Mobile Genetic Element SCCmec-encoded psm-mec RNA Suppresses Translation of agrA and Attenuates MRSA Virulence

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Abstract

Community acquired-methicillin resistant Staphylococcus aureus (CA-MRSA) is a socially problematic pathogen that infects healthy individuals, causing severe disease. CA-MRSA is more virulent than hospital associated-MRSA (HA-MRSA). The underlying mechanism for the high virulence of CA-MRSA is not known. The transcription product of the psm-mec gene, located in the mobile genetic element SCCmec of HA-MRSA, but not CA-MRSA, suppresses the expression of phenol-soluble modulin α (PSMα), a cytolytic toxin of S. aureus. Here we report that psm-mec RNA inhibits translation of the agrA gene encoding a positive transcription factor for the PSMα gene via specific binding to agrA mRNA. Furthermore, 25% of 325 clinical MRSA isolates had a mutation in the psm-mec promoter that attenuated transcription, and 9% of the strains had no psm-mec. In most of these psm-mec-mutated or psm-mec-deleted HA-MRSA, PSMα expression was increased compared with strains carrying intact psm-mec, and some mutated strains produced high amounts of PSMα comparable with that of CA-MRSA. Deletion of psm-mec from HA-MRSA strains carrying intact psm-mec increased the expression of AgrA protein and PSMα, and virulence in mice. Thus, psm-mec RNA suppresses MRSA virulence via inhibition of agrA translation and the absence of psm-mec function in CA-MRSA causes its high virulence property.

Introduction

CA-MRSA, especially the USA300 clone, causes severe infectious diseases in many people in the United States and in European countries. CA-MRSA is generally considered more virulent than most HA-MRSA [1]. Determining the molecular mechanism underlying the high virulence of CA-MRSA will be important toward establishing new therapeutic strategies against CA-MRSA infections. One reason for the high virulence of the CA-MRSA USA300 strains is suggested to be the high amounts of secreted toxins, including PSMα, α-hemolysin, δ-hemolysin (Hld), and the Panton-Valentine leukocidin (PVL) [1,2,3]. The USA300 strains show increased expression of the agr locus, which upregulates the production of PSMα, α-hemolysin, and PVL, compared with HA-MRSA strains [1,3,4,5]. The agr locus is essential for the virulence of the USA300 strains against animals [4,6]. The agr locus encodes agrBDCA, which functions in quorum sensing [7]. An extracellular quorum-sensing molecule made from AgrD activates the sensor protein AgrC. AgrC activates the transcription factor AgrA by phosphorylation. AgrA activates the transcription of agrBDCA, including agrA itself [8]. Thus, quorum sensing is under positive feedback regulation. The agr locus also encodes RNAIII, which is an mRNA of Hld as well as a regulatory RNA that upregulates the expression of various toxins, including α-hemolysin, and downregulates the expression of various cell surface proteins [9]. AgrA activates the transcription of RNAIII and other virulence genes, including the psmα operon, by direct binding to the promoter [8,10]. The mechanism that increases the expression of agr in the USA300 strains, however, is not known.

SCCmec is a mobile genetic element that confers methicillin resistance to MRSA strains. The structure of the SCCmec region differs between the CA-MRSA and HA-MRSA strains [11]. We previously reported that the psm-mec gene that exists in type-II and type-III SCCmec, which is found in most HA-MRSA, regulates the virulence of S. aureus [12]. The psm-mec gene is absent in type-IV SCCmec of the CA-MRSA USA300 strains [12]. Introduction of psm-mec into FRP3757, a CA-MRSA USA300 strain, or Newman, a methicillin-sensitive S. aureus strain that carries neither SCCmec nor psm-mec, decreases the secreted amount of PSMα, suppresses colony-spreading ability, and promotes biofilm formation [12,13].

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**Author Summary**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is resistant to various antibiotics, including β-lactams, thus causing serious clinical problems. Hospital-associated (HA)-MRSA infects immunocompromised patients in hospitals. Community-acquired (CA)-MRSA causes serious diseases in healthy people who have not had contact with hospitals in the United States, Canada, or Europe. CA-MRSA produces higher amounts of extracellular toxins and has higher virulence than HA-MRSA, although the reason for this is unclear. SCCmec is a foreign DNA integrated into the MRSA chromosome that contains several genes including the mecA gene that confers resistance against methicillin. The SCCmec of CA-MRSA does not contain the psm-mec gene that exists in the HA-MRSA SCCmec. In the present study, we found that the transcription product of psm-mec inhibits translation of the agrA gene encoding a positive transcription factor for many extracellular toxins by direct binding to the agrA mRNA, resulting in decreased extracellular toxin production. Furthermore, some HA-MRSA strains carry mutated psm-mec or no psm-mec and produce higher amounts of extracellular toxins than HA-MRSA strains carrying intact psm-mec. These findings suggest that psm-mec RNA negatively regulates agrA and mutation or absence of psm-mec leads to a high virulence capacity of MRSA.

Furthermore, the psm-mec-transformed strains attenuate virulence in a mouse model of systemic infection [12,13]. The finding first revealed that a factor encoded by a mobile-genetic element negatively regulates bacterial virulence. Because the USA300 strains have no psm-mec, we proposed that the absence of psm-mec is a genetic determinant of the high virulence of USA300 [12]. Suppression of colony-spreading and promotion of biofilm formation by the psm-mec gene are attributed to both the transcription product and the translation product of psm-mec [12]. In contrast, suppression of the expression of PSMzx leads to the inhibition of the translation of agrA and decreases the amount of AgrA in the cells. To examine this possibility, we first established a method to determine the amount of AgrA protein in cells. Anti-AgrA immunoglobulin (IgG) was prepared from a rabbit immunized with His-tagged recombinant AgrA and Western blot analysis was performed. In cell extracts of the Newman strain, the 28-kDa band of AgrA was detected, which was consistent with the predicted molecular mass of AgrA, 27.9 kDa (Fig. 2A). In contrast, the band was not detected in cell extracts of the agr-null mutant (Fig. 2A). In addition, the band was not detected using IgG from a non-immunized rabbit (data not shown). Therefore, we concluded that the 28-kDa protein detected by anti-AgrA IgG was the AgrA protein. We then performed Western blot analysis of AgrA in psm-mec-transformed Newman strain. In the psm-mec (pF)-transformed Newman strain, the AgrA band intensity was decreased compared with the vector (pND50)-transformed Newman strain (Fig. 2B). Decreased intensity of the AgrA band was also observed in Newman transformed with psm-mec containing a stop-codon (pC1) (Fig. 2B), which expresses psm-mec RNA without the expression of PSM-mec protein [12]. In contrast, the psm-mec gene, which has a -71>T mutation in the promoter (pM1) and does not express psm-mec RNA [12], did not decrease the AgrA band intensity (Fig. 2B). These findings suggest that the transcription product of psm-mec acts to decrease the amount of AgrA in S. aureus cells. Furthermore, we examined whether psm-mec RNA also decreases the amount of AgrA in the CA-MRSA strains MW2 (USA400) and FRP3757 (USA300). In both CA-MRSA strains, the introduction of a plasmid carrying psm-mec (pF) led to a decrease in the amount of AgrA (Fig. 2C). We further analyzed whether a single copy of psm-mec is enough to decrease the amount of AgrA in respective S. aureus strains. We previously reported the construction of a Newman strain integrated with psm-mec into the chromosomal DNA [13]. In this study, we constructed MW2 and FRP3757 strains into which psm-mec was integrated into the chromosomal regions near the mecA of SCCmec, where psm-mec is originally present in HA-MRSA strains (Fig. S1). In these three strains, integration of psm-mec into the chromosomal decreases the amount of AgrA in the cells (Fig. 2C). These findings indicate that introduction of psm-mec into CA-MRSA strains decreases the amount of AgrA and that a single copy psm-mec is enough to exert the repression effect.

