

***gyrB-225*, a Mutation of DNA Gyrase that Compensates for Topoisomerase I Deficiency: Investigation of its Low Activity and Quinolone Hypersensitivity**

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The B subunit of DNA gyrase (GyrB) consists of a 43 kDa N-terminal domain, containing the site of ATP binding and hydrolysis, and a 47 kDa C-terminal domain that is thought to play a role in interactions with GyrA and DNA. In cells containing a deletion of *topA* (the gene encoding DNA topoisomerase I) a compensatory mutation is found in *gyrB*. This mutation (*gyrB-225*) results in a two amino acid insertion in the N-terminal domain of GyrB. We found that cells containing this mutation are more sensitive than wild-type cells to quinolone drugs with respect to bacteriostatic and lethal action. We have characterised the mutant GyrB protein *in vitro* and found it to have reduced DNA supercoiling, relaxation, ATPase, and cleavage activities. The mutant enzyme is up to three-fold more sensitive to quinolones than wild-type. The mutation also increases the affinity of GyrB for GyrA and DNA, while the affinity of quinolone for the enzyme-DNA complex is unaffected. We propose that the loss in activity is due to misfolding of the GyrB-225 protein, providing an example in which misfolding of one protein, DNA gyrase, suppresses a deficiency of another, topoisomerase I. The increased quinolone sensitivity is proposed to be a consequence of an altered conformation of the protein that renders quinolones better able to disrupt, rather than generate, gyrase-drug-DNA complexes.

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Introduction

Type II topoisomerases are enzymes that alter the topological state of DNA by cleaving both strands and forming an intermediate phosphotyrosine linkage with the 5' phosphate group.^{1,2} DNA gyrase is a bacterial type II topoisomerase that introduces negative supercoils into DNA using ATP as an energy source. In the absence of ATP, gyrase can catalyse the relaxation of negative

supercoils. It consists of two proteins, GyrA and GyrB, that form a functional A₂B₂ tetramer.^{3,4} GyrA from *Escherichia coli* comprises two domains; an N-terminal domain (64 kDa) that contains the active-site tyrosine residue involved in DNA cleavage, and a C-terminal domain that is involved in the wrapping of a segment of DNA around the A₂B₂ complex. The GyrB protein also consists of two domains: a 43 kDa N-terminal domain, containing the site of ATP binding and hydrolysis, that is thought to act as a DNA clamp,^{5,6} and a 47 kDa C-terminal domain that is thought to interact with DNA and be involved in strand passage.

DNA gyrase is the target for a number of antibacterial agents, including the coumarin and quinolone drugs.⁷ Coumarins (e.g. novobiocin) inhibit the enzyme by binding to the N-terminal domain of GyrB and preventing ATP hydrolysis. Quinolones (e.g. oxolinic acid and ciprofloxacin) are potent antibacterial agents that trap DNA gyrase

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Abbreviations used: CFX, ciprofloxacin; GyrA, DNA gyrase A protein; GyrB, DNA gyrase B protein; OXO, oxolinic acid; SPR, surface plasmon resonance.

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(and DNA topoisomerase IV) on chromosomal DNA as complexes in which the DNA contains a pair of staggered single-stranded breaks.⁸ The complexes block DNA replication^{9,10} and transcription,¹¹ accounting for the bacteriostatic activity of the drugs. The quinolones also kill cells,¹² but at short incubation times, higher concentrations of drug are needed for lethal action than for blocking DNA replication.¹³ It has been suggested that cell death correlates with the release of pairs of single-stranded breaks from the complexes.¹³ Release of breaks mediated by quinolones such as nalidixic acid and oxolinic acid is blocked by inhibitors of protein or RNA synthesis,¹³ indicating that release is mediated by an as yet unidentified "suicide" protein. Since the lethal action of fluoroquinolones such as ciprofloxacin is blocked only partially by chloramphenicol, an inhibitor of protein synthesis, there also appears to be a lethal pathway that does not require the suicide protein. Little is known about the biochemistry of either lethal pathway.

Gyrase mutations that render both enzyme and cells more sensitive to quinolones offer a way to study lethal events. One of the best-characterized alleles is *gyrB*-225. This mutation was discovered

in a strain of *E. coli* that was deficient in *topA*, the gene encoding DNA topoisomerase I.^{14,15} Topoisomerase I counteracts the negative supercoiling function of gyrase by relaxing supercoiled DNA. In the absence of topoisomerase I, the action of gyrase leads to a build-up of negatively supercoiled DNA;¹⁵ excessive supercoiling is known to inhibit cell growth.^{14,15} The *gyrB*-225 mutation consists of a six base-pair insertion between nucleotides 1134 and 1135 of *gyrB*.¹⁶ This translates to two extra amino acids between residues 378 and 379 in the 43 kDa N-terminal region of GyrB (Figure 1) so that amino acid residues 377-382, including the insertion (bold), now comprise: Asp-Ala-**Ala-Arg**-Ala-Arg. The *gyrB*-225 mutation compensates for the lack of topoisomerase I by decreasing negative supercoiling and/or increasing relaxation activities of gyrase.¹⁷ The net result is that chromosomal DNA extracted from mutant cells has a lower level of negative supercoiling than that from wild-type cells.¹⁵ Mutants containing *gyrB*-225 form gyrase-DNA-quinolone complexes that appear to be more labile than those observed with wild-type cells, since nucleoids isolated from oxolinic acid-treated cells behave as though DNA

