Mechanisms of Quinolone Resistance in *Vibrio parahaemolyticus* Strains Isolated from Imported Shrimp

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Abstract

Sixty-one quinolone-resistant strains of *Vibrio parahaemolyticus* were isolated from 247 shrimp samples. These isolates had MICs of 256 and 4-8 µg/mL for nalidixic acid and ciprofloxacin respectively. Template DNA of the 61 isolates were screened by PCR for the quinolone resistance genes. Purified PCR amplicons of *gyr*A, *gyr*B and *par*C from these isolates were sequenced and analyzed for point mutations that confer resistance to these antibiotics. Point mutations in the quinolone resistance determining region (QRDR) of GyrA at positions 68, 83, 85 and 89 and in ParC at positions 85 as well as in the non QRDR of GyrA at positions 48 along with 4 different point mutations in GyrB at positions 311, 354, 360 and 374 conferred resistances to these antibiotics. Structural analysis were undertaken to determine the role of these four novel point mutations in *V. parahaemolyticus* at codons 48, 68, 83 and 89 on GyrA in enhanced MICs to quinolone antibiotics. Homology analysis indicated that all four mutations are localized in the vicinity of quinolone binding and DNA binding major grooves. These four mutations are known to play pivotal roles in readjusting the position of α-helices, stabilization of GyrA, loss of electrical charges or the formation of A2 dimers resulting in enhanced MICs to the quinolone antibiotics. Ethidium bromide uptake experiments indicated higher efflux pump activities in drug resistant *V. parahaemolyticus* than their sensitive counterparts. Our results indicate that imported shrimp is a reservoir of quinolone-resistant *V. parahaemolyticus*.

Key Words: Quinolone Resistance, *Vibrio parahaemolyticus*, Shrimp

1. Introduction

Shrimp is the most popular seafood in the United States and 80% of the shrimp is imported from Asia (Biao X, 2007). Infectious diseases curtail shrimp production by up to 70%, thus antibiotics such as fluoroquinolone, tetracyclines, macrolides and sulfanomides, are used in shrimp aquaculture ponds to reduce the incidence of diseases (Biao X, 2007; Bondad-Reantaso et al., 2005). The inadvertent abuse of these antibiotics in shrimp aquaculture may select bacteria resistant to multiple antibiotics. Since quinolones are drugs of choice for the treatment of gastroenteritis caused by *V. parahaemolyticus,* the ingestion of quinolone-resistant *V. parahaemolyticus* in shrimp may reduce the efficacy of quinolone drugs in clinical treatment of this infection. Additionally, resistant bacteria may transfer antibiotic resistance determinants to other pathogenic bacteria. Thus, U.S. regulatory agencies, such as United States Food and Drug Administration (USFDA) and Centers for Disease Control and Prevention (CDC), want to limit the prevalence of quinolone-resistant bacteria in food-producing animals to protect the efficacy of the quinolone drugs for human use (USFDA, 1997).

Several different kinds of mechanisms confer bacterial resistance to quinolone antibiotics (Baranwal et al., 2002; Ghosh et al., 1998; Hopkins et al., 2005; Karczmarczyk et al., 2011; Poole, 2000; Ruiz, 2003; Yoshida et al., 1991). In Gram-negative bacteria, resistance to quinolones is mainly due to chromosomal mutations in the quinolone resistance determining region (QRDR) of genes encoding the drug target enzymes (DNA gyrase and topoisomerase IV) and a majority of mutations in these bacteria have been found within the N termini of GyrA (between codons Ala67-Gln106), GyrB (between codons Asp426-Lys447) and ParC proteins (between codons 83 and 87). Recently several reports have indicated resistance through efflux pumps (Poole, 2000; Webber and Piddock, 2003) that are involved in the extrusion of toxic substrates from within cells into external environment and plasmid-mediated quinolone resistance (Hopkins et al. 2005; Karczmarczyk et al. 2011). However, limited information is available on the characterization of quinolone resistance mechanisms in *V. parahaemolyticus*. Kitiyodom et al. (2010) reported that a point mutation in the QRDR of GyrA at position 83 (Ser→Ile) conferred resistance to quinolones in four isolates of *V. parahaemolyticus* (Kitiyodom et al., 2010). None of these isolates had mutations in the QRDR of the *par*C gene. Another report indicated that point mutations in *gyr*A at position 83 (Ser→Ile) and *par*C at position 85 (Ser→Leu) conferred resistance to quinolones in seven strains of *V. parahaemolyticus* isolated from food samples (Liu et al., 2013). Since limited information is available on the prevalence and characterization of quinolone-resistant *V. parahaemolyticus* in imported shrimp, we decided to investigate the prevalence of quinolone-resistant *V. parahaemolyticus*.

2. Materials and Methods

2.1. Isolation, characterization and identification of the bacteria:

Unless otherwise stated, bacteria were isolated from 247 farm raised, frozen shrimp (*Penaeus monodon*) samples imported from Thailand and purchased from retail grocery stores in Little Rock, AR. Typically, 2 g of thawed shrimp sample was

homogenized with 10 mL of alkaline peptone water (APW) in a stomacher (Tekmur Co., Cincinnati, OH) for 5 min (Ragunath et al., 2009). The homogenate was incubated overnight at 37^{o}C. One loopful of enriched sample was streaked on thiosulfate citrate bile salts sucrose (TCBS agar, Remel, Lenexa, KS). Presumptive positive colonies of *V. parahaemolyticus* were biochemically characterized using the Vitek 2 compact system (bioMerieux Vitek, Hazelwood, MO). The isolates were further confirmed based on the fatty acid methyl ester analysis (MIDI, Newark, DE). All isolates were stored in LB containing 20% glycerol at -70^{o}C.

