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Cloning and Expression of the *norA* Gene for Fluoroquinolone Resistance in *Staphylococcus aureus*

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The *norA* gene for fluoroquinolone resistance in *Staphylococcus aureus* TK2566 was cloned from chromosomal DNA into fluoroquinolone-susceptible *Escherichia coli* TG1. The resulting transformant, NY11, contained a recombinant plasmid, designated pTUS1, with a cloned 5.5-kilobase (kb) *Hin*dIII fragment of staphylococcal DNA. The MIC of norfloxacin for the strain increased from 0.1 to 3.13 μg/ml. Furthermore, when the fragment was recloned into *S. aureus*, the transformant NY12, containing recombinant plasmid pTUS20, had the same level of resistance to norfloxacin as did the original strain, although it was less resistant to ofloxacin and ciprofloxacin. A single *Kpn*I-*Hae*III fragment was found to be the minimum size able to express norfloxacin resistance, suggesting that the *norA* gene is located within the 2.6- to 3.2-kb region of the original 5.5-kb fragment. The 5.5-kb fragment hybridized to DNA from a fluoroquinolone-susceptible *S. aureus* strain.

Unlike the older quinolones, compounds of the fluoroquinolone group are very active against gram-negative and gram-positive bacteria (6, 17, 26, 27, 32). These agents are noted for a low incidence of resistant mutants in gram-negative bacteria (5, 6, 15) and good oral absorbability. Because of these advantages, fluoroquinolones have been widely used against bacterial infections, but along with their greater use has come a rapid increase of fluoroquinolone-resistant strains, especially in *Staphylococcus aureus*. Since many of these strains are also resistant to methicillin (18), this has become an important problem for clinicians.

Although the mechanism of fluoroquinolone resistance in *Escherichia coli* (2, 13, 15, 25) and other gram-negative bacteria (1, 14) has been studied in detail, this is the first report concerning *S. aureus*. We describe here the genetic characterization of fluoroquinolone resistance caused by a mutation(s) on the chromosome of *S. aureus*.

MATERIALS AND METHODS

Strains and plasmids. Table 1 shows the strains and plasmids used. Fluoroquinolone-resistant *S. aureus* TK2566, resistant to norfloxacin, ofloxacin, and ciprofloxacin, was isolated in 1986 from the urine of a patient who received norfloxacin for 2 weeks at Teikyo University Hospital.

E. coli TG1 and *S. aureus* SA113 were used as recipients for transformation. Vector plasmid pSU40 for *S. aureus* was constructed by inserting a 45-base-pair *Eco*RI-*Hin*dIII fragment of a multiple cloning site into plasmid pSU20 encoding chloramphenicol resistance, which had been isolated from *S. aureus* TK567 (30). Plasmids pAW011 and pJB11, which contain *gyrA* (34) and *gyrB* (33) genes, respectively, from *E. coli* KL16, were used for Southern blot hybridization.

Preparation of chromosomal DNA and plasmid DNA. Chromosomal DNA from each strain was purified from cells grown to the late logarithmic phase in 100 ml of L broth, according to methods previously described (22). Rapid electrophoretic analysis of the recombinant plasmid in *E. coli* was performed by the alkaline lysis procedure (21). To isolate the DNA from *S. aureus*, lysostaphin (Sigma Chemical Co., St. Louis, Mo.), instead of lysozyme, and 0.5 M EDTA were added to the cell suspensions to give concentrations of 33 μg/ml (31). A large amount of plasmid DNA from bacteria cultured in 100 ml of L broth was purified by CsCl density gradient centrifugation with a 300L centrifuge (Beckman Instruments, Inc., Fullerton, Calif.) at 100,000 × *g* for 6 h at 15°C.

Transformation. *E. coli* TG1 was transformed by the method described by Mandel and Higa (20) under slightly modified culture conditions. That is, 0.2 ml of overnight-cultured bacteria was inoculated into 10 ml of SOB (21) broth and incubated at 37°C with vigorous shaking until the optical density at 550 nm increased to 0.5 for maximum transformation efficiency. *S. aureus* SA113 was transformed by modifying the method for *Bacillus subtilis* (4). The bacteria were cultured until the late logarithmic phase in 50 ml of L broth at 37°C with shaking and harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. They were washed once with 10 ml of SMMP solution, which contained equal volumes of SMM solution (0.7 M sucrose, 0.02 M maleate, 0.02 M $MgCl_2$, pH 6.5) and PB broth (antibiotic no. 3 [Difco Laboratories, Detroit, Mich.], concentrated four times), and were suspended in 5 ml of the same solution. After lysostaphin was added to a concentration of 20 μg/ml, the suspensions were gently shaken at 37°C for 1 to 2 h. When most of the cells were converted to protoplasts, they were collected by centrifugation at 12,000 × *g* for 10 min at 4°C, washed once, and carefully resuspended in 2 ml of SMMP solution without vortexing.