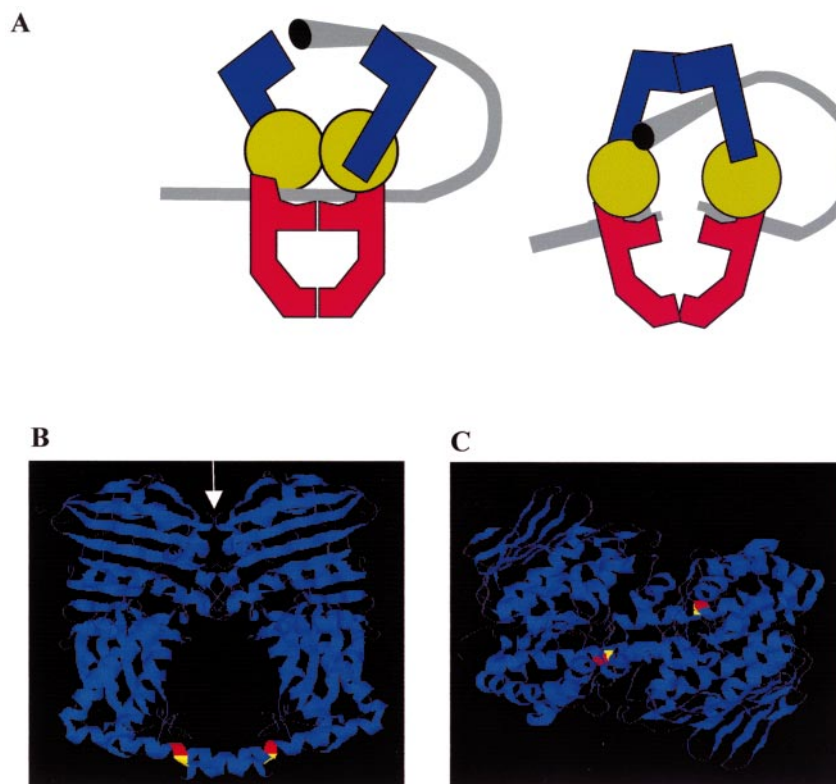


Figure 1. Structure of the DNA gyrase-DNA complex. (a) A representation of the proposed domain organisation of gyrase and its interaction with DNA. The N-terminal domain of GyrA is coloured red, the N-terminal domain of GyrB is blue, and the C-terminal domain of GyrB is green. The DNA is in grey and the C-terminal domain of GyrA is omitted for clarity. The complex is shown in both the clamp-open (left) and clamp-closed (right) conformations.⁴³ (b) and (c) The crystal structure of the 43 kDa N-terminal domain of GyrB,⁵ (b) from the "front", and (c) from "above" (the direction of the white arrow in (b)), showing amino acid residues 379 (red) and 380 (yellow); in GyrB-225 the inserted amino acids, Ala-Arg, lie between these two positions. The structures in (b) and (c) are in the clamp-closed conformation.

factors such as drug uptake and formation of gyrase-DNA-drug complexes on lethal activity, we measured lethal action at a fixed multiple (20-fold) of the concentration required to inhibit growth by 50%. The two strains were equally sensitive, unless chloramphenicol was present (Figure 2(c)). Thus, the *gyrB-225* allele preferentially facilitates chloramphenicol-insensitive lethal activity, which we speculate reflects gyrase subunit dissociation.¹⁹

A trivial explanation for the sensitivity of the mutant strain to the bacteriostatic action of oxolinic acid is that the mutant and wild-type enzymes are produced at different levels. For example, quinolones are thought to act by converting DNA gyrase into a poison;^{19,20} consequently, more enzyme could mean more complexes on the chromosome and hence increased killing. Conversely, compensation for the *topA⁻* phenotype could be achieved by producing less gyrase. In order to explore this possibility, we determined the level of GyrB production in wild-type and mutant cells by Western blotting (Figure 3). We found that SD104 and SD104-20 produce identical amounts of GyrB throughout their phases of growth. These experiments showed that the migration of the mutant protein was slightly slower in polyacrylamide gels, consistent with it having a slightly greater molecular mass and increased positive charge. The same experiments showed that a protein of approximately 45 kDa, visible in samples from wild-type cells, was apparently absent from the mutant cells (Figure 3). Determination of the first ten N-terminal amino acid residues of this protein revealed the sequence Met-Glu-Asn-Phe-Lys-His-Leu-Pro-Glu-Pro, showing it to be tryptophanase, an enzyme involved in the synthesis and breakdown of tryptophan.^{21,22} The reason for the apparent absence of tryptophanase from the mutant cells is unclear.

GyrB-225 shows reduced DNA supercoiling, DNA relaxation and ATPase activities

To facilitate purification, we cloned *gyrB-225* into plasmid pAG111, which over-expresses GyrB,²³ and purified the mutant protein to homogeneity using the same procedures as those used for the wild-type protein. The mutant protein was complexed with GyrA, and its activities compared to those of wild-type gyrase. The supercoiling activity of the mutant enzyme was sixfold lower than that of its wild-type counterpart, and its relaxation activity was lower by threefold (Table 1); typical supercoiling specific activities for wild-type GyrB are 4×10^4 - 4×10^5 units/mg. These results suggest that the mutant enzyme is less active than the wild-type enzyme in terms of both relaxation and supercoiling, although it should be noted that these results do not take account of any differences in the amounts of inactive or misfolded protein in the two preparations.

ATP hydrolysis experiments were carried out with mutant and wild-type gyrase (A₂B₂) in the presence of linear plasmid pBR322 DNA. The results show that the wild-type and mutant enzymes have similar ATP hydrolysis rates (Table 1). The experiment was also carried out using the 43 kDa N-terminal domain of GyrB, which has an intrinsic but not a DNA-stimulated ATPase activity.²⁴ The mutant 43 kDa protein exhibited an ATPase rate approximately twofold less than that of the wild-type protein (Table 1).

Supercoiling and relaxation activities of GyrB-225 are hypersensitive to quinolones

We determined the *in vitro* sensitivity to quinolones of the DNA supercoiling and relaxation reactions of the mutant and wild-type enzymes. In these experiments, we used concentrations of gyrase just sufficient to give maximal or slightly sub-maximal

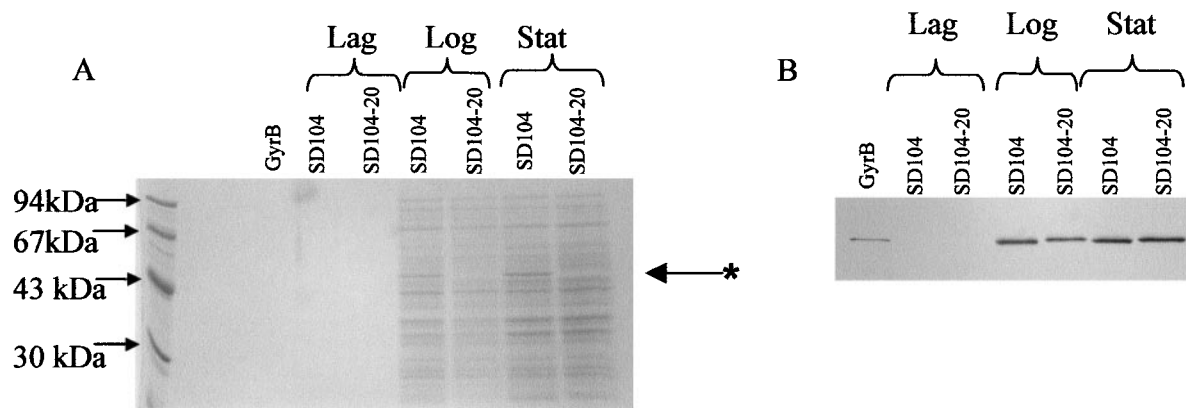


Figure 3. (a) SDS/polyacrylamide gel electrophoresis of proteins extracted from *E. coli* strains SD104 and SD104-20 at lag, log and stationary phases of growth. Note that a band (indicated with an asterisk) is present in SD104 cells and absent from SD104-20. (b) Western blot of the gel in (a) with 9G8, a GyrB-specific monoclonal antibody.⁴²

