Cloning and Sequencing of a Novel Gene (recG) That Affects the Quinolone Susceptibility of Staphylococcus aureus

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In a study of the quinolone resistance genes in Staphylococcus aureus, a recG homolog was cloned as a gene affecting quinolone susceptibility. Sequencing analysis revealed that the gene consists of 2,061 nucleotides and encodes a 686-amino-acid polypeptide, which shows 38, 39, and 50% amino acid identity with the RecGs of Escherichia coli, Haemophilus influenzae, and Streptococcus pneumoniae, respectively. Seven helicase motifs are well conserved in the gene product. A plasmid carrying the gene complemented a recG-deficient mutant of E. coli with respect to mitomycin hypersusceptibility, demonstrating that the gene product is functionally equivalent to E. coli RecG. These results indicate that the gene is the recG gene of S. aureus. S. aureus RCM101 (recG::Tn551), designated S. aureus 3f33, is four to eight times more susceptible to quinolones than the parent strain, RCM101. The transformation of strain 3f33 with a plasmid carrying the S. aureus recG gene made it as quinolone resistant as strain RCM101. These results suggest that the recG gene is involved in the repair of DNA damage resulting from quinolone treatment in S. aureus.

Two principal mechanisms by which Staphylococcus aureus acquires quinolone resistance have been characterized: (i) alteration of the target enzymes, DNA gyrase, and topoisomerase IV (1, 2, 6, 11, 29, 30, 33); and (ii) increase in quinolone efflux from bacterial cells caused by membrane protein NorA (12, 22, 34). Many in vitro studies concerning the isolation of quinolone-resistant mutants have demonstrated that high-level resistance is obtained through at least two steps (3, 8, 11). In previous reports concerning quinolone resistance mutations of the DNA gyrase gyrA and gyrB genes of S. aureus, we reported a low-level quinolone-resistant first-step mutant of S. aureus, RCM101, selected with ciprofloxacin (11). RCM101 has no mutation in the genes related to quinoline resistance such as gyrA, gyrB, grlA, and norA, as far as has been examined (23). In the course of a study to identify the gene conferring quinolone resistance on the mutant, a quinolone-resistant mutant, 3f33, was isolated from RCM101 by means of transposon mutagenesis. The transposon-inserted gene was cloned and sequenced, and it was found to be S. aureus recG because of its sequence homology and functional similarity to Escherichia coli recG (17). This is the first report showing that recG is a gene affecting quinolone susceptibility.

MATERIALS AND METHODS

Materials. Ciprofloxacin, norfloxacin, oxolinic acid, and sparfloxacin were synthesized at Discovery Research Laboratories II, Dainippon Pharmaceutical Co., Ltd. Ampicillin, chloramphenicol, erythromycin, kanamycin, and tetracycline were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction endonucleases, Klenow fragment, and TaKaRa Ex Taq polymerase were obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan). Oligonucleotides for PCR and DNA sequencing were synthesized by Takara Shuzo Co., Ltd. Other reagents (guaranteed grade) were purchased from Nakalai Tesque, Inc. (Kyoto, Japan), or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Bacterial strains and vectors. S. aureus RN4220 (14) and plasmid pRN3208 (13) were kindly provided by R. P. Novick. A suicide vector, pKTN701 (24), and E. coli SY327 λpir (21) were obtained from M. Nishibuchi, E. coli JM109 and the pT7Blue(R)T vector were purchased from Takara Shuzo Co., Ltd. Plasmids pACYC184 and pBR322 were purchased from Wako Pure Chemical Industries, Ltd. Plasmid pND50, an E. coli-S. aureus shuttle vector, was constructed in this laboratory as described previously (33). S. aureus RCM101 is a low-level quinolone-resistant strain, which was isolated from quinolone-susceptible S. aureus RN4220 by single-step selection with ciprofloxacin (11). E. coli strains were grown in Luria-Bertani broth, and S. aureus strains were cultivated in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.), unless otherwise indicated. Plasmid pKTN701 and its derivatives containing the replication origin of R6K were propagated in E. coli SY327 λpir.

Drug susceptibility tests. MICs were determined by the twofold agar dilution method recommended by the Japan Society of Chemotherapy.

