FOR THE RECORD

Temperature-sensitive suppressor mutations of the *Escherichia coli* DNA gyrase B protein

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Abstract: *Escherichia coli* strain LE316 contains a mutation in *gyrB* that results in the substitution of Val164 to Gly and confers both chlorobiocin resistance and temperature sensitivity. Selection for suppressors of the ts phenotype yielded second-site mutations in GyrB at His38 and Thr157. The properties of proteins bearing these mutations have been characterized, and a mechanism of suppression is proposed based upon structural considerations.

Keywords: coumarin; gyrase; novobiocin; suppressor; topo-isomerase

DNA gyrase is the bacterial enzyme that catalyzes DNA supercoiling (Reece & Maxwell, 1991; Wigley, 1995). It consists of two subunits, GyrA and GyrB, which form an A_2B_2 complex. The principal reaction catalyzed by gyrase is the ATP-dependent supercoiling of DNA. This involves the breakage of DNA in both strands and the passage of another segment of DNA through the break.
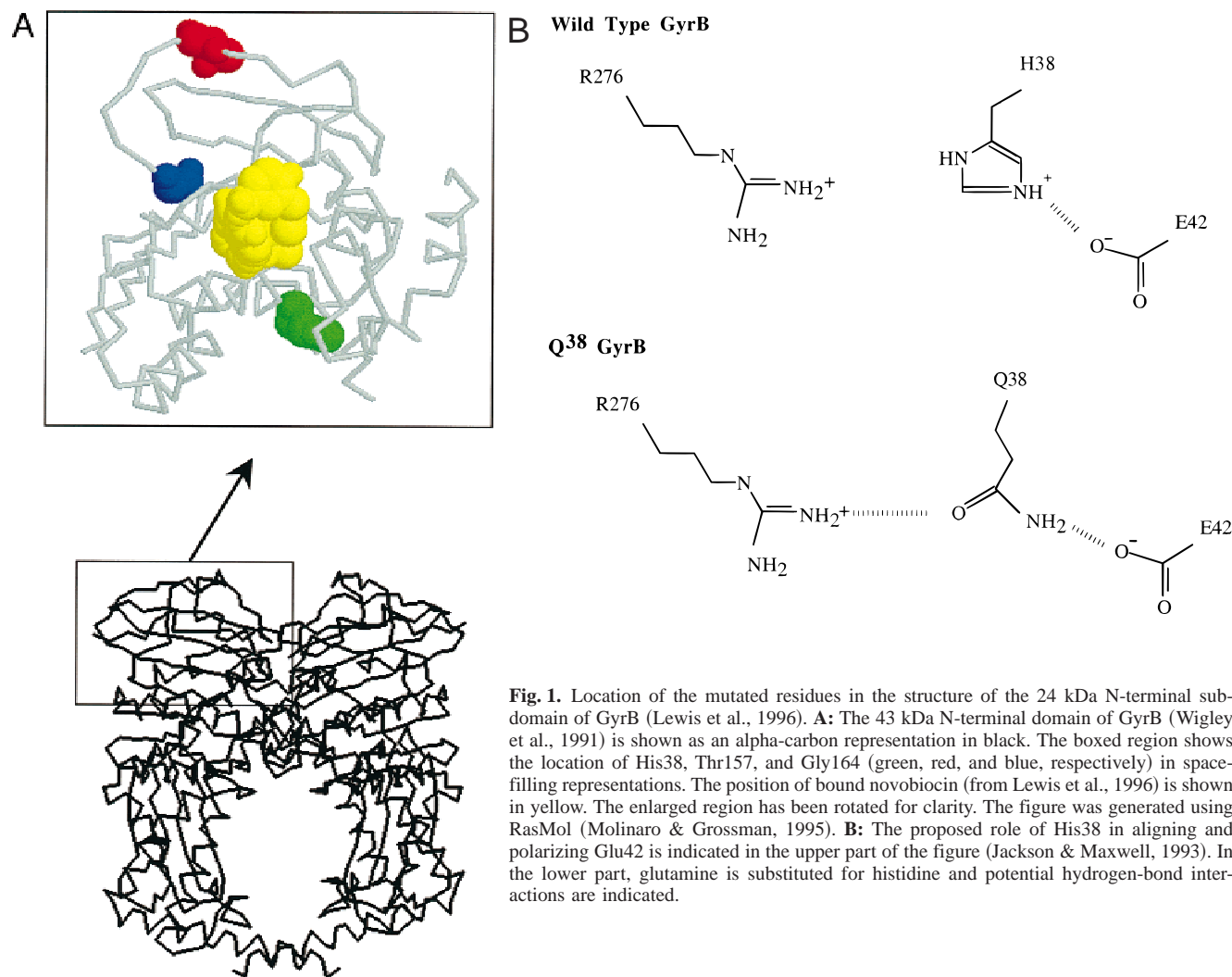
DNA gyrase is the target of a number of antibacterial agents including quinolones and coumarins (Maxwell, 1997). Quinolones inhibit the enzyme by stabilizing a cleavage complex between gyrase and DNA that is thought to arrest replication forks in vivo. Coumarin drugs are antibiotics isolated from *Streptomyces* species and include coumermycin A_1, chlorobiocin, and novobiocin. Coumarins have been shown to bind to GyrB and competitively inhibit ATP hydrolysis (Gormley et al., 1996; Lewis et al., 1996).