Table 1. The activities and quinolone sensitivities of GyrB and GyrB-225

Enzyme	Specific activity (units/mg) ^a		ATPase (s ⁻¹)		IC ₅₀ (μM)			
	Supercoiling	Relaxation	A ₂ B ₂ (DNA-dependent)	43 kDa domain ^b	Supercoiling		Relaxation	
					CFX	OXO	CFX	OXO
GyrB	6.7 × 10 ⁴	6.9 × 10 ³	0.95	6.5 × 10 ⁻⁴	0.103	5.0	9.2	380
GyrB-225	1.1 × 10 ⁴	2.3 × 10 ³	0.87	3 × 10 ⁻⁴	0.066	1.5	5.2	191

CFX, ciprofloxacin; OXO, oxolinic acid.

^a Supercoiling, relaxation and DNA-dependent ATPase activities were measured in the presence of GyrA and DNA.

^b The concentration of the 43 kDa domain was 5 μM dimer.

supercoiling and titrated in quinolone drugs in order to find the concentration required to inhibit the reaction by 50%. We found that GyrB-225 required up to about threefold less quinolone than the wild-type enzyme to inhibit the supercoiling and relaxation reactions by 50% (Figure 4; Table 1). In the case of ciprofloxacin, the differences were too small to be resolved unambiguously, but from several similar experiments we found the mutant strain to be consistently more sensitive than the wild-type. This result suggests that the mutant enzyme is

indeed hypersensitive to quinolone drugs, consistent with the *in vivo* observations.

Rate of DNA cleavage is reduced in GyrB-225

Aside from inhibiting the DNA supercoiling and relaxation reactions of gyrase, quinolones induce DNA cleavage;^{25,26} the release of cleaved DNA is thought to be the bactericidal event *in vivo*.¹³ It has been shown that the appearance of cleaved DNA is slow compared with the inhibition of supercoiling, which occurs very rapidly.²⁷ We have investigated the rate of trapping of the cleaved complex by wild-type and mutant proteins in the presence of ciprofloxacin. In these experiments, we found that fivefold more mutant enzyme than wild-type was needed in order to give comparable levels of maximal DNA cleavage. Cleavage with GyrB-225 was slower than with the wild-type enzyme (Figure 5;

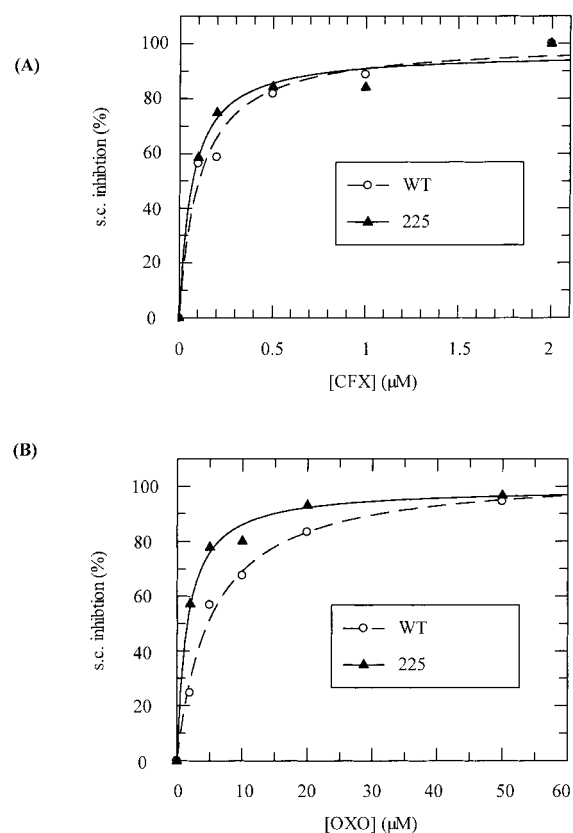


Figure 4. Inhibition of gyrase-mediated supercoiling of relaxed pBR322 by (a) ciprofloxacin (CFX), (b) oxolinic acid (OXO), for wild-type and mutant gyrases. Maximum supercoiling inhibition is normalised to 100% for purposes of comparison. IC₅₀ values: wild-type, 103 nM (CFX) and 5 μM (OXO); mutant, 66 nM (CFX) and 1.5 μM (OXO).

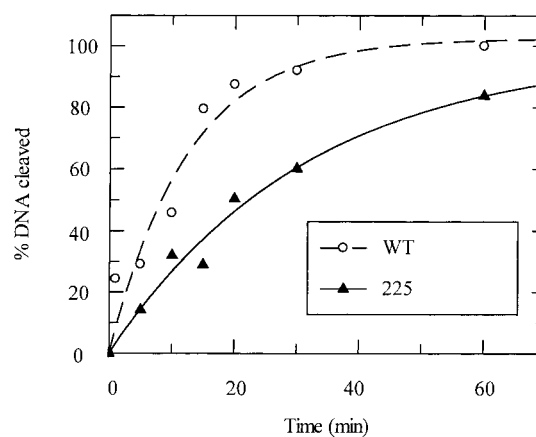


Figure 5. Time-course for cleavage of relaxed pBR322 to its linear form in the presence of ciprofloxacin, for wild-type and mutant gyrases. The DNA concentration was 3.4 nM, the enzyme concentrations were 20 nM (wild-type) and 100 nM (mutant). Reactions were incubated at 37 °C and samples were removed at the times indicated. After treatment with SDS and proteinase K (see Materials and Methods), samples were analysed on a 1% agarose gel in the presence of 10 μg/ml chloroquine. The amount of linear DNA was determined by analysing the digitized image using Syngene software. The extent of DNA cleavage is normalised to 100%; graphs were fitted using a first-order rate equation.

