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Correlation of Cell Membrane Lipid Profiles with Daptomycin Resistance in Methicillin-Resistant *Staphylococcus aureus*

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We compared the cell membrane (CM) lipid composition among nine well-characterized daptomycin-susceptible (Dap^s)/Dap-resistant (Dap^r) methicillin-resistant *Staphylococcus aureus* (MRSA) strain pairs. Compared to the 9 Dap^s parental strains, Dap^r strains (with or without *mprF-yycFG* mutations) exhibited significantly reduced phosphatidylglycerol (PG) content ($P < 0.01$), significantly increased total synthesis of lysyl-PG (LPG) ($P < 0.01$), and reduced carotenoid content ($P < 0.05$ for 5/9 strains). There were no significant changes in LPG flipping, cardiolipin content, or fatty acid composition among strain pairs.

Daptomycin (Dap) is a lipopeptide antibiotic, first FDA approved in 2003, which demonstrates excellent antibacterial potency and *in vivo* activity against susceptible Gram-positive pathogens (1–7). Although both the bacterial cell membrane (CM) and cell wall (CW) are felt to participate in its bactericidal pathway, Dap principally targets the CM in a strictly calcium-dependent manner, rapidly perturbing its integrity and dissipating its electrochemical gradient, leading to cell death (8). *Staphylococcus aureus* utilizes adaptations in both CM phospholipid (PL) content and CW composition to modulate its relative positive surface charge as a protective mechanism, presumably against the binding and insertion of positively charged (cationic) antimicrobial peptides (CAPs), such as Dap, and host defense peptides (HDPs) (8–14). In addition, *S. aureus* can alter its carotenoid profiles to calibrate its CM order (fluidity versus rigidity) to best resist the microbicidal action of CAPs (12, 15). In these regards, *S. aureus* strains have been shown to accumulate single nucleotide polymorphisms (SNPs) in two particular gene loci during evolution of Dap^r, *mprF* and *yycFG* (8, 12, 16). The *mprF* locus in *S. aureus* is involved in the lysinylation of CM phosphatidylglycerol (PG) to generate the positively charged species, lysyl-PG (LPG), and also promotes LPG translocation from the inner to outer CM leaflet (8, 10, 17–19). In addition, mutations in *yycFG* (involved in the CM stress response and fatty acid biosynthesis) is a well-known accompaniment of the Dap^r phenotype in *S. aureus* (8, 16). The aim of this study was to analyze the fatty acid (FA) and PL content of a well-characterized recent set of Dap^s/Dap^r strain pairs.

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Nine previously published Dap^s/Dap^r methicillin-resistant *S. aureus* (MRSA) clinical bloodstream strain pairs were used in this study (21). (Although the official terminology is "daptomycin nonsusceptible," the term "daptomycin resistant" is employed in this study for a more facile presentation.) The strain pairs were initially selected on the basis of whether or not the Dap^r isolate possessed an *mprF* SNP (with and without a concomitant *yyc* operon mutation) (21). Each Dap^s and Dap^r strain pair was identical on the basis of pulsed-field gel electrophoresis (PFGE) (21). In addition, the following detailed comparative genotyping assays strongly suggested genetic identity among strain pairs: *agr* typing (22), *spa* typing, clonal complex determinations (23), and staphylococcal cassette chromosome *mec* element (SCC*mec*) typing (24). As reported before (21), among these Dap^r isolates, the Dap MICs ranged from 4- to 16-fold higher than those of their respective parental Dap^s isolates (Table 1). In 4/10 Dap^r isolates, the VISA phenotype was observed (vancomycin MICs of 4 μg/ml) (21) (Table 1).

Seven of the 9 Dap^r strains exhibited SNPs within the *mprF* gene locus, with or without concomitant SNPs within *yycFG*, while in two of the 9 Dap^r strains, there were no mutations in either gene locus (Table 1) (21).

