Molecular Analysis of Isoleucyl-tRNA Synthetase Mutations in Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus* with Low-Level Mupirocin Resistance

Emergence and spread of low-level mupirocin resistance in staphylococci have been increasingly reported in recent years. The aim of this study was to characterize missense mutations within the chromosomal isoleucyl-tRNA synthetase gene (*ileS*) among clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) with low-level mupirocin resistance. A total of 20 isolates of MRSA with low-level mupirocin resistance (minimal inhibitory concentration, 16-64 μg/mL) were collected from 79 patients in intensive care units for six months. The isolates were analyzed for isoleucyl-tRNA synthetase (IleS) mutations that might affect the binding of mupirocin to the three-dimensional structure of the *S. aureus* IleS enzyme. All isolates with low-level mupirocin resistance contained the known V588F mutation affecting the Rossman fold, and some of them additionally had previously unidentified mutations such as P187F, K226T, F227L, Q612H, or V767D. Interestingly, Q612H was a novel mutation that was involved in stabilizing the conformation of the catalytic loop containing the KMSKS motif. In conclusion, this study confirms that molecular heterogeneity in *ileS* gene is common among clinical MRSA isolates with low-level mupirocin resistance, and further study on clinical mutants is needed to understand the structural basis of low-level mupirocin resistance.

Key Words : *Staphylococcus aureus; Mupirocin; Drug Resistance; Isoleucine-tRNA Ligase; Mutation, Missense*

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INTRODUCTION

Methicillin-resistance *Staphylococcus aureus* (MRSA) has been a major cause of nosocomial infections and has been isolated with increasing frequency from patients in hospitals and nursing homes (1). Nasal and extranasal carriage of MRSA is an essential step in invasive MRSA infections and plays a decisive role in the dissemination of these microorganisms (2).

Topical mupirocin has been widely used for managing MRSA infections, reducing certain types of surgical site infections, and clearing nasal MRSA carriage, especially among healthcare workers. In 1987, shortly after the introduction of mupirocin, clinical isolates of mupirocin-resistant *S. aureus* began appearing in the U.K. (3). Since then, such isolates have been increasingly reported in many countries (4-6). Increasing numbers of mupirocin-resistant MRSA isolates have been detected in patients who had previously received mupirocin treatment, an observation cautioning against extended use of mupirocin ointment for eradication of MRSA nasal carriage (5).

Mupirocin binds to the target enzyme, isoleucyl-tRNA synthetase (IleS), in the vicinity of an ATP-binding subsite and is a bifunctional inhibitor with characteristics of both isoleucine and ATP, i.e. an analogue of isoleucyladenylate (7). Mupirocin resistance is phenotypically divided into two groups: low-level (minimal inhibition concentration [MIC]=8 to 256 μg/mL) and high-level (MIC $\geq$ 512 μg/mL) (8). High-level resistance is mediated by a plasmid containing the *ileS-2* gene that encodes a novel isoleucyl-tRNA synthetase enzyme, whereas low-level resistance usually results from alteration of the native IleS as a consequence of spontaneous mutations in the *ileS* gene (8, 9). Since low-level mupirocin resistance is more prevalent among clinical isolates (10), concerns about treatment failure for nasal decolonization of MRSA are increasing (11).

Previous studies revealed the presence of molecular heterogeneity of mutations in the *ileS* gene among clinical isolates and low-level mupirocin resistant MRSA strains selected in vitro (12, 13). These studies reported that the V588F mutation affecting the Rossman fold could interact directly with mupirocin, causing emergence of low-level mupirocin resistance (12). Notably, this mutation was not absolutely necessary for the development of low-level mupirocin resistance, and the V631F mutation was found to have effects similar

to those of V588F on the structure IleS. Although V588F and V631F clearly play a role in low-level mupirocin resistance, it is unknown whether these mutations represent the full spectrum of *IleS* mutations that give rise to low-level mupirocin resistance.

In this study, we examined the *ileS* gene for molecular heterogeneity in low-level mupirocin resistant MRSA strains isolated from patients in intensive care units (ICUs), and analyzed how individual amino acid mutations affect the crystal structure of the complex of *S. aureus* IleS with mupirocin.

