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NOTES

Mutation at the "Exit Gate" of the *Salmonella* Gyrase A Subunit Suppresses a Defect in the Gyrase B Subunit

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In *Salmonella enterica* serovar Typhimurium, an S431P substitution in the B subunit of gyrase (allele *gyrB651*) confers resistance to nalidixic acid and causes reduced DNA superhelicity and hypersensitivity to novobiocin. Selection for novobiocin resistance allowed isolation of a mutation in the *gyrA* gene (allele *gyrA659*), a T467S substitution, which partially suppresses the supercoiling defect of *gyrB651*. Modeling analysis suggests that this mutation acts by destabilizing the GyrA bottom dimer interface. This is the first example of a *gyrA* mutation that compensates for a *gyrB* defect.

DNA gyrase is a member of the family of type II DNA topoisomerases, a group of enzymes that catalyze the interconversion of different topological forms of DNA. DNA gyrase introduces supercoils into DNA through the breakage of a DNA duplex, the passage of another DNA segment through the break, and the resealing of the break (8). This activity involves the opening and closing of a series of molecular gates, coupled to ATP hydrolysis. DNA gyrase is a heterotetramer of two A subunits (97 kDa) and two B subunits (90 kDa), encoded by the *gyrA* and *gyrB* genes, respectively (8). The 64-kDa N-terminal domain of GyrA is thought to intervene in the breakage-reunion reaction, whereas the 33-kDa C-terminal domain is involved in the stabilization of the gyrase-DNA complex. The GyrB protein contains a 43-kDa N-terminal ATP binding domain and a 47-kDa C-terminal domain that interacts with GyrA and DNA. Structural analysis indicates that the GyrA breakage-reunion domain resembles a clamp with two sets of jaws at opposite ends (18). Experimental studies, using yeast topoisomerase II or DNA gyrase, indicate that DNA is transported by a "two-gate" mechanism, entering the interior of the enzyme through an upper N gate at the GyrA head dimer interface and leaving the enzyme through an "exit gate" at the GyrA bottom dimer interface closer to the C termini (19, 20, 26).

DNA gyrase is the target of two classes of inhibitors, the quinolones, such as nalidixic acid (Nal), which trap DNA gyrase covalently bound to its cleaved substrate, and the coumarins, such as novobiocin (Nov), which inhibit the ATPase activity (15). Mutations conferring Nal resistance are for the most part located in a region of the *gyrA* gene specifying the N-terminal domain portion between amino acids 51 and 106, the so-called quinolone resistance-determining region (22). Resistance to quinolones (albeit to a lower level) can also result from mutations in the *gyrB* gene, and the affected sites identified are amino acids D426, K447, and S464 in the GyrB C-terminal domain (12, 27). In addition to their effect on quinolone sensitivity, mutations at sites D426 and K447 also inhibit the gyrase catalytic activity (13). It has been proposed that the quinolone-binding site is a pocket surrounded by surfaces involving the quinolone resistance-determining regions of both the GyrA and GyrB proteins (13, 27).

Isolation of the *gyrB651* allele and its phenotypic suppressor, *gyrA659*. Mutants of *Salmonella enterica* serovar Typhimurium resistant to low levels of nalidixic acid were isolated by plating 0.2 ml of an overnight liquid culture from strain MA251 (Table 1) on nutrient broth (Difco) agar containing 3 μg/ml Nal. Strain MA251 harbors a *his-lac* transcriptional fusion whose expression level negatively correlates with gyrase activity (3, 11). This effect can be easily monitored on *lac* indicator plates containing subinhibitory concentrations of novobiocin, such as MacConkey-lactose plates (Difco) containing 70 μg/ml Nov, on which strain MA251 forms deep red colonies (11). To identify new gyrase alleles affecting DNA supercoiling, mutants selected for growth on 3 μg/ml Nal were examined for their colony color on MacConkey plates containing 70 μg/ml Nov. This led to the identification of a clone (strain ABP280) incapable of growing on such plates. Transductional crosses showed the Nal^r mutation to be 50% linked to the zid-*748*::Tn*10* insertion previously mapped near *gyrB* (formerly zib-*748*::Tn*10* [11]). This suggested the mutation to be a *gyrB* allele mutation, which was named *gyrB651*. The *gyrB651* allele was moved into a wild-type background, selecting for tetracycline resistance and identifying Nal^r colonies among the Tc^r transductants. All the Nal^r colonies tested displayed hypersensitivity to novobiocin.