DNA methods. All standard DNA manipulations were performed according to the methods described by Sambrook et al. (26). Genomic DNA from S. aureus was prepared by the procedure of Novick (25). Isolation of plasmid DNA from S. aureus and electrotransformation of S. aureus were carried out as described previously (11). The antibiotic concentrations used for the selection of transformants were as follows: E. coli, ampicillin, 50 mg/ml, and chloramphenicol, 20 mg/ml; S. aureus, chloramphenicol, 15 mg/ml, and erythromycin, 100 mg/ml.

Transpositional mutagenesis. Transpositional mutagenesis was carried out with pRN3208, which contains Tn551 (carrying the erythromycin resistance gene) and is temperature sensitive with respect to replication at 43°C. S. aureus RCM101 was electroporated with pRN3208. The transformants were grown overnight at 30°C in BHI broth containing 100 mg/ml of erythromycin per ml. An overnight culture was diluted 1:10 with BHI broth, and then 100-μl aliquots of the suspension were plated on BHI agar containing 100 μg of erythromycin per ml. The plates were incubated at 43°C for 24 h. Erythromycin-resistant colonies were then screened to identify transpositions that had become quinolone susceptible. One of the erythromycin-resistant quinolone-susceptible mutants was selected (designated 3f33) for further study.

Identification of the Tn551 insertion fragment. Chromosomal fragments of 3f33 carrying the Tn551 insertion were identified by means of a Southern hybridization test. The 4.2-kb HpaI-BalI fragment internal to the Tn551 sequence and the 1-kb HindIII-Aval right-junction fragment of Tn551 (Fig. 1) were isolated from plasmid pRN3208 and used as Tn551 gene probes. Southern hybridization was carried out by a nonradioactive method with the ECL (enhanced chemiluminescence) direct nucleic acid labelling and detection system according to the manufacturer's protocol (Amersham International, Buckinghamshire, United Kingdom).

Cloning and sequencing strategies for the Tn551-inserted sequence. The two HindIII-digested chromosomal fragments of S. aureus 3f33 (each containing a part of the Tn551 sequence) were detected by means of a Southern hybridization test. In order to amplify only the chromosomal portions of these two fragments, a cassette-ligation-mediated PCR (10) was carried out with a TaKaRa LA PCR in vitro cloning kit and a Perkin-Elmer GeneAmp PCR system 9600. Briefly, the total DNA extracted from strain 3f33 was digested with HindIII and then ligated with the HindIII cassette. For amplification of the right-side fragment of the Tn551 insertion site, the first PCR was carried out with the cassette C1 primer (5′-GTACATATTGTGTTAGAACCGTATACGACTA-3′) containing the 3′-terminal sequence of Tn551, and the second PCR was performed with inner primers (i.e., the cassette
C2 primer [5′ CGTGAACGCGATACTGACTCTATAGGAGA 3′] and primer CLM2 [5′ GCCGCTGAAATGTTGATGAG 3′] were used for the first and second PCRs, respectively. CLM1, CLM2, CLM3, and CLM4 were designed on the basis of the sequence reported by Lloyd and Sharples (16). A genomic DNA preparation from one of the chloramphenicol-resistant and mitomycin-hypersusceptible mutants was examined by Southern hybridization analysis with the recG gene probe (the 1.1-kb MboI fragment internal to the recG gene) to confirm the destruction of the recG gene in the mutant. The analysis revealed that E. coli KL16 preparation contained a probe-positive 12-kb ClaI fragment (wild-type recG), while the mutant preparation contained a 19.5-kb ClaI fragment (recG::pIN208) (data not shown), suggesting that the 7.5-kb pIN208 was inserted into the chromosomal recG gene. In addition, the 7.0-kb KpnI fragment containing the recG gene was observed in wild-type KL16, while the KpnI digestion of the mutant preparation yielded instead the 11- and 3.5-kb fragments as expected (data not shown). A unique KpnI site is present on the vector plasmid. This recG-deficient mutant was designated E. coli KL16-P1.

Construction of plasmids carrying the recG genes of E. coli and S. aureus. Plasmid pIN102 for use for transformation with the S. aureus recG gene was constructed by inserting a 2.8-kb PstI-EcoRI fragment containing the recG gene of pIN101 between the PstI and EcoRI sites of the E. coli S. aureus shuttle vector, pND50. Plasmid pIN107 was obtained by ligating a 2.6-kb BamHI fragment containing the recG gene of pIN101 with the 4-kb BamHI-HindIII fragment of pBR322. Plasmids pIN203 carrying the E. coli recG gene was constructed by ligating a 2.6-kb BamHI-HindIII fragment from pIN201 with the 4-kb BamHI-HindIII fragment of pBR322.

