Accumulation of Mutations in both *gyrB* and *parE* Genes Is Associated with High-Level Resistance to Novobiocin in *Staphylococcus aureus*

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Coumarin-resistant mutants of *Staphylococcus aureus* were isolated by three-step selection with novobiocin at different concentrations. Sequencing analysis of the *gyrB* and *parE* genes of the first-, second-, and third-step mutants revealed that successive point mutations first occurred specifically in the *gyrB* gene, followed by a point mutation in the *parE* gene and then an additional point mutation in the *gyrB* gene. These findings demonstrate that DNA gyrase is the primary target and that topoisomerase IV is the secondary target for novobiocin and that the accumulation of point mutations in both the *gyrB* and the *parE* genes is associated with high-level resistance to novobiocin in *S. aureus*. Moreover, our results show that the amino acid substitutions (Asp-89 to Gly and Ser-128 to Leu) found in GyrB are associated with resistance to novobiocin but not to coumermycin A1, suggesting that the interactions of novobiocin and coumermycin A1 with GyrB differ at the molecular level.

DNA gyrase and topoisomerase IV (topo IV) are essential bacterial type II topoisomerases that play important roles in DNA replication, chromosome segregation and DNA compaction (14, 15). DNA gyrase, an A₂B₂ heterotetramer, introduces negative supercoils in DNA by stabilizing double-stranded DNA break and using ATP hydrolysis to pass another portion of DNA through this break (25). Topo IV, on the other hand, is a C₂E₂ tetramer (6) involved in decatenation of daughter chromosomes following DNA replication (31). As GyrA and GyrB of DNA gyrase share extensive sequence homology with ParC and ParE, respectively, of topo IV, both topoisomerases are targets of quinolones and coumarin antibacterial agents, such as novobiocin and coumermycin A1. The coumarins inhibit the ATPase activity of GyrB by competing with ATP for binding to the GyrB of DNA gyrase (16). On the other hand, the quinolones, which were first shown to inhibit DNA gyrase (23) and which were subsequently demonstrated to inhibit topo IV (21), form a ternary complex with topoisomerase in the presence of DNA, resulting in lethal double-stranded DNA breaks (11).

The mechanism of resistance to quinolones, especially alteration of target enzymes, has long been studied. Point mutations involved in quinolone resistance have been shown to occur in defined regions of GyrA and GyrB, termed quinolone resistance-determining regions (29, 30), and those in the *parC* and the *parE* genes of topo IV have been reported to take place in similar regions (4, 10). For instance, in *Escherichia coli*, a strain must have a mutation rendering DNA gyrase resistant in order to acquire a subsequent mutation in topo IV genes, since the primary target of quinolones in *E. coli* is DNA

gyrase (10). Interestingly, it has been demonstrated that in some gram-positive organisms, the hierarchy is reversed and topo IV is the primary target (5, 20). Other studies have shown that the primary target (DNA gyrase or topo IV) depends on the type of quinolone used (1, 24). Although the primary target is different among microorganisms, accumulation of alterations in DNA gyrase and topo IV confers high-level resistance to the quinolones (5, 10). On the other hand, the coumarins have long been known to inhibit DNA gyrase (8) and have also been shown to inhibit, at higher concentrations than those required for DNA gyrase inhibition, the activity of purified topo IV (21). Moreover, it has been reported that only *gyrB* mutations confer resistance to the coumarins and that all mutations reported occur at the periphery of the GyrB ATP-binding cleft (7, 17, 18, 22). Although recent studies have indicated that an amino acid substitution in the topo IV ParE subunit of *E. coli* confers resistance to novobiocin in vitro (9), no report has shown novobiocin- or coumermycin A1-resistant mutants with point mutations in the *parE* gene. Thus, detailed information on the mechanism of coumarin resistance is still lacking, and the availability of this information might help with the development of potent DNA gyrase inhibitors that are effective against quinolone-resistant strains with mutations in DNA gyrase genes.

In order to understand better the mechanism of acquisition of high-level coumarin resistance in *Staphylococcus aureus*, spontaneous novobiocin-resistant mutants of *S. aureus* were isolated by three-step selection and were characterized in this study.

MATERIALS AND METHODS

Materials and bacterial strains. Novobiocin and coumermycin A1 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sparfloxacin and norfloxacin were synthesized at our Chemistry Research Laboratories. Other reagents were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise indicated.

