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Limitations in the use of PSMγ, *agr*, RNAIII, and biofilm formation as biomarkers to define invasive *Staphylococcus epidermidis* from chronic biomedical device-associated infections

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Abstract

Staphylococcus epidermidis is a common cause of biomedical device-associated infections. *Agr* is the major quorum sensing system in staphylococci and regulates virulence factors. Four *agr*-specificity groups exist in *S. epidermidis,* and chronic *S. epidermidis* infections are hypothesised to select for *agr*-negative phenotypes. Therefore, we investigated *S. epidermidis* strains from prosthetic joint- and catheter-associated infections to establish i) whether an infection selects for an *agr*-negative phenotype; ii) the importance of PSMγ and iii) if the *agr*-specificity group is infection dependent. *S. epidermidis* nasal isolates from healthy volunteers were used as controls. The distribution of *agr*-specificity groups was significantly different between infection and control episodes, but did not distinguish between the infection types. PSMγ secretion was used to determine *agr*-activity and HPLC analysis showed that 44% of prosthetic and 32% of catheter-associated episodes produced no PSMγ in comparison to 8% of the control strains. However, PSMγ expression did not always correlate with RNAIII up-regulation, indicating that PSMγ synthesis is likely influenced by additional post-transcriptional control. The data suggests chronic *S. epidermidis* infections favour *agr*-specificity group 1 but the results suggest that they do not select for an *agr*-negative phenotype. Further studies are required to explore the mechanisms underlying the selection and survival of these *S. epidermidis* phenotypes isolated from biomedical device-associated infections.

Introduction

Infections are a major problem associated with biomedical devices, such as central venous catheters, prosthetic joints, cardiac pacemakers, heart valves and cerebrospinal fluid-shunts. A major concern in these infections is their chronic persistence and recalcitrance to antibiotics, making the removal of the infected device necessary. The commensal *Staphylococcus epidermidis* is a leading cause of nosocomial biomedical device-associated infections (1). *S. epidermidis* has evolved sophisticated regulatory systems and mechanisms allowing adaption to changing environmental conditions during colonization and, in particular during an infection. Critical to the pathogenesis of biomedical device-associated infections is the ability of the bacteria to adhere to the biomaterial surface and subsequently produce biofilm. Thereby, *S. epidermidis* successfully evades the host's immune system and becomes intrinsically resistant against most first line antibiotics (2).

An important factor suggested to influence the pathogenesis of *S. epidermidis* is its ability to produce pro-inflammatory peptides named phenol soluble modulins (PSMs) (3). The PSM family in *S. epidermidis* consists of PSMα, PSMβ1, PSMβ2, PSMδ, PSMε and PSMγ/δ-toxin (4). PSMs have been associated with a strong pro-inflammatory effect, as they can induce cytokine release in monocytes, activate the HIV-1 LTR in macrophage-like cells, and induce NFκB production (3). They are also chemotactic for leukocyte subsets and cause degranulation and inhibit human neutrophil apoptosis (5). PSMs like many toxins and surface proteins involved in the colonisation and persistence of *S. epidermidis* infections are regulated by the quorum sensing accessory gene regulator (*agr*) system (6, 7). PSMs are also involved in biofilm maturation and detachment as well as bacterial defence against human neutrophils (8, 9).

In staphylococci, the *agr* system is responsible for the regulation of various virulence factors (10). The *agr* system consists of four genes (*agrA, agrB, agrC* and *agrD*) that are divergently co-transcribed by RNAII (a density dependent autoinducing system), and RNA111 (an RNA effector molecule), which also includes an open reading frame encoding PSMγ (δ-toxin) (Supplemental Fig. 1) (10). AgrD contains the sequence of the autoinducing peptides (AIP) generated by proteolytic processing through AgrB. The AIP is bound by AgrC, which is a membrane-bound receptor of the two-component system AgrC/AgrA. At a threshold concentration, AgrC phosphorylates or dephosphorylates AgrA. Then, activated AgrA, in conjunction with SarA, activates the two *agr* promoters P2 and P3 leading to rapid autoinduction of the *agr* system and synthesis of the effector molecule RNAIII, which in turn controls the transcription of the *hld* gene leading to the expression of PSMγ. In *S. aureus* and *S. epidermidis*, *agr* is activated during transition from exponential growth to the stationary phase and attenuates expression of several cell surface proteins, whilst increasing the expression of many secreted virulence factors. *Agr* activity can be determined by the amount of PSMγ expressed, as the *hld* gene is encoded within RNAIII (6, 11). Previous studies have suggested that strains with *agr*-negative phenotypes are frequently associated with infections (10, 12), and the development of thicker biofilms (12). This is also supported by an *S. epidermidis* 1457Δ*agr*-mutant which colonised a subcutaneous catheter with significantly higher cell numbers consistent with its higher biofilm-forming capacity, whilst the wild type had higher cell numbers in the surrounding tissues, indicating a higher degree of invasiveness (12). In addition, the exogenous addition of PSMγ decreased biofilm formation, thus it was

