An *Escherichia coli* DNA Topoisomerase I Mutant Has a Compensatory Mutation That Alters Two Residues between Functional Domains of the DNA Gyrase A Protein

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Nucleotide sequence analysis revealed that the compensatory *gyrA* mutation in *Escherichia coli* DM750 affects DNA supercoiling by interchanging the identities of Ala-569 and Thr-586 in the DNA gyrase A subunit. These residues flank Arg-571, a site for trypsin cleavage that splits gyrase A protein between DNA breakage-reunion and DNA-binding domains. The putative interdomain locations of the DM750 mutation and that of *E. coli* DM800 (in gyrase B protein) suggests that these compensatory mutations may reduce DNA supercoiling activity by altering allosteric interactions in the gyrase complex.

DNA gyrase and DNA topoisomerase I are the best-characterized activities influencing bacterial DNA supercoiling (25). Gyrase, an A_2B_2 tetramer encoded by the *gyrA* and *gyrB* genes, catalyzes ATP-dependent DNA supercoiling and is essential for cell growth (7, 25). DNA topoisomerase I (topo I), encoded by *topA*, removes negative (but not positive) DNA supercoils and is one of several DNA-relaxing enzymes present in *Escherichia coli* (25). The expression of gyrase and topo I genes is sensitive to the supercoiled state of the chromosome: gyrase genes are downregulated by DNA supercoiling, whereas *topA* is up-regulated (12, 24). These results and others suggested that DNA supercoiling is homeostatically regulated by balancing the antagonistic activities of gyrase and topo I (12). However, transcription also affects DNA supercoiling, and it has recently been proposed that the primary role of gyrase and topo I is to remove the respective positive and negative supercoils formed ahead of and behind the tracking RNA polymerase (10, 16, 27). Whatever the detailed mechanism underlying regulation of DNA supercoiling, genetic studies of topo I mutant strains of *E. coli* emphasize the importance of balancing DNA supercoiling and relaxing activities. *topA* deletion strains are viable only because they acquire a secondary or "compensatory" mutation which in strains DM750 and DM800 has been mapped to the *gyrA* and *gyrB* genes, respectively (4, 17). These mutant alleles compensate for the loss of topo I by producing gyrase subunits with defective supercoiling activity (6).

Gyrase acts by passing a DNA segment through a transient enzyme-bridged double strand break in DNA (13). The two 97-kDa gyrase A (GyrA) subunits mediate transient DNA breakage-reunion which is somehow coupled to ATP binding and hydrolysis by the 90-kDa gyrase B (GyrB) subunits (25). Both A and B subunits appear to be organized into functional domains (5, 18–20, 23). The X-ray crystal structure has recently been reported for the ATP-binding N-terminal domain of GyrB (26). However, the large size of the enzyme complex and the absence of detailed structural information have hindered mechanistic studies using conventional strategies such as site-directed mutagenesis. As an

alternative approach, we have sought to understand the defective gyrases present in compensatory *E. coli* K-12 strains. The GyrA protein of *E. coli* DM750 is 20-fold less active than the wild type at low (1 mM) Mg^{2+} concentrations, while DM800 gyrase B protein is 10-fold less active than the wild-type subunit (6). Previously, we showed that the DM800 *gyrB* mutation results in an Ala-Arg insertion into the B protein (11). Here we characterize the DM750 *gyrA* gene and identify the novel compensatory mutation in GyrA protein.

