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Transcriptomic profiling of interacting nasal staphylococci species reveals global changes in gene and non-coding RNA expression

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Abstract

Interspecies interactions between bacterial pathogens and the commensal microbiota can influence disease outcome. In the nasal cavities, *Staphylococcus epidermidis* has been shown to be a determining factor for *Staphylococcus aureus* colonization and biofilm formation. However, the interaction between *S. epidermidis* and *S. aureus* has mainly been described by phenotypic analysis, and little is known about how this interaction modulates gene expression.

This study aimed to determine the interactome of nasal *S. aureus* and *S. epidermidis* isolates to understand the molecular effect of interaction. After whole-genome sequencing of two nasal staphylococcal isolates, an agar-based RNA sequencing setup was utilized to identify interaction-induced transcriptional alterations in surface-associated populations. Our results revealed differential expression of several virulence genes in both species. We also identified putative non-coding RNAs (ncRNAs) and, interestingly, detected a putative ncRNA transcribed antisense to *esp*, the serine protease of *S. epidermidis*, that has previously been shown to inhibit nasal colonization of *S. aureus*. In our study, the gene encoding Esp and the antisense ncRNA are

both downregulated during interaction with *S. aureus*. Our findings contribute to a better understanding of pathogen physiology in the context of interactions with the commensal microbiota, and may provide targets for future therapeutics.

Keywords

pathogen-commensal interaction; *Staphylococcus aureus*; *Staphylococcus epidermidis*; transcriptome; RNAseq; non-coding RNA

Introduction

Interactions between pathogenic bacteria and the commensal microbiota can influence disease phenotype or the clinical outcome of the infection (Wigneswaran *et al.* 2016). One example is the gut microbiota providing resistance to pathogen colonization by direct competition for nutrients, though also by activating the host immune system and inducing killing of the invading pathogens (Sassone-Corsi and Raffatellu 2015). Moreover, interactions with commensal bacteria have been shown to directly modulate virulence potential of bacterial pathogens. For example, *Enterococcus faecalis* has been shown to increase the virulence of *Escherichia coli* in urinary tract infections (Lavigne *et al.* 2008), while *Staphylococcus aureus* virulence can be reduced during co-infection with commensal bacterial species (Ngba Essebe *et al.* 2017). In the gut, Bifidobacteria limit infection by enteropathogenic *Escherichia coli* (Fukuda *et al.* 2011). These and other related examples of interactions between the commensal microbiota and potential pathogens underline the importance of understanding the molecular basis of interactions.

Identification and characterization of the bacterial gene products and metabolites that mediate microbe-microbe interactions have revealed an astonishing diversity of signals and molecules being exchanged among microbial species. The molecular basis of microbial interactive processes range from direct cell-cell signalling such as quorum sensing cross-talk and small molecule transfer (Eberl and Tümmler 2004; Fugère *et al.* 2014), effects induced by release of organic volatile compounds (Briard, Heddergott and Latgé 2016), interspecies transfer of mobile small RNAs (Liu *et al.* 2012; Zhou, Zhou and Chen 2017), to electrical communication among different microbial species (Prindle *et al.* 2015; Humphries *et al.* 2017). It is equally important to determine in which way pathogen-commensal interactions modify the behaviour of the pathogen. For example, application of RNA sequencing to analyse these interactive systems may reveal transcriptomic changes induced by microbe-microbe interactions.

The focus of this study is the clinically relevant interaction between pathogenic *S. aureus* and the commensal *S. epidermidis*, with the primary reservoirs of both being the nasal cavities. Individuals colonized by *S. aureus* are at a greater risk of disease development, as these sites can function as infection reservoirs (Moss, Squire and Topley 1948; Wertheim *et al.* 2005). *S. aureus* is the causative agent of many serious infections whereas *S. epidermidis* is the dominant commensal bacterium in the nasal cavities. *S. epidermidis* appears to be a determining factor for carriage of *S. aureus* and its production of a serine protease Esp has been reported to inhibit biofilm formation and limit nasal colonization by *S. aureus* (Iwase *et al.* 2010; Sugimoto *et al.* 2013; Vandecandelaere *et al.* 2014). However, more recent studies have suggested that the inhibitory effect of *S. epidermidis* might be more complex than initially assessed (Fredheim *et al.* 2015).

The insight into the interaction between nasal staphylococci strains has so far been based on phenotypic analyses, with limited knowledge about interactions at the molecular level. The clinical importance of the staphylococcal interaction as well as the unknown molecular basis of this interaction highlights the importance of using molecular methods to characterize the interactome of commensal and pathogen in the nasal cavities. In this study, we use RNA sequencing technology (RNAseq) to obtain molecular level insight into the functional consequences of microbe-microbe interactions in terms of effects on the transcriptome.

