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Mutations Causing Aminotriazole Resistance and Temperature Sensitivity Reside in *gyrB*, Which Encodes the B Subunit of DNA Gyrase

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Certain mutations in *gyrA* and *gyrB*, the genes encoding the two subunits of DNA gyrase, are known to influence expression of the *his* operon (K. E. Rudd and R. Menzel, Proc. Natl. Acad. Sci. USA 84:517–521, 1987). Such mutations lead to a decrease in $tRNA^{His}$ levels and consequently to an attenuator-dependent increase in *his* operon expression. This effect presumably is due to the dependence of the *hisR* promoter (*hisR* encodes $tRNA^{His}$) on supercoiling for maximal activity. We used a relaxed (Rel^-) strain of *Escherichia coli* to isolate *gyrB* mutants by selecting for resistance to the histidine antimetabolite 3-amino-1,2,4-triazole and then screening for temperature-sensitive growth on rich medium. Rel^- mutants, which generally have lower basal levels of ppGpp (a positive regulator of *his* operon transcription), are more sensitive than wild-type *E. coli* to aminotriazole. The chance of isolating *spoT* mutants, which can be selected with a similar procedure, was decreased by selecting in the presence of a multicopy plasmid that carries the wild-type *spoT* gene. Under these conditions, *gyrB* mutants were isolated preferentially. This scheme selects for loss of function of DNA gyrase, rather than for its alteration due to resistance to specific gyrase inhibitors, and thus a greater variety of gyrase mutations might be obtainable.

DNA gyrase is a type II topoisomerase that catalyzes a number of topological changes in DNA, including supercoiling, relaxation, knotting and unknotting, and catenation and decatenation. The *Escherichia coli* enzyme is an A_2B_2 tetramer; the A subunit is encoded by the *gyrA* gene, and the B subunit is encoded by the *gyrB* gene (8). Because of their pleiotropic nature, mutations in the *gyr* genes can influence the expression of a wide range of operons (5, 7). In particular, the *his* regulatory mutation *hisW* and a subset of *hisU* alleles lie in the genes encoding the subunits of DNA gyrase in *Salmonella typhimurium* (16). These *his* mutations were originally identified as a minority class that conferred resistance to the toxic histidine analog 1,2,4-triazole-3-alanine (3). Many of the *his* regulatory mutations, including *hisR*, *hisT*, *hisS*, *hisU*, and *hisW*, give rise to defects in various aspects of tRNA-His biosynthesis (23). They lead to a decrease in functional $tRNA^{His}$ (encoded by *hisR*), which allows read-through of the *his* attenuator, increased synthesis of histidine biosynthetic enzymes and consequent resistance to toxic histidine analogs (3). Presumably, the positive effect of *gyr* mutations on *his* expression is due to the dependence of the *hisR* promoter on supercoiling for maximal activity (6a, 16). Here we present a selection scheme that is based on acquired resistance to the histidine antimetabolite 3-amino-1,2,4-triazole (AT) and that preferentially yields mutations in the genes encoding DNA gyrase.

Strategy for the isolation of *gyr* mutants. The *his* operon is regulated by a transcription-attenuation mechanism that is sensitive to the level of charged tRNA-His in the cell and by a metabolic control mechanism that responds positively to increases in the intracellular concentration of ppGpp (2, 23). Mutations that lead to derepression of the *his* operon would be expected to show an AT-resistant phenotype (15). Those that reduce the basal level of ppGpp also decrease *his* operon expression and lower cellular resistance to the histidine analogs AT and 1,2,4-triazole-3-alanine (15, 21). Consequently, a strain carrying a mutation in the *relA* gene or in any other gene encoding a function that blocks the *relA*-dependent pathway for ppGpp synthesis (such as *relC*, used here) is more sensitive than an isogenic wild-type strain to AT. This sensitivity is exacerbated when the medium is supplemented with all amino acids except histidine (AT agar [15, 22]). Amino acid supplementation is thought to lower the basal level of ppGpp, thereby decreasing *his* expression such that cells are more sensitive to the toxic effects of the analog. Strains with mutations in the *spoT* gene that elevate *his* operon expression and thus confer resistance to AT were previously isolated (the *spoT* gene encodes a ppGpp-degrading activity [15]).

