Correlation between Mutations in liaFSR of Enterococcus faecium and MIC of Daptomycin: Revisiting Daptomycin Breakpoints

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Mutations in liaFSR, a three-component regulatory system controlling cell-envelope stress response, were recently linked with the emergence of daptomycin (DAP) resistance in enterococci. Our previous work showed that a liaF mutation increased the DAP MIC of a vancomycin-resistant Enterococcus faecalis strain from 1 to 3 μg/ml (the DAP breakpoint is 4 μg/ml), suggesting that mutations in the liaFSR system could be a pivotal initial event in the development of DAP resistance. With the hypothesis that clinical enterococcal isolates with DAP MICs between 3 and 4 μg/ml might harbor mutations in liaFSR, we studied 38 Enterococcus faecium bloodstream isolates, of which 8 had DAP MICs between 3 and 4 μg/ml by Etest in Mueller-Hinton agar. Interestingly, 6 of these 8 isolates had predicted amino acid changes in the LiaFSR system. Moreover, we previously showed that among 6 DAP-resistant E. faecium isolates (MICs of >4 μg/ml), 5 had mutations in liaFSR. In contrast, none of 16 E. faecium isolates with a DAP MIC of ≤2 μg/ml harbored mutations in this system (P < 0.0001). All but one isolate with liaFSR changes exhibited DAP MICs of ≥16 μg/ml by Etest using brain heart infusion agar (BHLA), a medium that better supports enterococcal growth. Our findings provide a strong association between DAP MICs within the upper susceptibility range and mutations in the liaFSR system. Concomitant susceptibility testing on BHIA may be useful for identifying these E. faecium first-step mutants. Our results also suggest that the current DAP breakpoint for E. faecium may need to be reevaluated.

Enterococci are leading causes of nosocomial infections in the United States, and multidrug-resistant (MDR) isolates have become an important clinical problem throughout the world. Enterococcus faecalis and Enterococcus faecium account for most of the clinically relevant enterococcal isolates recovered from infections in humans (20). The treatment of enterococcal infections is particularly challenging due to the emergence of MDR isolates and the paucity of newer therapeutic alternatives. Moreover, the dissemination of a hospital-associated clade of ampicillin- and vancomycin-resistant E. faecium (17) has dramatically reduced the options for the treatment of severe enterococcal infections (1).

Daptomycin (DAP) is a cyclic lipopeptide antibiotic that exhibits in vitro bactericidal activity against MDR enterococci and is one of the few therapeutic options available for these organisms. Although DAP has not been approved for the treatment of enterococcal bacteremia or vancomycin-resistant enterococcal (VRE) infections, it is frequently used off-label for these purposes (8,19). Interestingly, the DAP breakpoint is 4 μg/ml, which is four times higher than its breakpoint for staphylococci (12). However, clinical and microbiological failures have been described in subjects with E. faecium bacteremia and MICs close to the breakpoint (3 to 4 μg/ml) (4,35). Moreover, animal models of S. aureus endocarditis have shown decreased efficacy of DAP when treating susceptible microorganisms with MICs approaching the established staphylococcal breakpoint (1 μg/ml) (10). Additionally, several cases of DAP resistance emerging during therapy of DAP-susceptible enterococcal isolates have been documented (26,28), which suggests that selection of in vivo resistance during therapy may be an issue for the use of DAP against enterococci.

The mechanisms of enterococcal resistance to DAP remain to be fully elucidated. Nonetheless, recent data have shown that mutations in two distinct group of genes are important for the development of DAP resistance: (i) a three-component regulatory system (designated liaFSR) that orchestrates the cell envelope response to antibiotics and (ii) genes encoding proteins involved in phospholipid metabolism, including glycerolphosphoryl diester phosphodiesterase (gdpD) and cardiolipin synthase (cls) (3,31). Using allelic gene replacements, we recently demonstrated that a mutation in the liaF gene, which is part of the liaFSR system, produced a 3-fold increase in the DAP MIC of a DAP-susceptible, vancomycin-resistant clinical isolate of E. faecalis (3). Furthermore, mutations predicting alterations in LiaFSR proteins were commonly found in clinical isolates of DAP-resistant E. faecium. Thus, we postulate that mutations in the liaFSR system may be a pivotal, first-step event in the development of DAP resistance during therapy in clinical isolates of enterococci and that these mutations are associated with decreased DAP susceptibility, which sub-

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sequently may predispose deep-seated enterococcal infections to DAP failure.