Subsequently, 0.5 ml of the protoplast suspension and 50 μl of the ligated DNA were gently mixed, 1.5 ml of 40% (wt/vol) polyethylene glycol (in SMM) was immediately added, and the mixture was allowed to stand at 30°C for 2 min. Five milliliters of SMMP solution was then added, and the mixture was centrifuged. The sedimented protoplasts were suspended in 10 ml of regeneration medium, which consisted of 3.45 ml of base medium (5 g of Casamino Acids and 5 g of yeast extract per 350 ml), 5 ml of 1 M sodium succinate, 1 ml of potassium phosphate solution (0.1 M KH_2PO_4, 0.2 M K_2HPO_4), 0.25 ml of 20% glucose, 0.2 ml of 1 M $MgCl_2$, and 0.1 ml of 2% bovine serum albumin, and the suspension was gently shaken at 37°C for 2 h. The cells were harvested by centrifugation and spread onto selective agar plates containing 5 μg of chloramphenicol per ml (prepared by solidifying the regeneration medium with 1.5% agar).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype[a]	Source or reference
Strains		
E. coli		
TG1	K-12, Δ(*lac-pro*) *supE thi hsdD5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZΔM15*	M. Kanno
NY11	TG1(pTUS1)	This study
NY13	TG1(pTUS18)	This study
NY14	TG1(pTUS19)	This study
S. aureus		
SA113	r^- m^-	M. Inoue
TK2566	Nfx^r	Clinical isolate from Teikyo University Hospital
NY12	SA113(pTUS20)	This study
Plasmids		
pUC19	Amp^r	M. Inoue
pBR322	Amp^r Tc^r	M. Inoue
pAW011	Amp^r; *gyrA* gene from *E. coli* KL16 cloned into pBR322	H. Yoshida (34)
pJB11	Amp^r; *gyrB* gene from *E. coli* KL16 cloned into pBR322	J. Yamagishi (33)
pSU40	Cm^r; insertion of a 45-base-pair *Eco*RI-*Hin*dIII multiple cloning site	K. Ubukata (30)
pTUS1	Amp^r; 5.5-kb chromosomal DNA fragment from *S. aureus* TK2566 cloned into pUC19	This study
pTUS18	*Kpn*I-*Hae*III fragment from pTUS1 subcloned into pUC19	This study
pTUS19	*Eco*RV-*Eco*RV fragment from pTUS1 subcloned into pBR322	This study
pTUS20	Cm^r Nfx^r; 5.5-kb DNA fragment from pTUS1 recloned into pSU40	This study

[a] Resistance to the following drugs is indicated: Nfx^r, norfloxacin; Amp^r, ampicillin; Cm^r, chloramphenicol.

Another 4 ml of regeneration medium, containing 0.4% agar and 5 μg of chloramphenicol per ml, was overlaid on each plate, which was then incubated at 37°C for 3 to 4 days. One thousand colonies grown on the plates were randomly selected and inoculated into 2 ml of L broth, incubated overnight at 37°C, and used to measure the MICs of norfloxacin, ofloxacin, and ciprofloxacin.

Measurement of MICs. The MICs of nalidixic acid, norfloxacin, ofloxacin, ciprofloxacin, and novobiocin for the wild-type strains and transformants were determined by the spot method on sensitivity test agar plates (Eiken Co. Ltd., Tokyo, Japan) containing serial twofold dilutions of each compound. Bacteria cultured overnight were diluted to 10^5 CFU per spot and inoculated on the agar plates with a multipoint replicator. The MICs were determined after incubation for 24 h at 37°C.

Southern blotting. Chromosomal and plasmid DNA fragments were electrophoresed in a 0.8% agarose gel containing 0.5 μg of ethidium bromide per ml. DNA transfer to nylon filters (Ultrafine Filtration Co., East Hills, N.Y.) and hybridization with radioactive probes were performed as previously reported (21, 28). A 5.5-kilobase (kb) *Hin*dIII fragment from pTUS1, labeled with [α-^{35}S]dCTP (410 Ci/mmol; Amersham Japan Co., Tokyo, Japan), was used as a radioactive probe.

