Distribution and Regulation of the Mobile Genetic Element-Encoded Phenol-Soluble Modulin PSM-mec in Methicillin-Resistant Staphylococcus aureus

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Abstract

The phenol-soluble modulin PSM-mec is the only known staphylococcal toxin that is encoded on a mobile antibiotic resistance determinant, namely the staphylococcal cassette chromosome (SCC) element mec encoding resistance to methicillin. Here we show that the psm-mec gene is found frequently among methicillin-resistant Staphylococcus aureus (MRSA) strains of SCCmec types II, III, and VIII, and is a conserved part of the class A mec gene complex. Controlled expression of AgrA versus RNAIII in all 3 psm-mec-positive SCCmec types demonstrated that expression of psm-mec, which is highly variable, is controlled by AgrA in an RNAIII-independent manner. Furthermore, psm-mec isogenic deletion mutants showed only minor changes in PSMs peptide production and unchanged (or, as previously described, diminished) virulence compared to the corresponding wild-type strains in a mouse model of skin infection. This indicates that the recently reported regulatory impact of the psm-mec locus on MRSA virulence, which is opposite to that of the PSM-mec peptide and likely mediated by a regulatory RNA, is minor when analyzed in the original strain background. Our study gives new insight in the distribution, regulation, and role in virulence of the PSM-mec peptide and the psm-mec gene locus.

Introduction

Staphylococcus aureus is a significant human pathogen causing a series of acute and chronic, and frequently life-threatening diseases [1]. Widespread resistance to antibiotics severely complicates management of S. aureus infections [2]. S. aureus strains that are resistant to methicillin (methicillin-resistant S. aureus, MRSA) in particular are widespread in the hospital setting and have recently also caused a global epidemic of community-associated infections [3]. The molecular determinants that carry methicillin resistance are located on mobile genetic elements (MGEs) called staphylococcal cassette chromosome mec (SCCmec), which range in size from 21 to 67 kb. SCCmec elements contain genes involved in methicillin resistance and recombination, in addition to accessory elements such as transposons, integrated plasmids, and genes encoding resistance to heavy metals [4].

The virulence potential of S. aureus varies significantly among different strains as a result of the presence or absence of MGEs that contain genes coding for toxins and other virulence determinants [5]. In contrast, a series of core genome-encoded toxins such as alpha-toxin are present in virtually all S. aureus strains. Among those, phenol-soluble modulins (PSMs) have recently emerged as crucial virulence determinants that promote pro-inflammatory processes and lyse neutrophils and other human cell types [6,7]. In addition, some PSMs facilitate biofilm structuring and dispersal [8].

We have recently discovered a novel PSM peptide, PSM-mec, which is encoded on SCCmec elements, representing the first toxin gene of S. aureus co-localized on MGEs with antibiotic resistance determinants [9]. According to published MRSA genome sequences, the psm-mec gene is found predominantly within SCCmec elements of types II and III [9]. However, a broad-scale analysis of the distribution of psm-mec in SCCmec elements of MRSA strains has not been performed yet.

Many toxins in S. aureus are under control of the Agr global virulence regulator [10]. Until recently, it was believed that all Agr targets are regulated via a regulatory RNA, RNAII [11]. However, we have shown in a recent study that selected genes are controlled by the Agr system independently of RNAIII [12]. Genes coding for PSMs are the prototypes of such genes that are controlled by the AgrA DNA-binding protein in an RNAIII-independent fashion [12]. Like other PSMs, PSM-mec is under strict control by Agr, as evidenced by complete lack of production in agr deletion mutants [9]. However, it has remained unclear whether the psm-mec gene is under RNAIII- or AgrA-dependent control.

Previously, we demonstrated that in a strain that produces high relative amounts of PSM-mec compared to other PSMs, psm-mec significantly increases lysis of neutrophils and erythrocytes, and increases virulence in a mouse model of skin infection [9]. Furthermore, Kaito et al. recently showed that the psm-mec locus also has a regulatory function, presumably via a psm-mec-associated regulatory
RNA, whose impact on virulence is opposite to that mediated by the PSM-mec peptide [13]. This was demonstrated using plasmid-based expression of the psm-mec gene with or without an altered start codon. While this method allows conclusions on a regulatory function of psm-mec, an understanding of the role of psm-mec in virulence can only be gained using manipulation of the genome of the original strain background. However, the result of deleting the psm-mec gene in strains other than those of SCCmec type II has not been evaluated.