We isolated mutants that are resistant to AT and that are also temperature sensitive (Ts) for growth at 42°C. AT-resistant cells were selected by mutagenizing the *relC* strain *E. coli* JF368 (*relC metF recA ilv rpsL* [14]) that had been transformed with pGA1 (a multicopy plasmid carrying the *spoT* gene [1]). Mutagenized cells (50 μg/ml of nitrosoguanidine [13]) were spread directly onto M9-minimal agar medium supplemented with 19 amino acids (minus histidine), tetracycline, and 20 mM AT (AT agar). The plasmid pGA1 was present to reduce the probability of isolating *spoT* mutations (15). Colonies capable of growth on AT agar at 30°C ($1/10^6$) were patched on AT agar and LB agar at 30 and 42°C. Colonies that displayed a Ts phenotype on both media were subjected to coreversion analysis to determine whether the two characteristic phenotypes, temperature sensitivity and AT resistance, were due to a single mutation. Both

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TABLE 1. Bacterial strains and their characteristics

Strain	Growth[a] in:					Coreversion frequency	Suppressor plasmid(s)
	Minimal medium[b]	AT-agar		LB			
		30°C	42°C	30°C	42°C		
JF368 (*relC*)	++	−	−	++	++		
JF4737 [JF368(pGA1)] (*spoT*)	++	−	−	++	++		
Group I							
JF4776	++	++	−	++	−	2/10	Not determined
JF4777	++	++	−	++	−	6/11	pJF4777-2
JF4779	++	++	−	++	−	10/30	pJF4779-2
JF4780	++	++	−	++	−	4/25	pJF4780-1
Group II[c]							
JF6015	++	++	−	++	−	4/16	pMT-1, pMT-2
JF6020	++	++	−	++	−	6/25	pMT-6, pMT-7, pMT-8

[a] Positive notation indicates good patch growth at the designated temperature; colonies were patched from a master culture grown on LB.
[b] Minimal medium is described in text and in references 15 and 22.
[c] Described in reference 22.

phenotypes in six of the strains coreverted at a rate that is consistent with the existence of a single mutation (Table 1).

Characterization of genes that complement the mutant phenotypes. To find plasmids that could complement the Ts phenotypes, the six mutant strains were first cured of the *spoT*-carrying plasmid, pGA1, by selecting for tetracycline sensitivity (12). The cured cells retained both the Ts and AT-resistant phenotypes. Plasmid clones that complemented the Ts phenotype were subsequently isolated by transforming the six mutants with a pBR322-based *E. coli* library (provided by F. Wittinghofer [4]). Complementing clones for six of the mutations were isolated; some of these clones complemented more than one mutation. Based on this criterion, the six mutants were placed in two complementation groups, four in group I and two in group II. Plasmids that complemented mutants in group I were investigated further (Table 1). Group I strains that were transformed with these plasmids became temperature resistant and regained a level of AT sensitivity that was characteristic of the parental strain. Furthermore, all of these plasmids had common restriction fragments (Fig. 1). Maxicell analysis (17) of proteins encoded by three of the plasmids that can complement group I mutations showed that each plasmid encodes a protein of approximately 85 kDa (unpublished data).

Chromosomal location of genes that complement the mutant phenotypes. The genes contained on the plasmids of group I were mapped to the *E. coli* chromosome. The chromosomal location was approximated by hybridizing a ^{32}P-labelled DNA sequence, internal to the plasmid inserts (Fig. 1), to chromosomal DNA that was digested with restriction endonuclease *Not*I, separated by a pulsed-field gel electrophoresis, and blotted onto a nylon membrane (20). *E. coli* DNA is separated into 22 discernable restriction fragments by the *Not*I restriction enzyme (20). DNA blot hybridization with probes from three complementing plasmids all showed hybridization to the 203-kb L fragment (nomenclature of Smith et al. [20]; data not shown). This fragment was mapped previously and was shown to encompass a region between 80 and 85 min on the *E. coli* map (20). A more precise map location of these inserts was obtained by comparing restriction maps of the complementing plasmids with the high-resolution restriction map of the *E. coli* chromosome in the 203-kb region indicated by DNA blot analysis (9). A region of strong similarity was found near the *rnpA* gene at 82 min. This region contains genes for