In order to evaluate the mutations in the liaFSR system in DAP-susceptible clinical isolates, we determined the nucleotide sequence of liaFSR in clinical enterococcal isolates exhibiting MICs within the higher range of susceptibility (MICs between 3 and 4 μg/ml) and in a subset of the isolates with MICs of ≥2 μg/ml. We also compared the sequences of liaFSR in DAP-susceptible isolates of enterococci whose genomes have been sequenced and are publicly available. Finally, in an effort to improve the phenotypic detection of reduced DAP susceptibility, we compared DAP MICs determined by Etest of enterococcal isolates, including those with higher MICs (3 to 4 μg/ml), on standard Mueller-Hinton agar (MHA) with MICs determined on media that better support the growth of enterococci, such as brain heart infusion agar (BHIA) and Trypticase soy with 5% sheep blood (BAG). Our data suggest that the current DAP breakpoint for enterococci does not identify isolates with genetic changes in liaFSR that may predispose them to in vivo development of DAP resistance.

MATERIALS AND METHODS

Bacterial strains. The enterococcal clinical isolates used for this study were recovered from bacteremic episodes occurring in two large hospitals in the Texas Medical Center, Houston, TX, from January to June of 2011. A bacteremic episode was defined as the presence of at least one positive blood culture for Enterococcus spp. Species-specific identification of enterococci was confirmed by multiplex PCR targeting genes encoding the d-alanine–d-alanine ligase, as previously described (13).

TABLE 1 List of primers utilized to amplify the liaFSR system and cardiolipin synthase gene, cls

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>liaF</td>
<td>5’-CATCTAAAGTCTCATATGGCAAC (F)</td>
<td>756</td>
</tr>
<tr>
<td></td>
<td>5’-CAGCTGAGGTTGCTTGAGT (R)</td>
<td></td>
</tr>
<tr>
<td>liaS</td>
<td>5’-AACGGCGCTGTGTTGCGCAAC (F)</td>
<td>1,104</td>
</tr>
<tr>
<td></td>
<td>5’-CTCCGGGATTAAGTGGGCGGA (R)</td>
<td></td>
</tr>
<tr>
<td>liaR</td>
<td>5’-GACGTACTGAATCTGGAACCTC (F)</td>
<td>621</td>
</tr>
<tr>
<td></td>
<td>5’-TGCGTGGCCGAAAGTGCTT (R)</td>
<td></td>
</tr>
<tr>
<td>cls</td>
<td>5’-ACATCAAAAAGACCGCCCTCA (F)</td>
<td>1,446</td>
</tr>
<tr>
<td></td>
<td>5’-AAAGATGTCCTGTTTCACAA (R)</td>
<td></td>
</tr>
</tbody>
</table>

a liaF, liaS, and liaR are genes encoding the LiaFSR system. cls encodes a putative cardiolipin synthase.

b (F), forward; (R), reverse.

Those obtained using BMD methodology. E. faecalis ATCC 29212 and Staphylococcus aureus ATCC 29213 were used as controls in each set of tests. The CLSI DAP breakpoint (≤4 μg/ml) was used to define DAP susceptibility (12).

PCR amplification and DNA sequencing. The enterococcal homologues of the genes coding for the liaFSR regulatory system and the putative cardiolipin synthase gene (cls) were sequenced in their entirety from all clinical enterococcal isolates with DAP MICs between 3 and 4 μg/ml (Etest on MHA) and from a subset of isolates with MICs of ≥2 μg/ml. PCR amplification was performed using Taq polymerase (Promega, Madison, WI) from colony lysates with primers shown in Table 1. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA), and the amplicons were sequenced using both strands by the dideoxynucleotide chain termination method (34) with fluorescent cycle sequencing with dye-labeled terminators (Applied Biosystems BigDye Terminator version 3.1 cycle sequencing kit, Foster City, CA) on an ABI Prism 3730 automated DNA sequencer.