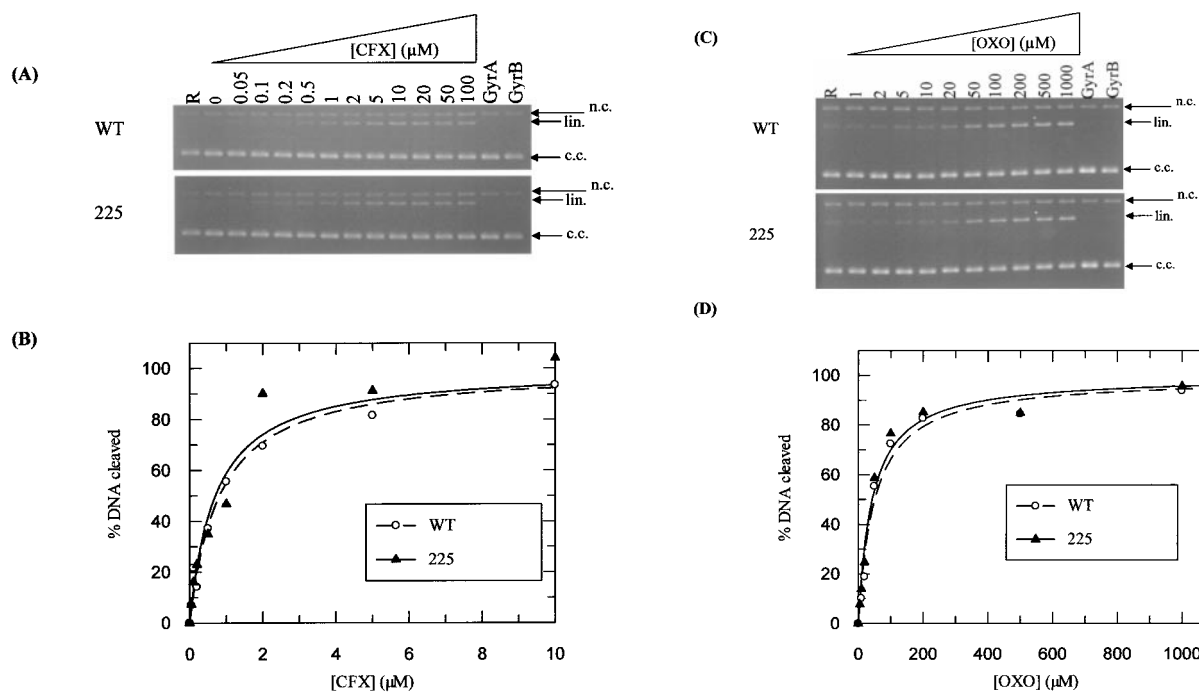


Figure 6. Gyrase-mediated cleavage of relaxed pBR322 in the presence of ciprofloxacin and oxolinic acid, and wild-type or mutant gyrases. The DNA concentration was 3.4 nM and the enzyme concentrations were 20 nM (wild-type) and 100 nM (mutant). Samples (a) and (c) were treated as described in the legend to Figure 5. The graphs (b) and (d) show the amount of DNA cleaved as the drug concentration is varied. The amount of DNA cleaved by each enzyme was normalised to 100%. K_d^{app} values: wild-type, 0.8 μ M (CFX) and 53.4 μ M (OXO); mutant, 0.7 μ M (CFX) and 44.0 μ M (OXO). rel., Relaxed DNA; lin., linear DNA; c.c., closed circular DNA; GyrA, GyrA protein alone; GyrB, GyrB protein alone.

the time taken to achieve 50% of maximal cleavage was 8.5 minutes for wild-type gyrase and 21.6 minutes for mutant gyrase). We also found that, at sub-saturating levels of ciprofloxacin, the rate of trapping of the cleaved complex was approximately first-order with respect to ciprofloxacin concentration. In the case of oxolinic acid, we carried out cleavage experiments at 8°C as cleavage occurred too rapidly at 37°C to measure the rates reliably; we found no significant difference between wild-type GyrB and GyrB-225 in the rate of trapping (data not shown).

The apparent affinity of quinolone drugs for GyrB-225 is similar to that for the wild-type enzyme

An enzyme that shows increased sensitivity to drugs might also be expected to bind the drugs more tightly. The apparent affinity of quinolone drugs for the gyrase-DNA complex can be estimated by determining the amount of cleaved DNA over a range of drug concentrations, i.e. the maximal level of cleavage can, in principle, be used as a measure of the level of drug binding at equilibrium. Fivefold more mutant than wild-type enzyme was used, as above, in order to get comparable levels of maximum DNA cleavage. Such an experiment shows that the apparent K_d values

(K_d^{app}) for both ciprofloxacin and oxolinic acid agree closely for both wild-type and mutant enzymes (Figure 6). This result was extended for ciprofloxacin in binding experiments using ³H-labelled drug and a 140 bp DNA fragment (Figure 7). In this experiment, the amount of drug bound to the gyrase-DNA complex was determined for a range of drug concentrations using rapid gel-filtration. A fivefold excess of enzyme over DNA was used for both wild-type and mutant enzymes. Under these conditions, we assume that the DNA concentration is equivalent to the concentration of gyrase-DNA complex. Earlier work has shown that drug binding to enzyme alone or DNA alone is negligible.^{28,29} By fitting K_d^{app} values to the binding curves, we found that ciprofloxacin has similar affinities for wild-type and mutant enzyme-DNA complexes, although the levels of maximum drug bound (as estimated from the plateaus of the binding isotherms) are slightly different (Figure 7). From these plateaus the stoichiometries of quinolone binding to the gyrase complex can be estimated as ~2 for the wild-type enzyme and ~1.5 for the mutant (Figure 7), consistent with previous studies that suggested a stoichiometry of two drug molecules per gyrase-DNA complex.³⁰ Note that the K_d^{app} values in Figures 6 and 7 are different; this is likely to be a consequence of the different DNA substrates used