The novobiocin-hypersensitive phenotype of *gyrB651* was

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TABLE 1. Strains used in this study

Strain[a]	Genotype	Source or reference[b]
KR2264	zej-*754*::Tn*10*	Ken Rudd
LB417	zid-*748*::Tn*10*	Lionello Bossi
MA251	*his*C*9968*::MudJ	11
ABP280	*his*C*9968*::MudJ *gyrB651*	This study
ABP290	*his*C*9968*::MudJ *gyrB651 gyrA659*	This study
ABP348	*his*C*9968*::MudJ *gyrB651* zej-*754*::Tn*10*	This study
ABP350	*his*C*9968*::MudJ *gyrA659* zid-*748*::Tn*10*	This study

[a] Strains are derived from *S. enterica* serovar Typhimurium strain LT2. Strains ABP348 and ABP350 were derived from strain ABP290 by transduction using a phase P22 lysate made on strain KR2264 or LB417, respectively.
[b] The genotype of strain MA251 is identical to that of strain MA463 in reference 11.

used to select for suppressor mutations. Novobiocin-resistant mutants were isolated by plating 0.2 ml of an overnight liquid culture from strain ABP280 on MacConkey agar containing 70 μg/ml Nov. One resistant colony, strain ABP290, was chosen for further analysis. Transductional crosses showed a 50% linkage between the Nov^r mutation and zej-*754*::Tn*10* (formerly zeh-*754*::Tn*10*), a Tc^r insertion previously mapped near *gyrA* (21). This result suggested that ABP290 harbored a compensatory *gyrA* mutation, which was named *gyrA659*.

Measurement of the MICs of nalidixic acid and ciprofloxacin indicated that the *gyrB651* mutant increases the levels of both acidic and amphoteric quinolones (Table 2), as reported for the D426N substitution in GyrB (27). Intriguingly, the increase is even higher when the *gyrB651* allele is combined with *gyrA659*. In contrast, the latter does not appear to affect the sensitivity to these drugs significantly (Table 2). Finally, *gyrB651* causes a slight increase in Nov sensitivity, and *gyrA659* suppresses this effect (Table 2). Exacerbation of these phenotypes in MacConkey medium (data not shown) accounts for our ability to isolate *gyrA659* as a lethality suppressor on 70 μg/ml Nov.

The *gyrA659* allele partially corrects the supercoiling defect of *gyrB651*. To assess the effects of *gyrA* and *gyrB* mutations on negative DNA supercoiling, relevant strains were transformed with a reporter plasmid and the supercoiling levels of plasmid DNA extracted from growing cells were compared by analyzing the distributions of DNA topoisomers in agarose gels containing chloroquine as the intercalating agent. Plasmid pKK232-8

TABLE 2. MICs of nalidixic acid, ciproflaxin, and novobiocin for the *gyrB651* mutant and the compensatory *gyrA659* allele

Strain	Relevant genotype	MIC (μg/ml)[a]		
		Nalidixic acid	Ciprofloxacin	Novobiocin
MA251	Wild type	3	0.016	100
ABP348	*gyrB651*	12	0.047	75
ABP290	*gyrB651 gyrA659*	32	0.094	100
ABP350	*gyrA659*	3	0.016	75

[a] The MICs of nalidixic acid and novobiocin were determined on LB solid media containing 1, 2, 3, 4, 8, 12, 16, 20, or 32 μg/ml Nal or 50, 60, 75, 100, 150, or 200 μg/ml Nov by spotting 10^4 CFU. The MIC of each antibiotic is defined as the concentration where no growth was visible after overnight incubation. The MIC of ciprofloxacin was determined by the E-test strip method according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

