

# Interactions between QnrB, QnrB Mutants, and DNA Gyrase

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**Plasmid-encoded protein QnrB1 protects DNA gyrase from ciprofloxacin inhibition. Using a bacterial two-hybrid system, we evaluated the physical interactions between wild-type and mutant QnrB1, the GyrA and GyrB gyrase subunits, and a GyrBA fusion protein. The interaction of QnrB1 with GyrB and GyrBA was approximately 10-fold higher than that with GyrA, suggesting that domains of GyrB are important for stabilizing QnrB1 interaction with the holoenzyme. Sub-MICs of ciprofloxacin or nalidixic acid reduced the interactions between QnrB1 and GyrA or GyrBA but produced no reduction in the interaction with GyrB or a quinolone-resistant GyrA:S83L (GyrA with S83L substitution) mutant, suggesting that quinolones and QnrB1 compete for binding to gyrase. Of QnrB1 mutants that reduced quinolone resistance, deletions in the C or N terminus of QnrB1 resulted in a marked decrease in interactions with GyrA but limited or no effect on interactions with GyrB and an intermediate effect on interactions with GyrBA. While deletion of loop B and both loops moderately reduced the interaction signal with GyrA, deletion of loop A resulted in only a small reduction in the interaction with GyrB. The loop A deletion also caused a substantial reduction in interaction with GyrBA, with little effect of loop B and dual-loop deletions. Single-amino-acid loop mutations had little effect on physical interactions except for a  $\Delta 105I$  mutant. Therefore, loops A and B may play key roles in the proper positioning of QnrB1 rather than as determinants of the physical interaction of QnrB1 with gyrase.**

Quinolones are antimicrobials that target the essential bacterial enzymes DNA gyrase and topoisomerase IV and form drug-enzyme-DNA complexes that block movement of the DNA replication complex (1, 2). Bacterial resistance mutations have been selected with wide clinical use of quinolones. These mutations reduce drug binding by altering the target enzymes or eliminate quinolones by increased expression of efflux pumps.

Unexpectedly, transferable resistance has also emerged to compromise the clinical utility of quinolones further. First recognized in an isolate of *Klebsiella pneumoniae* in 1998 (3), transferable quinolone resistance is now widespread in enteric Gram-negative bacteria. Such resistance is transmissible by plasmids which often contain additional genes conferring resistance to other classes of antimicrobial agents (4–7). Plasmid-mediated quinolone resistance (PMQR) is caused by genes encoding either components of quinolone efflux pumps, a quinolone-modifying enzyme, or Qnr proteins that can protect gyrase and topoisomerase IV from quinolone inhibition. Although low-level resistance results from the presence of PMQR genes alone, their presence facilitates selection of higher level mutational resistance (8), and these genes have been documented to have spread worldwide (9).

Qnr proteins are members of the pentapeptide repeat family of proteins. Six types of plasmid-encoded proteins (QnrA, -B, -C, -D, -S, and -VC), are known, some with multiple variants, as well as others encoded on the chromosome especially of aquatic bacteria (3, 10–14). QnrB variants appear to have the highest prevalence (14). QnrB1 dimerizes via its C-terminal domain to form a rod-like structure that mimics B-form DNA (15). Each monomer has two external loops, a smaller loop, loop A (8 amino acids, Y46-Q53) and a larger loop, loop B (12 amino acids, M102-S113), which are required for Qnr-mediated protection of gyrase from ciprofloxacin action.

Alanine mutagenesis and deletion analysis of QnrB1 have provided information on structural elements that are important for

the ability of the protein to confer resistance (16). By convention, the central amino acid in the pentapeptide unit is termed *i*, with neighboring amino acids  $i^{+1}$  and  $i^{-1}$  and  $i^{+2}$  and  $i^{-2}$  (17). Essential amino acids for protection of gyrase were found at the central amino acid positions *i* and  $i^{-2}$  in the pentapeptide repeat module and in the larger loop, loop B, where deletion of only a single amino acid compromised activity. For the single-alanine substitutions in the two loops, there was little effect on protective activity except for F111A in loop B. While alanine substitution of T106, T107, and R108 showed little effect on protection, their single or multiple deletions strongly reduced protection. Furthermore, deletion of 15 to 20 N-terminal amino acids and as few as 3 C-terminal amino acids also abolished resistance (16). Therefore, loop B seems to be a key for QnrB1's protective positioning on gyrase (18). Purified QnrA has been shown to bind to both the GyrA and GyrB subunits as well as the ParC and ParE subunits of topoisomerase IV (19, 20). QnrA also affects gyrase binding of DNA, consistent with a model in which the DNA-like structure of Qnr proteins and the related MfpA pentapeptide repeat protein position themselves in the

