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Interaction of the Plasmid-Encoded Quinolone Resistance Protein Qnr with *Escherichia coli* DNA Gyrase

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Quinolone resistance normally arises by mutations in the chromosomal genes for type II topoisomerases and by changes in the expression of proteins that control the accumulation of quinolones inside bacteria. A novel mechanism of plasmid-mediated quinolone resistance was recently reported that involves DNA gyrase protection by a pentapeptide repeat family member called Qnr. This family includes two other members, McbG and MfpA, that are also involved in resistance to gyrase inhibitors. Purified Qnr-His_6 was shown to protect *Escherichia coli* DNA gyrase directly from inhibition by ciprofloxacin. Here we have provided a biochemical basis for the mechanism of quinolone resistance. We have shown that Qnr can bind to the gyrase holoenzyme and its respective subunits, GyrA and GyrB. The binding of Qnr to gyrase does not require the presence of the complex of enzyme, DNA, and quinolone, since binding occurred in the absence of relaxed DNA, ciprofloxacin, or ATP. We hypothesize that the formation of Qnr-gyrase complex occurs before the formation of the cleavage complex. Furthermore, there was a decrease in DNA binding by gyrase when the enzyme interacted with Qnr. Therefore, it is possible that the reaction intermediate recognized by Qnr is one early in the gyrase catalytic cycle, in which gyrase has just begun to interact with DNA. Quinolones bind later in the catalytic cycle and stabilize a ternary complex consisting of the drug, gyrase, and DNA. By lowering gyrase binding to DNA, Qnr may reduce the amount of holoenzyme-DNA targets for quinolone inhibition.

Quinolones are synthetic compounds that have been used extensively for treatment of a variety of infectious diseases (12). Increasing use of fluoroquinolones has triggered an increase in bacterial resistance. At present, resistance to fluoroquinolones has been observed even in pathogens such as *Escherichia coli* that had been originally highly susceptible to this class of antibiotics. Previous studies have shown that quinolone resistance arises by mutations in the chromosomal genes for type II topoisomerases, the targets of quinolone action (6), and by changes in expression of efflux pumps and porins that control the accumulation of these agents inside the bacterial cell (29). A novel mechanism of plasmid-mediated quinolone resistance was recently reported that involves DNA gyrase protection by a protein from the pentapeptide repeat family called Qnr.

Topoisomerases are a large group of enzymes found in all organisms and are involved in maintaining the topological state of DNA. Type II topoisomerases such as DNA gyrase cleave both strands of DNA to allow one double-stranded DNA molecule to pass through another, followed by religation of the original strand (18). Gyrase is responsible for the maintenance of steady-state levels of negative supercoiling and is essential for chromosome condensation, transcription initiation, and enzyme complex movement in replication and transcription (2).

Gyrase, first discovered and characterized in 1976 (9), is only found in bacteria, and is distinguished by its ability to wrap DNA around itself, resulting in negative supercoiling. Gyrase consists of a heterotetramer of two 97-kDa gyrase A (GyrA) subunits and two 90-kDa gyrase B (GyrB) subunits. In an ATP-dependent reaction, gyrase binds and cleaves both strands of the first (G or gate) DNA segment in a 4-bp stagger (24, 35, 37), forming a transient gate, through which the second (T or transported strand) DNA segment is wrapped around gyrase and then passed through the gate, resulting in negative supercoiling. The C terminus of the GyrA subunit is responsible for the unique negative supercoiling activity of DNA gyrase, and mutants lacking that C terminus lose the ability to form negative supercoils (15, 17). The N terminus of the GyrA subunit is responsible for cleaving DNA via phosphodiester bonds between the 5′ phosphate group of DNA and two tyrosine 122 groups, one on each GyrA subunit. The N terminus of the GyrB subunit mediates its ATPase activity, and the C terminus of that subunit binds to the GyrA subunit and DNA (15).

Gyrase is an excellent target for quinolones because it is not present in eukaryotic cells and is essential for bacterial growth. DNA gyrase is the primary target for quinolones in gram-negative bacteria due to the higher sensitivity of that enzyme to quinolone inhibition and formation of drug-enzyme-DNA complexes in comparison to the sensitivity of other topoisomerases. The mechanism of quinolone inhibition of DNA gyrase occurs via formation of a cleavage complex, whereby quinolones create a stable, poisonous ternary cleavage complex among gyrase, DNA, and quinolone that blocks progression of the DNA replication fork (11, 39).

Until the first confirmed report in 1998 (21), transmissible resistance to quinolones had been claimed but not validated (5). Martínez-Martínez et al. discovered the gene, *qnr*, on a plasmid from a resistant clinical isolate of *Klebsiella pneumoniae* (21). The plasmid was found to increase resistance to

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ciprofloxacin and other fluoroquinolones four-to eightfold and supplemented resistance due to *gyrA*, *gyrB*, and porin or efflux pump mutations (20). In plasmid-containing strains no change in quinolone accumulation, outer membrane porins, or drug inactivation could be detected, implying a resistance mechanism different from those then known. Cloning and sequencing the *qnr* gene revealed a novel gene whose amino acid sequence (36) shared homology with a heterogeneous family of proteins called the pentapeptide repeat family, two members of which, McbG and MfpA, are involved in resistance to gyrase inhibitors (8, 23). Purified Qnr-His_6 fusion protein was shown to protect *E. coli* DNA gyrase from ciprofloxacin inhibition as measured by an in vitro supercoiling assay. How protection occurred was not known. In the present study, we describe the physical interaction between Qnr and gyrase and its subunits and demonstrate its effects on DNA binding by gyrase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains were routinely grown at 37°C in Luria-Bertani (LB) broth except where noted. Culture plates contained tryptic soy agar (TSA). Selective media contained ampicillin (100 μg/ml) or kanamycin (25 μg/ml) as required. *E. coli* strains used were XL1-Blue (*endA1 gyrA96 hsdR17 lac recA1 relA1 supE44 thi-1* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10*]), DH5α (F$^-$ Φ80d*lacZ*M15(*lacZYA-argF U169 deoR endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1 phoA*), and BL21(DE3) (B F$^-$ *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ (*DE3*).

