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Increased *In Vitro* Phenol-Soluble Modulin Production is Associated with Soft Tissue Infection Source in Clinical Isolates of Methicillin-Susceptible *Staphylococcus aureus*

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Abstract

Background—Phenol-soluble modulins (PSM) are amphipathic proteins produced by Staphylococcus aureus that promote virulence, inflammatory response, and biofilm formation. We previously showed that MRSA isolates from soft tissue infection (SSTI) produced significantly

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CONFLICTS OF INTEREST

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higher levels of PSM than MRSA isolates from hospital-acquired pneumonia (HAP) or infective endocarditis (IE). In this investigation, we sought to validate this finding in methicillin-susceptible *S. aureus* (MSSA) isolates.

Methods—MSSA isolates (n=162) from patients with SSTI, HAP, and IE were matched 1:1:1 based on geographic origin of the infection to form 54 triplets (North America n=27, Europe n=25, Australia n=2). All isolates underwent spa-typing and were classified using eGenomics. In vitro PSM production was quantified by high performance liquid chromatography/mass spectrometry. Fischer's Exact Test and the Kruskal-Wallis test were used for statistical analysis.

Results—Spa1 was more common in SSTI (14.81% SSTI, 3.70% HAP, 1.85% IE) ($p<0.03$). Spa2 was more common in HAP (0% SSTI, 12.96% HAP, 3.70% IE) ($p<0.01$). Levels of PSMα1-4 all differed significantly among the three clinical groups, with SSTI isolates producing the highest levels and IE producing the lowest levels of PSMα1-4. Spa1 isolates produced significantly more delta-toxin ($p<0.03$) than non-Spa1 isolates. No associations between PSM levels and clinical outcome of SSTI, HAP, or IE were identified.

Conclusion—Production of PSMα1-4 is highest in SSTI MSSA isolates, supporting the hypothesis that these peptides are important for SSTI pathogenesis. These findings are similar to those described in MRSA, and demonstrate that associations between PSM levels and type of infection are independent of the methicillin resistance status of the isolate.

Keywords

phenol-soluble modulin; Methicillin-susceptible *Staphylococcus aureus*; skin and soft tissue infection; pneumonia; endocarditis

INTRODUCTION

Staphylococcus aureus produces many virulence factors that affect its clinical manifestations (1, 2). One recently discovered family of virulence factors are phenol-soluble modulins (PSM). Members of this group of amphipathic, alpha-helical peptides may influence inflammation induction, biofilm production, erythrocyte lysis, neutrophil lysis after *S. aureus* has been phagocytosed, and even antimicrobial activity (3). *S. aureus* is currently known to produce eight PSMs. α-type PSMs are 20–25 amino-acids long and are subdivided into PSMα1-4, delta-toxin, and PSM-mec. β-type PSMs are 44 amino-acids long and include PSMβ1-2 (3). PSMα1-4, PSMβ1-2, and delta-toxin are encoded on the core genome while PSM-mec is encoded on the mobile genetic element that confers methicillin resistance to *S. aureus* (4). All PSMα family proteins are encoded on the *psmα* operon, while PSMβ1-2 are both encoded on the *psmβ* operons, facilitating co-transcription of PSMα family proteins and of PSMβ1-2 (5).

We previously showed that MRSA isolates from patients with SSTI produced significantly more PSMs *in vitro* than geographically-matched MRSA isolates from patients with either HAP or IE (6). However, our results could have been confounded by the presence of traits that were associated with the methicillin-resistance phenotype. For example, PSM-mec is present on the SCCmec element of MRSA isolates and decreases the production of PSMα peptides by inhibiting the positive regulator of PSMα-type production (7). MRSA and

MSSA isolates have also been shown to produce different amounts of enterotoxin A, alpha toxin, and Toxic Shock Syndrome Toxin (8, 9). Thus, potential associations between increased PSM production and specific infection types needs to be confirmed in MSSA isolates.

