The Mechanism of Quinolone Resistance in *Staphylococcus aureus*

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Clinical isolates of 8 ofloxacin resistant *Staphylococcus aureus* (ORSA) were subjected to MIC test, Southern analysis on *gyrA* locus and nucleotide sequence analysis of 290 bp of *gyrA* gene (*gyrA*-290) spanning amino acid 26 to 121 in order to understand the mechanism of quinolone resistance in *Staphylococcus aureus*. ORSAs showed high level resistance against quinolones (8-250 fold increase of MICs) and also significant resistance against β-lactams (2-32 fold increase of MICs). However, ORSAs did not show any change in sensitivity against vancomycin. Southern analysis of ORSAs with *Hind*III, *Pst*I and *Alu*I revealed RFLPs on *gyrA* locus. In order to further analyze the *gyrA* gene, *gyrA*-290 was amplified by PCR and cloned to pTZ vector. Subsequent nucleic acid sequence analysis of *gyrA*-290 demonstrated a point mutation of C to T resulting amino acid change of Ser-84 to Leu-84 in all 8 ORSA strains. The substitution at 84th amino acid of gyrase A might confer one mechanism of high level quinolone resistance in *Staphylococcus aureus*.

KEY WORDS □ ***Staphylococcus aureus*, *gyrA*, quinolone, resistance**

Quinolones are fully synthesized *in vitro*, potent antibacterial agents that have been widely used in the treatment of different bacterial infections including methicillin-resistant *Staphylococcus aureus*, a problematic pathogen resistant against varieties of antibiotics (16). Most widely known quinolones are norfloxacin, ofloxacin, ciprofloxacin and sparfloxacin. Quinolone usage is dramatically increasing because of its favorable pharmacokinetic and toxicological profile as well as its wide spectrum and potency (2).

Qunolones are known to inhibit gyrase A, thereby prevent bacterial DNA replication. The DNA gyrase is a topoisomerase which catalyzes the supercoiling of relaxed covalently closed circular DNA, which is coupled with the hydrolysis of ATP (4, 15). Most studies on gyrases are done with *E. coli* (1, 5, 11, 16). Gyrase is composed of 2 different subunits, A and B, which are encoded by *gyrA* and *gyrB* gene respectively. Gyrase A is the target for quinolones whereas gyrase B is that for novobiocin and coumermycin (10).

While the details of the quinolone-DNA-gyrase interaction are still unequivocal, it seems clear that enzyme-DNA complex is formed and subsequently both strands of DNA are cleaved in the presence of quinolones. This irreversible formation of DNA strand breakage triggers a sequence of events that lead to cell death ultimately (9, 18).

Staphylococcal infections, particularly methicillin resistant ones, are a serious medical problem (3). Unfortunately quinolone resistant *S. aureus* isolates seem to appear. The emergence of quinolone resistant *S. aureus* is of particular concern given that relatively few antimicrobial agents are effective against them (8, 13, 14).

The molecular basis of quinolone resistance in *S. aureus* is not well-understood. There has not yet been a report that quinolone resistance is mediated via plasmid. In *E. coli*, quinolone resistance seems to arise through mutations of gyrase A and, less often, gyrase B (2,6,7). In particular, amino acid substitutions near Tyr-122 of gyrase A, the site of DNA attachment, were observed in quinolone resistant *E. coli* strains. However it is worthy of mentioning that most of

these studies were done with mutants whose resistance was induced under the laboratory condition.

These observations led us to study the mechanism of *in vivo* quinolone resistance in S. aureus. In this work, we tested the possibility that amino acid substitution(s) near the residue of DNA attachment in gyrase A might be involved in the *in vivo* quinolone resistance in *S. aureus*. The complete nucleotide sequence of *gyrA* is not determined to date even though the N-terminal partial sequence reveals high level of homology to that of *E. coli*. Therefore we performed Southern analysis in order to see RFLPs on *gyrA* locus and amplified 290 bp region near Tyr-122 of *gyrA* of 8 ORSAs as well as 2 ofloxacin susceptible ones by PCR reaction and compared their sequences.