hypothesized that the toxin might interfere with biofilm accumulation mechanisms in the later stages of biofilm formation (12). There are four *agr*-specificity groups in *S. epidermidis*, that differ in the hypervariable region of *agrBCD* and influence the amino acid sequence of the AIP (Supplemental Fig. 1) (13). It has been shown that AIP peptides from different *agr*-specificity groups are mutually inhibitory (12). A study by Carmody and Otto (14), demonstrated that *agr*-specificity groups may be associated with certain *S. epidermidis* infections. However the isolates used were not well characterised. Thus the aim of this present study was to elucidate the relationship between the ability of previously well characterised *S. epidermidis* isolates from different biomaterial-associated infections to produce PSMγ (15, 16); and if variations exist, confirm their relationship with an *agr*-negative phenotype, specific *agr*-specificity group and/or specific biomaterial-associated infection.

Materials and Methods

Bacteria strains

A total of 100 previously characterised prosthetic joint infection (PJI) isolates from 50 patients, and 40 isolates from central venous catheter infections (16 patients) collected between 1998 and 2004 in the ENDO-Klinik and University Hospital Hamburg-Eppendorf were used (15, 16). In each PJI case, staphylococcal isolates were obtained under strict aseptic conditions from independent pure cultures of a preoperative hip or knee joint aspirate and an intraoperative tissue specimen. The mean time interval between joint aspiration and intraoperative tissue specimen recovery was 11 weeks (median = 9 weeks). The central venous catheter infection isolates were recovered from the blood cultures of 16 bone marrow transplantation patients (2-5 isolates per patient, recovered 2 to 72 days after bone marrow transplantation). These *S. epidermidis* strains were regarded as invasive strains, as at least two clonally identical or closely related isolates were obtained from cultures of blood drawn at different time points or from independent cultures of blood drawn at one time per patient (16). Twenty six isolates from the nasal swabs of healthy volunteers were used as controls. Isolates were streaked from frozen stocks onto Columbia agar supplemented with 5 % horse blood (Oxoid, Thermo Fisher Scientific, Loughborough, UK), and incubated overnight at 37 °C, and were used in the subsequent experiments.

Identification of agr-specificity groups

Genomic DNA was isolated from bacteria cultured overnight in PY broth (1% peptone, 0.5% yeast extract, and 0.5% NaCl; all from Thermo Fisher Scientific) using a QIAmp mini kit (Qiagen, Crawley, UK) following the manufactures instructions. The primers and PCR conditions are summarised in Supplemental Table 1. The *agr* hypervariable region was amplified using Taq polymerase (Invitrogen, Paisley, UK) with primers specific to *agr*-specificity group 1 and *agr*-specificity group 2, 3 and 4 (Invitrogen), resulting in a product of approximately 500bp. Restriction fragment length polymorphism (RFLP) using restriction enzymes, Dra1 and Dre1 (Promega, Southampton, UK) was used to confirm the *agr*-specificity groups, as Dde1 cuts all *agr*-specificity groups, whilst Dra1 cuts only *agr*-specificity groups 2, 3 and 4. Nucleotide sequencing (Eurofins MWG Operon, London, UK) was used to differentiate between *agr*-specificity groups 2, 3 and 4.

PSMγ -production analysis

To analyse the production of PSMγ, bacteria were cultured in basic media (0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4, and 0.1% glucose; all from Thermo Fisher Scientific) for 8h from a starting OD_{600} of 0.05, then centrifuged and the supernatant kept for High Performance Liquid Chromatography (HPLC) analysis. A Resource PHE 1-ml column (GE Healthcare, Little Chalfont, UK) and an Applied Biosystem Vision BioCAD HPLC instrument was used as described previously (11). *S. epidermidis* PSMγ elutes at a retention time of about 7.5 min (after sample injection) as a distinct peak. The identity of PSMγ was determined after peak fractionation using a MALDI-ToF/MS (Bruker Daltonik GmbH, Germany), should appear as a peak of approximately 2849Da (3).

Transcription analysis of RNAIII

Quantitative RT-PCR was used to analyse the transcription of RNAIII. RNA was extracted from 2 and 8h cultures of 22 pairs of prosthetic joint infection isolates, 22 catheter isolates (8 patients) and 9 control nasal swab isolates, using a modified protocol described by Kenny *et al.* (17) and the Promega SV Total RNA isolation kit (Promega, Southampton, UK). Cells were harvested by centrifugation for 10min at ambient temperature, resuspended in 3ml sterile PBS (Sigma, Dorset, UK), then a 1.5ml portion of the cell suspension was mixed with 3ml of RNA protect solution (Qiagen) and incubated for 5 min at ambient temperature. Cells were centrifuged for 10min at ambient temperature and the pellet frozen at -20°C until ready to analyse. The samples were thawed and resuspended in 180µl of TE buffer (10mM Tris;