Here, we analyzed a collection of 29 MRSA strains containing the most commonly isolated SCCmec and sequence types (STs) to analyze the distribution and location of the psm-mec gene in MRSA strains in detail. Furthermore, we investigated whether the psm-mec gene is under control of AgrA or RNAIII and to what extent deletion of psm-mec impacts virulence in strains of different SCCmec types.

Results

We first analyzed an MRSA strain collection containing all major SCCmec types for presence and location of the psm-mec gene. Characteristics of the analyzed MRSA strains, including clonal complex (CC), ST, spa type, and SCCmec type are shown in Table 1. We used two analytical PCR assays to determine whether the psm-mec gene is present and whether its location is conserved: the first PCR fragment amplified the psm-mec gene itself, while the second PCR tested for the connection between psm-mec and the adjacent mecI gene. Our results demonstrated presence of psm-mec in SCCmec types II, III, and VIII, i.e. those types that have a class A mec gene complex (containing the core genes of the SCCmec element in the order IS431-mecA-mecR1-mecI) [4,14] (Fig. 1), while it was absent from types I, IV, V, and VI. Importantly, all tested type II, III, and VIII strains contained psm-mec, and the psm-mec gene was always connected to the mecI gene. Meanwhile, strain BK16991, possessing a truncated SCCmec III element (pSCCmec1991) that carries a class A mec complex but lacks the ccr gene complex [14], was also positive for psm-mec (Tab. 1). Furthermore, production of PSM-mec peptide occurred only in strains positive for psm-mec and the psm-mec/mecI connection (Tab. 1). This indicates that psm-mec and its location are conserved features of the class A mec gene complex.

Table 1. Distribution of the psm-mec gene and PSM-mec peptide production in MRSA strains.

| Strain          | Geographic origin | CC   | ST  | spa types | spa motif | SCCmec type | psm-mec gene | psm-mec - mecI link | PSM-mec peptide |
|-----------------|-------------------|------|-----|-----------|-----------|-------------|---------------|--------------------|-----------------|
| NCTC10442       | United Kingdom    | 8    | 250 | 1         | YHGFMQBLO | I           |               |                   |                 |
| NRS35           | France            | 8    | 572 | 4         | YHGFMQBLO | I           |               |                   |                 |
| NRS36           | France            | 8    | 247 | 4         | YHGFMQBLO | I           |               |                   |                 |
| NRS59           | Scotland          | 8    | 247 | 40        | YFGFMQBLO | I           |               |                   |                 |
| Mu3             | Japan             | 5    | 5   | 2         | TJBMMDGMK | II          | +             | +                 |                 |
| NRS74           | United States     | 5    | 105 | 2         | TJBMMDGMK | II          | +             | +                 |                 |
| NRS382          | United States     | 5    | 5   | 2         | TJBMMDGMK | II          | +             | +                 |                 |
| NRS383          | United States     | 30   | 36-like | 16   | WKGKAOMQQQ | II          | +             | +                 |                 |
| NRS22           | United States     | 45   | 45  | 10        | A2AKEEMBKB | II          | +             | +                 |                 |
| NRS27           | United States     | 45   | 45  | 15        | A2AKEEMBKB | II          | +             | +                 |                 |
| BS/2082         | Japan             | 8    | 239 | 3         | WKGAMAQM   | III         | +             | +                 | +               |
| BK1406          | United States     | 8    | 239 | 3         | WKGAMAQM   | III         | +             | +                 | +               |
| BK16704         | Romania           | 8    | 239 | 351       | WKGAMAQ    | III         | +             | +                 | +               |
| BK16691         | Romania           | 8    | 239 | 351       | WKGAMAQ    | III         | +             | +                 | +               |
| NRS387          | United States     | 5    | 5   | 29        | TJBMMDGMK  | IV          |               |                   |                 |
| NRS119          | United States     | 8    | 507 | 7         | YHCMQBLO   | IV          |               |                   |                 |
| NRS386          | United States     | 8    | 72  | 49        | UUGFMGGM   | IV          |               |                   |                 |
| NRS385          | United States     | 8    | 8   | 7         | YHCMQBLO   | IV          |               |                   |                 |
| NRS271          | United Kingdom    | 22   | 22  | 382       | TJEJNP2MN   | IV          |               |                   |                 |
| NRS241          | United States     | 59   | 59  | 17        | ZDMMAKNB   | IV          |               |                   |                 |
| NRS255          | France            | 80   | 80  | 70        | U1GBPB     | IV          |               |                   |                 |
| BK19489         | United States     | 88   | 78  | 868       | UFKBPPB    | IV          |               |                   |                 |
| NRS265          | Switzerland       | 88   | 88  | 876       | UGFMBBPB   | IV          |               |                   |                 |
| BK23603         | United States     | 8    | 8   | 664       | YCQBQLBO   | V           |               |                   |                 |
| WIS             | Australia         | 45   | 45  | 6         | A2AKBEKBKB | V           |               |                   |                 |
| HT0826          | France            | 152  | 377 | 207       | UI2MGKPN   | V           |               |                   |                 |
| HDE288          | Portugal          | 5    | 5   | 45        | TJBMMDGMK  | VI          |               |                   |                 |
| BK20781         | United States     | 8    | 8   | 1         | YHFGBQBLO  | VIII        | +             | +                 |                 |
| BK23684         | Canada            | 8    | 8   | 544       | YHGCQBQLBO | VIII        | +             | +                 |                 |