In the present investigation, we sought to confirm any such association. Our *a priori* hypothesis was that levels of *in vitro* PSM are higher in MSSA isolates from SSTI. To test this hypothesis, we utilized a unique, globally representative collection of *S. aureus* from a variety of clinical syndromes.

MATERIALS AND METHODS

Definitions

SSTI was defined as the presence of at least 1 of the following conditions: major abscess requiring surgical drainage, cellulitis, infected wound or ulcer, or infected burn plus purulent drainage/collection or ≥ 3 of the following conditions: heat, localized warmth, fluctuance, erythema, swelling, induration, pain/tenderness to palpation, temperature ≥ 38°C, WBC ≥ 10,000 cells/mm^3, or ≥15% bands (10). HAP was defined as: 1) presence of new or progressive infiltrates or consolidation, with or without pleural effusion on chest radiograph or computed tomography; 2) adequate respiratory specimen for culture and Gram stain and identification of an organism consistent with a respiratory pathogen isolated from respiratory tract or blood, or >2 of the following: cough, purulent sputum, auscultation findings, dyspnea, tachypnea, or hypoxemia acquired after 48 h in an inpatient acute or chronic care facility or that developed within 7 days after being discharged. In addition, patients had >2 of the following: temperature ≥38°C or rectal/core temperature ≤35°C, respiratory rate ≥30 breathes per minute, pulse ≥120 beats per minute, altered mental status, mechanical ventilation, WBC >10,000 cells/mm^3, <4500 cells/mm^3, or >15% band neutrophils (11). IE was defined using the modified Duke criteria (12).

Isolates

SSTI isolates were acquired from two double-blinded, randomized, parallel group, identical method Phase-III trials (10, 13). HAP isolates were acquired from two double-blinded, randomized, parallel group, identical method Phase-III trials (11). IE isolates were acquired from the Microbiologic Repository of the International Collaboration on Endocarditis – Prospective Cohort Study (ICE-PCS). A total of 58 sites in 25 countries enrolled 2781 patients ≥18 years old with definite IE (14, 15). All clinical studies were approved by their respective Institutional Review Boards.

Matching of Bacterial Isolates According to Region of Origin

Isolates from SSTI, HAP, and IE were matched on a 1:1:1 basis to form a “triplet” of three MSSA isolates (one each from SSTI, HAP, and IE) that originated in the same geographic region. The total number of triplets per continent was determined by the clinical category with the fewest isolates. For studies with more isolates per continent than the number of isolates present in the numerically smallest category, we used every X’th isolate so that all

total number of isolates selected were equivalent, with X = (number of isolates in larger category) / (number of isolates in smallest category).

Growth and Supernatant Collection

Bacterial isolates originally frozen in tryptic soy broth (TSB)/glycerol stock were grown overnight at 37°C on trypticase soy agar (TSA) petri dishes. Approximately 1μL of bacterial growth was inoculated into 10ml liquid TSB stock in 50mL conical tubes, then incubated overnight at 37°C at 200 RPM. The OD_{600} of each sample was measured and the amount of TSB-bacteria needed to reach start OD_{600} equal to 0.1 was added to 10mL liquid TSB in 50mL conical tubes. The new stocks were grown for 8 hours at 37°C and 200 RPM to reach early stationary phase with limited growth variation among different isolates as previously reported (26), and then placed on ice to arrest growth. Samples were centrifuged at 4°C and 4000 RPM for 10 minutes. 4 × 2mL supernatant (8mL total) were collected from each sample and stored at −80°C.

High-Performance Liquid Chromatography (HPLC) / Mass Spectrometry

Reversed phase HPLC / electrospray ionization mass spectrometry (RP-HPLC/ESI-MS) was used to measure PSM production in supernatant as previously described (5). Briefly, 50μL of culture supernatant was injected into LC/MS (Agilent Technologies, Santa Clara, CA) equipped with C8 column (2.1 × 5 mm, 2.7μm; PerkinElmer, Waltham, MA). PSMs were separated in flow rate of 0.5mL/min with the following gradient program: eluent A, 0.1% trifluoroacetic acid (TFA) in 100% water; eluent B, 0.1% TFA in 100% acetonitrile; 10% eluent B for 0.5 minutes; a linear gradient from 25% to 100% for 3 minutes; 100% for 2.5 minutes; 0% for 2.5 minutes. Parameters for operating mass spectrometer were as recommended by the manufacturer. Two major ions of each PSM with different charge states were used to generate extracted ion chromatogram for quantification by integration. We did not test for PSM-mec production because it is genetically located on the methicillin-resistance cassette, which MSSA lacks. Each sample was assayed three times and the results were averaged for each isolate and PSM peptide.