Non-coding RNAs (ncRNAs) can alter the transcriptional and translational profiles of a range of bacterial species (Gottesman and Storz 2011). Several ncRNAs have been identified in *S. aureus* with the most well-studied being RNAIII, controlling the *agr* system that plays a central role in bacterial pathogenesis (Boisset *et al.* 2007; Felden *et al.* 2011; Carroll *et al.* 2016). Even though it has previously been shown that ncRNAs play a role in host-pathogen interaction by modulating the human signaling response (Koeppen *et al.* 2016; Westermann *et al.* 2016), very few studies have focused on the ncRNA pool in response to bacterial interspecies interactions (Miller *et al.* 2017).

In this study, we aim to characterize the molecular basis of interactions between *S. aureus* and *S. epidermidis* isolated from the nasal cavities of a healthy human. We use an agar plate-based assay to control the interaction and determine changes in gene expression during co-culture by RNAseq. Our analysis of this pathogen-commensal interactive system revealed that expression of several virulence genes

is responsive to microbe-microbe interactions. Furthermore, we detect putative ncRNAs implicated in the interaction and identify those that are differentially expressed.

Materials and methods

Bacterial strains and growth conditions

A collection of frozen nasal swabs from Danes sampled in a previous study (Andersen *et al.* 2012) was obtained. All swabs were spread onto *S. aureus* chromID selective plates and blood agar plates and incubated at 37 °C to identify staphylococci strains. *S. epidermidis* 8/9A and *S. aureus* 9B were cultivated in tryptic soy broth (TSB) with shaking (200 rpm) at 37 °C overnight (ON).

Characterization of staphylococci strains

The identification of *S. aureus* and *S. epidermidis* was initially done by colony morphology investigation after plating on blood agar. Subsequent confirmation of *S. aureus* strains and identification of clonal complexes were established by *spa* typing using Sanger sequencing as previously described (Fode, Stegger and Andersen 2011). Moreover, a PCR strategy targeting the *tuf* gene was performed to distinguish *S. epidermidis* from *S. aureus* strains (Delgado *et al.* 2009). The PCR-based results were confirmed with Gram staining as well as coagulase and catalase tests as previously described (Andersen *et al.* 2012). *S. epidermidis* strains were subjected to a PCR strategy to identify the presence of the serine protease gene *esp* as previously described (Ikeda *et al.* 2004). The strains were typed using the web-based Multi Locus Sequence Typing (Larsen *et al.* 2012) on the basis of their genome sequence.

Biofilm formation assay

Semi-quantitative determination of biofilm formation was performed in 96-well tissue culture plates (O'Toole 2011). Single colonies were inoculated in TSB and grown ON at 37 °C, 200 rpm. Cell-free supernatants were prepared by passing the ON cultures through 0.2 µM pore size syringe filters. Supernatants and ON cultures were adjusted to an optical density (600 nm) of 1.0 and 0.1, respectively. The ON cultures were added to individual wells of the 96-well plate to a starting density of OD₆₀₀ = 0.05 and cell-free supernatants were added onto the cells. The plates were incubated statically at 37 °C ON, allowing the cells to form biofilms. Subsequently, OD₆₀₀ was measured using a microplate reader before the plates were washed with 0.9 % sodium chloride solution three times, dried for 60 minutes at 60 °C and stained with 0.1

% crystal violet solution. After staining and three washes, the density of adherent stained cells was measured at OD₅₉₀. The assay was repeated twice and representative results are presented.

RNAseq setup

Samples for transcriptome sequencing were prepared as follows. ON cultures in TSB were adjusted to OD₆₀₀ = 1.0, followed by spotting 10 µl of the cultures on top of autoclaved 0.45 µm (HA) filters placed on Tryptic Soy Agar (TSA) plates. The plates were incubated at 37 °C ON. After incubation, the filters were removed from the plates, transferred to individual microfuge tubes and frozen at -80 °C prior to RNA isolation.

RNA extraction and cDNA library preparation

The samples were homogenized in two rounds by FastPrep using 300 mg acid-washed glass beads at speed 6 for 40 seconds followed by incubation on ice. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the supplier's instructions. To remove genomic DNA, Ambion®TURBO DNA-free™ kit (Invitrogen) was used according to the manual.

RNA integrity was determined using the Bioanalyzer 2100 (Agilent Technologies) and samples with RNA Integrity Number above eight were selected for cDNA library preparation. Four independent biological experiments were performed. Library preparation was performed using the Scriptseq™ v2 RNAseq Library Preparation Kit (Epicentre) and rRNA removal was performed using Ribo-Zero rRNA Removal Kit (Illumina). Library preparation comprises RNA fragmentation, synthesis of cDNA, terminal tagging of cDNA, amplification and library quality control. cDNA was sequenced as 150 bp paired-end reads on a HiSeq Sequencer (Illumina). Generated reads have been deposited to NCBI Sequence Read Archive (SRA) (BioProject PRJNA421413).

