The plasmid-borne quinolone resistance protein QnrB, a novel DnaA-binding protein, increases the bacterial mutation rate by triggering DNA replication stress

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Summary

Bacterial antibiotic resistance, a global health threat, is caused by plasmid transfer or genetic mutations. Quinolones are important antibiotics, partially because they are fully synthetic and resistance genes are unlikely to exist in nature; nonetheless, quinolone resistance proteins have been identified. The mechanism by which plasmid-borne quinolone resistance proteins promotes the selection of quinolone-resistant mutants is unclear. Here, we show that QnrB increases the bacterial mutation rate. Transcriptomic and genome sequencing analyses showed that QnrB promoted gene abundance near the origin of replication (oriC). In addition, the QnrB expression level correlated with the replication origin to terminus (oriC/ter) ratio, indicating QnrB-induced DNA replication stress. Our results also show that QnrB is a DnaA-binding protein that may act as an activator of DNA replication initiation. Interaction of QnrB with DnaA promoted the formation of the DnaA-oriC open complex, which leads to DNA replication over-initiation. Our data indicate that plasmid-borne QnrB increases bacterial mutation rates and that genetic changes can alleviate the fitness cost imposed by transmitted plasmids. Derivative mutations may impair antibiotic efficacy and threaten the value of antibiotic treatments. Enhanced understanding of how bacteria adapt to the antibiotic environment will lead to new therapeutic strategies for antibiotic-resistant infections.

Introduction

Bacterial antibiotic resistance is a significant threat to the prevention of infectious diseases (Davies, 1994; Berendonk et al., 2015; Kling et al., 2015; Morehead and Scarbrough, 2018) that generally results from plasmid transfer or genetic mutations (Toprak et al., 2011; San Millan, 2018). High mutation rates can occur in response to environmental factors (Bell and Gonzalez, 2011; Jolivet-Gougeon et al., 2011), including exposure to antibiotics, and increasing mutation rates have certainly contributed to the emergence of antibiotic resistance in the clinical setting (Kohanski et al., 2010; Krasovec et al., 2014). Plasmids drive the horizontal transfer of antibiotic resistance genes, and compensatory mutations can alleviate the fitness cost imposed by the transmitted plasmid (Gama et al., 2018; San Millan, 2018). Furthermore, interactions between plasmids and the bacterial chromosome impact the spread of antibiotic resistance (Gama et al., 2018). Understanding the mechanisms underlying these processes will provide insights into how bacteria adapt to an antibiotic environment and will aid the optimization of antimicrobial strategies.

Quinolones, used as anti-infection agents, are fully synthetic in origin (Mitscher, 2005). For a long time, resistance to quinolones was thought to be caused by mutation of their target genes (encoding DNA gyrase and DNA topoisomerase IV) and/or changes in cell wall permeability. It was assumed that no quinolone resistance genes existed naturally. It is now known that quinolone resistance (Qnr) proteins cause low-level quinolone resistance and facilitate the selection of resistant mutants (Redgrave
et al., 2014). Since the first plasmid encoding a Qnr protein was discovered (Martinez-Martinez et al., 1998; Tran and Jacoby, 2002), a large number of plasmid-borne Qnr proteins have been described, and bacterial species harboring these proteins are ubiquitous (Hooper and Jacoby, 2015; Ruiz, 2018). The mpfA gene, which affects fluoroquinolone susceptibility (Montero et al., 2001), was the first chromosomal qnr gene to be identified. Subsequently, Qnr chromosomal homologs have been identified in many organisms (Jacoby and Hooper, 2013). MpfA has a DNA-mimicking structure and is predicted to interact with DNA gyrase and protect it against drug-induced damage (Hegde et al., 2005). Indeed, previous studies have mainly focused on the interaction of Qnr proteins with DNA topoisomerases (Shah and Heddle, 2014; Rodriguez-Martinez et al., 2016), and the native functions of Qnr proteins are generally thought to be related to their effects on DNA gyrase; however, no direct experiments support this speculation. Several studies have shown that Qnr proteins facilitate the selection of antibiotic-resistant mutations (Redgrave et al., 2014; Vinue et al., 2015). However, the molecular mechanisms by which Qnr proteins facilitate genetic variations that affect susceptibility to the quinolone ciprofloxacin (CIP) are unclear.

In this study, we examined the effect of QnrB on the mutation rate of the Escherichia coli BW25113 strain (Baba et al., 2006) and the Klebsiella pneumoniae KP48 clinical strain (Jiang et al., 2010). Luria and Delbruck fluctuation analyses revealed that QnrB increases the bacterial mutation rate. Furthermore, transcriptomic and whole genome sequencing analyses showed that QnrB upregulates gene expression and increases the number of gene copies near the origin replication (oriC) in both E. coli and K. pneumoniae. In addition to an increase in qnrB mRNA, marker frequency analysis showed an increase in replication origin to terminus (oriC/ter) ratio, indicating DNA replication stress, in both E. coli and K. pneumoniae. Bacterial two-hybrid and in vitro pull-down assays showed that QnrB interacts with the DNA replication initiator DnaA. In addition, microscale thermophoresis (MST) and oriC unwinding assays showed that QnrB increases the affinity of DnaA for single-stranded (ss) oriC and promotes the formation of the DnaA-oriC open complex, which leads to DNA replication stress and an accumulation of mutations, including those promoting quinolone resistance. Overall, our results show that plasmid-borne QnrB promotes bacterial mutation and the selection of quinolone-resistant mutants.

