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Regulation of *mprF* in Daptomycin-Nonsusceptible *Staphylococcus aureus* Strains▽

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We used a well-characterized isogenic set of clinical bloodstream *Staphylococcus aureus* strains to study (i) regulation of *mprF*-mediated phosphatidylglycerol lysinylation in the contexts of in vitro daptomycin (DAP) nonsusceptibility and (ii) the role of *mprF* mutation in endovascular virulence. We observed a correlation between increased expression of a mutant *mprF* gene and reduced in vitro DAP susceptibility. There were no detectable fitness differences between strains in experimental infective endocarditis.

Several groups have reported an association between an increase in daptomycin (DAP) MICs and single-point mutations in the *Staphylococcus aureus mprF* gene acquired during in vitro or in vivo exposure to DAP (1, 4, 9). Since *mprF* contributes substantially to the staphylococcal net positive surface charge by lysinylation of membrane phosphatidylglycerol (PG), a gain-in-function mutation was postulated to impart DAP nonsusceptibility via a charge-repulsive mechanism (3, 7, 9, 12). However, detailed genotypic studies to define the transcriptional profiles and regulation of the *mprF* gene in the context of DAP nonsusceptibility have not been reported.

In the present study, we used a well-defined and previously characterized series of *S. aureus* isolates from a patient with persistent endocarditis (3, 10). The initial isolate (strain 616), the first strain to exhibit increases in DAP MICs (strain 629, termed the "transitional strain"), and two later isolates with DAP MICs within the nonsusceptible range (strains 701 and 703) were included in this investigation (3). The MICs of this strain set to DAP, as determined by standard Etest, were 0.5, 0.75, 2, and 2 μg/ml for strains 616, 629, 701, and 703, respectively (3).

Sequencing of the *mprF* open reading frames revealed a single-point mutation (C to A) at position 884 in the two DAP-nonsusceptible strains (701 and 703), resulting in an amino acid substitution at position 295 from serine-to-leucine (S295L) (data not shown). In contrast, analyses of the *mprF* promoter region showed no difference in sequence between DAP-susceptible and DAP-nonsusceptible strains (data not shown).

As shown in Fig. 1, Northern blot analysis revealed increased *mprF* transcripts in the two DAP-nonsusceptible strains (701 and 703) during stationary growth (12 h of incubation) compared to the DAP-susceptible (616) strain. This increased transcription could not be explained by altered *mprF* mRNA stability (half-lives) during the stationary phase (data not shown).

We next determined whether DAP-nonsusceptible strains are more or less fit than DAP-susceptible strains in vivo. One measurement of organism fitness is its ability to colonize, propagate, and disseminate within relevant animal models. Since many of the DAP-nonsusceptible clinical isolates have emerged during DAP therapy of vascular infections (2, 10, 11), we chose the infective endocarditis (IE) model (13, 14) to assess in vivo virulence. In agreement with previous observations (14, 15), as shown in Fig. 2, *S. aureus* cell densities achieved in vegetations were significantly higher than those in kidneys and spleens for each strain at 24 h postinfection ($P < 0.05$). Interestingly, both the DAP-susceptible and DAP-nonsusceptible strains were equivalent in their capabilities to induce experimental IE, proliferate within the vegetative lesion, and then disseminate hematogenously to distant target organs (Fig. 2).

Our prior data strongly suggested that DAP nonsusceptibility was associated with MprF gain in function, especially enhanced flippase activity (increasing the amount of lysyl-PG translocated to the outer cell membrane leaflets), rather than increased synthase function of this protein (3). This paradigm is compatible with the enhanced net positive surface charge and reduced DAP binding in the DAP-nonsusceptible strains previously documented and supported a charge repulsion mechanism for this strain set (3, 6, 8, 12). Importantly, Ernst et al. have localized the putative synthase domain of MprF to the hydrophilic C terminus plus the last six transmembrane helices of its C terminus, while the translocase domain appears to involve at least the first eight or perhaps all of the transmembrane helices of the N terminus (C. Ernst, P. Staubitz, G. Hornig, D. Kraus, and A. Peschel, presented at the International Symposium on Staphylococci and Staphylococcal Infections, Cairns, Australia, 2008). Based on this mapping and the location of the single nucleotide polymorphism (SNP) we observed in the present study, the amino acid substitution we