Detailed methods for PL and FA extractions, fluorescamine labeling of outer CM LPG to define LPG translocation, FA profiling, and carotenoid quantifications have been described in detail before (8, 10, 12, 15, 25–29). For PL compositional analysis, major CM PLs of *S. aureus* PG, LPG, and CL were separated by two-dimensional (2-D) thin-layer chromatography (TLC) using Silica 60 F254 HPTLC plates (Merck). A minimum of seven TLC plates were used from two different lipid extracts on different days for the PL analysis. Data were expressed as the mean (±SD) percentages of the three major PLs (LPG + PG + CL = 100%). Distinct FAs, i.e., total iso-branched-chain FAs (BCFA), anteiso-BCFAs, saturated FAs (SFA), and unsaturated FAs (UFA), were identified by a gas-liquid chromatography-based microbial identification system (Sherlock 4.5; courtesy of Microbial ID Inc., Newark, DE). FA data represent the means (±SD) from a minimum of two independent determinations from different FA extracts on different days. Data were expressed as the percentage of the major FAs (BCFA + SCFA + UFA = 100%). FAs present in less than 1% of the total were not included in the data analysis. For carotenoid assays, stationary-phase cultures (overnight) of *S. aureus* cells were subjected to methanol extraction. The absorbance profile of

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TABLE 1 Description of study strains[a]

Strain	MIC		USA group	*mprF* SNP	*yycG* SNP
	Daptomycin (μg/ml)	Vancomycin (μg/ml)			
CB1483	0.25	1	USA100		
CB185	4	2		L826F[b]	None
CB5079	0.5	1	USA300		
CB5080	2	2		L826F[b]	None
CB5083	0.25	1	USA100		
CB5082	4	2		L341S[c]	None
CB5088	0.5	1	USA300		
CB5089	2–4	2		S295L[c]	None
CB1631	0.5	2	USA100		
CB1634	4	4		L826F[b]	Frameshift
CB1663	0.5	1	ND		
CB1664	4	4		L826F[b]	R86H
CB5057	0.5	1	USA300		
CB5059	4	4		I420N[b]	T474I
CB5062	0.5	1	ND		
CB5063	8	2		None	None
CB5015	1	4	ND		
CB5016	4	4		None	None

[a] Data in this table have been previously published (21). ND, not determined; none, no mutation detected.
[b] Mutation in putative *mprF* C-terminal synthase domain.
[c] Mutation in putative *mprF* central bifunctional domain.

the extracts was measured at an optical density of 450 nm (OD_{450}) (15). Carotenoid analyses are reported as the means (±SD) from a minimum of three independent experiments for all strains on different days.

The two-tailed Student *t* test was used for statistical analyses of quantitative data. *P* values of ≤0.05 were considered significant.

Several interesting findings were noted in this study. The Dap^r MRSA strains demonstrated a significant enhancement in overall synthesis of LPG ($P < 0.01$), with a concomitantly reduced production of PG ($P < 0.01$) (Table 2). There were no statistically significant differences in CL production, and importantly, the amount of LPG which was translocated to the outer CM did not differ among strain pairs (Table 2). Of note, this same PL phenotype occurred in the presence or absence of mutations in *mprF*, suggesting that genetic networks outside *mprF* in Dap^r strains may well impact the expression and/or functionality of the latter locus. In previous studies, *mprF* SNPs were associated either with excess production or increased flipping of LPG to the outer layer of CM, depending on their location within either the synthase or translocase domains of this locus, respectively (10, 25, 30). In the current study, the major "gain-in-function" phenotype observed was in overall LPG synthesis but not in translocation function. Thus, it would be predicted that there would be no major differences in net surface positive charge in comparing the Dap^r strains with their respective Dap^s parental isolate. In this regard, in a recent publication using these same 9 strain pairs (21), there was no consistent pattern of surface charge differences in comparing the respective paired Dap^s and Dap^r isolates.