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FIG. 1. Relationships between *S. aureus* RN4220 and its resistant mutants isolated by stepwise exposure to novobiocin and between phenotype and genotype in first-, second-, and third-step mutants. G1, group 1; G2, group 2; G3, group 3; G4, group 4; G5, group 5; G6, group 6; G7, group 7. NOV^s and COU^s , mutant susceptibility to NOV and COU, respectively, did not change compared to that of the parent strain; NOV^r and COU^r , mutant susceptibility to NOV and COU, respectively, decreased compared to that of the parent strain; NOV, novobiocin; COU, coumermycin A1; superscript S, D, or T on *gyrB* and *parE*, the gene had a single, a double, or a triple point mutation, respectively.

S. aureus RN4220 was kindly provided by R. P. Novick. *E. coli* KL16 was obtained from B. Bachmann.

Preparation of genomic DNA. *S. aureus* genomic DNA was prepared as described by Hudson and Curtiss (12), except that 100 $\mu\text{g}/\text{ml}$ mutanolysin was replaced by 50 $\mu\text{g}/\text{ml}$ lysostaphin.

Drug susceptibility test. MICs were determined by the twofold agar dilution method recommended by CLSI (formerly NCCLS) (19).

Isolation of spontaneous coumarin-resistant mutants of *S. aureus* RN4220. For isolation of the first-step mutants, a portion of the overnight culture of *S. aureus* RN4220 (approximately 10^{10} CFU) was spread onto tryptic soy agar containing novobiocin at one to four times the MIC and incubated at 35°C for 48 h. For the second-step and third-step mutants, the first-step and second-step mutants were incubated on agar containing novobiocin (at one to two times the MIC) at 35°C for 48 h and 72 h, respectively.

PCR amplification and DNA sequence analysis. Genes corresponding to the amino-terminal 43-kDa regions (ATP-binding domain) of *GyrB* and *ParE* subunits from *S. aureus* RN4220 and novobiocin-resistant *S. aureus* mutants were amplified by PCR with the following primers: the *gyrB* forward primer (5'-GG GTGACTGCATTGTTCAGATGTA-3'; the 5' nucleotide is at position 184 [the amino acid is at position 2] in the sequence published by Ito et al. [13]), the *gyrB* reverse primer (5'-GGACGTGTTACTTCACGCGCTTTT-3'; the 5' nucleotide is at position 1392 [the amino acid is at position 404]), the *parE* forward primer (5'-GGAATAAACAAAATAATTATTCAGATGAT-3'; the 5' nucleotide is at position 388 [the amino acid is at position 2] in the sequence published by Yamagishi et al. [27]), and the *parE* reverse primer (5'-GGACGAGCATCT TCACGAGCTTTAC-3'; the 5' nucleotide is at position 1575 [the amino acid is at position 397]). PCR was performed with a GeneAmp PCR System 9600 with TAKARA Ex Taq polymerase (Takara Shuzo, Kyoto, Japan). The reactions were repeated for 30 cycles (15 s at 94°C for denaturation, 30 s at 60°C for annealing, and 1 min at 72°C for polymerization). PCR-amplified fragments were sequenced by the cycle sequencing method with an ABI Prism Big Dye terminator, version

3.1, cycle sequencing kit and then applied on a Genetic Analyzer 3100 (Applied Biosystems, Foster, Calif.).

Enzyme assays. *S. aureus* DNA gyrase supercoiling assays and topo IV decatenation assays were carried out by the method of Blanche et al. (3).

RESULTS AND DISCUSSION

First-step mutants. About 5,400 first-step mutants were isolated from *S. aureus* RN4220 at a frequency of about 10^{-7} by selection with novobiocin (at one to four times the MIC). The MICs of novobiocin and coumermycin A1 for 579 randomly selected isolates of the first-step mutants were determined, and the isolates tested were classified into seven groups according to their resistance patterns (Fig. 1; Table 1). Since the susceptibilities of the first-step mutants tested to other types of antibiotics, including levofloxacin, ampicillin, tetracycline, gentamicin, and erythromycin, did not change (data not shown), mutations occurred only in genes of the target enzyme of novobiocin, such as the *gyrB* gene. Hence, we sequenced the regions of the *gyrB* genes corresponding to the ATP-binding cleft (28) amplified from 1 to 10 strains of the mutants in each group. The regions of the *parE* genes were also sequenced to identify mutations in the *parE* gene, if there were any. The mutants in each group had mutations only in *gyrB*. Mutants with the same mutations always had the same resistance profiles. For reasons of clarity, only one strain with each type of