MATERIALS AND METHODS

Bacterial Strains and Antimicrobial Agents

The bacterial strains used in this study were ofloxacin resistant clinical isolates of *Staphylococcus aureus* as well as ofloxacin sensitive ones isolated and donated from Hoechst, Germany. Ciprofloxacin, norfloxacin, methicillin and vancomycin were purchased from Sigma. Ofloxacin were obtained from Jeil Pharmaceutical Co. Cefotaxime was donated by Hoechst, Germany. Sparfloxacin was given by Dainippon Pharmaceutical Co., Japan.

Antimicrobial Susceptibility Testing

The susceptibility of bacteria was tested by means of agar dilution method on Muller-Hinton agar (Difco, USA). Plates were innoculated with an automatic inoculator (Dynatech, USA) which delivered 10^4 CFU per spot of stationary, freshly diluted cultures of the strains concerned. The MIC was taken as the lowest concentration at which no visible growth could be detected after 18 h of incubation at 37°C.

Chromosomal DNA Isolation from *S. aureus*

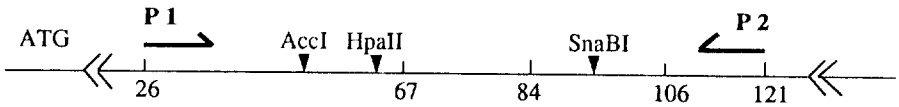
Bacterial genomic DNA was purified from cells harvested at late logarithmic phase. Cells were resuspended in 0.15 M NaCl, 0.1 M EDTA, 250 μg/ml lysostaphin and incubated for 30 min at 37 °C with gentle shaking. SDS was added to final concentration of 1% and incubated for 10 min at 60°C. Sodium perchlorate was added to final concentration of 1%. The bacterial proteins were removed by chloroform extraction. The DNA in the upper phase was precipitated with ethanol and harvested with sterilized glass rod. The DNA was treated with 50 μg/ml RNase A (Sigma) for 30 min at 37°C. The RNase was removed by chloroform extraction and the DNA was again precipitated and obtained with glass rod.

Southern Analysisc

Genomic DNA was digested with *Eco*RI, *Bam*HI, *Hind*III, *Pst*I, *Bgl*II, *Alu*I, *Hae*III, *Taq*I (KOSCO, Korea), *Cfo*I, *Hpa*II, *Mbo*I (BRL, USA), *Hha*I and *Rsa*I(NEB, USA) under the condition providers specify. DNAs treated with restriction enzymes were electrophoresed on 0.8% agarose gels and transferred to nitrocellulose (Hoefer, USA) with Trans-Vac TE80 (Hoefer, USA). The nitrocellulose was baked for 2 hrs at 80°C. pSLS 447, a plasmid with 7 kb *gyrA* gene insert and donated from Dr. J.C. Wang in Harvard University (16), was labeled with [α^{32}P]-dCTP (Amersham, USA) by means of random primed labeling (USBC, USA). Probe DNA with specific activity of approximately 10^7 cpm was used in each hybridization. Hybridization was done in 10 X Denhardt's solution, 0.1 mg/ml of salmon sperm DNA, 0.1% SDS and 0.4 M phosphate buffer (pH 6.0) for 18 hrs at 60°C. The blot was washed with 0.24 M phosphate buffer (pH 6.0), 0.1% SDS at room temperature for 15 min and subsquently with 0.4 M phosphate buffer (pH 6.0), 0.5% SDS for 30 min at 60°C. The blot was then dried and exposed to Kodak X-omat film for autoradiography.

Amplification of *gyrA*-290 by PCR

The oligonucleotide primer was synthesized by Operon Tech Corp. (USA). The sequence and position of primers is in Fig. 1. The PCR reaction was carried out with single block system thermal cycler (Ericomp, England) in 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.01% Tween 20, 0.01 % NP-40, 200 μM dNTPs (Pharmacia, USA), 1 μM of each primers, 5 units of Taq DNA polymerase (Perkin Elmer Cetus, USA) with 5 μg of



Primer 1 : 5' -GCGATGAGCGTTATCGTTGCTCGTGC- 3'
Primer 2 : 5' -CGCATTGCTGCTGCGCCATCTCC- 3'