RESULTS

Cloning of the *norA* gene for fluoroquinolone resistance. Figure 1 shows the results of cloning the locus for fluoroquinolone resistance in *S. aureus*. Total DNA extracted from fluoroquinolone-resistant *S. aureus* TK2566 was digested with *Hin*dIII, *Eco*RI, or *Bam*HI and ligated to vector plasmid pUC19, which had previously been digested with the same enzymes. Only from cells transformed with the ligated mixture of the *Hin*dIII fragments were transformants obtained on agar plates containing both 1 μg of norfloxacin per ml and 25 μg of ampicillin per ml. The transformant strain NY11 contained a recombinant plasmid, designated pTUS1,

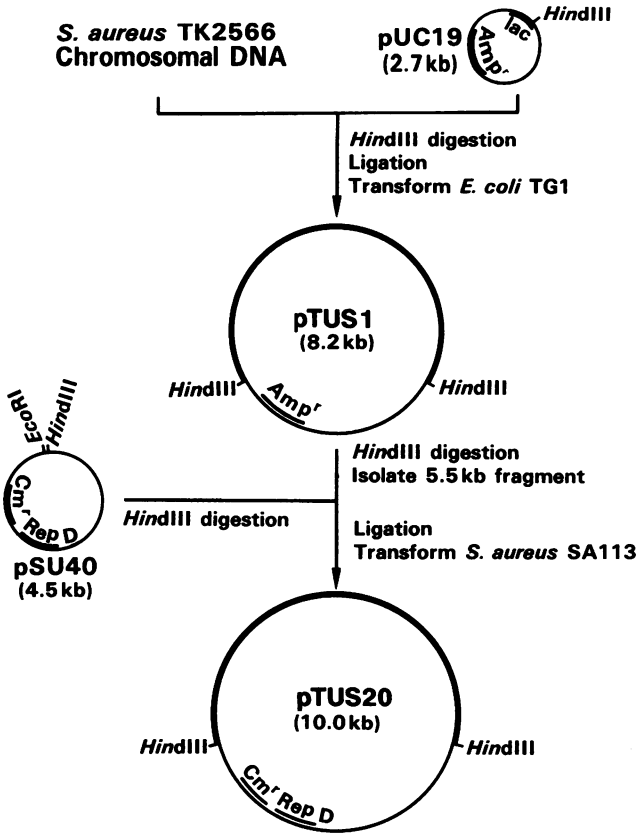

FIG. 1. Cloning of the fluoroquinolone resistance gene in *S. aureus*. Recombinant plasmid pTUS1 was constructed by inserting a 5.5-kb *Hin*dIII fragment of chromosomal DNA from *S. aureus* TK2566 into vector plasmid pUC19. A 5.5-kb *Hin*dIII fragment purified from pTUS1 was ligated at the *Hin*dIII site of vector plasmid pSU40 for *S. aureus* and used to transform cells of *S. aureus* SA113. The final recombinant plasmid, pTUS20, was constructed with a 5.5-kb chromosomal DNA fragment and plasmid pSU40, which encodes the *cat* (Cm^r) gene and replication functions (*repD*).

TABLE 2. Antimicrobial agent susceptibilities of strains used in this study

Strain	MIC (μg/ml)[a]				
	Norfloxacin	Ofloxacin	Ciprofloxacin	Nalidixic acid	Novobiocin
S. aureus TK2566	50	12.5	25	>400	0.1
E. coli					
TG1	0.1	0.1	0.1	3.1	>100
NY11(pTUS1)	3.1	0.2	0.4	3.1	>100
NY13(pTUS18)	3.1	0.2	0.4	3.1	>100
NY14(pTUS19)	0.1	0.1	0.1	3.1	>100
S. aureus					
SA113	0.8	0.2	0.2	25	0.1
NY12(pTUS20)	50	3.1	12.5	50	0.1

[a] MICs were determined with serial twofold dilutions of the agents in sensitivity test agar. Bacteria cultured overnight were inoculated onto the agar plates at 10^5 CFU per spot and incubated for 24 h at 37°C.

in which a 5.5-kb *Hin*dIII fragment of staphylococcal DNA was inserted.