Results

QnrB increases the bacterial mutation rate

Previous studies showed that, in the presence of qnr genes, a variety of mutations are selected to enhance CIP resistance (Cesaro et al., 2008; Vinue et al., 2015); therefore, we hypothesized that Qnr proteins can increase the bacterial mutation rate. To test this hypothesis, we amplified the qnrB gene from the K. pneumoniae multidrug-resistant pKP048 plasmid (Jiang et al., 2010), which carries several resistance-related genes, including qnrB, bla_SPC-2, bla_DHA-1 and armA (Jiang et al., 2010), and constructed E. coli BW25113 strains harboring pQE-qnrB (expressing the His_{6}QnrB protein) or pQE80L (empty vector) as a control. A Luria and Delbruck fluctuation analysis (Luria and Delbruck, 1943; Sarkar et al., 1992) was used to examine mutation of the rpoB gene, related to rifampicin resistance, in the two cell types, and the mutation rate was calculated using the MSS Maximum Likelihood Estimation method (Hall et al., 2009). Expression of QnrB in E. coli resulted in a twofold increase in the mutation rate (Fig. 1A and Table S1). Previous studies showed that overexpression of recombinant proteins may trigger bacterial stress, leading to an increased mutation rate (Hoffmann and Rinas, 2004; Krasovec et al., 2014). However, overexpression of MSMEG_2415 (pQE-msmeg_2415) (Li et al., 2014), a hemerythrin-like protein from Mycobacterium smegmatis, did not affect the mutation rate in E. coli BW25113 cells (Fig. 1A and Table S1), indicating that the increased mutation rate was caused by QnrB specifically, rather than general stress related to overexpression of a recombinant protein.

Next, we examined the effect of QnrB on the mutation rate of a clinical K. pneumoniae strain KP49 carrying the conjugated pKP048 plasmid. To avoid interference by other resistance-related genes, the native pKP048 plasmid in the strain was eliminated by treatment with sodium dodecyl sulfate at 42°C (El-Mansri et al., 2000). The KP49 was a cured strain that had lost its native pKP048 plasmid. Subsequently, the qnrB gene from pKP048 was amplified and cloned into the pACYC vector, which is useful for expressing recombinant proteins in K. pneumoniae (Xu et al., 2017), and strains containing the resulting construct (pACYC-qnrB/KP49), or the empty vector (pACYC/KP49) as a control, were generated. QnrB expression was detected using an anti-His antibody (Fig. S1). The mutation rate of the pACYC-qnrB/KP49 strain was more than twice that of the pACYC/KP49 strain (Fig. 1B and Table S1). Furthermore, as seen in E. coli, overexpression of MSMEG_2415 did not affect the mutation rate of K. pneumoniae cells (Fig. 1B and Table S1), indicating that the observed increase was attributed to QnrB specifically.

Subsequently, we examined the effect of QnrB on the mutation rate in K. pneumoniae cells treated with the quinolone CIP. Previous studies showed that quinolones can increase the mutation rate, at least in part, by inducing SOS responses (Ysern et al., 1990; Cirz and Romesberg, 2006). CIP treatment increased the mutation rate of all test strains (pACYC/KP49, pACYC-msmeg_2415/KP49 and
QnrB is an activator of DNA replication initiation

pACYC-qnrB(KP49) by approximately twofold (Fig. 1B and Table S1). Based on the increase in the overall mutation rate of all test strains, the mutation rate of pACYC-qnrB/KP49 was twofold higher than that of the strains lacking QnrB (Fig. 1B and Table S1). As a possible explanation for this result, QnrB is a DNA mimic protein that affects the action of gyrase and blocks progression of the DNA replication fork, resulting in increased mutation rates.

Next, we examined the effect of another antibiotic, trimethoprim (TRIM), on mutation rates in the pACYC/KP49, pACYC-msmeg_2415/KP48 and pACYC-qnrB/KP49 strains. TRIM causes thymineless death and an increased mutation rate due to an enhanced SOS response (Fonville et al., 2010), irrespective of gyrase function. TRIM treatment increased the mutation rate of all three test strains by approximately 8-10-fold (Fig. 1B and Table S1). Based on the increase in the overall mutation rate of all test strains, the mutation rate of pACYC-qnrB/KP49 was twofold higher than that of the strains lacking QnrB (Fig. 1B and Table S1). Overall, these results show that QnrB expression increases the bacterial mutation rate in a manner that may be independent of gyrase inhibition.

QnrB expression is correlated with upregulation of genes proximal to oriC

To determine the molecular mechanism by which QnrB increases bacterial mutation rates, we compared the global transcriptional responses of K. pneumoniae strains lacking or expressing qnrB. A previous study showed that CIP can induce replication stress and upregulate genes located close to oriC (Slager et al., 2014). Consistent with this finding, an increased transcript peak near oriC was observed in KP48 and KP49 cells treated with CIP, although the increase was more pronounced for the strain expressing QnrB (Fig. 2A). We hypothesized that, if QnrB overstimulates DNA replication, the copy numbers of genes near oriC would also increase. To test this hypothesis, we performed genome sequencing of KP48 and KP49 strains and found that QnrB expression was associated with a higher copy number of oriC-proximal genes (Fig. 2B). Taken together, these results show that QnrB promotes DNA replication stress and leads to upregulation of genes near oriC.

To confirm that QnrB upregulates oriC-proximal genes, we compared the copy numbers and expression levels of
genes near oriC in E. coli strains with and without QnrB expression. Genomic DNA and RNA were purified from cultures of E. coli BW25113 cells harboring a plasmid containing the qnrB gene under control of the araB promoter (Table S2). In this strain, arabinose and glucose induced and inhibited qnrB expression respectively. As seen in K. pneumoniae, QnrB expression upregulated oriC-proximal gene expression in this E. coli strain (Fig. S2), suggesting that it promotes DNA replication stress.