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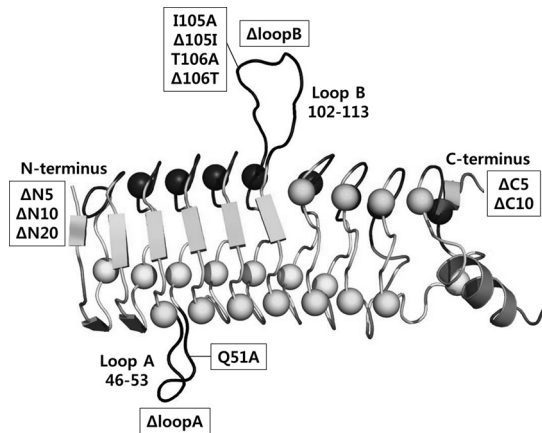
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**FIG 1** Illustration of QnrB1 and positions of the various mutations that were constructed in this study (modified from reference 15). Loop B is made up of the amino acids from positions 102 to 113, and loop A is made up of the amino acids from positions 46 to 53. Five, ten, or twenty amino acids were deleted from the N or C terminus to construct the  $\Delta N$  and  $\Delta C$  mutations.

gyrase DNA binding site (21). There are, however, no data on the effects of specific Qnr structural modifications on its physical interactions with gyrase proteins or on the effects of gyrase resistance mutations on this interaction. It also remains unclear how Qnr proteins are able to protect from quinolone action without impairing gyrase catalytic function.

In this study, we evaluated the physical interactions between QnrB1 and gyrase subunits, GyrA and GyrB, and a GyrBA fusion protein using a bacterial two-hybrid (B2H) system to analyze protein-protein interactions *in vivo* (22) and to assess the effects of QnrB and gyrase mutations on these interactions.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** *Escherichia coli* DH5 $\alpha$  or One Shot TOP10 (Invitrogen, Life Technology, CA, USA) was used for electrotransformation. BacterioMatch II validation reporter competent cells [ $\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \text{ endA1 hisB supE44 thi-1 recA1 gyrA96 (Nal)}^r \text{ relA1 lac}$  containing F' *lacI*<sup>q</sup> *HIS3 aadA Kan*<sup>r</sup>] (Agilent Technology, CA, USA) were used for chemical transformation and as the reporter strain in the bacterial two-hybrid (B2H) assay. The B2H system used 3.2-kb bait plasmid pBT (Cm<sup>r</sup>) and 4.4-kb target plasmid pTRG (Tc<sup>r</sup>) (Agilent Technology). Superoptimal broth with catabolic repressor (SOC) was used for bacterial growth and transformation (23).

**Cloning of QnrB1 and gyrase.** Primers used for cloning are listed in Table S1 in the supplemental material. Potentially significant amino acid positions for QnrB1-DNA gyrase interactions were selected, based on their effect on the protective activity of QnrB1 (Fig. 1) (16). Site-directed mutagenesis was performed by using a Phusion site-directed mutagenesis kit (Thermo Scientific, MA, USA) to delete or substitute alanines for amino acids Q51, I105, T106, and F111. This method was also used to make a GyrA:S83L (GyrA with the S83L substitution) mutant. Amino acids to be deleted in total loop deletion mutants were determined in a previous study: amino acids 46 to 51 for loop A deletion mutant, amino acids 104 to 113 for loop B deletion mutant, and both for dual-loop deletion mutant (15). Wild-type *qnrB1* and its N- or C-terminal deletion mutants were cloned into plasmid pBT using EcoRI and XhoI; *gyrA* or *gyrB* was similarly cloned into plasmid pTRG using BamHI and EcoRI or BamHI and XhoI, respectively. A GyrB-GyrA fusion protein with a 3-amino-acid (Gly-Ala-Pro) linker (GyrBA) was constructed, and its catalytic functions and interaction with quinolone were confirmed (C. Chen, R. Villet, G. A. Jacoby, and D. C. Hooper, unpublished data). These find-

