°C for 2 hours. sup is supercoiled pBR322 and rel is relaxed pBR322. WT and *gyrB*(Ts) respectively indicate AQ634 and MA249 strains that were used in the experiment. In (b), the amounts of protein used in the assays were 40 μ g (lanes 1 and 7), 20 μ g (lanes 2 and 8), 10 μ g (lanes 3 and 9), 5 μ g (lanes 4 and 10), 2.5 μ g (lanes 5 and 11) and 1.25 μ g (lanes 6 and 12). The reactions were incubated at 30°C for 4 hours with twice the amount of ATP. At the top of the ethidium bromide stained gel (top panel), sup is supercoiled pBR322 and rel is relaxed pBR322. The bottom panel is a picture of the gel probed with a random prime-labelled fragment hybridizing to the *bla* gene of pBR322. The strains used in the experiment were PS151 (*topA*⁻, *rnhA*⁻/pGB2nusB::kan and pBAD18*rnhA*) and PS152 (*topA*⁻, *rnhA*⁺/pGB2nusB::kan). They were grown in the absence of arabinose and the log phase cultures were obtained from the overnight ones diluted 1:1000. In (c), 2.5 μ g of proteins from a crude cell extract of the wild-type strain (AQ634) were mixed with 0, (lane 5), 5 (lane 1), 2.5 (lane 2), 1.25 (lane 3) or 0.63 μ g (lane 4) of proteins from the crude extract of PS151 strain used in (b) and with 5 (lane 6), 2.5 (lane 7), 1.25 (lane 8) and 0.63 μ g (lane 9) of proteins from the crude extract of PS152 strain used in (b). The reactions were incubated at 30°C for 2 hours. sup is supercoiled pBR322 and rel is relaxed pBR322.

Figure 8. Topoisomerase III overproduction does not change supercoiling in a *topA rnhA* null double mutant

SB224 (*topA*⁻, *rnhA*⁺/pPH1243) and SB383 (*topA*⁻, *rnhA*⁻/pPH1243) cells were grown in LB to log phase at 37°C. Spectinomycin was added to 400 µg/ml 15 min before the temperature downshift to 28°C. IPTG before spc means that IPTG was added to 1 mM during the growth at 37 °C whereas IPTG after spc means that IPTG was added to 1 mM 5 min after the temperature downshift. DNA was extracted 60 min after the downshift and used for 1-D (7.5 µg/ml chloroquine) gel analysis. The gel was hybridized with a probe to detect pPH1243. [--] points to hypernegatively supercoiled DNA. [rel] indicates extensively relaxed DNA.

topA rnhA null mutant/pPH1243

- ITPG

Figure 9. DAPI staining of *topA rnhA* null mutants reveals segregation defects that are corrected by topoisomerase III overproduction

Cells were grown and prepared for microscopy as described in Experimental procedures. In a), 1 and 2 are fluorescence pictures of DAPI stained SB383 cells (*gyrB*(Ts) Δ *topA rnhA* ::*cam*/pPH1243) respectively grown in the presence and the absence of IPTG both during overnight growth on plates and during growth in liquid. 3 and 4 are fluorescence pictures of DAPI stained PS151 cells (*gyrB*(Ts) *topA20*::Tn10 *rnhA* ::*cam*/pBAD18*rnhA*) respectively grown in the presence and the absence of arabinose both during overnight growth on plates and during growth in liquid. Superimposed pictures of phase contrast and fluorescence (by using Adobe Photoshop) of DAPI stained SB383 (no IPTG) cells are shown in b). Yellow and green arrows indicate respectively anucleate cells and cells with low DNA content. In c), 1 to 3 are fluorescence pictures of DAPI stained RFM430 (wild-type), PH379 (*rnhA* ::*cam*) and RFM475 (*gyrB*(Ts) Δ *topA*) cells, respectively.

Table 1*Escherichia coli* strains used in this study.

Strain	Genotype	Source or Ref.
AQ634	<i>ilv metB his-29 trpA9605 pro thyA deoB</i> (or <i>C</i>)	Hraiky <i>et al.</i> , 2000
MA249	AQ634 <i>gyrB221</i> (cou ^R) <i>gyrB203</i> (Ts) <i>zie-3163::Tn10kan</i>	Hraiky <i>et al.</i> , 2000
MA251	MA249 <i>gyrB221</i> (cou ^R) <i>gyrB203</i> (Ts) <i>zie-3163::Tn10kan</i> <i>topA20::Tn10</i>	Hraiky <i>et al.</i> , 2000
CM23	MA251 pBAD18 <i>rnhA</i>	This work
RFM430	<i>rpsL, galK2, AtrpE</i>	Drolet <i>et al.</i> , 1995
MIC1020	AB1157 <i>rnhA::cam</i>	Itaya and Crouch, 1991
PH379	RFM430 <i>rnhA::cam</i>	This work
PS147	CM23 <i>rnhA::cam</i>	This work
PS151	PS147 pGB2 <i>nusB::kan</i>	This work
PS152	MA251 pGB2 <i>nusB::kan</i>	This work
RFM445	<i>rpsL galK2 gyrB221</i> (cou ^R) <i>gyrB203</i> (Ts) Δ <i>lac74</i>	Drolet <i>et al.</i> , 1995
RFM475	<i>rpsL galK2 gyrB221</i> (cou ^R) <i>gyrB203</i> (Ts) Δ (<i>topA</i> <i>cysB</i>)204 Δ <i>lac74</i>	Drolet <i>et al.</i> , 1995
SB224	RFM475 pPH1243	This work
SB383	SB224 <i>rnhA::cam</i>	This work
LZ1	Hfr <i>thi gyrA</i> ^{L83} <i>zei-723::Tn10</i>	Zechiedrich <i>et al.</i> , 2000
PS158	RFM475 <i>gyrA</i> ^{L83} <i>zei-723::Tn10</i>	This work
VU21	PS158 pPH1243	This work
PS160	SB383 <i>gyrA</i> ^{L83} <i>zei-723::Tn10</i>	This work
VU48	MG1655 <i>sbmC::kan</i>	Carmen Gomez- Eichelmann
VU56	RFM475 <i>sbmC::kan</i>	This work
VU64	VU56 pPH1243	This work
VU70	VU64 <i>rnhA::cam</i>	This work