QnrB induces DNA replication stress

To confirm that QnrB promotes DNA replication stress, we performed a marker frequency analysis of a native clinical K. pneumoniae KP48 strain. The strain was treated with various concentrations of CIP (0, 32, 128, or 192 mg/l) to induce different levels of QnrB expression (Da Re et al., 2009; Wang et al., 2009), and the cells were harvested 90 min later. Genomic DNA and RNA were then extracted, and the oriC/ter ratio and qnrB mRNA level were determined. Compared with that in non-treated cells, the oriC/ter ratio of the KP48 strain was significantly higher in cells treated with all three concentrations of CIP (3.61 ± 0.14, 12.21 ± 0.17, 11.74 ± 0.14, and 10.63 ± 0.23 at 0, 32, 128, and 192 mg/l respectively) (Fig. 3A left panel). Similarly, the qnrB mRNA level was increased significantly by CIP treatment of the KP48 cells (1.28 ± 0.12, 9.40 ± 0.14, 15.56 ± 0.16, and 11.74 ± 0.14 at 0, 32, 128, and 192 mg/l respectively) (Fig. 3A right panel). These results show that the CIP-induced increase in qnrB mRNA expression was accompanied by increased DNA replication stress.

To determine whether increased QnrB expression directly leads to DNA replication stress, we compared the oriC/ter ratios in CIP-treated K. pneumoniae pACYC-qnrB/KP49 and pACYC/KP49 strains. After treatment with CIP (128 mg/l) for 90 or 120 min, the oriC/ter ratios in pACYC-qnrB/KP49 cells were 13.58 ± 0.17 and 12.07 ± 0.07, respectively, whereas those in pACYC/KP49 cells were 10.85 ± 0.27 and 8.67 ± 0.09, respectively (Fig. 3C), indicating that QnrB enhances the DNA replication stress caused by CIP. Next, we measured the oriC/ter ratio in K. pneumoniae pACYC-qnrB/KP49 and pACYC/KP49 strains treated with two other antibiotics: TRIM and tetracycline. As mentioned earlier, previous studies showed that TRIM blocks dihydrofolate reductase and causes thymineless death by inducing SOS responses and DNA replication stress (Fonville et al., 2010). As expected, TRIM induced qnrB mRNA expression (Fig. S3) and increased DNA replication stress. The oriC/ter ratios in pACYC-qnrB/KP49 and pACYC/KP49 cells were 11.50 ± 0.05 and 6.71 ± 0.09 respectively (Fig. 3B). Tetracycline, a protein synthesis inhibitor that does not increase the oriC/ter ratio (Slager et al., 2014), did not significantly induce qnrB mRNA expression (Fig. S3). Furthermore, the oriC/ter ratio was comparable in tetracycline-treated pACYC-qnrB/KP49 and pACYC/KP49 cells (4.00 ± 0.23 and 3.56 ± 0.17 respectively) (Fig. 3B). These findings indicate that QnrB can induce DNA replication stress.

Subsequently, we examined the effect of QnrB on DNA replication stress in an E. coli BW25113 strain expressing N-terminally His-tagged QnrB from the pBAD plasmid (pBAD-qnrB), in which the qnrB gene was under the control of the P_{araC} promoter. In this
QnrB is an activator of DNA replication initiation

Strain, L-arabinose and glucose induced and inhibited qnrB expression respectively. The oriC/ter ratio was 5.82 ± 0.04 in cells grown in LB containing L-arabinose, and 2.58 ± 0.16 in cells grown in glucose-containing medium (Fig. 3C). In a negative control strain carrying the empty pBAD33 vector (pBAD/BW25113), the oriC/ter ratio was 2.60 ± 0.04 in L-arabinose-containing medium and 2.32 ± 0.11 in glucose-containing medium (Fig. 3C). Overall, these findings confirm that QnrB promotes DNA replication stress.

QnrB-induced DNA replication stress is independent of DNA gyrase protection

QnrB-mediated replication stress could be related to the inhibition of DNA gyrase activity and the subsequent effect on DnaA-dependent replication initiation (Fuller and Kornberg, 1983; Schnos et al., 1988; Samadpour and Merrikh, 2018). However, as described above, TRIM, which exerts its effects independently of gyrase, induced QnrB-mediated DNA replication stress, resulting in an
increased mutation rate (Figs 2 and 3, and Table S1). Therefore, we tested the hypothesis that QnrB can cause DNA replication stress without the involvement of gyrase. DNA replication stress can affect DNA replication and the segregation of chromosomes (Passerini and Storchova, 2016; Zhang et al., 2018), thus influencing the DNA content of cells (Taylor et al., 2005). Consequently, we examined the genomic DNA and plasmid contents of two E. coli strains expressing (pQE-qnrB/BW25113) or lacking (pQE/BW25113) QnrB. As predicted, the genomic DNA and plasmid contents of the pQE-qnrB/BW25113 cells were 1.8 ± 0.3 and 2.8 ± 0.8 times higher, respectively, than those of the pQE/BW25113 cells. These results indicate a strong correlation between increased plasmid content and QnrB-mediated DNA replication stress.

QnrB has eight complete coils, each of which comprises a right-handed β helix, two extruding loops that are important for protection against CIP, and a C-terminal tail that is required for dimer formation (Shah and Heddle, 2014). We constructed various QnrB mutants containing deletions of coil 2, coil 3, coil 4, both loops, the C-terminal amino acid tail or the translation start site (designated Δcoil2, Δcoil3, Δcoil4, Δloop, ΔC-term or ΔN-term respectively; the corresponding regions are shown in Fig. S4), and expressed the mutant or wild-type QnrB proteins in E. coli. As seen in cells expressing wild-type QnrB (Fig. 4, lane 10), IPTG induction increased the plasmid content of cells expressing QnrBΔcoil3 (Fig. 4 lane 6), indicating an increase in DNA replication stress. By contrast, the plasmid contents of E. coli cells harboring empty vector (pQE80L) or the other QnrB mutants were not increased after IPTG induction (Fig. 4, lane 2; and Fig. S4).