Genetic analysis and molecular typing. DNA sequences were assembled using DNASTAR software’s SeqMan program (Lasergene, Madison, WI) and subsequently compared with all E. faecalis and/or E. faecium genomes available in NCBI (http://www.ncbi.nlm.nih.gov/genomes/geblast.cgi?taxid=1352). A mutation was considered significant if it predicted a unique change in an amino acid within LiaFSR and it was not present in any other daptomycin-susceptible enterococcal genomes publicly available in NCBI. Pulsed-field gel electrophoresis (PFGE) was performed for all strains whose liaFSR loci and cls genes were sequenced. Chromosomal DNA was digested with Smal following a previously described protocol (29). DNA fragments were separated by electrophoresis (CHEF-DR II system; Bio-Rad Laboratories, Inc., Richmond, CA) at 6 V/cm, with switch times ramped from 2 s to 28 s over 22 h at 14°C. Genetic relatedness among strains was assessed using previously published criteria (38).

Statistical analysis. Data were analyzed using STATA 10.0 (Stata Corp, College Station, TX). The difference between isolates with and without mutations was assessed using Fisher’s exact test. A P value of <0.05 was considered statistically significant.

RESULTS

Correlation between DAP MICs and mutations in the liaFSR system. A total of 80 clinical isolates were evaluated. Forty-two (52.5%) isolates were E. faecalis, and 38 (47.5%) were identified as E. faecium. Our initial hypothesis was that enterococcal isolates with higher DAP MICs (between 3 and 4 μg/ml, still within the susceptible range) are likely to harbor mutations in liaFSR which have been associated with decreased susceptibility to DAP. A total of 1 E. faecalis and 8 E. faecium isolates were found to have DAP MICs between 3 and 4 μg/ml using Etest in MH. We decided to focus our studies on E. faecium since DAP is more likely to be used...
against this enterococcal species. Six (75%) of these 8 E. faecium isolates had predicted amino acid changes in at least one of the members of the liaFSR system. The most common substitutions observed were Thr120→Ala in LiaS and Trp73→Cys in LiaR (Table 2). In contrast, no changes were found in the clp gene of any of the strains analyzed.

Subsequently, we sought to investigate the presence of mutations in liaFSR in isolates with DAP MICs of ≤2 μg/ml; among them, we arbitrarily chose 6 E. faecium isolates for sequencing from our collection of bacteremic episodes, including one strain from each month of the study period. None of these isolates with a DAP MIC of ≤2 μg/ml (range, 1 to 2 μg/ml) had mutations in the liaFSR genes. Additionally, we performed an Etest on MHA to determine the DAP MIC of 12 E. faecium clinical isolates whose genomes are published in the NCBI and whose liaFSR harbored no mutations. A total of 10 isolates (of 12) exhibited DAP MICs of ≤2 μg/ml. The 2 remaining isolates had MICs of 3 μg/ml. Overall, none of the 16 E. faecium isolates studied with DAP MICs of ≤2 μg/ml on MHA (10 from NCBI and 6 from our collection) harbored mutations in the genes encoding the liaFSR system, which is in clear contrast with the isolates with MICs of ≥3 μg/ml described above (P = 0.00019). PFGE analysis of isolates with MICs of ≥3 μg/ml showed that two isolates (105 and 119) (Table 2) were closely related (difference in 4 bands). The banding patterns of the remaining strains indicated that they were not clonally related by the criteria of Tenover et al. (>6-band difference in PFGE patterns).

**Determination of DAP MIC using brain heart infusion agar increases detection of isolates with mutations in the liaFSR system.** We sought to determine if performing Etest on BHIA, which better supports the growth of enterococci, would improve the identification of isolates harboring mutations in liaFSR. The distribution of MICs of all the enterococcal isolates under the different testing conditions is shown in Fig. 1. As previously observed (23), E. faecium strains exhibited higher MICs than E. faecalis strains regardless of the testing conditions, and the MICs by Etest using MHA were 3 μg/ml and 1 μg/ml for E. faecium and E. faecalis, respectively (data not shown). Etest on BHIA plates showed 3- to 4-fold increases in the MICs of both enterococcal species compared to the results of BMD or Etest on MHA, and 29 (36%) isolates on BHIA had MICs above 4 μg/ml. The relationship between DAP MICs by Etest on MHA and BHIA is shown in Fig. 2. Among the 10 isolates with DAP MICs in the upper range of susceptibility (MICs on MHA of 3 to 4 μg/ml), 8 from our collection and 2 of the NCBI strains, 6 had mutations in liaFSR and 4 did not. The MIC by Etest on BHIA was ≥16 μg/ml in 5 of the 6 strains with mutations, and the remaining isolate had a DAP MIC of 12 μg/ml. In contrast, the isolates without mutations all had DAP MICs on BHIA of ≤12 μg/ml (Fig. 2). No specific correlation was found between DAP MICs determined by BMD and mutations in liaFSR. Indeed, among the 10 isolates that exhibited DAP MICs between 3 and 4 μg/ml by Etest on MHA (Fig. 2), the MIC by BMD was 2 μg/ml for 7 isolates and 4 μg/ml for 3 isolates. However, as determined by Etest, all isolates lacking mutations in liaFSR had DAP MICs of ≤2 μg/ml by BMD.

