Published in final edited form as:

Int J Med Microbiol. 2014 July ; 304(0): 637–644. doi:10.1016/j.ijmm.2014.04.008.

Genome-wide analysis of the regulatory function mediated by the small regulatory *psm-mec* RNA of methicillin-resistant *Staphylococcus aureus*

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Summary

Several methicillin resistance (SCC*mec*) clusters characteristic of hospital-associated methicillin-resistant *Staphylococcus aureus* (MRSA) strains harbor the *psm-mec* locus. In addition to encoding the cytolysin, phenol-soluble modulins (PSM)-*mec*, this locus has been attributed gene regulatory functions. Here we employed genome-wide transcriptional profiling to define the regulatory function of the *psm-mec* locus. The immune evasion factor protein A emerged as the primary conserved and strongly regulated target of *psm-mec*, an effect we show is mediated by the *psm-mec* RNA. Furthermore, the *psm-mec* locus exerted regulatory effects that were more moderate in extent. For example, expression of PSM-*mec* limited expression of *mecA*, thereby decreasing methicillin resistance. Our study shows that the *psm-mec* locus has a rare dual regulatory RNA and encoded cytolysin function. Furthermore, our findings reveal a specific mechanism underscoring the recently emerging concept that *S. aureus* strains balance pronounced virulence and high expression of antibiotic resistance.

Keywords

Staphylococcus aureus; MRSA; virulence; antibiotic resistance

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Introduction

Staphylococcus aureus is a dangerous human pathogen, which produces a series of toxins and other factors to avoid elimination by human innate host defense (Lowy, 1998). Resistance to efficient anti-staphylococcal antibiotics such as the penicillins and methicillin further complicates treatment of infections with virulent *S. aureus* (Lowy, 2003). Methicillin resistance is encoded on mobile genetic elements called staphylococcal cassette chromosome (SCC) *mec* (Hiramatsu et al., 2001). Several types of SCC*mec* exist. Recently, we discovered that SCC*mec* types II, III, and VIII, found in a large number of epidemic hospital-associated MRSA strains, contain a gene encoding a potent cytolysin of the phenol-soluble modulins (PSM) family, called PSM-*mec* (Chatterjee et al., 2011; Queck et al., 2009). PSM-*mec* is encoded adjacent to the *mecA/mecR/mecI* methicillin resistance gene cluster. The PSM-*mec* peptide efficiently lyses human neutrophils and erythrocytes and has pro-inflammatory capacities (Queck et al., 2009), similar to several other members of the PSM family (Cheung et al., 2010; Wang et al., 2007). Thus, in a way not previously described for *S. aureus*, *psm-mec*-encoding SCC*mec* elements allow for simultaneous acquisition of antibiotic resistance and virulence determinants, representing an important element for the evolution of *S. aureus* as a highly successful pathogen (Otto, 2010b).

However, there have been contrasting results regarding the contribution of *psm-mec* to MRSA virulence. We previously showed that, unless overshadowed by strong production of other PSMs, the PSM-*mec* toxin has a significant contribution to MRSA virulence (Chatterjee et al., 2011; Queck et al., 2009). Accordingly, we found that the *psm-mec* locus increases virulence or has no effect, dependent on which strain is used (Chatterjee et al., 2011). In contrast, Kaito et al. reported that *psm-mec* has gene regulatory functions that ultimately decrease, rather than increase, MRSA virulence. First, they described and attributed gene regulatory functions to an open reading frame (ORF) called “*fudoh*”, which shows considerable overlap with *psm-mec*, but is encoded in the opposite direction (Kaito et al., 2008). They later found that the *psm-mec* locus has regulatory functions that are independent of the PSM-*mec* peptide (Kaito et al., 2011), notably including down-regulation of expression of the strongly cytolytic PSM α peptides (Wang et al., 2007). Rather than to “*fudoh*”, this was then attributed to a *psm-mec*-encoded RNA (Kaito et al., 2011). Recently, they reported that the *psm-mec* RNA interacts with the *agrA* transcript, thereby decreasing production of the PSM regulator AgrA (Queck et al., 2008), but only in some strains (Kaito et al., 2013). Accordingly, in strains that showed a *psm-mec*-dependent difference in AgrA expression, production of PSM α peptides was increased, in selected strains reaching a maximum of about factor four (Kaito et al., 2013). In three strains, in which this effect was most pronounced, an impact on virulence was observed. Despite the overall minor and highly strain-dependent effect of *psm-mec* on PSM α production and virulence, these authors concluded that absence of the *psm-mec* locus is responsible for the increased virulence potential of community- versus hospital-associated MRSA in a general fashion (Kaito et al., 2013).

