Cove Soyars

Role of hydrogen bonding in Ser-83 amino acid substitutions of DNA gyrase subunit A encoding gyrA

Introduction

Antibiotic resistance is a pressing problem in both healthcare and the food industry. Approximately 25,000 people die in Europe every year from bacterial infections acquired in hospitals that are resistant to antibiotics (Freire-Moran, et. al, 2011). It has been the opinion of recent studies that overuse of antibiotics is a contributing factor to this resistance (Zaman, et. al, 2017). In the context of the food industry, there is evidence supporting the notion that giving livestock antibiotics can lead to antibiotic resistant bacteria being transferred to people that have never taken the drugs themselves (Garau, et.al, 1999).

An example of antibiotics becoming less effective at killing bacteria are quinolones. Quinolones are a class of antibiotics that were once used only to treat simple urinary tract infections and have now, with new generations of the drugs, become very effective outside of the urinary system. Their improved utility is partially because of the addition of a fluorine group, creating the subclass of fluoroquinolones; including ciprofloxacin (Fig. 1), a now widely-prescribed antibiotic (Correia, et al. 2017). Quinolones are now used to treat a wide range of bacterial infections, but are still susceptible to resistant strains. Unfortunately, in every bacterial species that has been treated with quinolones, resistant strains have been observed (Aldred, et. al, 2014).

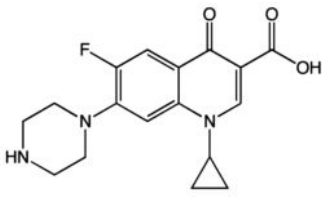


Fig. 1. Ciprofloxacin, a fluoroquinolone (Aldred et. al, 2017)

Quinolones target DNA replication by binding to the DNA/DNA gyrase complex. DNA gyrase is a bacterial type II topoisomerase. DNA gyrase, like other type II topoisomerases, acts by creating double-stranded breaks in DNA (using ATP) and then religating them later in order to relieve supercoiling tension so that DNA helicase can operate and initiate DNA replication. Unlike type I topoisomerases, DNA gyrase does not stop supercoiling once DNA is relaxed, instead it continues its function and introduces negative supercoils to the DNA (Schoeffler et. al, 2008). DNA gyrase consists of two non equivalent subunits: A and B, encoded by genes gyrA and gyrB, respectively. Quinolones insert themselves in between bases of the cleaved DNA and block religation. When the replication fork meets a gyrase that is interacting with a quinolone, gyrase is removed and the double-stranded break remains. Thus, quinolones operate by breaking apart the bacterial genome (Aldred, et. al, 2014).

There is substantial evidence that the Ser-83 position of the A subunit of DNA gyrase, coded by the gene gyrA, plays a crucial role in the binding of quinolones to DNA gyrase. First, a serine residue is highly conserved

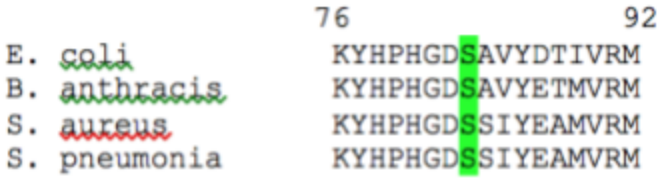


Fig. 2. Conservation of gyrA Ser-83 residue. The Serine residue in the 83rd position is colored green.

in the 83rd position of the DNA gyrase A subunit (Fig. 2). Second, a 2017 study (Qin, et. al) found that out of 400 quinolone resistant strains isolated, 302 (75.5%) had a mutation at the 83rd position. Third, Madurga et. al (2008) found Van der Waals attraction forces between quinolones and the Ser-83 residue using AutoDock 3.05 software.

It is believed that the Ser-83 residue of gyrA is involved in forming a water-metal ion bridge (Aldred et. al, 2014 & Correia et. al, 2017). The water-metal ion bridge, as described by Aldred et. al (2014), consists of a non-catalytic Magnesium ion surrounded by four water molecules (Fig. 3). The carbonyl oxygens of the C4 and C5 carbons of the drug (orange arrows) interact with the Magnesium ion (colored green). The four water molecules (colored blue) interact with the Magnesium ion as well as form hydrogen bonds (dashed red lines) with the hydroxy group of the Ser-83 residue (colored red). This proposed mechanism of quinolone binding could account for the Van der Waals forces detected by Madurga et. al (2008). Furthermore, it would be reasonable that mutations from Serine to amino acids without the ability to hydrogen bond would disrupt the binding of the drug while mutations that retain hydrogen bonds would not.