Purification of gDNA for sequencing of nasal isolates

Single colonies of the nasal *S. aureus* and *S. epidermidis* strains were inoculated in TSB and allowed to grow ON at 37 °C, 200 rpm. The purification of genomic DNA was performed using the Promega Wizard® Genomic DNA Purification kit according to the manufacturer's instructions. The genomic libraries were generated using modified (half-volumes of the reagents from the original protocol) Kapa Hyper Plus Library

Prep Kit (Roche Molecular Systems). Genomic libraries were sequenced using the MiSeq V2 300 Cycles Reagent kit (Illumina) as 150 bp paired-end reads. Generated reads have been deposited to NCBI SRA (BioProject PRJNA421413).

Genome assembly and annotation

Sequencing reads of nasal isolates were trimmed with the seqtk tool v1.2-r94 and assembled into contigs with SPAdes v3.11.0 (“careful” mode) (Bankevich *et al.* 2012). Contigs (>200 bp, >7x coverage) were rearranged with Mauve Contig Mover tool v2.4.0, based on the published reference genome sequence: *S. aureus* USA300 (NC_007795) and *S. epidermidis* RP62A (NC_004461) (Rissman *et al.* 2009). Contigs were concatenated using the “union” command from the EMBOSS package v6.6.0 (Rice, Longden and Bleasby 2000) and subsequently annotated with Prokka v1.12 (Seemann 2014).

RNAseq differential expression analysis

RNAseq reads were trimmed with seqtk and mapped with Bowtie2 v2.3.2 to genomes of respective nasal isolates (“very-sensitive-local” mode) (Langmead and Salzberg 2012). Mapped data were further processed with SAMtools v0.1.18 to generate sorted BAM file format (Li *et al.* 2009). BEDtools v2.26.0 was used to calculate read coverage of each annotated feature in the genome (Quinlan and Hall 2010). This was used as input for DESeq2 v1.16.1 to normalize the data and identify differentially expressed features at false discovery rate of 5 % (fold change ≥ 1.5 ; p-adjusted value ≤ 0.05) (Love, Huber and Anders 2014). RNAseq reads were visualized in a strand-specific manner in the Artemis genome browser (Rutherford *et al.* 2000).

Identification of ncRNAs

RNAseq read coverage for each nucleotide was calculated using BEDtools (Quinlan and Hall 2010). A Python script, named toRNA_{Do}, was written in-house to identify putative ncRNAs based on the nucleotide coverage (<https://github.com/pavsaz/toRNA_{Do}>). Both putative intergenic ncRNAs and those antisense to coding sequences were detected. The main criteria for ncRNA selection were: 1) normalized nucleotide expression value was above a defined expression threshold; 2) ncRNA contained a defined expression “peak”, with at least a 5-to-1 expression ratio between the highest and lowest points of the “peak”; 3) ncRNA was over 50 bp in size, in order to reduce the number of false positive hits and also due to most

ncRNAs being reported above 50 bp in size (Gottesman and Storz 2011). As another measure for improving detection accuracy, only those putative ncRNAs that appeared in all replicates for each RNAseq condition were included. When combining ncRNAs present in multiple samples, a minimum start position and a maximum end position were used to define the new putative ncRNA length.

Results

Nasal staphylococci strains isolated from healthy Danes

To be able to investigate the microbial interactions occurring in the nasal cavities of healthy individuals, we isolated staphylococci strains from nasal swabs of Danish adults (Andersen *et al.* 2012). We investigated a total of 108 nasal swabs, which were chosen because prior microbiome sequencing indicated the presence of staphylococcal strains (Liu *et al.* 2015). We were able to isolate staphylococci strains from 43 % of these samples (46/108), giving us a collection of 56 *S. aureus* and 27 *S. epidermidis* strains. The bacterial isolation was done by plating, while initial species identification was based on colony morphology and confirmed by Gram staining, PCR, catalase and coagulase testing as previously described (Andersen *et al.* 2012).

S. aureus and *S. epidermidis* were co-isolated from 7 % of the nasal swabs (3/46) (Table 1).

The *S. aureus* strains were *spa* typed and 18 different *spa* types were identified, with t021 (11 % of typed isolates) and t081 (11 %) being the most prevalent. The *spa*-typed isolates were classified into 10 different clonal complexes, with most isolates belonging to the CC30 (21.4 % of all isolates) and CC45 (17.9 %).

As the serine protease Esp has previously been implicated in the interaction between *S. aureus* and *S. epidermidis*, we used PCR to identify the *esp* gene in the *S. epidermidis* isolates. We detected the *esp* gene in 92.6 % (25/27) of the nasal *S. epidermidis* strains.