To analyze the regulatory impact of the *psm-mec* locus in detail, we here performed genome-wide transcriptional profiling of two *psm-mec* mutants in comparison with the corresponding isogenic MRSA wild-type strains. Furthermore, we produced single nonsense

basepair mutations in the *psm-mec* and (hypothetical) “*fudoh*” start codons to determine whether gene regulatory functions are due to the PSM-mec peptide, a putative *psm-mec* regulatory RNA, or “*fudoh*”. Notably, these mutations were produced in the genome of *psm-mec*-containing MRSA strains to analyze regulatory functions in the natural strain background, without an impact of plasmid-based artificial over-expression. Our results emphasize the strain dependence of *psm-mec* regulation, but identify protein A as the only target of *psm-mec* that is strong in extent and conserved in at least a series of *psm-mec*-positive MRSA strains.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains used in the present study are shown in Table. 1. MRSA252 is an epidemic SCCmec type II strain from the United Kingdom. MSA820, MSA890, MSA1601, and MSA3407 are MRSA strains recovered in the 1980s from Rhode Island (MSA820, MSA1601), Texas (MSA890) and New York (MSA3407), respectively (Fitzgerald et al., 2001; Musser and Kapur, 1992), also of SCCmec type II (Queck et al., 2009). All bacteria were grown in tryptic soy broth (TSB). A single base mutation of the start codon was introduced into *psm-mec* (resulting in *psm-mec**) and into *fudoh* (resulting in *fudoh**), with a procedure based on allelic replacement using plasmid pKOR1 (Bae and Schneewind, 2006). Growth patterns of the mutants in TSB were unchanged compared to the wild-type strain (Fig. 1). The *agr* mutant of strain MSA3407 was produced by phage transduction from strain RN6911, as described (Wang et al., 2007) and its *psm-mec* mutant was produced as described (Queck et al., 2009). For the construction of complementation plasmids, regions of DNA containing the respective promoter and gene were amplified from *S. aureus* genomic DNA of strain MSA3407, and cloned into plasmid pT181 via the EcoRI/BamHI sites. All oligonucleotides were synthesized by Sigma (Table 2).

Measurement of phenol-soluble modulins (PSMs)

PSM amounts were measured by reversed-phase high-pressure liquid chromatography/electrospray mass spectrometry (RP-HPLC/ESI-MS) using an 1100 HPLC system connected to an MSD Trap SL mass spectrometer (Agilent). Samples (100 µl) were injected onto a Zorbax SB-C8 2.3 × 30 mm column (Agilent) and a 0.1% trifluoroacetic acid (TFA) water/0.1% TFA acetonitrile gradient was applied as follows (flow rate, 0.5 ml/min); 10% acetonitrile from 0 to 2 min, 50% acetonitrile from 2 to 4 min, 50 to 100% acetonitrile linear gradient from 4 to 9 min, 100% acetonitrile from 9 to 13 min, 0% acetonitrile from 13 to 16 min. Relative PSM production was determined by measuring the peak area of the extracted ion chromatogram corresponding to multiply-charged m/z values of each PSM by using QuantAnalysis software (Agilent Technologies).

RNA isolation, transcriptional profiling, and quantitative RT-PCR

RNA was isolated as previously described using an RNeasy Mini Kit (Qiagen) as recommended in a standard protocol (Wang et al., 2007) from cultures grown in TSB to late exponential growth phase (8 h). Remaining DNA was removed using RNase-free DNase I (Amersham Biosciences). Removal of contaminant DNA was confirmed by PCR. The

reaction product was cleaned up with an RNeasy mini column. cDNA was synthesized and labeled according to the manufacturer's suggestions for Affymetrix antisense genome arrays (Affymetrix). A gel shift assay with NeutrAvidin (Pierce Biotechnology) was performed to estimate the labeling efficiency based on the instructions from Affymetrix. Biotinylated *S. aureus* cDNA was hybridized to custom Affymetrix GeneChips (RMLChip 3) with 96% coverage of genes from MW2 (2534 probe sets of 2632 ORFs) and scanned according to standard GeneChip protocols (Affymetrix). Each experiment was replicated at least 3 times. Affymetrix GeneChip Operating Software (GCOS v1.4, <http://www.affymetrix.com>) was used to perform the preliminary analysis of the custom chips at the probe-set level. Genes were regarded as differentially regulated when they passed significance tests and the factor of regulation was greater than two. The complete set of microarray data was deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and is accessible through GEO Series accession number GSE51366. Quantitative RT-PCR was performed as described (Wang et al., 2007).