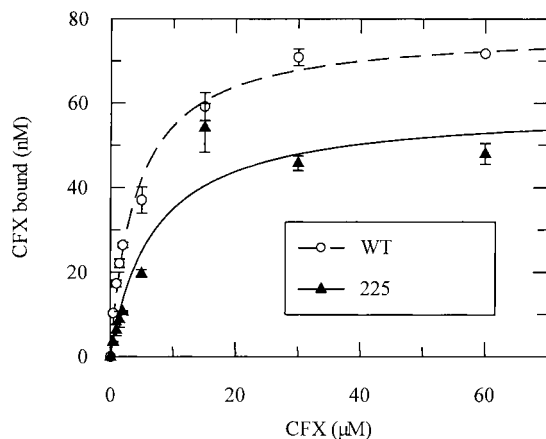


Figure 7. The binding of ³H-labelled ciprofloxacin to 40 nM complexes of 140 bp DNA and wild-type and mutant gyrases. Wild-type, maximum bound drug = 77 nM, $K_d^{app} = 4.2 \mu\text{M}$; mutant, maximum bound drug = 59 nM, $K_d^{app} = 6.8 \mu\text{M}$. The plateaus suggest stoichiometries of about two and about 1.5 molecules of ciprofloxacin per gyrase-DNA-drug complex for the wild-type and mutant enzyme, respectively.

(pBR322 and a 140 bp fragment) and the different methods used to make these measurements. The fact that the quinolone-sensitive GyrB-225 mutation has apparently no effect on the affinity of the drug for the enzyme-DNA complex suggests that the observed sensitivity is achieved by another mechanism.

Interaction of GyrB-225 with GyrA and DNA

The activities of DNA gyrase require that the GyrA and GyrB proteins form an A₂B₂ complex. We used the technique of surface plasmon resonance (SPR) to investigate the interaction of GyrA with wild-type and mutant GyrB proteins. The affinity of GyrB for GyrA was assessed using SPR by passing GyrB over a sensor chip to which GyrA was attached. The results (Figure 8) show that the affinity of GyrB-225 for its partner subunit is ~40 times greater than the affinity of wild-type GyrB.

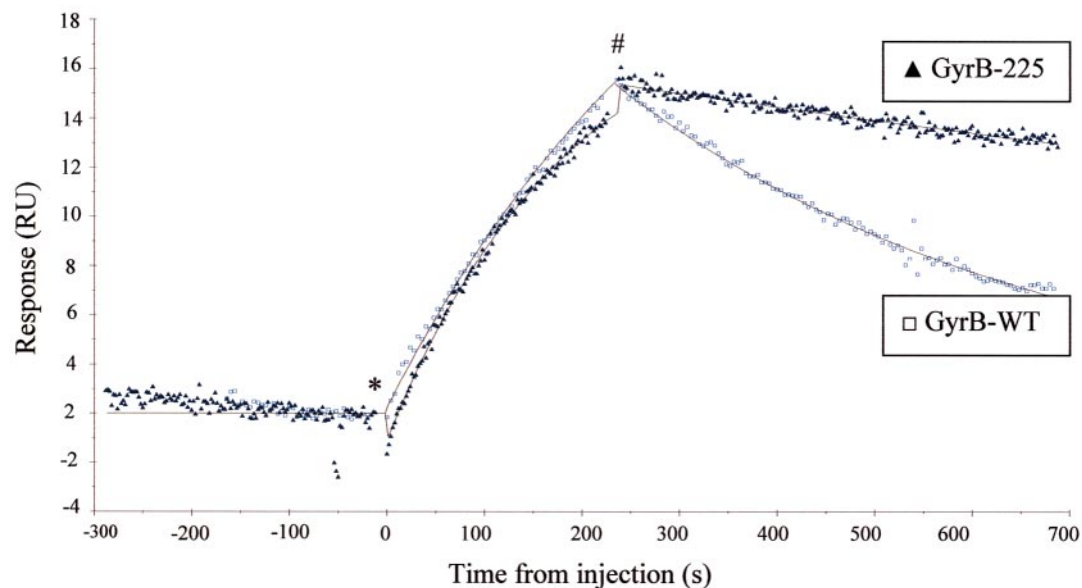
To form a complete enzyme-DNA complex, gyrase requires approximately 130 bp of DNA, of which the ~20 bp central region binds to the active site and the flanking regions wrap around the enzyme.³¹ DNA binding by gyrase can be measured using gel-retardation assays.³² We found by this method that the mutant gyrase (A₂B₂) binds DNA about twofold less effectively than the wild-type enzyme (data not shown). However, this method fails to take into account differences in the amount of active protein in the two preparations. In order to address this problem, the binding of DNA gyrase to 140 bp and 24 bp DNA fragments was measured using SPR. This technique can measure the affinity of enzyme for DNA indepen-

dent of the amounts of active protein. The results (Figure 9) show that the affinity of the mutant enzyme for DNA is increased about twofold compared to that of the wild-type enzyme. As the mutant has a higher affinity than the wild-type enzyme for both the 140 bp and 24 bp fragments, the increased binding seen with the mutant can be attributed to the central region of the gyrase-DNA complex, termed the G segment.³³ In these experiments, we found that the amplitude of the SPR signal with the mutant enzyme was ~0.25 that of the wild-type, consistent with the mutant enzyme preparation containing approximately one-quarter of the level of active, DNA-binding enzyme found in the wild-type preparation.

Discussion

gyrB-225 is a mutation that compensates for the loss of DNA topoisomerase I in *topA*-deficient *E. coli* strains.^{14,15} Strains containing this *gyrB* allele have subnormal levels of chromosomal and plasmid supercoiling,¹⁵ gyrase-DNA-quinolone complexes formed in those strains appear to be disrupted more easily,¹⁸ and the strains are hypersensitive to quinolones when bacteriostatic and bactericidal effects are measured (Figure 2). To better understand these phenotypes, we cloned the *gyrB-225* gene and overexpressed the GyrB protein. The purified GyrB-225 protein had reduced supercoiling and relaxing activities (sixfold and threefold, respectively) and, although the DNA-dependent ATPase activity was near-normal, the intrinsic ATPase activity of the 43 kDa N-terminal domain of GyrB was reduced about twofold (Table 1). The *in vitro* supercoiling and relaxation activities were somewhat hypersensitive to quinolones (Figure 4). The ciprofloxacin-induced DNA cleavage activity was found to be slower than that of the wild-type enzyme (Figure 5). However, as measured by a DNA cleavage assay or by direct binding studies, the mutant and wild-type enzyme appeared to bind a similar amount of drug. In DNA cleavage experiments, we had to use about fivefold more of the mutant enzyme to get levels of cleavage that were comparable with that of the wild-type enzyme, suggesting that only about a fifth of the mutant protein was folded correctly, by comparison with wild-type GyrB. This conclusion is supported by SPR experiments in which the mutant enzyme bound ~25% as much DNA as did the wild-type gyrase, even though the mutant binding to DNA was tighter (Figure 9). Binding of GyrB to GyrA was also tighter for the mutant than for the wild-type GyrB (Figure 8).