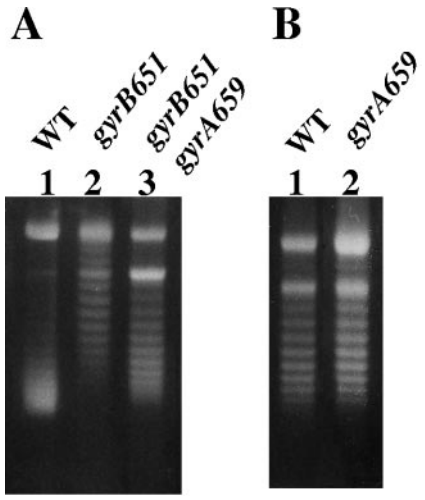

FIG. 1. Effect of gyrase mutations on plasmid DNA supercoiling. Cells harboring plasmid pKK232-8 were processed as previously described (25). The topoisomer distributions of pKK232-8 DNA isolated from different strains were analyzed in a 1.2% agarose gel containing chloroquine at 10 μg/ml (A) or 15 μg/ml (B). At these chloroquine concentrations, topoisomers with a lower linking number migrate faster in the gels. A. Lane 1, MA251 (wild type); lane 2, ABP348 (*gyrB651*); lane 3, ABP290 (*gyrB651 gyrA659*). B. Lane 1, MA251 (wild type); lane 2, ABP350 (*gyrA659*).

(4) was chosen for this study because its low overall transcriptional activity has no detectable influence on DNA supercoiling (10). Results in Fig. 1A show that *gyrB651* causes a dramatic reduction in the linking deficit ($\Delta Lk > 10$). Mutation *gyrA659* corrects this defect to a significant extent, although not completely. On the other hand, *gyrA659* by itself does not increase the supercoiling level but rather causes a slight negative supercoiling deficiency that is completely epistatic to that of *gyrB651* (Fig. 1B). As an independent verification of these

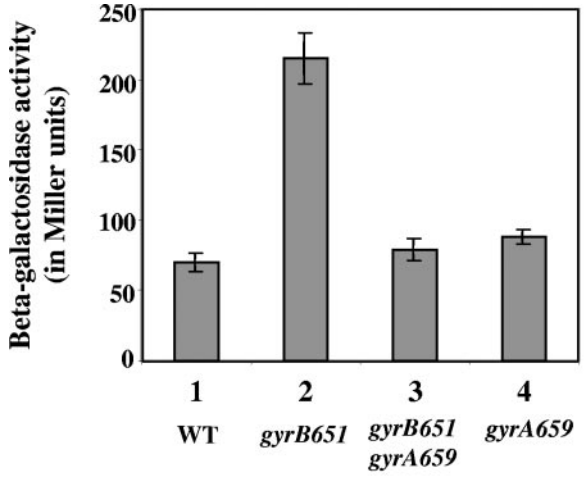

FIG. 2. Effect of gyrase mutations on expression of a *his-lac* operon fusion. Beta-galactosidase assays were carried out in different strains in LB medium at 37°C as described by Miller (17). Activities are averages of three independent experiments. Lane 1, MA251 (wild type); lane 2, ABP348 (*gyrB651*); lane 3, ABP290 (*gyrB651 gyrA659*); lane 4, ABP350 (*gyrA659*).