Western blot analysis

Harvested bacterial pellets were washed with phosphate-buffered saline (PBS) and then resuspended in PBS to the same OD_{600nm}. Cells were treated with lysostaphin for 5 min at 37°C and then centrifuged (13,000 rpm, 4°C, 5 min). Supernatants were mixed with 2 × SDS PAGE loading buffer and samples were heated for 5 min at 95°C. Protein bands were transferred from 12% bis/acrylamide SDS-PAGE gels onto nitrocellulose membranes using an iBlot Dry Blotting System (Invitrogen). Blots were incubated with anti-protein A antibody (Sigma) at 1:5,000 dilution and IRDye800CW-conjugated anti-rabbit antibody at 1:10,000 dilution. Signal was visualized using an Odyssey Infrared Imaging System (LICOR Biosciences). A positive control of staphylococcal surface protein A (Sigma) was used at 25 µg per lane. Membranes were washed in between incubations with Tris-buffered saline (pH 7.4, KD Medical) containing 0.1% Tween-20 (Sigma). All antibodies were diluted in Odyssey blocking buffer (Licor Biosciences).

Methicillin resistance

Resistance to methicillin was determined using oxacillin (Sigma) using a microtiter plate-based assay. Two hundred µl of a 2.048 mg/ml oxacillin solution was pipetted into a well, from which 100 µl were transferred to the next well. Then, 100 µl were transferred from that well to the next. This process was repeated another ten times. Then, 100 µl of a 1:100 dilution of a pre-culture grown overnight was added to each well, resulting in oxacillin concentrations ranging from one to 1024 µg/ml. Plates were grown for 24 h with shaking at 180 rpm at 37°C. Then, optical density at 600 nm was measured using an ELISA plate reader. The analysis was performed in quadruplicate (four wells per strain and concentration).

5'- and 3'-rapid amplification of cDNA ends (RACE)

5'- and 3'- RACE was performed to identify the full length of the *psm-mec* RNA transcript. 3'- RACE was performed as described previously (Argaman et al., 2001). Briefly, a 3' RNA adapter, primer E1 was ligated to calf intestine alkaline phosphatase treated-RNA with T4

RNA ligase (New England Biolabs) according to the manufacturer's instructions. Reverse transcription was performed with primer E4. PCR was performed using primers E4 and *psm-mec* GSP3. PCR products were purified and then sequenced with primer *psm-mec* GSP3. 5'- RACE was performed using the 5'- RACE System for Rapid Amplification of cDNA Ends Kit (Invitrogen). Primer PSM-mec GSP6 was used as the RNA adapter. PCR was performed using primers PSM-mec GSP6 and PSM-mec GSP7. PCR products were purified and then sequenced with PSM-mec GSP7. Mutations were confirmed by DNA sequencing.

Statistics

1-way ANOVA with Bonferroni's post-tests or unpaired *t* tests were used to calculate 2-tailed *P* values using Graph Pad Prism 6 software. *P* < 0.05 was considered significant. Error bars depict mean ± SEM.

Results

To analyze the gene regulatory function of the *psm-mec* locus, we used two *psm-mec*-positive MRSA strains and their isogenic *psm-mec* deletion mutants in genome-wide transcriptional profiling (microarray) analyses. Strain MSA3407 is characteristic of the subset of PSM-mec-positive strains that produce high amounts of PSM-mec and other PSMs (Queck et al., 2009). The other, MRSA252, produces lower amounts of PSM-mec and other PSMs (Queck et al., 2009), due to the recently described *agrC* mutation in contemporary isolates of clonal complex 30 strains (DeLeo et al., 2011) and the fact that all PSMs including PSM-mec are regulated by Agr (Chatterjee et al., 2011; Queck et al., 2008). MRSA252 was included in the analysis because it is a genome-sequenced strain (Holden et al., 2004). Notably, we confirmed by DNA sequencing that none of the strains used in the present study contains the -7 T>C mutation in the *psm-mec* promoter shown by Kaito et al. to lead to absence of the suppression of AgrA and PSMα expression (Kaito et al., 2013).

According to the results of our microarray analyses, a series of genes were regulated by the *psm-mec* locus in the two strains (Tab. 3). The factors of regulation were overall higher in MSA3407 than MRSA252, most likely due to the fact that *psm-mec* is more strongly expressed in MSA3407 (Queck et al., 2009). However, the *psm-mec* regulons of the two strains only showed very limited similarity to each other; and the only gene with pronounced *psm-mec*-dependent expression changes that was detected in both regulons was the *spa* gene coding for protein A. Other strongly regulated genes comprised purine, sugar, and amino acid metabolism genes. However, these effects were not consistent among the tested strains. Of note, we did not detect genes of the Agr regulon, such as toxin genes, to be significantly changed in expression in the fashion that the results by Kaito et al. would suggest (Kaito et al., 2013). This suggests that the effects of *psm-mec* in this strain are not mediated via an effect on AgrA.