ings are consistent with those previously reported in which the catalytic function of the same construct was shown (24) and is consistent with the similarity of the crystal structure of a GyrBA fusion and native gyrase (25). This GyrBA construct *gyrBA* was cloned into pTRG using BamHI and XhoI. After sequencing to confirm mutagenesis, pBT-QnrB1, pTRG-GyrA, pTRG-GyrB, and pTRG-GyrBA were transformed into the *E. coli* reporter strain by chemical transformation. Tetracycline (12.5  $\mu\text{g/ml}$ ) was used to select pTRG derivatives, chloramphenicol (50  $\mu\text{g/ml}$ ) for pBT constructs, and both antibiotics for pBT-pTRG cotransformants. Colony PCR was performed to confirm successful cotransformation of both plasmids.

**Bacterial two-hybrid assay.** The B2H assay was performed using the BacterioMatch II two-hybrid system vector system (Agilent Technology) according to the manufacturer's instructions (26). In brief, this system uses a *HIS3-aadA* reporter cassette, and detection of protein-protein interaction is based on transcriptional activation of the *HIS3* reporter gene, which allows growth in the presence of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of *HIS3* protein. When both proteins (bait and target) in the pBT and pTRG plasmids interact, they recruit and stabilize the binding of RNA polymerase at the promoter and activate the transcription of the *HIS3* gene. The reporter strain is able to grow on media lacking histidine and containing 5 mM 3-AT, as transcriptional activation increases expression of the *HIS3* gene product to levels that are sufficient to overcome the competitive inhibition by 3-AT.

After cotransformation was confirmed, 100- $\mu\text{l}$  aliquots of serially diluted cotransformants were spread on agar containing nonselective screening medium (NS medium), selective screening medium containing 5 mM 3-AT (3-AT medium), and 3-AT medium with sub-MIC of ciprofloxacin (CIP medium) or nalidixic acid (NAL medium). The NS medium was made using 1.5% Bacto agar with 1 $\times$  M9 minimal salts (Sigma-Aldrich, MO, USA), 0.4% glucose, 0.2 mM adenine HCl, 1 $\times$  His dropout amino acid supplement (Clontech, CA, USA), 1 mM MgSO<sub>4</sub>, 1 mM thiamine HCl, 10  $\mu\text{M}$  ZnSO<sub>4</sub>, 100  $\mu\text{M}$  CaCl<sub>2</sub>, 50  $\mu\text{M}$  isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), 25  $\mu\text{g/ml}$  of chloramphenicol, and 12.5  $\mu\text{g/ml}$  of tetracycline. The plates were incubated at 37°C for 24 h and at 30°C for an additional 2, 4, and 6 days for NS, 3-AT, and CIP/NAL medium, respectively. The numbers of CFU were then counted.

The strength of the interaction between two constructs was calculated as the ratio of colonies obtained on 3-AT medium compared to those on NS medium. Self-activation was evaluated by performing the B2H assay with an empty vector, e.g., empty pBT vector with pTRG-gyrase subunits or wild-type or mutant pBT-QnrB1 with an empty pTRG vector. The cutoff for noninteraction for the empty vectors was defined as a ratio of  $\leq 0.1\%$ , a value more stringent than that recommended by the manufacturer ( $\leq 1\%$ ). The fold changes were calculated by comparing the ratio for each cotransformant construct with the ratio in the corresponding control with an empty pBT and pTRG-gyrase vector. In the case of CIP medium or NAL medium, the strength of the interaction was compared by ratios of the colony count on CIP or NAL medium to the count on NS medium instead of the fold change because only a small number of colonies of control strains having an empty vector grew in the presence of ciprofloxacin or nalidixic acid. Independent assays were performed at least 5 times, and data for fold changes are presented as median and interquartile range (IQR). MICs of ciprofloxacin and nalidixic acid were determined by broth microdilution using Mueller-Hinton II broth (Becton Dickinson, Franklin Lakes, NJ) with an additional 50  $\mu\text{M}$  IPTG, 25  $\mu\text{g/ml}$  of chloramphenicol, and 12.5  $\mu\text{g/ml}$  of tetracycline in the serially diluted quinolone-containing media.

Fold change of QnrB1 mutant interactions with gyrase was compared with that of wild-type QnrB1. Wilcoxon signed-rank test was performed for statistical analyses using IBM SPSS Statistics for Windows, version 20.0. (Armonk, NY, USA). *P* values of  $< 0.05$  were considered statistically significant.