Quinolones form a complex with DNA and gyrase, and the gyrase-cleaved DNA cannot be reeled (Hooper and Jacoby, 2015). QnrB is a DNA mimic that is thought to protect DNA gyrase against quinolone damage and reduce quinolone-mediated DNA cleavage (Merens et al., 2009; Shah and Heddle, 2014). Monitoring plasmid cleavage using agarose gels can be used to detect the protective effects of QnrB on gyrase. Following CIP treatment, plasmid cleavage was observed in E. coli cells expressing pQE-qnrB (Fig. 4, lane 12), but not in those expressing the empty pQE80L vector (Fig. 4, lane 4). In addition, plasmid cleavage was not observed in cells expressing pQE-qnrBΔcoil3 (Fig. 4 lane 8). These results indicate that QnrBΔcoil3 increases DNA replication stress but has no protective effect on gyrase (Fig. 4). In addition, glutathione S-transferase pull-down experiments showed that intact GST-QnrB interacted with His6-GyrA/GyrB, but GST-QnrBΔcoil3 did not (Fig. S4). Overall, these findings indicate that QnrB can increase DNA replication stress regardless of an interaction with gyrase.

QnrB interacts with DnaA

DNA replication is strictly regulated to ensure that genetic material is accurately inherited by offspring. In all organisms, the DNA replication initiation protein binds the replication origin to initiate DNA replication. In E. coli, DnaA forms a complex with oriC, resulting in the unwinding of oriC to initiate DNA replication (Ozaki and Katayama, 2012; Ozaki et al., 2012; Zorman et al., 2012; Katayama et al., 2017; Hansen and Atlung, 2018). Since QnrB was found to upregulate the expression of oriC-proximal genes (Fig. 2) and increase the oriC/ter ratio (Fig. 3), we hypothesized that it might affect the formation of the DnaA-oriC complex. To test this hypothesis, we examined the interaction between DnaA and QnrB using a bacterial two-hybrid system. The coding regions of qnrB and dnaA were cloned into pUT18c and pKT25 to generate pUT18c-qnrB and pKT25-dnaA, respectively, which were co-transformed into the E. coli BTH101 strain. We evaluated the interaction of QnrB with DnaA by monitoring the growth

Fig. 4. QnrB promotes plasmid propagation in a manner that is independent of DNA gyrase protection. E. coli strains harboring pQE80L (empty vector), pQE-qnrB or pQE-qnrBΔcoil3 were cultured to an OD600 of 0.6 in LB at 25°C, and then 0.5 mM IPTG and/or 5 mg/l CIP was added. After culture for 1 h, the plasmids were isolated and dissolved in 50 μl of water. Equal volumes were analyzed by gel electrophoresis. M, DNA marker.
QnrB is an activator of DNA replication initiation.

Next, we investigated how QnrB affects the formation of the DnaA-oriC complex, leading to DNA replication stress. DnaA is a DNA-binding protein that binds to the oriC region and initiates DNA replication (Kaguni, 2011). QnrB is a DNA mimic protein that regulates DNA-protein interactions (Wang et al., 2014). Our results described above indicated that QnrB interacts with DnaA but not oriC (Fig. S6), inferring that QnrB affects the affinity of DnaA for oriC. The Kd of DnaA for ssDNA differs depending on the specific DNA motif (Katayama et al., 2017), so we used MST to measure the affinity of DnaA for various oriC fragments in the presence or absence of QnrB (Wienken et al., 2010; Lippok et al., 2012). First, we examined the effect of QnrB on the affinity of DnaA for double-stranded (ds) oriC and oriC-truncates. In the absence of QnrB, the Kd values for ds-oriC and the ds-oriC duplex-unwinding element-R1 (F1) fragment were 18.6 ± 3.8 nM and 0.13 ± 0.05 μM respectively. In the presence of QnrB, these values were 22.6 ± 1.5 nM and 0.22 ± 0.08 μM, respectively (Fig. S7), indicating that QnrB has little or no effect on the affinity of DnaA for ds-oriC/oriC-truncates (Fig. S7A and B).

Previous studies showed that binding of DnaA to ssDNA plays an important role in DNA replication initiation (Duderstadt et al., 2011; Katayama et al., 2017).

Fig. 5. QnrB interacts with DnaA.

A. Bacterial two-hybrid system analysis showing that QnrB associates with DnaA. E. coli cells were co-transformed with the pUT18c-zip and pKT25-zip plasmids (1) as a positive control. Cells harboring the pUT18c and pKT25 (2), pUT18c-qnrB and pKT25 (3), and pUT18c and pKT25-dnaA (4) plasmid pairs were included as negative controls. Cells harboring pUT18c-qnrB and pKT25-dnaA were used to detect the interaction between QnrB and DnaA (5). Bacteria were cultured on LB containing 100 mg/l ampicillin (Amp) and 50 mg/l kanamycin (Kan) (left panel), or M63 containing 0.2% maltose (right panel).

B. Affinity tagged pull-down assays to evaluate the interaction between QnrB and DnaA. The proteins were induced and purified from BL21(DE3) cells expressing His$_6$-DnaA-FLAG or His$_6$-Myc-QnrB. The purified proteins were diluted 100-fold, and 2 μl samples were used as the input. The interactions were determined by western blotting using an antibody against FLAG (for DnaA) or Myc (for QnrB). Data represent the results of three independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]
DnaA binds the oriC region containing the DUE and DnaA-binding sites (DnaA boxes) (Fig. 6A), unwinds the double-stranded oriC DUE, and provides a ssDNA region for DnaB helicase loading, which is required for further DNA replication (Katayama et al., 2017). In addition, other studies showed that the oriC DUE-M, DUE-R, R1, IHF-binding site (IBS) and R5M motifs may play important roles in binding of DnaA to ssDNA. However, the role of the putative DnaA-trio in E. coli has not yet been identified. Therefore, we synthesized a series of 6-FAM-labeled ss-oriC fragments carrying different motifs (termed DUE and F1-6; Fig. 6A and Table S3), and measured the affinity of DnaA for these fragments in the presence and absence of QnrB. As shown in Tables 1 and 2, QnrB increased the affinity of DnaA for the F2 ss-oriC fragment containing DUE-R, DnaA-trio and IBS, but had no effect on the affinity of DnaA for fragments lacking these motifs (Fig. 6B and Tables 1 and 2). The TTATT sequence in DUE-R is a specific ssDNA-binding site of DnaA, and the DnaA-trio sequence is located within the DUE-R motif (Fig. 6B); therefore, we could not conclude that the DnaA-trio motif is necessary to promote the formation of the DnaA-oriC complex by QnrB.