Given the phenotype of strains carrying the *gyrB-225* mutation, it is expected that the mutant GyrB protein would have reduced activities compared with wild-type GyrB. Indeed, we have found reduced supercoiling and relaxation activities. We propose that the reduced activity of GyrB-225 derives from an increased propensity of this



	k_a ($M^{-1} \cdot s^{-1}$)	k_d (s^{-1})	K_d (M)
GyrB-WT	1.23×10^4	2.32×10^{-3}	1.89×10^{-7}
GyrB-225	1.03×10^5	4.61×10^{-4}	4.47×10^{-9}

Figure 8. SPR trace showing association and dissociation responses when 50 nM GyrB or GyrB-225 were flowed over approximately 600 RU of GyrA immobilised on the chip surface. The Table shows equilibrium and rate constants, which suggest that GyrB-225 has a higher affinity for GyrA than the wild-type protein. The asterisk (*) shows the point at which GyrB was injected across the sensor chip. The hash (#) shows the point at which the injection ceased and dissociation of GyrB from the chip began.

protein to misfold. Wild-type GyrB also has a tendency to misfold^{34,35} and we suggest that the insertion mutation in GyrB-225 increases that tendency. This proposal is supported by the observation that the amplitude in SPR experiments measuring mutant GyrB-DNA binding was ~25% that seen with the wild-type protein (Figure 9). If only ~25% of the GyrB-225 preparation (compared to wild-type) is competent to bind DNA, supercoiling and relaxation activities would be lower. Supercoiling may be affected more profoundly than relaxation because of an additional decrease in the intrinsic ATPase activity of the protein. (We have previously noted that the DNA-dependent ATPase activity of gyrase is largely invariant, irrespective of the quality of the GyrB preparation.) DNA cleavage assays also required about fivefold more GyrB-225 complex to achieve levels of cleavage comparable to those observed with the wild-type complex, consistent with the level of active protein being lower. Since the level of GyrB-225 protein produced *in vivo* is the same as that of the wild-type (Figure 3), the mutation would reduce the

level of active gyrase and hence compensate for the deficiency in *topA*.

A further consequence of the *gyrB-225* mutation is its effects on the sensitivity of gyrase to quinolones. These effects are complicated but can be interpreted with reference to the proposed mode of interaction of gyrase with the drugs. It has been suggested that quinolone interaction with gyrase proceeds *via* (at least) two distinct steps.²⁷ In the first step, quinolones bind to the gyrase-DNA complex and form an initial complex that inhibits strand passage activity. In the second (slower) step, a further complex is formed that leads to trapping of cleaved DNA. *In vivo*, this cleavage complex can arrest replication forks. The present work suggests that the *gyrB-225* mutation does not alter the binding of quinolones to the gyrase-DNA complex (Figures 6 and 7), but that the initial complex that inhibits strand passage is formed in the mutant more readily (as evidenced by the increased sensitivity of the supercoiling and relaxation reactions to quinolones). Conversion of this initial complex to the cleavage complex is apparently slower with GyrB-225 than with wild-type