MATERIALS AND METHODS

Bacterial isolates

A total of 79 MRSA isolates were consecutively collected from clinical cultures taken from patients in ICUs in a tertiary care hospital from November 2003 to April 2004. The specimens originated from blood, gastric juice, pus, skin, central venous catheter tip, sputum, stool, swab, tracheal tip, urine, and wounds. Isolates from one patient that were recovered from different cultures were included only once. The strains were stored in brain-heart infusion broth plus 20% glycerol at -70°C until studied.

MRSA detection and mupirocin susceptibility

MRSA isolates were identified by the standard disk diffusion method (14) and *mecA* was detected by polymerase chain reaction (PCR). The primer pair used was MecA1 (5′- ATG CTA AAG TTC AAA AGA GTA TTT ATA A) and MecA2 (5′-TGA TGA TTC TAT TGC TTT TAA GTC), yielding a 400-bp product. MICs of mupirocin were determined by a standardized agar dilution method in Mueller-Hinton agar (Oxoid, Basingstoke, United Kingdom) with inocula in Mueller-Hinton agar (Oxoid) of 10^6 CFU per spot. After 24 hr of incubation at 37°C, the MIC was deemed the lowest concentration of antibiotic that prevented visible colonial growth. MIC determination was evaluated according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines (15). *S. aureus* ATCC 29213 was used as the control for the susceptibility test.

PCR amplification and sequencing of the *ileS* gene

To overcome the difficulty of long-PCR amplification of the 3,044 bp- *ileS* gene sequence, we selected three parts of the gene to be amplified which might contain the potential mutations related to mupirocin resistance based on previous studies (12, 13) and the three-dimensional structure of the IleS enzyme active site. Three primer pairs were designed from the *S. aureus ileS* gene (GenBank accession number X74219); one pair was Smr1 (5′ -ATA AAG GTA AAA AGC CAG

TTT ATT GGT) and Smr2 (5′ -TAA TCG CAA CAT TTG ATG GAA TTG TC) for the fragment of 200 bp (nt 450 to 650), the second was Mrm1 (5′ -TCC CAG CAG ATA TGT ATT TAG AAG GT) and Mrm2 (5′ -AAC CAC TTG GTC AGG TAC AAT CAC A) for the fragment of 450 bp (nt 800 to 1,250), and the third was Lmr1 (5′ -GTA AAT CTT TAG GTA ATG TGA TTG TAC) and Lmr2 (5′ -TCT TCT TTA ACA TGT GGT GTA TGA GA) for the fragment of 690 bp (nt 1,730 to 2,420). Genomic DNA was prepared, and the *ileS* gene fragments were amplified by PCR, as previously described (12). PCR products were purified with QIAquick-spin PCR purification kit (Qiagen, GmbH, Germany) and sequenced by Bioneer Corporation (Daejeon, Korea). To detect the *ileS-2* gene conferring high-level mupirocin resistance, a 410-bp region in the *ileS-2* gene was amplified using another primer pair Mup1 (5′ -TAT ATT ATG CGA TGG AAG GTT GG) and Mup2 (5′ -AAT AAA ATC AGC TGG AAA GTG TTG) (10).

Structural analysis of IleS mutations

To better understand the interactions of IleS with mupirocin, we used the crystal structure of IleS from *S. aureus* (Protein Data Bank code: 1FFY) (16).

Pulsed-field gel electrophoresis (PFGE)

Chromosomal DNA for the *Sma*I restriction digest was purified from 20 clinical MRSA isolates with low-level mupirocin resistance as described previously (17). Electrophoresis was performed with the GeneNavigator™ System (GE Healthcare U.K. Ltd. Buckinghamshire, United Kingdom) at 130 V and at 16°C; 5 sec pulse time for 4 hr, 25 sec pulse time for 6 hr, 45 sec pulse time for 20 hr, and 75 sec pulse time for 6 hr. Total running time was 36 hr. PFGE λ ladder* (New England Biolabs Inc.) was used to provide molecular size markers. After staining with ethidium bromide, the band patterns were compared using the criteria for bacterial strain typing (18).

RESULTS

Isolation of clinical MRSA strains with low-level mupirocin resistance

We isolated a total of 79 MRSA strains from patients in ICUs in our hospital for the six-month study period. The isolation rate of MRSA strains was approximately 75%. MRSA isolates were mostly recovered from sputum cultures. Determination of MICs of mupirocin by the agar dilution method revealed that twenty strains (Kua 1 to 20) (25.3% of total isolates) displayed low-level mupirocin resistance from 16 μg/mL to 64 μg/mL (Table 1). There were no MRSA isolates with high-level mupirocin resistance.

