Mupirocin-Induced Mutations in ileS in Various Genetic Backgrounds of Methicillin-Resistant Staphylococcus aureus

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Topical mupirocin is widely used for the decolonization of methicillin-resistant Staphylococcus aureus (MRSA) carriers. We evaluated the capacity of various MRSA clonotypes to develop mutations in the ileS gene associated with low-level mupirocin resistance. Twenty-four mupirocin-sensitive MRSA isolates from a variety of genotypes (determined by a multilocus variable-number tandem-repeat assay) were selected. Mupirocin MICs were determined by Etest. The isolates were then incubated in sub-inhibitory concentrations of mupirocin for 7 to 14 days. Repeat MIC determinations and sequencing of the ileS gene were then performed. Doubling times of isolates exposed to mupirocin and of unexposed isolates were compared. We found that exposure to mupirocin led to rapid induction of low-level resistance (MICs of 8 to 24 μg/ml) in 11 of 24 (46%) MRSA isolates. This phenomenon was observed in strains with diverse genetic backgrounds. Various mutations were detected in 18 of 24 (75%) MRSA isolates. Acquisition of mutations appeared to be a stepwise process during prolonged incubation with the drug. Among the five isolates exhibiting low-level resistance and the highest MICs, four tested sensitive after incubation in the absence of mupirocin but there was no reversion to the susceptible wild-type primary sequence. Resistance was not associated with significant fitness cost, suggesting that MRSA strains with low-level mupirocin resistance may have a selective advantage in facilities where mupirocin is commonly used. Our findings emphasize the importance of the judicious use of this topical agent and the need to closely monitor for the emergence of resistance.

Nasal carriage of Staphylococcus aureus, including methicillin-resistant S. aureus (MRSA), has been identified as a risk factor for staphylococcal infection, with approximately 10% to 30% of MRSA carriers subsequently developing infection (1, 2). In addition, MRSA carriers act as reservoirs for the transmission of MRSA between hospitalized patients. Preoperative S. aureus screening and decolonization regimens for those undergoing selected surgeries have been advocated to decrease the infection risk in carriers (3, 4). Decolonization therapy can also be a successful infection control strategy to limit MRSA transmission within hospitals (5).

Decolonization protocols to eradicate S. aureus carriage commonly use intranasal mupirocin and chlorhexidine body washes (6). However, increasing resistance to these agents with associated reduction in the effectiveness of the decolonization strategy is being reported (7–9). In contrast to the high-level mupirocin resistance (MICs of ≥512 μg/ml) which results from the acquisition of a mobile genetic element carrying the mupA gene (also designated ileS-2), low-level resistance is due to acquisition of a point mutation(s) in tRNA synthetase for isoleucine (ileS) (10) and is defined by MICs between 8 and 256 μg/ml.

We recently described increasing mupirocin resistance in MRSA bloodstream isolates in our institution (11). After the introduction of routine decolonization of MRSA carriers with intranasal mupirocin and chlorhexidine body washes in 1994, mupirocin resistance increased from 0% in 1999 to 79% in 2008. In addition, the prevalence of resistance in a given year closely correlated with hospital-wide mupirocin consumption. The majority of mupirocin-resistant isolates exhibited low-level resistance, harbored the V588F point mutation, and belonged to the ST228 (clonal complex 5 [CC5]) clone which is the dominant MRSA clone in our region.

This study aimed to explore the genetic evolution underlying the emergence of low-level resistance in MRSA by investigating the development of mutations under selective pressure in vitro in MRSA isolates with diverse genetic backgrounds. These genetic changes could explain in vivo observations and predict reductions in the effectiveness of decolonization regimens with increasing mupirocin use.

MATERIALS AND METHODS

Strain selection and MIC determination. A panel of 24 clinical MRSA isolates from various genotypes were selected from a collection of strains from the international Mastering Hospital Antimicrobial Resistance (MOSAR) research study (12) as follows: CC152 (n = 1), CC5 (n = 8), CC7 (n = 1), CC8 (n = 4), CC59 (n = 1), CC80 (n = 1), CC30 (n = 3), CC22 (n = 1), CC45 (n = 1), CC398 (n = 2), and CC1 (n = 1). The 24 isolates were maintained in Mueller-Hinton broth (MHb). All isolates were confirmed to be MRSA using a latex agglutination test, a tube coagulase test, and meca PCR. Mupirocin MICs were determined by using Etests (AB bioMérieux, France).

Genotyping. Strains were genotyped and characterized as previously described by using a multilocus variable-number tandem-repeat assay (13, 14) and multilocus sequence typing (15).