Next, liposome-based data from our laboratories have also

TABLE 2 PL content and asymmetry of LPG of 9 study strain pairs

Strain	Cell membrane PL composition (% of total PL [mean ± SD])				
	I-LPG	O-LPG	Total LPG	PG	CL
CB1483	13.39 ± 3.5	1.91 ± 1.6	15.30 ± 3.7	77.21 ± 4.2	7.49 ± 1.8
CB185	32.10 ± 6.6[a]	3.85 ± 2.3	35.96 ± 6.5[b]	52.31 ± 4.9[b]	11.73 ± 7.9
CB5079	13.63 ± 3.9	1.80 ± 0.4	15.43 ± 3.9	72.12 ± 8.0	12.44 ± 6.5
CB5080	24.78 ± 4.4[a]	1.39 ± 0.4	26.18 ± 4.1[b]	64.08 ± 7.3[b]	9.75 ± 4.2
CB5083	10.15 ± 4.8	1.92 ± 0.7	12.07 ± 5.0	83.3 ± 6.1	4.63 ± 2.4
CB5082	19.24 ± 5.1[a]	2.37 ± 0.9	21.61 ± 5.9[b]	73.58 ± 7.2[b]	4.82 ± 2.6
CB5088	13.61 ± 1.6	1.75 ± 1.3	15.36 ± 2.5	77.66 ± 4.1	6.97 ± 3.7
CB5089	22.62 ± 6.0[a]	2.29 ± 1.4	24.91 ± 7.2[b]	65.93 ± 4.8[b]	9.16 ± 5.4
CB1631	10.25 ± 3.2	1.83 ± 0.6	12.08 ± 3.2	80.41 ± 4.3	7.51 ± 2.3
CB1634	18.68 ± 3.1[a]	1.93 ± 1.0	20.61 ± 3.6[b]	71.75 ± 4.8[b]	7.63 ± 2.4
CB1663	10.24 ± 2.7	1.72 ± 0.4	11.96 ± 3.0	83.20 ± 4.6	4.84 ± 2.5
CB1664	14.69 ± 1.2[a]	1.36 ± 0.5	16.05 ± 1.0[b]	81.33 ± 1.7	2.62 ± 1.8
CB5057	14.32 ± 1.6	1.59 ± 0.8	15.91 ± 1.9	79.25 ± 2.9	4.85 ± 2.0
CB5059	24.77 ± 3.9[a]	2.44 ± 1.5	27.22 ± 4.9[b]	69.92 ± 4.5[b]	2.87 ± 1.8
CB5062	11.49 ± 2.0	1.21 ± 0.70	12.71 ± 2.1	79.90 ± 1.6	7.40 ± 2.6
CB5063	29.23 ± 6.5[a]	2.33 ± 1.43	31.55 ± 7.8[b]	59.01 ± 6.3[b]	9.44 ± 2.4
CB5015	12.73 ± 1.15	1.32 ± 0.7	14.06 ± 1.0	83.31 ± 1.4	2.63 ± 1.0
CB5016	17.61 ± 3.12[a]	1.66 ± 0.38	19.27 ± 3.2[b]	77.20 ± 3.1[b]	3.53 ± 1.7

[a] *P* value <0.005 versus parent strain.
[b] *P* value <0.01 versus parent strain.

TABLE 3 Carotenoid profiles of study strain pairs

Strain	OD_{450} of carotenoids	*P* value
CB1483	0.616 ± 0.1	
CB185	0.705 ± 0.07	0.19
CB5079	1.102 ± 0.08	
CB5080	0.616 ± 0.10	0.003
CB5083	0.994 ± 0.10	
CB5082	0.531 ± 0.11	0.006
CB5088	0.922 ± 0.15	
CB5089	0.604 ± 0.01	0.06
CB1631	0.638 ± 0.03	
CB1634	0.405 ± 0.06	0.02
CB1663	1.12 ± 0.06	
CB1664	0.798 ± 0.10	0.01
CB5057	0.564 ± 0.01	
CB5059	0.336 ± 0.05	0.01
CB5062	0.143 ± 0.04	
CB5063	0.122 ± 0.02	0.5
CB5015	0.606 ± 0.07	
CB5016	0.489 ± 0.04	0.07