Next, we examined whether psm-mec represses the translation of agrA. AgrA functions in a positive feedback loop to activate the transcription of agrBDC4, including agrA itself. To exclude the effect on the transcription initiation of agrBDC4 from its native promoter, we transformed the agr-null mutant of Newman with pMNS-agrBDC4, which expresses agrBDC4 from an IPTG-inducible promoter. The amount of AgrA was increased by increasing the IPTG concentration in the strain transformed with pMNS-agrBDC4 and empty vector (pKE516), whereas the introduction of a plasmid carrying psm-mec (pKE516-F) into the strain transformed with pMNS-agrBDC4 diminished AgrA expression in the presence of IPTG (Fig. 2D). Thus, psm-mec...
inhibits AgrA expression without the transcriptional regulation of the agrBDCA promoter. To examine the effect of psm-mec on the agrA coding region, we constructed a reporter gene-fusion construct in which the Shine-Dalgarno sequence of agrA and agrA ORF was fused in frame with luc under the control of the recF promoter (Fig. 2E). The introduction of psm-mec did not decrease the luciferase activity of luc-fusion with the 20–27 sequence of agrA (pGP-luc), although it decreased the luciferase activity of luc-fusion with the 20–717 sequence of agrA (pGP-agrA-luc) (Fig. 2F). Decreased luciferase activity of pGP-agrA-luc was also observed in Newman integrated with a single copy of psm-mec (Fig. 2G). These findings indicate that psm-mec inhibits the translation of agrA.

psm-mec RNA specifically binds agrA mRNA

We next searched for mRNA interacting with psm-mec RNA using the in silico programs sRNATarget [18] and RNAhybrid [19], and identified agrA mRNA as a candidate (Fig. 3A). We hypothesized that the inhibition of agrA translation by psm-mec is caused by the direct binding of psm-mec RNA to agrA mRNA. Primer extension [12] and nuclease S1 protection analyses (Fig. S2) revealed that the size of psm-mec RNA was 157 bases. To examine the direct binding of psm-mec RNA to agrA mRNA, we performed a gel shift analysis using 157 bases of psm-mec RNA that was synthesized by in vitro transcription. The addition of the 20–717 sequence of agrA mRNA retarded the mobility of the radiolabeled psm-mec RNA fragment in a dose-dependent manner (Fig. 3B). The retardation was cancelled by the addition of nonlabeled psm-mec RNA, although not by the addition of a 1000-fold amount of yeast tRNA (Fig. 3B). Thus, the binding of psm-mec RNA to agrA mRNA detected by gel shift assay was specific. Furthermore, to identify the region of agrA mRNA required for binding to psm-mec RNA, we examined whether the 20–267 sequence of agrA mRNA (agrA1), which contains the predicted binding region, and the 20–198 sequence of agrA mRNA (agrA2), which partially disrupts the predicted binding region, bind psm-mec RNA. This finding demonstrated that agrA1 RNA binds psm-mec RNA, although agrA2 RNA does not bind psm-mec RNA (Fig. 3C). Thus, the +199–+267 sequence of agrA mRNA is required for binding to psm-mec RNA. To determine whether the binding of psm-mec RNA to agrA mRNA is required for the inhibition of agrA translation, we performed a reporter gene-fusion analysis with and without the +199–+267 region of agrA. We compared the luciferase activity of

Figure 1. psm-mec RNA increased the amount of HutU, Spa, and Ddh in CA-MRSA FRP3757 (USA300). (A) The nucleotide sequence of the psm-mec ORF in pF, the stop-codon introduced sequence of psm-mec ORF in pC1, and the synonymous-codon substituted sequence of psm-mec ORF in pFP are shown. The substituted nucleotides are colored in red. The amino acid sequence of PSM-mec protein is shown below the respective nucleotide sequence. (B) Cell extract of FRP3757 strain that was transformed with empty vector (pND50), psm-mec (pF), mutated psm-mec harboring a stop codon (pC1), or mutated psm-mec harboring synonymous codon substitutions (pFP) was analyzed by two-dimensional electrophoresis. Proteins were stained with Coomassie Brilliant Blue. The protein spot was excised and identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Table S1). doi:10.1371/journal.ppat.1003269.g001
**Figure 2.** *psm-mec* RNA inhibits *agrA* translation. (A) Cell extracts of overnight cultures of Newman strain (WT) and the *agr*-null mutant (Δagr) were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels. One gel was stained with Coomassie Brilliant Blue (Left panel). Proteins in another gel were transferred to a membrane and used for Western blotting by anti-AgrA IgG (Right panel). (B) Cell extracts of 24 h-cultures of Newman strains transformed with empty vector (pND50), a plasmid carrying wild-type *psm-mec* (pF), a plasmid carrying *psm-mec* with a stop-codon (pC1), and a plasmid carrying *psm-mec* with the -7T→C promoter mutation (pM1) were subjected to Western blotting by anti-AgrA IgG. Each lane contains 3.5 μg proteins of cell extracts. (C) Cell extracts of 24 h-cultures of Newman, MW2 (USA400), and FRP3757 (USA300) strains that were transformed with pF carrying *psm-mec* (multi-copy), or integrated with *psm-mec* into the chromosome (single-copy) were subjected to Western blotting by anti-AgrA IgG (Upper panel). Each lane contains 3 μg proteins of cell extracts. Band intensities of AgrA were measured and are presented in the lower graph. The vertical axis represents the relative value against the AgrA band intensity of the parent strain in each Newman, MW2, and FRP3757 genetic background. Means ± standard deviations from four independent experiments are presented. Student t-test P-values between the parent strain and the *psm-mec*-introduced strain in each genetic background are presented. (D) The *agr* null mutant of Newman transformed with *pMNS-agrBDCA* carrying IPTG-inducible *agrBDCA* and pKE516 (empty vector), or *pMNS-agrBDCA* and pKE516-F carrying wild-type *psm-mec* was cultured in the presence or absence of IPTG. Cell extracts of 24-h cultures were subjected to Western blotting by anti-AgrA IgG. Each lane contains 6 μg proteins of cell extracts. (E) Schematic representation of luc-fusions of the recF promoter, *agrA* SD, the *agrA* ORF, and the luc ORF. Bold gray lines represent the plasmid construct. Horizontal dotted lines represent the regions deleted from the plasmids. Putative binding region means the region predicted to bind to the *psm-mec* RNA by in silico analysis. SD means Shine-Dalgarno sequence of *agrA*. (F) Luciferase activities of Newman strains that were transformed with the luc-fusion plasmids with *psm-mec* (+F) or without *psm-mec* (−F) were measured. The vertical axis represents the
luc-fusions with agrA that can bind psm-mec RNA (pGP-agrA1-luc) and agrA2 that cannot bind psm-mec RNA (pGP-agrA2-luc) (Fig. 2E). The expression of agrA1-luc was suppressed by psm-mec in a similar manner as agrA-luc, although the expression of agrA2-luc was not suppressed by psm-mec (Fig. 2F). These findings suggest that the binding of psm-mec RNA to agrA mRNA leads to the inhibition of agrA translation.

To identify the region of psm-mec RNA that is required for binding agrA mRNA, we constructed a psm-mec-D disrupting 22–52 sequence that is a partial region of the in silico predicted binding region and a psm-mec-M carrying a mutated 21–39 sequence, which are not complementary to agrA mRNA (Fig. 3D). Wild-type psm-mec RNA retarded the migration of agrA1 RNA, whereas these mutated psm-mec RNA did not retard the migration of agrA1 RNA (Fig. 3E). Thus, the 21–52 sequence of psm-mec RNA is required for binding to agrA mRNA. To verify whether binding of psm-mec RNA to agrA mRNA is required to repress the translation of agrA and inhibit PSM mRNA, we examined the effect of psm-mec-D and psm-mec-M on the activity of agrA-luc fusion, the amount of AgrA, and the expression of PSM23. The luciferase activity of agrA-luc fusion was partially restored in the presence of psm-mec-D or psm-mec-M compared with wild-type psm-mec (Fig. 3F).

The amount of AgrA was partially relieved in Newman transformed with psm-mec-D or psm-mec-M compared with that in Newman transformed with wild-type psm-mec (pF), which inhibited the expression of agrA (Fig. 3G). The expression of PSM23 was repressed by wild-type psm-mec, whereas the repression effect was attenuated in psm-mec-D or psm-mec-M (Fig. 3H). Therefore, binding of psm-mec RNA to agrA mRNA is required to repress the translation of agrA and to inhibit PSM23 expression by psm-mec.