Spa Genotyping

Spa genotyping was performed as previously described (6, 17). The eGenomics online platform (http://tools.egenomics.com) was used to define *spa* type.

Statistical Methods

Spa type distribution among SSTI, HAP, and IE was evaluated with contingency tables and Fisher's Exact Test. The level of *in vitro* production of each PSM type for a particular MSSA isolate was defined as the average of three separate assays of the same sample. Differences in production across the three clinical categories and by *spa* types were compared using means and the Kruskal-Wallis test. $P<0.05$ was threshold for statistical significance in all tests.

Institutional Review

This study was approved by the Duke University Medical Center Institutional Review Board.

RESULTS

A total of 162 MSSA isolates from patients with SSTI, HAP, and IE were divided 1:1:1 into 54 geographically-matched triplets. Of the 54 triplets, 27 (50%) came from North America, 25 (46.3%) came from Europe, and 2 (3.7%) came from Australia.

Spa Genotyping According to Infectious Source

Among the 162 isolates, 65 known *spa* types were identified. The most common *spa* types were *spa*1 (n=11, 6.8%), *spa*2 (n=9, 5.6%), *spa*33 (n=8, 4.9%), and *spa*73 (n=8, 4.9%). Collectively, these 4 *spa* types accounted for 22.2% (n=36) of all isolates. The 61 other known *spa* types each was found in 6 or fewer isolates, and constituted 60.5% of isolates. New *spa* types (n=28) comprised the remaining 17.3% of isolates. *Spa*1 was significantly more common in SSTI (SSTI 8/54 [14.81%], HAP 2/54 [3.70%], IE 1/54 [1.85%]) (p=0.0301), while *Spa*2 was significantly more common in HAP (SSTI 0/54 [0%], HAP 7/54 [12.96%], IE 2/54 [3.70%]) (p=0.0111) (Table 1).

Association between Infectious Source and *in vitro* PSM production

Among the SSTI, HAP, and IE isolates, significant differences existed in levels of *in vitro* production of PSMα1 (p=0.0262), PSMα2 (p=0.0164), PSMα3 (p=0.0021), and PSMα4 (p=0.0016). SSTI isolates produced the highest levels of all four types of PSMα while IE isolates produced the lowest. There were no significant differences in the production of PSMβ1, PSMβ2, or delta-toxin among the three clinical groups (Figure 1).

Because Spa1 was the most common single genotype in this study, we considered the possibility that its predominance in the SSTI cohort might confound results. PSM production patterns between *Spa*1 and non-*Spa*1 isolates were similar. *Spa*1 did not produce significantly different quantities of PSMα1-4 and PSMβ1-2 compared with non-*spa*1 isolates. After excluding *spa*1 isolates from analysis, statistically significant in-group differences remained in PSMα1 (p=0.0293), PSMα2 (p=0.0224), PSMα3 (p=0.0050), and PSMα4 (p=0.0042) (data not shown).

Association between clinical outcome and *in vitro* PSM production

Next, we explored the possible association between PSM level in MSSA isolates and the clinical outcome of the patients they infected. No statistically significant associations were identified between the clinical outcomes of patients with SSTI, HAP, or IE and the *in vitro* PSM level of the MSSA isolates causing the infection (Table 2).

DISCUSSION

The role of PSMs in the pathogenesis of *S. aureus* infections is increasingly recognized. Previously, we demonstrated an association between levels of *in vitro* PSM production of

MRSA isolates and the clinical source from those isolates were obtained. The current investigation confirmed this association, and showed that levels of *in vitro* PSMα1-4 production were higher in MSSA from patients with SSTI than similar isolates from geographically-matched patients with HAP or IE.