To elucidate the importance of the DnaA-trio motif to DnaA-oriC complexes, we synthesized four 6-FAM-labeled F2 derivatives containing mutations in the DNA-trio motif (ss-F2m1–4) and performed MST analyses to measure the Kd values of DnaA for these derivatives in the presence of QnrB. When the TTATTAG or TAG sequence was deleted (ss-F2m1 or ss-F2m2 respectively; Fig. 6B), both the DUE-R and DnaA-trio motifs were incomplete, and QnrB had no effect on the affinity of DnaA for these derivatives (Table 2). By contrast, QnrB affected the affinity of DnaA for the ss-F2m3 derivative (Table 2), in which the AG sequence was deleted, resulting in disruption of the DnaA-trio and DUE-R motifs but retention of the TTATT sequence. Similarly, QnrB affected the affinity of DnaA for fragments lacking these motifs (Fig. 6B and Tables 1 and 2).
the ss-F2m4 derivative (Table 2), in which the TA sequence was replaced by CG (F2m4). Overall, these results showed that the DnaA-trio motif is not required for QnrB to promote the affinity of DnaA for oriC (Tables 1 and 2). Our results also showed that QnrB increases the affinity of DnaA for ss-oriC fragments carrying DUE-R and IBS motifs.

The AAA+ domain of DnaA contributes to ssDNA formation during oriC unwinding (Fujikawa et al., 2003; Duderstadt et al., 2011; Richardson et al., 2016); therefore, we examined the effect of QnrB on the ATPase activity of DnaA. Unlike QnrB, DnaA has ATPase activity, but this activity was not affected by QnrB (Fig. S8). Along with the evidence that QnrB does not interact directly with oriC (Fig. S6), these results suggest that QnrB promotes the binding of DnaA to ss-oriC, thereby increasing DNA replication initiation (Keyamura et al., 2007).

Next, we performed an oriC unwinding assay to investigate whether QnrB promotes the formation of oriC open complexes. P1 nuclease specifically degrades ssDNA, and the pUC-oriC plasmid contains a unique AlwNI restriction site in the replication origin (ori) of the plasmid (Fig. 7A). When pUC-oriC was treated with P1 nuclease followed by AlwNI, 2.2 and 1.2 kb fragments were produced (Fig. 7A). In the presence of limited amounts of DnaA, QnrB promoted the formation of the 2.2 and 1.2 kb fragments in a concentration-dependent manner, indicating increased oriC open complex formation (Fig. 7B, upper panel). Similarly, when pUC-oriC was treated with P1 nuclease followed by the SspI restriction enzyme, the recognition site of which is located in the backbone of pUC-oriC, QnrB increased the formation of the expected 2.3 and 1.1 kb fragments (Fig. S9B). In the absence of DnaA, QnrB did not affect oriC unwinding (Fig. 7B, lower panel). Taken together, these results show that QnrB facilitates the formation of an open DnaA-oriC complex, which leads to over-initiation of DNA replication.

**Discussion**

Antibiotic resistance is a global health threat, and plasmid transmission is one way in which bacteria can obtain resistance (San Millan, 2018). Transmitted plasmids cause compensatory mutations that allow bacteria to adapt to their presence; however, the molecular mechanisms underlying this process are not fully understood (Carroll and Wong, 2018). In this study, we found that the plasmid protein QnrB (quinolone resistance protein) increased the bacterial mutation rate by interacting with DnaA, which promoted the DnaA-oriC interaction and formation of the DnaA-oriC interaction, and by supporting the formation of the DnaA-oriC open complex, resulting in over-initiation of DNA replication. Our findings may explain why QnrB promotes the selection of high-level quinolone resistance mutants; the increased mutation rate caused by QnrB could increase the chances of selecting bacterial antibiotic resistance mutants (Rando and Verstrepen, 2007).

Our studies have delineated a molecular mechanism by which plasmids not only spread antibiotic resistance genes, but also enhance the bacterial mutation rate. An advanced understanding of plasmid-mediated bacterial antibiotic resistance will provide insights into new strategies for combatting bacterial infections.

Since the discovery of the first plasmid Qnr (Martinez-Martinez et al., 1998; Tran and Jacoby, 2002), many other plasmid-borne and chromosomal-encoded Qnr proteins have been identified in different strains (Tran and Jacoby, 2002; Hooper and Jacoby, 2015). Qnr proteins are distributed widely in bacteria (Table S4) (Jacoby and Hooper, 2013), suggesting widespread existence suggests that they share important biological functions. DnaA is a highly

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**Table 1.** Microscale thermophoresis analyses of the affinity of DnaA for oriC with and without QnrB.

| DnaA motif | DUE-L | DUE-M | DUE-R | DnaA-trio | R1 | IBS | R5M | QnrB effect |
|------------|-------|-------|-------|-----------|----|-----|----|-------------|
| DUE        | +     | +     | +     | +         | +  | +   | +  | −           |
| F1         | +     | +     | +     | +         | +  | +   | +  | +           |
| F2         | +     | +     | +     | +         | +  | +   | +  | +           |
| F3         |       | +     | +     | +         | +  | +   | +  | −           |
| F4         |       | +     | +     | +         | +  | +   | +  | −           |
| F5         | +     | +     | +     | +         | +  | +   | +  | +           |
| F6         |       | +     | +     | +         | +  | +   | +  | −           |