suggested that LPG plays an additional key role beyond surface charge regulation in Dap-CM interactions (31). Thus, increases in LPG CM content (as in the current study) concomitantly reduce the proportionality of CM PG (31). It appears that the latter negatively charged PG (as well as negatively charged CL) are important participants in the initial "docking" of CAPs within target CMs. In support of this notion, Dap^r strains of enterococci and *Bacillus subtilis* also exhibit reductions in CM PG (27, 32); in *B. subtilis*, derived as Dap^r by serial *in vitro* passage in Dap, such PG content reductions are associated with an acquired mutation in *pgs* (the PG synthase gene locus) (32). Further, PG appears to have an independent and pivotal function in the capacity of Dap to oligomerize within target CMs (32). Thus, there are at least two mechanisms by which increases in LPG synthesis, with reciprocal decreases in PG production, may impact Dap^r in a "noncharge"-based manner.

Further, our prior investigations with the same strain pairs confirmed that the Dap^r isolates had more fluid CMs than their respective Dap^s parental strains (21). It is known that extremes of CM order (highly fluid or highly rigid CMs) can alter susceptibility to a variety of CAPs, presumably by modifying the capacity of such molecules to bind to and/or oligomerize within target CMs (33). We therefore performed a detailed FA compositional analysis (a major contributor to CM order) (8, 12, 15, 34), especially the proportionality of total iso-BCFAs, anteiso-BCFAs, SFAs, and UFAs. The Dap^r strains did not exhibit any consistently or significantly altered FA content pattern compared to that of their respective Dap^s parental strains (data not shown).

In addition, since we have noted before that carotenoid content of the *S. aureus* CM affects not only its fluidity properties but also susceptibility profiles to CAPs (15), the comparative carotenoid content among strain pairs was determined. Most of the Dap^r isolates (excluding CB185) exhibited less CM carotenoid content than their respective Dap^s parental strains (Table 3). In 5/9 strain pairs, this difference reached statistical significance (Table 3). As carotenoids can influence CM order by rigidifying their architecture, our observation of lowered carotenoid content among Dap^r strains fits with their previously observed increases in CM fluidity profiles (27). A recent investigation from our laboratory involving the evolution of Dap^r during *in vitro* passage (12) also confirmed a parallelism between CM order and carotenoid content. Thus, in the latter study, progressive evolution of Dap^r during such serial *in vitro* passages correlated with both enhanced CM rigidity and increased carotenoid content.

In summary, the major CM lipid perturbation demonstrated in the current study among Dap^r isolates was a hyperproduction of the positively charged PL species, LPG. This was accompanied by a concomitant reduction in CM PG content. Of interest, this unique phenotype occurred in Dap^r strains both with and without *mprF* mutations, suggesting that gene loci and/or networks outside *mprF* can have a major influence on ultimate LPG biosynthesis.

