Inhibition of Exotoxin Production by Mobile Genetic Element SCCmec-Encoded psm-mec RNA Is Conserved in Staphylococcal Species

Mariko Ikuo*, Gentaro Nagano*, Yuki Saito, Han Mao, Kazuhisa Sekimizu, Chikara Kaito*

Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan

Abstract

Staphylococcal species acquire antibiotic resistance by incorporating the mobile-genetic element SCCmec. We previously found that SCCmec-encoded psm-mec RNA suppresses exotoxin production as a regulatory RNA, and the psm-mec translation product increases biofilm formation in Staphylococcus aureus. Here, we examined whether the regulatory role of psm-mec on host bacterial virulence properties is conserved among other staphylococcal species, S. epidermidis and S. haemolyticus, both of which are important causes of nosocomial infections. In S. epidermidis, introduction of psm-mec decreased the production of cytolytic toxins called phenol-soluble modulins (PSMs) and increased biofilm formation. Introduction of psm-mec with a stop-codon mutation that did not express PSM-mec protein but did express psm-mec RNA also decreased PSM production, but did not increase biofilm formation. Thus, the psm-mec RNA inhibits PSM production, whereas the PSM-mec protein increases biofilm formation in S. epidermidis. In S. haemolyticus, introduction of psm-mec decreased PSM production, but did not affect biofilm formation. The mutated psm-mec with a stop-codon also caused the same effect. Thus, the psm-mec RNA also inhibits PSM production in S. haemolyticus. These findings suggest that the inhibitory role of psm-mec RNA on exotoxin production is conserved among staphylococcal species, although the stimulating effect of the psm-mec gene on biofilm formation is not conserved.

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* E-mail: kaito@mol.f.u-tokyo.ac.jp

These authors contributed equally to this work.

Introduction

Pathogenic bacteria produce exotoxins that damage host immune cells to facilitate bacterial survival and proliferation in the host environment. Pathogenic bacteria also form a biofilm to resist host immune factors and antibiotics [1]. Understanding the molecular mechanisms of exotoxin production and biofilm formation is important for establishing therapeutic strategies against infectious bacterial diseases. Bacteria possess virulence factors encoded on the core genome and also acquire virulence factors by incorporating plasmids, phages, or transposons, which are known as mobile genetic elements [2]. Mobile genetic elements encode various virulence factors such as exotoxins and superantigens that directly interact with host factors [3,4]. Mobile genetic elements also encode a regulatory factor against core genome encoded-virulence genes [5,6,7].

We recently found that the psm-mec gene located in the mobile genetic element SCCmec, which carries antibiotic resistant genes, regulates the virulence properties of S. aureus, a serious human pathogen [8]. In the S. aureus core genome, the agr locus encodes agrBDC41 and RNAIII, which regulate the expression of various virulence genes according to cell density [9]. The agrC gene encodes a positive transcription factor for phenol-soluble modulins (PSMs), which are cytolysins essential for S. aureus virulence [10,11,12]. The transcription product of psm-mec acts as a regulatory RNA to inhibit the translation of agrC, resulting in decreased PSMs production [13]. In contrast, the translation product of psm-mec stimulates biofilm formation [8]. The psm-mec gene exists in type-II and type-III SCCmec of hospital-associated methicillin-resistant S. aureus (HA-MRSA), but not in type-IV SCCmec of community-acquired MRSA with higher virulence than HA-MRSA [14,15,16]. Furthermore, 25% of HA-MRSA strains carry promoter-deficient psm-mec and produce higher amounts of a cytolytic exotoxin, PSMα3, than the strains carrying intact psm-mec [13]. These findings indicate that psm-mec is a genetic determinant of the virulence capacity of MRSA. The function of psm-mec to inhibit exotoxin production and to increase biofilm formation might contribute to alleviate excess damage to host animals and to survive in the host environment. The psm-mec exists not only in S. aureus, but also in other staphylococcal species [17]. It has remained unclear, however, whether the regulatory function of psm-mec is conserved among the staphylococci species carrying SCCmec, which needs the conserved interaction between the mobile genetic element-encoded psm-mec and core genome encoded-virulence factors.

Most staphylococcal species other than S. aureus do not produce coagulase and are called coagulase-negative staphylococci. S. epidermidis is a commensal bacterium present on human skin...
surfaces that often contaminates catheters and other surgical implants [18,19]. The recent emergence of methicillin- or vancomycin-resistant S. epidermidis is a serious clinical problem [20]. S. haemolyticus is also a commensal bacterium on human skin surfaces and in domestic animals [21] that causes various infectious diseases, including skin infections and meningitis in humans [21]. In particular, S. haemolyticus bacteremia in a neonatal intensive care unit and the emergence of drug-resistant S. haemolyticus were recent serious clinical issues [22,23]. In the present study, we investigated the effect of psm-mec in S. epidermidis and S. haemolyticus and found that the inhibitory function of psm-mec RNA on exotoxin production was conserved among these species.