1mM EDTA (pH 8.0); Thermo Fisher Scientific), centrifuged to remove any residual RNA protect solution, and resuspended in 180µl TE buffer containing 200 µg/ml lysostaphin (Sigma), 400 U/ml mutanolysin (Sigma) and 40 µg/ml proteinase K (Qiagen). Samples were incubated at 37°C for 1-2h, with occasional shaking, then 75µl of RNA lysis buffer and 350µl of RNA dilution buffer from the SV RNA isolation kit were added to the suspension and mixed gently by inversion. The subsequent extraction of RNA was performed according to the instructions of the manufacturer. Extracted RNA was quantified with a NanoDrop ND100 spectrophotometer (Thermo Fisher Scientific, UK), and diluted to 10µg/ml. 4µg/ml of RNA was used for first-strand cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad, Hemel Hempstead, UK) in a total volume of 20µl. 3µl of cDNA was used as a template in real-time PCR analysis with a SensiMix SYBR mix (Bioline, London, UK) and primers for RNAIII and *gyrB* in a RotorGene 6000 instrument (Qiagen) using the conditions outlined in Supplemental Table 1. All RT-PCR analyses were performed in triplicate for at least two independent experiments. Relative transcriptional levels within distinct experiments were determined using the $2^{-\Delta\Delta}$Ct method which compares RNAIII transcription at 2 and 8h (18) and *gyrB* as the reference housekeeping gene. A 3-fold difference in $2^{-\Delta\Delta}$Ct was used as a cut-off point for RNAIII transcription up-regulation between 2 and 8h.

Biofilm assay

The biofilm phenotype of the *S. epidermidis* isolates was determined with a semi-quantitative adherence assay using 96-well tissue culture plates (Nunc, UK) to measure attachment and accumulation of biofilm on the plastic surface, as described previously (19). Biofilm-positive strains were defined as strong biofilm: mean OD_{570} greater than 0.7; biofilm positive: mean OD_{570} 0.5-0.69; and weak biofilm positive: mean OD_{570} 0.2 - 0.49. Biofilm negative strains had a mean OD_{570} less than 0.19.

Statistical analysis

Data were analysed using a Fisher exact test and ANOVA using SPSS software, with the level of significance set at $p < 0.05$.

Results

Identification of agr-specificity groups.

The *agr*-specificity groups (Table 1) in the prosthetic joint infection (PJI; 50 pairs), catheter infection (16 patients) and control nasal swab (n = 26) isolates were established using

specific PCR primers and RFLP to differentiate between the 4 *agr*-specificity groups. The results showed that *agr* specificity group 1 was most common among the PJI and catheter episodes, with 74 and 90% prevalence respectively. Isolates with *agr* specificity group 2 and 3 were also identified in 16 and 10% of PJI episodes respectively, whilst the remaining catheter isolates (10%) were all *agr* specificity group 2. *Agr-specificity* group 4 was only found in one PJI patient. In comparison, the control nasal swab isolates analysed were equally distributed in *agr* groups 1-3 and none in 4 (Table 1). The *agr*-specificity group of isolates from the same patients were identical in all cases. The distribution of *agr*-specificity groups was significantly different between infection episodes and control nasal swab isolates ($p < 0.05$), whilst the *agr*-specificity group distribution between prosthetic and catheter episodes was not ($p > 0.05$).

PSMγ production analysis

PSMγ production was used to establish *agr* activity within the isolates using HPLC (Table 2 and Supplemental Figure 2) and thus determine if isolates had an *agr* negative phenotype. In *agr* active isolates (Supplemental Figure 2), a peak was observed as expected after about 7.5 min in HPLC separation (11), and analysis of this peak using MALDI-ToF/MS showed a distinct peak of 2840 Da (not shown), thus confirming the presence of PSMγ. Similar analysis of all isolates (Table 2) confirmed that 44% of the PJI ones and 32% of those from catheters produced no PSMγ in comparison to only 8% of control nasal swab isolates; and no PSMγ production was found in any *agr*-specificity groups irrespective of isolate source. The differences observed in PSMγ production between infection (PJI or Catheter) and control nasal swab isolates was significant ($p < 0.05$), but no significant difference was observed between the PJI and catheter isolates ($p > 0.05$). Interestingly, a difference in PSMγ production was observed between 26% of the PJI pairs and 19% of the catheter isolates from the same patient, that is one isolate produced PSMγ whilst the other did not; and such a phenotypic difference was observed consistently in replicate experiments.

Transcription analysis of RNAIII

RNAIII is only transcribed if the *agr* locus is active, therefore RNAIII up-regulation was analysed in 22 pairs of PJI isolates, 22 catheter isolates (8 patients), and 9 control nasal swab isolates), and a 3-fold difference between in $2^{-\Delta\Delta}$Ct used as the cut-off point. The results showed that RNAIII was transcribed by 65 of the isolates tested (Supplemental Table 2), despite the fact that 24 of these isolates did not produce PSMγ according to the HPLC results.

No significant association was found between RNAIII transcription and PSMγ production for the PJI, catheter or control isolates ($p > 0.05$) (Figure 1). However, the 3-fold increase in the RNAIII transcribed by the catheter isolates was significantly greater than that observed in the PJI and control isolates ($p < 0.05$). Furthermore, median values for RNAIII transcription in PSMγ positive isolates were higher than in PSMγ negative isolates for both infection types but was not statistically significant ($p > 0.05$).