The results of this study add to a growing body of evidence linking PSM production with specific manifestations of staphylococcal disease. Experimentally, PSMα-deficient MRSA isolates form smaller soft tissue infections in rabbit and murine models of SSTI than MRSA with normal PSMα production (5, 18). PSMα peptides have also been shown to promote bacterial spread across surfaces (19), destroy neutrophils (5) and erythrocytes (20), and inhibit dendritic cell activity (21) that could potentiate detection by immune response on the skin. Clinically, MRSA isolates from patients with SSTI exhibited higher levels of PSM than MRSA from other distinct clinical syndromes (6). The current study extends this observation into MSSA isolates as well, and provides further evidence for a key role for PSM in the pathogenesis of SSTI.

MSSA IE isolates produced less PSMα1-4 than SSTI isolates did, while HAP isolates made intermediate amounts. These patterns are consistent with those in MRSA isolates from the same infection types (6). Nonetheless, the role of PSM in bloodstream infections remains unclear. Mice infected by PSMα-deletion or delta-toxin-deletion strain *S. aureus* showed decreased TNF-α inflammation and improved septicemia survival rates (5). PSM production is positively controlled by the *agr* regulatory system (22). *S. aureus agr* knockout mutants have diminished ability to induce experimental IE at low concentrations and adhere to valve vegetation (23). In addition, a unique PSMα3 variant that elicits an attenuated cytolytic and inflammatory host response is associated with increased hematogenous spreading among the Clonal Complex 30 strain of *S. aureus* (24), which is associated with IE (25). Finally, both the *agr* regulatory system and PSMs themselves are neutralized by LDL and VLDL lipoproteins at physiologic conditions (26, 27), potentially decreasing the impact of PSMs secreted by *S. aureus* as it enters the host bloodstream. Collectively, these data suggest that attenuated PSM production by specific strains of *S. aureus* could reduce host recognition and response to *S. aureus* bacteremia (28). The data also provides a rationale for the hypothesis that MSSA isolates from HAP and IE produce less PSMα1-4 to evade detection from and prevent destruction by the host immune system. Overall, more research is necessary to elucidate how PSMs affect pathogenicity of *S. aureus* within different host body sites.

The role of PSMβ1-2 in *S. aureus* infection remains unclear. PSMβ peptides are far less cytolytic than the PSMα family (5), do not affect *S. aureus* ability to escape lysosomes (29), and are not bactericidal against other pathogens (3). In the current study and in MRSA (6), levels of PSMβ1-2 were lower than those of either PSMα or delta-toxin, and were not significantly associated with clinical types of infection. Taken together, these results suggest that PSMβ production may be less important in the pathogenesis of SSTI than other forms of PSM peptides, such as PSMα.

The absence of PSM-mec in MSSA isolates may affect the total production levels of PSMs. PSM-mec is located on a regulatory RNA that inhibits *agrA* positive regulation of PSMα,

PSMβ, and delta-toxin (7). MRSA strains with their PSM-mec deleted produced more *agrA* and PSMα family peptides, and had increased virulence in mice models (7). Berlon *et al* showed that decreased PSM-mec production is associated with significantly increased PSMα, β, delta-toxin in MRSA SSTI isolates compared with HAP and IE (6). Moreover, PSM-mec vector-transformed *S. epidermidis* and *S. haemolyticus* had a 30% reduction in their combined PSMα and delta-toxin output compared with their null-vector transformed counterparts, suggesting that the PSM-mec downregulation of PSM production is conserved in the Staphylococcus family (30). Given that MSSA lacks the SCCmec cassette and by extension PSM-mec, total MSSA PSM production may be higher than total MRSA PSM production, even though the relative proportions of PSMs produced may have similar profiles. Thus, while the PSM production patterns appear independent of methicillin-resistance, the total amount of PSM produced may be influenced by methicillin-resistance acquisition.

