ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1997, p. 1770–1774 Vol. 41, No. 8
0066-4804/97/$04.00+0

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

TOSHIYUKI NIGA, HIROAKI YOSHIDA, HIROAKI HATTORI, SHINICHI NAKAMURA, AND HIDEAKI ITO*

Discovery Research Laboratories II, Dainippon Pharmaceutical Co., Ltd., Suita, Osaka 564, Japan

Received 21 March 1997/Returned for modification 16 May 1997/Accepted 11 June 1997

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*::Tn*551*), 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 quinolone 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-susceptible 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, mitomycin, 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 Nacalai 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 λ *pir* (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 λ *pir*.

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 μg/ml, and chloramphenicol, 20 μg/ml; *S. aureus*, chloramphenicol, 15 μg/ml, and erythromycin, 100 μg/ml.

Transpositional mutagenesis. Transpositional mutagenesis was carried out with pRN3208, which contains Tn*551* (carrying the erythromycin resistance gene) and is temperature sensitive with respect to replication at 43°C. *S. aureus* RCM101 was electrotransformed with pRN3208. The transformants were grown overnight at 30°C in BHI broth containing 100 μg 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 Tn*551* insertion fragment. Chromosomal fragments of 3f33 carrying the Tn*551* insertion were identified by means of a Southern hybridization test. The 4.2-kb *Hpa*I-*Xba*I fragment internal to the Tn*551* sequence and the 1-kb *Hin*dIII-*Ava*I right-junction fragment of Tn*551* (Fig. 1) were isolated from plasmid pRN3208 and used as Tn*551* 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 Tn*551*-inserted sequence. The two *Hin*dIII-digested chromosomal fragments of *S. aureus* 3f33 (each containing a part of the Tn*551* 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 *Hin*dIII and then ligated with the *Hin*dIII cassette. For amplification of the right-side fragment of the Tn*551* insertion site, the first PCR was carried out with the cassette C1 primer (5′ GTACATATTGTCGTTAGAACGCGTAATACGACTCA 3′) containing the sequence which is present in the cassette and primer CLM1 (5′ CTCGTCAAAATAGATCCCGA 3′) containing the 3′-terminal sequence of Tn*551*, and the second PCR was performed with inner primers (i.e., the cassette

* Corresponding author. Mailing address: Discovery Research Laboratories II, Dainippon Pharmaceutical Co., Ltd., Enoki 33-94, Suita, Osaka 564, Japan. Phone: 81.6.3375909. Fax: 81.6.3387656.