TABLE 1. Coumarin resistance mutations in *gyrB* gene in first-step mutants

Group	No. of strains	Representative isolate	MIC ($\mu\text{g/ml}$) ^a				Mutation in:	
			NOV	COU	SPX	NOR	<i>gyrB</i>	<i>parE</i>
		RN4220 ^b	0.5	0.008	0.125	2		
1	220	N167	4	0.008	0.125	2	S128 (TCA)→L (TTA)	None
2	7	N349	4	0.125	0.125	2	T173 (ACT)→A (GCT)	None
3	1	N295	8	0.032	0.125	2	I102 (ATT)→S (AGT)	None
4	331	N175	16	0.5	0.125	2	R144 (AGA)→S (AGT)	None
		N465	16	0.5	0.125	2	G85 (GGT)→S (AGT)	None
5	18	N506	16	1	0.125	2	R144 (AGA)→I (ATA)	None
6	1	N580	32	1	0.125	2	I56 (ATC)→S (AGC) ^c	None
							R144 (AGA)→S (AGT)	
7	1	N276	32	16	0.125	2	I102 (ATT)→S (AGT)	None
							R144 (AGA)→S (AGT)	

^a NOV, novobiocin; COU, coumermycin A1; SPX, sparfloxacin; NOR, norfloxacin.^b Parent strain.^c Mutation not previously reported.

mutation is shown in Table 1. First-step mutants in groups 1 to 5 had 8- to 32-fold decreased susceptibilities to novobiocin and single mutations in their *gyrB* genes: group 1, Ser-128 (TCA) to Leu (TTA); group 2, Thr-173 (ACT) to Ala (GCT); group 3, Ile-102 (ATT) to Ser (AGT); group 4, Arg-144 (AGA) to Ser (AGT) or Gly-85 (GGT) to Ser (AGT); and group 5, Arg-144 (AGA) to Ile (ATA). All first-step novobiocin-resistant mutants except those in group 1 were also less susceptible to different levels of coumermycin A1 (4- to 128-fold increased MICs compared with that for the parent strain). Strains of groups 6 and 7, which were resistant to higher levels of novobiocin and coumermycin A1, had two amino acid substitutions in *GyrB*: group 6, Ile-56 (ATC) to Ser (AGC) and Arg-144 (AGA) to Ser (AGT); group 7, Ile-102 (ATT) to Ser (AGT) and Arg-144 (AGA) to Ser (AGT). The amino acid changes Gly-85 to Ser, Ile-102 to Ser, Ser-128 to Leu and Arg-144 to Ile have been reported previously (7, 22); and the amino acid substitutions Arg-144 to Ser and Thr-173 to Ala are analogous to mutated residues found in previously characterized resistance-associated *gyrB* genes from *E. coli* and *Bartonella bacilliformis*, respectively (2, 17). It has also been demonstrated that two amino acid substitutions in *GyrB* confer higher levels of resistance to coumarins (7, 22). Thus, our results for the first-step mutants are in agreement with those of previous reports. In group 6, we found an amino acid substitution of Ile-56 to Ser in *GyrB*. Ile-56 in *S. aureus* is equivalent to Ile-48 in *E. coli* and is located in the ATP-binding cleft. These findings strongly suggest that the amino acid substitution of Ile-56 to Ser is involved in coumarin resistance. In group 7, strain N276 had double amino acid substitutions, Ile-102 to Ser and Arg-144 to Ser, in *GyrB*. The MICs of coumarins for strains carrying the individual mutations Ile-102 to Ser (N295) and the Arg-144 to Ser (N175) were 8 and 16 $\mu\text{g/ml}$ of novobiocin and 0.032 and 0.5 $\mu\text{g/ml}$ of coumermycin A1, respectively. The MICs of novobiocin and coumermycin A1 for N276 were 32 and 16 $\mu\text{g/ml}$, respectively, and compared to the parent strain, N276 had 64- and 2,048-fold decreased susceptibilities to novobiocin and coumermycin A1, respectively. We cannot explain the difference in the susceptibility changes. However, the results might indicate that the double amino acid substitutions Ile-102 to Ser and Arg-144 to Ser make the ATP-binding cleft narrowed and

the bulkier molecule, coumermycin A1, enters the cleft harder than the smaller molecule, novobiocin.