We then verified the microarray results using qRT-PCR, focusing on strain MSA3407, owing to the strong extent of *psm-mec* expression and observed *psm-mec*-dependent regulatory effects in that strain. In these analyses, we included the complete *psm-mec* deletion strain and single genomic basepair mutants abolishing translation of the *psm-mec*

gene and the hypothetical “*fudoh*” gene (*psm-mec** and *fudoh** strains, respectively). The qRT-PCR results confirmed the extremely strong effect of the *psm-mec* locus on transcription of the *spa* gene (factor of ~ 175) and demonstrated that this effect was not due to the hypothetical protein product of the “*fudoh*” ORF (Fig. 1A). Importantly, *spa* expression levels in the *psm-mec** strain with abolished production of the PSM-mec peptide were only slightly lower than those in the wild-type strain, indicating that the regulatory effect is largely due to the *psm-mec* RNA rather than the PSM-mec peptide. Furthermore, we attempted to analyze the direct, according to our microarray results, AgrA-independent effect of *psm-mec* on *spa* transcription using an *agr* mutant of strain MSA3407. However, we could not detect significant differences in *spa* transcript levels between the *agr* and *agr*/*psm-mec* deletion strains (Fig. 1C), likely because the effect of the *agr* deletion on *spa* is too strong in comparison. The overwhelming effect of *agr* became evident in the lack of significance computed by ANOVA for the wild-type vs. *psm-mec* comparison (compared to the significance attributed to the wild-type vs. *agr* or *agr*/*psm-mec* comparisons). Using a t-test directly comparing these two strains, the difference was, however, significant ($p=0.0003$), in accordance with the data shown in Fig. 1A.

Western blot analysis showed increased expression of protein A in the MSA3407 *psm-mec* wild-type compared to the *psm-mec* deletion strains, in accordance with up-regulation of the *spa* transcript (Fig. 2). We found similar effects on protein A production in strains MSA820 and MSA1601, while no difference was found between the MSA890 and MRSA252 wild-type and corresponding *psm-mec* deletion mutants. These results indicate that strong expression of *psm-mec*, as present in strains MSA3407, MSA1601, and MSA820, but not MSA890 and MRSA252 (Queck et al., 2009), is a prerequisite for an impact on protein A expression. Furthermore, they show that *psm-mec* RNA-dependent regulation is conserved among different *S. aureus* strains.

In addition, we analyzed by qRT-PCR a series of further mostly virulence-associated genes that showed *psm-mec*-dependent regulation in the microarray results. The effects of *psm-mec* on the expression of those genes were overall low and sometimes not significant. Notably, the hypothetical “Fudoh” protein did not have a significant impact on the expression of any of the investigated genes. In these assays, we also included the *psmA* operon, which according to Kaito et al. is regulated by the *psm-mec* locus independently of the PSM-mec peptide (Kaito et al., 2013; Kaito et al., 2011), and because the short *psmA* genes were not included in the microarray chip used. We could confirm that *psmA* transcripts were influenced in a PSM-mec peptide-independent manner. However, in accordance with our previous results (Chatterjee et al., 2011) and those of Kaito et al. (Kaito et al., 2013), this effect was minor (factor of ~ 3). Furthermore, measurement of PSM peptide production indicated that there was no considerable impact of the *psm-mec* locus, either by the *psm-mec* RNA, the PSM-mec peptide, or the hypothetical “Fudoh” protein, on the production of any PSM peptide (Fig. 3). As expected, PSM-mec production was abolished in the *psm-mec* deletion strain and unchanged in the *fudoh** strain.

Interestingly, we reproducibly detected very small amounts of PSM-mec peptide in the *psm-mec** strain (Fig. 3), indicating that the mutated codon ATA may be used as non-canonical start codon, albeit in a strongly reduced fashion. While to our knowledge use of ATA as

start codon has not been described in staphylococci, it was reported in other bacteria, for example *E. coli* (Kopke and Leggatt, 1991). The fact that the *psm-mec* gene is one of the most strongly translated genes in *S. aureus* may play a role for accepting a non-canonical start codon to a certain extent. This does not change the basis of our conclusions; it may only cause a slight under-estimation of the effect mediated by the PSM-mec peptide.