Sample B was chosen for detailed study as it included an *esp*-positive *S. epidermidis* (strain 8/9A), which is DT-218, and a *S. aureus* strain (strain 9B), which is ST-59 (Table 1). The strains were whole-genome sequenced and serotyping was based on these sequences.

S. aureus biofilm formation is not affected by *S. epidermidis* and vice versa

To investigate the interaction phenotype of the nasal *S. aureus* and *S. epidermidis* strains, we assayed biofilm formation of the two strains in a microplate setup according to previous protocols (O'Toole 2011). Cells were allowed to form biofilms with addition of cell-free supernatants and we found that the addition

of cell-free supernatant from *S. epidermidis* did not alter biofilm formation of *S. aureus* in our setup (Figure 1). The addition of cell-free *S. aureus* supernatant to *S. epidermidis* cells also did not affect biofilm formation properties. The same was true for the TSB medium supplemented with 1 % glucose, except that the *S. aureus* growth was inhibited by addition of its own cell-free supernatant (Supplemental figure S1).

A simple agar-based RNAseq setup reveals the interactome of *S. aureus* and *S. epidermidis*

To map the interactome of *S. aureus* and *S. epidermidis* nasal isolates, we developed a simple agar-based setup where the strains were incubated either separately (mono-culture) or in close proximity to each other (co-culture) on the same agar plate, with cultures applied on filter paper (Figure 2). Following the incubation, bacterial colonies were easily removed and prepared for downstream processing by transferring the filters to microfuge tubes. This enables separate processing and data analysis of the different strains and culture conditions. We used the agar-based RNAseq assay to determine the interactome of the nasal *S. aureus* and *S. epidermidis* pair when grown in co-culture compared to mono-cultures. Total RNA was isolated from four biological replicates for each condition: mono- vs. co-culture. We obtained from 26.5 to 61.8 million total reads per sample and the average percentage of reads mapped to genomes of respective nasal isolates was 83 %.

S. aureus showed 10 significantly differentially expressed (DE) genes, which were all upregulated in co-culture with *S. epidermidis* (Table S1, Figure 2). *S. epidermidis* showed 50 genes significantly DE in response to co-culture with *S. aureus*. Of these, 12 genes were upregulated and 38 downregulated (Table S1, Figure 2)

70 % (7 of 10 DE genes) of the *S. aureus* genes upregulated during co-culture with *S. epidermidis* are related to the cell membrane and transmembrane transport according to the functional category analysis tool on the DAVID database (Huang, Sherman and Lempicki 2008). The remaining three genes fall into the categories: Biosynthesis of Antibiotics (SAOUHSC_00579), Pyruvate Metabolism (SAOUHSC_00533) and Unknown (SAOUHSC_02726). Two genes of the *mnhABCDEFGF* operon, *mnhA* and *mnhD*, which encode subunits of a putative multi-subunit Na⁺ /H⁺ antiporter (Hiramatsu *et al.* 1998), are significantly upregulated in response to *S. epidermidis*.

Analysis of the *S. epidermidis* DE genes using the DAVID database revealed that the genes fall into four main functional categories: Metabolic Pathways (20 of 50 DE genes), Infection Related (5/50), Membrane-associated (10/50) and Nucleotide/ATP-binding (15/50) (Figure 2). 59 % of the genes of the Metabolic

Pathway category are part of the Biosynthesis of Amino Acids sub-category. Some of the genes of the “Infection Related” category have previously been described as *S. epidermidis* virulence factors (Otto 2009). For example, it is evident that all genes of a putative operon SE0405-SE0407 of *S. epidermidis* are highly upregulated in co-culture with *S. aureus*, with fold-change values ranging from 7.8 to 9.0. This putative operon contains the previously described virulence factors *sitA*, *sitB* and *sitC* (Otto 2009), which encode an iron-regulated ABC-transporter (Cockayne *et al.* 1998). Intriguingly, the Esp serine protease that has been shown to inhibit *S. aureus* colonization (Iwase *et al.* 2010) is significantly downregulated in co-culture conditions (fold change = -2.3).

Repertoire of non-coding RNAs in interacting staphylococci

It has been previously shown that staphylococci strains possess a range of ncRNAs involved in gene regulation (Boisset *et al.* 2007). We therefore attempted to identify the expression of ncRNAs in the nasal staphylococcal isolates during mono- and co-culture based on RNAseq data.

Using a computational approach we identified 40 putative ncRNAs in the *S. aureus* nasal isolate and 150 putative ncRNAs in the *S. epidermidis* nasal isolate (Figure 3, materials and methods). Both intergenic and antisense ncRNAs were identified. 67 % (100/150) and 63 % (25/40) of the *S. epidermidis* and *S. aureus* ncRNAs, respectively, were identified in both mono- and co-culture conditions. Average size of ncRNAs is 598 bp for *S. aureus* and 1164 bp for *S. epidermidis* (Table S2).