Results and Discussion

psm-mec Alters the Virulence Phenotype of S. epidermidis

To examine whether the alteration of the S. epidermidis phenotype by psm-mec is caused by the transcription product or the translation product of psm-mec, we transformed S. epidermidis with psm-mec carrying either a stop codon mutation or a deficient promoter, which we previously used in S. aureus [8] (Fig. 1A). The strains transformed with pC1, pC2, or pC3, which carries a mutated psm-mec with a stop codon, did not produce PSM-mec protein (Fig. 1B), but expressed an amount of psm-mec RNA indistinguishable from that of the intact psm-mec-transformed strain (Fig. 1C). In contrast, the strains transformed with pM1 or pM2, which carried the promoter-deficient psm-mec, expressed little amount of psm-mec RNA or PSM-mec protein (Fig. 1B and 1C). These findings suggest that the translation of psm-mec was inhibited by pC1, pC2, and pC3, whereas the transcription of psm-mec was inhibited by pM1 and pM2 in S. epidermidis.

We transformed S. epidermidis ATCC12228 strain, which does not possess psm-mec, with a plasmid carrying psm-mec (pF). Analysis of extracellular proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis revealed that the expression pattern of extracellular proteins was altered in the psm-mec-transformed strain compared with the vector (pND50)-transformed strain (Fig. 1D). S. epidermidis secretes PSMs that are involved in biofilm maturation and have cytolytic activity against mammalian cells [24,25]. High-performance liquid chromatography (HPLC) analysis revealed that the introduction of psm-mec decreased the amount of PSMs in culture supernatants (Fig. 1E). In the psm-mec-transformed strain, the combined amount of PSM1 and PSM5, which were not separated by our HPLC conditions, was decreased to 70% of that in the vector-transformed strain (Fig. 1F). The amounts of PSM2, PSM3, and PSM4 in the psm-mec-transformed strain were decreased to 10% of that in the vector-transformed strain (Fig. 1G, 1H, and 1I). In contrast, the psm-mec-transformed strain exhibited increased biofilm formation compared with the vector-transformed strain (Fig. 1J). These findings suggest that psm-mec alters the expression pattern of extracellular proteins, decreases the expression of PSMs, and stimulates biofilm formation in S. epidermidis.

The mutated psm-mec with a stop codon in pC1, pC2, and pC3 retained the activities to alter the expression pattern of extracellular proteins and to decrease the amount of PSMs (Fig. 1D, 1F, 1G, 1H, and 1I). In contrast, the mutated psm-mec with a stop codon lost the ability to increase biofilm formation (Fig. 1J). The promoter-deficient psm-mec in pM1 and pM2, which did not express either psm-mec RNA and PSM-mec protein, did not induce the phenotypic alteration of S. epidermidis, which was caused by intact psm-mec (Fig. 1D, 1F, 1G, 1H, 1I, and 1J). These results suggest that the psm-mec translation product promotes biofilm formation, whereas the psm-mec transcription product inhibits PSM expression and alters the expression pattern of extracellular proteins in S. epidermidis.

psm-mec Alters the Virulence Phenotype of S. haemolyticus

The agr locus encoding agrBDCA and RNAIII is involved in regulating S. aureus virulence properties [9]. Although the agr function is not elucidated in S. haemolyticus, the sequence of agrBDCA is conserved in S. haemolyticus [26]. We examined whether psm-mec decreases the expression of agr in S. haemolyticus. Introduction of psm-mec decreased the amount of AgrA in the S. haemolyticus JCM2146 strain that does not carry psm-mec (Fig. 2A). In addition, the psm-mec-transformed strain decreased hemolysin production (Fig. 2B). Although there are no reported analyses of the PSMs of S. haemolyticus, genome analysis revealed the presence of genes encoding PSMβ1, PSMβ2, and PSMβ3 [27]. We performed HPLC analysis of the culture supernatant of S. haemolyticus and identified several molecules with absorbance at 215 nm within the retention time in which the PSMs of S. aureus and S. epidermidis are eluted (Fig. 2C). The molecules at 9, 12, and 13 min were identified as PSMβ3, PSMβ2, and PSMβ1 by liquid chromatography/mass spectroscopy analysis, respectively (Fig. 2C). The psm-mec-transformed strain exhibited decreased amounts of PSM3, PSMβ2, and PSMβ1 (Fig. 2C, 2D, 2E, and 2F). Introduction of the mutated psm-mec with a stop codon (pC1) also decreased the amounts of AgrA, hemolysin, and PSMs (Fig. 2A, 2B, 2D, 2E, and 2F). In contrast, introduction of the promoter-deficient psm-mec (pM1) did not cause the phenotypic alterations (Fig. 2A, 2B, 2D, 2E, and 2F). Thus, the psm-mec transcription product decreased the expression of AgrA, hemolysin, and PSMs in S. haemolyticus.