TABLE 1 Effects of sub-MIC amounts of ciprofloxacin or nalidixic acid on interaction of wild-type QnrB1 with gyrase<sup>a</sup>

Gyrase gene on pTRG	MIC ( $\mu\text{g/ml}$ )		Strength of interaction <sup>b</sup> (%)														
	CIP	NAL	No drug (control, <sup>c</sup> fold change <sup>d</sup> )	CIP ( $\mu\text{g/ml}$ )					NAL ( $\mu\text{g/ml}$ )								
				0.0156	0.0313	0.0625	0.125	0.25	1	2	4	8	16	32			
No gyrase gene (empty)	0.5	64															
GyrA	0.125	16	3.83 (0.01, 383)			0.008								0.001			
GyrA:S83L	0.5	32	2.74 (0.01, 274)			19.3	18.3	0.529						0.176	0.030		
GyrB	0.5	64	12.5 (0.00434, 2,865)			71.9	41.9	1.59						17.7	4.36	0.685	
GyrBA	0.125	8	0.679 (0.000161, 4,219)	0.016	0.001	<0.001				0.040	0.003	<0.001					

<sup>a</sup> The effects of sub-MIC amounts of ciprofloxacin and nalidixic acid in *E. coli* BacterioMatch II validation reporter competent cells with plasmids pBT-QnrB1 (bait plasmid) and pTRG (target plasmid). Abbreviations: CIP, ciprofloxacin; NAL, nalidixic acid.

<sup>b</sup> The strength of interaction represents the ratio (as a percentage) of numbers of colonies in selective screening medium containing 3-amino-1,2,4-triazole to those on nonselective screening medium.

<sup>c</sup> The first value in the parentheses, the control value, represents the strength of interaction in cotransformants having empty pBT and pTRG-gyrase.

<sup>d</sup> The second value in the parentheses, fold change, was calculated by comparing the ratio in each cotransformant with the ratio in the control strain.

## RESULTS

The number of colonies on plates with empty vector controls was always well within the acceptable limits for the procedure (i.e.,  $\leq 0.1\%$ ). QnrB1 interacted with individual gyrase subunits (Table 1); QnrB1 interacted most strongly with GyrB (median fold change, 2,865) and GyrBA (median fold change, 4,219) and less strongly with GyrA (median fold change, 383). In individual experiments, which were performed 9 times, including 3 wild-type cotransformants simultaneously, the GyrB interaction was consistently higher (5.5-fold median [IQR, 3.7- to 8.1-fold]) than that with GyrA and similar to the interaction with GyrBA (1.0-fold [IQR, 0.6- to 1.6-fold]).

The magnitude of QnrB protection of gyrase supercoiling varies inversely with quinolone concentration, suggesting a potential competitive interaction in the binding of the two components (27). We thus evaluated the effects of quinolones on the interaction of QnrB with the gyrase subunits and GyrBA. The interactions of QnrB1 with gyrase components were measured after adding sub-MICs of ciprofloxacin (0.0156 to 0.25  $\mu\text{g/ml}$ ) or nalidixic acid (1 to 32  $\mu\text{g/ml}$ ) to evaluate the effect on the interaction of the two proteins. In comparison to the MIC of ciprofloxacin or nalidixic acid in the presence of plasmids with wild-type QnrB1 and empty pTRG, cotransformants with QnrB1 and either pTRG-GyrA, pTRG-GyrB, or pTRG-GyrBA had MICs that were 4-fold lower, the same, or 4- to 8-fold lower, respectively (Table 1). Since the reporter strain has a chromosomal quinolone resistance *gyrA96* mutation, the lowered MIC is in keeping with the poison mechanism of quinolone action in which increases in quinolone-susceptible target expression increase the likelihood of lethal enzyme-DNA complex formation and consequently lower the MIC (28). In order to adjust for the different MICs of the various cotransformant constructs, interaction signals were measured in the presence of a range of sub-MICs of ciprofloxacin. There were substantial reductions ( $< 0.1\%$ ) in the interaction signals between QnrB1 and GyrA and GyrBA after adding sub-MICs of ciprofloxacin or nalidixic acid (Table 1). In contrast, quinolones somewhat stimulated the interaction of QnrB1 with GyrB. Notably, the interaction of QnrB1 with quinolone-resistant GyrA:S83L was also substantially less affected than with wild-type GyrA. GyrA resistance mutations reduce the binding of quinolones to the gyrase-DNA complex (29). The quinolone-mediated reduced binding of QnrB1 to GyrA was lost in the presence of such a resistance mu-

tation in our study. This finding is consistent with the concept that quinolones and QnrB each competitively affect the binding of the other to gyrase.