Notably, the *psm-mec* locus had a moderate, but significant negative impact on the expression of the neighboring *mecA* gene (~ 6-fold by qRT-PCR and ~ 2-fold by microarray in MSA3407; ~ 3-fold by microarray in MRSA252), apparently mediated at least in part via the PSM-mec peptide, suggesting that PSM-mec expression limits expression of methicillin resistance. Accordingly, resistance to oxacillin was increased significantly in the MSA3407 *psm-mec* deletion strain and the isogenic *psm-mec** strain, compared to the corresponding wild-type strain (MICs: MSA3407, 49 µg/ml; *psm-mec* mutant, 80 mg/ml; *psm-mec**, 87 µg/ml) (Fig. 4).

We also performed qRT-PCR analyses of the same genes as analyzed for the MSA3407 strain and derivatives using the *psm-mec* deletion, *psm-mec**, and *fudoh** mutants of strain MSA890 (Fig. 1). We showed previously that this strain only produces very low absolute, but high amounts of PSM-mec relative to those of other PSMs, resulting in a measurable virulence-enhancing effect of the *psm-mec* locus (Queck et al., 2009). In strain MSA890, we did not detect considerable effects on the expression of *spa* or any other of the selected genes, indicating that the *psm-mec* locus must be expressed at high levels for gene regulatory effects to occur, in accordance with the results we obtained on protein A production.

Together, our experiments identified the *spa* gene as the only conserved target of *psm-mec*-dependent regulation that is considerable in extent and occurs independently of the PSM-mec peptide. As our results indicated that the *psm-mec* gene is embedded within a regulatory RNA, we set out to precisely determine the extensions of that RNA. 5'- and 3'- RACE experiments showed that the *psm-mec* RNA only slightly extends from the boundaries of the *psm-mec* gene (Fig. 5), in accordance with the results obtained by Kaito et al. (Kaito et al., 2013; Kaito et al., 2011). To verify that this RNA has the regulatory capacity determined in the microarray and qRT-PCR experiments, we complemented the *psm-mec* deletion mutant of strain MSA3407 with a plasmid expressing the corresponding sequence (Fig. 6). This construct increased the low-level expression of *spa* of the mutant strain by about the same factor as detected by comparison of the MSA3407 wild-type and *psm-mec* deletion strains, confirming that this RNA confers the regulatory features described above. In addition, we produced constructs with single basepair mutations of the *psm-mec* gene, similar to those introduced in the genomes of strains MSA3407 and MSA890. Results with these constructs confirmed those obtained with the genomic mutants, inasmuch as abolished translation of *psm-mec* did not affect *spa* transcription. Finally, we performed qRT-PCR with the complementation clones also with the other selected genes (see Fig. 1). Clones with the plasmid expressing *psm-mec* showed results opposite to those shown in Fig. 1 in strains with deletion of *psm-mec*, confirming the results shown in Fig. 1 (Fig. 6). Furthermore, clones with plasmids containing the single basepair mutations of the *psm-mec* genes showed similar

results, with no significant difference to the respective clones expressing the PSM-mec peptide, confirming that the effects are mediated by the *psm-mec* RNA.

Discussion

Results obtained in the present study reveal the *spa* gene encoding protein A as the primary conserved regulatory target of the *psm-mec* srRNA. Kaito et al. previously also noted *psm-mec*-dependent up-regulation of protein A expression in some strains. However, these authors attributed this effect to a general inhibition of the Agr system by the *psm-mec* RNA (Kaito et al., 2013). In contrast, our genome-wide transcriptional profiling analyses did not show gene regulatory changes, such as decreased production of toxins, that would be expected from an inhibition of AgrA translation and thus, functionality of the Agr system, as reported by Kaito et al. (Kaito et al., 2013). Of note, our findings do not challenge the results by Kaito et al., but the claim by those authors on the general applicability of their results and the conclusion that the mechanism they described would explain the previously noted increased virulence of CA- versus HA-MRSA (Kaito et al., 2013; Otto, 2010a). Rather, the impact of *psm-mec* on virulence appears to be highly strain-dependent.

We noted previously that the acquisition of *psm-mec*-containing SCCmec elements may simultaneously enhance virulence and antibiotic resistance properties in some MRSA strains such as MSA890 (Otto, 2010b). However, we also show here that expression of the PSM-mec peptide may lead to decreased expression of the *mecA* gene, resulting in decreased oxacillin resistance. This finding is in accordance with a recently emerging concept indicating that expression of methicillin resistance in MRSA strains is balanced versus virulence gene expression (Cheung et al., 2011; Rudkin et al., 2012). Possibly, considerable expression of both PBP2a and virulence determinants, PSM-mec in the present case, would cause too strong a metabolic burden for the bacteria.