Second-step mutants. In order to elucidate the mechanism of acquisition of higher levels of resistance to coumarins, second-step mutants were isolated from the first-step mutants (N167, N175, N276, and N465) by selection with novobiocin (at one to two times the MIC) and characterized. Strains N167, N175, and N465 (groups 1 and 4) were the most frequently isolated; and strain N276 had two amino acid substitutions in *GyrB*. From first-step mutants N167, N175, N276, and N465, 116, 15, 26, and 2 second-step mutants were isolated, respectively. The MICs of novobiocin and coumermycin A1 for all second-step mutants were determined, and the second-step mutants were classified according to their resistance patterns: N167 derivatives, six resistance patterns; N175 derivatives, three resistance patterns; N276 derivatives, two resistance patterns; N465 derivatives, one resistance pattern (Fig. 1). The regions of the ATP-binding cleft of both the *gyrB* and the *parE* genes of one to three strains of the second-step mutants in each group were analyzed. Since the mutants with the same resistance pattern had the same mutation(s) in the *gyrB* and/or the *parE* gene, the results for representative second-step strains with each type of mutation are shown in Table 2. Compared with the parent strains, all second-step mutants were 2 to 16 times more resistant to novobiocin. In addition, all second-step mutants except N791, N891, and N586 were 4 to 256 times more resistant to coumermycin A1. Without exception, additional one or two point mutations were found in the *gyrB* and the *parE* genes of the second-step mutants. The mutations in *parE* gene were only seen in mutants that had two *gyrB* mutations. The amino acid substitution in *GyrB* (Asp-89 [GAT] to Gly [GGT]) found in N791, N891, and N586 was associated with higher levels of resistance to novobiocin but not to coumermycin A1, suggesting that the Asp-89 to Gly substitution and the Ser-128 to Leu substitution found in first-step mutants are not involved in coumermycin A1 resistance. These results demonstrate the molecular-level difference in the modes of action between novobiocin and coumermycin A1; i.e., Asp-89 and Ser-128 in *GyrB* could interact with novobiocin but not with coumermycin A1. In *E. coli*, the coumarin ring in both novobiocin and coumermycin A1 forms hydrogen bonds

TABLE 2. Additional mutations in *gyrB* and *parE* genes found in second- and third-step mutants

Strain (mutation)	MIC ($\mu\text{g/ml}$) ^a				Additional mutation in:	
	NOV	COU	SPX	NOR	<i>gyrB</i>	<i>parE</i>
Second-step mutants						
N167 (S128L in <i>GyrB</i>)	4	0.008	0.125	2		
N167-derived mutants						
N791 (9) ^c	32	0.008	0.125	2	D89 (GAT)→G (GGT) ^b	None
N754 (51)	16	0.25	0.125	2	T173 (ACT)→A (GCT)	None
N846 (1)	32	0.063	0.125	2	I102 (ATT)→S (AGT)	None
N776 (51)	32	0.5	0.125	2	G85 (GGT)→S (AGT)	None
N811 (3)	32	2	0.125	1	R144 (AGA)→I (ATA)	None
N825 (1)	64	0.5	0.125	1	D89 (GAT)→G (GGT) ^b	None
					T173 (ACT)→A (GCT)	
N175 (R144S in <i>GyrB</i>)	16	0.5	0.125	2		
N175-derived mutant						
N891 (9)	64	0.5	0.125	1	D89 (GAT)→G (GGT) ^b	None
N893 (3)	32	2	0.125	2	I102 (ATT)→T (ACT)	None
N888 (3)	32	16	0.125	2	I102 (ATT)→S (AGT)	None
N465 (G85S in <i>GyrB</i>)	16	0.5	0.125	2		
N465-derived mutant						
N586 (2)	64	1	0.125	1	D89 (GAT)→G (GGT) ^b	None
N276 (I102S and R144S in <i>GyrB</i>)	32	16	0.125	2		
N276-derived mutant						
N742 (1)	128	64	0.125	2	None	G78 (GGT)→S (AGT) ^b
N884 (25)	128	64	0.125	2	None	R136 (CGA)→G (GGA) ^b
Third-step mutants						
N742 (I102S and R144S in <i>GyrB</i> , G78S in <i>ParE</i>)	128	64	0.125	2		
N742-derived mutant						
N899 (1)	512	64	0.125	2	D89 (GAT)→G (GGT) ^b	None
N884 (I102S and R144S in <i>GyrB</i> , R136G in <i>ParE</i>)	128	64	0.125	2		
N884-derived mutant						
N898 (1)	256	64	0.125	2	D89 (GAT)→G (GGT) ^b	None