To demonstrate expression of the locus supposed to correspond to fluoroquinolone resistance, a 5.5-kb fragment was purified from plasmid pTUS1 and inserted at the *Hin*dIII site of vector plasmid pSU40 for *S. aureus*. This ligation mixture was used to transform protoplasts derived from *S. aureus* SA113. Norfloxacin-resistant colonies, containing recombinant plasmid pTUS20 with a 5.5-kb *Hin*dIII DNA fragment, were selected as transformant strain NY12.

Fluoroquinolone susceptibilities of transformant strains. The MICs of norfloxacin, ofloxacin, ciprofloxacin, nalidixic acid, and novobiocin for the recipient strains, *E. coli* TG1 and *S. aureus* SA113, and the transformant strains, *E. coli* NY11, NY13, and NY14 and *S. aureus* NY12, are shown in Table 2. The MIC of norfloxacin for the *E. coli* transformant NY11 was about 30 times higher than that for the recipient TG1, whereas the MICs of ofloxacin and ciprofloxacin were only two to four times higher, and those of nalidixic acid and novobiocin were the same or nearly so for either strain. In contrast, for *S. aureus* transformant NY12, the MIC of norfloxacin was the same as that for the wild-type strain TK2566, and the MICs of norfloxacin and ciprofloxacin were 30 times higher than those for the recipient. With nalidixic acid and novobiocin, the MICs for either strain were again about the same.

Physical map of 5.5-kb *Hin*dIII DNA fragment. A physical map of the 5.5-kb *Hin*dIII fragment is shown in Fig. 2. An MIC of norfloxacin about 10 times higher was observed for the *E. coli* transformants containing pTUS18 but not for those containing pTUS19. Plasmid pTUS18 contained the 3.2-kb *Kpn*I-*Hae*III DNA fragment, but plasmid pTUS19 lacked the 0.6-kb *Eco*RV-*Hae*III portion of the fragment. From these results, it was presumed that the locus for fluoroquinolone resistance, designated *norA*, may be located on the 2.6- to 3.2-kb region of the fragment.

Southern blot hybridization. Figure 3 shows the results of Southern blot hybridization when a ^{35}S-labeled 5.5-kb *Hin*dIII fragment purified from recombinant plasmid pTUS1 was used as a probe. Chromosomal DNAs from *S. aureus* wild-type strain TK2566 (lane B), transformant strain NY12 (lane C), and recipient strain SA113 (lane D) were digested with *Hin*dIII, separated by electrophoresis, and hybridized with a radioactive probe. The DNA fragments from the three strains clearly hybridized with the probe, and their sizes were the same as that of the probe DNA (lane A).

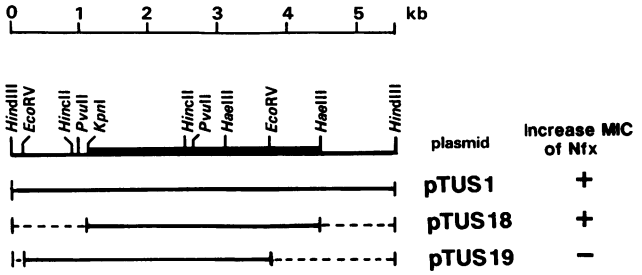

FIG. 2. Restriction map of the cloned 5.5-kb *Hin*dIII fragment containing the *norA* gene. Subclones are indicated below the map, and the increase (+) or lack of an increase (−) in the MIC of norfloxacin is shown at the right. The approximate extent of the *norA* region is indicated by a heavy line from the *Kpn*I to *Hae*III sites.

Figure 4 shows the results of hybridization of plasmids pAW011 (lane a) and pJB11 (lane b), which contain the *gyrA* and *gyrB* genes, respectively, from *E. coli* KL16, with the radioactive probe from pTUS1. The two DNA fragments, a 5.8-kb *Bam*HI-*Spl*I fragment from pAW011 and a 3.4-kb *Eco*RI-*Ava*I fragment from pJB11, seemed to hybridize with the probe (lane c) but not with another chromosomal DNA (3) cloned from *E. coli* K-12 (data not shown).

DISCUSSION

DNA gyrase of *E. coli* consists of two subunits, A and B, the products of the *gyrA* and *gyrB* genes, respectively (7, 9, 11, 23, 24). These subunits are considered to be the molecular targets of the quinolones (8, 29), and their nucleotide sequences have been determined, along with the sites of the mutations resulting in quinolone resistance (33, 34). Another mechanism of quinolone resistance, related to a decrease of cell membrane permeability, has also been reported (10, 13, 15, 16).