Translational inhibition of agrA by psm-mec might be due to the destabilization of agrA mRNA by psm-mec. We examined the stability of agrA mRNA in the presence or absence of psm-mec. In the vector (pND50)-transformed Newman strain, the half-life of agrA mRNA was 11 min, whereas in the psm-mec (pF)-transformed Newman strain, the half-life of agrA mRNA was 5 min (Fig. 4A). Thus, psm-mec slightly decreased the stability of agrA mRNA. RNase III is an endoribonuclease that catalyzes double-stranded RNA and contributes to repress gene expression by the regulatory RNA, RNAIII [20,21,22]. We examined whether RNase III encoded by the rnc gene contributes to the alteration of the half-life of agrA mRNA by psm-mec. In the rnc-deleted mutant, introduction of psm-mec (pF) did not decrease the half-life of agrA mRNA compared with introduction of the vector (pND50) (Fig. 4B). Therefore, RNase III is required for the destabilization of agrA mRNA by psm-mec. We then examined the inhibitory effect of psm-mec on the translation of agrA under the rnc-deletion background, in which psm-mec did not decrease the stability of agrA mRNA. In the rnc-deleted mutant, introduction of psm-mec (pF) decreased the amount of AgrA (Fig. 4C). These results suggest that psm-mec represses the translation of agrA independently of the decrease in stability of agrA mRNA, although psm-mec acts to decrease the stability of agrA mRNA via RNase III. Next, we examined whether the stability of psm-mec RNA was affected by agrA. psm-mec RNA was expressed from an anhydrotetracycline-inducible promoter, because psm-mec transcription is positively regulated by AgrA [23]. There was no difference in the half-life of psm-mec RNA between Newman strain and the agrA-null mutant and the half-life was approximately 20 min (Fig. 4D). In this condition, psm-mec RNA slightly decreased the stability of agrA mRNA, which is consistent with the finding using psm-mec expressed from the native promoter (Fig. 4E). These results suggest that psm-mec RNA is stable and the stability is not affected by agrA mRNA.

Mutation or absence of psm-mec correlates with high expression of extracellular PSMs in MRSA clinical isolates

Because psm-mec inhibits agrA translation, resulting in the repression of PSM3 expression, we hypothesized that the psm-mec mutation was related to the high expression levels of PSM3 in clinical MRSA isolates. We collected 325 clinical MRSA strains from three hospitals in the Kanto area in Japan, and sequenced their psm-mec genes. Eighty-one strains (25%) carried the -7T>C mutation in the psm-mec promoter and one strain carried an insertion of 2.2 kbp and the -4G>A mutation in the psm-mec promoter, both of which repressed the expression of psm-mec in the Newman strain (Table 1). Twenty-eight strains (9%) did not carry psm-mec (Table 1). As we reported previously [12], -7T>C-mutated psm-mec lacked the ability to inhibit PSM3 expression and colony spreading, and to promote biofilm formation in the Newman strain (Fig. S3). The psm-mec carrying an insertion of 2.2 kbp and the -4G>A mutation also lacked these abilities (Fig. S3). In contrast, in other psm-mec mutations, such as D2, D4, and D5, which did not decrease the expression of psm-mec, the inhibitory abilities of PSM3 expression and colony spreading, and the promotion of biofilm formation in the Newman strain were maintained (Fig. S3). We next examined whether 81 strains carrying -7T>C psm-mec and 28 strains without psm-mec had higher amounts of PSM3 than the other 193 strains carrying intact psm-mec. The amount of PSM3 in the culture supernatant of each strain was determined by high performance liquid chromatography analysis. The findings revealed that these strains carrying mutations in psm-mec expressed higher amounts of PSM3 in the culture supernatant than the strains carrying intact psm-mec (Fig. 5). Some strains carrying the mutations produced higher amounts of PSM3 than the CA-MRSA FRP3757 strain (USA300) (Fig. 5). These findings suggest that the psm-mec mutations increase the amount of PSM3 in HA-MRSA isolates, and that there are some strains in the psm-mec-mutated isolates that produce even higher amounts of PSM3 than produced by CA-MRSA.

To investigate whether the genetic backgrounds of the clinical isolates carrying -7T>C psm-mec or no psm-mec differ from those carrying intact psm-mec, we determined the SCCmec types and spa types of all tested strains. One hundred and twenty-seven strains (65%) of the isolates carrying intact psm-mec had type II SCCmec (Table 2). Seventy-five of 81 strains (93%) carrying -7T>C psm-mec had type II SCCmec (Table 2). The results of SCCmec typing were in high agreement with the previously reported data that psm-mec is closely related to the class A mec gene complex carried by either types II, III, or VIII of the SCCmec element [13,23,24]. Most of the strains carrying intact psm-mec or -7T>C psm-mec had type 2 spa (122 (63%) of the isolates carrying intact psm-mec; 69 (85%) of the isolates carrying -7T>C psm-mec (Table 3). In contrast, all isolates not carrying psm-mec also did not carry class A mec and the majority of them carried either type I or IV SCCmec elements (Table 2). Sixteen strains (55%) of the isolates not
Negative Regulation of agrA by psm-mec
Figure 3. psm-mec RNA specifically binds agrA mRNA and inhibits its translation. (A) Hybridization between psm-mec RNA and agrA mRNA was predicted by an in silico program RNA hybrid. Black and gray lines represent strong and weak hydrogen bonds, respectively. (B) Binding between psm-mec RNA and agrA RNA (−20–717) was analyzed using a gel retardation assay. Various amounts of nonlabeled agrA RNA were added to 32P-labeled psm-mec RNA (0.13 pmol), and electrophoresed in 6% native polyacylamide gel. In the right six lanes, nonlabeled psm-mec RNA or yeast tRNA was added to compete with the binding between agrA RNA and 32P-labeled psm-mec RNA. (C) Binding experiment between psm-mec RNA and deletion mutants of agrA RNA. Various amounts of nonlabeled agrA RNA (−20–267) or agrA2 RNA (−20–198) were added to 32P-labeled psm-mec RNA (0.13 pmol). (D) Nucleotide sequences of psm-mec RNA, a deletion mutant of psm-mec RNA (psm-mec-D), and a nucleotide-substituted psm-mec RNA (psm-mec-M) are presented. Red dotted line in psm-mec-D indicates the deleted region. Red letters in psm-mec-M indicate the substituted nucleotides that are not complementary to agrA RNA. (E) Various amounts of nonlabeled psm-mec RNA, psm-mec-D RNA, or psm-mec-M RNA were added to 32P-labeled agrA1 RNA (−20–267), and electrophoresed in 6% native polyacylamide gel. (F) Luciferase activities of Newman strains that were transformed with pG-agrA-luc carrying no psm-mec (−F), psm-mec (+F), psm-mec-D, or psm-mec-M were measured. The vertical axis represents the relative luciferase activity against that of pG-agrA-luc carrying no psm-mec. Means ± standard deviations from three independent experiments are presented. Student t-test P-values are presented. (G) Cell extracts (3 μg protein) of 24 h-cultures of Newman strains transformed with pND50 (empty vector), pMage carrying psm-mec, p-psm-mec-D carrying psm-mec-D, or p-psm-mec-M carrying psm-mec-M were subjected to Western blotting by anti-AgrA IgG (left panel). Band intensities of AgrA were measured (right graph). Means ± standard deviations from three independent experiments are presented. Student t-test P-values are presented. (H) Amounts of PSM3 in the supernatants of 24 h-cultures of Newman strains transformed with pND50 (empty vector). Student t-test P-values are presented.

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carrying psm-mec had type 855 spa and three strains (10%) had type 2 spa (Table 3). These results indicate that most isolates carrying -7T>C psm-mec and intact psm-mec have closely related genetic backgrounds, whereas most isolates not carrying psm-mec have different genetic backgrounds compared to isolates carrying intact or -7T>C psm-mec. In addition, there are various spa types in the isolates carrying -7T>C psm-mec or no psm-mec, indicating that the isolates carrying the psm-mec mutation are polyclonal.

psm-mec is required for suppression of the virulence in MRSA clinical isolates

In our previous study, we transformed S. aureus strains carrying no psm-mec with psm-mec and investigated the function of psm-mec. Use of this method to evaluate gain of function cannot, however, establish the requirement of psm-mec to suppress HA-MRSA virulence. From 193 HA-MRSA strains carrying intact psm-mec, we selected 18 strains that produce the PSM-mec protein and are susceptible to antibiotics, from which psm-mec can be deleted by the antibiotics resistance gene. The psm-mec-deleted mutants of these 18 strains were constructed (Fig. S4), and were examined whether their PSM2 production, AgrA expression, and colony spreading ability were increased, or biofilm formation was decreased. In 13 of 18 strains, each psm-mec-deleted mutant produced more PSMz3 than the respective parent strain (Fig. 6A). In 14 of 18 strains, each psm-mec-deleted mutant produced more PSMz1 and Hld than the respective parent strain (Fig. 6B). In 14 of 18 strains, each psm-mec-deleted mutant expressed more AgrA than the respective parent strain (Fig. 6C). In 15 of 18 strains, each psm-mec-deleted mutant had a greater colony spreading capacity than the respective parent strain (Fig. 6D). In contrast, in 8 of 18 strains, each psm-mec-deleted mutant produced less biofilm than the respective parent strain (Fig. 6E). Therefore, in most HA-MRSA strains, psm-mec is required for the suppression of PSMz2 production, AgrA expression, and colony spreading, as well as the promotion of biofilm formation.