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REFERENCES

1. **Fuchs PC, Barry AL, Brown SD.** 2002. *In vitro* bactericidal activity of daptomycin against staphylococci. J. Antimicrob. Chemother. **49**:467–470.
2. **Kirkpatrick P, Raja A, LaBonte J, Lebbos J.** 2003. Daptomycin. Nat. Rev. Drug Discov. **2**:943–944.
3. **Safdar N, Andes D, Craig WA.** 2004. *In vivo* pharmacodynamic activity of daptomycin. Antimicrob. Agents Chemother. **48**:63–68.
4. **Petersen PJ, Bradford PA, Weiss WJ, Murphy TM, Sum PE, Projan SJ.** 2002. *In vitro* and *in vivo* activities of tigecycline (GAR-936), daptomycin, and comparative antimicrobial agents against glycopeptide-intermediate *Staphylococcus aureus* and other resistant Gram-positive pathogens. Antimicrob. Agents Chemother. **46**:2595–2601.
5. **Sakoulas G, Eliopoulos GM, Alder J, Eliopoulos CT.** 2003. Efficacy of daptomycin in experimental endocarditis due to methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47**:1714–1718.
6. **Schriever CA, Fernandez C, Rodvold KA, Danziger LH.** 2005. Daptomycin: a novel cyclic lipopeptide antimicrobial. Am. J. Health Syst. Pharm. **62**:1145–1158.
7. **Steenbergen JN, Alder J, Thorne GM, Tally FP.** 2005. Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. J. Antimicrob. Chemother. **55**:283–288.
8. **Mishra NN, Yang SJ, Sawa A, Rubio A, Nast CC, Yeaman MR, Bayer AS.** 2009. Analysis of cell membrane characteristics of *in vitro*-selected daptomycin-resistant strains of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **53**:2312–2318.
9. **Bertsche U, Weidenmaier C, Kuehner D, Yang SJ, Baur S, Francois P, Schrenzel J, Yeaman MR, Bayer AS.** 2011. Correlation of daptomycin-resistance in a clinical *Staphylococcus aureus* strain with increased cell wall teichoic acid production and D-alanylation. Antimicrob. Agents Chemother. **55**:3922–3928.
10. **Jones T, Yeaman MR, Sakoulas G, Yang SJ, Proctor RA, Sahl HG, Schrenzel J, Xiong YQ, Bayer AS.** 2008. Failures in clinical treatment of *Staphylococcus aureus* infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding. Antimicrob. Agents Chemother. **52**:269–278.
11. **Koprivnjak T, Peschel A.** 2011. Bacterial resistance mechanisms against host defense peptides. Cell. Mol. Life Sci. **68**:2243–2254.

