2017

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Recommended Citation

Wu, Eugene Y., and Angela K. Hilliker. "Identification of Rifampicin Resistance Mutations in Escherichia Coli, Including an Unusual Deletion Mutation." *Journal of Molecular Microbiology and Biotechnology* 27, no. 6 (2017): 356-62. https://doi.org/10.1159/000484246.

Identification of Rifampicin Resistance Mutations in *Escherichia coli*, Including an Unusual Deletion Mutation

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Key words: Antibiotic, rifampicin, Escherichia coli, mutation

Abstract

Rifampicin is an effective antibiotic against mycobacterial and other bacterial infections, but resistance readily emerges in laboratory and clinical settings. We screened *Escherichia coli* for rifampicin resistance and identified numerous mutations to the gene encoding the β chain of RNA polymerase (*rpoB*), including an unusual nine nucleotide deletion mutation. Structural modeling of the deletion mutant indicates locations of potential steric clashes with rifampicin. Sequence conservation in the region near the deletion mutation suggests a similar mutation may also confer resistance during the treatment of tuberculosis.

Introduction

Rifampicin (RIF) is a member of rifamycin antibiotics used in the treatment of tuberculosis and other bacterial infections [Sensi, 1983]. It targets bacterial RNA polymerases by binding near the polymerase active site and inhibiting the elongation of nascent mRNA [Campbell et al., 2001]. The catalytic core of bacterial RNA polymerases consists of α_2, β, β′, and ω chains, and rifampicin binds to a pocket formed by the β chain (Fig. 1A) [Campbell et al., 2001; Molodtsov et al., 2013]. Although rifampicin binds in the RNA tract, many of the residues in the rifampicin binding pocket do not directly contact the RNA. Numerous mutations in the RNA polymerase β chain confer rifampicin resistance (RIF^R) in *Escherichia coli* and *Mycobacterium tuberculosis* [Xu et al., 2005; Makiela-Dzbenska et al., 2011; Zhou et al., 2013; Jin, and Gross, 1989; Sandgren et al., 2009; Barrick et al., 2010]. These mutations are clustered in four regions of RNA polymerase β, termed RIF resistance-determining regions (RRDRs) N, I, II, and III (Molodtsov et al. 2016). Crystal structures of rifampicin bound to RNA polymerases of *Thermus aquaticus* and *E. coli* show that all four RRDRs consist of residues located within ~10 Å of rifampicin [Campbell et al., 2001; Molodtsov et al., 2013]. RIF^R mutations and structural studies have provided detailed information on how *M. tuberculosis* and other bacteria evade the antibiotic action of rifampicin and other rifamycins [Artsimovitch et al., 2005; Molodtsov et al., 2017].

In this study, we selected *E. coli* for rifampicin resistance in the absence of mutagens and sequenced a region of RNA polymerase β chain encompassing RRDRs I, II, and III to identify new RIF^R mutations. Nonpathogenic *E. coli* serves as an excellent experimental system to probe rifampicin resistance because of its ease of handling in the

laboratory in contrast to *M. tuberculosis*, and there exists an abundance of structural and biochemical information on its RNA polymerase [Molodtsov et al., 2013; Campbell et al., 2001; Kang et al., 2017; Gill, and Garcia, 2011]. While most of the *E. coli* mutants contained previously characterized mutations, we identified a new mutation located in RRDR-I, an unusual in-frame deletion that did not result in misfolding of RNA polymerase.

Results & Discussion

Rifampicin resistance in E. coli. Selection of *E. coli* K-12 MG1655 laboratory strain bacteria on LB plates containing rifampicin readily produced a large and variable number of rifampicin-resistant clones (results not shown). The number of RIF^R colonies and the total live bacteria in each of 64 cultures were counted and used to compute the mutation rate from Luria-Delbrück fluctuation tests [Luria, and Delbrück, 1943; Gillet-Markowska et al., 2015; Ycart, and Veziris, 2014]. We estimate the mutation rate of the MG1655 strain at 2.0×10^{-9}, which is in general agreement with other studies on *E. coli* lab strains and rifampicin-resistance [Bjedov et al., 2003; Galán et al., 2007] but lower than rates from clinical isolates [Baquero et al., 2004].