Cloning of DNA gyrase subunits. The *gyrA* and *gyrB* genes were amplified by PCR from genomic DNA isolated from *E. coli* KL16 by using the primers 5′-CATGGATCCAGCGACCTTGCGAGAG and 5′-CATGGAATTCTCTATTCTTCTTCTGGCTCG and the primers 5′-CATGCTGCAGGTCGAATTCTTATGAC and 5′-CATGAAGCTTCGCCATTAAATATCGA, respectively. The BamHI/EcoRI-digested *gyrA* and PstI/HindIII-digested *gyrB* PCR products were cloned separately into expression vector pTrcHisA (ampicillin resistant; Invitrogen) that contained N-terminal His_6 and Express epitopes (Invitrogen) used to facilitate isolation and detection of the fusion proteins. The correct in-frame placement of *gyrA* and *gyrB* in the pTrcHisA-*gyrA* and pTrcHisA-*gyrB* recombinant plasmids was confirmed by DNA sequencing by the Sanger method (34) (Tufts Core Facility).

Expression and purification of gyrase subunits. The pTrcHisA-*gyrA* and pTrcHisA-*gyrB* plasmids were introduced into *E. coli* BL21(DE3) by electroporation. Transformants were selected on TSA plates containing ampicillin. Transformed cells were grown to mid-logarithmic phase in 500 ml of LB medium with ampicillin, and expression was induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. After cell lysis with 0.02% lysozyme, 0.12% Brij-58, and 1× EDTA-free protease inhibitor mix (Roche Diagnostics, Mannheim, Germany) and centrifugation at 100,000 × *g* for 1 h at 4°C, the supernatant was collected and filtered through a syringe filter to remove any residual cellular debris. A HiTrap Chelating HP column (Amersham/Pharmacia Biotech, Piscataway, N.J.) was equilibrated with 1 ml of 0.1 M $NiSO_4$, followed by 10 ml of TGN_{150} buffer containing 20 mM Tris-HCl (pH 7.5), 10% glycerol, and 150 mM NaCl. The filtered supernatant was loaded onto the column, which was then washed twice with 10 ml of TGN_{150} buffer containing 10 mM imidazole, followed by a wash with 10 ml of TGN_{300} buffer containing 300 mM NaCl and 10 mM imidazole. Successive elutions were performed with 5 ml of TGN_{150} containing imidazole concentrations of 50, 100, 200, and 300 mM. Eluted fractions (1 ml) were dialyzed against TDEN buffer (50 mM Tris-HCl [pH 7.5], 5 mM dithiothreitol, 1 mM EDTA, 150 mM NaCl) overnight at 4°C with several changes in buffer, divided into aliquots, quick-frozen in ethanol-dry ice, and stored at −70°C. Protein concentration was determined in each of the elution fractions by a Protein Assay kit (Bio-Rad). Protein fractions were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with an anti-Express antibody (Invitrogen) as described below.

Gyrase supercoiling assay. DNA supercoiling assays were performed as previously described (36) with the following modifications. The gyrase holoenzyme was reconstituted by preincubating GyrA and B subunits on ice for 30 min. We added 2.8 nM gyrase holoenzyme in a 40-μl reaction mixture containing 0.5 μg of relaxed pBR322 plasmid DNA. Purified Qnr was then added, followed by 6 μM ciprofloxacin and 1.4 mM ATP. The reactions were incubated for 2 h at 25°C for maximal interactions between gyrase and Qnr and loaded on a 1% TAE agarose gel for electrophoresis.

Cloning of *qnr* for gel retardation assays. *qnr* was amplified by PCR from plasmid pMG254 (36) with the primers 5′-GATCGGATCCGATATTATTG ATAA and 5′-GATCGTCGACAATCCGGCAGCACTA. The BamHI/PstI-digested *qnr* insert was cloned into the plasmid vector pQE-100 DoubleTag (Qiagen), which contained N-terminal His_6 and C-terminal Tag100 epitopes used to facilitate isolation and detection of translated fusion proteins.

Expression and purification of Qnr for gel retardation assays. The pQE100-*qnr* plasmid was introduced into *E. coli* BL21(DE3) by electroporation, and transformants were selected on TSA plates containing ampicillin. Transformed cells were grown in 750 ml of LB media with ampicillin to mid-logarithmic phase, and Qnr expression was induced with 1 mM IPTG. Protein isolation was carried as described above. Eluted fractions were dialyzed against 50 mM Tris-HCl (pH 7.5)–10% glycerol overnight at 4°C with several changes in buffer and then divided into aliquots, flash frozen with ethanol-dry ice, and stored at −70°C.