2.2. Determination of antimicrobial susceptibility and minimum inhibitory concentrations (MICs):

Antimicrobial susceptibility of each *V. parahaemolyticus* isolate was determined using a disk diffusion assay (Bauer et al., 1966). The susceptibility of each isolate was determined as per the criteria specified by the Clinical and Laboratory Standards Institute (NCCLS, 2002). The concentration ranges for ciprofloxacin were 0.125 to 16 µg/mL and for nalidixic acid they were 4 to 512 µg/mL.

2.3. Genomic DNA extraction:

Genomic DNA was extracted from cells grown overnight at 37^{o}C with the QIAamp DNA Mini kit (Qiagen, Valencia, CA).

2.4. Primer design and detection of quinolone-resistance genes by PCR:

The presence of quinolone-resistance genes (*gyr*A,B, *par*C and *qnr*A,B,S,) was investigated in the template DNA by PCR (Table 1). The detection and amplification of the quinolone-resistance genes were carried out as detailed elsewhere (Nawaz et al., 2012; Shakir et al., 2012).

2.5. Detection of mutations in the quinolone resistance determining region (QRDR):

The target genes (*gyr*A-B, *par*C, *qnr*A,B,S) were amplified by PCR and purified by the QIAquick PCR purification kit (Qiagen). The mutations (Table 2) of the quinolone resistance determining regions (QRDR), the amino acid sequences of the QRDR were analyzed by BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared to the protein sequences of GyrA, GyrB, ParC and ParE proteins from the quinolone-susceptible strains deposited in the GenBank. s

2.6. Determination of efflux pump activity in the quinolone-sensitive and resistant strains of *V. parahaemolyticus*:

Efflux pump activity was determined as described previously (Baranwal et al., 2002; Karczmarczyk et al., 2011). For determination of the presence of active efflux, ethidium bromide at a final concentration of 2 µg/ml was added immediately before the reading in a Synergy 2 Multi-Mode Microplate Reader (BIOTEK Instruments, Winooski, VT). Fluorescence was read every 1 min at excitation 530 nm and emission 600 nm for the next 30 min. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), an inhibitor of the efflux pump (Baranwal et al., 2002; Webber and Piddock, 2003), at a final concentration of 100 µM, was added to the assay mixture. The natural fluorescence of the cells was subtracted and the fluorescence intensity was expressed in relative fluorescence units (RFU). All experiments were performed in triplicate.

2.7. Homology modeling:
The homology model was generated using an automated PHYRE2 server and a Swiss homology modeling suite (Arnold et al., 2006; Kelly and Sternberg, 2009) and another homology model was also constructed using Win Coot (Emsley et al., 2009). To generate the homology model we selected a high resolution crystal structure of *S. aureus* gyrase (pdb:2XCS) for the *V. parahaemolyticus gyr*A-B structure. The PHYRE2 homology modeling program then built a *V. parahaemolyticus gyr*A model based on the amino acid sequence identity and structural similarity (Laponogov et al., 2009).
The generated model was energy minimized in Sybyl (www.tripus.com) and analyzed by superimposing known crystal structures of bacterial DNA topoisomerase and DNA gyrase structures (Bax et al., 2010; Okuda et al., 1999; Ouabdesselam et al., 1995; Yoshida et al., 1991). After revising the model, the relevant mutation was introduced in the DNA GyrA structure according to a quinolone-resistant *V. parahaemolyticus* strain DNA sequence 48 (Val→Phe), 68 (Arg→Cys), 83 (Ser→Ile) and 89 (Ile→Phe).

3. Results

3.1. Isolation and identification of *V. parahaemolyticus* from imported shrimp:
Typical *Vibrio* colonies that are round, 2-3 mm in diameter and green or blue-green in color were selected from TCBS plates after 24 h of incubation for further biochemical characterization and identification. Sixty-one isolates from 247 imported shrimp samples, were tentatively identified as *V. parahaemolyticus*. These isolates were positive for Ala-Phe-Pro-Arylamidase (APPA), L-proline arylamidase (ProA), L-lactate alkalinisation (ILATK), Glu-Gly-Arg-arylamidase (GGAA), Beta-N-acetyl-glucosaminidase (BNAG), L-pyrrolidonyl-arylamidase (PyrA), tyrosine arylamidase (TyrA) and beta galactosidase (BGLU). These isolates were also positive for the utilization of D-glucose, D-maltose, D-mannose, D-trehalose, D-mannitol and for the assimilation of L-malate and L-lactate (data not shown). Results from the Vitek database indicated that the percent probabilities of identification of these strains as *V. parahaemolyticus* were 99.0%.

3.2. Antimicrobial resistance profiles of *V. parahaemolyticus*:
The antibiotic resistance profiles of 61 isolates of *V. parahaemolyticus* to 9 different antibiotics were determined. All 61 isolates were resistant to ampicillin and nalidixic acid. Thirty four percent (21/61) also were resistant to tetracycline. Eighteen of the sixty-one (29.0%) isolates were resistant to tetracycline, erythromycin, chloramphenicol, and rifampicin. Fifteen percent of the isolates (9/61) were resistant to tetracycline, erythromycin, chloramphenicol, rifampicin, trimethoprim/sulfamethamazole, and cipro-floxacin. Twenty one percent (13/62) of the isolates were only resistant to ampicillin and nalidixic acid. All strains were resistant to >256 µg of nalidixic acid. Strains resistant to ciprofloxacin had a MIC of 4-8 µg of ciprofloxacin.