Identity of RIF-resistance mutations. The chromosomal rpoB gene fragment containing the region that codes for residues 473-720 of the β chain of RNA polymerase was directly amplified from RIF^R colonies using the polymerase chain reaction, resulting in a 744 base pair band (results not shown). This section of *E. coli* RNA polymerase encompasses the rifampicin-binding site and RIF resistance-determining region, including clusters I (residues 507-533), II (residues 563-572), and III (residue 687), but not the N-terminal

cluster (residue 146). Sequencing of PCR products revealed 16 different mutations in clusters I and II (Table 1). All but one of the cluster I mutations have previously been reported in rifampicin-resistant *E. coli* mutants. The 9 bp deletion had not been previously reported, to our knowledge, but is similar to a previously characterized deletion that replaces 507-GSSQL-511 with V [Jin, and Gross, 1989]. The deleted sequence (nucleotides 1517-1525 in the *rpoB* open reading frame, TCGGTTCCA) contained no obviously unusual features (e.g. A-T rich, long tracts of single nucleotides, self-annealing sequences). Mutations at I572 and S574 in cluster II have also previously been identified in RIF-resistant *E. coli* clones [Jin, and Gross, 1989; Zhou et al., 2013; Makiela-Dzbenska et al., 2011; Sandgren et al., 2009; Barrick et al., 2010]. Several of these mutations occurred in positions that increased transcript deletions (Q513) or decreased transcriptional elongation slippage (D516 and I572) by *E. coli* RNA polymerase [Zhou et al., 2013].

Two of the clones (out of 81 total) contained no mutations to the coding region for residues 473-720. It is possible that these contained a mutation to the N-terminal cluster of rpoB or other regions of genes encoding RNA polymerase. Alternatively, mutations of other *E. coli* genes could lead to rifampicin secretion or breakdown could also result in resistance. We did not subject these clones to genome sequencing to determine if this was the case. Of the sequenced clones, the H526Y mutant was the most abundant (29/81). The mutations of nucleotides 1546 (GAC to AAC; D516N), 1547 (GAC to GGC; D516G), 1576 (CAC to TAC; H526Y), and 1592 (TCC to TTC; S531F), were the only transition mutations observed (35/81).

Relationship of mutations to the rifampicin binding site in RNA polymerase.

Rifampicin-resistant mutants were mostly clustered to the rifampicin binding site in RNA polymerase β (Fig. 1B). Several of the residues where mutations occur are adjacent to where rifampicin binds (Q513, D516, H526, S531, I572). Mutations to these first shell residues are expected to alter the shape and/or chemical characteristics of the rifampicin binding site without substantially affecting the structure or function of RNA polymerase. According to the RNA polymerase-rifampicin complex structure [Molodtsov et al., 2013], the serine 512 and 574 side chains do not contact rifampicin, but point towards the interior of the protein. Mutation to a larger tyrosine at residue S512 or at S574 would require a change to the backbone structure, leading to possible changes in the rifampicin binding surface.

Unusual in-frame deletion near rifampicin binding site.

The deletion of nine base pairs (1517-1525; Δ9) from the *rpoB* gene changes 506-FGSS-509 to C506. Because RNA polymerase is essential to the survival and growth of *E. coli*, we concluded that the Δ9 mutation did not create a non-functional protein and that the folding and structure of the enzyme was largely retained. In wild-type RNA polymerase, residues 506-509 reside at the end of an α-helix adjacent to the rifampicin binding site (Fig. 1B and 2A). However, none of the residues contact rifampicin in the crystal structure.

To understand the impact of the three-residue deletion on the structure of RNA polymerase, the translated protein sequence from the Δ9 mutant of *rpoB* was threaded into known crystal structures of *E. coli* RNA polymerase using Iterative Threading ASSEmbly Refinement (I-TASSER; [Yang et al., 2015]). The Δ9 mutant model with the highest confidence score (C-Score of 1.65) produced by I-TASSER was highly similar to

known *E. coli* RNA polymerase crystal structures with identical secondary structural elements and a similar rifampicin binding site (Fig. 2A). The 506-FGSS-509 sequence lies at the C-terminal end of an α helix pointing to where rifampicin binds, with glycine 507 marking the end of the helix and the start of a loop. The threaded model generated by I-TASSER indicates that shortening of the β chain sequence by three residues leads to the truncation of the α helix by one turn, but no substantial changes to the rest of the protein backbone. A comparison of the RNA polymerase-rifampicin complex crystal structure (4KMU; [Molodtsov et al., 2013]) with the *E. coli* RNA polymerase elongation complex cryo-electron microscopy structure (5UPC; [Kang et al., 2017]) indicates that rifampicin inhibits RNA synthesis by blocking the path of the nascent RNA chain [McClure, and Cech, 1978; Campbell et al., 2001; Lin et al., 2017] and that residues 506-509 of the β-chain lie near where the backbone of the transcript and template strand would lie in the transcriptional elongation complex (Fig. 2B). The side chain of Q510 (wild type numbering) in the Δ9 mutant makes contact with the RNA transcript backbone (Fig. 2B), but the clash could be alleviated by a rotamer change. The observation that Δ9 *rpoB* mutant formed a colony suggests that RNA polymerase activity is not substantially decreased by the unusual mutation.