To further verify whether psm-mec is needed to suppress HA-MRSA virulence in animals, we examined the virulence of the psm-mec-deleted mutants of NI-13, SR-1, and NIR-34, which produced higher amounts of PSMz3 than their respective parent strain, using mouse models of skin infection and systemic infection. In the skin infection model, bacterial virulence was quantitatively evaluated by measuring the dermonecrosis area formed by the S. aureus infection [25]. The psm-mec-deleted mutants of NI-13, SR-1, and NIR-34 formed a larger area of dermonecrosis than the respective parent strain (Fig. 7A). In the mouse systemic infection model, the psm-mec-deleted mutants of NI-13 and NIR-34 killed mice faster than the respective parent strain (Fig. 7B), although the psm-mec-deleted mutant of SR-1 killed mice in the same manner as the parent strain (data not shown). These results suggest that psm-mec suppresses the virulence of these HA-MRSA strains against animals.

It was recently revealed that psm-mec is located between two regulatory loci, mecI and mecR2, which are transcribed in the opposite direction of psm-mec and regulate the expression of mecA [26]. Expression of mecA encoding penicillin binding protein 2a interferes with the agr system, reduces toxin expression, and attenuates virulence in mice [27,28]. Deletion of psm-mec between mecI and mecR2 might alter the expression of mecR1-mecI-mecR2 mRNA, resulting in the altered expression of mecA. We examined whether psm-mec-deleted mutants of 18 HA-MRSA strains alter the expression of mecA. In 16 of 18 strains, each psm-mec-deleted mutant expressed an amount of mecA comparable with that of the respective parent strain (Fig. S5). In CR-11 and SR-1 strains, each psm-mec-deleted mutant expressed less mecA than the respective parent strain (Fig. S5). These results indicate that in most HA-MRSA strains, the effect of psm-mec-deletion on virulence phenotypes are not related to mecA expression, whereas in CR-11 and SR-1 strains, the reduced expression of mecA in the psm-mec-deleted mutants might contribute to the observed phenotype.

Concluding remarks

Here, we revealed that psm-mec RNA specifically binds agrA mRNA encoding an S. aureus virulence regulatory factor and inhibits its translation. We further demonstrated that the deletion of psm-mec from HA-MRSA strains carrying intact psm-mec led to increased expression of AgrA and PSMz2. Furthermore, we demonstrated that one-third of HA-MRSA isolates from the Kanto area of Japan harbored -7T>C mutated psm-mec or did not carry psm-mec. These HA-MRSA strains produced high amounts of PSMz3. These findings support the notion that the mutation or absence of psm-mec in HA-MRSA strains leads to the high expression of AgrA, resulting in the high production of exotoxins and high virulence, whereas in almost two-thirds of HA-MRSA strains carrying intact psm-mec, the expression of AgrA is inhibited by psm-mec, resulting in attenuated virulence. In addition, we demonstrated that the integration of psm-mec into the chromosomes of the CA-MRSA strains, which do not carry psm-mec, led to a decrease in the expression of AgrA. Thus, we propose that the absence of psm-mec is a genetic determinant of the high virulence property of CA-MRSA, i.e., the high expression of agr locus. Identification of the psm-mec mutation could be a novel method for predicting the virulence properties of MRSA strains. We have
Figure 4. *psm-mec* affects the stability of *agrA* mRNA. (A) Northern blot analysis was performed to measure *agrA* mRNA stability in Newman strain transformed with an empty vector (pND50) or pF carrying *psm-mec*. Total RNA was extracted from cultures (A600 = 3) at the indicated time point after rifampicin treatment. *agrA* mRNA (RNAII) was detected by 32P-labeled DNA probe. rRNA was stained with ethidium bromide. The amounts of...
agrA mRNA were normalized with the amount of 16S rRNA at each time-point and the amounts of agrA mRNA relative to the amount at 0 min are shown in graph. The half-life at which 50% of agrA mRNA remained was determined by exponential approximation. Data are representative from three independent experiments. (B) agrA mRNA stability was measured in the mrc-deleted mutant transformed with pND50 or pF. Total RNA was extracted from cultures (A600 = 3) at the indicated time point after rifampicin treatment. Data presentation and the calculation of the RNA half-life are the same as in (A). Data are representative from three independent experiments. (C) Cell extracts (4.2 µg protein) of 24-h cultures of Newman strains transformed with pND50 or pF and the mrc-deleted mutant transformed with pND50 or pF were subjected to Western blotting by anti-AgrA IgG (Left panel). Band intensities of AgrA were measured (Right graph). Means ± standard deviations from two independent experiments are presented. Student t-test P-values are presented. (D) psm-mec RNA stability was measured in Newman and the agr-null mutant, which were transformed with anhydrotetracycline-inducible psm-mec (pNDX1-F). S. aureus cells were grown to A600 = 2 in the presence of 0.4 µg/ml of anhydrotetracycline. Total RNA was extracted after rifampicin treatment and electrophoresed. Data presentation and the calculation of the RNA half-life are the same as in (A). Data are representative from two independent experiments. (E) agrA mRNA stability was measured in Newman transformed with empty vector (pNDX1) or anhydrotetracycline-inducible psm-mec (pNDX1-F). S. aureus cells were grown to A600 = 2 in the presence of 0.4 µg/ml of anhydrotetracycline. Total RNA was extracted after rifampicin treatment and electrophoresed. Data presentation and the calculation of the RNA half-life are the same as in (A). Data are representative from two independent experiments.

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revealed that HA-MRSA isolates carrying -7T>C psm-mec had closely related genetic backgrounds with isolates carrying intact psm-mec. Thus, the -7T>C mutation of psm-mec may frequently appear from intact psm-mec. In contrast, HA-MRSA isolates carrying no psm-mec have genetic backgrounds that differ from those carrying intact or -7T>C psm-mec, indicating that isolates not carrying psm-mec are evolutionarily distant from isolates carrying intact or -7T>C psm-mec. It will be interesting to see whether the ratio of these three MRSA groups in hospitals changes over time in relation to clinical outcome.

AgrA activates the transcription of psm-mec [23,29]. We also confirmed that the expression of psm-mec was diminished in the agr-null mutant and that psm-mec expression was restored by the introduction of agrBDC. This study revealed that psm-mec RNA negatively regulates agrA translation. These results indicate that AgrA increases the amount of psm-mec RNA, psm-mec RNA inhibits agrA translation, and the decreased amount of AgrA leads to decreased expression of PSMα as well as of psm-mec RNA itself. Thus, the expression of psm-mec RNA and AgrA is assumed to be maintained in a steady balance by this feedback loop, and the presence of psm-mec might moderately suppress AgrA expression.

Queck et al. revealed that psm-mec has a positive effect on virulence in mouse skin and systemic infection models of the HA-MRSA strain MSA890, in which the amount of PSMc, which has cytolysin activity against human neutrophils, was higher than that of PSMα peptides [29]. Furthermore, the same group revealed that psm-mec had no effect on the virulence of HA-MRSA strains Sanger252, BK1406, and BK23684, in which the amount of PSMc was not higher than that of PSMα peptides [23,29]. In the present study, we constructed the psm-mec-deleted mutants from 18 clinical isolates of HA-MRSA and demonstrated that psm-mec represses the expression of PSMα and AgrA in most of these strains. In addition, we revealed that psm-mec suppresses virulence in mouse skin and systemic infection models of at least two HA-MRSA strains. To examine whether the discrepancy between our results and the results by Queck et al. was due to differences in the experimental procedure, we examined the virulence of MSA890, Sanger252, and their psm-mec-deleted mutants in a mouse systemic infection model. We found that the psm-mec-deleted mutant of the MSA890 strain showed decreased virulence compared with the parent strain, whereas the psm-mec-deleted mutant of Sanger252 did not show decreased virulence compared with the parent strain [Fig. S6], consistent with the reports by Queck et al. Therefore, the genetic backgrounds of the HA-MRSA strains, not differences in the assay system, might explain the discrepancy between our results and the results reported by Queck et al.