Table 1. Distribution of mupirocin MICs among MRSA isolates from 79 patients in ICUs during November 2003 through April 2004

MIC (μg/mL)	No. of isolate (%)	Remark
<8	59 (74.7%)	Susceptible
16	1 (1.2%)	
32	13 (16.5%) 20 (25.3%)	Low-level resistant
64	6 (7.6%)	
>256	0 (0%)	High-level resistant
Total	79 (100%)	

Table 2. Mupirocin MICs and PFGE patterns of 20 MRSA isolates with low-level mupirocin resistance

Strain	Source	MIC (μg/mL)	PFGE pattern
Kua 1	Wound	32	A1
Kua 2	Sputum	64	A
Kua 3	Sputum	32	A
Kua 4	Sputum	64	B
Kua 5	Sputum	64	B
Kua 6	Sputum	64	B
Kua 7	Sputum	64	A
Kua 8	Sputum	32	C
Kua 9	Sputum	32	C
Kua 10	Sputum	32	C
Kua 11	Sputum	32	C
Kua 12	Tracheal aspirate	32	D
Kua 13	Sputum	32	C
Kua 14	Sputum	32	D
Kua 15	Sputum	32	D
Kua 16	Sputum	32	E
Kua 17	Sputum	32	C
Kua 18	Tracheal aspirate	32	C
Kua 19	Urine	16	A
Kua 20	Sputum	64	C

Table 3. Heterogeneity of IleS mutations among 20 MRSA isolates with low-level mupirocin resistance

Mutation identified	Isolates (%) containing individual mutation
P187F	Kua 4-12, 14, 15, 19 (60)
K226T	Kua 3 (5)
F227L	Kua 3 (5)
V588F	Kua 1-20 (100)
Q612H	Kua 8-11, 13-15, 17, 18, 20 (50)
V767D	Kua 1 (5)

P, Proline; F, Phenylalanine; K, Lysine; T, Threonine; L, Leucine; V, valine; Q, glutamine; H, histidine; D, Aspartic acid.

Fig. 1. Structure of IleS from *S. aureus* Oxford. Distribution of mutated residues identified from MRSA isolates with low-level mupirocin resistance. (**A**) Overall IleS structure showing bound tRNA[Ile] (violet) and mupirocin (slate/red) (22). The backbone fold of IleS is shown as a ribbon, with individual sites of mutation highlighted displaying 6 residues' side chains (red) and sequence numbers, and yellow-colored structure with the Q612H mutation. (**B**) Enlarged view of the beta-helix region adjacent to the Rossman fold and residues 588 and 612. Note that the Q612H mutation has potential to interact with mupirocin. Q612 sits close to the core of IleS Rossman fold motif (yellow/blue), where it forms a water-bridged hydrogen bond with the side chain of D635 (arrow).

Identification of missense mutations in the *ileS* gene sequence in clinical MRSA isolates