Four pairs of oligonucleotides were used to amplify segments of the DM750 *gyrA* gene by polymerase chain reaction (PCR), in each case by the following method (Table 1 and Fig. 1). A single colony of DM750 cells was resuspended in PCR mix, which consisted of 10 mM Tris hydrochloride (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.01% gelatin containing 200 µM each deoxynucleoside triphosphate and 1 to 5 µM each oligonucleotide primer (total volume, 49.5 µl). Tubes were incubated at 94°C for 5 min and allowed to cool to room temperature, and 1.5 U of *Taq* polymerase was added. PCR conditions were as follows: 92°C, 30 s; 64°C (55°C for oligonucleotides 1-2 and 7-8), 2 min; and 74°C, 3 min; 30 cycles followed by a final 74°C incubation for 10 min. The four pairs of oligonucleotides each produced single PCR products (Fig. 1, A through D), which were extracted with phenol and precipitated with ethanol. Product A was digested with *Eco*RI and *Bam*HI, B was digested with *Sac*I-*Sma*I, C was digested with *Sma*I-*Eco*RI and then either *Pst*I or *Bgl*II, and D was digested with *Kpn*I-*Eco*RI and then *Bgl*II. Restriction fragments were isolated and purified by electrophoresis in 2% low-gelling agarose and ligated into appropriately cut M13mp18 and M13mp19 replicative-form DNAs prior to transformation of *E. coli* XL1 Blue *recA*. For each fragment, recombinant clones derived from at least two independent PCRs were sequenced by the dideoxy chain termination method, using the Sequenase version 2.0 protocol (United States Biochemicals) (21). The DNA sequences obtained for each complementary strand of the gene gave concordant results (Fig. 1).

The DM750 *gyrA* gene sequence was identical to that reported for the wild-type *E. coli* K-12 gene (22) except for three nucleotide changes: G→A (nucleotide 1705) and A→G (1756) changes resulted in Ala-569→Thr and Thr-586→Ala

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TABLE 1. Oligonucleotide pairs used to amplify *gyrA*
gene segments by PCR

Oligo-nucleotide	Sequence (nucleotide position)[a]
1	5'GTGGATCCTATATCAGGCATTGGA (−118−−95)
2	TCGAATTCCGTCGATAGAACCGAA (348–325)
3	TACACCGGTCAACATTGAGG (24–43)
4	TTAATGATTGCCGCCGTCGG (671–652)
5	AGACATCAGCATTGAAGGGC (603–622)
6	CGCAGACGGTTGAATTCGGTGAGG (2000–1977)
7	GGCGTGAAAGTCTTCATGGCG (1927–1947)
8	GCCTGGACTTTTGAATTCAAACAAGGG (2687–2661)

[a] Based on the *gyrA* sequence determined by Swanberg and Wang (22). Oligonucleotides 2, 4, 6 and 8 are based on non-coding-strand *gyrA* sequences. Artifical *Bam*HI or *Eco*RI sites introduced to facilitate cloning of PCR products are underlined.

substitutions at the protein level (a C→T change at position 1785 was silent, retaining the wild-type Ser-595 codon). No differences in *gyrA* promoter sequences were seen. Neither coding change observed in the DM750 GyrA sequence is present in the gyrase A proteins of other *E. coli* strains, including K-12 derivatives KL16 (28 and references therein), KNK453 and D110 (14, 15a), and clinical isolate 227 (2). Therefore, we assume that both DM750 GyrA changes contribute to the altered phenotype. Thus, the DM750 compensatory mutation appears to be a novel structural change that affects GyrA activity by interchanging the identities of residues 569 and 586.

The two closely spaced substitutions in DM750 GyrA protein flank a site for trypsin cleavage (Arg-571) that generates two protein fragments with distinct functions (Fig. 2) (18). The 33-kDa C-terminal fragment (residues 572 to 875) has no enzymatic activity but is involved in stabilizing the
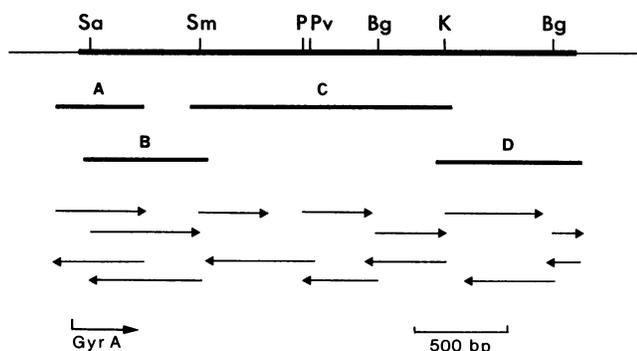