^a NOV, novobiocin; COU, coumermycin A1; SPX, sparfloxacin; NOR, norfloxacin.^b Mutations not previously reported.^c The number of isolates that had the same resistance pattern is shown in parentheses.

with Gly-77 and Arg-136 in *GyrB* (equivalent to Gly-85 and Arg-144, respectively, in *S. aureus*) (16). Therefore, in *S. aureus*, it is suggested that mutations in these residues direct removal of the hydrogen-bonded interactions associated with resistance to novobiocin and coumermycin A1. Alternatively, since the 3'-isopentenyl-4'-hydroxybenzoate moiety and the 3'-carbamoyl group of novobiocin have specific interactions with residues Asp-89 and Ser-128, respectively, substitutions of these residues can be associated with resistance to novobiocin but not to coumermycin A1. Interestingly, the mutants isolated from N276, N742, and N884, which had two amino acid substitutions in the *GyrB*, had additional amino acid changes (Gly-78 [GGT] to Ser [AGT] and Arg-136 [CGA] to Gly [GGA], respectively) in *ParE*. Alignment of *GyrB* and *ParE* revealed that Gly-78 and Arg-136 in *ParE* were equivalent to Gly-85 and Arg-144 in *GyrB*, respectively, and were in the region of *ParE* that corresponds to the ATP-binding domain of *GyrB* (Fig. 2). Moreover, the amino acid changes in *GyrB*, i.e.,

Gly-85 to Ser and Arg-144 to Ile, were associated with resistance to the coumarins. These findings strongly suggest that the amino acid residues Gly-78 and Arg-136 in *ParE* interact with the coumarins and are involved in their resistance and that the mechanism of inhibition of topo IV by the coumarins is the same as that of inhibition of DNA gyrase, namely, blocking of ATP binding.

Third-step mutants. Next, to examine additional amino acid changes in *GyrB* or *ParE*, we isolated much higher-level coumarin-resistant mutants from the second-step mutants that already had amino acid changes in *ParE* (N742 and N884). Only one third-step mutant was isolated from each second-step mutant. The third-step mutants, N899 and N898, derived from N742 and N884, respectively, were two to four times more resistant to novobiocin but not to coumermycin A1 than the parent strains. In these mutants, analysis of the *gyrB* and the *parE* genes demonstrated that an additional amino acid change (Asp-89 [GAT] to Gly [GGT]) occurred in *GyrB* (Table 2).

Characterization of the first-, second-, and third-step mutants revealed that the mechanism of acquisition of high-level novobiocin resistance in *S. aureus* is as follows: (i) first, successive point mutations specifically occurred in *gyrB*; (ii) next, a point mutation occurred in *parE*; (iii) finally, a point mutation occurred in *gyrB* again (Fig. 1). Therefore, the accumulation of mutations in both the *gyrB* and the *parE* genes is associated with high-level resistance to novobiocin.

The concentrations of the compounds that inhibited 50% of the enzymatic activity of novobiocin (0.2 µg/ml) and coumermycin A1 (0.05 µg/ml) for *S. aureus* DNA gyrase were 125 to 32 times lower than those for *S. aureus* topo IV (25 µg/ml for novobiocin and 1.56 µg/ml for coumermycin A1), suggesting that the coumarins interact primarily with DNA gyrase. Furthermore, Hardy and Cozzarelli (9) reported that in *E. coli*, a mutation in the *parE* gene homologous to a commonly mutated residue in *gyrB* alleles confers novobiocin resistance. Although these in vitro studies suggest that topo IV is a secondary target for coumarins, our results directly indicate that, in vivo, DNA gyrase is the primary target and topo IV is the secondary target for the coumarins. It would be interesting to determine whether the priority of target is reserved for other bacteria, as is the case with quinolones. Hardy and Cozzarelli (9) also tried, without success, to isolate a high-level novobiocin-resistant *parE* mutant by plating an *E. coli* strain on increased levels of novobiocin. The reason for this is unclear.

It is necessary to develop new antibacterial agents that are also active against existing antibiotic-resistant strains of bacteria. DNA gyrase is a clinically validated target, and coumarins are also potent against quinolone-resistant strains. Hence, several pharmaceutical companies have been researching a GyrB inhibitor. Because ParE is the secondary target for coumarins and the first-step mutants that have mutations only in GyrB are commonly isolated, a compound that inhibits both GyrB and ParE at the same level should be explored. It is expected that the emergence of resistant mutants is rare when the compound is used clinically.

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