**Table 2.** Microscale thermophoresis analyses to determine the importance of the DnaA-trio to the DnaA-oriC complex in the presence of QnrB.

| Sequence    | DnaA-trio | QnrB effect |
|-------------|-----------|-------------|
| F2          | GATCTCTTATTAG | +           |
| F2m1        | GATCTC• • • • • • − − | −           |
| F2m2        | GATCTCTTATT• • − − | −           |
| F2m3        | GATCTCTTATTG | −           |
| F2m4        | GATCTCTTATCGG | −           |
conserved DNA initiator protein that binds to the replication origin and initiates DNA replication in all organisms (Katayama et al., 2017; Hansen and Atlung, 2018). In the current study, we found that QnrB not only interacts with DnaA, but affects its affinity for ss-oriC (Ozaki et al., 2008; Katayama et al., 2017), binding to which requires DUE-R as well as the IBS element (Tables 1 and 2, and Fig. S7). Given the conserved structure of DnaA and the common specific recognition motifs within the replication origin oriC, regulation by Qnr may occur in multiple organisms carrying the qnr gene. Furthermore, we found that QnrB results in DNA replication stress (Fig. 3). DNA replication stress is an 'evolvability factor' that microbes use to acquire mutations and evolve into drug-resistant strains. In Streptococcus pneumoniae, antibiotics such as CIP over-initiate DNA replication and trigger bacterial competence, allowing cells to take up antibiotic genes from the environment (Slager et al., 2014). By triggering DNA damage, DNA replication stress plays an important role in cancer development (Di Micco et al., 2006; Bielas et al., 2006; Hills and Diffley, 2014; Wang et al., 2016). These observations suggest that replicative stress-induced mutagenesis may lead to the development of resistant bacteria and cancer cells. Further examination of the mechanisms involved in this process will aid the development of new strategies for the prevention or treatment of bacterial infections and cancer.

Previous studies showed that qnrB expression is controlled by the SOS system and induced by damaging agents (Da Re et al., 2009). In the current study, CIP induced expression in the qnrB (Fig. 3) mRNA. In addition, TRIM, but not tetracycline, induced QnrB-mediated DNA replication stress (Fig. S3). Like CIP, TRIM induced a SOS response (Slager et al., 2014). Furthermore, we found that (i) QnrB leads to DNA replication stress, and (ii) the copy numbers and expression levels of oriC-proximal genes were higher in CIP-treated cells expressing QnrB than in those lacking QnrB (Fig. 2). In addition, QnrB expression increased the oriC/ter ratio in K. pneumoniae and E. coli strains (Fig. 3). We suggest that these effects are caused by QnrB-mediated promotion of an open DnaA-oriC complex, resulting in over-initiation of DNA replication (Fig. 7), which may damage the replication fork and trigger SOS and DNA repair pathways. A recent study also showed that bacteria undergo chromosomal gene mutations to adapt to transmitted plasmids (Loftie-Eaton et al., 2017). Further studies are required to understand the mechanism by which genes involved in the SOS and DNA repair pathways are involved in QnrB-mediated bacterial mutation, and how QnrB-containing plasmids interact with the genome to affect the spread of antibiotic resistance genes.

In conclusion, this study found that the plasmid protein QnrB is a DnaA-binding protein that promotes DNA replication stress, resulting in an increased bacterial mutation rate. Our results explain how QnrB promotes the accumulation of mutations, including quinolone resistance mutators. In addition, our findings suggest that QnrB produces heterogeneity in bacterial populations by increasing the DNA mutation rate and promoting the ability to survive antibiotic exposures.

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Experimental procedures

Bacterial strains and growth conditions

All strains and plasmids used in this study are shown in Table S2. Briefly, E. coli BW25113 strains harboring pQE-qnrB or QnrB mutants and pBAD-qnrB were used for measurements of genomic DNA/plasmid contents and examination of bacterial growth. The E. coli BL21-Gold (DE3) strain was used for protein expression and purification. All E. coli strains were grown in LB supplemented with 50 mg/l kanamycin sulfate or 100 mg/l ampicillin as necessary. The K. pneumoniae KP48 strain harboring the pKP048 plasmid was grown in LB containing 32 mg/l CIP from frozen stock, and the K. pneumoniae KP49 strain, lacking the pKP048 plasmid was grown in LB.

Mutation rate examination

A Luria and Delbruck fluctuation analysis (Luria and Delbruck, 1943; Sarkar et al., 1992; Hall et al., 2009) was performed to determine the genomic mutation rates of K. pneumonia and E. coli. All strains were tested in at least three independent biological replicates. This method is commonly used to measure the spontaneous mutation rate in tested strains, and rifampicin resistance, mediated via mutations in the rpoB gene, was used as a marker. E. coli BW25113 strains carrying pQE80L (as a blank control), pQE-msmeg_2415 (Li et al., 2014) (as a negative control) and pQE-qnrB were used to measure mutation rates. The strains were grown in LB at 37°C overnight, with mixing at 200 rpm. The cultures were reinoculated at 1:100 in LB to an OD \(_{600}\) of 0.5, diluted 100-fold and then reinoculated at 1:100 in 40 ml of LB. After shaking for 6.5 h, a 200-ml aliquot of bacteria was collected and resuspended in 2 ml of fresh LB. Next, a 50-μl aliquot of the resuspended culture was plated onto LB plates containing 50 mg/l rifampicin and the number of colonies was counted after incubation at 37°C for 36 h. The initial inoculation number and mutation number were recorded as N0 and M0 respectively.