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Induction of mutations. Strains were incubated in Mueller-Hinton broth (MHB) (control condition) as well as MHB supplemented with 0.5 MIC of mupirocin according to the original MIC of the isolate (Sigma-Aldrich, Saint Louis, MO). After overnight incubation, the suspensions were calibrated to 0.5 McFarland standard and diluted 1:100 in fresh medium. This was repeated on a daily basis for 7 days to reflect the duration of *in vivo* exposure to mupirocin in decolonization protocols, which usually last for 5 to 14 days (16–18). After 7 days, strains underwent MIC determinations by Etest before being subjected to another 7-day antimicrobial exposure cycle, with the mupirocin concentration adjusted according to the new MIC. To assess the stability of mutations after withdrawal of the selection pressure with mupirocin, samples of resistant strains were reincubated for a further 2 weeks with daily subculturing in fresh medium without mupirocin, corresponding to a total of approximately 350 cellular generations.

DNA extraction and sequence analysis. Genomic DNA from the MRSA strains isolated after incubation with and without mupirocin was obtained as previously described (13) by using a DNeasy kit (Qiagen, Basel, Germany). The *ileS* sequence was obtained by manually assembling the 8 sequencing results (LG Genomics, Berlin, Germany) obtained from 8 distinct PCRs performed with primers (Table 1) designed using Primer 3 (http://biotools.umassmed.edu/biapps/primer3_www.cgi). Sequences were compared by using blastX (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and alignments were displayed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). All mutations were detected by visual inspection.

Fitness cost. To evaluate whether the emergence of a point mutation(s) in *ileS* affects the growth of the resulting mutants with low-level resistance, isolates that were exposed to mupirocin as well as their parent strains were grown in MHB overnight in a Tecan Infinite F200pro microtiter plate reader (Tecan, Männedorf, Switzerland). The optical density was assessed and doubling times of susceptible and mupirocin-exposed strains were calculated as the doubling time of the susceptible isolate divided by the doubling time of the resistant (or mupirocin-exposed) isolate. To assess whether there was a difference in growth rates, the Wilcoxon signed-rank test was used to determine whether the ratios of the doubling times were significantly different from 1 (19) and the Wilcoxon rank sum test was used to compare the mean ratios of the doubling times of isolates that remained sensitive to the doubling times of those that developed resistance after mupirocin exposure. A two-sided *P* value of <0.05 was considered statistically significant.

The analysis was conducted with STATA V.11.0 (STATA Corp.).

**Table 1** List of primers used to sequence the *ileS* gene.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>5′→3′ sequence</th>
<th>Length (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ileS_56_F</em></td>
<td>GTTAAAGGTTGATCCGCGAG</td>
<td>23</td>
</tr>
<tr>
<td><em>ileS_996_F</em></td>
<td>AGATGCTATATTTGCTGTC</td>
<td>20</td>
</tr>
<tr>
<td><em>ileS_1700_F</em></td>
<td>TCACAATTCCAGTGTCACAAGGGA</td>
<td>26</td>
</tr>
<tr>
<td><em>ileS_2504_F</em></td>
<td>ATCAAATATGCTAATGCGAA</td>
<td>20</td>
</tr>
<tr>
<td><em>ileS_57_R</em></td>
<td>GATTAGAAGAGACCTTAA</td>
<td>20</td>
</tr>
<tr>
<td><em>ileS_482_R</em></td>
<td>AAAATTAGATTTTGTCAG</td>
<td>20</td>
</tr>
<tr>
<td><em>ileS_1174_R</em></td>
<td>TCTCCAGCTGTGGATGATCATG</td>
<td>24</td>
</tr>
<tr>
<td><em>ileS_2254_R</em></td>
<td>AGATTGGTGCTAACAACCTGGTAT</td>
<td>25</td>
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</table>

*a* Conditions for amplification were as follows: first step (t1), 2 min at 95°C; t2, 20 s at 95°C; t3, 10 s at 60°C; t4, 25 s at 72°C (t2 to t4, repeated 35 times); and t5, 10 min at 74°C. The total volume of each PCR mixture (KOD Hot Start; Novagen) was 20 μl and contained 0.8 U of KOD polymerase and primers at 0.3 μM.

**Stability of mutations in the absence of selection pressure.** In a sample of five isolates showing low-level mupirocin resistance that were selected to assess the stability of the mutations, all mutations detected after the first period of mupirocin exposure persisted even after a prolonged period of growth in the absence of mupirocin (Table 3). Four of 5 strains reverted to a sensitive phenotype with an MIC less than or equal to 4 μg/ml. However, the previously detected mutations were still present in these now phenotypically sensitive isolates. Interestingly, in two of these isolates, additional mutations were detected.