Selection for spontaneous coumermycin-resistance mutants in *Escherichia coli* frequently yields mutations that map to *gyrB*, resulting in the mutation of the amino acid Arg136 (del Castillo et al., 1991; Contreras & Maxwell, 1992). The only exception is a chlorobiocin-resistance mutation Gly164 to Val that confers low-level coumermycin resistance and temperature sensitivity (Orr et al., 1979; Contreras & Maxwell, 1992). Recent site-directed mutagen-esis work based on crystal structures of the N-terminal domain of GyrB complexed with novobiocin (Lewis et al., 1996) and chlorobiocin (Tsai et al., 1997) has shown that other mutations of GyrB (e.g., at Asn46 and Asp73) can result in coumarin-resistant proteins (Kampranis et al., 1999). However, the mutations render the enzyme catalytically inactive, hence strains bearing such mutations would be inviable. This is a consequence of the overlap of the ATP- and coumarin-binding sites in GyrB (Lewis et al., 1996) and suggests that Arg136 and Gly164 are the only amino acids in this region that can be mutated to confer coumarin resistance without a seriously deleterious effect on the protein. In the case of Arg136 mutations, changes to Cys, His, and Ser led to decreases in the in vitro supercoiling activity to 6–13% of wild-type activity (Contreras & Maxwell, 1992); such a reduction in supercoiling activity can presumably be accommodated in vivo. In the case of Gly164, attempts to produce the mutant GyrB protein at the permissive temperature (30 °C) failed to yield soluble protein (Contreras & Maxwell, 1992), suggesting that even at this temperature, the mutant GyrB is an unstable protein. This is reflected by the low growth rate of the strain carrying this mutation (LE316 (Contreras & Maxwell, 1992)).

In the crystal structure of the novobiocin-GyrB complex, Gly164 does not make a contact with the bound drug, although the adjacent residue, Thr165, makes a water-mediated hydrogen bond to the carbonyl oxygen of the carbamoyl group at the 3′ position of the novobiose sugar. In the chlorobiocin structure, this contact is more important as the 3′ carbamoyl group is replaced by a 3′-(5-methyl-2-pyrroylcarbonyl) group with which Thr165 makes a direct contact (Tsai et al., 1997). It seems likely that the effect of the Gly164 to Val mutation is indirect, due to the alteration of the conformation of adjacent amino acids, resulting in a less stable complex with the coumarin drug. It is likely that this altered conformation also results in the temperature-sensitive (ts) phenotype. In other words, the effect of the Gly164 to Val mutation is twofold. First, the valine would increase the conformational constraint on this turn (Fig. 1), which lies adjacent to the coumarin-binding site and would be commensurate with a less stable interaction with the coumarin molecule. Second, the ts phenotype may result from the increased thermodynamic constraint leading to stabilization of a less-favored conformation.

Fig. 1. Location of the mutated residues in the structure of the 24 kDa N-terminal subdomain of GyrB (Lewis et al., 1996). **A:** The 43 kDa N-terminal domain of GyrB (Wigley et al., 1991) is shown as an alpha-carbon representation in black. The boxed region shows the location of His38, Thr157, and Gly164 (green, red, and blue, respectively) in space-filling representations. The position of bound novobiocin (from Lewis et al., 1996) is shown in yellow. The enlarged region has been rotated for clarity. The figure was generated using RasMol (Molinaro & Grossman, 1995). **B:** The proposed role of His38 in aligning and polarizing Glu42 is indicated in the upper part of the figure (Jackson & Maxwell, 1993). In the lower part, glutamine is substituted for histidine and potential hydrogen-bond interactions are indicated.