*pSCCmec1991* is a truncated SCCmec III element, with a 24-kb deletion encompassing the right chromosomal junction downstream of the class A mec element; BK16704 possesses an SCCmec 3A.1.4 structure, with a dcs gene locus located at the J3 region of SCCmec element [19].

*single locus variant of ST36, with the MLST allelic profile 2-2-52-2-3-3-2.

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Expression of psm genes can vary considerably, with functionality of the Agr system having a strong effect on PSM production [7,15]. Analysis of the presence of the psm-mec gene alone therefore only allows a preliminary assessment of the potential of a given strain to produce the PSM-mec peptide. Furthermore, our previous results indicated that the extent to which PSM-mec contributes to virulence is influenced to a large extent by the relative level of production compared to that of other PSMs [9]. Therefore, we first determined concentrations of all PSM peptides in culture filtrates of every MRSA strain of our collection that was positive for the psm-mec gene (Fig. 2). Absence of PSM-mec production in psm-mec-positive strains occurred in two strains (Mu3 and NRS74). Both these strains lacked production of any PSM peptide, indicating that this phenotype is due to Agr being non-functional. In strains with production of PSM-mec, concentrations relative to those of other PSMs varied. Strains BK16691 and BK23684 for example showed moderate absolute, but high relative production of PSM-mec compared to other PSMs, similar to strain MSA3407 (SCCmec type II), in which PSM-mec has been shown in a mouse skin infection model to contribute significantly to virulence [9].

PSM-mec concentration was strongly correlated with concentrations of AgrA-controlled PSMα peptides (PSMα1, p = 0.0002; PSMα2, p = 0.0008; PSMα3, p = 0.014; PSMα4, p = 0.0048; Pearson’s correlation test) and the RNAIII-embedded δ-toxin (p<0.0001). Correlation with production of PSMβ peptides was not or barely significant, likely owing to the low level of PSMβ peptide concentration under the investigated conditions. Finally, there was no apparent correlation between SCCmec type and level of PSM production.

As the correlation analysis did not allow a clear distinction of whether expression of the psm-mec gene is under control of AgrA or RNAIII, we constitutively expressed agrA or RNAIII from plasmids in an agr-negative strain background, using the naturally agr-dysfunctional, psm-mec-positive MRSA strain N315 [9,16]. Furthermore, we produced agr mutations in strain BK23684 (SCCmec type VIII) and strain BK1406 (SCCmec type III). We then determined expression of psm-mec on the transcript and protein levels. These analyses clearly showed that psm-mec is under transcriptional control by the DNA-binding AgrA response regulator in all 3 strains, while RNAIII had no influence on psm-mec expression (Fig. 3A,B). Despite its unusual location on an
MGE, the *psm-mec* gene is thus regulated in the same fashion as the other *psm* operons, *psmA* and *psmB* [12]. Interestingly, the putative *psm-mec* promoter region does not contain sequences with strong homology to those of the *psmA*, *psmB*, or *agr* P2 and P3 promoter regions. This finding is in keeping with our previous analysis of those promoters [12], which showed only little conservation of AgrA binding consensus sequences, underlining that AgrA-regulated genes cannot be identified based on sequence analysis alone.