The psm-mec-transformed strain of S. haemolyticus exhibited the same level of biofilm formation as the vector-transformed strain (Fig. 2G). Introduction of either the mutated psm-mec with a stop codon (pC1) or the promoter-deficient psm-mec (pM1) did not alter the biofilm formation by S. haemolyticus (Fig. 2G). These findings suggest that psm-mec does not affect the biofilm formation by S. haemolyticus.

Conclusions

In this study, we revealed that the inhibitory effect of the psm-mec transcript against exotoxin production is conserved in S. epidermidis and S. haemolyticus. This is the first report to reveal that the regulatory role of a mobile genetic element-encoded factor on host bacterial virulence is conserved among the species incorporating the mobile genetic element. Because the SCCmec carrying psm-mec is widely observed among staphylococcal species, including S. aureus, S. epidermidis, S. haemolyticus, S. hominis, S. pseudintermedius, S. saprophyticus, S. flavescens, S. vitulinus, and S. simulans [14,17], the inhibitory effect of psm-mec against exotoxin production is assumed to confer conserved advantages to staphylococcal bacteria in their survival in host animals and may cause a positive effect on the dissemination and maintenance of SCCmec.

In S. haemolyticus, the intact psm-mec or the stop codon-mutated psm-mec decreased the amount of AgrA. Thus, psm-mec RNA is assumed to inhibit translation of AgrA, as in the case of S. aureus [13]. We were not able to examine the effect of psm-mec on AgrA expression in S. epidermidis ATCC12228 because the expression level was too low. Based on the reports that the agr locus is required for PSM expression in S. epidermidis [28,29] and our result that psm-mec RNA inhibited PSM expression of S. epidermidis, we assume that psm-mec RNA inhibits AgrA expression in S. epidermidis. Because the agr locus is conserved among staphylococcal species [26], the inhibition of agr function by psm-mec RNA is probably
Figure 1. Alteration of virulence phenotype in the *psm-mec*-transformed *S. epidermidis*. (A) Schematic representation of the *psm-mec* mutations in pC1, pC2, pC3, pM1, and pM2 is shown. (B) The amount of PSM-mec protein was measured by reversed-phase HPLC in the *S. epidermidis* strains transformed with plasmids listed in (A). Data are means ± standard deviations from three independent experiments. Asterisks indicate Student’s t-test p value less than 0.05 between the pF-transformed strain and the others. ND means not detected. (C) The amount of *psm-mec* RNA...
was measured by quantitative RT-PCR in the *S. epidermidis* strains transformed with plasmids listed in (A). Data shown are means ± standard deviations from three independent experiments. Asterisk indicates Student’s t-test p value less than 0.05 between the pF-transformed strain and the others. ND means not detected. (D) Extracellular proteins of overnight culture of *S. epidermidis* ATCC12228 strain, which was transformed with pND50 as an empty vector, pF carrying intact psm-mec, or plasmids carrying mutated psm-mec (pC1, pC2, pC3, pM1, or pM2) were electrophoresed in a 10% SDS polyacrylamide gel and stained with Coomassie brilliant blue. A representative result from three independent experiments is shown. (E) Expression of PSMs in the psm-mec-transformed strain (pF, magenta line) or empty vector-transformed strain (pND50, black line) was analyzed by reversed-phase HPLC. The PSM species were identified by LC/ESI-MS. (F, G, H, I) The amounts of PSMs + PSMδ were measured by reversed-phase HPLC. The vertical axis represents the relative value to the amount of PSMs in the pnd50-transformed strain. Data are shown as means ± standard deviations from three independent experiments. Asterisks indicate Student’s t-test with a p value less than 0.05 between the pND50-transformed strain and others. (J) Biofilm formation of the *S. epidermidis* strains transformed with pND50 as an empty vector, pF carrying intact psm-mec, or plasmids carrying mutated psm-mec (pC1, pC2, pC3, pM1, or pM2) was measured. Data shown are means ± standard deviations from three independent experiments. Asterisks indicate Student’s t-test with a p value less than 0.05 between the pF-transformed strain and the others.

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**Materials and Methods**

**Bacterial Strains, Plasmids, and Culture Conditions**

*S. aureus* RN4220 strain was used as a host for pND50 and its derivatives. Plasmids were extracted from the RN4220 strain and used for the transformation of *S. epidermidis* ATCC12228 [37] or *S. haemolyticus* JCM2416 [38]. These strains were transformed by electroporation as in the case of *S. aureus* [39]. *S. epidermidis* and *S. haemolyticus* strains were aerobically cultured in tryptic soy broth at 37°C. When culturing the transformed strains, chloramphenicol was added to the broth to maintain plasmids.