3.3. PCR amplification of *gyr*A, B, *par*C and *qnr*A, B, S and analysis of gene sequences:
Synthetic oligonucleotide primers (Table 1) specific for the amplification of the 433-bp *gyr*A, 479-bp *gyr*B, and 437-bp *par*C genes amplified the respective PCR products from

the template DNA of the isolates. Sequence analysis of the quinolone resistance determining region (QRDR) of *gyr*A, *gyr*B and *par*C amplicons indicated mutations that may contribute to the quinolone resistance observed in these isolates (Table 2). Five different kinds of mutations were observed in these sequences: (i) Template DNA of a majority of the isolates (33/61, 54%) was found to simultaneously harbor single point mutations in the QRDR of *gyr*A and *par*C, respectively. Analysis of the mutations in the *gyr*A amplicons of isolates indicated a serine residue at position 83 replaced by isoleucine. In *par*C amplicons a serine phenylalanine residue at position 85 was replaced by henylalanine. (ii) Amplicons from 6/61 (10.0%) of the isolates having point mutations in the QRDR (83Ser→Ile) and non QRDR (48Val→Phe) region of *gyr*A accompanied by a point mutation in *par*C (85Ser→Phe) (Table 2) (iii) Amplicons from 3/61 (5.0%) of the isolates having triple mutations in the QRDR of *gyr*A accompanied by a point mutation in *par*C. Sequence analysis of the *gyr*A amplicons indicated triple point mutations in the QRDR region at positions 83 (Ser→Ile), 89 (Ile→Phe) and 68 (Arg→Cys) was detected (Table 2). Mutation in *par*C was detected at position 85 (Ser→Phe). (iv) Quinolone resistance in 10/61 isolates (ca. 16.0%) was regulated by mutation exclusively in the QRDR of *par*C only (Table 2). These isolates had a mutation at position 85 (Ser→Phe). (v) Sequence analysis of *gyr*B amplicons from nine isolates (15.0%) indicated 4 different kinds of point mutations at codons 311 (Glu→Lys), 354 (Leu→Met), 360 (Ala→Gly) and 374 (Glu→Lys) accompanied by a point mutation in *par*C at position 85 (Ser→Phe) that conferred resistance to high levels of quinolone resistance (Table 2). Primers (*qnr*A-B and *qnr*S) specific for the amplification (Table 1) of the plasmid-mediated quinolone resistance failed to amplify the genes from the template DNA of any of the isolates.

3.4. Homology model:

The structural superposition indicates that the homology model retains all the secondary structures (α-helices and β-strands) that have been observed in *S. aureus* gyrase and topoisomerase structures, except for a few connecting loop regions (Fig. 1A-B). The role of the unique double mutations at codons 48 and 83 and triple mutations at codons 68, 83 and 89 on MICs for nalidixic acid and ciprofloxacin were structurally analyzed. Structural analysis indicates that N-terminal region of GyrA forms three α-helical structures (helix-1 contains residues from 43-52, helix-2 contains residues from 66-78 and helix-3 contains residues 82-93). Furthermore, all four mutations are localized near the vicinity of quinolone binding and DNA binding major groove (Fig. 2A). Ser83 mutation is located at the starting of helix-3 which is an essential residue and contributes for hydrogen bond interaction with quinolones (Fig. 2B). Ile89 mutation is 10 A^{o} away from Ser83 mutation. However, it makes unfavorable interaction with side chain residues of helix-1 (Fig. 2B). Ile89 mutation causes hydrophobic clash that result in readjustment of helix conformation leading to favorable interaction and stabilization of GyrA. The high MIC values observed for nalidixic acid and ciprofloxacin could be attributed to the repositioning of the helix. Similarly, the high MICs of Val48 mutation could also be attributed to the repositioning of the helix and stabilization of GyrA (Fig. 3A).

Structural analysis of Arg68 mutation indicates it is located 13.6A^{o} away from the Ser83 mutation and does not participate in quinolone or DNA binding. However, it does participate in the formation of A2 dimer (Fig. 3B). Arg→Cys replaces the larger side chain into smaller side chain and in the process loses a positive charge. The loss of charge and bulkiness of the long side chain of Arg may decrease the stability of homodimer of GyrA, which may play a role in enhanced the MICs to these drugs.

3.5. Ethidium bromide accumulation assay:

Accumulation of ethidium bromide in the quinolone-sensitive (*V. parahaemolyticus* ATCC 17802) and resistant (*V. parahaemolyticus* strains 114 and 115) isolates was tested in the presence and absence of CCCP. The accumulation of ethidium bromide increased gradually with incubation time in both the quinolone-sensitive and resistant isolates (Fig. 4A). In the absence of CCCP, the intracellular uptake of ethidium bromide in *V. parahaemolyticus* ATCC 17802 was significantly higher than the uptake of ethidium bromide in all strains of quinolone-resistant *V. parahaemolyticus* (Fig. 4A). However, when CCCP was added at the beginning, it induced a significant increase of ethidium bromide uptake in the quinolone-resistant isolates, indicating the involvement of an active efflux pump (Fig. 4B). The uptake of ethidium bromide was far greater in strains 114 and 115 than in quinolone-sensitive reference strain ATCC 17802.