Our study is limited by its *in vitro* nature, as *in vivo* factors, such as the presence of LDL and VLDL in blood, could alter PSM production. Although we geographically matched the SSTI, HAP, and IE isolates by continent of origin, this strategy may be insufficiently precise, as specific regions and even individual hospitals or hospital wards may often be affected by a handful of dominant bacterial clones. Furthermore, we were unable to investigate MSSA from regions other than North America, Europe, or Australia. Finally, our results confirm an association between *in vitro* levels of PSM and SSTI versus HAP or IE but do not establish the molecular basis for this association.

In conclusion, the results of this investigation confirm that *S. aureus* isolates from patients with SSTI exhibit significantly higher levels of *in vitro* PSM production than similar isolates from patients with either HAP or IE. This finding supports the hypothesis that PSMs play a significant role in SSTI pathogenesis. Future studies of this type are needed to begin to bridge the gap between the bench and the bedside by providing a better understanding of the determinants of virulence in *S. aureus*.

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Appendix

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Key Point

In vitro production of most phenol-soluble modulin proteins is significantly higher among methicillin-susceptible *S. aureus* isolates obtained from skin and soft tissue infection when compared to *S. aureus* isolates obtained from patients with hospital acquired pneumonia or infective endocarditis.

Highlights

- 162 MSSA isolates from SSTI, HAP, and IE were geographically matched and *spa*-typed
- PSM production was quantitated by liquid chromatography/mass spectrometry.
- SSTI isolates produced the most PSMα1-4, supporting a PSM role in SSTI pathogenesis
- PSM production patterns are similar in MRSA and MSSA

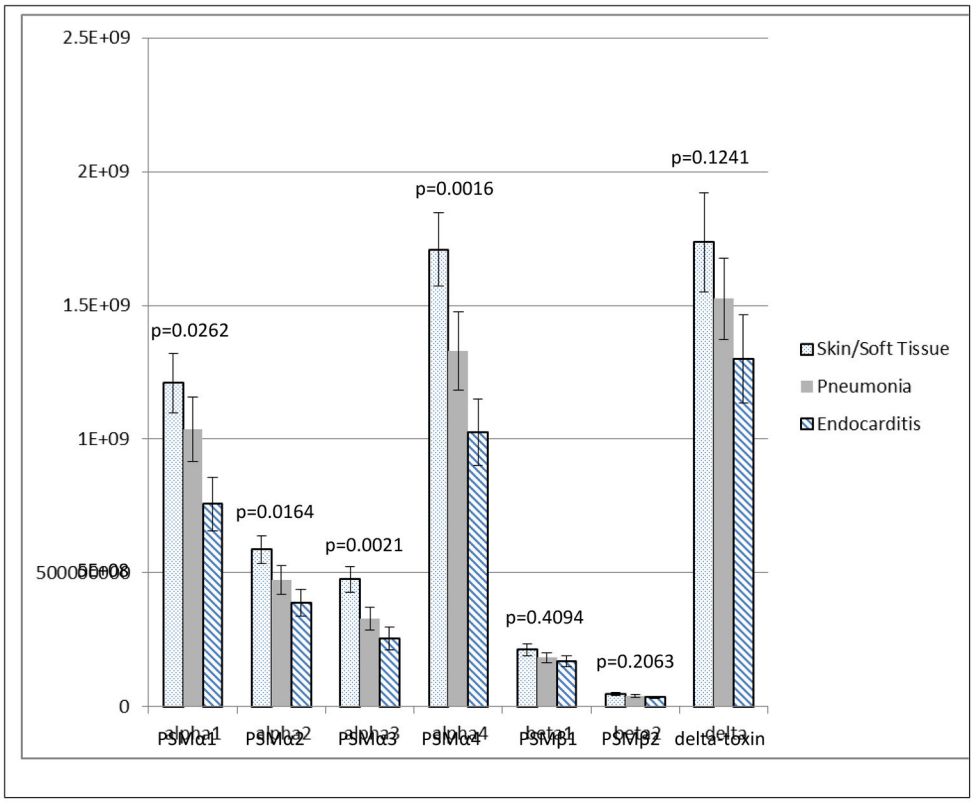

Figure 1. Mean Levels of *In Vitro* Phenol-Soluble Modulin Production by Clinical Source of Isolates of Methicillin-susceptible *S. aureus*