Biofilm formation

The ability of the PJI, catheter and nasal isolates to form biofilms was tested using a semi-quantitative adherence assay (19). Biofilm-positive isolates were defined as strong, positive or weak biofilm formers and the results are shown in Table 3. The ability of the isolates to form a biofilm was also correlated to their *agr* type and whether they were PSMγ positive or negative. Of the PJI isolates 66% were biofilm positive and all the strong biofilm producers (13 isolates) belonged to *agr* specificity group 1, with isolates from *agr* specificity group 2, 3 and 4 producing weaker biofilms. In comparison, 83% of the catheter isolates were biofilm-positive, with 9 isolates forming strong biofilms, again all belonging to *agr* specificity group 1. Only 1 of the nasal isolates produced a positive biofilm, whilst 9 isolates were weak biofilm producers and 16 were biofilm negative, from *agr* specificity groups 1, 2 and 3. No significant differences were seen between *agr* specificity group and ability to form a biofilm ($p > 0.05$).

An *agr*-negative phenotype has been associated with stronger biofilms. However, of the 13 PJI strong biofilm producers, only 4 were *agr*-negative, and of the 9 catheter-associated strong biofilm producers only 4 were *agr*-negative (Table 3). No significant difference was observed between PJI, catheter or nasal isolates and whether they had an PSMγ positive/negative phenotype ($p = > 0.05$).

Discussion

The *agr* quorum sensing system is responsible for the regulation of many factors involved with *S. epidermidis* colonisation, immune evasion and activation (5, 20). Four different *agr*-specificity groups have been identified in *S. epidermidis* (13) and have been linked with specific clinical infection entities in *S. aureus* (21). This study investigated the relationship between the differential ability of *S. epidermidis* strains isolated from prosthetic joint

infections (PJI) and catheter infections to produce PSMs, and whether any variations identified were associated with a specific *agr*-specificity group, *agr*-negative phenotype and~~/or~~ biomaterial-associated infection.

Firstly, the distribution of *agr*-specificity groups in *S. epidermidis* isolates from a strain collection of 100 prosthetic joint infections (50 pairs) and 40 catheter infections (16 patients) showed *agr*-specificity group 1 as the most prominent type in both prosthetic joint infection and catheter isolates (74 and 90%), whereas there was no significant specificity group in the control nasal swab isolates from healthy volunteers (38, 31 and 31%). Three other studies looking at *agr*-specificity groups in *S. epidermidis* infections, also found *agr*-specificity group 1 common in infection isolates (4, 14, 22) with fewer *agr*-specificity group 2 and 3 isolates. Two of these studies had similar values of *agr*-specificity group 1-3 within the commensal isolates, thus substantiating the results obtained in this current study. However, in the Li *et al.* (22) study, *agr*-specificity group 2 was significantly more dominant at 51%. A possible a*gr*-specificity group 4 was found in a PJI episode in this current study and in a commensal isolate in the Hellmark *et al.* study (4). *Agr*-specificity groups in relation to other specific *S. aureus* infections have also been investigated, and have been found to correlate broadly with strain genotypes (23-25). Campoccia et al. [37] found differences in *agr*-specificity group prevalence in orthopaedic implant infections which were dependent on whether the isolate was from a sporadic or epidemic infection. Hence, there is strong evidence to suggest that specific *agr*-specificity groups in *S. epidermidis* are also associated with particular clinical infections.

Agr-negative phenotypes have been proposed to be common in infection isolates in comparison to commensal ones (10) and have been associated with increased biofilm formation, an important virulence factor in *S. epidermidis* implant associated infections (6, 12). Thus a second hypothesis was that infection isolates may select for an *agr*-negative phenotype. Firstly, PSMγ production was analysed, as its production is assumed to signify an active *agr* system. The results showed significantly fewer of the infection isolates produced PSMγ in comparison to the control nasal swab ones ($p < 0.05$), suggesting that they have an *agr*-negative phenotype, thus correlating to previous studies (10). Specifically, these results correlate with Vuong *et al*. (12, 20), as they also found significantly more *agr*-negative phenotypes in their clinical isolates than in their healthy volunteer isolates. It has also been suggested that having an inactive *agr* system would be advantageous during prosthetic joint

and catheter infections as both are associated with biofilm formation, and PSMγ expression is known to affect biofilm formation (6). However, this current study found no significant difference between being *agr*-negative/positive and biofilm formation in any of the PJI, catheter or nasal isolates ($p > 0.05$). A possible reason for this is the fact that the *agr* system is not the only regulatory system that influences biofilm formation in *S. epidermidis*, as at least four unlinked regulatory gene loci have been identified to have a role in *S. epidermidis* biofilm formation (26, 27).