Results and discussion: The fact that the Gly164 to Val mutation confers both ts and chlorobiocin-resistance phenotypes presents the opportunity to select for mutations that increase the stability of GyrB. Overnight cultures of *E. coli* strains LE316 and LE234 (the isogenic wild-type strain (Orr et al., 1979)) were grown in L-Broth and aliquots (3×10^7–5×10^8 cells) were plated onto L-Broth agar plates containing 0, 50, and 80 μg/mL chlorobiocin at 30 and 42 °C. LE234 grew at 30 and 42 °C in the absence of drug but showed little or no growth in the presence of drug. LE316 grew at 30 °C under all conditions but at 42 °C showed only a few (12 in total) isolated colonies. Replating of these colonies showed that they were indeed viable at 42 °C. Determination of the minimum inhibitory concentration (MIC) for chlorobiocin using the slope plate method showed that 9 out of 12 had MICs similar to that of LE316 ($\sim$175 μg/mL under these conditions). Five of these strains were subjected to colony polymerase chain reaction to generate DNA fragments that encompassed the *gyrB* gene. Subsequent DNA sequencing showed that all five strains contained the Gly164 to Val mutation (GGC to GTC). Two out of the five contained the additional mutation His38 to Gln (CAC to CAA), and the other three contained the additional mutation Thr157 to Ile (ACC to ATC). No other mutations were found throughout the entire length of the

gyrB gene. Therefore, we conclude that the mutations His38 to Gln and Thr157 to Ile are second-site suppressors of the ts phenotype of the Gly164 to Val mutation, and that they retain the chlorobiocin-resistance phenotype.

Using site-directed mutagenesis (QuikChange, Stratagene, La Jolla, California), the mutations encoding the changes Gly164 to Val, His38 to Gln, and Thr157 to Ile were introduced into plasmid pAG111, which overexpresses the GyrB protein (Hallett et al., 1990). In addition, plasmids making GyrB containing the double mutations (Gly164 to Val plus His38 to Gln, and Gly164 to Val, plus Thr157 to Ile) and the triple mutation were also constructed. The *gyrB* sequence in each plasmid was verified and the mutant GyrB proteins were overexpressed and, where possible, purified (Maxwell & Howells, 1999). Table 1 shows the properties of the mutant proteins. Consistent with previous results, the strain expressing GyrB containing the Gly164 to Val mutation produced very little soluble protein and large amounts of GyrB protein was found in the insoluble pellet, both at 37 and 30 °C. The double mutants containing this ts mutation, and the triple mutant, also produced mostly insoluble proteins, although in these cases the fraction of soluble protein was somewhat greater, suggesting that the suppressor mutation improved the solubility of GyrB. The

Table 1. *Properties of mutant GyrB proteins*

Mutation (s)	Expression[a]	Supercoiling activity[b] (U/mg)
None (WT)	Soluble	4×10^4
G^{164}V	Insoluble	NA
H^{38}Q	Soluble	8×10^4
T^{157}I	Soluble	8×10^4
G^{164}V + H^{38}Q	Insoluble	NA
G^{164}V + T^{157}I	Insoluble	NA
G^{164}V + H^{38}Q + T^{157}I	Insoluble	NA

[a]Cultures were grown at 37 °C and cell extracts prepared after induction. Soluble/insoluble indicates whether most of the protein was in the soluble or insoluble fraction.
[b]Supercoiling assays were carried out at 37 °C for 30 min (Reece & Maxwell, 1989). NA = not applicable.

suppressor mutations on their own (His38 to Gln, and Thr157 to Ile) led to GyrB, which was largely soluble and had a supercoiling activity consistently greater than that of the wild-type protein (Table 1). Taken together, the results suggest that both suppressor mutations (His38 to Gln, and Thr157 to Ile) lead to a modest stabilization of GyrB, which is manifested in the slightly improved solubility of the proteins bearing the double mutations and the greater specific activity of GyrB proteins with the single mutations (Table 1). Moreover, we have found that GyrB containing the Thr157 to Ile mutation has activity (as measured by supercoiling assays) that is more stable to temperature than the wild-type protein, as evidenced by greater activity at 42 °C and retention of activity with prolonged incubation at 37 °C (data not shown).

Inspection of the crystal structures of the N-terminal subdomain of GyrB complexed with novobiocin and chlorobiocin (Lewis et al., 1996; Tsai et al., 1997) shows the locations of Thr157 and Gly164 to be at either end of a turn, which sits above the coumarin-binding site (Fig. 1A). The effects of the Gly164 to Val mutation, namely temperature sensitivity and coumarin resistance, can be explained on the basis of the introduction of a tighter constraint on the loop region and the consequent effect on the conformation of the adjacent amino acids, including the coumarin-binding site. The insolubility of proteins bearing this mutation is likely to be due to this effect on protein folding and the introduction of a nonpolar residue into a solvent-accessible region of the protein. The substitution of Thr157 by Ile may lead to a favorable hydrophobic interaction that helps to constrain the loop in a conformation that is more stable. For example, Ile at position 157 could make hydrophobic interactions with Val156 and/or Ile140 to stabilize such a conformation and thus compensate for the effects of Val164. Ile140 is located immediately beneath residues 156 and 157 on the turn interior to that containing 157 and 164 (Fig. 1A). A molecular surface calculation using the program GRASP (Nicholls et al., 1991) shows that the profile of the surface in the region of position 157 undergoes only minimal changes when the Val substitution is made.