4.0. Discussion

The isolation and characterization of 61 quinolone-resistant *V. parahaemolyticus* strains from imported shrimp in this study indicates that quinolone antibiotics may be widely used in shrimp aquacultural practices in Thailand (Biao and Kaijin, 2007, Bondad-Reantaso et al., 2005). Furthermore, the aquaculture ecosystem may be a reservoir of quinolone-resistant bacteria that may transfer their resistance determinants to other bacteria. Thus the prevalence of quinolone-resistant bacteria may adversely affect the health of both humans and animals and thus become a public health problem.

Results from our studies indicate that simultaneous point mutations on *gyr*A at position 83 (Ser→Ile) and *par*C at position 85 (Ser→Phe) conferred high levels of resistance to both nalidixic acid and ciprofloxacin. In addition, the amino acid substitution in *par*C due to point mutation at position 85 (Ser→Phe) in our study was different from earlier findings (Liu et al., 2013) that reported a point mutation in *par*C at position 85 (Ser→Leu). The unique double mutations on *gyr*A at positions 83 and 48 accompanied by a missense on *par*C at position 85 (Ser→Phe) was also responsible for high levels of resistance to these antibiotics. Furthermore, a novel triple point mutations on the QRDR of *gyr*A at positions 68, 83 and 89 accompanied by point mutation on *par*C at position 85 also conferred high levels of resistance to nalidixic acid and ciprofloxacin. The triple point mutations on the non QRDR and QRDR of *gyr*A, accompanied by point mutation in *par*C, may be an additional mechanism involved in the regulation of high-level quinolone/fluoroquinolone resistance in *V. parahaemolyticus*.

Several reports have indicated the association of mutations in the QRDR of *gyr*B with decreased susceptibility to

fluoroquinolones (Hopkins et al., 2005; Ouabdesselam et al., 1995; Yoshida et al., 1991). Yoshida et al. (1991) identified mutations in *E. coli gyr*B at positions 426 (Asp→Asn) and 447 (Lys→Glu). The mutation at position 426 conferred resistance to all quinolones, while the mutation at position 447 conferred only resistance to nalidixic acid and not to ciprofloxacin. Sequence analysis of the amplified *gyr*B from quinolone-resistant strains of *V. parahaemolyticus* in this investigation indicated that four different point mutations on the non QRDR regions of *gyr*B at positions 311 (Glu→Lys), 354 (Leu→Met), 360 (Ala→Gly) and 374 (Glu→Lys) conferred resistance to high levels of nalidixic acid and low levels of resistance to ciprofloxacin. These mutations on *gyr*B, independent of mutations in *gyr*A may be an additional mechanism involved in the regulation of resistance to nalidixic acid and fluoroquinolones in *V. parahaemolyticus*.

The homology model of quinolone-resistant *V. parahaemolyticus* $gyrA_2B_2$ isolated from imported shrimp has structural similarities with models of other quinolone-resistant bacteria (Bax et al., 2010; Okuda et al., 1999; Ouabdesselam et al., 1995). Missense at codons 83 contributes to the introduction of bulky, hydrophobic residues that reduce the bacterial susceptibility to these drugs. Missense at codons 48 and 89 on the GyrA readjusts the position of the helix and stabilizes the GyrA and contributes to enhanced resistance to these drugs. Mutation at position 68 on the GyrA results in loss of charge, bulkiness and stability of GyrA and increases the MIC values to these drugs.

Point mutations exclusively in the QRDR regions of topoisomerase genes may not be the sole contributing factor for bacterial resistance to quinolone antibiotics, suggesting that other possible mechanisms may also play a role in conferring resistance to these antibiotics (Baranwal et al., 2002; Ghosh et al., 1998; Hopkins et al., 2005 Karczmarczyk et al., 2011; Poole, 2000; Webber and Piddock, 2003). Since none of the isolates examined in this study contained any of the plasmid-mediated quinolone resistance genes (*qnr*), high levels of quinolone resistance could be a function of decreased accumulation of the antibiotics due to active efflux pumps. Indeed, data from our studies indicate the presence of active efflux pumps in quinolone-resistant *V. parahaemolyticus*, as indicated by the increased accumulation of ethidium bromide in the presence of the efflux pump inhibitor CCCP. Thus, high level quinolone resistance in *V. parahaemolyticus* may be due to a combination of multiple point mutations in the QRDR and non QRDR regions accompanied by the involvement of active efflux pumps.

Results from our study indicate that imported shrimp is a reservoir of quinolone-resistant *V. parahaemolyticus*. The prevalence of quinolone resistance in seafood is mainly due to indiscriminate use of quinolone antibiotics in aquaculture farms. The widespread occurrence of quinolone-resistant bacteria in the environment would lead to increased difficulty in treating bacterial infections. In addition the potential health risks due to the consumption of improperly cooked shrimp harboring quinolone-resistant pathogenic bacteria should not be underestimated. Although adequate cooking should eliminate quinolone-resistant pathogenic bacteria, undercooking or cross contamination during preparation could be of concern. Thus the

prevalence of quinolone-resistant pathogenic bacteria in seafood could pose a potential threat to public health.