Fig. 1. *The sequence and position of gyrA-290 primers. The numbers below the line represent amino acid numbers of S. aureus gyrase A.*

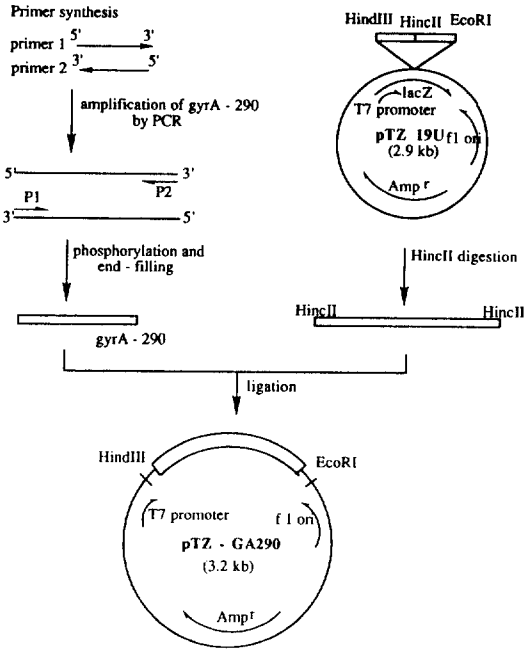


Fig. 2. *Cloning strategy of gyr A-290 by PCR amplification.*

chromosomal DNA. We routinely programmed the reaction as follows; 1 cycle of step 1 (15 min at 94°C, 1 min at 55°C), 33 cycles of step 2 (1 min at 94°C, 1 min at 55°C, 1 min at 72°C) and 1 cycle of step 3 (1 min at 94°C, 1 min at 55°C, 5 min at 72°C). The amplified product was checked on 1.5% agarose gel and the *gyrA*-290 fragments were isolated with electroelutor (Hoefer, USA).

Nucleotide Sequence Analysis of gyrA-290

The *gyrA*-290 fragments amplified by PCR were phosphorylated and treated with Klenow enzyme (KOSCO, Korea) in order to make blunt ends. pTZ vector (obtained from Genetic Engineering Research Institute) was digested with HincII (KOSCO, Korea) to make blunt ends. Thus, blunt end ligation was performed between the *gyrA*-290 and pTZ vector. The cloning scheme is shown in Fig. 2. The cloned *gyrA*-290 was subjected to sequence analysis. DNA sequences were determined by dideoxy chain termination method with the DNA sequencing kit (USBC, USA).

RESULTS

Antimicrobial Susceptibilities of *S. aureus*

The MICs of OSSA and ORSA against seven antibiotics were determined (Table 1). ORSAs showed 15-30 fold higher MICs against ofloxacin, 10-60 fold against ciprofloxacin and norfloxacin than those of OSSAs. The resistance of ORSAs against hydrophobic quinolone, sparfloxacin, was most dramatic i.e. 60-250 fold higher MICs than those of OSSAs. In case of cefotaxime and methicillin, which are β-lactams, the MIC of ORSAs was also increased 2-30 fold than those of OSSAs. It is noteworthy that vancomycin was highly effective against ORSAs as well as OSSAs. Among ORSAs, *S. aureus* 17613 was most resistant against three hydrophilic quinolones.

Southern Analysis of *gyrA* gene

The possibility that the nucleotide sequence changes in gyrase A gene, thereby resulting RFLPs in that locus, might be involved in quinolone resistance mechanism was examined. Since we did not have the *gyrA* clone of *S. aureus*, *E. coli gyrA* clone was used to detect restriction fragments of *S. aureus gyrA*. *E. coli gyrA* probe hybridized to *S. aureus gyrA* under the condition described above. However the hybridization signal

Table 1. *Antimicrobial agent susceptibility of S. aureus strains. Numbers represent MIC values (μg/ml). OFX: ofloxacin, NFX: norfloxacin, CPFX: ciprofloxacin, SPFX: sparfloxacin, CFX: cefotaxime, MTCN: methicillin, VCMN: vancomycin.*