RNAIII was the first identified regulatory RNA in S. aureus [9] and regulates the expression of various genes [7]. RNAIII inhibits the translation of spa encoding protein A [20], coa encoding staphylocoagulase [21], and rot encoding a transcription factor [30]. RNAIII carries hairpin loops with a C-rich motif that binds the G-rich sequence of the SD region of target mRNA and inhibits its translation [22,30]. RNAIII forms an imperfect duplex with target mRNA, which is digested by RNase III, and decreases the stability of target mRNA [20,31]. RNAIII is stable with a half-life of over 20 min [20,30]. In the present study, we predicted that the C-rich motif (ACCC) of agrA mRNA binds the SD region (GGGU) of psm-mec RNA [Fig. 3A]. We revealed that psm-mec RNA slightly destabilizes agrA mRNA in an RNase III-dependent manner.

### Table 1. Identification of mutations of the psm-mec gene from MRSA strains.

| Name | Mutation of psm-mec | Expression (%) | Number of isolates |
|------|---------------------|---------------|--------------------|
| D1   | -7T>C               | 0             | 81                 |
| D2   | -42A>G              | 150           | 3                  |
| D3   | -70–71 insertion of 2.2 kbp<sup>1</sup>; -4G>A | 0 | 1 |
| D4   | -74–75 insertion of T | 125           | 18                 |
| D5   | -242–243 insertion of 1.3 kbp<sup>2</sup> | 70           | 1                  |
| Absence | psm-mec            | 0             | 28                 |
| Intact | psm-mec            | 100           | 193                |
| Total |                    | 325           | 100                |

*Mutation of psm-mec is presented as a number of nucleotides from the transcription start site of psm-mec and nucleotide substitutions. T>C means that thymine was exchanged with cytosine. Expression of the respective mutated psm-mec gene in the Newman strain was examined (Fig. S3) and is presented in the column ‘Expression’. 1, DNA fragment of 2206 bp (GenBank, AB 729111). 2, DNA fragment of 1332 bp (GenBank, AB 729110). doi:10.1371/journal.ppat.1003269.g004
Figure 5. MRSA clinical isolates harboring a \( {\text{psm-mec}} \) mutation produce high amounts of PSM\(\text{a}_3\). Nucleotide sequences of \( {\text{psm-mec}} \) genes of 325 MRSA isolates were determined (Table 1). MRSA strains harboring intact \( {\text{psm-mec}} \) (Intact), -7T>C-mutated \( {\text{psm-mec}} \) (-7T>C), or no \( {\text{psm-mec}} \) (Absence) were cultured for 15 h. The amounts of PSM\(\text{a}_3\) in the culture supernatants were measured. The vertical axis represents the relative amount of PSM\(\text{a}_3\) against that of Newman strain. Closed circles represent the amounts of PSM\(\text{a}_3\) of each MRSA strains, which are the means from two independent experiments. Magenta lines represent the averaged amount of PSM\(\text{a}_3\) of each MRSA groups. Cyan dotted line represents the amount of PSM\(\text{a}_3\) of CA-MRSA strain FRP3757 (USA300). Student t-test P-values are presented. ND, not detected.

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Table 2. Typing of SCC\text{mec} of MRSA clinical isolates.

| Number of isolates belonging to each SCC\text{mec} type | \( {\text{ccr}} \) type | II | n.a. | IV | I | n.a. | n.a. | NT\(^2\) |
|--------------------------------------------------------|----------------|-----|-----|----|---|-----|-----|-----|
| Name                                                   | \( {\text{mec}} \) class | A   | A   | B  | B | C2  | A   |
| D1 (-7T>C)                                             | 81             | 75  |     | 2  | 2+5| 2    | 1    | 6    |
| D2                                                     | 3              | 2   |     | 2  | 2+5| 2    | 1    | 1    |
| D3                                                     | 1              |     |     | 2  | 2+5| 2    | 1    | 2    |
| D4                                                     | 18             | 14  | 2   |    |    |      |      |      |
| D5                                                     | 1              |     |     | 2  | 2+5| 2    | 1    | 2    |
| Absence                                                | 28             | 21  | 1   | 1  | 1  |      |      | 5    |
| Intact                                                 | 193            | 127 | 48  | 2  | 1  |      |      | 16   |

\( {\text{ccr}} \) genes and \( {\text{mec}} \) gene complex were identified by multiplex PCRs [48]. All isolates were \( {\text{mecA}} \) positive. SCC\text{mec} types, I, II, and IV were assigned by the combination of types of \( {\text{ccr}} \) gene and \( {\text{mec}} \) gene complex. Abbreviations are as follows:

1n.a., SCC\text{mec} type could not be assigned from the experiments;

2Total, total number of isolates;

3NT, non-typed, since DNA fragment was not amplified by PCR identifying either \( {\text{ccr}} \) genes or \( {\text{mec}} \) gene complex. ‘2+5’ in \( {\text{ccr}} \) type means that both type 2 and type 5 \( {\text{ccr}} \) were identified, indicating that 48 strains (25%) carry type II SCC\text{mec} and SCC carrying \( {\text{ccrC}} \). ‘2+4’ in \( {\text{ccr}} \) type indicates that 2 strains (1%) carry type II or type VIII SCC\text{mec}.

The combination of type 2 \( {\text{ccr}} \) and class C2 \( {\text{mec}} \) gene complex suggests that it might be a novel SCC\text{mec} element. Since it was out of scope of this paper, we classified it in the group of not assigned.

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The psm-mec RNA was stable with a half-life of approximately 20 min (Fig. 4D). Therefore, the molecular mechanism underlying the interaction between psm-mec RNA and agrA mRNA is similar to that of RNAIII. Because the half-life of psm-mec RNA was not changed by the presence of agrA mRNA (Fig. 4D), it is possible that anhydrotetracycline induced excess amounts of psm-mec RNA relative to the amount of agrA mRNA, and thus digestion of the psm-mec RNA/agrA mRNA duplex structure did not affect the half-life of the psm-mec RNA. Furthermore, psm-mec inhibited the expression of AgrA in an RNase III-independent manner (Fig. 4C). These findings suggest that the destabilization of agrA mRNA by psm-mec RNA and RNase III occurs after the inhibition of agrA translation by psm-mec RNA. This is consistent with previous reports that translational repression by a small RNA does not require mRNA destabilization in E. coli and S. aureus [32,33]. Most small RNAs bind the SD sequence of target RNA, although recent reports indicate that some regulatory RNAs bind the coding region of target mRNA [34,35]. A unique feature of the interaction between psm-mec RNA and agrA mRNA is that both RNAs encode proteins and duplex formation involves the coding sequence of both RNAs, i.e., mRNA-mRNA interaction. Because psm-mec RNA binds the coding region of agrA mRNA, which is far from the ribosome binding site, it is possible that the conformational change of agrA mRNA by psm-mec RNA prevents ribosome recruitment or the RNA-RNA pairing inhibits translation elongation. Further studies are needed to clarify whether the interaction between psm-mec RNA and agrA mRNA leads to conformational alteration of the SD structure and to examine the effects of psm-mec RNA on the translation initiation and elongation of agrA mRNA. An mRNA-mRNA interaction might have a broad role in the regulation of gene expression and should be investigated further.

Materials and Methods

Ethics statement

This study was performed in strict accordance with the recommendation of the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology in Japan, 2006. All mouse protocols followed the Regulations for Animal Care and Use of the University of Tokyo and were approved by the Animal Use Committee at the Graduate School of Pharmaceutical Science at the University of Tokyo (approval number: 19–28). All clinical isolates of MRSA were obtained in accordance with the protocols approved by the ethics committee of Nippon Medical School Hospital (approval number: 18-03-49). All patients provided informed consent prior to donating S. aureus isolates. All clinical isolates of MRSA were anonymized because clinical data were not used.