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Table 1. Oligonucleotide primers used in the amplification of quinolone-resistance genes from *V. parahaemolyticus* isolated from imported shrimp.

Primers	**Nucleotide Sequence**	**Target**	**T_m (^{0}C)**	**Size (bp)**
*gyr*AF	5'-CAGTACACCGCCGCGTTTTATTC-3'	*gyr*A	60	433
*gyr*AR	5'-AGGTTATGTGGCGGGATGTTGGT-3'			
*gyr*BF	5'-GTAAAGCGCGTGAAATGACTCGT-3'	*gyr*B	60	479
*gyr*BR	5'-GTACTGCTCTTGTTTGCCCTTCTT-3'			
*par*CF	5'-GGACCGTGCATTGCCATACATT-3'	*par*C	60	437
*par*CR	5'-CGATACCCGTTACGCCATTCAA-3			
*qnr*AF	5'-ATTTCTCACGCCAGGATTTG-3'	*qnr*A	57	516
*qnr*AR	5'-GATCGGCAAAGGTCAGGTCA-3			
*qnr*BF	5'-ATGGATAAAACAGACCAGTTAT-3'	*qnr*B	52	660
*qnr*BR	5'-AAATTTAGTCAGGAACTACTAT-3'			
*qnr*SF	5'-ATTTTAAGCGCTCAAACCTCCG-3'	*qnr*S	61	521
*qnr*SR	5'-CCTGTTGCCACGAGCATATTTT-3'			

The quinolone resistance genes were amplified after an initial denaturation at 94^{O}C for 2 min. PCR was performed with 35 cycles and after completion of the 35 cycles, a final extension step of 10 mins at 72^{O}C was included in all protocols.

Table 2. Correlation of MICs for nalidixic acid (Na) and ciprofloxacin (Cip) with target gene mutations in quinolone-resistant *V. parahaemolyticus* isolated from imported shrimp.

No. of Isolates	Mutation in			MIC (mg/L)	
	***gyr*A**	***gyr*B**	***par*C**	**Nalidixic acid**	**Ciprofloxacin**
33/61 (54%)	83 (Ser→Ile)		85 (Ser→Phe)	>256	2.0
6/61 (10%)	83 (Ser→Ile) 48 (Val→Phe)		85 (Ser→Phe)	>256	8.0
3/61 (5%)	83 (Ser→Ile) 89 (Ile→Phe) 68 (Arg→Cys)		85 (Ser→Phe)	>256	8.0
10/61 (16%)			85 (Ser→Phe)	>256	4.0
9/61 (15%)		311 (Glu→Lys) 354 (Leu→Met) 360 (Ala→Gly) 374 (Glu→Lys)	85 (Ser→Phe)	>256	2.0

The mutations of the quinolone resistance determining regions (QRDR), the amino acid sequences of the QRDR BLASTx and compared to the protein sequences of GyrA, GyrB, ParC and ParE proteins from the quinolone-sus deposited in the GenBank.

Figure Legends

Figure 1A-B. Homology model of the *V. parahaemolyticus* *gyr*A_2B_2 complex structure; secondary structure of the *gyr*A dimer is shown in orange, and *gyr*B is shown in red color. The DNA double helix is shown in green color. A) Side view of the A_2B_2complex and B) Top view of A_2B_2 complex.

Fig. 2. Location of the mutations in the QRDR region of GyrA of *V. parahaemolyticus***.** Mutational residues are shown in red sphere and all are located in α-helices in the N- terminal region**.** The Val48 is located in helix-1, Arg68 located in helix-2 and Ser83 and Ile89 are located in helix-3. The distance between these mutational residues are also shown in red color.

Fig. 3A-B: (**A**) The cartoon diagram of GyrA N-terminal region, which forms three α-helices (green color). The figure shows the superposition of WT residues with mutated residues on top of each other. The wild type residues are shown in green color and mutated residues are shown in pink color. These helices form a platform providing suitable platform for DNA binding. (**B**) Indicates the Arg68→Cys mutation. In both subunits (A and B) Arg68 involves in protein-protein interaction which is located in helix-2. The Cys reduces the dimer interaction.

Figure 4A-B: The active efflux pump activity was monitored by measuring the accumulation of ethidium bromide in quinolone-sensitive and resistant isolates in the absence (**A**) and presence (**B**) of CCCP. Ethidium bromide at a final concentration of 2 µg/mL was added immediately before reading in a Synergy 2 multi-mode microplate reader (Biotek Instruements, Winooski, VT). CCCP, an inhibitor of the efflux pump, at a final concentration of 100 µM, was added to the assay mixture. Circle, triangle, and square symbols represent ATCC 17802, *V. parahaemolytics* strain 115, and *V. parahaemolyticus* strain 114, respectively.

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Fig. 1A.