Although the Δ9 mutant does not result in large structural rearrangements near the mutation site, the sequence change does lead to rifampicin resistance. The backbone of the next five residues after Δ9 mutation (QLSQF, numbered residues 510-514 in the wild type sequence) follows closely to the structure of full length, wild-type *rpoB* (Fig. 2C), but subtle structural changes in the Δ9 mutant model lead to steric clashes with rifampicin, particularly at Q513, F514, and I572 (wild type numbering). The strong

overlap between rifampicin atoms and the Δ9 mutant model denoted by red disks in Figure 2C suggest that rifampicin may not bind well to the mutant without alterations to the protein surface. One such change could be the rotations of side chains away from rifampicin if there is space. The clashes between rifampicin and F514 and I572 can be resolved by rotamer changes in the two side chains. However, the model indicates that the side chain of Q513 forms hydrogen bonds with the Q510 backbone and H526 side chains. We note that the hydrogen bond between Q510-Q513 in the Δ9 mutant model is not present in wild type crystal structures (4KMU.pdb and 5UPC.pdb). This interaction could prevent the Q513 side chain from swinging out of the way of rifampicin. Without an experimental atomic structure of this Δ9 mutant of RNA polymerase, we cannot be sure how this mutation confers rifampicin resistance, but we speculate that the subtle changes in structure immediately after residue 506, specifically the formation of a new backbone-side chain hydrogen bond, lock the β-chain into a conformation that has a lower binding affinity to rifampicin, thereby conferring resistance.

Implications for rifampicin resistance in M. tuberculosis *and other bacteria.*

Rifampicin resistance is a major problem for treatment of human infections by *Staphyloccocus aureus* and *M. tuberculosis*. Many rifampicin-resistant clinical isolates of *Staphyloccocus* contain nonsynonymous single nucleotide substitutions that result amino acid substitutions in the rifampicin binding site (e.g. S455A, L466S, H481N) [Zhou et al., 2012; Murugan et al., 2015; Hellmark et al., 2009], but a three-nucleotide insertion mutant of *rpoB* has also been isolated [Hellmark et al., 2009]. A nine-nucleotide deletion similar to the Δ9 mutant identified in our study may also generate a RIF^R mutant, as the RNA polymerase β amino acid sequence corresponding to 506-

FGSS-509 in *E. coli* is the same in *S. aureus* (Table 2). A comparison of crystal structures of rifampicin bound to RNA polymerases of *M. tuberculosis* (*Mt*rpoB) and *E. coli* (*Ec*rpoB) indicates that rifampicin binds to highly conserved sites with nearly identical structures in both enzymes [Lin et al., 2017]. The most clinically important mutation in the treatment of tuberculosis, serine 450 (or 531 in *E. coli*) to leucine [Williams et al., 1998; Telenti et al., 1993], was not observed in our screen due to the codon encoding serine (TCT) requiring two mutations to mutate the residue to leucine. We did observe a similar S531F mutant, which required only a single mutation (TCC to TTC). The Δ9 rifampicin resistant mutant in *E. coli* converts 506-FGSS-509 to C506. Homologous residues in RNA polymerase β chains of 10 other bacteria are almost identical to *E. coli* RNA polymerase (Table 2). A similar nine nucleotide deletion in *Mt*rpoB would change the corresponding sequence to 425-SQLSQF-428 instead of CQLSQF in *Ec*rpoB. Serine and cysteine differ only by one atom (oxygen replaced by sulfur) and are chemically similar. Thus, we hypothesize that a similar nine nucleotide deletion mutation may also confer resistance in *M. tuberculosis* and other bacteria.