Mean Levels of *In Vitro* Phenol-Soluble Modulin Production by Clinical Source of MSSA Isolate. PSM production in culture supernatant from SSTI, HAP, and IE isolates were quantified and averaged in technical triplicate (n=54 per group). Error bars show SEM. Kruskal-Wallis analysis was used to calculate differences in production. There were significant in-group differences in the production of PSMα1-4, with SSTI isolates producing the highest levels in all four PSMα subtypes.

Table 1

Spa Type Distribution Among Methicillin Susceptible *S. aureus* Isolates from Skin/Soft Tissue Infection, Hospital Acquired Pneumonia, and Infective Endocarditis.

Spa Type	Skin and Soft Tissue% (n)	Pneumonia % (n)	Endocarditis % (n)	p-value
1	14.8% (8)	3.7% (2)	1.9% (1)	0.030
2	0.0% (0)	13.0% (7)	3.7% (2)	0.011
33	1.9% (1)	9.3% (5)	3.7% (2)	0.278
73	7.4% (4)	3.7% (2)	3.7% (2)	0.732
NEW	18.5% (10)	18.5% (10)	14.8% (8)	0.900
OTHER	57.4% (31)	51.9% (28)	72.2% (39)	0.089

Spa typing was performed on 162 MSSA isolates from SSTI, HAP, and IE. All *spa* types with n ≥ 8 are listed. *Spa*-type NEW are isolates with genomic *spa* sequences that did not match a known *spa* type on eGenomics. *Spa*-type OTHER consist of all isolates with known *spa* types but with total n < 8. Fischer's Exact Test showed that SSTI isolates are significantly more likely to be *spa* type 1 (p=0.030) and significantly less likely to be *spa* type 2 (p=0.011).

Table 2

Associations between Patient Outcome and Average Levels of *In Vitro* Phenol-Soluble Modulin Production from methicillin susceptible *S. aureus* isolates causing Skin and Soft Tissue Infection, Hospital-Acquired Pneumonia, and Infective Endocarditis.

Phenol Soluble Modulin Subtype	Average PSM production in Skin and Soft Tissue Infection				Average PSM production in Hospital-Acquired Pneumonia				Average PSM production in Infective Endocarditis*		
	Cure n=41	Failure n=9	Unknown n=4	p-value	Alive at 28 days n=34	Dead at 28 days n=7	Unknown n=13	p-value	Alive at Discharge n=40	In-hospital Mortality n=11	p-value
PSMα1	1259.0	999.0	1168.4	0.77	1151.1	709.6	910.4	0.53	800.4	700.1	0.96
PSMα2	616.0	481.6	523.5	0.58	525.0	338.1	407.9	0.58	402.9	361.5	0.89
PSMα3	493.8	427.3	403.3	0.78	379.7	209.4	263.8	0.56	271.8	226.6	0.93
PSMα4	1745.6	1736.0	1267.6	0.65	1453.3	1012.0	1177.3	0.57	1083.1	828.0	0.61
PSMβ1	222.9	162.6	214.3	0.64	189.1	125.4	196.9	0.58	172.2	173.5	0.71
PSMβ2	49.0	38.4	44.3	0.90	40.6	29.5	45.7	0.85	34.6	38.3	0.79
δ-toxin	1752.6	2100.0	1375.7	0.73	1653.0	1367.0	1420.3	0.78	1308.4	1362.4	0.77

Averaged PSM production classified by clinical outcomes (SSTI = treatment cure or failure; HAP = alive or death at 28 days; IE = alive at discharge or in-hospital mortality). Kruskal-Wallis analysis was used to calculate differences in production. There were no statistically significant differences. Values are in millions of units.

* Information for 3 subjects not available.