In K. pneumonia, the pACYC/KP49 (Blank), pACYC-msmeg_2415/KP49 (negative control, Ctr) and pACYC-qnrB/KP49 strains were used to measure the mutation rate. The strains were grown in LB at 37°C overnight. The cultures were reinoculated at 1:50 in LB medium to an OD \(_{600}\) of 0.5, and then reinoculated at 1:400 in 40 ml of LB medium. Mutation rates were examined following 12 h of growth in the presence of antibiotic. The onefold minimum inhibitory concentration of antibiotic was used for the mutation rate assay. The initial inoculation and mutation colonies were recorded and the final culture number and mutant colonies were counted. MSS Maximum Likelihood Estimation methods (FALCOR, http://www.keshavsingh.org/protocols/FALCOR.html) (Hall et al., 2009) were used to calculate the mutation rates. The mutation rate was compared in strains with and without QnrB under different treatments. Mutation rates were statistically compared using a two-tailed t-test.

Construction of expression vectors

The dnaA, gyrA, gyrB, hupA and hupB genes were amplified from E. coli BW25113 genomic DNA. The qnrB gene was amplified from K. pneumoniae KP48 strain harboring the pKP048 plasmid, a gift from Dr. Jiang (Jiang et al., 2010), and the PCR fragment was cloned into the pQE80L, pGEX-5x-3, pACYCDuet1 and pBAD33 vectors. Deletion mutants, including qnrBΔcoil2, qnrBΔcoil3, qnrBΔcoil4, qnrBΔloop, qnrBAC-term and qnrBAN-term, were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA). The corresponding deletion positions in qnrB are shown in Figure S4. The primers used in this study are shown in Table S3. The constructed vectors were confirmed by DNA sequencing (BGI Company, China).

Protein expression and purification

QnrB, GyrA, GyrB, DnaA and MSMEG_2415 were overexpressed in E. coli BL21-Gold(DE3) cells. The cells were grown at 37°C, and protein expression was induced by the addition of 0.5 mM IPTG when the cells reached an OD \(_{600}\) of 0.5. Subsequently, the cells were collected by centrifugation after 4 h of extended growth at 23°C. Protein purification and concentration measurements were performed as described previously (Zawilak-Pawlik et al., 2006; Harper and Speicher, 2011; Tao et al., 2013). Details of the protein purification protocol are available on request. The quality of the purified QnrB and DnaA proteins is shown in Figure S10.

Isolation and curing of the large plasmid from KP48

The K. pneumoniae KP48 strain was cultured in LB at 37°C overnight. The cells were then reinoculated at 1:500 into LB containing 5% sodium dodecyl sulfate and grown at 42°C for 10–12 h. Three to five rounds of inoculation and growth were performed. Next, a 50 μl aliquot of culture was diluted 10,000-fold and plated onto LB plates. Colonies that had lost pKP048 were detected by colony PCR using primers specific for QnrB (see Table S2).

RNA sequencing

Overnight cultures of KP48 and KP49 cells were diluted 1:100 in LB medium and treated with 256 mg/l CIP for 30 or 90 min when growth reached an OD \(_{600}\) of 0.25. The cells were then harvested by centrifugation (11,000 g for 10 min) and frozen. RNA extraction, library preparation and whole-mRNA sequencing were performed by Novogene (Beijing, China). For RNA sequencing, libraries were generated using the NENext Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA). Sequencing of cDNA libraries was performed with an Illumina HiSeq platform, and paired-end reads were generated. The reference genome and gene model annotation files were downloaded from the genome website. Building of the reference genome index and alignment of clean reads to the reference genome were performed with Bowtie 2–2.2.3 (Langmead and Salzberg, 2012). The results obtained at different times showed a very high correlation (R \(^2\) > 0.9). The FPKMs (Fragments Per Kilobase of transcripts sequence per Millions base pairs) of genes for the 30 min CIP-treated group were used when the FPKMs of genes showed the same trend at 30 and 90 min.
DNA sequencing

For DNA sequencing, overnight cultures of KP49 and KP48 cells were diluted 1:100 in LB containing CIP and grown to an OD_{600} of 0.25 (approximately 60 min). The cells were then harvested by centrifugation (11,000 g for 10 min) and frozen. DNA was fragmented by ultrasonication, and library preparation was performed using the Illumina TruSeq DNA Sample Preparation Kit (Illumina, USA). Pair-end sequencing was performed on an Illumina HiSeq 2000 system (Illumina, USA). Short reads were assembled using SOAPdenovo (http://soap.genomics.org.cn), a genome assembler specifically developed for next-generation short-read sequences (Luo et al., 2012). The algorithm was sensitive to sequencing errors, such that low-quality reads were filtered, and high-quality reads were used for de novo assembly. The sequences were filtered for low-quality reads using the DynamicTrim Perl script within SolexaQA. The SOAP GapCloser package was used to close gaps after assembly. The sequences were filtered for low-quality reads using the DynamicTrim Perl script within SolexaQA. The SOAP GapCloser package was used to close gaps after assembly. The genome sequence was uploaded to the Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008) server for annotation.

E. coli BW25113 strains harboring pBAD33 or pBAD-qnrB were grown in LB at 37°C overnight. The cultures were then inoculated at 1:100 in LB supplemented with 1% glucose (w/v) at 30°C. At an OD_{600} of 0.25, the cells were collected by centrifugation at 20°C, and immediately resuspended in LB. L-arabinose (0.5%) was then added to the resuspended cells, and the cultures were grown with aeration at 30°C for 30 min. Next, 10^8 bacteria were collected and used for genomic DNA isolation. DNA sequencing was performed as described above.

A number of windows were used to split the reference genome, and these values were used to compute the graphs that plot information across the reference genome. Basically, reads falling in the same window were aggregated in the same bin. The higher the number, the bigger the resolution of the plots but also the longer the data processing time. By default, 400 windows were used. Visualization and statistical analysis were performed using R statistical software (http://www.r-project.org/).