Fluoroquinolone compounds such as norfloxacin, ofloxacin, and ciprofloxacin, first synthesized in the 1980s, were found to have broad antimicrobial spectra and to possess strong activities against quinolone-resistant bacteria. This appears to be because the fluoroquinolones are more strongly inhibitory than quinolones for DNA gyrase (15; K.

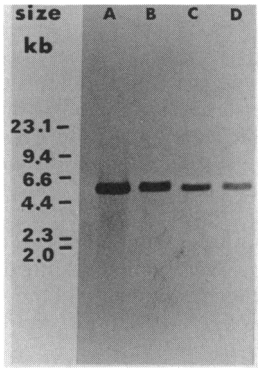

FIG. 3. Southern hybridization of chromosomal DNA prepared from staphylococcal cells. DNAs were digested with *Hin*dIII, electrophoresed in a 0.8% agarose gel, transferred to a nylon filter, and sequentially hybridized to a ^{35}S-labeled 5.5-kb *Hin*dIII fragment from plasmid pTUS1. Lanes: A, probes; B, norfloxacin-resistant wild-type strain TK2566; C, norfloxacin-resistant transformant strain NY12; D, norfloxacin-susceptible recipient strain SA113.

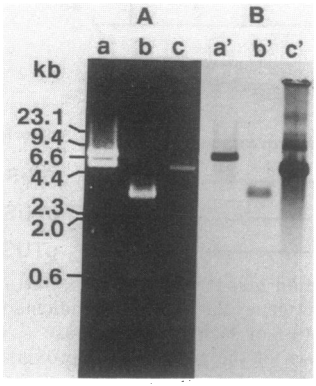

FIG. 4. Agarose gel electrophoresis (A) and Southern hybridization (B) of plasmids pAW011 (a and a') and pJB11 (b and b'), which encode *gyrA* and *gyrB* genes, respectively, from *E. coli* KL16, with a probe (c and c'). A 5.5-kb *Hin*dIII fragment purified from pTUS1 was used as a radioactive probe. Plasmid pAW011 was double digested with *Bam*HI and *Spl*I, and plasmid pJB11 was digested with *Eco*RI and *Ava*I. These samples were electrophoresed in a 0.8% agarose gel and transferred to a nylon filter, and the blot was hybridized under low-stringency conditions. The standard was λ DNA digested with *Hin*dIII. Sizes (in kilobases) are shown at the left.

Sato, Y. Inoue, S. Yamashita, M. Inoue, and S. Mitsuhashi, Proc. 14th Int. Congr. Chemother., p. 21–25, 1986) and also have an excellent capacity to permeate bacterial cells (12). However, their use for inpatients has been followed by an increase of the fluoroquinolone-resistant strains of *S. aureus*, *Serratia marcescens*, and *Pseudomonas aeruginosa* (18). Nevertheless, the mechanism of fluoroquinolone resistance in *S. aureus* has not yet been explored, whereas the mechanism in *E. coli* has been described previously (2, 13, 15, 25).

Therefore, we attempted to elucidate the mechanism of fluoroquinolone resistance in clinical isolates of *S. aureus*. One fundamental approach is to purify DNA gyrase and analyze its activity, but attempts to use wild-type strains have not been successful because the DNase activity is too high. In another approach, we tried to isolate the gene for fluoroquinolone resistance to observe its expression and to verify its hybridizability with the *gyrA* and *gyrB* genes of *E. coli*.

A homologous DNA fragment of the same size as the cloned 5.5-kb *Hin*dIII fragment from the fluoroquinolone-resistant strain was detected in the fluoroquinolone-susceptible strain. Furthermore, it seemed to hybridize with the DNA fragments which contained the *gyrA* and *gyrB* genes from *E. coli*. These results suggest the possibility that the *gyrA* and *gyrB* genes encoding the DNA gyrase subunits (A and B) are contained on the 5.5-kb DNA fragment. It is not yet clear whether both genes are encoded completely on the fragment or not. However, similar results demonstrating that the *gyrA* and *gyrB* genes are located in the same region of the DNA fragment, at about a 3.9-kb *Sal*I-*Eco*RI fragment, have been reported for *B. subtilis* (19).

On the other hand, although the genes encoding gyrases A and B in wild-type *E. coli* are dominant over the quinolone-resistant mutation (13, 15), the apparently higher MICs of fluoroquinolones for *S. aureus* transformant NY12 do not support this possibility. Further analysis of the *norA* gene will enable us to elucidate the mechanism of fluoroquinolone resistance in *S. aureus* in greater detail.

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