Materials and Methods

Selection of RIF^R strains of Escherichia coli. *E. coli* K-12 MG1655 was streaked on Luria-Bertani Broth (LB)-agar plates to yield single colonies. Colonies were picked and dissolved in 200 µl sterile saline. 10 µl of bacteria were diluted to individual cultures of 25 ml of LB and shaken overnight at 37°C. 200 µl of each bacterial culture was plated on LB-agar plates containing 100 µg/mL rifampicin and cultured at 37°C overnight. Plates generally contained between 5 and 250 colonies. In addition, each individual 25 mL

culture was serially diluted to 10^{-5} or 10^{-6} and plated on LB-agar plates without antibiotics to determine the concentration of live bacteria in the culture.

Sequencing of E. coli rpoB gene fragments form Rif^R strains. Colonies were restreaked onto LB+rifampicin plates to obtain freshly grown bacteria. Single colonies were picked and dissolved in 1X GoTaq Green Master Mix (Promega Corporation, Madison, Wisconsin) with 1 μM rpoBforward (5′- CGT GCG GTG AAA GAG CGT CTG TCT -3′) and rpoBreverse (5′- ACG TTT AGC TAC CGC AGT TAC ACC -3′) primers. The primers are designed to amplify nucleotides 1417-2160 of the *E. coli* K-12 MG1655 rpoB open reading frame. The rpoB fragment was amplified directly from colonies with 30 cycles of 95°C for 30 seconds, 57°C for 45 seconds, and 72°C for 1 minute after initial denaturation at 95°C for 2 minutes. Polymerase chain reaction (PCR) amplification was confirmed by agarose gel electrophoresis. PCR products were purified using the MinElute PCR Purification Kit (Qiagen Sciences, Germantown, Maryland) and sequenced by Sanger DNA sequencing using the rpoBforward primer (Genewiz, Inc., South Plainfield, New Jersey).

Acknowledgments

These experiments were part of a Genetics course taught at the University of Richmond. We wish to acknowledge the contributions of the students and instructors of the class in performing the experiments. In particular, we wish to thank K. Zoghby and Dr. F. Gomez for their assistance in supervising the students' execution of experiments. The work was funded by the University of Richmond School of Arts & Sciences.

Table 1. Mutations to rpoB gene identified from rifampicin-resistant *E. coli*.

Residue number	Protein sequence change	Mutation*	Number of observed clones
506-509	FGSS to C	9 bp (1517-1525) deletion	1
512	S to Y	TCT to TAT (1534)	1
513	Q to K	CAG to AAG (1537)	2
	Q to L	CAG to CTG (1538)	4
	Q to P	CAG to CCG (1538)	1
516	D to Y	GAC to TAC (1546)	3
	D to N	GAC to AAC (1546)	1
	D to V	GAC to GTC (1547)	11
	D to G	GAC to GGC (1547)	2
526	H to Y	CAC to TAC (1576)	29
	H to N	CAC to AAC (1576)	8
	H to D	CAC to GAC (1576)	1
	H to L	CAC to CTC (1577)	4
531	S to F	TCC to TTC (1592)	3
572	I to F	ATC to TTC (1714)	6
	I to L	ATC to CTC (1714)	1
574	S to Y	TCT to TAT (1721)	1

* Nucleotide number in *E. coli rpoB* open reading frame in parentheses.

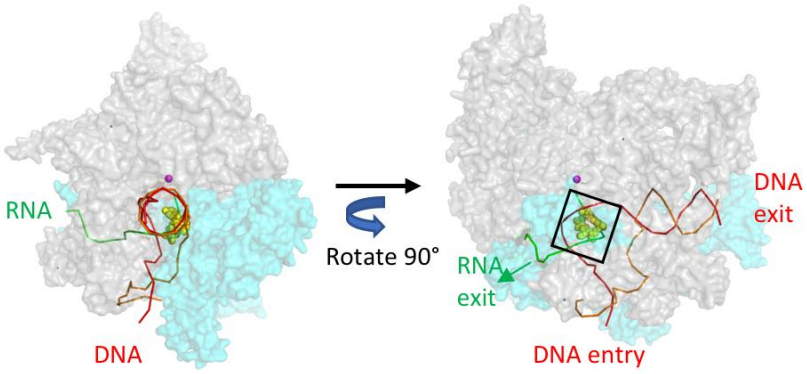
Table 2. Protein sequence alignment of several bacterial RNA polymerases in RRDR I & II.