Recently, Kaito et al. demonstrated a regulatory effect of the *psm-mec* RNA when over-expressed on a plasmid in hosts without SCCmec or with SCCmec type IV not harboring *psm-mec* [13]. Furthermore, under those circumstances the *psm-mec* transcript down-regulated expression of the cytolysic *psm*-encoded peptide and led to decreased virulence at least in part for that reason [13]. Of note, this report replaced, or added to, a previous hypothesis by the same authors, attributing that regulatory effect to a putative gene named *fudoh* [17], which overlaps with almost the entire *psm-mec* gene. Given the clear evidence for *psm-mec* expression [9], “*fudoh*” very likely is a pseudogene. In contrast, in our previous study, deletion of *psm-mec* in an SCCmec type II strain with high relative production of PSM-mec compared to other PSMs (MSA890) resulted in increased virulence and hemolysis, while in an SCCmec type II strain with lower relative production of PSM-mec (Sanger 252), virulence was not significantly changed [9]. Furthermore, we demonstrated a function of the PSM-mec peptide in cytolysis, indicating an important role in promoting disease [9]. To gain further insight into the role of the *psm-mec* locus in its different original strain backgrounds, we here analyzed two strains of SCCmec types not previously included in our analyses: one strain each of SCCmec type III and VIII (BK1406, BK23684), with moderate to high relative production of PSM-mec. We did not detect a significant change in virulence upon deletion of the *psm-mec* locus (Fig. 4). Additionally, we investigated in those 4 *psm-mec* deletion strains, containing examples of all SCCmec types in which *psm-mec* occurs, whether the genomic deletion of the *psm-mec* locus led to a change in the production of PSM peptides. We found only minor and inconsistent changes in PSM production of *psm-mec* deletion versus the corresponding wild-type strains (Fig. 5).

**Figure 4. Mouse abscess model.** Isogenic *psm-mec* deletion mutants of strains BK1406 (SCCmec type III) and BK23684 (SCCmec type VIII) were compared to their corresponding wild-type strains in a mouse abscess model that was performed as previously described for strain MSA890 and its *psm-mec* deletion mutant [9]. Abscesses formed by strain BK1406 usually presented with open lesions, whereas those formed by strain BK23684 did not. There were no significant differences (using t-tests) between corresponding wild-type and *psm-mec* deletion mutant strain abscess sizes.

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**Figure 5. Impact of the *psm-mec* locus on the production of genome-encoded PSMs.** Four MRSA strains of 3 different SCCmec types and their isogenic *psm-mec* deletion mutants were analyzed for production of all *S. aureus* PSM peptides (a1, a2, a3, a4, b1, b2, δ-toxin, mec) by RP-HPLC/MS. Production levels are shown for all peptides except for β1 and β2, for which production levels were in general very low.

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**Discussion**

The staphylococcal PSM-mec toxin is unique in its link to an antibiotic resistance element. This coupling allows simultaneous acquisition by *S. aureus* strains of key antibiotic resistance together with virulence determinants, significantly facilitating the spread of antibiotic resistance and virulence among the *S. aureus* population [9,18]. The present study was undertaken because, despite the immense importance of such a mechanism for public health, we lack knowledge about distribution and location of the *psm-mec* gene.

Results from the present study show that the MGE-encoded *psm-mec* gene is widely distributed among MRSA strains and its expression is highly variable. The *psm-mec* gene is linked to the class A mec gene complex present in SCCmec types II, III, and VIII, with a conserved location next to the *mec* gene. The fact that the *psm-mec* gene was always found among strains harboring SCCmec elements of types II, III, and VIII at the same location indicates that it is an original part of these elements, representing a possible marker for analytical purposes.

RNAIII-independent regulation via the Agr system has only recently been discovered, and the *psm-mec* and *psmB* operons are the only genetic elements for which a direct binding of and regulation by the AgrA response regulator has been shown on a molecular level [12]. These findings indicated that PSMs represent evolutionarily early parts of the Agr regulon, while other targets of Agr regulation likely were added later, via evolution of the regulatory RNAIII and its connection to the Agr system. This raised the interesting question whether *psm-mec* is under the same, direct regulation of AgrA despite its location on an MGE. We demonstrate here that *psm-mec* shares with the other, core-genome encoded *psm* genes the characteristic Agr-dependent, but RNAIII-independent regulation. Thus, based on this similarity, it is tempting to speculate that core genome-encoded *psm* genes and *psm-mec* have a common origin.