FIG. 1. Restriction map and DNA sequencing strategy for the *E. coli* DM750 *gyrA* gene. Bg, K, P, Pv, Sa, and Sm, sites for *Bgl*II, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, and *Sma*I, respectively. Segments of the *gyrA* gene (heavy line) were amplified by PCR. PCR products A through D, obtained by using oligonucleotide pairs 1-2, 3-4, 5-6, and 7-8 (Table 1), respectively, were cut with appropriate restriction enzymes (see text), cloned into M13mp18 and M13mp19, and sequenced by the chain termination method (15, 21). Each region indicated by arrows was sequenced at least twice on complementary strands from independent PCR products. The *Bgl*II-*Kpn*I fragment of product C was isolated from three independent PCR reactions and in each case was sequenced on both top and bottom strands.

gyrase complex: an overexpressed recombinant form of the fragment was found to bind and wrap DNA in an Mg^{2+}-independent process (18, 20). The N-terminal 64-kDa tryptic fragment (residues 7 to 571) when complemented with GyrB supported all the known reactions of gyrase, including DNA supercoiling (albeit less efficiently) (18, 19). A genetically engineered N-terminal GyrA fragment (residues 7 to 523) mediated quinolone-dependent DNA cleavage and passive ATP-independent DNA relaxation but not DNA supercoiling (19). It has been suggested that GyrA (residues 7 to 523) represents the functional DNA breakage-reunion domain and that residues 523 to 571 are somehow involved in coupling subunit interactions necessary for DNA supercoiling (19). The DM750 Ala-569→Thr mutation (and possibly the Thr-586→Ala change) lies in this same interdomain region (Fig. 2).

At present, we do not know how the DM750 change affects gyrase activity. The interchange of Thr for Ala at position 569 and the inverse substitution at 586 occur in a region (residues 568 to 588 inclusive) predicted to form an alpha-helical segment (not shown), but it is not clear how these changes alter the Mg^{2+} dependence of gyrase reconstituted with DM750 GyrA protein (6). Mg^{2+} ions are required for A subunit binding to DNA (20) and have also been located in the ATP-binding site of gyrase B protein (26). One or both mutations in DM750 GyrA could affect a direct interaction involving Mg^{2+} binding or, more likely, could operate indirectly by altering the coupling between A-A or A-B subunit domains, e.g., with the ATPase sites of GyrB. Resolution of these questions will require enzymatic studies with purified gyrase subunits.

The position of the DM750 mutation between functional GyrA domains is analogous to that of the DM800 compensatory mutation in GyrB (Fig. 2) (11). The Ala-Arg insertion in DM800 GyrB occurs after residue 382 adjacent to an in vivo proteolytic cleavage site (Arg-393) defining a 50-kDa C-terminal fragment (residues 394 to 804) that binds and activates GyrA (1, 5) (Fig. 2b) and a 43-kDa N-terminal fragment (residues 2 to 393) that binds ATP and may form part of the protein gate through which the translocated DNA strand passes during DNA supercoiling (26). Previously, we proposed that the DM800 mutation reduces GyrB activity by affecting domain spacing within the gyrase complex (11). Consistent with this view, the X-ray structure analysis of GyrB fragment (residues 2 to 393) shows that residues 366 to 392 form an extended alpha helix (26) which (if present in the intact protein) could serve to separate GyrB domains. The putative domain-spacing regions with their trypsin-sensitive sites share only weak sequence homology, although each has a dipeptide repeat (Fig. 2b). It is interesting that the location of compensatory mutations in gyrase proteins contrasts with the locations of those conferring resistance to gyrase inhibitors. Mutations determining resistance to 4-quinolones and to coumarin drugs map within domains of the gyrase subunits (Fig. 2a) (2, 3, 15, 28, 29).