Bacterial species	*rpoB* residue	RDRR I and II Protein sequence†
Escherichia coli	505-533, 571-575	F**FGSS**QLSQFMDQNNPLSEITHKRRISAL LINSL
Pseudomonas aeruginosa	508-538, 574-578	F**FGSS**QLSQFMDQNNPLSEITHKRRVSAL LINSL
Neisseria meningitidis	527-557, 598-602	F**FGSS**QLSQFMDQTNPLSEVTHKRRVSAL LINSL
Clostridioides difficile	479-509, 548-552	F**FGSS**QLSQFMDQTNPLSELTHKRRLSAL LINSL
Streptococcus pneumoniae	475-505, 544-548	F**FGSS**QLSQFMDQHNPLSELSHKRRLSAL LINNL
Staphylococcus aureus	459-488, 525-529	F**FGSS**QLSQFMDQANPLAELTHKRRLSAL LINSL
Mycobacterium tuberculosis	422-452, 490-494	F**FGTS**QLSQFMDQNNPLSGLTHKRRLSAL LIGSL
Mycobacterium smegmatis	419-449, 487-491	F**FGTS**QLSQFMDQNNPLSGLTHKRRLSAL LIGSL
Chlamydia pneumoniae	448-478, 516-519	F**FGRS**QLSQFMDQTNPVAELTHKRRLSAL LITSL
Thermus thermophilus	383-413, 451-455	F**FSRS**QLSQFKDETNPLSSLRHKRRISAL LITSL
Thermus aquaticus	383-413, 451-455	F**FSRS**QLSQFKDETNPLSSLRHKRRISAL LITSL
Sequence conservation‡		**: ****** * **: : ****:*** ** **
Mutations in this study		____ ^^ ^ ^ ^ ^ ^ ^

† Protein sequence shown in one-letter code. Sequence corresponding with the Δ9 deletion mutation is shown in bold and denoted by dashes in the last row. Point mutations observed in this study are denoted by ^ in the last row.

‡ Complete sequence identity amongst the RPOB sequences represented here denoted by "*"; Conservation of amino acid type (positively or negatively charged, hydrophobic, hydrophilic, side chain size) denoted by ":".

A



B

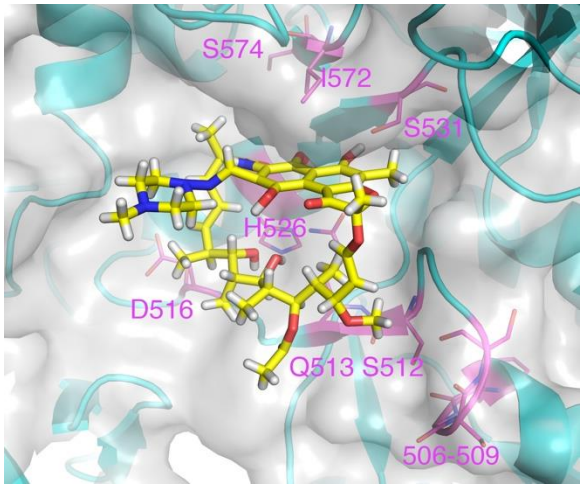


Figure 1. Rifampicin binding to the bacterial transcriptional machinery. (A) Rifampicin (yellow spheres) binds inside the DNA-RNA tunnel of RNA polymerase elongation complex (gray surface representation; 5UPC.pdb) to the surface of the β chain (cyan). The rifampicin binding site overlaps with the path of the nascent RNA chain (green). The template (red) and non-template (orange) DNA backbones are shown as lines, and the polymerase active site is shown as a magenta sphere. (B) The rifampicin (Sticks representation; yellow carbons, blue nitrogens, red oxygens, white hydrogens) binding site (black box in (A)) and its solvent-exposed surface (gray) in *E. coli* RNA polymerase β chain (cyan) from 4KMU.pdb. Locations of mutations identified in this study are depicted as magenta lines and labeled.