Electrophoresis and Western blotting. Protein purity was assessed by SDS-PAGE by using Ready-Gel Precast gels (Bio-Rad). After electrophoresis, proteins were transferred for 1.5 h onto a nitrocellulose membrane by using a 1-mA/cm^2 electric field and transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol. The membrane was incubated in blocking buffer consisting of 3% bovine serum albumin (BSA) dissolved in TBST buffer (20 mM Tris-HCl [pH 7.5], 140 mM NaCl, 0.25% Tween 20) and incubated with primary antibody diluted 1:5,000 in blocking buffer. The primary antibodies used were anti-Express (for GyrA and GyrB) and Tag100 (Qiagen) (for Qnr). The membrane was then washed three times in 10 ml of TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G1 antibody (Southern Biotechnology Associates, Birmingham, Ala.) diluted 1:3,300 in blocking buffer, and then washed with TBST. Color detection of the positive signal was accomplished by incubation of the membrane in solution containing 0.4 mg of 3-amino-9-ethyl carbazole/ml, 50 mM sodium acetate (pH 5.0), and 0.015% hydrogen peroxide. The Coomassie blue-stained gel and blot images were recorded digitally by using Fotodyne Archiver software.

Gel retardation assays. A standard gyrase assay was performed as described above, with the following modifications. The gyrase holoenzyme was reconstituted by preincubating GyrA and -B subunits on ice for 40 min. The reconstituted gyrase holoenzyme was added to the reaction mix containing 1 μg (4.4 nM) of relaxed pBR322 DNA, followed by Qnr, 6 μM ciprofloxacin, and 1.4 mM ATP. The amount of gyrase subunits and Qnr added was specified for each reaction in the figure legends. The reaction mix was incubated for 25°C for 40 min. The reaction was divided equally for gel electrophoresis analysis by using Coomassie blue staining and for Western blotting. All samples for the gel retardation assays were run under native conditions (without SDS or 2-mercaptoethanol) to preserve protein-protein interactions. The transfer was carried out overnight at 4°C at constant voltage of 7 V, with transfer buffer containing 51 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol to enhance transfer of large protein complexes. The detection of Qnr was performed as described above.

DNA filter-binding assays. DNA filter binding was a modification of the procedure of Roca and Wang (32). The gyrase supercoiling assay was performed as previously described (36) with the following modifications. BSA was at a concentration of 20 μg/ml in the reaction buffer, 2 mM AMPPNP was used to lock the DNA-gyrase complex, and 31.2 ng (0.27 nM) of pBR322 and 1.9 μM Qnr were incubated with 26 nM gyrase. The Qnr used contained only the His_6 tag and not the Tag100 epitope, since the latter was not necessary for this assay. Qnr-His_6 was isolated similarly as for the His_6-Qnr-Tag100 fusion protein used in the gel retardation assays. The plasmid from which this Qnr-His_6 fusion protein was expressed was pQE60, as previously described (36). Before filtration, 200 μl of filtration buffer (1 M NaCl in 1× gyrase reaction buffer without 20 μg of BSA/ml) was added to 40 μl of gyrase reaction, and the total volume was added to a presoaked GF/C glass microfiber filter that had been blocked with 100 μg of sheared herring sperm DNA/ml and placed in a microcentrifuge tube. Centrifugations were carried out at 500 × *g* for 5 to 20 s, and the flowthroughs were collected. Three washes of 200 μl, each consisting of 1× gyrase reaction buffer with 1.5 M NaCl, were added, followed by elutions of gyrase-bound pBR322 with 2 volumes of 200 μl of 0.25% SDS in 50 mM Tris (pH 8.0)–1 mM EDTA. Eluted fractions from the filter-binding assays were separated in 1% TAE (40 mM Tris acetate [pH 7.6], 1 mM 1 mM disodium EDTA) agarose gels by electrophoresis at 70 V for 3 to 4 h. After denaturation and neutralization of the gels, the DNA was transferred onto Hybond N+ nylon membranes (AP Biotech) by capillary blotting overnight, followed by UV cross-linking to fix the DNA onto the membranes. Hybridization was performed with a pBR322 probe that was chemically labeled to HRP by using the ECL direct nucleic acid detec-