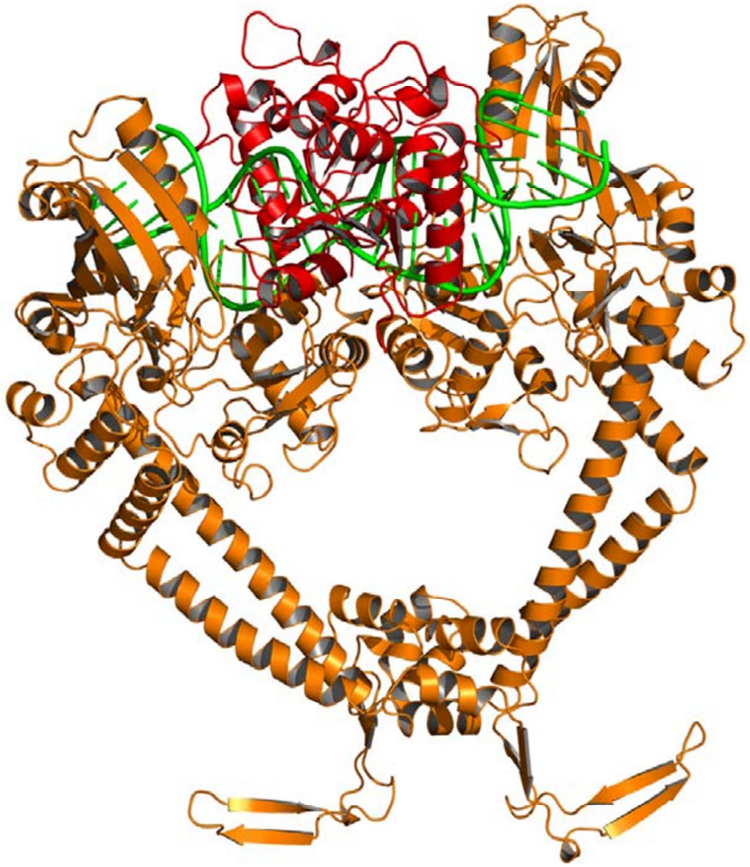
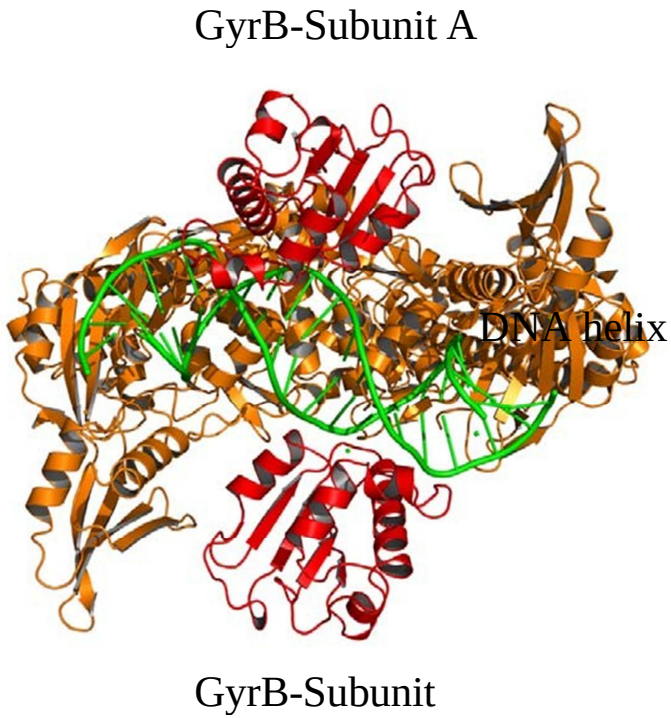


Fig. 1B.



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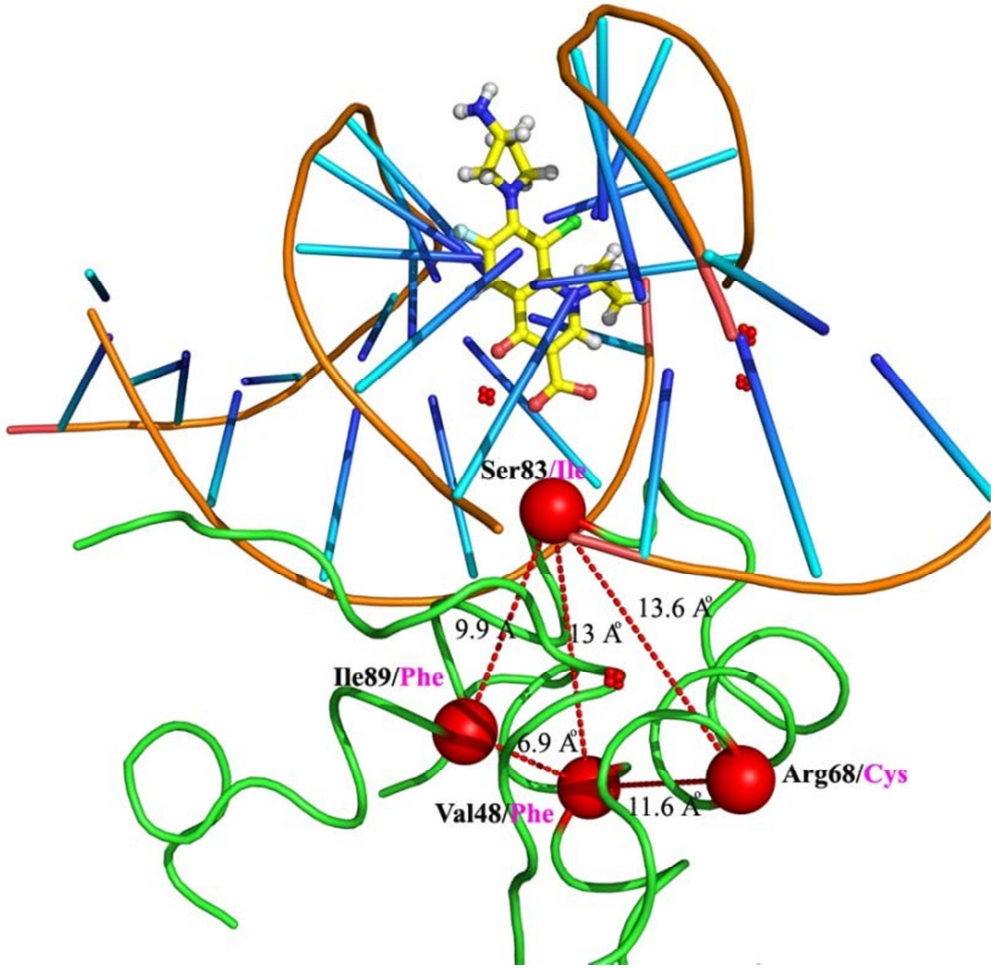