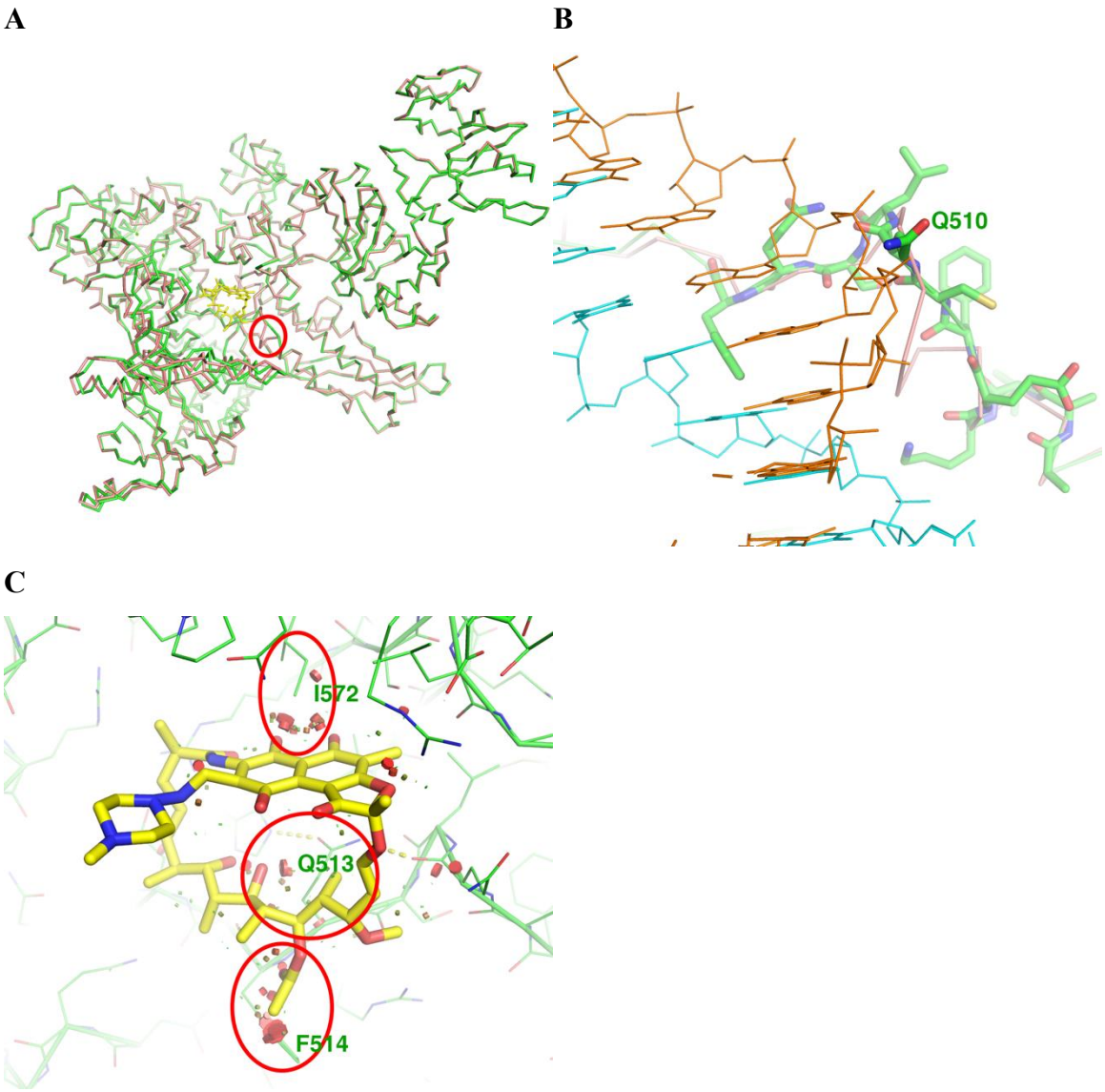


Figure 2. The structure of the I-TASSER model of the Δ9 mutant. (A) The Δ9 mutant model protein backbone (green lines connecting α-carbons) is superimposed on the crystal structure of rifampicin (pink lines) bound to the wild type *E. coli* RNA polymerase β chain (4KMU.pdb; gray ribbon). Rifampicin is show in yellow sticks. The red oval highlights the location of 506-FGSS-509 in the wild type RNA polymerase, which is mutated to C506 in the Δ9 mutant. (B) The Δ9 mutant (green lines connecting α-carbons) is superimposed on the crystal structure of a DNA (blue)-RNA (brown) hybrid bound to the *E. coli* RNA polymerase transcriptional elongation complex (5UPC.pdb; pink lines). Residues 500-514 of the Δ9 mutant model are show in sticks. (C) Superimposition of rifampicin (sticks with yellow carbons) onto the Δ9 mutant model indicates steric clashes (red disks) with Q513, F514, and I572 (highlighted in red ovals). The side chain of Q513 forms a hydrogen bond (yellow dotted line) with the backbone carbonyl of Q510 (wild type numbering). Figure 1B, 2A, 2B, and 2C are viewed in the same orientation. Figures and clashes were generated in PyMol [DeLano, 2014].

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