To identify missense mutations that might contribute to the low-level mupirocin resistance phenotype, we compared the sequences of the *ileS* gene fragments amplified from 49 MRSA isolated from patients in ICUs, including 20 low-level mupirocin resistanc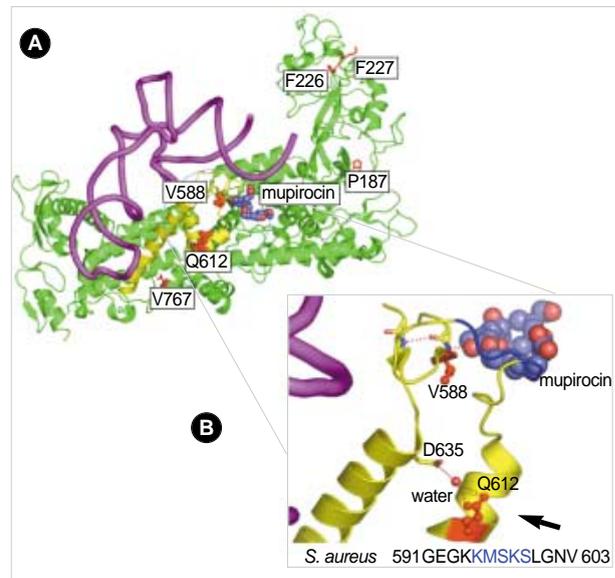e and 29 mupirocin-susceptible isolates, with the published *S. aureus ileS* gene sequence (GenBank accession no. X74219). We identified six missense mutations, P187F, K226T, F227L, V588F, Q612H, and V767D, in the strains with low-level mupirocin resistance. Those were not identified in any of the mupirocin susceptible MRSA isolates. Except for V588F, none of these mutations have been reported previously. The V588F and Q612H mutations are located near the Rossman fold motif, whereas the others are located outside this motif. All of the strains with low-level mupirocin resistance possessed the V588F, and some of them had an additional one to three other mutations (Table 2, 3). An A213D mutation was identified in all 49 strains analyzed, irrespective of the MIC level of mupirocin resistance. Additionally, we identified various null mutations (T594C, A602C, A643T, A685C and T1679C) that were present only in the *ileS* genes of low-level mupirocin-resistant strains and some (T1701C, T1974C, T1980C, T1995C and T2031G) that were present in both mupirocin-resistant and -susceptible strains (data not shown).

Structural analysis of amino acid mutations

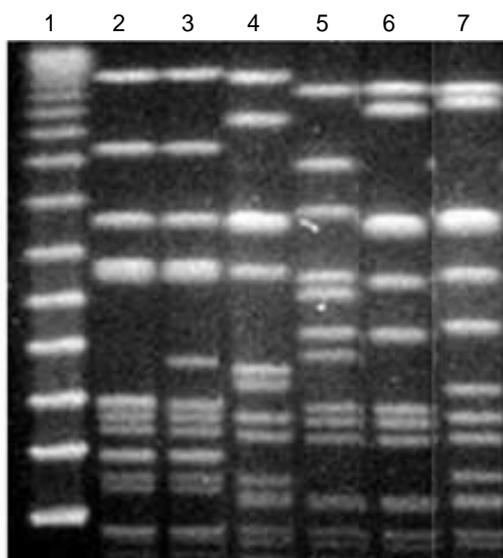To better understand the significance of the novel mutations,

Fig. 2. Representative PFGE patterns of *Sma* I macrorestriction fragments of genomic DNA of MRSA strains with low-level mupirocin resistance isolated from patients in ICUs. Lane 1, λ ladder marker; lane 2 to lane 7 show PFGE patterns A0, A1, B, C, D, and E, respectively.

we used a computer program to superimpose them on the crystal structure of the *S. aureus* IleS. With the exception of V588F, all the missense mutations we identified in this study are novel mutations. Two of them, V588F and Q612H, are located near the Rossman fold motif, and the others are located outside (Fig. 1A). Because V588 is located in a hydrophobic pocket that interacts directly with the fatty acid side chain of mupirocin, mutation of this residue causes steric repulsion to mupirocin. The Q612H mutation has potential to interact with mupirocin. Q612 sits close to the core of IleS Rossman fold motif, where it forms a water-bridged hydrogen bond with the side chain of D635 (Fig. 1B). Since it is located at about 15 Å from the binding site of mupirocin, it is not clear how the Q612H mutation could directly affect the interaction with mupirocin. The Q612H mutation appeared to affect the catalytic loop configuration during substrate binding, thus leading to the differences in the inhibitory activity of mupirocin.

Contributions of other mutations to mupirocin resistance are unlikely to be as direct as that of V588F, and these mutations might indirectly affect the interaction with mupirocin. K226T and F227L are on the outer surface adjacent to the tRNA molecule about 20 Å from the editing active site. P187F, about 20 Å distant from the 3′ end of the tRNA, is located near the CP2 domain. In contrast, V767D lies in the interior surface of the cylindrical central helical domain of the IleS, distal to the tRNA binding site.