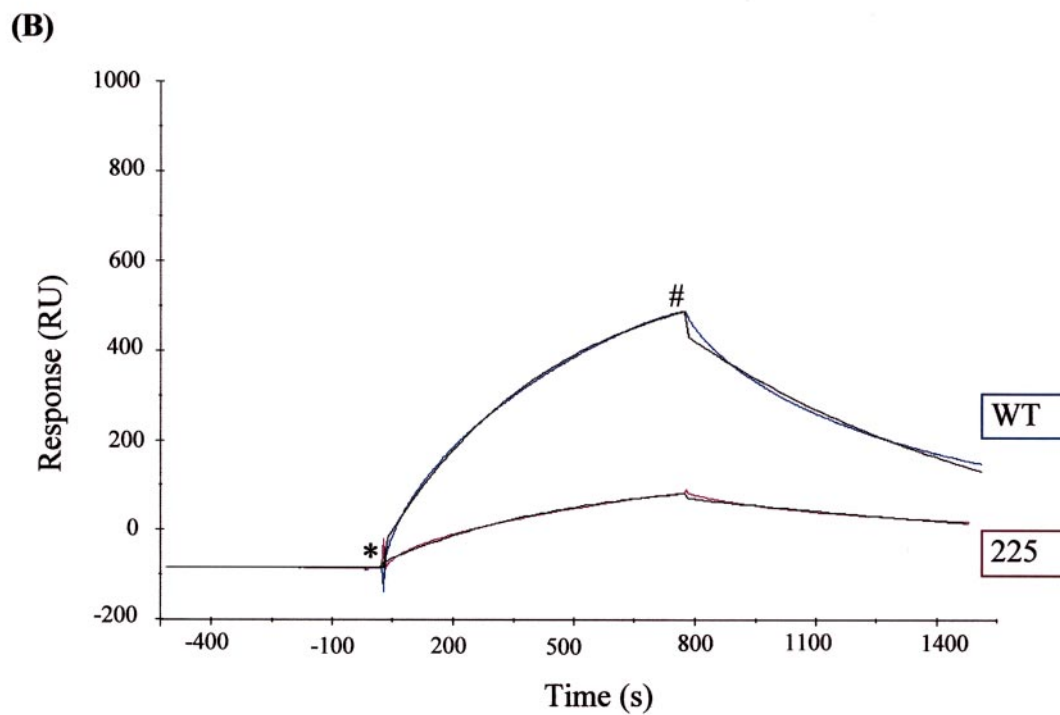
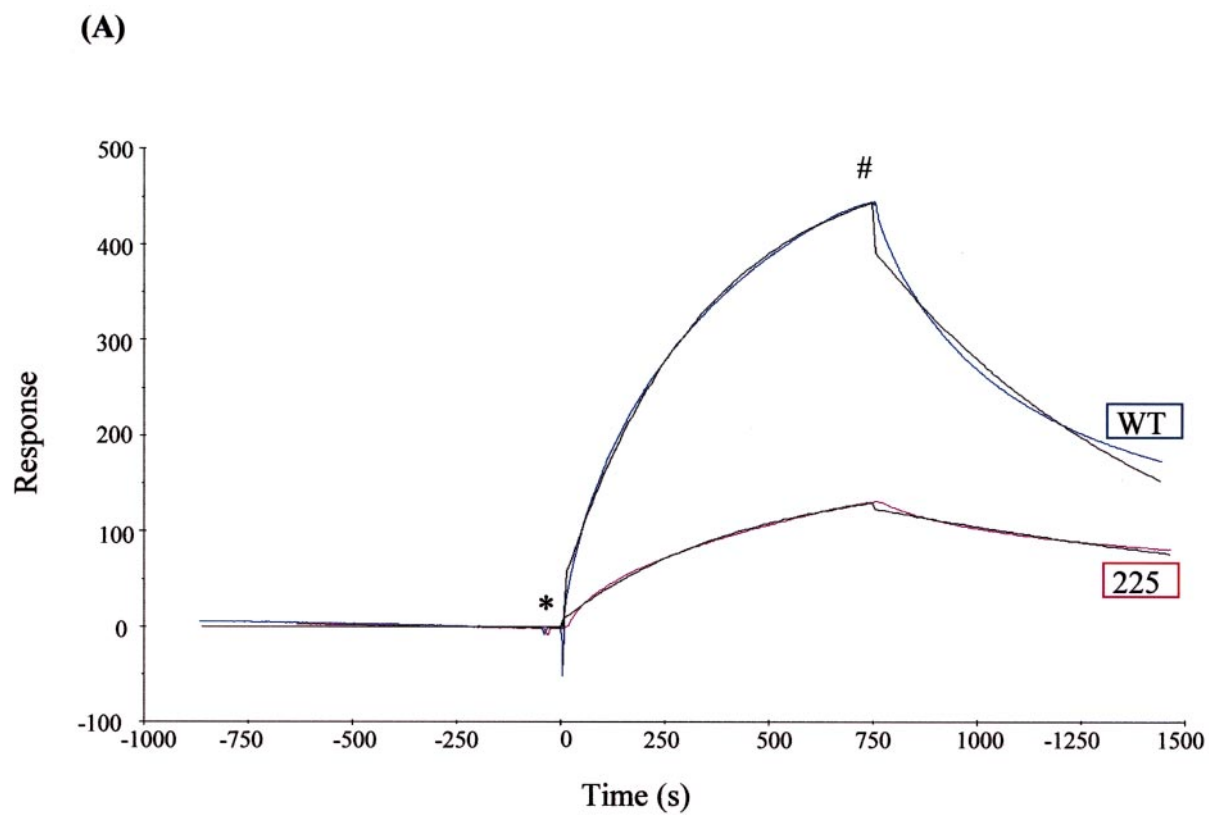


Figure 9 (legend opposite)

GyrB (Figure 5). *In vivo*, the trapped cleavage complex with GyrB-225 is disrupted more easily than the wild-type complex, leading to the observed

hypersensitivity. The origin of this effect is not clear but may be due to altered GyrA-GyrB interactions, which promote a conformation of gyrase

	140 bp fragment:			24 bp fragment:		
	k_a ($M^{-1}.s^{-1}$)	k_d (s^{-1})	K_d (M)	k_a ($M^{-1}.s^{-1}$)	k_d (s^{-1})	K_d (M)
WT gyrase	1.62×10^4	1.38×10^{-3}	8.49×10^{-8}	1.17×10^4	1.2×10^{-3}	1.02×10^{-7}
mutant gyrase	1.57×10^4	6.73×10^{-4}	4.28×10^{-8}	1.47×10^4	6.31×10^{-4}	4.29×10^{-8}

Figure 9. SPR traces showing 100 nM wild-type and mutant gyrases binding to (a) 140 bp DNA and (b) 24 bp DNA. The asterisk (*) shows the point at which gyrase was injected across the sensor chip. The hash (#) shows the point at which the injection ceased and dissociation of gyrase from the chip began. The fitted lines are shown in black. The data were fitted to a simple 1:1 binding model using the BIAevaluation software (BIAcore) as described.⁴⁴ The Table shows association and dissociation rates, and equilibrium dissociation constants (k_a , k_d , K_d). K_d values suggest an approximately twofold tighter binding to DNA for mutant compared to wild-type gyrase.

that, in the presence of quinolones, is disrupted by *in vivo* processes more easily.

The complex of GyrB-225 with GyrA is more stable than the wild-type GyrA-GyrB complex. The amplitude of the SPR trace in this experiment (Figure 8) suggests that all the mutant protein binds GyrA, i.e. not just the "active" fraction; the lack of a "biphasic" response would suggest that there are not two distinct interactions. It is possible that the increased binding affinity is a manifestation of the altered conformational state of the GyrB-225 protein, e.g. *via* increased exposure of hydrophobic surfaces. Although more or less all of the GyrB-225 protein is able to bind GyrA, it seems that only about 25% of this complex is able to bind DNA and remain catalytically active. These data exclude weakened GyrA-GyrB interactions as an explanation for the ease of disruption of drug-gyrase-DNA complexes¹⁸ and the hypersensitivity of lethal action in the presence of chloramphenicol (Figure 2(c)). A remaining possibility is that the GyrB-225 allele weakens GyrB-GyrB (or GyrA-GyrA) interactions. That would allow GyrA-GyrB dimers, each attached to an end of cleaved DNA, to dissociate more readily. If that process is stimulated by quinolones, as appears to be the case when assayed by illegitimate recombination,³⁶ it would explain how some quinolones can be highly lethal even in the absence of protein synthesis.³⁷ Attempts to probe the GyrB-GyrB interaction have so far been inconclusive.

In summary, the origins of the effects of the GyrB-225 mutation in compensating for the effects of a *topA*⁻ mutation appear to lie in its increased propensity to misfold, thereby generating an enzyme that is inactive in supercoiling. A further consequence of the mutation is strengthened

GyrA-GyrB interactions that lead to increased quinolone sensitivity, perhaps by promoting a conformation that is affected more readily by the inhibitory action of quinolones. The details of the gyrase-quinolone interaction are subjects for further investigation.