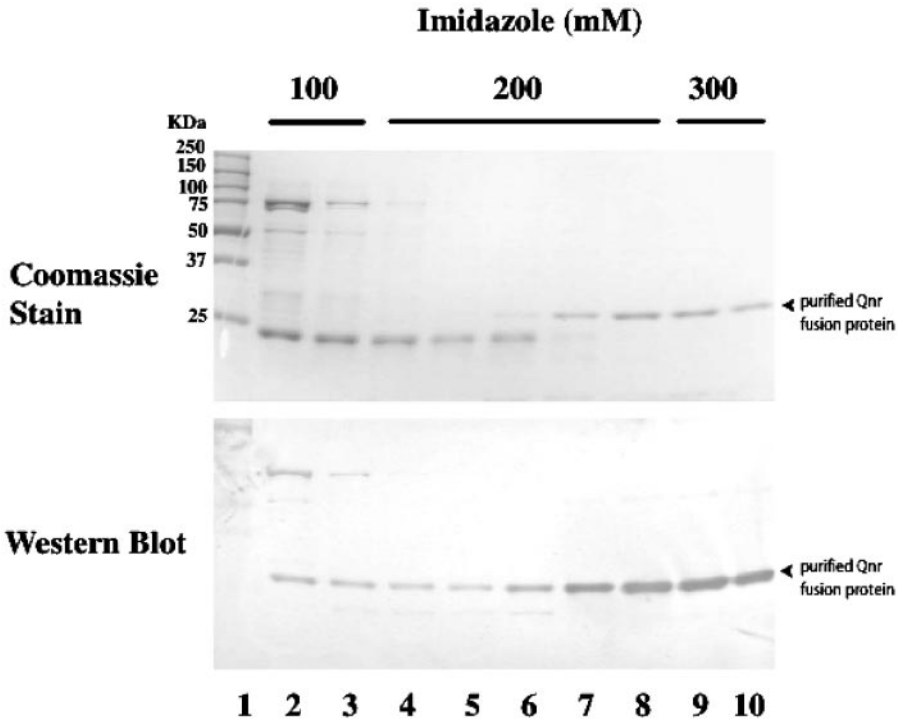

FIG. 1. Purification of the Tag100-Qnr-His_6 fusion protein. A cell extract was prepared from IPTG-induced *E. coli* cells carrying pQE-100 DoubleTag-*qnr* and the Qnr fusion protein was purified as described in Materials and Methods. Proteins (12.5 μl) were analyzed by SDS-PAGE (A) and by Western blotting (B). Lane 1, precision broad molecular weight standards (Bio-Rad); lane 2, the fourth 1-ml fraction eluted with 100 mM imidazole; lane 3, the fifth 1-ml fraction eluted with 100 mM imidazole; lane 4, the first 1-ml fraction eluted with 200 mM imidazole; lane 5, the second 1-ml fraction eluted with 200 mM imidazole; lane 6, the third 1-ml fraction eluted with 200 mM imidazole; lane 7, the fourth 1-ml fraction eluted with 200 mM imidazole; lane 8, the fifth 1-ml fraction eluted with 200 mM imidazole; lane 9, the first 1-ml fraction eluted with 300 mM imidazole; lane 10, the second 1-ml fraction eluted with 300 mM imidazole.

tion system (AP Biotech). Signals from HRP-catalyzed breakdown of luminol were detected by direct exposure to Hyperfilm (AP Biotech).

Binding was calculated from band intensities by using 1D Image Analysis Software (version 3.0; Kodak Digital Science, Rochester, N.Y.). DNA binding by gyrase, Qnr, or both was measured as the intensity of the SDS-eluted DNA. Background values in the presence of Qnr alone were subtracted from those in the presence of gyrase plus Qnr. The change in DNA binding was calculated as the difference between DNA binding by gyrase alone and by gyrase with Qnr, expressed as a percent reduction in DNA binding by gyrase in the presence of Qnr.

RESULTS

Purification of Qnr fusion protein. The *gyrA* and *gyrB* genes were inserted into the pTrcHisA expression vector, adding N-terminal His_6 and Express tags that allowed purification and detection of GyrA and GyrB fusion proteins. The *qnr* gene was inserted into the pQE-100 DoubleTag expression vector, adding an N-terminal His_6 and a C-terminal Tag100 epitope. The Tag100 epitope was added for unique identification of Qnr, since the gyrase subunits both contained His_6 epitopes as well. The fusion protein His_6-Qnr-Tag100 (Qnr) was purified to homogeneity as analyzed by SDS-PAGE and Western blotting (Fig. 1). The 29-kDa Qnr fusion protein was eluted predominantly by the fourth 1-ml wash with 200 mM imidazole and continued to elute with 300 mM imidazole. All subsequent purifications of Qnr yielded similar results. Gyrase subunits were also purified to homogeneity as analyzed by SDS-PAGE and Western blotting (data not shown).

The Qnr fusion protein protected gyrase from quinolone inhibition of supercoiling activity. When various concentrations of Qnr were added to a supercoiling reaction containing 2.8 nM gyrase and 6 μM ciprofloxacin, reversal of supercoiling inhibition was observed with Qnr at 0.27 or 0.54 μM (Fig. 2). No effect on supercoiling was seen in the absence of ciprofloxacin inhibition (data not shown). These results confirm the earlier report of reversal of quinolone inhibition of gyrase supercoiling by Qnr (36). In that study, a Qnr fusion protein with C-terminal His_6 was used. In this case, addition of an N-terminal His_6 and C-terminal Tag100 epitopes did not interfere with the ability of Qnr to protect gyrase from ciprofloxacin.

Qnr binding to DNA gyrase. Since Qnr protects gyrase from inhibition by ciprofloxacin, we analyzed the ability of Qnr to bind to DNA gyrase. The physical interaction between purified Qnr and DNA gyrase was studied by a nondenaturing gel retardation assay and Western blotting. In this method, a standard gyrase assay was performed and analyzed by PAGE under nondenaturing conditions to preserve protein-protein interactions. At the end of the electrophoresis, proteins were transferred to a membrane that was probed with a Tag100 antibody specifically recognizing the fusion protein His_6-Qnr-Tag100 (Qnr). This assay demonstrated binding of Qnr to DNA gyrase by the appearance of a band migrating at the position of gyrase that reacted with the Tag100 antibody in lanes containing

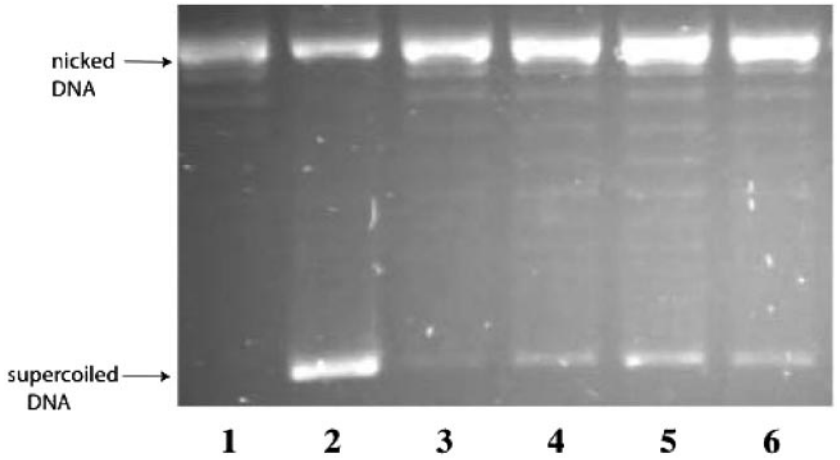

FIG. 2. Tag100-Qnr-His_6 fusion protein protects gyrase from quinolone inhibition of supercoiling. Reactions of 40 μl were analyzed by agarose gel electrophoresis. Reaction mixtures contained 0.5 μg of relaxed pBR322 plasmid DNA (lanes 1 to 5), 2.8 nM gyrase (lanes 2 to 5), 6 μM ciprofloxacin (lanes 3 to 5), and purified Qnr at 0.54 μM (lane 4) or 0.27 μM (lane 5).