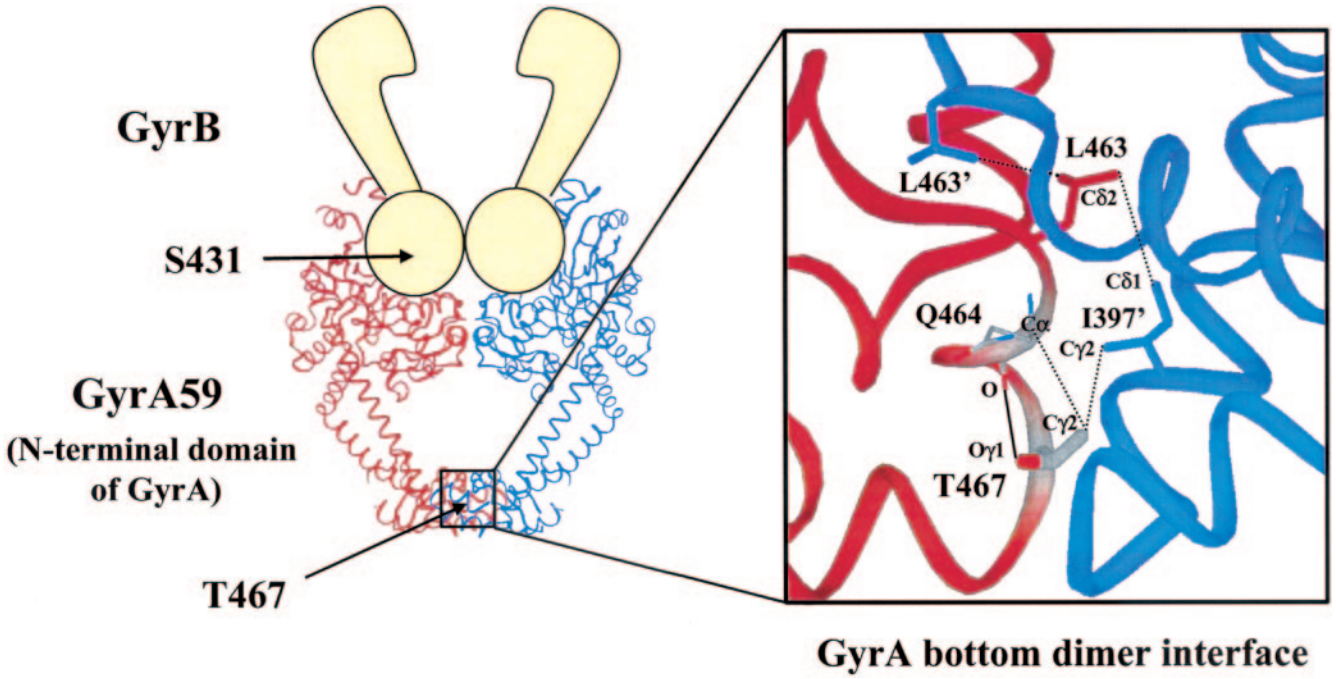

FIG. 3. Location of the T467 residue in the GyrA bottom dimer interface. The approximate relative positions of the GyrA T467 and GyrB S431 residues are shown in the left part of the figure. The two GyrB subunits are schematically represented in yellow, and the C-terminal domain is drawn as a circle. The structure of the N-terminal GyrA dimer (59-kDa fragment; PDB 1AB4 [18]) was drawn with the program ViTO (6). The environment of residue T467 at the GyrA bottom dimer interface is shown in detail in the enlarged box. The Cα trace of each GyrA subunit is drawn as a red or blue ribbon. Side chains of relevant residues (see text) are shown in thick wire frames. The Q464 residue is entirely represented in the thin wire frame using the atom-code color. Van der Waals interactions and hydrogen bonds are shown as dotted and plain lines, respectively. All relevant residues mentioned in the paper are conserved and located at the same position in *E. coli* and *S. enterica* serovar Typhimurium gyrase subunits. The *Salmonella* GyrA59 protein exhibits 96.5% identity and 99.9% similarity with the *E. coli* protein (16).

data, we examined the effects of *gyr* mutations on *his* operon expression, which is inversely correlated with the degree of negative supercoiling of chromosomal DNA (11). Results in Fig. 2 show that levels of β-galactosidase activity originating from a *his-lac* operon fusion increase substantially in the presence of *gyrB651* and that, again, *gyrA659* suppresses these effects. In this test, the *gyrA659* mutant does not differ significantly from wild type. In conclusion, these data show that allele *gyrB651* affects the ability of DNA gyrase to negatively supercoil DNA and that *gyrA659* partially compensates for this defect without improving by itself the activity of wild-type gyrase.

Sequence changes resulting from *gyrB651* and *gyrA659* mutations. The *S. enterica* serovar Typhimurium *gyrA* and *gyrB* genes are 2,637 bp and 2,415 bp long, respectively (16). Overlapping portions of each of the two genes (500 to 700 bp in length) were amplified by colony PCR from strain ABP290 (*gyrB651 gyrA659*). Amplified fragments were purified and subjected to sequence analysis. A single base-pair change was found in each of the two genes. The *gyrB* gene showed a T-to-C transition at the first base of codon 431, causing an amino acid change from serine to proline. Thus, *gyrB651* represents a novel Nal^r allele within the quinolone resistance-determining region of the B subunit. The mutation is also part of the B subunit 47-kDa domain, proposed to contact both the A subunit and the DNA (14).