By cross-referencing to the Staphylococcal Regulatory RNA Database (Sassi *et al.* 2015), we noted that 13 of the ncRNAs identified in *S. aureus* in our setup overlap with those previously described in other studies. In fact, two of the ncRNAs identified here have been found to play a role when *S. aureus* is interacting with *P. aeruginosa* (Miller *et al.* 2017). However, as only a single study has reported on putative ncRNAs in *S. epidermidis* (Broach, Weiss and Shaw 2016), the overlap between previously described ncRNAs and those identified here is limited.

In this study, we are specifically interested in the ncRNAs that show altered expression in response to co-culturing and we therefore performed DE analysis on the ncRNAs. We identified one DE ncRNA in *S. aureus* and seven DE ncRNAs in *S. epidermidis* (Table 2), with ncRNA lengths ranging from 365 bp to 4669 bp. As we saw in the gene expression analysis, there is also a tendency for upregulation of ncRNAs in *S. aureus* and downregulation in *S. epidermidis*. 75 % (6/8) of the DE ncRNAs are encoded antisense to annotated genes and in three cases we see a correlation between the differential expression of the gene and its

antisense ncRNA in the RNAseq data. All these examples display downregulation of both protein-coding gene and ncRNA (Figure 4). One of these cases, ncRNA epi80, is transcribed antisense to the *esp* gene, which, as mentioned above, has been implicated in the interaction between *S. aureus* and *S. epidermidis* (Iwase *et al.* 2010). The two remaining examples are ncRNA epi34, antisense to *dltABCD*, a previously described virulence factor (Otto 2009) responsible for D-alanylation of teichoic acids (Peschel *et al.* 1999) and implicated in resistance to phage attacks (Fallico *et al.* 2011), and epi116, which is antisense to a Co-enzyme A disulphide reductase gene.

Discussion

Previous studies have shown that virulence phenotypes such as biofilm formation and colonization of the nasal cavities by *S. aureus* are inhibited by the nasal commensal *S. epidermidis* (Iwase *et al.* 2010). In this study, we applied RNAseq to obtain molecular insight into the functional consequences regarding effects on the transcriptome of this pathogen-commensal interaction.

Our experimental setup involved co-cultivation of *S. aureus* and *S. epidermidis* isolates on agar surfaces. Although agar-based models do not accurately mimic the natural habitat, such models nevertheless enable systematic in-depth analysis of the mechanisms and genes that underlie microbe-microbe interactions as well as the associated molecular effects (Frydenlund Michelsen *et al.* 2016). We also note that our *in vitro* study of the interaction between surface-associated staphylococci populations has some parallels to the bacterial organization found in the nasal cavities where colonizing bacteria are indeed surface-associated and growing in microcolonies (Morawska-Kochman *et al.* 2017). Moreover, our experimental setup gives technical advantages as the downstream processing can be performed separately for each culture, aiding the data analysis. We recognize that bacterial colonies on agar surfaces are composed of clonal cells that can exist in many different physiological states depending on their spatial position. This population heterogeneity will most likely mean that we will not be able to identify subtle changes in gene expression, as these will be masked by noise. Despite these constraints, we were nevertheless able to capture new elements of the interaction response between nasal *S. aureus* and *S. epidermidis* isolates. We identified 10 differentially expressed genes in *S. aureus* and 50 in *S. epidermidis*, when these organisms were co-cultured. It is likely that these interaction-responsive genes represent a subset of a larger transcriptional response that may be detected with the advent of more sensitive transcriptomic techniques, which enable analysis of spatially resolved transcriptomes from surface-associated microbial populations (Heacock-Kang *et al.* 2017).

It has previously been shown that exposure to *S. epidermidis* culture supernatant leads to downregulation of virulence in *S. aureus* (Otto *et al.* 1999; Iwase *et al.* 2010). In our experimental system, we show that the general response of *S. aureus* to *S. epidermidis* co-culturing is upregulation of gene expression, whereas the opposite trend is seen in *S. epidermidis*, where most altered genes are downregulated. Interestingly, the three-gene operon *sitABC*, which encodes an iron-regulated ABC-transporter involved in virulence in *S. epidermidis* (Cockayne *et al.* 1998), is highly upregulated in co-culture with *S. aureus*. Yet, other *S. epidermidis* virulence factors are downregulated in co-culture conditions. The *dltABCD* operon (SE0624-SE0627) and *mprF* (SE1041), which are responsible for D-alanylation of teichoic acids (Peschel *et al.* 1999) and lysylation of phospholipids, respectively (Peschel *et al.* 2001), are all downregulated in co-culture. Overall, these results indicate that interactions between the nasal staphylococcal strains result in more complex responses than previously described and have the potential to modulate virulence in multiple ways.