The structural features of QnrB1 that are important for its ability to confer resistance to quinolones have been evaluated (15, 16), but mutant QnrB proteins have not been studied specifically for their physical interactions with gyrase. Deletion mutations at the C or N terminus in QnrB1 resulted in a marked decrease in interactions with GyrA, a limited or no effect on GyrB interactions, and an intermediate effect on GyrBA interactions compared with wild-type QnrB1 (Table 2). Each of these mutations, with the exception of the  $\Delta\text{N}5$  (five N-terminal amino acids deleted) mutation, was previously shown to eliminate the resistance phenotype of QnrB1 (16). These findings together suggest that N-terminal and C-terminal amino acids of QnrB1 are more important for binding to domains of GyrA rather than GyrB. In addition, the reduced but residual binding to GyrBA, possibly representing an overall domain structure most closely related to that of gyrase holoenzyme, may reflect binding of QnrB incorrectly positioned to effect resistance.

The QnrB1  $\beta$ -helix structure is interrupted by two external loops, loops A and B, that extend from the helix (15). Deletion of these loops also results in reduced resistance conferred by QnrB1, with the deletion of larger loop B causing the greater reduction in resistance (15). Deletion of loop B and both loops, but not deletion of loop A, significantly reduced the interaction signal with GyrA by three- to fourfold (Table 3). For interaction with GyrB, the QnrB1 loop A deletion had a significant but small reduction (30%), and the loop B and dual-loop deletions were associated with an increased interaction. Surprisingly, the loop A deletion had a substantial reduction (17-fold) in interaction with GyrBA, with little effect of the loop B and dual-loop deletions. Thus, reductions in the overall interaction signal of QnrB1 loop mutants with GyrA exhibited the best correlation with reductions in resistance phenotype seen in these mutants.

Several single-amino-acid changes in QnrB1 loops have also been associated with a reduced resistance phenotype (16). We chose five mutations in loop B (I105A,  $\Delta$ 105I, T106A,  $\Delta$ 106T, and F111A) and one in loop A (Q51A) that varied in their resistance phenotype to test for interaction with gyrase. The largest reductions in mutant interaction signal, although modest (two- to threefold), were seen with GyrA, with lesser or no effects seen with

TABLE 2 Interaction of DNA gyrases with QnrB1 and QnrB1 mutants having C- or N-terminal deletions

QnrB1 mutant (ciprofloxacin MIC [ $\mu\text{g}/\text{ml}$ ]) <sup>a</sup>	No. of expts	Fold change <sup>b</sup>		P value <sup>c</sup>
		Median (ratio <sup>c</sup> )	Interquartile range <sup>d</sup>	
Interaction with GyrA				
Wild type (0.25)	14	404 (1)	317–602	
$\Delta\text{C}5$ (0.032)	5	3 (<0.01)	1–47	<b>0.043</b>
$\Delta\text{C}10$ (0.032)	5	3 (<0.01)	2–32	<b>0.043</b>
$\Delta\text{N}5$ (0.25)	5	22 (0.05)	14–159	<b>0.043</b>
$\Delta\text{N}10$ (0.064)	5	4 (0.01)	1–116	<b>0.043</b>
$\Delta\text{N}20$ (0.032)	6	86 (0.21)	61–201	<b>0.046</b>
Interaction with GyrB				
Wild-type QnrB1	14	3,587 (1)	1,582–10,826	
$\Delta\text{C}5$	5	3,482 (0.97)	2,930–7,482	0.893
$\Delta\text{C}10$	5	2,689 (0.75)	1,610–4,333	<b>0.043</b>
$\Delta\text{N}5$	10	1,451 (0.40)	1,058–2,839	0.241
$\Delta\text{N}10$	5	1,958 (0.55)	1,526–5,684	0.893
$\Delta\text{N}20$	5	4,998 (1.39)	2,180–8,920	0.345
Interaction with GyrBA				
Wild type	9	3,777 (1)	2,368–5,436	
$\Delta\text{C}5$	5	831 (0.22)	788–2,843	<b>0.043</b>
$\Delta\text{C}10$	5	485 (0.13)	320–1,441	<b>0.043</b>
$\Delta\text{N}5$	5	444 (0.12)	149–1,352	<b>0.043</b>
$\Delta\text{N}10$	5	239 (0.06)	20–1,598	<b>0.043</b>
$\Delta\text{N}20$	9	2,547 (0.67)	584–3,340	0.051