12. **Mishra NN, Rubio A, Nast CC, Bayer AS.** 2012. Differential adaptations of methicillin-resistant *S. aureus* to serial *in vitro* passage in daptomycin resulting in evolution of daptomycin resistance: role of cell membrane carotenoid content and fluidity. Int. J. Microbiol. **2012**:683450.
13. **Rubio A, Moore J, Varoglu M, Conrad M, Chu M, Shaw W, Silverman JA.** 2012. LC-MS/MS characterization of phospholipid content in daptomycin-susceptible and -resistant isolates of *Staphylococcus aureus* with mutations in *mprF*. Mol. Mem. Biol. **29**:1–8.
14. **Xiong YQ, Mukhopadhyay K, Yeaman MR, Adler-Moore J, Bayer AS.** 2005. Functional interrelationships between cell membrane and cell wall in antimicrobial peptide-mediated killing of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **49**:3114–3121.
15. **Mishra NN, Liu G, Yeaman MR, Nast CC, Proctor RA, McKinnell J, Bayer AS.** 2011. Carotenoid related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. Antimicrob. Agents Chemother. **55**:526–531.
16. **Friedman L, Adler JD, Silverman JA.** 2006. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **50**:2137–2145.
17. **Ernst CM, Peschel A.** 2011. Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. Mol. Microbiol. **80**:290–299.
18. **Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A, van Kessel KP, van Strijp JA.** 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. J. Exp. Med. **193**:1067–1076.
19. **Yang SJ, Xiong YQ, Dunman PM, Schrenzel J, François P, Peschel A, Bayer AS.** 2009. Regulation of *mprF* in daptomycin-nonsusceptible *Staphylococcus aureus* strains. Antimicrob. Agents Chemother. **53**:2636–2637.
20. **Mishra NN, Rubio A, Bayer AS.** 2012. Cell membrane lipid profiles correlate with daptomycin resistance in methicilin-resistant *Staphylococcus aureus*, abstr C1-1744. Abstr. 52nd Annu. Intersci. Conf. Antimicrob. Agents Chemother. (ICAAC). American Society for Microbiology, Washington, DC.
21. **Mishra NN, McKinnell J, Yeaman MR, Rubio A, Nast CC, Chen L, Kreiswirth BN, Bayer AS.** 2011. *In vitro* cross-resistance to daptomycin and host defense cationic antimicrobial peptides in clinical methicillin-resistant *Staphylococcus aureus* isolates. Antimicrob. Agents Chemother. **55**:4012–4018.
22. **Lina G, Boutite F, Tristan A, Bes M, Etienne J, Vandenesch F.** 2003. Bacterial competition for human nasal cavity colonization: role of staphylococcal *agr* alleles. Appl. Environ. Microbiol. **69**:18–23.
23. **Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN.** 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. **37**:3556–3563.
24. **Chen L, Mediavilla JR, Oliveira DC, Willey BM, de Lencastre H, Kreiswirth BN.** 2009. Multiplex real-time PCR for rapid Staphylococcal cassette chromosome *mec* typing. J. Clin. Microbiol. **47**:3692–3706.
25. **Yang SJ, Nast CC, Mishra NN, Yeaman MR, Fey PD, Bayer AS.** 2010. Cell wall thickening is not a universal accompaniment of the daptomycin nonsusceptibility phenotype in *Staphylococcus aureus*: evidence for multiple resistance mechanisms. Antimicrob. Agents Chemother. **54**:3079–3085.
26. **Li M, Rigby K, Lai Y, Nair V, Peschel A, Schittek B, Otto M.** 2009. *Staphylococcus aureus* mutant screen reveals interaction of the human antimicrobial peptide dermcidin with membrane phospholipids. Antimicrob. Agents Chemother. **53**:4200–4210.
27. **Mishra NN, Bayer AS, Tran TT, Shamoo Y, Mileykovskaya E, Dowhan W, Guan Z, Arias CA.** 2012. Daptomycin resistance in enterococci is associated with distinct alteration of cell membrane phospholipid content. PLoS One **7**(8):e43958. doi:10.1371/journal.pone.0043958.
28. **Mukhopadhyay K, Whitmire W, Xiong YQ, Molden J, Peschel A, McNamara PJ, Proctor RA, Yeaman MR, Bayer AS.** 2007. Reduced *in vitro* susceptibility of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein-1 is associated with alterations in cell membrane phospholipid composition and asymmetry. Microbiology **153**:1187–1197.
29. **Yang SJ, Bayer AS, Mishra NN, Meehl M, Ledala N, Yeaman MR, Xiong YQ, Cheung AL.** 2012. The role of the *Staphylococcus aureus* GraRS two-component regulatory system in sensing of and resistance to cationic antimicrobial peptides. Infect. Immun. **80**:74–81.
30. **Ernst CM, Staubitz P, Mishra NN, Yang SJ, Hornig G, Kalbacher H, Bayer AS, Kraus D, Peschel A.** 2009. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. PLoS Pathog. **5**(11):e1000660. doi:10.1371/journal.ppat.1000660.
31. **Kilelee E, Pokorny A, Yeaman MR, Bayer AS.** 2010. Lysyl- phosphatidylglycerol attenuates membrane perturbation rather than surface association of the cationic antimicrobial peptide 6W-RP-1 in a model membrane system: implications for daptomycin resistance. Antimicrob. Agents Chemother. **54**:4476–4479.
32. **Hachmann AB, Sevim E, Gaballa A, Popham DL, Antelmann H, Helmann JD.** 2011. Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*. Antimicrob. Agents Chemother. **55**:4326–4337.
33. **Muraih JK, Harris J, Taylor SD, Palmer M.** 2012. Characterization of daptomycin oligomerization with perylene excimer fluorescence: stoichiometric binding of phosphatidylglycerol triggers oligomer formation. Biochim. Biophys. Acta **1818**:673–678.
34. **Bayer AS, Kupferwasser LI, Brown MH, Skurray RA, Grkovic S, Jones T, Mukhopadhay K, Yeaman MR.** 2000. *In vitro* resistance of *Staphylococcus aureus* to thrombin-induced microbicidal protein is associated with alterations in membrane fluidity. Infect. Immun. **68**:3548–3553.