Adding to the complexity of the interaction-induced transcriptional changes, our analysis of putative ncRNAs in the nasal staphylococcal isolates revealed that ncRNAs also play a part in this pathogen-commensal interaction. ncRNAs have been identified in many different organisms to play a range of regulatory roles during transcription as well as translation (Gottesman and Storz 2011). A common staphylococcal ncRNA pool has been described, but different staphylococcal species also encode unique ncRNAs (Broach, Weiss and Shaw 2016). In our analysis, we find a substantial overlap with previously identified ncRNAs of *S. aureus*, though a more modest overlap with *S. epidermidis* ncRNAs. This probably reflects the fact that ncRNAs in *S. epidermidis* are generally understudied, and our setup is very different from the single previous study on the subject (Broach, Weiss and Shaw 2016). Analysis of more strains and conditions is required to capture a more complete spectrum of ncRNAs in *S. epidermidis*. However, in the current setup we identify two ncRNAs in *S. aureus* that have previously been shown to play a part during interspecies interactions (Miller *et al.* 2017).

Despite general interest in ncRNAs and their regulatory roles, not much is known about the role of ncRNAs in connection to microbe-microbe interactions (Miller *et al.* 2017). In our study, we focused on ncRNAs differentially expressed in co-culture conditions and identified eight staphylococcal ncRNAs that play potentially important roles during interaction. Most of these ncRNAs are antisense to protein-encoding genes and in three cases the differentially expressed (antisense) ncRNAs followed the differential expression of their corresponding (sense) protein-encoding genes. For example, we identify a previously undescribed ncRNA (epi80), which is antisense to the *esp* gene in *S. epidermidis*. Expression of both the *esp*

gene and the corresponding epi80 antisense ncRNA is downregulated in response to interaction. Previous studies have shown that the Esp serine protease plays a central role in prevention of nasal *S. aureus* colonization and that the purified protease inhibits biofilm formation and disassembles previously formed biofilms (Iwase *et al.* 2010). Our finding that both *esp* and the epi80 antisense ncRNA are downregulated when *S. epidermidis* is co-cultured with *S. aureus* points towards an ncRNA-based positive regulatory mechanism for *esp* expression, which could explain why we do not observe an inhibitory effect on *S. aureus* biofilm grown with supernatant of the nasal *S. epidermidis* strain. Other studies have found that ncRNAs can have positive effects on gene regulation (Fröhlich and Vogel 2009). For example, antisense RNAs can protect mRNA from degradation by RNase E by masking endonuclease recognition sites (Stazic, Lindell and Steglich 2011). Future experiments will address if the epi80 antisense ncRNA functions in a similar way to regulate expression of *esp*.

Our results, together with the notion that *esp* is not expressed in all *S. epidermidis* strains (Fredheim *et al.* 2015), indicate that the observed inhibitory effect on *S. aureus* biofilm formation might be more complex than previously assumed. We recognize the possibility that our biofilm formation setup would not reveal effects that are exclusively induced during co-culture of the staphylococcal strains. It is possible that clonal variation of the staphylococcal strains and differences in experimental setups are all contributing factors, and further studies are needed to fully determine the factors that control *esp* expression. Moreover, our data show a self-inflicting inhibition of *S. aureus* when glucose is added to the medium, indicating difficulties in this experimental setup. The self-inflicting inhibition could be due to external alterations such as changes in pH of the medium.

With this study, we provide the first attempt to identify changes in gene expression as a result of interaction between *S. aureus* and *S. epidermidis* isolated from the nasal cavities of healthy individuals. We identified clear effects on the transcriptomic profile of both interacting partners and discovered ncRNAs potentially involved in interspecies interactions. Further studies are needed to determine the role of the differentially expressed genes and ncRNAs and to map the interactome in more detail. A comprehensive understanding of how microbe–microbe interactions change the physiology and gene expression of pathogenic bacteria may contribute to the identification of novel targets of interference.

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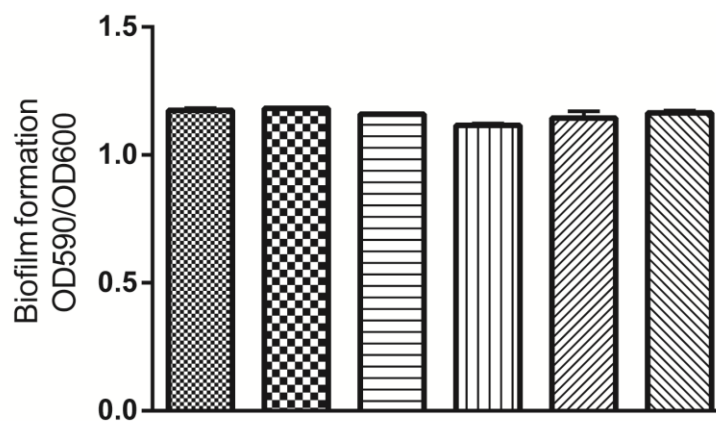
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<i>S. aureus</i> cells	+	+	+	-	-	-
<i>S. epidermidis</i> cells	-	-	-	+	+	+
<i>S. aureus</i> supernatant	-	+	-	-	-	+
<i>S. epidermidis</i> supernatant	-	-	+	-	+	-

Figure 1: *S. aureus* biofilm formation is not affected by *S. epidermidis* supernatant and vice versa. Biofilm formation when grown with (+) and without (-) cell-free supernatants after 22 hours of incubation at 37 °C.