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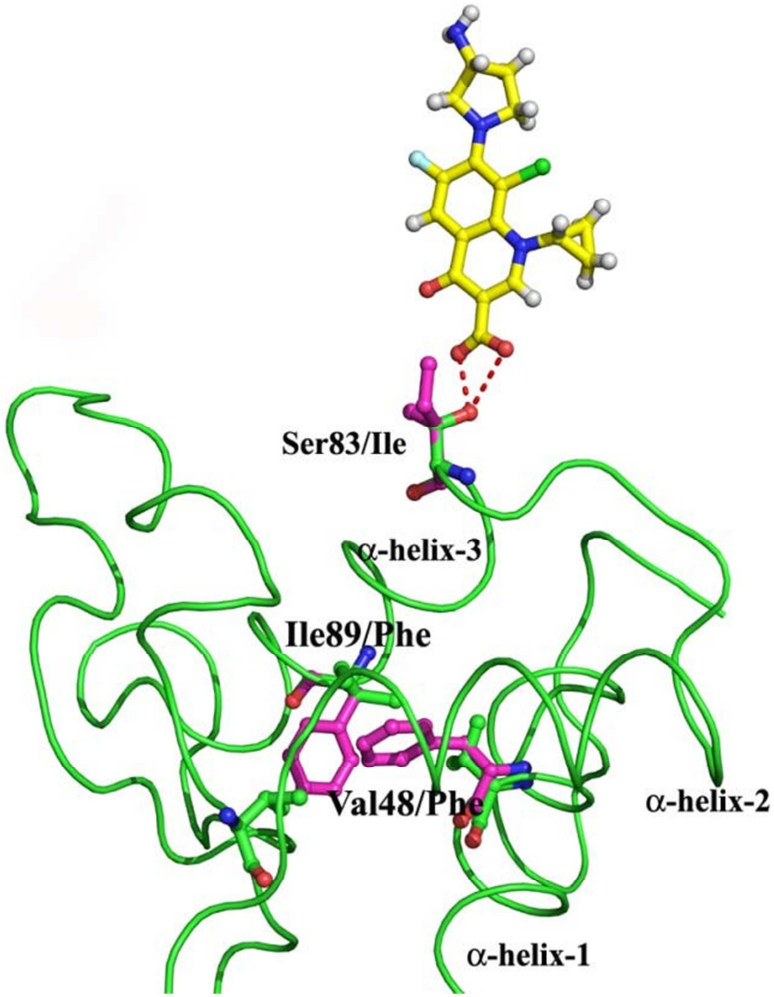

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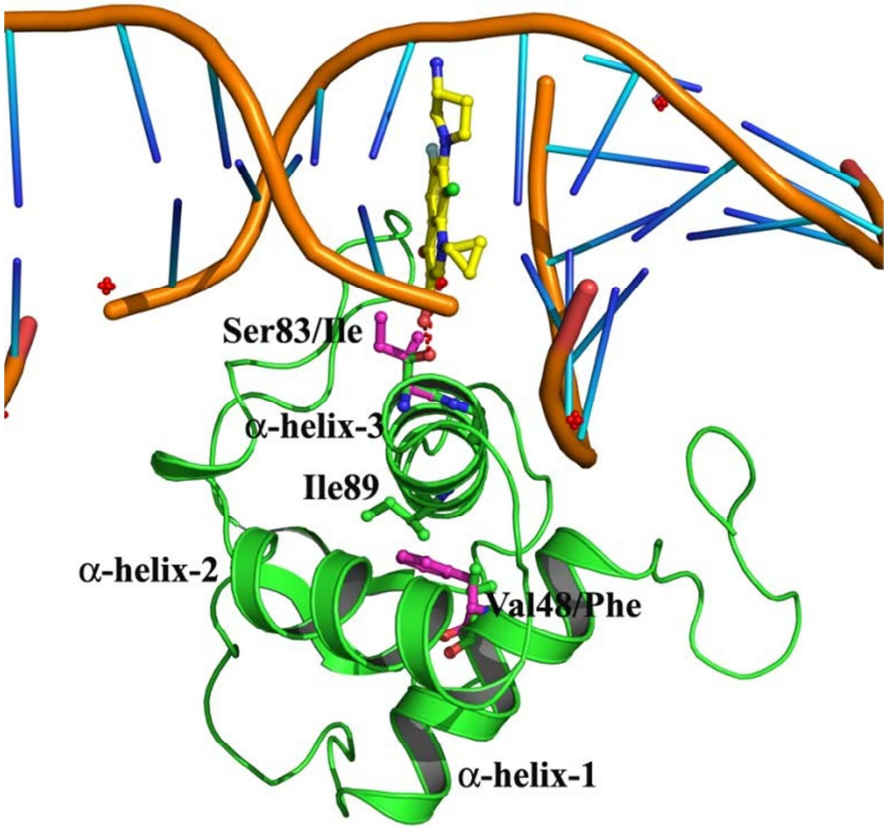