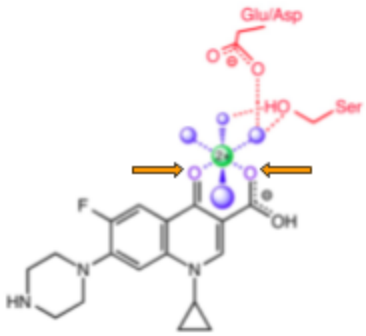


Fig. 3. Water-metal ion bridge (Aldred et. al, 2017)

Several point mutations from Serine to another amino acid have been found in quinolone resistant strains of bacteria. Mutations of Ser-83 to Alanine, Isoleucine, Leucine, Tryptophan, Tyrosine, and Valine have been found (Correia et. al 2017) in addition to Phenylalanine (Ngoi et al 2014). Of these resistance-conferring mutations, only Tryptophan and Tyrosine have hydrogen bonding groups. However, these have a much higher molecular weight than Serine, and are thereby much larger amino acids (Table 1). This suggests that large amino acids inhibit the binding of quinolones even if they contain hydrogen bonding groups.

Table 1. Amino acid size	Difference in size (MW_X -MW_{Ser})
Tryptophan	99da
Tyrosine	76da
Glycine	-30da
Methionine	44da
Cysteine	16da
Aspartic Acid	28da
Promega Corporation	

There are amino acid substitutions that have not been found in the literature that could serve to elucidate the proposed metal-ion bridge. These include Glycine and Methionine, which do not have hydrogen bonding groups and Cysteine and Aspartic acid, which do have hydrogen bonding groups (-SH and -COOH, respectively), all of which are smaller than Tryptophan and Tyrosine (Table 1).

If the proposed metal-ion bridge is correct, it would follow that mutations in the 83rd position to amino acids without hydrogen-bonding groups (Glycine and Methionine) would result in the drugs having inhibited action on gyrase. It would also follow that mutations to amino acids with hydrogen bonding groups (Cysteine and Aspartic acid) would result in a similar ability for quinolones to act on gyrase that the wild type Sereine exhibits.

Experiment

This study seeks to explore the role of hydrogen bonding between the Ser83 residue of gyrA and quinolones. To do this, mutants of the gyrase Subunit A with the mutations S83G, S83M, and S83C, and S83D will be purified and the quinolone resistance of the mutant gyrases will be measured by observing the function of gyrase at different levels of ciprofloxacin.

DNA supercoiling assay

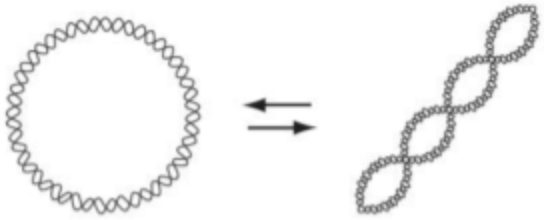


Fig. 4. Gyrase can convert relaxed DNA (left) to supercoiled DNA (right) (Nitiss et. al, 2012)

Gyrase relieves tension in DNA by relaxing positive supercoils. Gyrase continues to induce negative supercoils to relaxed DNA (Schoeffler et. al, 2008), a property which is exploited by the DNA supercoiling assay (Fig. 4). To test whether or not gyrase is functioning, it can be placed (both subunits) in a buffer with relaxed a relaxed DNA plasmid and ATP for a period of time and then the solution run on agarose gel (Nitiss et. al, 2012). DNA that is supercoiled by a functioning gyrase will travel further on the gel because the DNA is in a more compact conformation than a relaxed plasmid (Stellwagen, et. al, 2010).

The supercoiling assay was used in the context of antibiotic resistance in Yokoyama et. al (2011). Here the researchers sought to test if

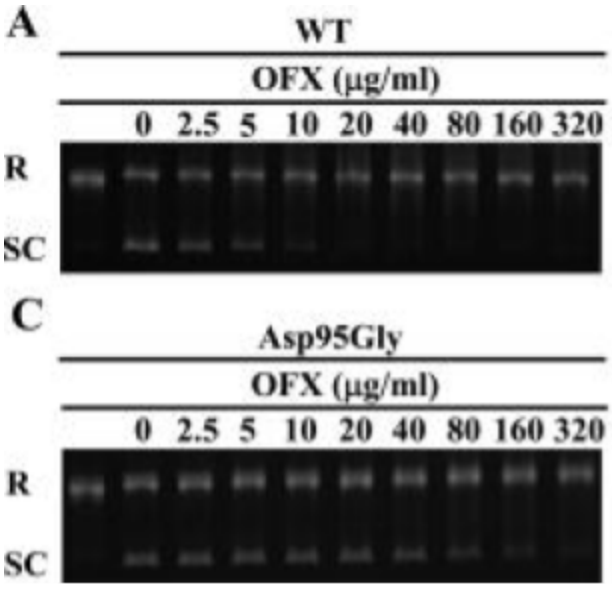


Fig. 5. Yokoyama et. al (2011). Supercoiling activity at various concentrations of ofloxacin. The bands labelled 'SC' represent supercoiled DNA.

homologous quinolone resistance conferring amino acid substitutions of M. tuberculosis gyrase also conferred resistance in M. Leprae gyrase. To do this, they placed both A and B subunits of M. Leprae gyrase, a relaxed DNA plasmid, and ATP in a buffer with a variable amount of the quinolone ofloxacin and incubated the solution. The solution was then run on agarose gel to give the results seen in Figure 5. One of the two homologous mutations being tested (Asp95Gly), in square C showed supercoiling bands at higher ofloxacin concentrations than the wild-type gyrase (square A).