**Fitness cost.** No significant fitness cost was demonstrated in the isolates that developed resistance after exposure to mupirocin (Table 3). Comparison of the growth rates of the original susceptible isolates, subsequent generations of isolates showing low-level mupirocin resistance, and susceptible revertants demonstrated only marginal (1% to 34%), nonsignificant differences in doubling times (*P* = 0.33). In addition, there was no significant difference in the mean ratios of the doubling times of isolates that remained mupirocin sensitive (mean, 1.04; standard deviation [SD], 0.20; range, 0.82 to 1.34) to the doubling times of those that developed mupirocin resistance (mean, 1.04; SD, 0.14; range, 0.82 to 1.22) after exposure to the antibiotic (*P* = 0.76).
<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Isolate source</th>
<th>Sequencetype</th>
<th>Clonalcomplex SCC</th>
<th>meca</th>
<th>spa type</th>
<th>Common name(s) for MRSA clone</th>
<th>Epidemiological classification</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Geneva, Switzerland</td>
<td>ST105 CC5</td>
<td>II</td>
<td>t002</td>
<td>New York/Japan related</td>
<td>HA</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Belgrade, Serbia</td>
<td>ST228 CC5</td>
<td>I</td>
<td>t041</td>
<td>Southern German related</td>
<td>CA</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Athens, Greece</td>
<td>ST8 CC8</td>
<td>IVa</td>
<td>t008</td>
<td>USA300</td>
<td>CA</td>
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</tr>
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</table>

a SCC meca, staphylococcal cassette meca.
b HA, health care associated; CA, community associated; LA, livestock associated.
c agr, accessory gene regulator.
d pvl, Panton-Valentine leukocidin.
DISCUSSION

Our study found that all MRSA strains exposed to mupirocin in vitro developed an increase in mupirocin MIC and that the development of resistance mutations was common, rapid, and stable. This is a concern, as recent evidence supporting the use of decolonization therapy as an intervention to reduce health care-associated infections (3, 18) will likely lead to an increase in mupirocin use. Mupirocin selection pressure was the driver of resistance mutations in our MRSA isolates. In contrast to a previous report of the emergence of the N213D mutation without exposure to mupirocin (21), no spontaneous mutations were detected in our study in the absence of the drug. In the nasal mucosa, where concentrations of the antibiotic are likely to be high (about 20,000 μg/ml with 2% mupirocin), the induction of resistance mutations may be uncommon. However, there have been reports of resistance developing in the pharynx in patients who have undergone nasal decolonization with mupirocin (22). Pharyngeal concentrations of the antibiotic are less predictable, and subinhibitory levels may promote resistance. Similar effects may be seen when mupirocin is used on the skin or at catheter exit sites. Indeed, widespread community use of mupirocin on the skin is thought to have contributed to the increase in mupirocin resistance in some regions (23).

Detection of mutations was common after exposure to mupirocin, seen in 75% (18 of 24 sequences) of our isolates, with the development of a phenotype of low-level resistance in 46% (11 of 24) of the isolates. The most frequent mutations observed in our study were V588F, which is well recognized for its association with low-level resistance (10), and V631F. We identified two hot spots, covering the protein sequences between amino acids 200 to 350 and amino acids 450 to 650, in which most mutations were located. The region of the IleS protein around amino acid 600 was particularly variable, as demonstrated by our ability to detect mutations at positions 588, 593, 631, 632, 633, and 634 among our 24 strains. This part of the protein is in the region of the Rossman fold which contains the active site responsible for the biological activity of the protein (10, 21). Many of the mutations affecting the Rossman fold have previously been well characterized, and a number of mutations that we detected have been associated with reduced mupirocin susceptibility in prior studies (10).

Reversion to the wild type is described when selection pressure is removed. Previous ecological studies have demonstrated a reduction in mupirocin resistance after restriction of its use at an institutional level (24). In our sample of five isolates that developed low-level resistance due to the acquisition of point mutations previously shown to alter susceptibility, four tested sensitive after incubation without mupirocin. However, it was concerning that the point mutations in these isolates persisted despite subsequent growth in the absence of the antibiotic, and there was accumulation of additional mutations in two isolates. The importance of these additional mutations is unclear as they did not lead to significant increases in mupirocin MIC. However, this observation may indicate that strains with low-level resistance are more...
prone to the development of spontaneous mutations. For strains that reverted to the sensitive phenotype despite the persistence of resistance mutations, the presence of mupirocin may be required to induce expression of the mutations. In these strains, phenotypic tests for mupirocin resistance may not predict resistance when exposure to the antibiotic occurs \textit{in vivo}. Thus, in settings where mupirocin use is relatively common and accumulation of resistance mutations in circulating MRSA strains may be expected, genotypic tests for resistance may be more reliable than phenotypic tests.