DNA gyrase holoenzyme and at least 146 nM Qnr (Fig. 3, lanes 4 to 6).

Since ciprofloxacin binds to a complex of both gyrase holoenzyme and cleaved DNA substrate, thereby converting the enzyme-DNA complex into a poisonous cleaveable complex, we next sought to determine whether Qnr requires the drug, DNA, or ATP to recognize the enzyme. The interaction of Qnr with gyrase holoenzyme was found also to occur in the absence of relaxed pBR322 substrate (Fig. 4, lane 5), as well as without ciprofloxacin and ATP (Fig. 4, lane 6). Thus, the formation of the ternary complex of gyrase, DNA, and ciprofloxacin in the presence of ATP was not necessary for Qnr recognition of gyrase.

When GyrA and GyrB were incubated individually with Qnr, an extra band reacting with Tag100 antibody was also observed. The position of those bands was subunit specific and could be distinguished from one another (Fig. 5, compare lane 2 with lane 4). Since it was determined that Qnr could bind to the individual subunits, we next sought to determine whether such interactions between Qnr and an individual gyrase sub-

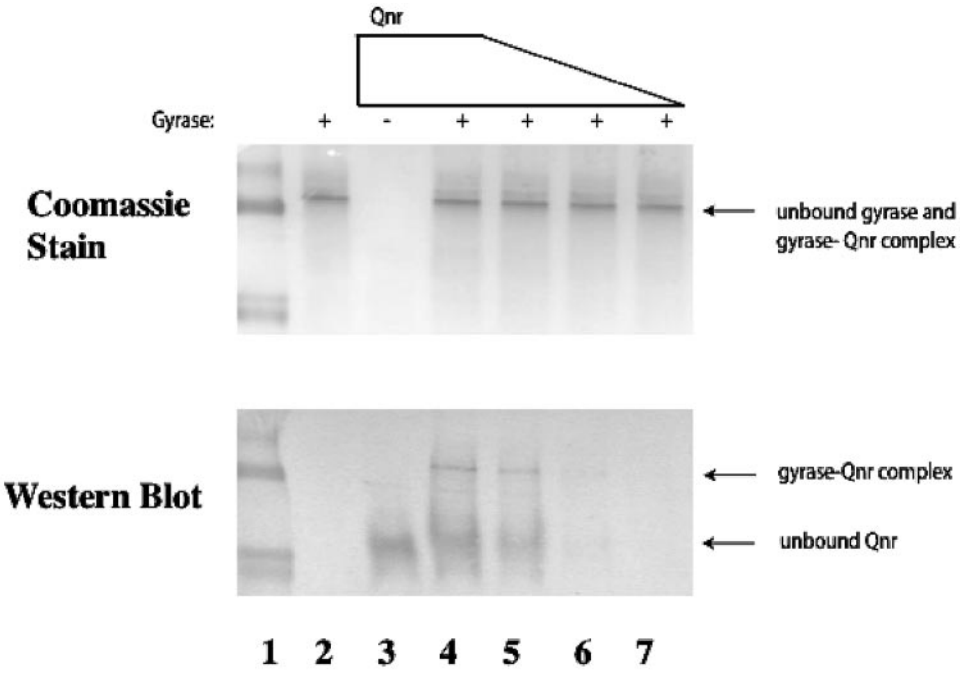

FIG. 3. Interaction of Qnr and DNA gyrase. Reactions of 80 μl (40 μl per gel) were analyzed by native gel electrophoresis (A) and by Western blotting with Tag100 antibody (Invitrogen) (B). Reaction mixtures contained 385 nM gyrase holoenzyme (lanes 2 and 4 to 7) and 6 μM ciprofloxacin (lanes 2 to 7). The assays were performed in the presence of Qnr at concentrations of 1,462 nM (lane 3 and 4), 731 nM (lane 5), 146 nM (lane 6), and 29 nM (lane 7). Prestained broad range molecular weight markers (Bio-Rad) were run in lane 1 to allow comparison of the two gels and not for molecular sizing.