Interestingly, the *psm-mec* locus has striking similarities with RNAIII, one of the best-studied srRNAs, which represents the intracellular regulatory effector molecule of the *S. aureus* accessory gene regulator (Agr) quorum-sensing system (Novick et al., 1993). Both the RNAIII and *psm-mec* srRNAs regulate the expression of virulence-associated genes and encode a cytolytic PSM peptide, PSM-mec in the case of the *psm-mec* srRNA and δ -toxin in the case of RNAIII. Based on the comparison of *psm* gene loci and their regulation, we proposed previously that RNAIII evolved around the *hld* gene coding for the PSM δ -toxin, thus linking quorum-sensing control of virulence genes to that of PSMs (Queck et al., 2008). The *psm-mec* srRNA might have evolved around the *psm-mec* gene in a similar fashion. Of note, the *psm-mec* locus so far represents the only known example, in addition to RNAIII, that combines virulence-enhancing properties mediated by an srRNA and an encoded protein. Together with the *Escherichia coli* *srgS* RNA (Wadler and Vanderpool, 2007), RNAIII and *psm-mec* form rare examples of srRNAs that also encode proteins.

The mechanism by which the *psm-mec* RNA controls the level of *spa* transcript is unclear. We speculated that it may bind to the *spa* promoter region; and bioinformatics analysis in fact suggested base pairing with the region upstream of the *spa* gene with the *psm-mec* RNA. However, electrophoretic mobility assays that we performed did not reveal specific

binding (data not shown). Furthermore, in a previous report, the mapped *spa* promoter sequence did not include the region for which we detected basepairing in silico (Gao and Stewart, 2004).

A main function of protein A is to non-specifically bind immunoglobulins, thus producing a “camouflage coat” preventing opsonization via specific antibodies (Forsgren and Nordstrom, 1974). This control may lead to extended protection from antibody-mediated host defenses. However, it is in general debatable whether the acquired immune system plays a significant role in controlling *S. aureus* infection (Lin et al., 2009). Still, protein A has multiple functions in pathogenesis that are not dependent on evading the acquired immune defense (Gomez et al., 2004; Merino et al., 2009). We performed mouse models of bacteremia and pneumonia using the MSA3407 strain and its isogenic *psm-mec* mutant, in addition to the skin infection model performed previously (Queck et al., 2009), but were unable to detect significant differences (data not shown). Likely, the effect of *psm-mec*-dependent differential protein A expression on virulence may be too low and overshadowed by other virulence factors, such as α -toxin, leukocidins, and other PSMs.

Finally, it has remained unclear whether the gene product of the “*fudoh*” ORF first believed to be involved in gene regulatory effects attributed to the *psm-mec* locus (Kaito et al., 2008) participates in gene regulation. Our results using genomic and plasmid constructs with a mutated start codon abolishing “*fudoh*” translation clearly demonstrate that the hypothetical gene product of that ORF is not involved in the gene regulatory functions described here and elsewhere (Kaito et al., 2008; Kaito et al., 2011). Furthermore, it is highly likely that there is no “*fudoh*” gene or protein, given the wealth of data now available on the function of the overlapping *psm-mec* gene and its product, the PSM-mec peptide (Chatterjee et al., 2011; Kaito et al., 2008; Kaito et al., 2011; Queck et al., 2009).

In conclusion, we identified the *spa* gene as a conserved and strongly regulated target of the *psm-mec* locus. The negative impact of PSM-mec on the expression of *mecA* represents a specific mechanistic example underlining the recent concept that MRSA strains balance methicillin resistance versus virulence gene expression. However, our results also underline the strain dependence of the impact of *psm-mec* on virulence and methicillin resistance.

Acknowledgments

This study was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, U. S. National Institutes of Health.

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Fig. 1.

Effects of *psm-mec* on virulence gene expression. (A) Expression of selected virulence-associated genes in wild-type, *psm-mec* deletion and *psm-mec* and *fudoh* start codon deletion mutants in strains MSA3407 and MSA890. wt, wild-type strain; *psm-mec*, *psm-mec* gene deletion strain; *psm-mec*^{*}, strain harboring a *psm-mec* start codon mutation abolishing translation; *fudoh*^{*}, *fudoh* (hypothetical) start codon mutation abolishing translation of hypothetical Fudoh protein. (B) Corresponding growth curves. (C) Effect of *agr* and *agr/psm-mec* deletion on *spa* transcription in strain MSA3407. (A, C) Gene expression was determined using qRT-PCR in late exponential phase (8-h) cultures. Values are from measurements performed at least in triplicate (three biological replicates, measured three times each) and relative to expression of the housekeeping gene *gyrB*. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Fig. 2.