We found no significant fitness cost associated with the resistance mutations. Previous studies have documented loss of fitness specific to certain mutations. In a recent paper, Mongkolrattanothai and colleagues recorded some alteration of growth rate in strains harboring the V588F mutation (25). Previous investigations have also shown that common mutations associated with low-level resistance, including V588F, may have a small effect on growth rate but that accumulation of further mutations that lead to higher levels of resistance can significantly decrease fitness (26). This suggests that there may be a selective advantage for MRSA strains with low-level resistance in facilities where mupirocin is commonly used.

We detected low-level resistance rather than high-level resistance. Although the clinical relevance of high-level resistance has been recognized for some time, there is recent evidence to suggest that low-level resistance is also associated with a reduction in effectiveness of this antibiotic with respect to eradicating MRSA carriage (9, 17, 27). Note that the mutations which resulted in low-level resistance emerged rapidly, within days of exposure to subinhibitory concentrations of the antibiotic. Although previous reports identified prolonged exposure to mupirocin as a risk factor for resistance (8), our data suggest that even a single short treatment course is sufficient to induce clinically relevant resistance mutations.

Not all mutations detected in \textit{ileS} result in low-level mupirocin resistance. Certain mutations, including N213D, have been described in isolates and are thought to have no effect on the function of the enzyme (21). The ST59 isolate in our study exhibited an increase in its MIC from 0.25 \(\mu\)g/ml to 1.5 \(\mu\)g/ml but remained in the susceptible range despite the acquisition of four point mutations, demonstrating that the number of mutations did not correlate with the level of resistance. We found that the same mutations could correspond to phenotypes of sensitivity or low-level resistance in different isolates. The S634F mutation, for example, was detected in MRSA isolates with drug MICs ranging from 1 to 24 \(\mu\)g/ml. This again highlights that some isolates that have previously been exposed to sub-MICs of mupirocin may test susceptible despite harboring resistance mutations. However, it is possible that these MRSA strains may become phenotypically resistant after repeated exposure to the antibiotic.

We studied diverse strains of MRSA belonging to the most common clonal complexes in human isolates. We detected the V588F mutation in different genetic backgrounds, including CC398 and various STs from CC8, and noted that it was particularly frequent in CC5 isolates, including our dominant ST228 clone (11). Isolates from CC5 also developed a diverse range of other mutations, while those from CC398 were more homogeneous. Strains in these clonal complexes rapidly developed resistance after a single selection cycle. In contrast, we were not able to induce resistance in strains from all sequence types (e.g., ST247, ST45, and ST36). We noted that the USA300 isolate in our study did not develop mutations conferring low-level resistance after prolonged exposure to mupirocin. Although previous studies found that 20% of USA300 isolates were mupirocin resistant, the resistance was plasmid-mediated high-level resistance (28). It is possible that the USA300 strain has a higher barrier to the development of chromosomal mutations leading to low-level resistance. These strain differences may account for the predominance of high-level rather than low-level resistance in the United States (29) and predict variation in the longer-term effectiveness of topical mupirocin in different geographical settings. Our collection contained only one USA300 isolate (from Greece). Further studies, including a larger number of isolates of this strain type, particularly in the United States, where this strain is dominant, may be warranted. Our findings are consistent with the published literature, which reports that low-level mupirocin resistance occurs more frequently in MRSA strains belonging to certain clonal complexes (including various STs from CC5 and CC8) than in those belonging to other clonal complexes, suggesting that specific genome content may be associated with the stability of \textit{ileS} in \textit{S. aureus} exposed to mupirocin.

In conclusion, MRSA strains exposed to mupirocin \textit{in vitro} develop rapid and persistent mutations associated with low-level resistance. Strains with these mutations do not exhibit significant fitness loss; therefore, there is potential for these strains to emerge as the dominant clone in institutions with ongoing and widespread use of mupirocin. Resistance mutations may be more common in certain clones but can be seen in MRSA strains with a variety of genetic backgrounds; thus, emergence of resistance associated with routine use of this antibiotic is likely to be a widespread problem. The clinical benefits of mupirocin need to be balanced with these data, which demonstrate the ease with which resistance occurs. In order to retain the clinical utility of mupirocin for staphylococcal eradication therapy, it is necessary to implement rational prescribing policies. These policies should be coupled with long-term surveillance for the emergence of genotypic and phenotypic resistance as well as ongoing monitoring of the clinical effectiveness of this antibiotic.

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