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 tRNAHis 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 tRNAHis) 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 unknotted, and catenation and decatenation. The Escherichia coli enzyme is an A2B2 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 tRNAHis (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 antime- tabolite 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 nitrosogua- dinine [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 (1/106) 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
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 32P-labelled DNA sequence, internal to the plasmid inserts (Fig. 1), to chromosomal DNA that was digested with restriction endonuclease NotI, 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 NotI 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

![Restriction map of the E. coli chromosome in the region of the gyrB gene](chart)

**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 EcoRI-PvuII 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.

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

* Positive notation indicates good patch growth at the designated temperature; colonies were patched from a master culture grown on LB.

* Minimal medium is described in text and in references 15 and 22.

* Described in reference 22.
mpA4, dnaA4, 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).

Tus mutations in complementation group I map to the gyrB locus. We determined the genetic linkage of the Tus mutations to a transposable element (zip-3162::Tn10kan), 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 Tus 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::Tn10kan) was used to select kanamycin-resistant transductants of the four mutant strains in group I. In each case, KanR 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 rpsL 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 A1 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 multi-copy 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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REFERENCES