The fact that PSMγ negative isolates were detected signifies that it is not critical to the survival of *S. epidermidis in vivo*, but would influence the ability of the bacteria to disperse as this is thought to be PSMγ dependent (6, 8). Surprisingly, differences within prosthetic joint infection pairs and catheter patient samples were observed. This observation was unexpected, as different infection episodes from the same patients had the same *agr*-specificity group and were clonal according to previous work (15, 16). Hence to substantiate the PSMγ results RNAIII transcription was analysed in a selection of the isolates. They showed that 64 out of 72 isolates transcribed RNAIII, despite 24 of these not producing PSMγ. The amount of RNAIII transcribed in the PSMγ negative isolates was lower than in the PSMγ positive ones but not significantly, thus suggesting a possible delay in RNAIII transcription resulting in no PSMγ production or not enough being produced for detection using HPLC. It has previously been shown in *S. epidermidis* and *S. aureus* that there is a 1-2h delay between RNAIII transcription, *hld* translation and PSMγ expression, due to the fact that the *hld* gene is embedded in the gene encoding RNAIII (20). Another study has also suggested that in *S. aureus*, using PSMγ production as an indicator of *agr* activity was not always accurate when dealing with clinical isolates (28). Hence in this present study it is possible that in some cases, PSMγ was produced but to a sub-optimum level for HPLC detection, thus suggesting that despite RNAIII being transcribed to comparable amounts, an unknown post-transcription or post-translational mechanism may influence PSMγ secretion.

Another factor that has not been considered so far, is that in *S. aureus* it has been shown that AgrA rather than RNAIII influences PSMγ expression, and that the expression of the *hld* gene despite being embedded within RNAIII is under the strict regulation of the *agr* locus and not RNAIII expression (29). Such a relationship has yet to be elucidated in *S. epidermidis*, but would certainly explain the discrepancy observed in this study between PSMγ expression and RNAIII transcription. In *S. aureus*, mutation in the 3'end of *agrA* have

been shown to result in a further 2-3 hours delay, leading to the lack of PSMγ expression (28), whilst mutations in *S. epidermidis agrC* and *agrA* have previously been suggested to result in no *agr* expression (12). However, despite finding SNPs within the 3'end region of *agrA* and *agrC* in isolates within the current study (results not shown), there was no particular correlation between the presence of these SNPs and PSMγ expression.

To conclude, the results presented in this study indicate that in *S. epidermidis*, specific *agr*-specificity groups are associated with particular clinical infections. Assuming that no PSMγ production is indicative of an *agr*-negative system, it may be concluded that chronic infections such as prosthetic joint infections are frequently associated with *agr*-negative phenotype in comparison to nasal isolates from healthy volunteers. This would be advantageous to the persistence of the bacteria as it could produce thicker biofilms which aid in evasion of the host immune system. However, if RNAIII transcription is used to define the activity of the *agr* system, chronic infection isolates are not associated with an *agr*-negative phenotype. Thus the discrepancy observed between RNAIII transcription and PSMγ production warrants further study if we are to fully understand the role of the *agr* system in infections.

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Figure and Table Legends

Table 1. Distribution of *agr*-specificity groups among PJI, Catheter and Nasal swab isolates

The number in brackets refers to the overall percentage of isolates from each source in each *agr*-specificity group.

Table 2. Distribution of PSMγ-production among the infection types and nasal swab isolates

Fisher's Exact results: PJI vs. Control, $p < 0.05$; Catheter vs. Control, $p < 0.05$; PJI vs. Catheter, $p = 0.042$

Table 3. Biofilm formation results with correlation to *agr* specificity group and *agr* positive/negative phenotype based on the PSMγ results.

No significant differences between *agr* specificity group and ability to form a biofilm in PJI, catheter or nasal isolates, $p > 0.05$.

Statistics of PJI ability to form a biofilm vs. PSMγ positive/negative, $p = 0.284$; Catheter ability to form a biofilm vs. PSMγ positive/negative, $p = 0.599$; Control ability to form a biofilm vs. PSMγ positive/negative, $p = 0.129$

Figure 1. Box-plot analysis correlating PSMγ production with RNAIII transcription between 2 and 8h

Supplemental Table 1. Primers used in this study, and the PCR/qRT-PCR conditions used