Real-time quantitative PCR analyses of the oriC/ter ratio and qnrB mRNA levels

Overnight cultures of K. pneumoniae KP48 and KP49 strains were diluted 1:100 in LB containing CIP (0, 32, 128 or 256 mg/l) and grown at 37°C for 90 min. Other samples were treated with CIP (64 mg/l) for 90 or 120 min, or TRIM (0.7 mg/l) or tetracycline (1 mg/l) for approximately 90 min at 37°C. Cells at an OD_{600} of 1.0 (approximately 10^8 bacteria) were collected and used for extraction of genomic DNA.

E. coli BW25113 cells bearing plasmids were grown with aeration in LB supplemented with 1% glucose (w/v) at 37°C. At an OD_{600} of 0.3, the cells were collected by centrifugation at 20°C and immediately resuspended in LB. Glucose (1%) or L-arabinose (0.5%) was then added to equal portions of the resuspended cells, and the cultures were grown with aeration at 30°C for 40 min.

Genomic DNA was extracted from K. pneumoniae and E. coli cells using the TIANamp Bacteria DNA Kit. The oriC/ter ratio and qnrB mRNA levels were determined by quantitative PCR as described previously (Simmons et al., 2004; Felczak and Kaguni, 2009), using the primers listed in Table S3, and data analyses were performed as described previously (Simmons et al., 2004; Felczak and Kaguni, 2009). Each 20 μl sample consisted of 50 ng of cDNA, 2 pmol of each primer (Table S3) and 10 μl of 2 × SYBR Green Supermix. Amplification was performed in an iQ5 Real-Time PCR Detection System, using the following program: 95°C for 1.5 min; 40 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 15 s; and then 72°C for 6 min. The real-time PCR results were calculated as the mean ± SD of three replicates. Overnight cultures of the K. pneumoniae qnrB^+KP48 strain were used as a reference.

Examination of plasmid DNA yields in E. coli

Recombinant bacterial strains harboring individual vectors were grown at 37°C overnight in 5 ml of LB containing ampicillin (100 mg/l). Next, 50 μl aliquots of the cultures were cultured to an OD_{600} of 0.6 at 37°C in 5 ml of LB broth containing ampicillin (100 mg/l) and split into two identical sets of cultures. One set of cultures was cultured for 1 h with or without CIP (5 mg/l), and the other set was cultured for 1 h with IPTG (1 mM) and with or without CIP (5 mg/l) at 25°C. All cultures were subjected to plasmid DNA isolation using the Plasmid Miniprep Kit. Equal volumes (15 μl) were directly analyzed by gel electrophoresis or digested with BamHI and then analyzed by the same method.

Pull-down and co-immunoprecipitation assays

For GST pull-down assays, mixtures of His_{6}-GyrA/GyrB and GST-QnrB were incubated for 3 h at 25°C, and then incubated with glutathione-Sepharose beads for 4 h at 4°C. After three washes of the beads with buffer comprising 50 mM Tris-HCl (pH 8), 150 mM NaCl and 0.1-0.5% NP-40, the samples were resuspended in SDS-PAGE loading buffer and boiled for 5 min before western blotting analysis. Assays were performed with 1 × TBS containing 1 mM PMSF for FLAG-QnrB and Myc-DnaA.

Microscale thermophoresis

MST experiments were performed using a Monolith NT.115 instrument (NanoTemper Inc., Germany), as described previously (Seidel et al., 2012). Fluorescent 6-FAM-labeled oligonucleotides were purchased from Sangon Biotech (Shanghai, China). The labeled DNA sequences are shown in Table S3. Once dissolved in water, the oligonucleotides were stored in light-protected vials at −20°C. An infrared laser with 30% intensity setting. The labeled DNA sequences are shown in Table S3. Once dissolved in water, the oligonucleotides were stored in light-protected vials at −20°C. A thermal gradient of 4°C was set up over a period of 30 s using an infrared laser with 30% intensity setting. The labeled oriC fragments of DNA (200 nM) were incubated with purified ATP-DnaA at a gradient concentration (ranging from 0 or 0.0012 to 80 μM) in buffer (20 mM HEPES, 50 mM NaCl, 10 mM MgCl₂, pH 7.6). The MST assay was performed with 30% LED power and 80% MST power. The Kd Fit function of the Nano Temper Analysis Software (v.1.5.41) was used to fit curves and calculate the value of the dissociation constant.
(Kd). A positive effect was assigned when the ratio of Kd values was larger than 1.5.

oriC unwinding assays

The oriC unwinding assays were performed as described previously (Keyamura et al., 2007), with a few modifications. Briefly, the required amounts of QnrB were incubated for 5 min at 37°C in buffer (20 μl) containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 8 mM DTT, 100 mg/l BSA, 10% glycerol, 150 mM potassium glutamate, 30 ng of HU protein, 1 mM ATP and the pUC-orlC plasmid (500 ng; 0.22 pmol) in the presence or absence of DnaA (0.04 nmol), and then incubated for a further 3 min in the presence of P1 nuclease (10 U, Sigma). After chloroform extraction and ethanol precipitation, the plasmids were digested with AlwNI or SspI and the DNA fragments were analyzed by electrophoresis on a 1% agarose gel.

Bacterial growth on LB agar plates

Growth of E. coli BW25113 cells harboring the empty pQE80L vector (pQE-qnrB) was evaluated on LB plates supplemented with or without 0.2 mM IPTG at 28°C. Each spot was inoculated with a 2-μl aliquot from a 10-fold dilution series of stationary cells (10−1-, 10−2-, 10−3- and 10−4-fold). Photographs were taken after incubation at 37°C for 24 h.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0c software. Significant differences were determined by t-tests.

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Author contributions

XL, YZ, XZ, XH, YZ, DL performed all the experiments. KM designed the study and performed the bioinformatics analyses. KM wrote a preliminary draft of the manuscript. KM and AM revised the manuscript. All authors reviewed and approved the manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting information section at the end of the article.