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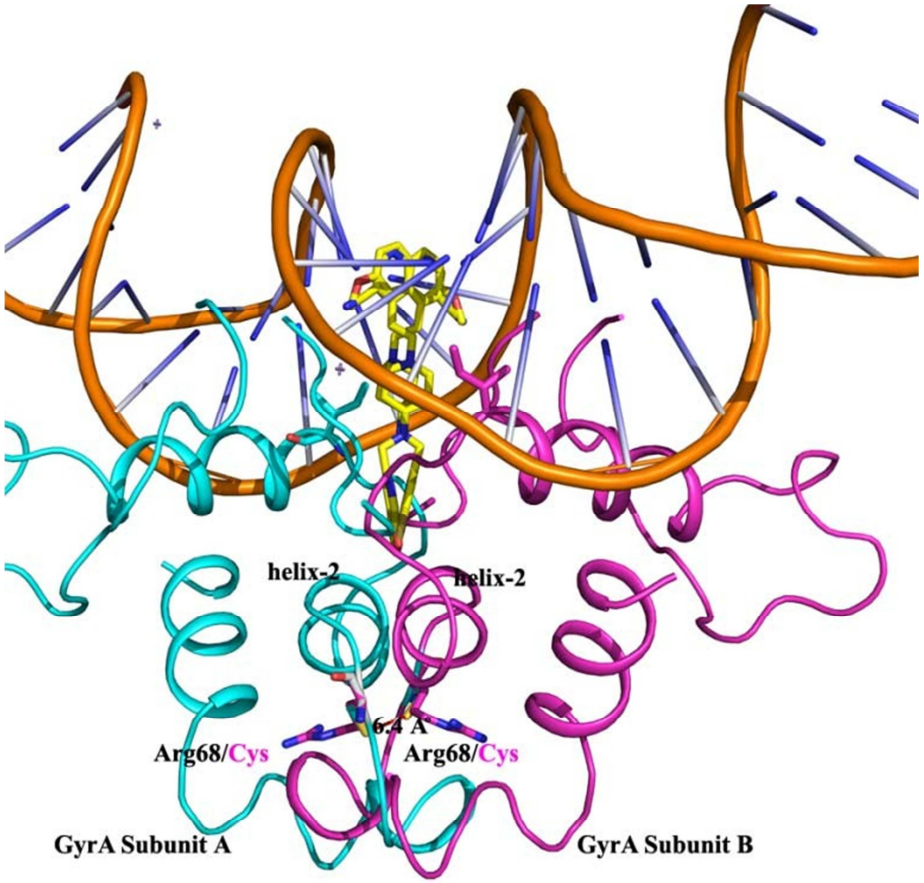

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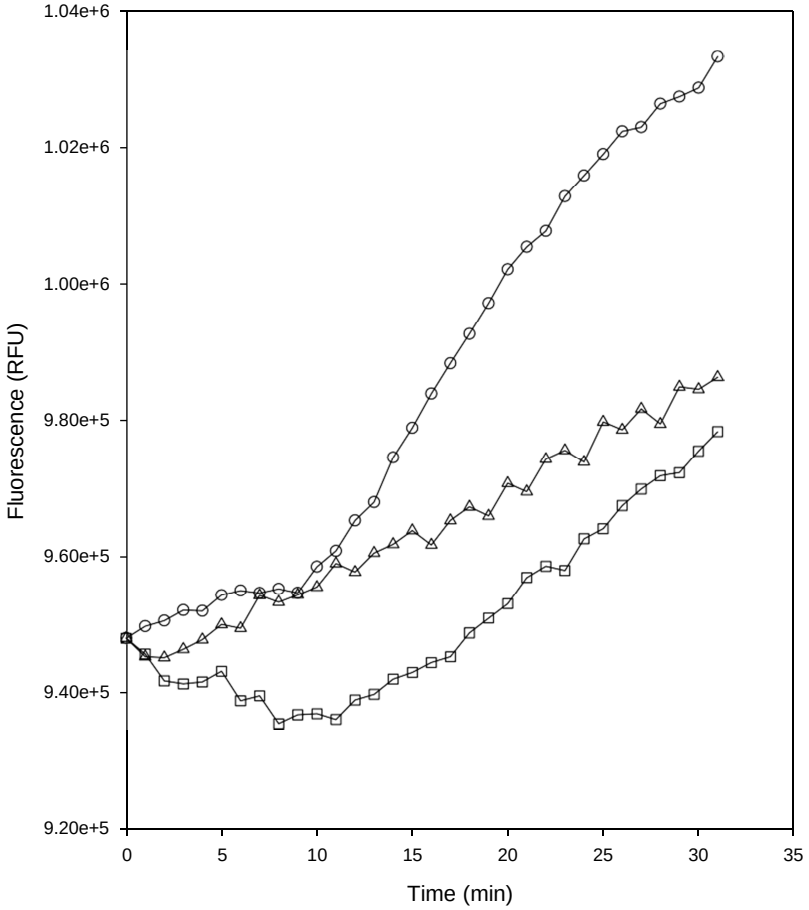

Fig. 4B.