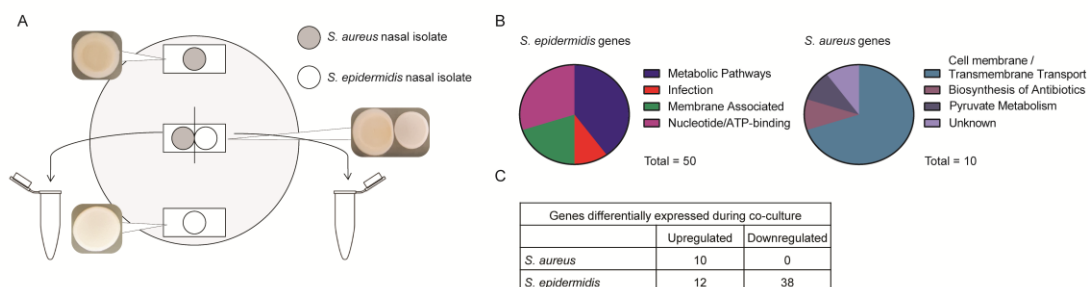


Figure 2: Co-culturing of staphylococci strains leads to altered gene expression. A) Agar-based RNAseq setup used to map staphylococci interactome. *S. aureus* and *S. epidermidis* are grown in mono- and co-culture and the setup allows for separate downstream processing of samples. B) The DAVID database (Huang, Sherman and Lempicki 2008) was used to divide the DE genes into functional categories. Pie charts showing the distribution of functional categories of *S. aureus* and *S. epidermidis* DE genes. C) Overview of DE genes. Cut-off: $\log_2\text{foldchange} \geq 1.5$ and adjusted $p\text{-value} > 0.05$.

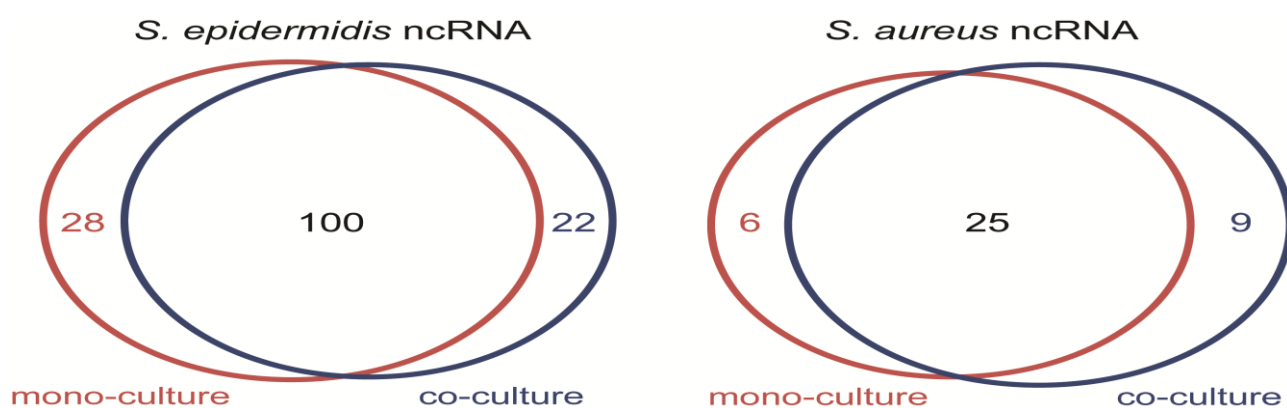


Figure 3: ncRNA repertoire of nasal staphylococci strains. Venn diagram showing the putative ncRNAs identified in the nasal *S. aureus* and *S. epidermidis* strains during mono- and co-culture.