The *gyrA* gene had a C-to-G mutation at the second base of codon 467, resulting in an amino acid change from threonine to serine. This mutation is located at the C-terminal end of the DNA breakage-reunion domain of GyrA. The T467 residue is highly conserved among known type II topoisomerases, including yeast and human topoisomerase II enzymes (5). To date, only one spontaneous mutation has been described in this region of DNA gyrase: an R462C substitution which confers resistance to the bacterial toxin CcdB (2).

How *gyrB651* and *gyrA659* mutations might affect gyrase structure and function. The approximate relative positions of the GyrA T467 and GyrB S431 residues are shown in Fig. 3. The structure of the C-terminal subdomain of GyrB protein, encompassing the S431 residue, has not been solved yet but it can be modeled based on the structure of the B′ subdomain of the yeast topoisomerase II (1). The percent identity between both domains is approximately 20%, but it reaches 34% in a region of 120 amino acids that includes the quinolone resistance-determining region (amino acids 420 to 540). A theoretical three-dimensional model of the *Salmonella* C-terminal subdomain of GyrB was built using the server @TOME (7) and the program MODELLER (23). Evaluation using PROSA (24) and Verify 3D (9) tentatively confirmed the validity of the model (data not shown). The S431 residue is part of an alpha-helix that would be destabilized by the substitution with proline. Such a local conformational change could alter the quinolone-binding pocket (thus increasing resistance to quinolones) and affect the overall structure in a deleterious way for supercoiling activity. The hypothesis of a structural change is in

agreement with the fact that the mutation increases resistance to both acidic and amphoteric quinolones.

The monomer of the 59-kDa N-terminal subdomain of GyrA from *Escherichia coli*, encompassing the T467 residue, is formed by an N-proximal head and a C-terminal tail (18). The crystal structure reveals that the GyrA59 dimer exhibits two regions of interface, the head dimer interface and the bottom dimer interface located at the tails. The role of the bottom interface (the "exit gate") in DNA strand passage has been shown using cysteine cross-linking (26). In the model of the corresponding region of the *Salmonella* enzyme, the residues T467 and L463 appear to be among the most buried residues at the primary dimer interface. The T467 residue is involved in an intricate interaction network (Fig. 3). T467 of one GyrA molecule interacts with I397′ of the second GyrA molecule (distance Cγ2 to Cγ2, 3.6 Å) through van der Waals contacts. In addition, the threonine side chain interacts with the backbone atoms of the residue Q464 by both hydrogen bond (distance Oγ1 to O, 2.8 Å) and van der Waals contact (distance Cγ2 to Cα, 4.5 Å). Furthermore, the hydrophobic side chain of the L463 from one monomer is sandwiched between the L463′ and the I397′ from the second monomer. The T467S mutation, resulting in the loss of the carbon Cγ2, would create an empty space as well as an unfavorable conformational mobility of the serine side chain. This would in turn destabilize the small helical segment bearing the L463 residue. As a whole, the subtle threonine-to-serine amino acid change could significantly alter the interactions between the two GyrA subunits by destabilizing the primary interface. The increased quinolone resistance of the *gyrA gyrB* double mutant compared to the *gyrB* mutant might be due to an effect on the reaction cycle of the enzyme, such as a change in the amount of gyrase-DNA complex that could change the frequency of a quinolone-sensitive state.

Conclusions. The data presented here constitute the first example in which a sequence change in *gyrA* compensates for a *gyrB* mutation. Since the mutations affect regions of the two proteins not thought to be in direct contact in the enzyme quaternary structure, it seems likely that the suppression mechanism involves independent compensatory effects. In particular, we propose that by destabilizing the bottom dimer interface, the T467S substitution in *gyrA659* might facilitate the passage of the DNA strand through the "exit gate," thus alleviating the possible energy transduction deficit resulting from the *gyrB651* allele.

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