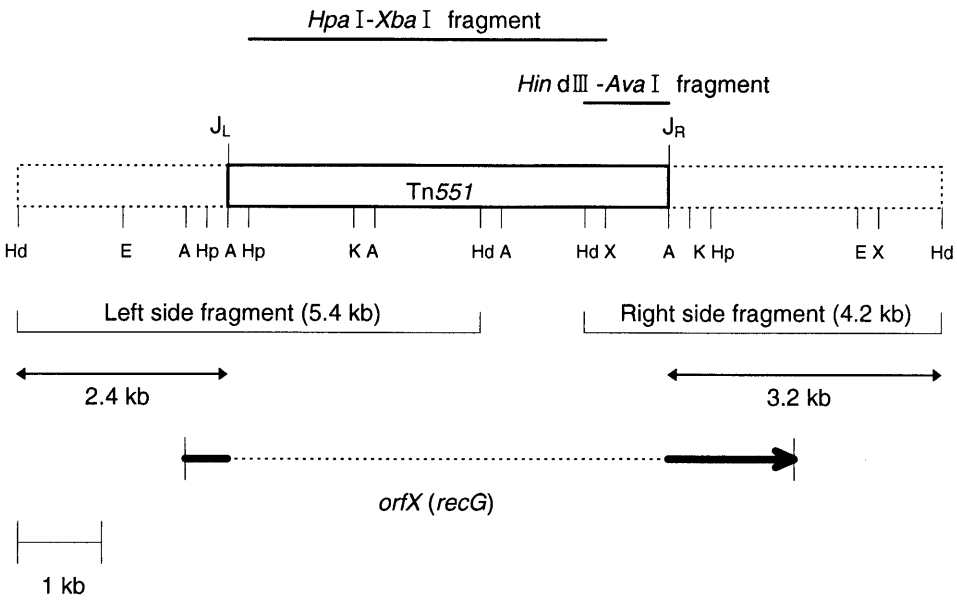

FIG. 1. Restriction map of the chromosomal fragment of *S. aureus* 3f33 around the Tn*551* insertion site. The 4.2-kb *Hpa*I-*Xba*I and 1-kb *Hin*dIII-*Ava*I probes are indicated by bold lines. J_L and J_R indicate the left and right junctions of Tn*551*, respectively. Two *Hin*dIII fragments, the left-side fragment (5.4 kb) and the right-side fragment (4.2 kb), detected in a Southern hybridization assay are shown. The left (2.4 kb) and right (3.2 kb) PCR-amplified portions are shown by arrows. The direction of *orfX* (*recG*) is indicated by a bold arrow. A, *Ava*I; E, *Eco*RV; Hd, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; X, *Xba*I.

C2 primer [5′ CGTTAGAACGCGTAATACGACTCACTATAGGGAGA 3′] and primer CLM2 [5′ GCCTTGAAACATTGGTTTAG 3′]). Thermal cycling of these PCRs was performed for 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 1.5 min), and extension (72°C, 3 min), and finally a 3.2-kb fragment was obtained. For amplification of the 2.4-kb left-side fragment of the Tn*551* insertion site, the cassette primer C1 and primer CLM3 (5′ CTGCAATAACCGTTACCTG 3′) containing a sequence complementary to the 5′-terminal sequence of Tn*551*, and the cassette primer C2 and primer CLM4 (5′ CACCGTCAAGTTAAATGTAC 3′) were used for the first and second PCRs, respectively. CLM1, CLM2, CLM3, and CLM4 were designed on the basis of the Tn*917* sequence, which is almost identical to the Tn*551* sequence (27); CLM1, nucleotide positions 5134 to 5153; CLM2, nucleotide positions 5281 to 5300; CLM3, nucleotide positions 285 to 267; and CLM4, nucleotide positions 181 to 162. The PCR-amplified fragments were sequenced by the cycle sequencing method with an ABI Prism dye terminator cycle sequencing ready reaction kit with Ampli*Taq* DNA polymerase, FS (Perkin-Elmer, Foster City, Calif.), and then applied on a Applied Biosystems sequencer (model 377). Oligonucleotide primers for sequencing, based on sequences already determined, were used to generate a series of overlapping sequences. In order to amplify the intact genes corresponding to the Tn*551*-inserted sequences from *S. aureus* RN4220 and RCM101 by PCR, a forward primer, 5′ GGTCAAGATGCAGAGCAAG 3′, and a reverse primer, 5′ GTGCGATCTAAACGAATTG 3′, were designed on the basis of the sequence data for the 2.4-kb left-side fragment and the 3.2-kb right-side fragment, respectively. A PCR was carried out with genomic DNA of each strain for 25 cycles, under conditions of 30 s at 94°C for denaturation, 30 s at 54°C for annealing, and 1.5 min at 72°C for polymerization. The PCR-generated fragments were ligated with the pT7Blue(R)T vector and then transformed into *E. coli* JM109. The recombinant plasmids contained the 2.8-kb fragments from strains RN4220 and RCM101, which were designated pIN101 and pIN104, respectively. These plasmids were used as templates for the automatic DNA sequencing described above.