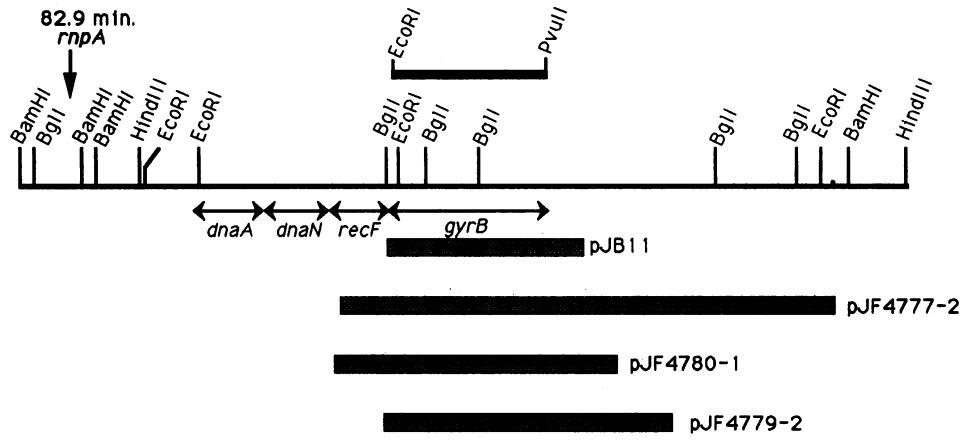

FIG. 1. Restriction map of the *E. coli* chromosome in the region of the *gyrB* gene; data were taken from Kohara et al. (9) and Yamagishi et al. (24). The 2.4-kb *Eco*RI-*Pvu*II fragment used to probe an orthogonal-field-alternation gel electrophoresis DNA blot is indicated. The region cloned into each of the complementing plasmids (Table 1) is shown, as are the locations of some of the known genes in the *gyrB* region.

rnpA, *dnaA*, *dnaN*, *recF*, and *gyrB*, which are all located in close proximity to each other (24). Closer examination of the published restriction maps for these genes revealed that all three plasmids that confer temperature resistance to group I mutations contain the *gyrB* gene (Fig. 1). In addition, plasmid pJB11 (provided by S. Nakamura [24]; Fig. 1), containing only the *gyrB* gene under its own promoter, conferred a temperature-resistant, AT-sensitive phenotype upon all mutant strains in complementation group I. Maxicell analysis of cells containing pJB11 showed a major protein band of the same size as the 85-kDa protein encoded by the group I complementing plasmid clones (data not shown).

Ts mutations in complementation group I map to the *gyrB* locus. We determined the genetic linkage of the Ts mutations to a transposable element (*zid-3162*::Tn*10kan*), known to map near *gyrB* (19), that confers kanamycin resistance. Since all of the original strains were Rec$^-$, all genetic mapping was done in the presence of pDR1453, a pBR322-based plasmid that carries a wild-type copy of the *recA* gene (18). Transformation of the mutant strains with this plasmid caused them to become UV resistant, as expected, but did not affect the AT resistance or Ts phenotypes. Bacteriophage P1 grown on (MG1655 *zid-3162*::Tn*10kan*) was used to select kanamycin-resistant transductants of the four mutant strains in group I. In each case, Kan^r transductants inherited a temperature-resistant phenotype at a frequency of between 80 and 90% (data not shown), consistent with the Ts mutations residing in the *gyrB* gene. Genetic mapping of the Ts mutations in the two strains in complementation group II demonstrated that these strains carried a mutation in the *rpsB* locus (22).

Previous *gyr* mutants have been selected on the basis of resistance to various inhibitors of DNA gyrase (7). Antibiotics such as nalidixic acid and oxolinic acid affect the *gyrA* subunit, whereas novobiocin and coumermycin A_1 inhibit the function of the *gyrB* subunit (5). Here we show that *gyrB* mutants can be isolated at a high frequency by selecting for mutations in a Rel$^-$ host that confer both resistance to AT and Ts. A Ts phenotype ensures that mutations that affect only histidine biosynthesis, with the possible exception of *hisS* (encoding histidyl tRNA synthetase), are screened out. Our mutants were also selected in the presence of a multicopy plasmid carrying the *spoT* gene, which substantially decreases the chances of isolating *spoT* mutants. Because this scheme selects for a loss of function, rather than for resistance to gyrase inhibitors, it widens the spectrum of *gyr* mutations that can be obtained by selecting for resistance to gyrase inhibitors alone. Although only *gyrB* mutations were found in our selection, we expect that a further search would reveal *gyrA* mutations as well.

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