**DISCUSSION**

The use of antimicrobial agents with bactericidal activity against enterococci is of paramount importance to treat endocarditis and, potentially, other severe infections caused by these microorganisms. Unfortunately, the emergence of resistance to ampicillin and vancomycin and high-level resistance to aminoglycosides has limited the therapeutic alternatives for serious enterococcal infections over the past few decades (6). DAP is a lipopeptide antibiotic with *in vitro* bactericidal activity against enterococci which is often used by clinicians in the treatment of vancomycin-resistant E. faecium infections due to the lack of bactericidal alternatives (2). Therefore, strategies to optimize the use of DAP against enterococci would be important for the management of seriously ill patients who are often predisposed to develop infections by MDR enterococci.

One of the limitations for DAP use against enterococci appears to be the decreased susceptibility of clinical isolates compared to the susceptibility of staphylococci; indeed, the CLSI DAP breakpoint for enterococci is 4-fold higher than that for staphylococci (≥4 μg/ml versus ≤1 μg/ml, respectively). This difference may have clinical consequences and has led to the hypothesis that higher doses of DAP may be needed to overcome its lower potency, particularly in the setting of infective endocarditis (9). Moreover, several cases of DAP failure and emergence of resistance have been reported (4, 25, 26, 28, 35), suggesting that the best strategy for the use of DAP against enterococci is still unknown.
The liaFSR system, or its homologs, encodes a three-component regulatory system that has been shown to be involved in the regulation of the cell envelope stress response in a wide range of Gram-positive organisms, including Streptococcus mutans, Streptococcus pneumoniae, Bacillus subtilis, and S. aureus (14, 18, 27, 37). Furthermore, its involvement seems to be crucial for some types of resistance to be expressed (7, 21). We previously demonstrated that a deletion of a codon in liaF produced a 3-fold decrease in DAP susceptibility in a clinical strain of vancomycin-resistant E. faecalis (3). The change in susceptibility was not enough to increase the MIC above the current breakpoint (4 μg/ml) but was required for full expression of the resistance phenotype after a subsequent mutation in a gene encoding an enzyme involved in phospholipid metabolism (gdpD) was introduced (3).

Thus, our previous data suggested that mutations in the liaFSR system may be a critical initial event in the development of in vivo DAP resistance. Therefore, we postulated that mutations in liaFSR may be present in a significant proportion of isolates with MICs close to the breakpoint and that the identification of these isolates may be important to recognize isolates predisposed to develop DAP resistance. Indeed, we found that 6 of 8 E. faecium isolates (75%) from our bacteremia collection with DAP MICs of $\geq$3 μg/ml harbored mutations in one of the genes encoding the liaFSR system. Interestingly, the substitutions Trp120→Ala and Trp73→Cys in LiaS and LiaR, respectively, have been previously associated with DAP-resistant clinical isolates of E. faecium (3). Among 12 E. faecium isolates whose genomes are available, two had a DAP MIC of 3 μg/ml, although neither of these harbored mutations in liaFSR. Interestingly, 1 of these 2 isolates that lacked liaFSR mutations (TX1033, MIC of 3 μg/ml) was recovered from a patient with endocarditis who failed DAP monotherapy (4), supporting our hypothesis that a DAP MIC of 3 μg/ml may be an indication of possible failure even in the absence of mutations in liaFSR, since other genes may be involved in decreasing the susceptibility to this antimicrobial peptide. Conversely, none of the isolates with DAP MICs of $\approx$2 μg/ml harbored mutations in liaFSR. The relevance of the selective pressure of DAP and its role in the selection of these mutations is unknown.