Nucleotide sequence accession number. The sequence obtained in this study has been assigned DDBJ, EMBL, and GenBank nucleotide sequence accession no. AB000439.

RESULTS AND DISCUSSION

Isolation of Tn551 insertional mutants with increased quinolone susceptibility. Introduction of Tn551 into the chromosome of S. aureus RCM101, which is a low-level quinolone-resistant mutant isolated from S. aureus RN4220, was carried out as described in Materials and Methods. About 1,300 erythromycin-resistant colonies which were considered to contain Tn551 were examined for ciprofloxacin susceptibility; 65 ciprofloxacin-resistant strains were obtained. Among these 65 strains, most of which were only two times more susceptible to ciprofloxacin than strain RCM101 (data not shown), 1 strain, S. aureus 3f33, was eight times more susceptible to ciprofloxacin.
than RCM101 and was as ciprofloxacin susceptible as the original quinolone-susceptible strain, *S. aureus* RN4220 (Table 1). Its norfloxacin susceptibility was four times higher than that of RCM101, and it was two times more resistant than strain RN4220. Interestingly, strain 3f33 was two times more susceptible to oxolinic acid and sparfloxacin than RN4220, respectively. Moreover, the susceptibility of 3f33 to other types of antibiotics did not change at all (Table 1). These results suggest that the mechanism in RCM101. However, the fact that *S. aureus* 3f33 in which the recG gene of RCM101 was inactivated is ciprofloxacin susceptible suggests that recG is actually one of the genes affecting quinolone susceptibility. In order to determine whether or not the recG gene per se is involved in the change in quinolone susceptibility, 3f33 was transformed with plasmid pN102 carrying the wild-type recG gene cloned from RN4220. As shown in Table 1, a transformant of strain 3f33 with pN102 was as quinolone resistant as RCM101, indicating that the destruction of the recG gene caused an increase in quinolone susceptibility. This effect was also observed in *E. coli* KL16-P1, the recG-deficient mutant; this strain was four times more hypersusceptible to ciprofloxacin and sparfloxacin than KL16 (data not shown). Strain 3f33 was hypersusceptible to DNA-damaging agents, such as mitomycin and methyl methanesulfonate, as well (data not shown), in agreement with the previous studies that demonstrated increases in the susceptibilities to these agents on inactivation of the recG genes in *E. coli* and *S. pneumoniae* (16, 20). The RecG proteins of *E. coli* and *S. pneumoniae* are needed for normal recombination and DNA repair, and they have been shown to help Holliday junction intermediates change into mature products by catalyzing branch migration (16, 20, 31). Considering the role of the RecG proteins, it is conceivable that destruction of the recG gene causes an increase in quinolone susceptibility, in addition to hypersusceptibility to mitomycin and methyl methanesulfonate. Howard et al. (9) demonstrated that the recA13 mutant, deficient in both recombinational DNA repair (error-free repair) and SOS mutagenic DNA repair (error-prone repair), was more susceptible to new quinolones such as ciprofloxacin than the lexA3 mutant, deficient only in SOS DNA repair, which was hypersusceptible to the agents, and they suggested that the DNA damage resulting from the new quinolones is subject not only to error-free recombinational repair but also to SOS error-prone repair. Interestingly, error-free recombination repair, but not an inducible SOS system, is responsible for the repair of nalidixic acid-induced DNA damage (15). These findings suggest that some genes related to DNA repair are involved in the repair of quinolone-induced DNA damage and that the recG gene is one such repair gene. Normal-level expression of the gene may be enough for repair of the quinolone-induced DNA damage, because the recG gene on a multicopy plasmid did not confer resistance on wild-type RN4220.