Materials and Methods

Bacterial strains and bacteriological methods

E. coli K-12 strain SD104-20 is a *gyrB*-225 derivative of SD104 constructed by P1-mediated transduction of *gyrB* from strain DM800.¹⁴ For bacteriostatic and bactericidal measurements, cells were grown in LB liquid medium³⁸ overnight, unless otherwise indicated. For viable cell determinations, cultures were applied to LB agar plates followed by colony-forming-unit determination after overnight incubation. The incubation temperature in all experiments was 37°C.

Proteins and DNA

Wild-type gyrase was prepared as separate subunits (GyrA and GyrB), as described.³⁹ GyrA was a gift from Mrs A.J. Howells (University of Leicester). The *gyrB*-225 mutant gene was cloned into pAG111, the plasmid encoding GyrB,²³ and the mutant protein was expressed in DH5a cells (Stratagene). Protein was purified to homogeneity using the same method as that used for wild-type GyrB. Plasmid pBR322 in relaxed and supercoiled forms was a gift from Mrs A.J. Howells. A 140 bp fragment containing the major preferred gyrase cleavage site from pBR322⁴⁰ was synthesised by PCR using the following primers: 5'-TCG GGG AAT TCG CAT GGC G and 5'-TGG ACA GCA TGG CCT GCA A. A 24 bp fragment containing the gyrase cleavage site from pBR322 was prepared by annealing two synthetic oligonucleotides (5'-GAG GCT GGA TGG CCT TCC CCA TTA, and

its complement). All oligonucleotides were made by PNAOL (University of Leicester).

Supercoiling and relaxation assays

The supercoiling activity of GyrB in the presence of GyrA was determined using a method similar to that described elsewhere.⁴¹ GyrA and GyrB were added to 30 ml reactions containing 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM MgCl₂, 1.8 mM spermidine, 5 mM DTT, 6.5% (w/v) glycerol, 0.36 mg/ml bovine serum albumin, 9 µg/ml tRNA, 1.4 mM ATP and 10 µg/ml pBR322 DNA. Reaction mixtures were incubated at 37°C for 30 minutes and stopped by the addition of 30 µl of chloroform/isoamyl alcohol (24:1, v/v) and 15 µl of 40% (w/v) sucrose, 0.1 M Tris-HCl (pH 8), 0.1 M EDTA, 1% (w/v) bromophenol blue. Samples (15 µl) of the aqueous phase were loaded onto 1% (w/v) agarose gels and typically run at 80 V for two hours. Relaxation assays were the same as supercoiling assays except that ATP and spermidine were omitted and supercoiled pBR322 was used instead of the relaxed form.

ATPase assays

ATPase reactions were followed using an enzyme-linked assay system.²⁴ Reactions were carried out in a volume of 150 µl at 25°C containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM ATP, 10% (w/v) glycerol, 1 mM EDTA, 5 mM DTT, 5 mM MgCl₂, 400 µM phosphoenolpyruvate, 250 µM NADH and 1.5 µl of phosphokinase/lactate dehydrogenase (PK/LDH) enzyme mix (in 50% (w/v) glycerol, 100 mM KCl, 10 mM Hepes (pH 7.0), 0.1 mM EDTA; Sigma). Reactions were initiated by the addition of ATP and the decrease in A_{340 nm} was measured over time. Typically, 50 nM GyrB was used or 5 µM GyrB 43 kDa domain. ATPase activities of GyrB were measured in the presence of excess GyrA and linear pBR322.

Quinolone-induced DNA cleavage assays

Conditions were as for supercoiling reactions except that ATP and spermidine were omitted and ciprofloxacin (a gift from Bayer, Germany) or oxolinic acid (Sigma) was included at a range of concentrations, depending on the nature of the assay. Cleaved DNA was revealed after digestion of gyrase by the addition of 0.2% (w/v) SDS and 0.1 mg/ml proteinase K and incubation for 30 minutes at 37°C. Reactions were stopped and samples analysed as described for supercoiling reactions, except that the agarose gels contained 10 µg/ml chloroquine.

Quinolone binding assays

The binding of quinolone drugs to DNA gyrase was assessed using rapid gel-filtration.²⁸ Samples contained 50 mM Tris-HCl (pH 7.5), 55 mM KCl, 4 mM MgCl₂, 5 mM DTT, 5% (w/v) glycerol, 50 or 100 nM 140 bp DNA fragment, 500 nM gyrase and varying amounts of ³H-labelled ciprofloxacin (Amersham). Samples were incubated at 25°C for three hours, 80 µl was withdrawn and applied to a Sephadex G50 column (Pharmacia). Columns were centrifuged at 4°C and 1700 rpm in a Mistral bench-top centrifuge (MSE) for ten minutes. The volumes of the eluants were measured and the amount of tritiated drug retained was determined by scintillation counting.

Western blot analysis

Detection of GyrB was carried out using the mouse monoclonal anti-GyrB antibody 9G8 as described.⁴² To detect the secondary antibody, an ECLTM chemiluminescence kit (Amersham) was used. The membrane was dried between two sheets of 3MM paper (Whatman) and soaked in each of the two luminescent reagents, which had been mixed prior to use, for one minute. The membrane was dried and exposed to X-ray film (Fuji) for approximately five seconds.

Surface plasmon resonance

SPR measurements were made on a BIAcore 2000 apparatus (BIAcore). Interactions were measured between gyrase proteins using the following method. One of the two proteins of interest (the ligand) was attached to a CM5 sensor chip (BIAcore) as per the manufacturer's instructions; this entails activating the chip by applying 35 µl of *N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS). Once the chip was activated, the ligand was passed across and bound covalently to the chip surface. Typically, 100 µl of 100 nM protein was passed across the chip at 5 µl/minute. This generally results in 500-1000 relative units (RU) of protein attaching to the chip. Unoccupied sites for protein binding were blocked by applying 35 µl of ethanolamine-HCl at 7 µl/minute. Analyte protein was passed across the chip in 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM MgCl₂, 5 mM DTT, 6.5% (w/v) glycerol, 0.002% (v/v) Tween 20, at a flow-rate of 25 µl/minute. DNA (140 bp or 24 bp fragment) was synthesised with biotin at its 5'-end and coupled to a streptavidin-coated chip (sensor chip SA; BIAcore) and protein flowed across as described above.

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