Bacterial isolates

We collected 325 clinical isolates of MRSA strains from Nippon Medical School Hospital (Bunkyo, Tokyo, Japan), Nippon Medical School Chiba Hokusoh Hospital (Inzei, Chiba, Japan), and Sekino Clinical Pharmacology Clinic (Toshima, Tokyo, Japan) from 2008-2010. These strains were streaked on mannitol sodium chloride plates (Eiken Chemical Inc., Tokyo, Japan) and their utilization of mannitol and high-salt resistance were confirmed. Their minimum inhibitory concentration values against oxacillin were examined and resistance to oxacillin was also confirmed. Bacterial strains used in this study are shown in Table 4.
Figure 6. Deletion of psm-mec in MRSA clinical isolates increases the PSMa production, agrA expression, and colony spreading, whereas decreases biofilm formation. (A, B) The amounts of PSMa3 (A) and Hld + PSMa1 (B) of 18 MRSA isolates and its psm-mec-deleted mutants were measured. White bar represents the clinical isolate used as the parent strain. Black bar represents the psm-mec-deleted mutant of the clinical isolate. The vertical axis represents the amount of PSMa in arbitrary units based on A215. Means ± standard deviations from three independent experiments are shown. Student t-test P-values between the parent strain and the psm-mec-deleted mutant are presented. NS, P > 0.05. (C) Cell extracts (3.7 μg protein) of 15 h-cultures of clinical MRSA isolates and the psm-mec-deleted mutants were subjected to Western blotting by anti-AgrA IgG (Upper panel). Band intensities of AgrA were measured and are presented as relative values against that of the parent strain (Lower graph). Means ± standard deviations from three independent experiments are presented. Student t-test P-values between the parent strain and the psm-mec-deleted mutant are presented. NS, P > 0.05. (D) Colony spreading abilities of clinical MRSA isolates and the psm-mec-deleted mutants were evaluated. Overnight cultures were spotted onto soft agar plates and incubated for 24 h at 37°C. The vertical axis represents diameters of giant colonies. Means ± standard deviations from three independent experiments are shown. Student t-test P-values between parent strain and the
DNA manipulation
To regulate the expression of AgrA protein under the control of IPTG, pMNSs was constructed by fusing pMutinT3 [36] and pTetON [37]. pMNSs contains the transcription terminators, Papc promoter, lacZα and lacI from pMutinT3 and pE194 ori, pUC ori, and the spectinomycin resistance gene from pTetON. pMNS is compatible with pKE516 [38]. Plasmids used in this study are shown in Table 4.

Preparation of a polyclonal antibody against AgrA
A DNA fragment containing the agrC gene was amplified by polymerase chain reaction (PCR) using oligonucleotide primer pairs agrA-HisC-F and agrA-HisC-R and pW as a template [39]. The amplified DNA fragment was phosphorylated by T4 polynucleotide kinase and self-ligated, resulting in pW-agrAHis. The DNA fragment was amplified by PCR using oligonucleotide primer pairs Agra-F-Nde and Agra-R-BamHI and pW-agrAHis as a template. The amplified DNA fragment was inserted into pET-9a at the NdeI and BamHI sites, resulting in pET-9a-agrA-His. E. coli BL21(DE3) carrying pLysS was transformed with pET-9a-agrA-His. Transfomants were cultured in Terrific broth containing 1 M sorbitol and 10 mM betaine at 25°C according to Koenig RL et al. [8]. Isopropyl β-D-1-thiogalactopyranoside (0.4 mM) was added to the culture at A600 = 0.3 and cultured further for 2.5 h. Cells were collected and lysed by freezing and thawing, and subsequent sonication in a lysis buffer (20 mM Tris-HCl [pH 7.9], 6 M guanidine hydrochloride, 0.5 M NaCl). His-tagged AgrA was purified using a Ni column (ProBond Resin, Life Technologies, Tokyo, Japan) according to the manufacturer’s protocol. His-tagged AgrA (0.5 mg) was subcutaneously injected into a Japanese white rabbit 5 times at 2-week intervals. Blood was collected from the rabbit and used for IgG purification by protein G-Sepharose.

Western blotting for AgrA
S. aureus overnight culture was inoculated into a 100-fold amount of fresh tryptic soy broth (TSB) and cultured for 24 h or 15 h at 37°C. S. aureus cells were collected by centrifugation from 650 μl of culture and suspended in a lysis buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], 25 μg/ml lysostaphin) and incubated at 37°C for 30 min. The sample was sonicated and centrifuged at 10,000 g for 10 min. The amount of protein in the supernatant was measured by the Bradford method and the protein concentration of different samples was equalized by adding a buffer. Proteins were electrophoresed in 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel at 100 V for 3 h. Proteins were transferred from the gel to a membrane (Immobilon-P, Millipore) in buffer (10 mM CAPS, 20% methanol) at 150 mA for 3 h. The membrane was treated with a blocking buffer (Tris-buffered saline with Tween 20 [TBST] containing 5% Easy Blocker [GeneTex, Irvine, CA]) at room temperature for 1 h. The membrane was treated with a blocking buffer containing 1:100 anti-AgrA IgG at room temperature for 1 h. After washing with TBST, the membrane was treated with a blocking buffer containing 1:2000 anti-rabbit IgG conjugated with alkaline phosphatase at room temperature for 1 h. After washing with TBST, the membrane was treated with a staining buffer (100 mM Tris-HCl [pH9.5], 100 mM NaCl, 50 mM MgCl2, 2% nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate) for 5 min. The band intensity was measured by densitometry scanning [Image J 1.45 s, NIH].

Gel shift assay
DNA fragments encoding psm-mec mRNA or agrC mRNA were amplified by PCR using the primers listed in Table S2 and pE194 and pGP-agrA-luc as a template, and used as templates for in vitro transcription. In vitro transcription was performed using the T7 RiboMAX Express Large Scale RNA Production System (Promega). The 5’ end-labeling of dephosphorylated RNA was performed with T4 polynucleotide kinase and [γ-32P]-ATP. Gel shift assays were performed with 0.13 pmol of labeled RNA (final 19 nM) and various doses of nonlabeled RNA in 7 μl of binding buffer (10 mM Tris-HCl, pH8.0, 30 mM KC1) using our protocol modified from Kawamoto et al. and Antal et al. [40,41]. The samples were incubated at 95°C for 1 min and at 37°C for 90 min. Two microliters of 50% glycerol were added to the samples, which were then electrophoresed in a 6% polyacrylamide gel in 45 mM Tris-borate at 4°C. The gels were dried and RNA-RNA interactions were analyzed by phosphoimaging using Typhoon (GE) and Image Gauge v. 4.23 software (Fujifilm).

Mouse model
The mouse skin infection experiment was performed according to Bunce et al. [25]. Female 6-week old Hos:HR-1 mice were purchased from Hoshino Laboratory Animals (Ibaraki, Japan). S. aureus overnight culture was inoculated into 100-fold amounts of fresh TSB and cultured to A600 = 0.5. Cells were centrifuged and suspended in phosphate-buffered saline (PBS). Colony forming units (CFU) were measured by spreading the suspended cells on TSB agar plates. Mice were anesthetized with pentobarbital and subcutaneously injected with the suspended bacterial cells containing 5% microbeads (Cytodex 1, GE Healthcare). The inflamed area around the injection site was measured daily (length [L] x width [W]). For mouse systemic infection, S. aureus overnight culture was inoculated into 100-fold amounts of fresh TSB and cultured for 20 h. S. aureus cultures were centrifuged and cells were suspended in PBS. Bacterial suspension (10 μl) was injected into the tail vein of 7-week-old female ICR mice. Survival after injection was monitored. All mice were killed after the experiment.

Protein separation by 2-DE
Overnight cultures of S. aureus strains were inoculated into 100-fold amounts of fresh TSB containing 12.5 μg chloramphenicol/ml and cultured for 14 h at 37°C. One milliliter of culture was centrifuged at 10,000 g for 5 min at 4°C and the pellet was frozen in liquid nitrogen. Pellets were resolved with 360 μl of PBS containing 4.8 Unit D Nase, 9.6 μg RNase A, and 9.6 μg lystaphin, and incubated for 60 min at room temperature. Cell lysates were centrifuged at 10,000 g for 5 min at 4°C. TCA was added to the samples (final 10%) and the samples were incubated for 60 min at 4°C. The lysates were centrifuged at 10,000 g for 5 min at 4°C. TCA was added to the samples (final 10%) and the samples were incubated for 60 min at 4°C. The lysates were centrifuged at 10,000 g for 5 min at 4°C. TCA was added to the samples (final 10%) and the samples were incubated for 60 min at 4°C. The precipitates were washed with ethanol twice, and resolved with sample buffer (7 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS, 0.2% bio-lyte 3/10 ampholyte [Bio-rad], 0.001% BPB). The first isoelectric focusing was performed using 11 cm pH 4–7 IPG strips [Bio-rad] and the PROTEAN IEF system [Bio-rad]. The
Negative Regulation of \textit{agrA} by \textit{psm-mec}

**A**

NI-13

- **NI-13** (Parent)
- **NI-13D** (Δ\textit{psm-mec})

![Graph showing dermonecrosis area over time for NI-13 and NI-13D](image)

SR-1

- **Parent** (Open circle)
- **Δ\textit{psm-mec}** (Filled circle)

![Graph showing dermonecrosis area over time for SR-1](image)

NIR-34

- **Parent** (Open circle)
- **Δ\textit{psm-mec}** (Filled circle)

![Graph showing dermonecrosis area over time for NIR-34](image)

**B**

NI-13

- **Parent** (Open circle)
- **Δ\textit{psm-mec}** (Filled circle)

![Graph showing survival of mice over time for NI-13](image)

NIR-34

- **Parent** (Open circle)
- **Δ\textit{psm-mec}** (Filled circle)

![Graph showing survival of mice over time for NIR-34](image)
samples (60 μg protein/200 μl sample buffer) were applied to an IPG strip rehydrated for 12 h, and isoelectrically focused at 250 V for 15 min, 8000 V for 6 h, and 500 V for 24 h. Each IPG gel strip was equilibrated in buffer (375 mM Tris-HCl [pH 8.8], 6 M urea, 20% glycerol, 2% SDS, 130 mM DTT) for 20 min and in 2.5% iodoacetamide for 10 min. The IPG gel strips were embedded onto 12.5%-SDS polyacrylamide gel (16 cm×16 cm) using 1% low-melting agarose. The second dimension electrophoresis was performed at a constant 200 V for 3 h at 4°C. Gels were subsequently stained with Coomassie Brilliant Blue.