Antibiotics / Strains	Quinolones				β-lactams		
	OFX	NFX	CPFX	SPFX	CFX	MTCN	VCMN
Ofloxacin susceptible							
S. aureus 285	0.391	1.563	1.563	0.098	3.125	3.125	0.781
S. aureus 503	0.391	1.563	0.781	0.098	0.781	1.563	0.391
Ofloxacin resistant							
S. aureus 179	6.250	50.000	12.500	6.250	6.250	6.250	0.781
S. aureus 241	6.250	50.000	12.500	6.250	12.500	6.250	0.781
S. aureus 293	12.500	50.000	12.500	6.250	25.000	25.000	0.781
S. aureus 303	6.200	50.00	12.500	12.500	6.250	6.250	0.781
S. aureus 1763	25.000>	200.000	12.500	6.250	6.250	0.781	
S. aureus 17740	6.250	50.000	12.500	25.000	12.500	12.500	0.781
S. aureus 17746	12.500	50.000	12.500	6.250	6.250	3.125	0.781
S. aureus 17845	6.250	50.000	12.500	6.250	12.500	6.250	0.781

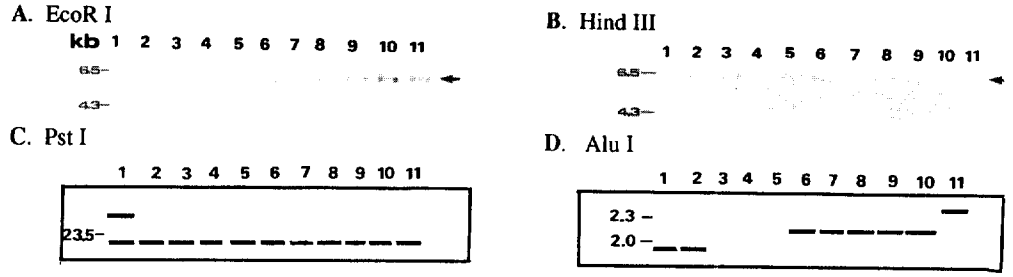


Fig. 3. *Southern analysis of RFLPs of gyrA locus of S. aureus. Chromosomal DNAs of S. aureus was digested with A: EcoRI, B: HindIII, C: PstI and D: AluI. In case of PstI and AluI digestion, figures are drawn according to the autoradiogram becuse of the weak signal.*
Lane 1-9: *S. aureus* 179, *S. aureus* 241, *S. aureus* 293, *S. aureus* 303, *S. aureus* 17613, *S. aureus* 17740, *S. aureus* 17746, *S. aureus* 17845, *S. aureus* 8236 (ORSAs), lane 10, 11: *s. aureus* 285, *S. aureus* 503 (OSSAs).

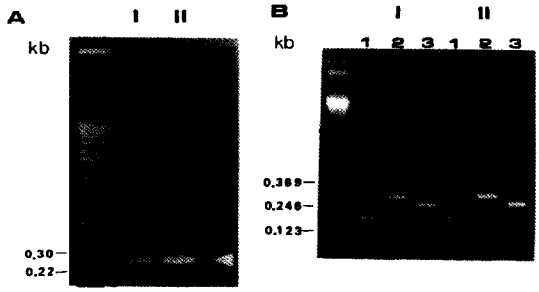
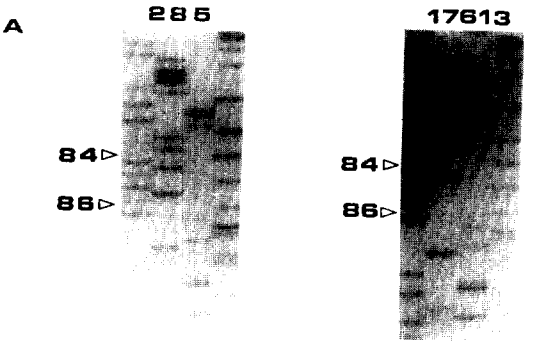


Fig. 4. *Identification of amplified product of gyrA-290 of S. aureus.*
A. amplified PCR product of *gyrA*-290 from *S. aureus* strains. M: λ*BstE*II, I: *S. aureus* 285, II: *S. aureus* 17613; B, I: digestion of PCR product from *S. aureus* 285 with 1: *Hpa*II, 2: *Sna*BI, 3: *Acc*I, II: digestion of PCR product from *S. aureus* 17613 with 1: *Hpa*II, 2: *Sna*BI, 3: *Acc*I.