Construction of a *recG*-deficient mutant of *E. coli*. The *recG* gene of *E. coli* KL16 (19) was amplified by PCR with chromosomal DNA as a template and primers 5′ GATCAGGACATCATCATTCC 3′ (nucleotide positions 132 to 151) and 5′ CGATCACTGAACGCTTTGG 3′ (nucleotide positions 2808 to 2790), which were designed on the basis of the sequence reported by Lloyd and Sharples (17). Thermal cycling was performed for 25 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s), and extension (72°C, 1 min). The PCR-generated fragment was cloned into the pT7Blue(R)T vector, resulting in pIN201. A truncated *recG* gene (ca. 1.0-kb *Nsp*I-*Pvu*II fragment) was isolated from pIN201. The 1.0-kb *Nsp*I-*Pvu*II fragment was ligated with *Xba*I-digested pKTN701 (a suicide vector carrying a chloramphenicol resistance gene) by blunt-end ligation, following refilling of the sticky ends with Klenow fragment, and then transformed into *E. coli* SY327 λ *pir*, resulting in pIN208 (7.5 kb in size). Plasmid pIN208 was transformed into *E. coli* KL16, and 14 chloramphenicol-resistant transformants were obtained. Since the *pir* gene necessary for replication of plasmid pIN208 containing the R6K replication origin is absent in *E. coli* KL16, pIN208 was expected to be recombined into the *recG* locus of strain KL16 through a single crossover event in the transformants. The 14 transformants were then screened for their hypersusceptibility to mitomycin, because a *recG*-deficient mutant has been reported to be hypersusceptible to mitomycin (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 *Mlu*I fragment internal to the *recG* gene) to confirm the destruction of the *recG* gene in the mutant. The analysis revealed that the *E. coli* KL16 preparation contained a probe-positive 12-kb *Cla*I fragment (wild-type *recG*), while the mutant preparation contained a 19.5-kb *Cla*I 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 *Kpn*I fragment containing the *recG* gene was observed in wild-type KL16, while the *Kpn*I digestion of the mutant preparation yielded instead the 11- and 3.5-kb fragments as expected (data not shown). A unique *Kpn*I 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 *Pst*I-*Eco*RI fragment containing the *recG* gene of pIN101 between the *Pst*I and *Eco*RI sites of the *E. coli-S. aureus* shuttle vector, pND50. Plasmid pIN107 was obtained by ligating a 2.8-kb *Bam*HI-*Hin*dIII fragment containing the *S. aureus recG* gene of pIN101 with the 4-kb *Bam*HI-*Hin*dIII fragment of pBR322. Plasmid pIN203 carrying the *E. coli recG* gene was constructed by ligating a 2.6-kb *Bam*HI-*Hin*dIII fragment from pIN201 with the 4-kb *Bam*HI-*Hin*dIII 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 Tn*551* insertional mutants with increased quinolone susceptibility. Introduction of Tn*551* 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 Tn*551* were examined for ciprofloxacin susceptibility; 65 ciprofloxacin-susceptible 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

TABLE 1. Quinolone and antibiotic susceptibilities of a Tn*551* insertional mutant, *S. aureus* 3f33, and its transformant with the *S. aureus recG* gene

S. aureus strain	MIC (μg/ml)[a]						
	CPFX	NFLX	SPFX	OA	ABPC	KM	TC
RN4220[b]	0.2	0.78	0.1	1.56	0.05	0.78	0.1
RCM101[c]	1.56	6.25	0.2	3.13	0.05	0.78	0.1
3f33[d]	0.2	1.56	0.025	0.78	0.05	0.78	0.1
3f33(pND50)[e]	0.2	1.56	0.025	0.78	0.05	0.78	0.1
3f33(pIN102)[f]	1.56	6.25	0.1	3.13	0.05	0.78	0.1

[a] CPFX, ciprofloxacin; NFLX, norfloxacin; SPFX, sparfloxacin; OA, oxolinic acid; ABPC, ampicillin; KM, kanamycin; TC, tetracycline.
[b] Parental strain.
[c] Low-level quinolone-resistant mutant of RN4220 selected with CPFX (11).
[d] Tn*551* insertional mutant of RCM101.
[e] Plasmid vector.
[f] Plasmid carrying the wild-type *S. aureus recG* gene.