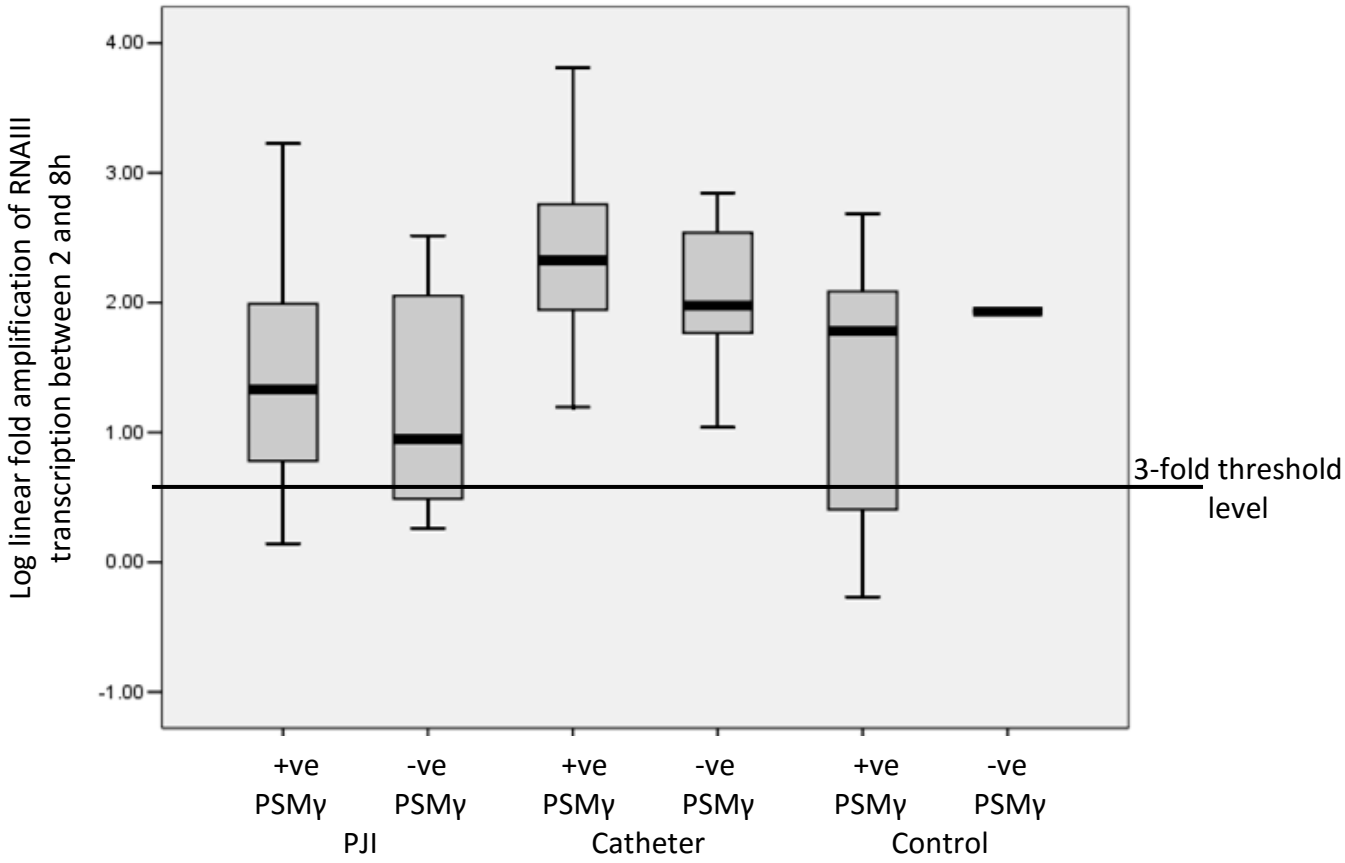
Supplemental Table 2. Detailed analysis of the HPLC and qRT-PCR results

No significant difference in RNAIII transcription or PSMγ between Prosthetic episodes / Catheter episodes & Control, $p > 0.05$

Supplemental Figure 1. Schematic representation of the function of the *agr* system of *Staphylococcus*, including the amino acid peptides of *S. epidermidis* specificity groups 1-4

Supplemental Figure 2. Spectral results from HPLC analysis of *S. epidermidis* 8h culture supernatants showing the production of PSMγ (peak) or no production (flat-line)

Figure 1. Box-plot analysis correlating PSMγ production with RNAIII transcription between 2 and 8h



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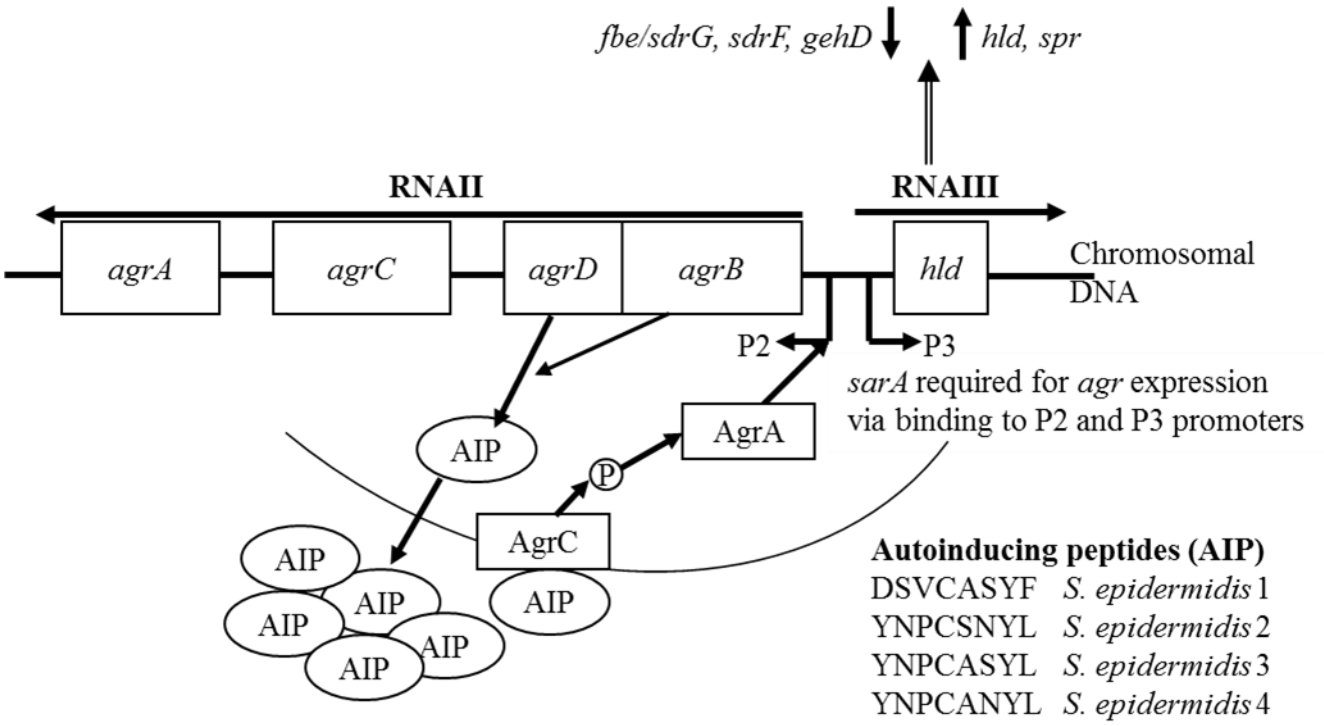