Compensatory mutations are an adaptive response that allow bacteria to survive the loss of topo I by redressing the balance of DNA supercoiling and relaxing activities. For those mutations that act on gyrase, it is tempting to suggest that changes affecting the coupling of gyrase subunit domains offer a subtle way of reducing but not abolishing enzyme activity. However, not all compensatory mutations operate to reduce gyrase activity; some appear to increase intracellular DNA relaxing activities. For example, *toc* mutations act by amplifying the *parC* and *parE* genes, which encode topoisomerase IV, a type II enzyme related to gyrase
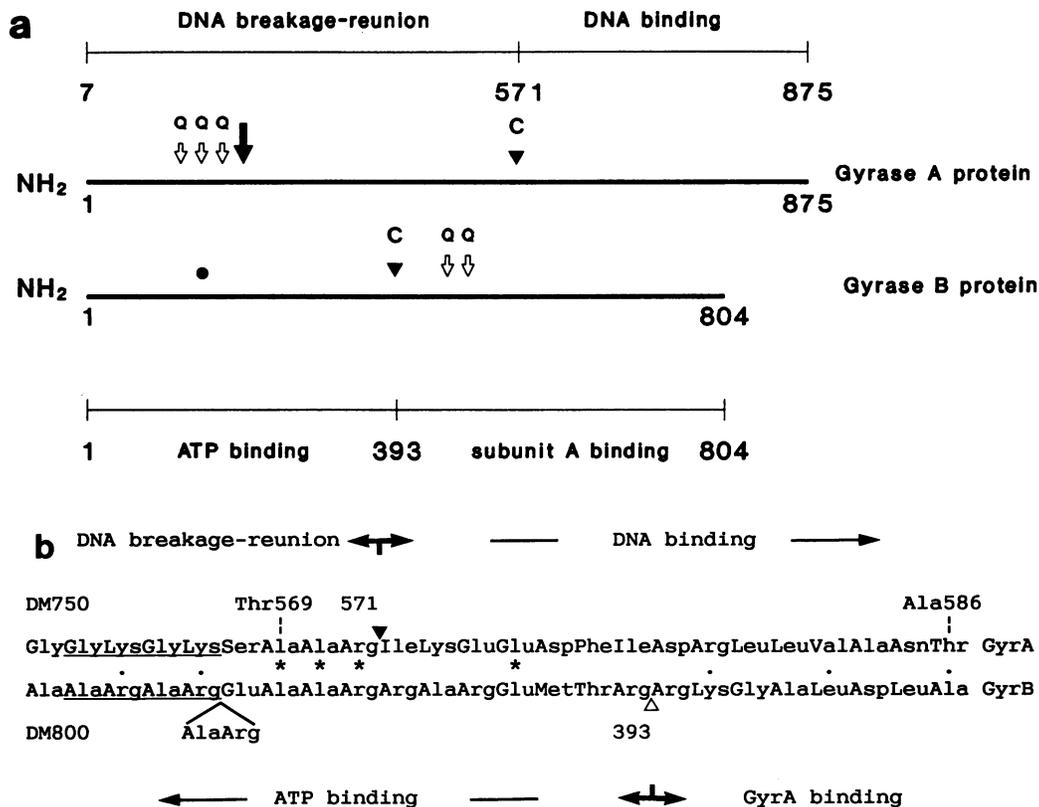
FIG. 2. Analysis of compensatory mutations in DM750 GyrA and DM800 GyrB proteins. (a). Schematic diagram showing the domain structure of *E. coli* gyrase A and B subunits. C denotes the locations of the DM750 mutation in GyrA and the DM800 mutation in GyrB (11). Solid arrow, catalytic Tyr-122 in GyrA (8). Solid circle and Q indicate positions of mutations conferring resistance to coumarins and to 4-quinolone gyrase inhibitors, respectively (2, 3, 15, 28, 29). Numbers at left and right indicate N-terminal and C-terminal residues of gyrase subunits: residues 7 and 571 are tryptic cleavage sites in the native GyrA protein (18); proteolysis of GyrB at residue 393 has been observed in vivo (1, 5). (b). Sequence of *E. coli* GyrA and GyrB proteins and locations of compensatory mutations. *, identity between protein residues; •, conserved residues. Tandem repeat sequences are underlined. Solid triangle, site of tryptic cleavage of GyrA producing 64-kDa DNA breakage-reunion and 33-kDa DNA-binding fragments (18, 20). Open triangle, site of presumed proteolytic cleavage of GyrB, generating a 50-kDa GyrA-binding fragment (1, 5). The 43-kDa N-terminal GyrB fragment binds ATP (23, 26). The sequences of wild-type *E. coli* K-12 GyrA and GyrB proteins have been reported previously (1, 22, 28).

but which catalyzes DNA relaxation (9). Clearly, analysis of compensatory mutations should provide valuable insight into the structure and functions of gyrase and the other bacterial topoisomerases.

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