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 and four 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 suggested that Tn*551* was inserted into the gene, whose inactivation specifically induced quinolone susceptibility. The chromosome of 3f33 was analyzed by means of a Southern hybridization test with a 4.2-kb *Hpa*I-*Xba*I DNA fragment of Tn*551* as a gene probe. When the chromosomal DNA of strain 3f33 was digested with *Eco*RV, whose recognition site is absent in Tn*551*, a single 8.8-kb fragment hybridized with the probe. On the other hand, digestion with *Hin*dIII, which has two recognition sites within Tn*551*, produced 5.4-, 4.2-, and 1.3-kb (internal to Tn*551*) fragments hybridizing with the probe (data not shown), and the digest of the chromosomal DNA of RCM101 gave no signal. These results showed that *S. aureus* 3f33 has a single copy of Tn*551* in its chromosome. The 5.4-, and 4.2-kb fragments were considered to be the left-side and right-side fragments with reference to the Tn*551* insertion site, respectively, since the 4.2-kb fragment hybridized with the 1-kb *Ava*I-*Hin*dIII right-junction fragment of Tn*551* (data not shown).

Cloning and sequencing of the Tn*551*-inserted gene. Attempts to clone the 8.8-kb *Eco*RV fragment and the 5.4- and 4.2-kb *Hin*dIII fragments of strain 3f33 into plasmids pBR322 and pACYC184 were not successful. Hence, in order to determine the nucleotide sequence around the Tn*551* insertion site, only the chromosomal portions of the 5.4- and 4.2-kb *Hin*dIII fragments were amplified by PCR and sequenced. Sequencing analysis of these PCR-generated fragments revealed that Tn*551* 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 pIN104, 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 38, 39, and 50% amino acid identity with the RecGs of *E. coli* (17), *Haemophilus influenzae* (4), and *Streptococcus pneumoniae* (20), respectively (Fig. 2). Identical residues are spread all over the sequences, with increased densities in stretches of amino acids corresponding to the seven helicase motifs defined by Gorbalenya et al. (5). *E. coli* KL16-P1, the *recG*-deficient mutant which was four times more hypersusceptible than KL16 to mitomycin, like a mutant carrying *recG* 258 (16), was made mitomycin resistant to the level of wild-type KL16 on transformation with pIN107 carrying the presumed *S. aureus recG* (*orfX*) gene as well as with pIN203 carrying the wild-type *E. coli recG* gene (data not shown). These results indicate that the OrfX protein is functionally equivalent to *E. coli* RecG, and thus *orfX* is considered to be the *recG* gene of *S. aureus*, being classified as a member of the DExH helicase family (18).

Increase in quinolone susceptibility caused by inactivation of the *recG* gene. Contrary to our expectations, the nucleotide sequence of the *recG* gene of RCM101 did not differ from that of the wild-type RN4220 throughout the *recG* coding region, or the sequences upstream (ca. 300 bp) and downstream (ca. 300 bp) of the coding region (data not shown), suggesting that the *recG* gene is not involved in the ciprofloxacin resistance 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 pIN102 carrying the wild-type *recG* gene cloned from RN4220. As shown in Table 1, a transformant of strain 3f33 with pIN102 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.