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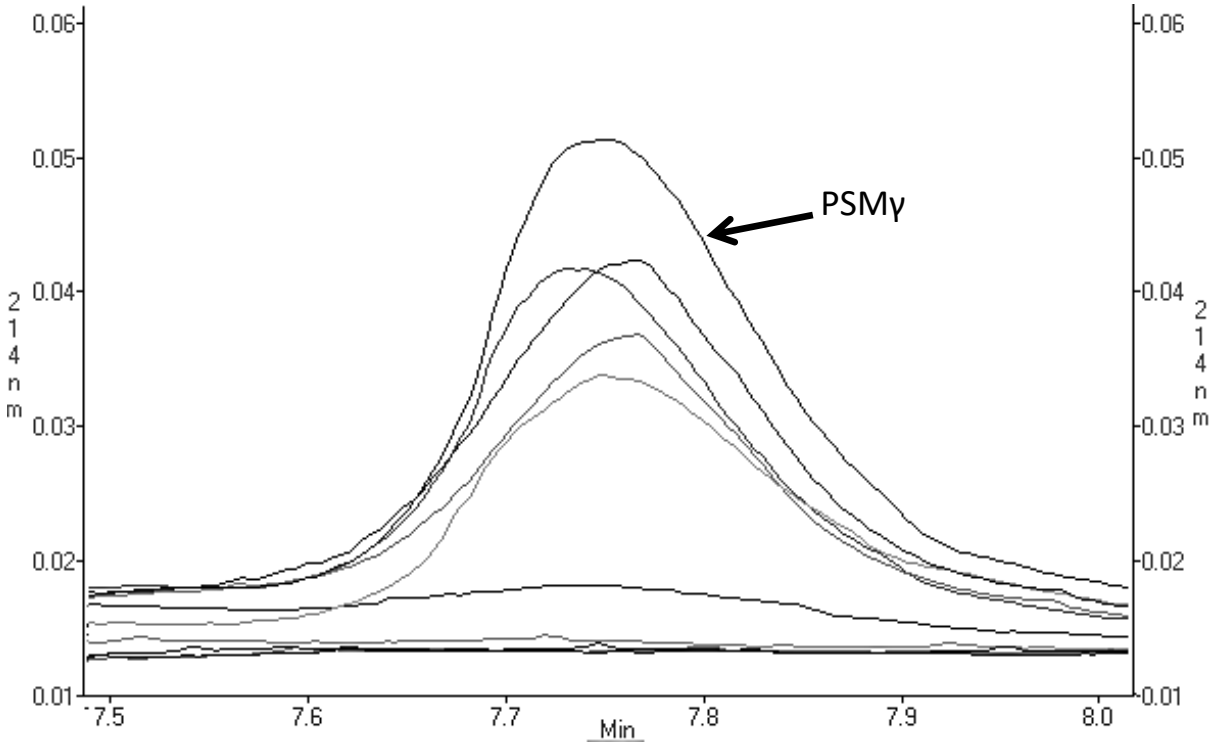

Table 1. Distribution of *agr*-specificity groups among PJI, Catheter and nasal swab isolates.

	agr group 1	*agr* group 2	*agr* group 3	*agr* group 4
PJI	37 (74%)	8 (16%)	4 (8%)	1 (2%)
Catheter	15 (94%)	1 (6%)	0	0
Control	10 (38%)	8 (31%)	8 (31%)	0

The number in brackets refers to the overall percentage of isolates from each source in each *agr*-specificity group.