<sup>a</sup> The interaction of DNA gyrases with wild-type QnrB1 and the QnrB1 mutants with C- or N-terminal deletions is shown. The ciprofloxacin MICs of *E. coli* M15(pREP4) carrying pQE-60-QnrB1 derivatives in the presence of 100  $\mu\text{M}$  IPTG in a previous study (16) are shown in the parentheses.

<sup>b</sup> Fold changes for the interaction of wild-type QnrB1 in this table and subsequent tables differ slightly from the values in Table 1 since they include data from additional repetitions.

<sup>c</sup> The ratio represents the median fold change in the mutant compared to that in the wild type.

<sup>d</sup> The interquartile range is the 25th percentile to the 75th percentile.

<sup>e</sup> The P value compares the value for the mutant to the value for the wild type. A boldface P value indicates a statistically significant decrease in interaction.

GyrB and GyrBA overall (Table 4). No significant reductions were seen with the Q51A small-loop mutant, which had a wild-type resistance phenotype. Of the loop B mutants, the I105A mutant, which had a four- to eightfold reduction in resistance, and the

$\Delta\text{I}105\text{I}$  mutant were particularly notable, exhibiting a reduced interaction signal with both GyrA and GyrBA, although the difference reached statistical significance only for the  $\Delta\text{I}105\text{I}$  mutant. The T106A mutant, which exhibited wild-type resistance, had no

TABLE 3 Interaction of DNA gyrases with QnrB1 and QnrB1 mutants having loop deletions<sup>a</sup>

QnrB1 mutant	No. of expts	Fold change		P value <sup>c</sup>
		Median (ratio <sup>b</sup> )	Interquartile range	
Interaction with GyrA				
Wild type	14	404 (1)	317–602	
$\Delta\text{loopA}$	5	372 (0.92)	161–844	0.893
$\Delta\text{loopB}$	8	175 (0.43)	128–294	<b>0.012</b>
$\Delta\text{loopA+B}$	8	104 (0.26)	56–230	<b>0.012</b>
Interaction with GyrB				
Wild type	14	3,587 (1)	1,582–10,826	
$\Delta\text{loopA}$	6	2,621 (0.73)	1,523–6,572	<b>0.028</b>
$\Delta\text{loopB}$	5	16,517 (4.60)	6,435–18,909	0.080
$\Delta\text{loopA+B}$	5	14,242 (3.97)	6,797–25,222	0.080
Interaction with GyrBA				
Wild type	9	3,777 (1)	2,368–5,436	
$\Delta\text{loopA}$	5	219 (0.06)	63–455	<b>0.043</b>
$\Delta\text{loopB}$	9	3,285 (0.87)	2,419–6,625	0.441
$\Delta\text{loopA+B}$	9	4,926 (1.30)	3,962–6,450	0.110

<sup>a</sup> Table 3 is set up like Table 2 unless indicated otherwise. See Table 2 footnotes.

<sup>b</sup> The ratio is the median fold change in the mutant compared to that in the wild type.

<sup>c</sup> A boldface P value indicates a statistically significant decrease in interaction.

**TABLE 4** Interaction of DNA gyrases with QnrB1 and QnrB1 mutants having alanine substitutions or deletions in loop A or B amino acids<sup>a</sup>