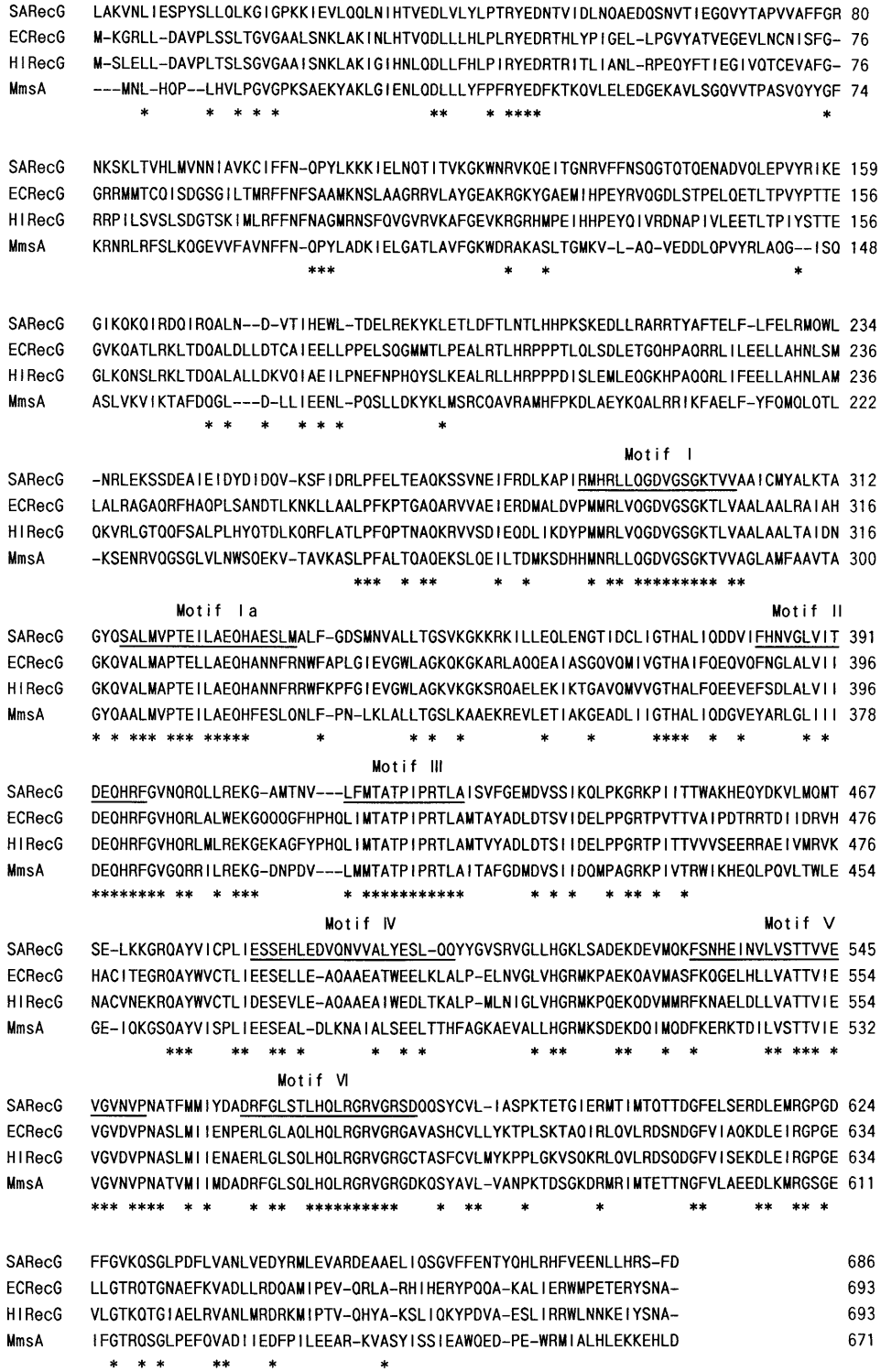

FIG. 2. Alignment of the sequence of *S. aureus* OrfX (RecG [SARecG]) with those of *E. coli* (ECRecG [GenBank accession no. M64367]), *H. influenzae* (HIRecG [GenBank accession no. U32847]), and *S. pneumoniae* (MmsA [GenBank accession no. Z49988]). Identical amino acid residues are indicated by asterisks. 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 on DNA, resulting in recovery from DNA damage in bacterial cells. Since *recG* is not related to excision and error-prone repair, being different from *recA* (16), the repair mechanism of *recG* is considered to be different from that of *recA*. The antibacterial activity of quinolones seems to depend not only on their affinities to the target enzymes but also on the degrees of repair of DNA damage due to DNA gyrase (or topoisomerase IV)-DNA-quinolone complexes.

ACKNOWLEDGMENTS

We thank M. Nishibuchi for supplying the *E. coli* strain and plasmid used in this study.

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