The present study will employ a similar experimental strategy to that of Yokoyama et. al (2011) except with the S83G, S83M, S83C, and S83D mutations of E. coli gyrase and ciprofloxacin.

Mutant Protein Synthesis and Purification

The Polymerase Chain Reaction (PCR) will be used to create mutant gyrA plasmids from cloned wild-type gyrA in an expression vector (Fig. 6). obtained from an outside vendor. The vector will use a T7 promoter and T7 terminator and will be induced with Isopropylβ-D-1-thiogalactopyranoside (IPTG) and will include a 6xHis tag portion. The primers for this reaction were designed using Agilent QuikChange Primer Design (Fig. 7). PCR will also be used to insert 6x Histidine tags for purification, since Yokoyama et. al (2011) found that histidine tags to not interfere with the catalytic function of gyrase. gyrB will need to be cloned as well for the supercoiling assay.

```
S83D    5′-AAATACCATCCCCATGGTGACGATGCGGTCTATGACACGATTGTC-3′
S83M         5'-CCATCCCCATGGTGACATGGCGGTCTATGACACG-3'
S83C   5'-TAAATACCATCCCCATGGTGACTGCGCGGTCTATGAC-3'
S83C         5′-CATCCCCATGGTGACGGGGCGGTCTATGACAC-3′
```

Fig. 7. Primer design. Mutated bases are in bold.
(Agilent Primer Design)

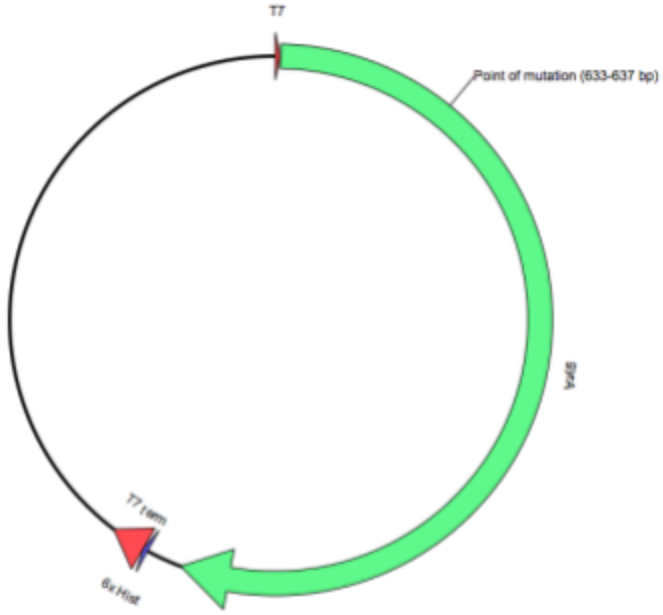


Fig. 6. gyrA expression vector. gyrA is colored green, the T7 promoter and terminators in red, and the 6xHis tag in blue. The point of mutation is indicated at the 633-637 bp mark of gyrA.

The mutated expression vector will then be transformed into E. Coli cells, which will express the mutant proteins. IPTG will be used to induce transcription.The cells will be sonically lysed and the protein will be collected using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin column chromatography. chromatography since the produced proteins are tagged. The resin in the columns has an affinity for the 6xHis tags and causes the protein to remain in the column while

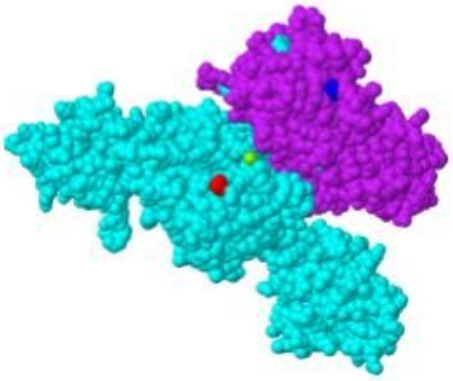
those without the tags, along with other debris, pass through. The 6xHis tag sequence will consist of 6 repeats of the Histidine codon: CAT.