### Table 1. Quinolone and antibiotic susceptibilities of a Tn551 insertional mutant, *S. aureus* 3f33, and its transformant with the *S. aureus* recG gene

<table>
<thead>
<tr>
<th><em>S. aureus</em> strain</th>
<th>CPFX</th>
<th>NFLX</th>
<th>SPFX</th>
<th>OA</th>
<th>ABPC</th>
<th>KM</th>
<th>TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN4220</td>
<td>0.2</td>
<td>0.78</td>
<td>0.1</td>
<td>1.56</td>
<td>0.05</td>
<td>0.78</td>
<td>0.1</td>
</tr>
<tr>
<td>RCM101</td>
<td>1.56</td>
<td>6.25</td>
<td>0.2</td>
<td>3.13</td>
<td>0.05</td>
<td>0.78</td>
<td>0.1</td>
</tr>
<tr>
<td>3f33</td>
<td>0.2</td>
<td>1.56</td>
<td>0.025</td>
<td>0.78</td>
<td>0.05</td>
<td>0.78</td>
<td>0.1</td>
</tr>
<tr>
<td>3f33(pND50)</td>
<td>0.2</td>
<td>1.56</td>
<td>0.025</td>
<td>0.78</td>
<td>0.05</td>
<td>0.78</td>
<td>0.1</td>
</tr>
<tr>
<td>3f33(pIN102)</td>
<td>1.56</td>
<td>6.25</td>
<td>0.1</td>
<td>3.13</td>
<td>0.05</td>
<td>0.78</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*CPFX*, ciprofloxacin; NFLX, norfloxacin; SPFX, sparfloxacin; OA, oxolinic acid; ABPC, ampicillin; KM, kanamycin; TC, tetracycline.

### Cloning and sequencing of the Tn551-inserted gene.

Cloning and sequencing of the Tn551-inserted gene. Attempts to clone the 8.8-kb EcoRV fragment and the 5.4- and 4.2-kb *Hind*III fragments of strain 3f33 into plasmids pHBr322 and pACYC184 were not successful. Hence, in order to determine the nucleotide sequence around the Tn551 insertion site, only the chromosomal portions of the 5.4- and 4.2-kb *Hind*III fragments were amplified by PCR and sequenced. Sequencing analysis of these PCR-generated fragments revealed that Tn551 was inserted into a sequence which could form a large open reading frame (tentatively designated orfX). The fragment containing orfX was then amplified from the *S. aureus* RCM101 chromosome by PCR and cloned into the pT7Blue(R)T vector, resulting in pN104, which was used as a template for the DNA sequencing. orfX has turned out to consist of 2,061 nucleotides and encodes a 686-amino-acid polypeptide with a predicted molecular mass of 78,3 kDa.

### Identification of the OrfX protein as a RecG homolog.

A search of available data banks revealed that the OrfX protein exhibits significant homology with RecG proteins, which catalyze the branch migration of Holliday junctions. OrfX shows

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*Note: The table and content are extracted and reformatted for clarity and readability. The original text contains more extensive details and context than shown here.*
FIG. 2. Alignment of the sequence of S. aureus OrfX (RecG [SARecG]) with those of E. coli (ECRecG [GenBank accession no. M64367]), H. influenzae (HIRecG [GenBank accession no. U32847]), and S. pneumoniae (MmsA [GenBank accession no. Z49988]). Identical amino acid residues are indicated by asterisks. Conserved helicase motifs I to VI, defined by Gorbalenya et al. (5), are indicated. Motif II contains the highly conserved DExH residues characteristic of this DNA-RNA helicase subfamily (18).
It has been well established that DNA gyrase and topoisomerase IV are the targets of quinolones and that quinolones bind with the DNA gyrase-DNA complex, forming ternary complexes (28, 35). Willmott et al. (32) have shown that the DNA gyrase-DNA-quinolone ternary complex acts as a barrier for transcription by RNA polymerase and that blocking of the transcription leads to cell death. The ternary complexes themselves are considered to be reversible, but collision with the replication or transcription complex is supposed to make them irreversible and probably to start them on a killing pathway (7). However, little is known about such downstream mechanisms of quinolones related to bacterial cell death. The RecA and RecG repair systems might remove the barrier of the ternary complexes (28, 35). Willmott et al. (32) have shown that the DNA gyrase-DNA-quinolone ternary complex acts as a barrier for transcription by RNA polymerase and that blocking of the transcription leads to cell death. The ternary complexes them- selves are considered to be reversible, but collision with the replication or transcription complex is supposed to make them irreversible and probably to start them on a killing pathway (7).

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REFERENCES