Western blot analysis of protein A expression. Surface protein preparations obtained by lysostaphin digestion from 8-h cultures grown in TSB were probed using a specific anti-protein A antibody. 3407, strain MSA3407; similar results were obtained with strains MSA1601 and MSA820. 252, strain MRSA252; similar results were obtained with strain 890. Strain LAC and isogenic *spa* (protein A) mutant were used as controls.

Fig. 3.

PSM production in MSA3407 wild-type, *psm-mec* deletion and *psm-mec* and *fudoh* start codon deletion mutants. Culture filtrates from 16-h cultures grown in TSB were analyzed for PSM production by HPLC/MS. Parts of bars with cross stripes represent N-deformylated portions. wt, wild-type strain; *psm-mec*, *psm-mec* gene deletion strain; *psm-mec**, strain harboring a *psm-mec* start codon mutation abolishing translation; *fudoh**, *fudoh* (hypothetical) start codon mutation abolishing translation of hypothetical Fudoh protein.

Fig. 4.

Impact of the *psm-mec* locus on oxacillin resistance. Resistance to oxacillin was measured in a microtiter plate assay in quadruplicate. 3407, MSA3407 wild-type strain; *psm-mec*, *psm-mec* gene deletion strain; *psm-mec**, strain harboring a *psm-mec* start codon mutation abolishing translation. Dotted lines show connecting curves; solid lines are non-linear best-fit calculated curves.

Fig. 5.

The *psm-mec* RNA. The extensions of the *psm-mec* RNA were determined by 5'- and 3'-RACE. Secondary structure prediction was performed using the RNAfold Webserver at the University of Vienna (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>). Color bar shows basepair probability. The N-terminus ("Met") and C-terminus ("Stop") of the PSM-mec-encoding region are depicted.

Fig. 6.

Plasmid-based complementation of the MSA3407 *psm-mec* deletion mutant with *psm-mec* and *psm-mec**. Values are from measurements performed at least in triplicate (three biological replicates, measured three times each) and relative to expression of the housekeeping gene *gyrB*. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

Table 1

Bacterial strains used in the present study.

Strain	Antibiotic	Source
<i>S. aureus</i> MRSA252		(Queck et al., 2009)
<i>S. aureus</i> MRSA252 <i>psm-mec</i>		(Queck et al., 2009)
<i>S. aureus</i> MSA820		(Queck et al., 2009)
<i>S. aureus</i> MSA820 <i>psm-mec</i>		(Queck et al., 2009)
<i>S. aureus</i> MSA890		(Queck et al., 2009)
<i>S. aureus</i> MSA890 <i>psm-mec</i>		(Queck et al., 2009)
<i>S. aureus</i> MSA1601		(Queck et al., 2009)
<i>S. aureus</i> MSA1601 <i>psm-mec</i>		(Queck et al., 2009)
<i>S. aureus</i> MSA3407		(Queck et al., 2009)
<i>S. aureus</i> MSA3407 <i>psm-mec</i>		(Queck et al., 2009)
<i>S. aureus</i> MSA890 <i>psm-mec</i> *		This study
<i>S. aureus</i> MSA890 <i>fudoh</i> *		This study
<i>S. aureus</i> MSA3407 <i>psm-mec</i> *		This study
<i>S. aureus</i> MSA3407 <i>fudoh</i> *		This study
<i>S. aureus</i> MSA3407 (pT181)	Tet 12.5 µg/ml	This study
<i>S. aureus</i> MSA3407 (pT <i>psm-mec</i>)	Tet 12.5 µg/ml	This study
<i>S. aureus</i> MSA3407 (pT <i>psm-mec</i> *)	Tet 12.5 µg/ml	This study

Table 2

Oligonucleotides used in the present study.