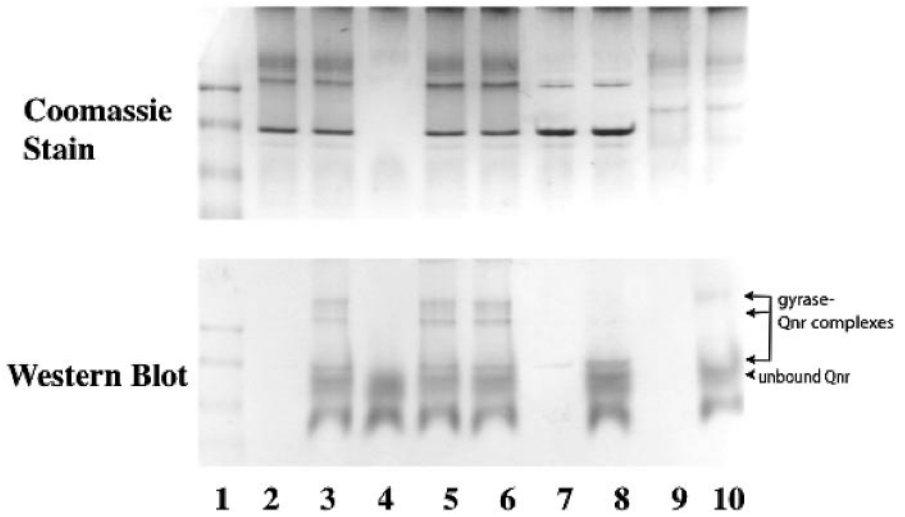

FIG. 4. The formation of the Qnr-DNA gyrase complex does not depend on the presence of ATP, ciprofloxacin, or DNA. Reactions of 80 μl (40 μl per gel) were analyzed by native gel electrophoresis (A) and by Western blotting with Tag100 antibody (Invitrogen) (B). Reaction mixtures contained 411 nM gyrase holoenzyme (lanes 2, 3, 5, and 6), and 212 nM Qnr (lanes 3 to 6). The assays were performed in the absence of DNA (lane 5), ATP (lane 6), or ciprofloxacin (lane 6). Prestained Precision broad range molecular weight markers (Bio-Rad) were run in lane 1 to allow comparison of the two gels and not for molecular sizing.

unit could prevent holoenzyme formation. If Qnr interferes with gyrase holoenzyme formation, it could reduce the levels of available targets for quinolones in the cell. In order to answer this question, Qnr was preincubated with either the GyrA or GyrB subunit, followed by addition of the second subunit. The formation of holoenzyme was not affected by the preincubation of Qnr with either GyrA (Fig. 6, compare lanes 3 and 4) or GyrB (Fig. 6, compare lanes 3 and 5). Furthermore, Qnr bound to the holoenzyme regardless of which gyrase subunit was preincubated with it prior to the formation of holoenzyme (Fig. 6, lanes 3 to 5).

In addition, the removal of individual reaction buffer components from the reaction mixture did not affect the formation of the Qnr-gyrase complex (data not shown), suggesting that none of the individual buffer components was necessary for the interaction. Furthermore, increases of ciprofloxacin of up to 600 μM did not interfere with Qnr and gyrase binding, suggesting that Qnr does not compete with ciprofloxacin for binding sites on gyrase as its mechanism of protection. The replacement of $MgCl_2$ with $CaCl_2$, which has been shown to increase DNA cleavage by gyrase (30), also did not inhibit the formation of the Qnr-gyrase complex.

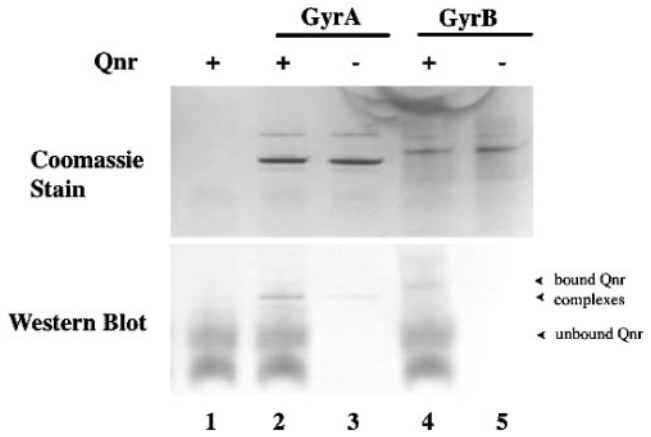

FIG. 5. Qnr binds to DNA gyrase subunits. Reactions of 80 μl (40 μl per gel) were analyzed by native gel electrophoresis (A) and by Western blotting with Tag100 antibody (B). Reaction mixtures contained 770 nM GyrA (lanes 2 and 3), 940 nM GyrB (lanes 4 and 5), 192 nM Qnr (lanes 1, 2, and 4), and 6 μM ciprofloxacin (lanes 1 to 5).

Due to its regularly repeating structure (36), Qnr has been postulated to have a structural or binding function rather than enzymatic properties. In order to ascertain whether the binding to gyrase was specific, several other proteins were incubated with Qnr. Incubation of Qnr with BSA (data not shown), myosin, and carbonic anhydrase did not result in formation of complex-specific distinctive bands (Fig. 7), suggesting that the physical interaction between Qnr and gyrase was specific and not a general property of protein binding by Qnr.

The demonstration of Qnr binding to gyrase holoenzyme and its individual subunits, GyrA and GyrB, provides a physical basis for the protection of gyrase against quinolone inhibition. In order to ascertain further whether this binding could affect an intrinsic enzymatic property of gyrase, such as its binding to DNA, we used a filter-binding assay in which protein-bound DNA but not unbound DNA was retained on filters (19, 31, 32). In this assay, the presence of the nonhydrolyzable analog of ATP, AMPPNP, is crucial to lock the gyrase clamp, since ATP is thought to be vital to enzyme cycling and release of DNA. Only in the presence of AMPPNP is the binding between DNA and gyrase stabilized enough to allow the bound DNA to be retained on the filter after three 1.5 M NaCl washes (data not shown). The addition of 0.25% SDS allows the retained protein to be eluted from the filter with the bound DNA. Fractions were then separated by agarose gel electrophoresis and eluted DNA was detected after Southern blotting with HRP-labeled pBR322 DNA as a probe. The binding of