Finally, we analyzed the contribution of *psm-mec* to virulence in SCCmec type III and VIII strains. We do not believe that the type of SCCmec element impacts the contribution of *psm-mec* to virulence, as the region around *psm-mec* is well conserved in SCCmec elements of class A. Rather, these experiments were performed to gain insight into the role of the *psm-mec* locus in additional strains. Our previous results on the cytolytic role of the
PSM-mec toxin [9] and those by Kaito et al. [13] who found a regulatory effect of the psm-mec locus, including on genome-encoded PSMs, indicate that the contribution to virulence of the RNA and the peptide it encodes are opposite. However, the results by Kaito et al. were based entirely on plasmid-based over-expression in strains not naturally harboring psm-mec, prompting us to investigate the role of the psm-mec locus deletion by allelic replacement strategies, which are not influenced by plasmid copy effects and allow conclusions on the role of the locus in its natural strain background. In the 4 clinical strains that we investigated in our previous and the present study, absence of psm-mec either did not significantly alter virulence in a mouse skin infection model, or virulence was decreased. Furthermore, there were only very minor effects of the psm-mec deletion on the expression of other PSM peptides. Notably, this included the strongly cytolytic PSMx3, whose production was reported as decreased upon plasmid-based expression of psm-mec by Kaito et al. [13]. These findings suggest that the virulence-promoting effect of the PSM-mec peptide balances or supersedes the reported virulence-diminish effect of the psm-mec RNA, depending on the strain background. In the future, a thorough analysis of the roles of psm-mec on the RNA and peptide levels will have to be accomplished using manipulations of psm-mec-containing clinical strains on the genome that allow a distinction between the roles of the psm-mec transcript and its product.

Methods

Ethics statement

Animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee (IUCAC number ASP LHBP 1E), National Institute of Allergy and Infectious Diseases.

Bacterial strains, typing, and growth conditions

The bacterial strains used in the present study are shown in Table 1. In addition, we used strains N315 and MSA3407, which were analyzed in our previous study on psm-mec [9]. SCCmec and spa typing were performed as previously described [14,19]. All bacteria were grown in tryptic soy broth (TSB). For xylose induction experiments, glucose-free TSB was used. Antibiotics were added at appropriate concentrations (chloramphenicol, 10 μg/ml, tetracycline 12.5 μg/ml).

Production of psm-mec and agr mutants

Isogenic deletion mutants of the psm-mec locus in strains BK1406 and BK23684 were produced using allelic replacement with plasmid pKOR1 as described [9,20]. Mutants in the agr and BK23684 were produced using allelic replacement with plasmid pKOR1 as described [9,20]. Mutations in the agr, psm-mec, and mecI were accomplished using manipulations of psm-mec-containing clinical strains on the allele, including on genome-encoded PSMs, indicating that the contribution to virulence of the RNA. For RT-PCR mecI-rev TACAAATGCAAAAGGACTGGAGTCC

Table 2. Oligonucleotides used in this study.

| Name                  | Sequence                                                                 |
|-----------------------|--------------------------------------------------------------------------|
| For psm-mec analytical PCR |                                                                          |
| Psmmec-for            | ATGGATTCTACGTGGTTATATCATACAGGC                                           |
| Psmmec-rev            | TTACCGGAAGGCTGTAAGCAGGAGATCTCTG                                          |
| For mecI link analytical PCR |                                                              |
| Psmmec-for            | ATGGATTCTACGTGGTTATATCATACAGGC                                           |
| mecI-rev              | TACAAATGCAAAAGGACTGGAGTCC                                                 |
| For RT-PCR            |                                                                          |
| psmmecRfor            | TGGTATGGAATGGTATCATGGTTA                                                 |
| psmmecRrev            | ATTTAATCAAGACTTGCAATTAG                                               |
| psmmecRProbe          | CCTGGAATATTCTTCTGTTTTTTAGTTG                                             |

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Analysis of PSM-mec production

Production of PSM-mec was analyzed by reversed-phase high pressure liquid chromatography/electrospray mass spectrometry (RP-HPLC/ESI-MS) of culture filtrates as described [7,9].

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed as described [21], using the house-keeping gene gyrB as control.

Animal skin infection model

The mouse skin infection (abscess) model was performed as described previously [22].

Statistics

Statistical analysis was performed using graph Pad Prism Version 5.04.

Author Contributions

Conceived and designed the experiments: SSC BNK MO. Performed the experiments: SSC GC SYJ GYCC. Analyzed the data: SSC H-SJ BNK MO. Wrote the paper: MO.

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