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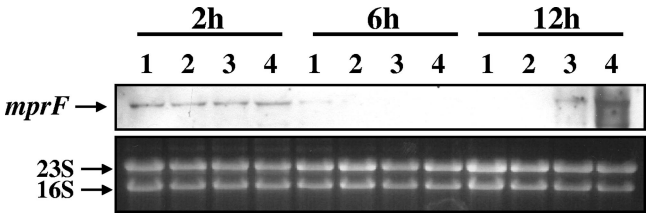

FIG. 1. Northern blot analyses of *mprF* transcription. Total cellular RNA samples from strain 616 (lane 1), 629 (lane 2), 701 (lane 3), and 703 (lane 4) cells grown in Trypticase soy broth media were isolated at 2, 6, and 12 h postinoculation, as described previously (16, 17). Five micrograms of each RNA sample was separated in a 1% (wt/vol) agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized to *mprF*-specific digoxigenin (DIG)-labeled probe synthesized using a PCR-based DIG probe synthesis kit (Roche) with primers mprF-F (5′-GTAGTAATCACATTGTATCGGGAGT-3′) and mprF-R (5′-GATGCATCGAAAACATGGAATAC-3′).

identified likely resides within the translocase domain of *mprF* and accounts for an *mprF* gain in function, as reflected by increased lysyl-PG flipping in our DAP-nonsusceptible mutant strains.

Our Northern blot data, in combination with the sequencing data, indicate that DAP-nonsusceptible strains, 701 and 703, express more net *mprF* transcripts [in its mutated form, MprF(S295L)] over a standard in vitro growth cycle compared to the parental strain, 616. Recent studies have shown that neither exposure to DAP nor host defense cationic peptides from mammalian polymorphonuclear leukocytes or platelets (hNP-1 or RP-1, respectively) (8) induced expression of *mprF* in strains 616 and 703 (data not shown). Thus, it appears that DAP-nonsusceptible *S. aureus* strains with *mprF* SNPs and basal *mprF* gain-in-function phenotypes do not require additional *mprF* induction to affect nonsusceptibility to DAP.

Current studies are in progress to further examine the relationship of *mprF* SNPs and DAP resistance, as well as to define the genetic regulatory pathway or pathways that influence DAP resistance. Of note, Li et al. have recently shown that the *aps* (*graRS*) system in *S. aureus* positively regulates *mprF* expression via a cationic sensor function (5). The potential involvement of this latter locus in the *mprF* gain-in-function expression in our DAP-resistant mutants is under active investigation. Finally, the equivalent fitness of our strain set in vivo emphasizes the role of DAP treatment in divulging the clinical impacts of DAP nonsusceptibility.

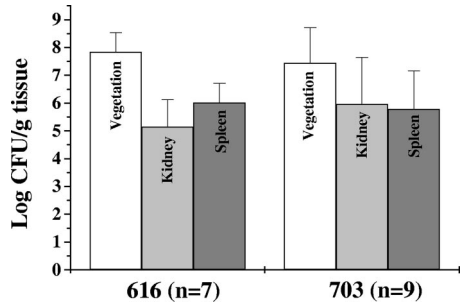

FIG. 2. In vivo virulence of DAP-susceptible and DAP-nonsusceptible strains. IE was produced using the DAP-susceptible (strain 616) or DAP-nonsusceptible (strain 703) strain, and microbiologic evaluation was performed at 24 h after infection as described previously (13, 14). IE was produced by intravenous injection of the selected *S. aureus* isolate (2×10^4 CFU/animal) at 48 h postcatheterization.

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