Differential adaptive responses of *Staphylococcus aureus* to *in vitro* selection with different antimicrobial peptides.

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**Running title:** antimicrobial peptides and resistance

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Abstract

We subjected Staphylococcus aureus ATCC 29213 to serial passage in the presence of sub inhibitory concentrations of magainin 2 and gramicidin D for several hundred generations. We obtained S. aureus strains with induced resistance to magainin 2 (55MG) and gramicidin D (55GR) that showed different phenotypic changes in membrane properties. Both exhibited change in membrane phospholipid content and increase in membrane rigidity, while net charge alteration occurred only in case of 55MG as compared to control.
The growing problem of resistance in pathogens like *Staphylococcus aureus* to conventional antibiotics and the need for new alternatives has stimulated interest in the development of antimicrobial peptides (AMPs) as human therapeutics (1). This has led many AMPs like pexiganan (analogue of magainin), gramicidins, polymyxin, nisin, daptomycin, and defensin-mimetic molecule to enter the clinical trials (2-6). However, it has been argued in several *in vitro* studies and recent reports that usage of AMPs in therapeutic amounts over an extended period of time might lead to reduced susceptibility along with adaptive changes in phenotypic and genotypic characteristics of the organism (7-13). Therefore, the aim of our study was to examine whether *S. aureus* on continuous exposure to sub-lethal concentrations of certain well studied AMPs (magainin 2 and gramicidin D) alter their susceptibility and whether resistant strains are selected. Since the putative mechanism of action of the studied AMPs involve targeting bacterial membrane, analyses of various cell membrane (CM) parameters were performed to understand their role in the *in vitro* selected strains.

To generate AMP-resistant strains, *S. aureus* ATCC 29213 was chosen and two cell lines were maintained for each peptide in Mueller-Hinton broth (MHB). Serial passage was done in two cell lines for each AMP; one in the presence of non-inhibitory and increasing AMP concentrations (positive selection line) and the other grown in the absence of peptide (control selection line) as described elsewhere (10). The experiment was conducted for 55 serial passages, constituting 600-700 bacterial generations. Each transfer was given strain designation to indicate serial passage number. After every 5 transfers, evolution of resistance in positive selection line against magainin 2 and gramicidin D was identified by determining the minimum inhibitory concentration (MIC)
in MHB media following CLSI guidelines (14). Further confirmation of resistance was done by performing bactericidal assay as described before (15-16).

Development of resistance occurred for both the studied AMPs, as indicated by a gradual rise in MIC value with increasing passage number in the positive selection lines from respective control selection lines. At 55th passage, both the strains (55MG and 55GR) showed ≥ 8- and 128- fold increase in MIC value from the control for magainin 2 and gramicidin D respectively (Fig. 1A and B). The MICs of the selected strains were stable after daily passaging on peptide-free media for 10 consecutive transfers (data not shown). Further, in vitro bactericidal assay (Fig. 1C and D) showed a substantial increase in cfu/ml value in both selected strains (55MG and 55GR) as compared to control (55C) after treatment with different concentrations of the respective AMPs for 1 h.

In order to understand the phenotypic modifications that the in vitro selected strains had undergone, analyses of various membrane parameters were done. This included membrane order, total phospholipid content, flipping of the cationic phospholipid, lysyl phosphatidylglycerol (LPG) to outer membrane leaflet and net cell charge. Membrane order of S. aureus cells were determined by measuring fluorescence polarization (P value) (Shimadzu RF-5301 PC spectrofluorimeter) using the fluorescent probe DPH (1, 6-diphenyl-1, 3, 5-hexatriene) as described earlier (17-18). The net charge of the S. aureus strains were determined by measuring zeta potential on an electrophoresis instrument (ZC-2000, Microtec, Japan) as described elsewhere (19-20). For membrane phospholipid (PL) compositional analysis, major CM PLs of S. aureus - phosphatidylglycerol (PG), cardiolipin (CL) and LPG, were separated by two
dimensional (2D) thin-layer chromatography (TLC) using Silica 60 F254 HPTLC plates (Merck) and quantified as described before (21-22). Outer leaflet LPG was detected and measured using fluorescamine, a fluorescent probe and quantified spectrophotometrically as detailed before (21-22). All assays were done in triplicate and repeated in three independent experiments on different days and the results were plotted as mean ± standard deviation. Statistical analysis (multiple comparisons among data sets) was performed using one-way analysis of variance (ANOVA) with Minitab™ (15). A p-value of ≤ 0.01 was considered significant.