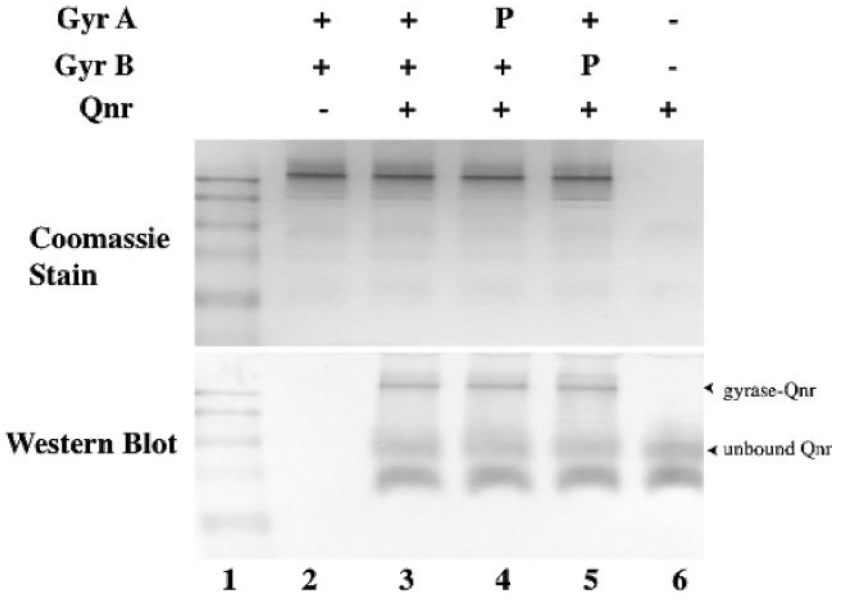

FIG. 6. Qnr does not interfere with DNA gyrase holoenzyme formation. Reactions of 80 μl (40 μl per gel) were analyzed by native gel electrophoresis (A) and by Western blotting (B) with Tag100 antibody. Reaction mixtures contained 385 nM gyrase holoenzyme (lanes 2 to 5) and 192 nM Qnr (lanes 3 to 6). Qnr was preincubated with 770 nM GyrA (lane 4) or 1.2 μM GyrB (lane 5) before addition of the cognate subunit. Prestained Precision broad range molecular weight markers (Bio-Rad) were run in lane 1 to allow comparison of the two gels and not for molecular sizing. P, preincubation with the indicated subunit.

gyrase to DNA appeared to be salt stable: pBR322 DNA was retained even after three 1.5 M salt washes and was present in the SDS elution (Fig. 8, lanes 2 and 3). With the addition of Qnr to the reaction, there was a 60% decrease in the binding of DNA, as indicated by the reduction in the intensity of DNA retained by gyrase and eluted in the SDS wash (Fig. 8, lanes 4 and 5). The percent reduction was calculated from two duplicate experimental lanes. The DNA was therefore released by gyrase in the presence of Qnr and was washed out in the previous 1.5 M salt washes (data not shown). This change was specific for Qnr, since the equivalent concentration of BSA did not cause a decrease in DNA binding by gyrase (data not shown).

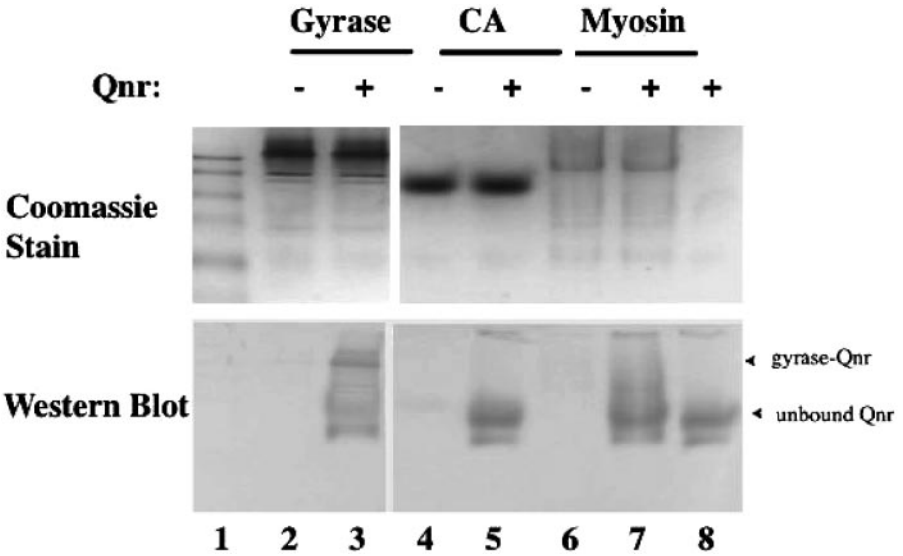

FIG. 7. Qnr does not interact with myosin and carbonic anhydrase. Reactions of 80 μl (40 μl per gel) were analyzed by native gel electrophoresis (A) and by Western blotting with Tag100 antibody (B). Reaction mixtures contained 1.1 μM gyrase holoenzyme (lanes 2 and 3), 192 nM Qnr (lanes 3, 5, 7, and 8), 5 μM carbonic anhydrase (CA) (lanes 4 and 5), and 1.4 μM myosin (lanes 6 and 7). The assays were performed in the absence of DNA, ciprofloxacin, and ATP. Prestained Precision broad range molecular weight markers (Bio-Rad) were run in lane 1 to allow comparison of the two gels and not for molecular sizing.