QnrB1 mutant (ciprofloxacin MIC <sup>b</sup> [μg/ml])	No. of expts	Fold change		P value <sup>d</sup>
		Median (ratio <sup>c</sup> )	Interquartile range	
<b>Interaction with GyrA</b>				
Wild type (0.032)	14	404 (1)	317–602	
Q51A (0.032)	10	218 (0.54)	63–979	0.799
I105A (0.008)	5	125 (0.32)	51–430	0.345
Δ105I (NA)	5	197 (0.49)	135–232	<b>0.043</b>
T106A (0.064)	5	465 (1.15)	250–661	0.893
Δ106T (0.001)	9	156 (0.39)	72–731	0.374
F111A (0.004)	6	1116 (2.76)	240–6,599	0.173
<b>Interaction with GyrB</b>				
Wild type	14	3,587 (1)	1,582–10,826	
Q51A	5	3,180 (0.89)	2,247–6,223	0.500
I105A	8	2,457 (0.68)	2,037–12,010	0.674
Δ105I	5	1,550 (0.43)	1,364–9,722	0.686
T106A	5	7,029 (1.96)	4,164–34,599	0.080
Δ106T	5	5,521 (1.54)	2,550–9,529	0.138
F111A	6	3,229 (0.90)	1,300–16,498	0.345
<b>Interaction with GyrBA</b>				
Wild type	9	3,777 (1)	2,368–5,436	
Q51A	5	6,408 (1.70)	4,961–8,371	<b>0.043<sup>e</sup></b>
I105A	9	2,377 (0.63)	1,321–3,859	0.110
Δ105I	9	2,150 (0.57)	1,492–3,548	<b>0.038</b>
T106A	9	4,494 (1.19)	2,601–5,870	0.214
Δ106T	9	3,463 (0.92)	2,657–5,524	0.953
F111A	5	4,180 (1.11)	3,241–7,086	0.138

<sup>a</sup> Table 4 is set up like Table 2 unless indicated otherwise. See Table 2 footnotes.

<sup>b</sup> Ciprofloxacin MICs of *E. coli* BL21(DE3) carrying pET28a:QnrB1 derivatives in the presence of 100 μM IPTG from reference 16. NA, not available.

<sup>c</sup> The ratio represents the median fold change in the mutant compared to that in the wild type.

<sup>d</sup> A boldface P value indicates a statistically significant decrease in interaction.

<sup>e</sup> The QnrB1:Q51A mutant showed a significantly higher fold change than the wild type.

reduction in interaction signal with GyrA, GyrB, or GyrBA, whereas the Δ106T mutant, which exhibited an 8- to 32-fold reduction in resistance, had a 2.5-fold reduction in interaction signal with GyrA but no reduction with GyrB or GyrBA. In contrast, the F111A mutant, which like the Δ106T mutant had an 8- to 32-fold reduction in resistance, had a similar or increased interaction signal relative to the wild type. Thus, correlations of overall interaction signals of single-amino-acid loop mutants with their resistance phenotypes vary and suggest that key structural determinants of QnrB1 affecting resistance are not mediated by their determining overall physical interaction between QnrB1 and gyrase.

## DISCUSSION

Bacterial two-hybrid systems have been useful tools to evaluate protein-protein interactions within intact cells and to map the interaction domain of these proteins after site-directed mutagenesis (22). Using this system, we investigated the physical interactions between the quinolone resistance protein QnrB1 and its target DNA gyrase and the effects of quinolones and structural changes in QnrB1 on that interaction. This approach has the advantage of studying the protein-protein interactions in the cellular milieu in which they occur.

Purified QnrA has been shown to bind to the holoenzyme and its individual GyrA and GyrB subunits and to alter gyrase binding to DNA (20). Because of the structural similarity of QnrB and

other pentapeptide repeat proteins, such as MfpA, with DNA, one model of the interaction of MfpA with GyrA has positioned MfpA in the GyrA DNA binding site (30). Direct data on the nature of interaction of the pentapeptide repeat proteins and gyrase, however, have been lacking. Notably, MfpA can inhibit gyrase function but is not able to protect gyrase from quinolone action *in vitro* (21), highlighting the need to explain how Qnr proteins can protect gyrase from quinolones at low concentrations without inhibiting its catalytic activity.

QnrB1 exhibited *in vivo* interaction signals with GyrA and GyrB, in keeping with data for the purified proteins, as well as with GyrBA, a catalytically active fusion protein capable of complementing thermosensitive gyrase mutants *in vivo* (Chen et al., unpublished). The GyrA interaction was notable for its complete disruption in the presence of subinhibitory concentrations of ciprofloxacin and nalidixic acid and its dependence on intact C-terminal and N-terminal domains and A and B loops of QnrB1, domains that are necessary for its resistance phenotype. GyrB interactions with QnrB1, in contrast, required substantially higher concentrations of quinolones for disruption and were not reduced by C- and N-terminal and loop deletions of QnrB1, suggesting that the interactions are less specific for the resistance phenotype. Notably, the interaction of GyrBA with QnrB1 was 7- to 11-fold higher than the interaction signal of GyrA alone but retained sensitivity to the presence of quinolones and partial dependence of binding on the C- and N-terminal domains. These findings sug-