Discussion

It is expected that the Glycine and Methionine mutants will produce supercoiling bands at higher drug concentrations than the wild type and the Cysteine and Aspartic acid mutants. This result would support Aldred et. al (2014)'s proposed metal-ion bridge. Since Glycine and Methionine lack the ability to hydrogen bond, on which the ion bridge depends. It is also expected that the Cysteine mutant will show supercoiling bands at lower drug concentrations than the wild type and Aspartic acid mutant since its thiol group has weaker hydrogen bonding than the hydroxyl group of Serine and the Carboxyl group of Aspartic acid.

Alternatively, all mutants and the wild type could show supercoiling bands at similar drug concentrations. This could imply that mutations of the Ser-83 residue are not enough on their own to confer resistance. This could also serve to contradict the metal ion-bridge proposed by Aldred et. al (2014).

The Cysteine mutant poses a potential limitation resulting from the formation of disulfide bridges between the mutant residue and another Cysteine residue. However, viewing the the structure of Gyrase A and B subunits using Jmol Protein Explorer 0.5 shows that there are no nearby cysteine residues with which this type of bond could form (Fig. 8).



Structure of gyrase A (cyan) and B (purple) subunit complex with respect to Cysteine residues. Ser-83 is colored red, cysteine residue blue, and noncatalytic Magnesium green.

References

Aldred, K. J., Kerns, R. J., & Oshero, N. (2014). Mechanism of Quinolone Action and Resistance. *Biochemistry* 2014, 53, 1565−1574

Correia, S., Poeta, P., Hébraud, M., Capelo, J. L., & Igrejas, G. (2017). Mechanisms of quinolone action and resistance: where do we stand? *Journal of Medical Microbiology*, *66*(5), 551–559. https://doi.org/10.1099/jmm.0.000475

Freire-Moran, L., Aronsson, B., Manz, C., Gyssens, I. C., So, A. D., Monnet, D. L., & Cars, O. (2011). Critical shortage of new antibiotics in development against multidrug-resistant bacteria - Time to react is now. *Drug Resistance Updates*, *14*(2), 118–124. https://doi.org/10.1016/j.drup.2011.02.003

Garau, J., Xercavins, M., Rodríguez-Carballeira, M., Gómez-Vera, J. R., Coll, I., Vidal, D., … Ruíz-Bremón, A. (1999). Emergence and dissemination of quinolone-resistant Escherichia coli in the community. *Antimicrobial Agents and Chemotherapy*, *43*(11), 2736–2741.

Madurga, S., Sánchez-Céspedes, J., Belda, I., Vila, J., & Giralt, E. (2008, September). *Mechanism of Binding of Fluoroquinolones to the Quinolone Resistance-Determining Region of DNA Gyrase: Towards an Understanding of the Molecular Basis of Quinolone Resistance*. *ChemBioChem* 2008, 9, 2081 – 2086 https://doi.org/10.1002/cbic.200800041

Ngoi, S. T., & Thong, K. L. (2014). High resolution melting analysis for rapid mutation screening in gyrase and topoisomerase IV genes in quinolone-resistant salmonella enterica. *BioMed Research International*, *2014*. Article ID 718084, 8 pages. https://doi.org/10.1155/2014/718084

Nitiss, J. L., Soans, E., Rogojina, A., Seth, A., & Mishina, M. (2012). *NIH Public Access*. *Curr Protoc Pharmacol 2012.* June; CHAPTER: Unit3.3. doi:10.1002/0471141755.ph0303s57. https://doi.org/10.1002/0471141755.ph0303s57.

Qin, T., Qian, H., Fan, W., Ma, P., Zhou, L., Dong, C., … Huo, X. (2017). Newest data on fluoroquinolone resistance mechanism of Shigella flexneri isolates in Jiangsu Province of China. *Antimicrobial Resistance & Infection Control*, *6*(1), 97. https://doi.org/10.1186/s13756-017-0249-1

Schoeffler, A. J., & Berger, J. M. (2008). DNA topoisomerases: harnessing and constraining energy to govern chromosome topology. *Quarterly Reviews of Biophysics*, *41*(1), 41–101. https://doi.org/10.1017/S003358350800468X

Stellwagen, N. C., & Stellwagen, E. (2010). NIH Public Access, *1216*(10), 1917–1929. https://doi.org/10.1016/j.chroma.2008.11.090.Effect

Yokoyama, K., Kim, H., Mukai, T., Matsuoka, M., Nakajima, C., & Suzuki, Y. (2012). Amino acid substitutions at position 95 in GyrA can add fluoroquinolone resistance to Mycobacterium leprae. *Antimicrobial Agents and Chemotherapy*, *56*(2), 697–702. https://doi.org/10.1128/AAC.05890-11

Zaman, S. Bin, Hussain, M. A., Nye, R., Mehta, V., Mamun, K. T., & Hossain, N. (2017). A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Cureus*, *9*(6): e1403. https://doi.org/10.7759/cureus.1403

Agilent QuikChange Primer Design: https://www.genomics.agilent.com/primerDesignProgram.jsp