Oligonucleotide name	Oligonucleotide sequence		
<i>Construction of start codon mutation in fudoh</i>			
MecFw	GGGGACAAGTTTGTACAAAAAGCAGGCTTTAATGGCATTTCGACCAAAA		
MecRv	GGGGACCACTTTGTACAAGAAAGCTGGGTAACGCTGAAAGATTTCGCTTC		
MecFwmut	GTTGAAAAAATTAACCGAAAGCCTGAATACAAGTCTTG		
MecRvmut	CAAGACTTGTATTCAGGCTTTCGGTTAATTTTTTCAAC		
<i>Oligonucleotides to test for presence of start codon mutation in fudoh</i>			
MecFwseq	GGCGGTTTCAATTCACCTGT		
<i>PSM-mec, PSM-mec* and fudoh* complementation plasmids</i>			
F-eco	ATGCGAATTCTATTTTATTTTCCATAATTGCCTACCCCATAG		
F-Bam	ATGCGGATCCCAATTCACCTTGTCTTAACTTTGTAGAAAAAGAAG		
<i>5' RACE</i>			
PSM-mec GSP6	AAACAATCAAGTCGTTGAATATTTCTCTG		
PSM-mec GSP7	ATTAACCGAAAGCCTGAATGCAAGTCTTGAT		
<i>3' RACE</i>			
PSM-mec GSP3	ATGGATTTCACTGGTGTATTAC		
E1	5'-phosphate-UUCACUGUUCUUAGCGGCCGAUGCUC-idT-3'		
E4	GAGCATGCGGCCGCTAAGAACAGTGAA		
<i>qRT-PCR</i>			
Gene	Forward	Reverse	Probe
<i>lip</i>	ACCGCCCAATAATGAGCTA	AGTGCATGGTTTCAATGGGTTT	CACTGAAGGATTAATATC
<i>thrC</i>	GGTAATGGCTGTGACAAAAGCAA	TTTCCAGTCGAAGCGCATATT	AGAGCAAGGTAAG AAAA
<i>lrgB</i>	AGGCGAAGTCGAAAAAGTAGGA	ACCGGCTGGTACGAAGAGTAAG	CGACACTAACAAATAAC
<i>spa</i>	CAGCAAACCATGCAGATGCTA	GCTAATGATAATCCACCAAATACAGTTG	CATTACCAGAACTGGTGAAGAAAATCCATTCATTG
<i>psma</i>	TATCAAAAGCTTAATCGAACAAATTC	CCCCTTCAAATAAGATGTTTCATATC	AAAGACCTCCTTTGTTTGTATGAAATCTTATTTACCAG
<i>mecA</i>	TTCCACATTGTTTCGGTCTAAAATT	AATGCAGAAAGACCAAAGCATACA	CCACGTTCTGATTTTAAA
<i>gyrB</i>	CAAATGATCACAGCATTTGGTACAG	CGGCATCAGTCATAATGACGAT	AATCGGTGGCGACTTTGATCTAGCGAAAAG

Table 3

Genes strongly regulated by *psm-mec* (microarray analysis, all genes with up- or down-regulation by a factor > 10 are shown for MSA3407 and > 5 for MRSA252)¹.

Gene number ²	Function	Factor of regulation
<i>MSA3407, up-regulated</i>		
02548 (COL)	Protein A	43.2
SAV2377 (Mu50)	PTS system, sucrose-specific IIBC component	20.7
SAV2538 (Mu50)	PTS system, glucose-specific II ABC component	16.4
SAV2507 (Mu50)	Gluconate operon transcriptional repressor	14.1
SAV1068 (Mu50)	Phosphoribosylformylglycinamide synthase I	11.5
SAV1065 (Mu50)	Phosphoribosylaminoimidazole carboxylase	10.1
<i>MSA3407, down-regulated</i>		
SAV1330 (Mu50)	Homoserine kinase	11.6
SAV1329 (Mu50)	Threonine synthase	11.3
<i>MRSA252, up-regulated</i>		
SAV2190 (Mu50)	PTS system, lactose-specific IIBC component	10.0
SAV2194 (Mu50)	Galactose-6-phosphate isomerase LacB subunit	6.6
01835 (COL)	Tagatose-6-phosphate kinase (LacC)	6.5
02548 (COL)	Protein A	5.7
<i>MRSA252, down-regulated</i>		
SAV1071 (Mu50)	Phosphoribosylformylglycinamide cyclo-ligase	8.2
SAV1069 (Mu50)	Phosphoribosylformylglycinamide synthetase	7.4
00597 (COL)	Phosphoribosylglycinamide formyltransferase	7.1
708 (EMRSA16)	Phosphoribosylaminoimidazole- succinocarboxamide synthetase	6.5
SAV1068 (Mu50)	Phosphoribosylformylglycinamide synthase I	6.4
SAV1073 (Mu50)	Bifunctional purine biosynthesis protein	5.9
SAV1074 (Mu50)	Phosphoribosylamine-glycine ligase	5.4
SAV1065 (Mu50)	Phosphoribosylaminoimidazole carboxylase	5.3

¹The entire list of differentially regulated genes can be accessed using the GEO database (XXX).

²Gene number according to strain Mu50 (Kuroda et al., 2001), unless gene not present in Mu50.