gest that domains of GyrB are important for stabilizing the QnrB1 interaction with the holoenzyme but that QnrB1 interaction with the domains of GyrA is most important for affecting quinolone interactions, either by blocking drug access or altering the conformation of the quinolone binding site in GyrA. Furthermore, the interaction of quinolones and QnrB1 with gyrase appears to be competitive. Such a model of competitive binding is supported by prior work (12) indicating the reciprocal relationship of quinolone concentration and Qnr protection of gyrase and specifically by our finding here that the interaction of QnrB1 with the quinolone-resistant S83L GyrA mutant, which demonstrates less quinolone binding (29), was substantially less affected by ciprofloxacin. Thus, GyrA domains determine the ability of quinolones to disrupt QnrB1 binding, a property that is inconsistent with a model in which QnrB acts simply by binding quinolones rather than by disrupting their interaction with gyrase.

The structural determinants of the ability of QnrB1 and other Qnr proteins to confer quinolone resistance have been studied previously (15, 16, 18, 31, 32). Deletion of portions of the C-terminal and N-terminal domains of QnrB1 results in four- to eight-fold reductions in resistance (15). The C-terminal domains are also important for the formation of tail-to-tail Qnr dimers (17). C-terminal deletions have the greatest effect on QnrB1 binding to GyrA and GyrBA with little or no effect on binding to GyrB, which suggests that the C termini themselves, dimerization, or both are important for the overall interaction with GyrA and holoenzyme domains. A similar pattern was seen with N-terminal deletions, although the N20 deletion, in contrast to the N5 and N10 deletions, retained substantial interaction with GyrB and GyrBA, suggesting a mode of binding different from that of the shorter deletions.

The A and B external loops of QnrB and other Qnr proteins from Gram-negative bacteria are required for their ability to cause quinolone resistance (15, 31) and are absent on pentapeptide repeat proteins that do not confer quinolone resistance *in vitro* (17). The effects of loop deletions on QnrB1 binding to GyrA and GyrBA were, with the exception of the loop A deletion mutant interaction with GyrBA, substantially less than the effects of deletion of the C and N termini. Similarly, alanine substitutions or deletions of amino acids of a single loop had limited effects on the interaction signals. Taken together, these findings indicate that despite their importance in determining resistance, loops A and B are not strong determinants of the overall physical interaction of QnrB1 with gyrase. The findings would then further imply that the role of the loops may be instead as key determinants of the proper positioning of QnrB1 to interfere with the binding of quinolones to the gyrase-DNA complex without interfering with catalytic function. One model for such a mechanism could be envisioned to allow QnrB1 to bind specifically in the quinolone binding pocket formed in one particular enzyme conformation in such a way so as to block quinolone access but with sufficiently low affinity to be displaced upon further changes in conformation as the enzyme proceeded through its catalytic cycle.

There is a possibility that chromosome-encoded gyrase subunits could interact with plasmid-encoded gyrase subunits or QnrB1 in this study. However, the two-hybrid interaction signals are predominantly driven by the interaction of the additional domains that are fused to bait and prey plasmid-encoded proteins in order to generate transcription of the HIS3 gene in the Bacterio-Match II two-hybrid system (26). These points are also borne out

in Table 1 with the MIC values of ciprofloxacin and nalidixic acid for the various constructs. Cells with empty vectors have a MIC of ciprofloxacin of 0.5  $\mu\text{g/ml}$ , a finding consistent with the *gyrA96* resistance mutation in this background. In contrast, cells containing plasmids with both wild-type *gyrA* and *gyrBA* have MICs of 0.125  $\mu\text{g/ml}$ , consistent with the known dominance of quinolone sensitivity in diploids, particularly those in which the copy number is greater for the quinolone sensitivity allele relative to the quinolone resistance alleles, and as expected, cells with a quinolone resistance *gyrA* allele have a MIC of 0.5  $\mu\text{g/ml}$ . In addition, any such effect by chromosome-encoded gyrase subunits if present would likely be similar across QnrB mutants in this study.

Two-hybrid assays and other methods of studying protein-protein interactions are limited even with analyses of mutants, because they measure only overall protein-protein binding. Thus, more-definitive information on how Qnr proteins position themselves on gyrase to confer quinolone protection without enzymatic inhibition must await cross-linking or cocrystallization analyses.

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