Our study showed interesting but different phenotypic changes in the membrane parameters of magainin 2 and gramicidin D resistant strains (55MG and 55GR). Although their membranes were found to be significantly more rigid than control (p ≤ 0.01), both the strains behaved differently. For example, 55MG showed a modest increase in rigidity (P = 0.28 ± 0.02) whereas 55GR showed a substantial increase (P = 0.32 ± 0.03) from the control, 55C (P = 0.23 ± 0.02) as shown in Table 1. This characteristic of altered membrane order was also observed in various *S. aureus* strains non-susceptible to other cationic AMPs like, daptomycin, tPMP-1 (23-25). It has been postulated that for each specific AMP and bacterial membrane interaction, there is an optimum relative membrane order at which AMPs exert maximum activity. Altered membrane order is an adaptation under the continuous selective pressure of the peptide and this could be due to shifts in fatty acid unsaturation indices or branched chain species (26).

Another notable disparity observed was the relative net charge of the *in vitro* selected strains measured by zeta potential. As seen from Fig. 2, 55MG exhibited a remarkable
increase in its cationic charge (3mV), while in 55GR, zeta potential was almost same (-14mV) as compared to control (-18mV). The difference in zeta potential among the S. aureus strains reached statistical significance ($p \leq 0.001$). The increase in net charge in 55MG was manifested by a substantial increase in the synthesis of the cationic phospholipid LPG in its membrane as compared to control, 55C (Fig. 3A). In addition, ≥3 fold higher ($p \leq 0.01$) translocation of LPG to the outer membrane leaflet was observed in 55MG than the control (Fig. 3B). These findings are corroborated by previous reports that increased LPG content and its flipping to outer membrane leaflet contribute to increased positive charge in the staphylococcal cell surface (27). This underscored the potential for a charge-mediated repulsion of the peptide as one possibility for magainin 2 resistance phenotype. On the contrary, translocation of the cationic phospholipid to outer membrane leaflet did not differ in 55GR from control (Fig. 3D), in spite of increased synthesis of total LPG (Fig. 3C). This could be one of the reasons why the net charge remained almost same as control in 55GR. This phenomenon of increased LPG content without affecting bacterial net charge was also reported by us and other scientists (17,28). A liposome-based data suggested that apart from surface charge regulation in AMP-bacterial CM interaction, LPG plays an additional role of stabilizing membrane integrity (29). It is also to be noted that, since gramicidin D unlike magainin 2 (having net charge +4) is a neutral AMP, its mechanism of bactericidal action is not driven by electrostatic interaction. Rather, other factors like membrane rigidity, cell wall alteration or hydrophobic interactions might contribute to resistance to gramicidin D in S. aureus. It is postulated that the negatively charged phospholipids are required for the initial “docking” of AMPs within target CMs (30).
Therefore it was not surprising that the increase in LPG content in both 55MG and 55GR concomitantly reduced the proportion of negatively charged PG in our study.

In summary, the current study demonstrated the emergence of resistance in *S. aureus* under consistent *in vitro* exposure of magainin 2 and gramicidin D. *S. aureus* cells adapted differently to defend themselves from the lethal action of the test AMPs. Increase in net charge along with a rigid membrane may account for resistance towards the cationic AMP, magainin 2; while an increase in membrane rigidity combined with an alteration of membrane composition may contribute to adaptive response to the neutral gramicidin D in *S. aureus*. Considering the urgent need of introducing AMP as an alternative therapy to combat bacterial infection, a study of this kind is of utmost importance to minimize the emergence of resistant organisms against AMPs and to develop AMPs as potentially useful antimicrobial agents.

**ACKNOWLEDGEMENTS**

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FIGURE LEGENDS

FIG 1. Upper panel shows MIC (mg/liter) values for *S. aureus* ATCC 29213 during serial passage in absence of AMP (control selection line) and with increasing concentration of AMP (positive selection line): (A) magainin 2 and (B) gramicidin D. Lower panel shows cfu/ml in control and selected *S. aureus* strains exposed to different concentrations of (C) magainin 2 and (D) gramicidin D. **indicates $p \leq 0.01$ (one-way ANOVA, Minitab™).

FIG 2. Zeta potential of control and selected *S. aureus* strains. * indicates $p \leq 0.001$ (one-way ANOVA, Minitab™).

FIG 3. Phospholipid composition of 55MG (A) and 55GR (C) with respect to control (55C). Asymmetry of LPG in 55MG (B) and 55GR (D) with respect to control (55C). * and ** indicates $p \leq 0.001$ and 0.01 respectively (one-way ANOVA, Minitab™).
TABLE 1. Polarization value (P) of control and selected *S. aureus* strains.

<table>
<thead>
<tr>
<th><em>S. aureus</em> strains</th>
<th>Polarization value (P)</th>
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<tbody>
<tr>
<td>55C</td>
<td>0.23 ± 0.02 **</td>
</tr>
<tr>
<td>55MG</td>
<td>0.28 ± 0.02 **</td>
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<tr>
<td>55GR</td>
<td>0.32 ± 0.03 **</td>
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