Our data support an association between DAP MICs of $\geq$3 μg/ml and mutations in at least one of the genes encoding the liaFSR system. This association is further supported by the fact that 5 of 6 previously reported DAP-resistant E. faecium clinical isolates (DAP MICs of $\geq$4 μg/ml) with no epidemiological relationship with our clinical collection also harbored mutations in at least one component of the liaFSR system (3). Thus, our data strongly suggest that the current breakpoint of 4 μg/ml includes isolates in the susceptible category in which initial genetic changes associated with the development of DAP resistance have already occurred. We speculate that changes in liaFSR are the first-step mutations that may eventually lead to failures during DAP therapy. Of note, none of the E. faecium isolates with DAP MICs between 3 and 4 μg/ml exhibited any changes in cls, encoding a cardiolipin synthase, an enzyme linked to DAP resistance in an in vitro-selected DAP-resistant strain of E. faecalis (31) and, more recently, in clinical isolates of S. aureus (32). The only strain with mutations in cls shown in Fig. 2 was a DAP-resistant clinical isolate of E. faecium with a DAP MIC of $>24$ μg/ml (3).

Since BMD is the gold standard method to determine DAP MICs in enterococci, we attempted to establish a correlation between MIC values obtained by BMD and mutations in liaFSR. Our results showed that 50% of the isolates with mutations in liaFSR would have been missed if the DAP MIC were determined by BMD.
BMD (MICs of 2 μg/ml), supporting the fact that MIC determination by BMD is less sensitive than Etest to identify enterococcal isolates that may have developed decreased susceptibility to DAP.

Enterococci are known to have important nutritional requirements for growth (30), and MH medium is not the ideal medium to support the growth of these organisms (24). For this reason, we also tested DAP MICs with Etest on enriched media, such as BHIA and Trypticase soy agar supplemented with blood, which better support the in vitro growth of enterococcal isolates. Higher DAP MICs were observed in BHIA than were determined on MHA (Etest), a phenomenon previously described (23). Since we identified that a MIC of 2 μg/ml in MHA may be associated with mutations in liaFSR (see above), we attempted to determine if the use of Etest on BHIA could help identify isolates with such mutations. Indeed, a MIC of ≥16 μg/ml on BHIA identified 11 of the 12 isolates (all of them also had an MHA Etest MIC of ≥3 μg/ml) with mutations, of which 10 had changes in liaFSR and 1 had a mutation in cls; no isolate without a mutation had a MIC of ≥16 μg/ml. A BHIA MIC of ≥12 μg/ml identified the one other isolate with a mutation (12 of 12 total isolates with mutations), but 3 isolates with this MIC did not have a mutation. An MHA Etest MIC of ≥3 μg/ml also identified all 12 isolates with mutations, but 4 other isolates without mutations also had a MIC of 3 μg/ml. In brief, with the use of a breakpoint of 16 μg/ml on BHIA, no false-positive results were observed, whereas only one isolate with a mutation was “missed” (Fig. 2). This finding may serve as the basis of a potential microbiological tool to screen and fully identify isolates that have already sustained a first step in the pathway to resistance and which have the potential of becoming fully resistant during therapy with a single subsequent mutation. However, there are important considerations that have to be addressed. DAP MICs vary widely with different concentrations of calcium in the testing medium (15), and this variation has been observed not only between different types of agars but also among different brands and lots of the same medium (16, 22, 23). Since the amount of calcium in BHIA is not standardized, the use of the DAP Etest on this medium is likely to have issues of reproducibility.

In summary, we confirmed a strong association between mutations in genes encoding the liaFSR system and DAP MICs within the higher susceptibility range (3 to 4 μg/ml) by Etest on MHA. Also, concomitant susceptibility testing on BHIA may be a useful tool to identify E. faecium isolates with a first-step mutation, which are predisposed to develop resistance during therapy, requiring only one additional mutation for this to occur. Our findings suggest the need to reevaluate the current breakpoints for DAP in E. faecium. We believe our results may have the potential to optimize the use of DAP against enterococci in clinical settings. A correlation between the presence of liaFSR mutations and clinical outcomes is the subject of our ongoing studies.

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REFERENCES