**Evaluation of Biofilm Formation**

*S. epidermidis* and *S. haemolyticus* strains were cultured in tryptic soy broth containing 0.25% glucose in 96-well polystyrene microplates (Cat No. 3803-096, Iwaki, Tokyo, Japan) at 37°C for 3 days. After removing the cultures, the plate was stained with 0.1% safranin. Absorbance at 490 nm was measured using a microplate reader (MTP900, CORONA, Ibaraki, Japan).

**Measurement of the Amount of PSMs**

Fifty-microliter of overnight cultures of *S. epidermidis* or *S. haemolyticus* strains were inoculated into 5 ml of tryptic soy broth and aerobically cultured at 37°C for 18 h or 14 h. The culture supernatants were dried using a centrifugal evaporator. The precipitates were dissolved in 40% acetonitrile and the solute fraction was dried. The precipitates were dissolved in water and analyzed in reversed phase HPLC using SOURCE SRPC ST 4.6/150 column (GE Healthcare, Tokyo, Japan) at 37°C. PSMs were identified by liquid chromatography/electrospray-ionization mass spectroscopy (LC 1100 series, Agilent Technologies, Santa Clara, CA; ESI-MS, Bio-TOFQ, Bruker Daltonics, Billerica, MA) and the predicted molecular masses of PSMs [25,27]. *S. epidermidis* PSMα and PSMδ were eluted at the same retention time in this assay condition.

**Measurement of the Amount of psm-mec RNA**

*S. epidermidis* strains were aerobiocly cultured to A₆₀₀ = 1 at 37°C and the cells were collected by centrifugation at 20,000 g for 1 min. The cells were treated with RNaseA to protect Bacteria Reagent (Qiagen) and the total RNA was extracted using an RNaseasy Mini Kit (Qiagen). RNA was reverse-transcribed to cDNA using Multiscribe Reverse Transcriptase (Roche), Quantitative polymerase chain reaction (PCR) was performed using cDNA, SYBR Premix ExTaq (Takara Bio, Tokyo, Japan), and primers for 16 S rRNA or psm-mec according to the previously described method [13]. Signals were detected using the Step One Plus Real Time PCR System (Applied Biosystems, Tokyo, Japan).

**Detection of *S. haemolyticus* AgrA**

*S. haemolyticus* AgrA was detected according to the previously described method [13]. Briefly, *S. haemolyticus* was aerobiocly cultured for 15 h at 37°C and the cells were collected by centrifugation at 20,000 g for 1 min. The cells were lysed in lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10 μg/ml lysozyme) at 37°C for 30 min. The samples were sonicated and centrifuged at 20,000 g for 5 min. The protein concentration of the supernatants was determined by the Bradford assay. The protein was electrophoresed in a 15% SDS polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was subjected to detection of AgrA by using anti-AgrA antibody.
Figure 2. Alteration of virulence phenotype of the *psm-mec*-transformed *S. haemolyticus*. (A) AgrA expression in *S. haemolyticus* strain transformed with pND50 as an empty vector, pF carrying intact *psm-mec*, pC1 carrying a stop codon-mutated *psm-mec*, or pM1 carrying a promoter-deficient *psm-mec* was examined. Protein (2.8 μg) was electrophoresed in each lane and subjected to Western blotting using anti-AgrA IgG. A representative result from two independent experiments is shown. (B) Hemolysin production of *S. haemolyticus* strain transformed with pND50, pF, pC1, or pM1 was measured on tryptic soy agar plates containing 5% sheep erythrocytes. A color-changed region around the colonies reflects the lysis of erythrocytes. (C) PSMs in *S. haemolyticus* strains transformed with pND50 or pF were detected by reversed-phase HPLC. Respective PSM species were identified by LC/ESI-MS. (D, E, F) The amounts of PSM* b*3, PSM* b*2, and PSM* b*1 in the *S. epidermidis* strain transformed with pND50, pF, pC1, or pM1 were measured by reversed-phase HPLC. The vertical axis represents the relative value to the amount of PSMs in the pND50-transformed strain. Data shown are means ± standard deviations from three independent experiments. Asterisks indicate a Student’s t-test p value less than 0.05.
between the pH50-transformed strain and the others. (G) Biofilm formation of the S. haemolyticus strain that was transformed with pH50, pF, pC1, or pM1 was examined. Data shown are means ± standard deviations from three independent experiments.

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**Author Contributions**

Conceived and designed the experiments: CK. Performed the experiments: MI GN YS HM. Analyzed the data: MI GN YS HM KS CK. Wrote the paper: CK.

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