Construction of the psm-mec integrated CA-MRSA strains

DNA fragments containing the kanamycin resistance gene, psm-mec, and upstream and downstream genomic regions of the desired integration site were spliced together by overlap extension PCR. The psm-mec-I-cassette was inserted into the SmaI site of pKOR3a, resulting in pKOR3a-psm-mec-I (Fig. S1A). MW2 and FRP7577 strains were transformed with the plasmid and the psm-mec-integrated mutants were obtained using the previous method [12]. The desired integration by double recombination event was confirmed by Southern blot analysis (Fig. S1B). We confirmed that the 5' and 3' ends of psm-mec RNA transcribed from the genome-integrated psm-mec were the same as those transcribed from plasmid-encoded psm-mec (pF) by a circularized RACE experiment [42].

Determination of the 3'-terminus of psm-mec mRNA by S1 mapping

The DNA fragment containing psm-mec was amplified by PCR using primers S2 and F5, and pF as a template. The DNA fragment was digested with Nde I and its 3'-terminus was labeled with [α-32P]-dATP using a Klenow fragment. The labeled DNA fragment was electrophoresed in 5% native polyacrylamide gel in 0.5 x TBE. A single strand (242 bases) of the 32P-labeled DNA was subsequently stained with Coomassie Brilliant Blue. The reaction was terminated by adding PCI (phenol:urea-7.5% polyacrylamide gel. The gels were dried and DNA was resolved by phosphoimaging using Typhoon (GE).

Determination of RNA half-life

The half-life of mRNA was determined as previously described with slight modification [46]. Overnight cultures of S. aureus were inoculated into 100-fold amounts of fresh TSB and cultured to A600 = 1. Cells were collected by centrifugation at 10,000 g for 1 min. Cells were lysed and luciferase activity was measured according to Hanada et al. [45].

Sequencing of psm-mec of clinical isolates

DNA fragments containing psm-mec were amplified by PCR using genomic DNAs of clinical isolates as a template and primer pairs of S2 and S3 (Table S2). DNA fragments containing intact psm-mec and mutated psm-mec were amplified by PCR using primer pairs of S2-XbaI and S3-SacI, and inserted into the XbaI and SacI sites of pND50, resulting in pF, pM1, pD2, pD3, pD4, and pD5. The effects of these plasmids on the Newman strain were evaluated (Fig. S3).

spa typing

Typing of the polymorphic region of the protein A gene (spa) was performed as described previously [47]. Purified spa PCR products were sequenced, and short-sequence repeats were assigned using the spa database website (http://tools.eigenomics.com./Public/Login.aspx).

SCCmec typing

Multiplex PCRs were performed to identify the SCCmec types according to the established method [48]. Primer sets M-PCR1

Figure 7. Deletion of psm-mec in MRSA clinical isolates increases virulence in mice. (A) Mouse skin infection experiments using NI-13, SR-1, NIR-34, and the respective psm-mec-deleted mutants were performed. Mice (HR-1, n = 5) were subcutaneously injected with S. aureus cells and the dermonecrosis area was measured. Means ± standard deviations from the dermonecrosis areas of five mice are shown. Injected CFUs were as follows; NI-13 and its psm-mec-deleted mutant, 4×10^7 CFU; SR-1 and its psm-mec-deleted mutant, 8×10^7 CFU; NIR-34 and its psm-mec-deleted mutant, 2×10^6 CFU. Black stars indicate that Student’s t-test P-values between the parent strain and the psm-mec-deleted mutant were less than 0.05. Upper right panel is a representative image of a mouse injected with NI-13 and the psm-mec-deleted mutant at 143 h after bacteria infection. (B) Mouse systemic infection experiments were performed. ICR mice (n = 10) were intravenously injected with S. aureus cells. Injected CFUs was as follows; NI-13 and its psm-mec-deleted mutant, 4×10^7 CFU; NIR-34 and its psm-mec-deleted mutant, 4×10^6 CFU. Log-rank test P-values between the parent strain and the psm-mec-deleted mutant in NI-13 and SR-1 are 0.0005 and <0.0001, respectively.

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Table 4. Bacterial strains and plasmids used.

| Strains or plasmid | Genotypes or characteristics | Source or reference |
|--------------------|-------------------------------|---------------------|
| **Strains**        |                               |                     |
| S. aureus          | NCTC8325-4, restriction mutant | [15]                |
| Newman             | Laboratory strain, High level of clumping factor | [51]                |
| MW2                | CA-MRSA (USA400)              | [52]                |
| FRP3757            | CA-MRSA (USA300)              | [53]                |
| NI strains         | 40 clinical MRSA isolates from Nippon Medical School Hospital | [13]                |
| NIR strains        | 126 clinical MRSA isolates from Nippon Medical School Hospital | This study |
| CR strains         | 52 clinical MRSA isolates from Nippon Medical School Chiba Hokusoh Hospital | This study |
| SR strains         | 107 clinical MRSA isolates from hospital Sekino Clinical Pharmacology Clinic | This study |
| MN1844             | Newman Δagr::tetM (transduction from RN6911) | [54]                |
| MN1076             | Newman Δncp::pT1076           | This study          |
| MSA890             | MRSA strain carrying type-II SCCmec | [29]                |
| MSA890Δpsm-mec     | MSA890 Δpsm-mec without an antibiotic resistance marker | [29]                |
| Sanger252          | MRSA strain carrying type-II SCCmec | [29]                |
| Sanger252Δpsm-mec  | Sanger252 Δpsm-mec without an antibiotic resistance marker | [29]                |
| **E. coli**        |                               |                     |
| JM109              | General purpose host strain for cloning | Takara Bio |
| BL21(DE3)pLysS     | General purpose host strain for expression of recombinant proteins | Takara Bio |
| **Plasmids**       |                               |                     |
| pET-9a             | T7 promoter based expression vector, Amp' | Novagen |
| pET-9a-agrAHis     | pET-9a with His-tagged agrA | This study          |
| pKOR3a             | Vector for allelic replacement in S. aureus, Cm' | [55]                |
| pKOR3a-psm-mecT    | pKOR3a with psm-mec-cassette; Cm', Tet' | This study |
| pKOR3a-psm-mecP    | pKOR3a with psm-mec-cassette; Cm', Phleo' | This study |
| pKOR3a-psm-mec-I   | pKOR3a with psm-mec-I-cassette; Cm', Kan' | This study |
| pND50             | E. coli-S. aureus shuttle vector; Cm' | [56]                |
| pF                 | pND50 with intact psm-mec from N315 | [13]                |
| pM1                | pND50 with promoter deficient psm-mec (-7T>C) | [13]                |
| pC1                | pND50 with F3 Stop psm-mec | [12]                |
| pFP                | pND50 with codon-replaced psm-mec | [12]                |
| p-psm-mec-D        | pND50 with partial-deleted psm-mec | This study          |
| p-psm-mec-M        | pND50 with nucleotides-substituted psm-mec | This study |
| pGP-luc            | pND50 with recF promoter, –20–27 of agrA, and luc | This study |
| pGP-agrA-luc       | pND50 with recF promoter, –20–717 of agrA, and luc | This study |
| pGP-agrA1-luc      | pND50 with recF promoter, –20–267 of agrA, and luc | This study |
| pGP-agrA2-luc      | pND50 with recF promoter, –20–198 of agrA, and luc | This study |
| pCK20              | S. aureus suicide vector; Cm' | [57]                |
| pInt               | pCK20 with partial genomic region from RN4220 that can integrate into S. aureus chromosome | [13]                |
| pIntF              | pInt with intact psm-mec from N315 | [13]                |
| pW                 | pInt with agr region from Newman | [39]                |
| pMNS               | E. coli-S. aureus shuttle vector carrying Pspac; Amp', Spc' | This study |
| pMNS-agrBDCA       | pMNS with agrBDCA from Newman | This study          |
| pMutinT3           | S. aureus suicide vector; Erm' | [36]                |
| pT1076             | pMutinT3 with partial mc from NCTC8325-4 | This study |
| pIntE              | pMutinT3 with partial genomic region from RN4220 that can integrate into S. aureus chromosome | This study |
| pIntE-F            | pIntE with intact psm-mec from N315 | This study |
| pD2                | pND50 with D2-mutated psm-mec | This study          |
and M-PCR2 were used. When DNA was not amplified by using one of the primer set, the stain was classified as non-typed.