B

	80			83				87			90
E. coli K12	His CAT	Gly GGT	Asp GAC	Ser TCG	Ala GCG	Val GTC	Tyr TAT	Asp GAC	Thr ACG	Ile ATT	Val GTA
	81			84	85						91
S. aureus 81231	His CAT	Gly GGT	Asp GAC	Ser TCA	Ser TCT	Ile ATT	Tyr TAT	Glu GAA	Ala GCA	Met ATG	Val GTA
S. aureus 285	---	---	---	---	---	--C	---	---	---	---	---
S. aureus 17613	---	---	---	-T- (Leu)	---	---	---	---	---	---	---

Fig. 5. *DNA sequence comparison of ofloxacin susceptible and resistant S. aureus. The sequence of S. aureus 81231 was reported proviously (8).*

was weak in general probably beacuse of the low homology (60%) between *E. coli.* and *S. aureus* (14).

6 kb band was found with *EcoRI* digestion both in ORSAs and OSSAs (Fig 3A). With *Hind*III digestion, 6.5 kb band appeared in ORSAs but none in OSSAs (Fig 3B). With *Pst*I digestion, *S. aureus* 179 showed unique >23 kb band as well as <23 kb band present in all 10 *S. aureus* strains (Fig. 3C). A high molecular weight band (>23 kb) was found with *Bam*HI and with *Bgl*II both in ORSAs and OSSAs (data not shown). However it was difficult to size the fragments and to judge the possible size polymorphism because the fragments were too big to be analyzed accurately with the 0.8% agarose gel system.

Seven 4-cutter enzymes, *Alu*I, *Mbo*I, *Hha*I, *Rsa*I, *Cfo*I, *Hpa*II and *Taq*I, were also used. However with the exception of *Alu*I, no band was detected with 2 week exposure time (data not shown). In case of *Alu*I, *S. aureus* 179 and 241 showed 1.9 kb band and *S. aureus* 17740, 17746, 17845 and 8236 showed 2.2 kb band (Fig 3D). Among 2 OSSA strains, strain 285 showed 2.2 kb band and strain 503 showed 2.3 kb band.

Therefore RFLPs between ORSAs and OSSAs were detected with *Hind*III, *Pst*I and *Alu*I digestions.

Sequence Comparison of *gyrA*-290

The 290 bp region of gyrA of ORSAs and

OSSAs between amino acid 26 and 121 was amplified by PCR (Fig 4A). The amplified products were digested with *Hpa*II, *Sna*BI and *Acc*I (Fig 4B). The sizes of digestion products were the same as those could be predicted by the published *S. aureus* sequence (8).

The sequence comparison of ORSA and OSSA demonstrated two base difference with 290 bp region (Fig 5). A point mutation of C to T at the second base of amino acid 84 resulting Ser to Leu substitution was observed in all ORSA strains. Another base change, C to T, resulting no amino acid change was observed in the third base of amino acid 86 in all ORSA strains. Also the sequence of *gyrA*-290 of OSSAs was compared to that of published sequence of *S. aureus* of that region. A single base change resulting silent mutation was revealed in the third base of amino acid 86.

DISCUSSIONS

OSSAs were highly susceptible to quinolones and vancomycin and moderately susceptible to cefotaxime and methicillin. However MIC test result of clinical isolates of eight ORSA strains revealed that ORSAs were resistant not only to quinolones but also, even though with lesser degree, to cefotaxime and methicillin. Only vancomycin was equally effective against OSSAs and ORSAs. The antibiotic spectra of ORSA strains were similar to each other in general. However strain 17613 draws attention in that the degree of resistance against hydrophilic quinolones was most high among nine ORSAs. Sparfloxacin proved to be the most efficient agent against OSSAs among seven antibiotics used in this study. The potent antibacterial activity of sparfloxacin might be related to its lipophiliciity which facilitates its permeation. The fact that the resistance was most severely developed against sparfloxacin (up to 250 fold increase of MIC) in *S. aureus* in our study is surprising because sparfloxacin is one of the newest quinolones and its usage in the treatment of *S. aureus* infections seems to increase. Further studies seem to be necessary to understand the frequency and degree of sparfloxacin resistance development in *S. aureus*.