In relation to His38, it is thought that this residue makes a hydrogen bond to the catalytic glutamate for the ATPase reaction (Glu42) that helps to polarize and orientate this residue (Jackson & Maxwell, 1993). It is possible that the side-chain amino group of glutamine at position 38 could still fulfill this function while the carbonyl oxygen of the amide could engage in other interactions. For example, modeling suggests that it is feasible that this carbonyl could form a hydrogen bond to Arg276, using preferred rotamers, and thus help to stabilize the protein structure (Fig. 1B).

In conclusion, we have confirmed that the Gly164 to Val mutation in GyrB results in a protein that is unstable but that nonetheless confers coumarin resistance in vivo. Using an in vivo selection procedure we found two mutations, His38 to Gln, and Thr157 to Ile, which can compensate for the loss of stability while retaining the resistance phenotype. The stabilizing effects of these mutations can be rationalized in terms of protein structure considerations.

References

Contreras A, Maxwell A. 1992. *gyrB* mutations which confer coumarin resistance also affect DNA supercoiling and ATP hydrolysis by *Escherichia coli* DNA gyrase. *Mol Microbiol 6*:1617–1624.

del Castillo I, Vizan J, Rodriguez-Sainz M, Moreno F. 1991. An unusual mechanism for resistance to the antibiotic coumermycin A$_1$. *Proc Natl Acad Sci USA 88*:8860–8864.

Gormley NA, Orphanides G, Meyer A, Cullis PM, Maxwell A. 1996. The interaction of coumarin antibiotics with fragments of the DNA gyrase B protein. *Biochemistry 35*:5083–5092.

Hallett P, Grimshaw AJ, Wigley DB, Maxwell A. 1990. Cloning of the DNA gyrase genes under *tac* promoter control: Overproduction of the gyrase A and B proteins. *Gene 93*:139–142.

Jackson AP, Maxwell A. 1993. Identifying the catalytic residue of the ATPase reaction of DNA gyrase. *Proc Natl Acad Sci USA 90*:11232–11236.

Kampranis SC, Gormley NA, Tranter R, Orphanides G, Maxwell A. 1999. Probing the binding of coumarins and cyclothialidines to DNA gyrase. *Biochemistry 38*:1967–1976.

Lewis RJ, Singh OMP, Smith CV, Skarzynski T, Maxwell A, Wonacott AJ, Wigley DB. 1996. The nature of inhibition of DNA gyrase by the coumarins and the cyclothialidines revealed by X-ray crystallography. *EMBO J 15*:1412–1420.

Maxwell A. 1997. DNA gyrase as a drug target. *Trends Microbiol 5*:102–109.

Maxwell A, Howells AJ. 1999. Overexpression and purification of bacterial DNA gyrase. In: Bjornsti M-A, Osheroff N, eds. *Protocols for DNA topoisomerases I. DNA topology and enzyme purification*. Towata, New Jersey: Humana Press. pp 135–144.

Molinaro M, Grossman G. 1995. UCB enhanced RasMol version 2.6 2.6 edit. Roger Sayle, Berkeley, California.

Nicholls A, Sharp KA, Honig B. 1991. Protein folding and association: Insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins 11*:281–296.

Orr E, Fairweather NF, Holland IB, Pritchard RH. 1979. Isolation and characterisation of a strain carring a conditional lethal mutation in the *cou* gene of *Escherichia coli* K12. *Mol Gen Genet 177*:103–112.

Reece RJ, Maxwell A. 1989. Tryptic fragments of the *Escherichia coli* DNA gyrase A protein. *J Biol Chem 264*:19648–19653.

Reece RJ, Maxwell A. 1991. DNA gyrase: Structure and function. *CRC Crit Rev Biochem Mol Biol 26*:335–375.

Tsai FTF, Singh OMP, Skarzynski T, Wonacott AJ, Weston S, Tucker A, Pauptit RA, Breeze AL, Poyser JP, O'Brien R, et al. 1997. The high-resolution crystal structure of a 24-kDa gyrase B fragment from *E. coli* complexed with one of the most potent coumarin inhibitors, chlorobiocin. *Protein Struct Funct Eng 28*:41–52.

Wigley DB. 1995. Structure and mechanism of DNA gyrase. In: Eckstein F, Lilley DMJ, eds. *Nucleic acids and molecular biology*, Vol. 9, Berlin: Springer-Verlag. pp 165–176.

Wigley DB, Davies GJ, Dodson EJ, Maxwell A, Dodson G. 1991. Crystal structure of an N-terminal fragment of the DNA gyrase B protein. *Nature 351*:624–629.