Table 2. Distribution of PSMγ-production among the infection types and nasal swab isolates

	PJI			Catheter			Control	
	Distribution per isolate (n = 100)		Variation between episodes (n = 50)	Distribution per isolate (n = 40)		Variation between episodes (n = 16)	Distribution per isolate (n = 26)	
	PSMγ positive	PSMγ negative		PSMγ positive	PSMγ negative		PSMγ positive	PSMγ negative
agr group 1	41	33	12	27	9	3	10	0
agr group 2	10	6	0	0	4	0	6	2
agrgroup 3	3	5	1	0	0	0	8	0
agrgroup 4	2	0	0	0	0	0	0	0
Total	56 (56%)	44 (44%)	13 (26%)	27 (68%)	13 (32%)	3 (19%)	24 (92%)	2 (8%)

Fisher's Exact results: PJI vs. Control, $p < 0.05$; Catheter vs. Control, $p < 0.05$; PJI vs. Catheter, $p = 0.042$

Table 3. Biofilm formation results with correlation to *agr* specificity group and *agr* positive/negative phenotype based on the PSMγ results.

		Strong biofilm (OD_{570} > 0.7)	Positive biofilm (OD_{570} 0.5-0.69)	Weak biofilm (OD_{570} 0.2-0.49)	Negative biofilm (OD_{570} <0.19)
PJI	*agr* group 1	13	6	46	9
	agr group 2	2	4	7	3
	agr group 3	0	0	7	1
	agr group 4	0	0	2	0
	PSMγ positive	11	7	32	6
	PSMγ negative	4	3	30	7
Catheter	*agr* group 1	9	5	22	0
	agr group 2	0	0	4	0
	agr group 3	0	0	0	0
	agr group 4	0	0	0	0
	PSMγ positive	5	5	17	0
	PSMγ negative	4	0	9	0
Control	*agr* group 1	0	1	3	6
	agr group 2	0	0	4	4
	agr group 3	0	0	2	6
	agr group 4	0	0	0	0
	PSMγ positive	0	1	7	16
	PSMγ negative	0	0	2	0

No significant differences between *agr* specificity group and ability to form a biofilm in PJI, catheter or nasal isolates, $p > 0.05$. Statistics of PJI ability to form a biofilm vs. PSMγ positive/negative, $p = 0.284$; Catheter ability to form a biofilm vs. PSMγ positive/negative, $p = 0.599$; Control ability to form a biofilm vs. PSMγ positive/negative, $p = 0.129$

Supplemental Table 1. Primers used in this study, and the PCR/qRT-PCR conditions used

Gene	**Primers**	**PCR/qRT-PCR conditions**
agr-specificity group 1	Forward 5'-GAAACAACCTATACCTA-3' Reverse 5'-GCAGAAGGGATTACAATCGT-3'	2 min, 94°C; 30 cycles of 15s, 94 72°C; final extension 72°C for 7m
agr-specificity group 2, 3 and 4	Forward 5'-GAAACAACCTATACCTA-3' Reverse 5'-GCAGAAAGGATTATAATTCCA-3'	2 min, 94°C; 30 cycles of 15s, 94 72°C; final extension 72°C for 7m
hld	Forward 5'-GTTATGATGGCAGCAGA-3' Reverse 5'-GGATGGCTCAACAACTCA-3'	3min, 95°C; 35 cycles of 30s, 94°
gyrB	Forward 5'-CTGACAATGGCCGTGGTATTC-3' Reverse 5'-GAAGATCCAACACCGTGAAGAC-3'	3min, 95°C; 35 cycles of 30s, 94°

Supplemental Table 2. Detailed analysis of the HPLC and qRT-PCR results

Prosthetic joint (n = 44 isolates; n = 22 episodes)

	Distribution per isolate		Variation	Distribution per isolate	
	RNAIII positive	RNAIII negative	between episodes	PSMγ positive	PSMγ negative
agr group 1	27	5	5	23	9
agr group 2	4	2	2	4	2
agr group 3	4	0	0	3	1
agr group 4	2	0	0	2	0
Total	37 (84%)	7 (16%)	7 (16%)	32 (73%)	12 (27%)

Catheter (n = 22 isolates; n = 8 episodes)

	Distribution per isolate		Variation	Distribution per isolate	
	RNAIII positive	RNAIII negative	between episodes	PSMγ positive	PSMγ negative
agr group 1	17	1	1	15	3
agr group 2	4	0	0	0	4
agr group 3	0	0	0	0	0
agr group 4	0	0	0	0	0
Total	21 (95%)	1 (5%)	1 (5%)	15 (68%)	7 (32%)

Nasal swabs (n = 9)

	Distribution per isolate		Variation	Distribution per isolate	
	RNAIII positive	RNAIII negative	between episodes	PSMγ positive	PSMγ negative
agr group 1	3	0	n/a	3	0
agr group 2	2	1	n/a	2	1
agr group 3	2	1	n/a	3	0
agr group 4	0	0	n/a	0	0
Total	7 (78%)	2 (22%)	n/a	8 (89%)	1 (11%)

No significant difference in RNAIII transcription or PSMγ between Prosthetic episodes / Catheter episodes & Control, $p > 0.05$