We searched possible RFLPs first in order to group ORSAs and secondly to find mutation(s) between OSSAs and ORSAs. *E. coli gyrA* probe was homologous enough to allow us examine RFLPs in *S. aureus gyrA* locus with six cutter enzymes. However not much information was obtained with four cutter enzymes which were expected to reveal more length polymorphism. It may not be surprising that we failed to detect *S. aureus gyrA* gene fragments probing *E. coli gyrA* with many four cutters except *Alu*I because the sequence homology between *E. coli* and *S. aureus gyrA* genes was relatively low and the possibility to contain homologous region(s) within the shortened fragments became even lower. *Pst*I and *Alu*I digestions presented some possibilities of sequence variation(s) in the *gyr*A locus of ORSAs. However it is insufficient to know whether those sequence variations are within and/or outside the gyrA gene and are expressed as real amino acid changes with the limited RFLP informations.

Sequencing the entire gyrA genes of each *S. aureus* strain would be able to answer above questions. However that is not a simple task. Therefore we decided to examine 290 bp region which contains N-terminal amino acid residues 26 to 121 of gyrase A. A single point mutation at the second base of amino acid 84 which resulted an amino acid change Ser to Leu was found in every ORSA strains sequenced. The possibility that all ORSA isolates is the same strain should be excluded because differences were observed in RFLPs and in their antibiotic spectra even though we can not completely exclude the possibility that some of them might be the same origin. Mutations of *E. coli gyrA* gene were observed in Ala-67, Ser-83 and Gln-106 in quinolone resistant strains (12, 19, 20). The amino acids in these three amino acids are all conserved in *S. aureus*. Studies in *E. coli* indicate mutations were most frequently found in Ser-83 of gyrA. These suggest the possible involvement of Ser-84 residue of gyrase A in quinolone-gyrase interaction and thereby quinolone resistance development.

In this study only *gyrA* was examined in ORSA strains. Other possibilities such as mutations in *gyrB* and changes in permeabilities and in efflux systems involving *norA* gene (17, 21) need to be studied.

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초 록: *Staphylococcus aureus*에서의 Quinolone 내성 기작에 관한 연구

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*Staphylococcus aureus*의 퀴놀론계 약물에 대한 내성 기작을 이해하고자 ofloxacin 에 내성을 보이는 임상 채취 8 균주의 *Staphylococcus aureus* (ORSA)에 대하여 MIC 검사, *gyrA* 유전자 부근의 Southern 분석 및 아미노산 26번에서 121번까지를 포함하는 290 bp의 *gyrA* 유전자 일부(*gyrA*-290) 의 염기 서열 분석을 행하였다. ORSA 들은 퀴놀론계 약물에 대하여 높은 수준의 내성을 보였으며(8-250 배의 MIC 증가), β-lactam계 약물에 대하여도 상당한 수준의 내성(2-32 배의 MIC 증가)을 보였다. 하지만 ORSA 들은 vancomycin 에 관한 감수성의 변화를 보이지는 않았다. ORSA 에 대하여 Southern 분석을 실시한 결과, *Hind*III, *Pst*I 및 *Alu*I의 경우에는 *gyrA* 유전자 부근에서 RFLP 가 발견되었다. *GyrA* 유전자를 더 분석하고자 *gyrA*-290 부분을 중합효소 연쇄반응(PCR)으로 증폭하여 pTZ 벡터에 클론하였다. *gyrA*-290 의 염기 서열을 분석한 결과, 8 ORSA 균주 모두에 관하여 점 돌연변이의 결과로 Ser-84 이 Leu-84 으로 치환됨이 밝혀졌다. 이로써 *gyrA* 유전자의 84 번째 아미노산의 치환이 *Staphylococcus aureus*의 퀴놀론 내성 발현의 중요한 기작 중 하나일 가능성이 있다고 생각된다.