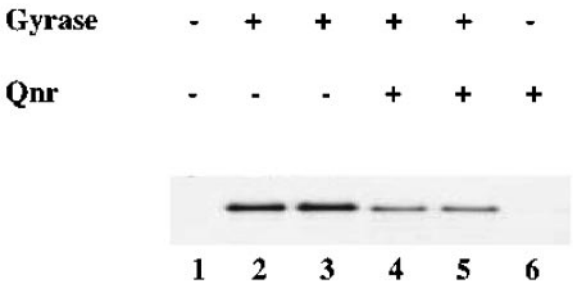

FIG. 8. Qnr decreases gyrase binding to DNA. Gyrase binding to DNA was assessed by filter-binding assay and the elution fractions were analyzed by Southern blotting (Materials and Methods). Reaction mixtures contained 26 nM gyrase (lanes 2 to 5) and 1.9 μM Qnr-His_6 (lanes 4 to 6). No protein was added to lane 1. Lane 3 was a duplicate of lane 2, and lane 5 was a duplicate of lane 4.

DISCUSSION

In previous work, purified Qnr-His_6 was shown to protect *E. coli* DNA gyrase directly from inhibition by ciprofloxacin. Gyrase protection was proportional to the concentration of Qnr-His_6, inversely proportional to the concentration of ciprofloxacin, and shown not to involve quinolone inactivation or independent gyrase activity (36).

In the present study, we have provided a biochemical basis for the mechanism of quinolone resistance. We have shown that Qnr can bind specifically to the gyrase holoenzyme, as well as to its respective subunits, GyrA and GyrB. This binding occurred in the absence of relaxed DNA, ciprofloxacin, or ATP, indicating that binding of Qnr to gyrase did not require the presence of the ternary complex of enzyme, DNA, and quinolone. Therefore, the formation of the Qnr-gyrase complex could occur before the formation of the cleavage complex and did not depend on conformational changes induced by the binding of the enzyme to ATP, DNA, or ciprofloxacin. Further evidence for this hypothesis can be found in the decrease in DNA binding of gyrase when the enzyme was allowed to interact with Qnr. The blot of the eluted fractions indicated that the addition of Qnr to gyrase resulted in a 60% decrease in retained DNA signal (Fig. 8, lanes 4 to 5), suggesting that interaction of Qnr with gyrase alters the DNA-binding properties of gyrase. Therefore, it is possible that the reaction intermediate recognized by Qnr is one early in the gyrase catalytic cycle, in which gyrase has just begun to interact with DNA. Quinolones are thought to bind later in the catalytic cycle in a pocket in which residues of GyrA, GyrB, and DNA are in proximity (7, 41) and to stabilize a ternary complex consisting of the drug, gyrase, and DNA, prior to the actual cleavage of DNA (17). However, the exact nature of the effect of Qnr binding on gyrase and its subunits remains to be determined. Qnr could bind to gyrase and alter the drug-binding pocket conformation such that quinolones recognize the target enzyme less efficiently. A similar mechanism has been implicated in resistance caused by mutations in DNA gyrase (40). These mutations interfere with quinolone binding to DNA gyrase and reduce the probability of formation of the protein-drug-DNA cleavage complex (1). Alternatively, it has been proposed that these mutations destabilize the cleavage complex without changing the probability of drug binding (11). In terms of the mechanism of Qnr action, we cannot exclude either of these two mechanisms. It is also possible that Qnr interferes with quinolone binding and reduces the formation of poisonous cleavage complexes. Qnr could, however, destabilize the cleavage complex without affecting quinolone binding directly, allowing for replication fork progression.

Previous studies have identified several proteins that bind DNA gyrase. Microcin B17, the toxin produced in conjunction with its immunity protein McbG, binds to gyrase (10), whereas CcdB, a toxin encoded on the F plasmid, binds to the GyrA subunit (16). Furthermore, recent work on the parDE postsegregational killing system of plasmid RK2 (14) established yet another inhibitory gyrase-binding protein, ParE (distinct from the ParE subunit of topoisomerase IV), which binds and inhibits gyrase. These three proteins lock gyrase in a cleavage complex formation in a manner similar to ciprofloxacin. Recently, GyrI, an 18-kDa protein encoded on the chromosome of *E. coli*, was shown to inhibit gyrase supercoiling activity by binding to the gyrase holoenzyme and weakly to individual GyrA and GyrB subunits (25, 26). Similarly to Qnr, the formation of the GyrI-gyrase complex did not require ATP, DNA, or ciprofloxacin. Initially, GyrI, which does not have a pentapeptide repeat structure (33), was considered strictly a gyrase inhibitor, because it reduced gyrase supercoiling activity. However, later studies demonstrated that GyrI protects gyrase against toxins, including microcin B17 and CcdB by reducing the binding between gyrase and DNA (3). Furthermore, GyrI was shown to protect against quinolones (4). Evidently, the potential harm from gyrase inhibition is outweighed by the benefits of avoiding cell death by DNA breakage mediated by gyrase inhibitors. It was shown previously that bacterial cells can tolerate some reduction in gyrase activity, whereas only a few double-strand breaks could be lethal (22, 27). The protection afforded by Qnr could have a similar basis. By lowering gyrase binding to DNA, Qnr may reduce the amount of holoenzyme-DNA targets for quinolone inhibition, a mechanism of quinolone resistance that has been demonstrated for topoisomerase IV in *Staphylococcus aureus* (13) and for amsacrine inhibitors of mammalian topoisomerase II (28, 38). Further studies to differentiate these possibilities and to assess further the interactions of Qnr with *E. coli* topoisomerase IV are in progress.

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