Characterization of low-level mupirocin resistant MRSA isolates by PFGE analysis

To determine whether the emergence of low-level mupirocin

resistant MRSA isolates in ICUs was derived from a clone with epidemic potential, we analyzed the genetic relatedness of 20 MRSA isolates with low-level mupirocin resistance by PFGE analysis. Related isolates were identified by *Sma*I macrorestriction patterns. All twenty isolates were separated into five PFGE types (A, B, C, D, and E) and a subtype, A1 (Fig. 2). PFGE type A was considered an endemic clone, since it was isolated persistently from the beginning to the end of the six-month study. Other clones (PFGE types, B, C, D and E) had colonized or infected patients for a few months and, once introduced into the ICUs, spread among patients. Interestingly, PFGE types were not associated with individual mutations of IleS enzymes. Isolates with the identical PFGE pattern contained different IleS mutations, and isolates from different PFGE types contained the same IleS mutations. Notably, the Q612H mutation was only identified in PFGE types C and D (Table 2).

DISCUSSION

In this study, we investigated the molecular heterogeneity of IleS mutations in low-level mupirocin resistant MRSA strains isolated from patients in ICUs. We characterized the identified mutations by superimposition on the crystal structure of the *S. aureus* IleS. We identified six missense mutations (P187F, K226T, F227L, V588F, Q612H, and V767D) that were only present among MRSA isolates with low-level mupirocin resistance. Based on previously reported *ileS* gene mutations associated with low-level mupirocin resistance in MRSA strains originating in Europe, North America (12), and Japan (13), five of the six missense mutations identified in this study are novel. In particular, the Q612H is a novel mutation that seems to be involved in stabilizing the conformation of catalytic loop containing the KMSKS motif. Until now, only amino acid substitutions at two sites (V588F and V631F) in IleS have been detected in clinical *S. aureus* isolates expressing low-level mupirocin resistance (12, 13). Whether these are the only lleS mutations that confer mupirocin resistance is unknown. The V588F mutation is known to disrupt the hydrophobic pocket within the mupirocin-binding Rossman fold possessing the consensus HXGX and GXKMSKS motifs, and the V631F substitution has an effect similar to that of V588F. Our results suggest that the mutations located at some distance from the KMSKS motif could have a significant effect on the conformational changes of IleS, leading to the low-level mupirocin resistance.

A recent study showed that other novel mutations linked to emergence of mupirocin resistance (G593V, R816C, H67Q, and F563L in various combinations) could be generated from laboratory *S. aureus* isolates with incremental increases in resistance after repeated exposure to increasing concentrations of mupirocin (19). However, in our study we only detected the V588F mutation and failed to detect either the novel muta-

tions that originated from the laboratory stains or other IleS mutations including the V631F mutation identified in previous clinical isolates with low-level mupirocin resistance (12, 13). On the other hand, the contributions of the other four mutations newly identified in this study are unlikely to be those of V588F or Q612H, and further studies on these mutations are needed. These findings confirm that the molecular heterogeneity of *S. aureus* IleS mutations are common among clinical isolates of MRSA with low-level mupirocin resistance.

Our study has revealed an unexpectedly high rate (25.3%) of isolation of MRSA strains with low-level mupirocin resistance, compared to rates reported previously in Japan (20) or in Korea (10). This probably stems from the targeted surveillance of patients in ICUs where MRSA is endemic. The PFGE analysis revealed different PFGE patterns among MRSA isolates with low-level mupirocin resistance. Several isolates with identical PFGE patterns contained different IleS mutations, whereas isolates from different PFGE types contained the same IleS mutations. The Q612H mutation was identified only in PFGE types C and D. Therefore, it is likely that individual IleS mutations might occur in several PFGE clones. It is also possible that a few of PFGE clones with the same IleS mutation were spread. It is not known whether the patients in the ICUs were exposed to mupirocin.

Although low-level mupirocin resistance in *S. aureus* is more prevalent in clinical isolates than high-level resistance (4, 10, 20), its clinical significance is still unknown. It is possible, however, that the increasing frequency of IleS mutants might cause failure for nasal decolonization of MRSA carriers (4). Alternatively, it might suggest that a low fitness cost is associated with the high prevalence of low-level mupirocin resistant strains (19).

In conclusion, molecular heterogeneity of IleS mutations conferring low-level mupirocin resistance is commonly present among clinical MRSA strains in ICUs in a setting where MRSA is endemic. In addition to the V588F, the novel Q612H mutation is expected to be linked to low-level mupirocin resistance. Further study of several IleS structures from mutant clinical isolates is necessary to better understand the structural basis of low-level mupirocin resistance.

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