**Construction of the psm-mec-deleted MRSA strains and the nrc-deleted Newman strain**

DNA fragments containing antibiotic-resistant gene and the upstream and downstream regions of psm-mec were spliced together by overlap extension PCR, resulting in a psm-mec-cassette. The psm-mec-cassette was inserted into the SmI site of pKOR3a, resulting in pKOR3a-psm-mec. MRSA strains were transformed with the plasmid and the psm-mec-deleted mutants were obtained using the previously reported method [12]. The desired deletion of psm-mec by double homologous recombination was confirmed by Southern blot analysis (Fig. S4). To disrupt mec in the Newman strain, a single-crossover recombination method was used, as reported previously [49].

**Measurement of PSMs**

The amount of PSM was measured as previously described with slight modification [12]. Overnight bacterial cultures (50 μl) were inoculated into 5 ml fresh tryptic soy broth and aerobically cultured at 37°C for 15 h without antibiotics. The cultures were centrifuged and the supernatants were evaporated using a centrifuge evaporator, and the evaporated products were solved in 40% acetonitrile and evaporated using a centrifuge evaporator (CC-105, TOMY, Tokyo, Japan). The molecular mass in the respective product was determined using liquid chromatography-electrospray ionization mass spectrometry (LC 1100 series, Agilent Technologies, Santa Clara, CA; ESI-MS, Bio-TOFQ, Bruker Daltonics, Billerica, MA) and the respective PSMs were identified as previously described [12]. Hld and PSMx1 were not separated in this system.

**Colony spreading assay**

The colony spreading assay was basically performed according to our previous method [50]. Two microliters of *S. aureus* overnight culture were spotted onto soft TSB agar plates containing 0.24% agar, and was incubated for 24 h at 37°C. The diameter of the giant colony was measured.

**Biofilm formation assay**

The biofilm formation assay was basically performed according to our previous method [12]. *S. aureus* overnight culture was inoculated into a 200-fold amount of fresh TSB containing 0.25% glucose in 96-well polystyrene plates and cultured for 3 days at 37°C. Cells attached to the plate were stained with safranin and measured by A490.

**Supporting Information**

**Figure S1** Integration of psm-mec into chromosomes of CA-MRSA strains. (A) The genomic region that was integrated with psm-mec is schematically represented as the psm-mec-integrated CA-MRSA strain. Probe DNA regions, construct of targeting plasmid, genomic region of CA-MRSA (type-IV SCCmec) are presented above. Predicted lengths of DNA fragments that were digested with *Sph* I are presented. (B) Genomic DNAs of MW2, FRP1757, and their psm-mec-integrated mutants were digested with *Sph* I and subjected to Southern blot analysis using probes 1 and 2. P indicates the parent strain. A, B, and C means independently obtained psm-mec integrated mutants. (TIF)

**Figure S2** Determination of 3′-terminus of psm-mec RNA. The 3′-terminus of psm-mec RNA was determined by S1 mapping. S1-digested products and Maxam-Gilbert sequencing ladders were electrophoresed in 8 M urea-7.5% polyacrylamide gel. Lanes 1 and 2 represent products that were digested with 75 U and 450 U of S1 nuclease, respectively. Lanes AG, CT, and C represent Maxam-Gilbert sequencing ladders. The nucleotide sequences of psm-mec RNA and the antisense RNA are presented on the left side of the panel. Black stars represent the 3′- terminus of psm-mec RNA determined by the migration of the S1-digested product. (TIF)

**Figure S3** Effect of the mutated psm-mec sequences found in clinical MRSA isolates on *S. aureus* Newman strain. (A) The amount of PSM-mec in the culture supernatant of the Newman strain were transformed with empty vector (pND50), intact psm-mec (pF), D1-mutated psm-mec (pM1), D2-mutated psm-mec (pD2), D3-mutated psm-mec (pD3), D4-mutated psm-mec (pD4), or D5-mutated psm-mec (pD5) was measured. The vertical axis represents the relative amount of PSM-mec against that of Newman transformed with pF. Means ± standard deviations from four independent experiments are shown. Student t-test P-values between pF-transformed Newman and other strains are presented. NS, p-value ≥ 0.05.

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**Table 4. Cont.**

| Strain or plasmid | Genotypes or characteristics | Source or reference |
|--------------------|-------------------------------|---------------------|
| pD3                | pND50 with D3-mutated psm-mec | This study          |
| pD4                | pND50 with D4-mutated psm-mec | This study          |
| pD5                | pND50 with D5-mutated psm-mec | This study          |
| pNDX1              | pND50-based *S. aureus-*E. coli shuttle vector carrying TetR and xyl/tet from pWH353; Cm² | [58] |
| pNDX1-F            | pNDX1 with intact psm-mec from N315 | [55] |
| pKE16              | *S. aureus-*E. coli shuttle vector, Emr¹, Amp² | [38] |
| pKE16-F            | pKE16 with intact psm-mec from N315 | This study |

*aAmp, ampicillin; Cm, chloramphenicol; Tet, tetracycline; Phleo, phleomycin; Kan, kanamycin; Spc, spectinomycin.
doi:10.1371/journal.ppat.1003269.t004
P>0.05, ND, not detected. (B, C) The amount of PSMx3 (B) and PSMz1+Hld (C) of the psm-mec-transformed strains described above was measured. The vertical axis represents the relative amount of PSMx3 against that of Newman transformed with pND50. Means ± standard deviations from four independent experiments are shown. Student t-test P-values between Newman and other strains are presented. NS, P>0.05. (D) Colony spreading ability of the above psm-mec-transformed strains was evaluated. Two microliters of S. aureus overnight cultures was spotted onto soft agar plates and incubated at 37°C for 8 h. Diameter of the giant colony was measured. Means ± standard deviations from four independent experiments are shown. Student t-test P-values between pF-transformed Newman and other strains are presented. NS, P>0.05. (TIF)

Figure S4 Development of psm-mec from clinical MRSA isolates. (A) Schematic representation of the genomic region around psm-mec in type-II SCCmec. psm-mec was deleted by tetL in NI-13, NI-18, CR-11, CR-12, CR-18, CR-29, CR-38, SR-8, NIR-34, NIR-45, and NI-7 strains. psm-mec was deleted by the phleomycin resistance gene in NI-4, NI-22, NI-36, SR-1, NIR-121, NI-3, and NI-38 strains. DNA fragment lengths that were digested by Bgl II are presented. (B) Genomic DNAs of 18 clinical MRSA strains and their psm-mec-deleted mutants were digested with Bgl II and subjected to Southern blot analysis using the probes presented in (A). P indicates the parent clinical strain. A, B, and C indicate independently obtained psm-mec-deleted mutants. (TIF)

Figure S5 Expression of mecA in the psm-mec-deleted mutants of clinical isolates. Northern blot analysis was performed to detect mecA mRNA in the psm-mec-deleted mutants and clinical isolates. Total RNA was extracted from cultures at the log phase (A600 = 0.5) and electrophoresed. rRNA stained with ethidium bromide is shown. Data are representative from three independent experiments. (TIF)

Table S1 Identification of proteins upregulated by psm-mec RNA in the FRP3757 strain. (DOC)

Table S2 Primers used in the study. (DOC)

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Author Contributions

Conceived and designed the experiments: CK. Performed the experiments: CK. Analyzed the data: CK KS. Contributed reagents/materials/analysis tools: KO CK YS MI YO HM GN TF SN XH. Wrote the paper: CK KS.

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