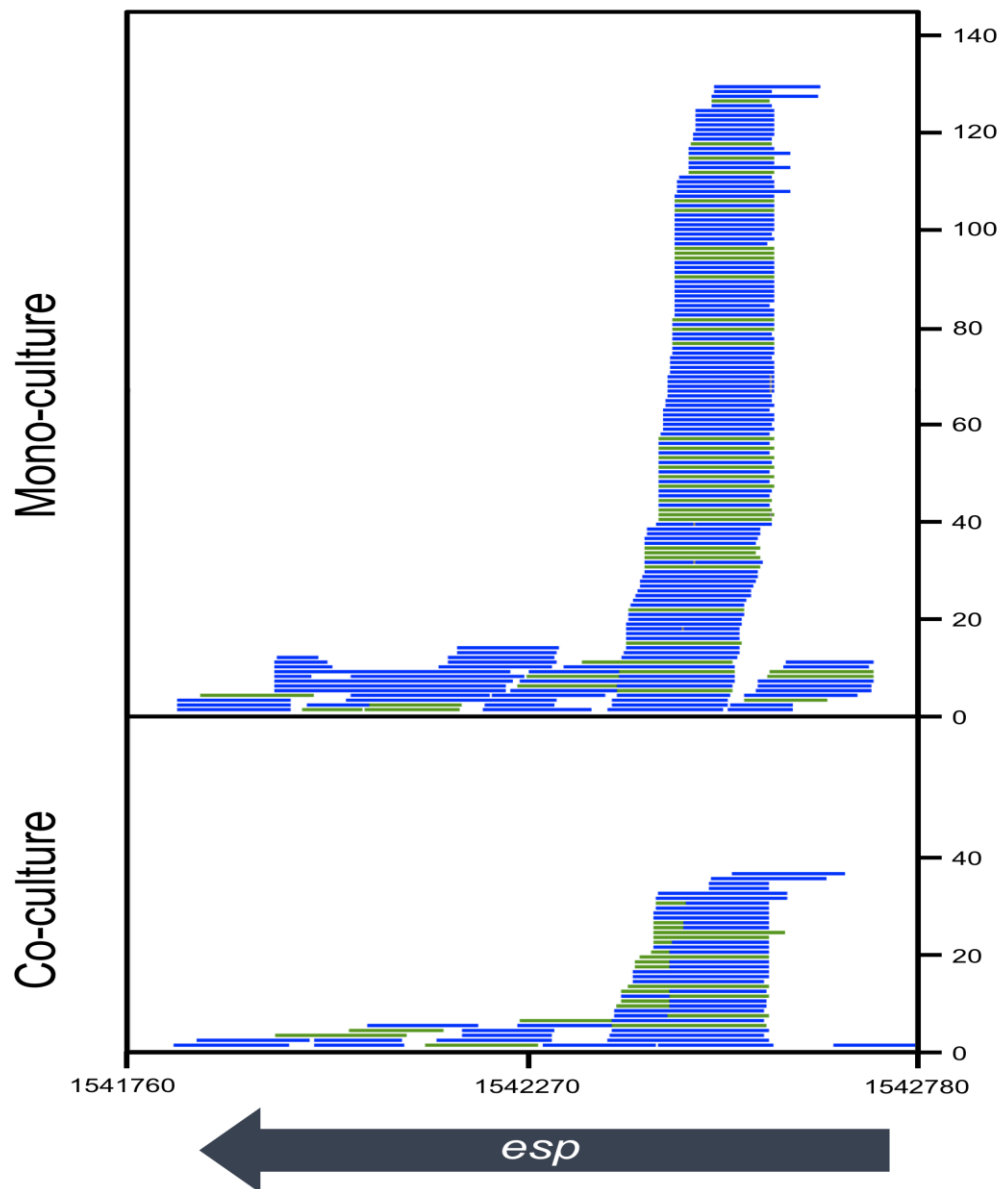


Figure 4: The expression of antisense ncRNA epi80 in mono- and co-culture conditions in the nasal *S. epidermidis* strain. Paired-end RNAseq reads (blue and green) are shown mapped antisense to the *esp* gene (grey arrow). Genomic coordinates shown on the x-axis refer to the position in the genome of the nasal *S. epidermidis* strain. The number of reads mapping antisense to the *esp* gene is shown on the y-axis.

Table 1: Nasal swab samples where *S. aureus* and *S. epidermidis* are co-isolated. Overview of the three samples, from which both *S. aureus* and *S. epidermidis* strains were isolated. G+: Gram-positive; G-: Gram-negative; C+: catalase-positive; C-: catalase-negative; Co+: coagulase-positive; Co-: coagulase-negative; CC: Clonal Complex; NA: not applicable. Negative for Esp means that we were unable to detect the *esp* gene.

Sample	Strain name	Species (PCR tuf gene)	Gram test	Catalase test	Coagulase test	Esp gene (<i>S. epidermidis</i>)
A	5A	<i>S. aureus</i>	G+	C+	Co+	NA
	6A	<i>S. epidermidis</i>	G+	C+	Co-	negative
B	9B	<i>S. aureus</i>	G+	C+	Co+	NA
	8/9A	<i>S. epidermidis</i>	G+	C+	Co-	esp
C	39A	<i>S. aureus</i>	G+	C+	Co+	NA
	39/40AB	<i>S. epidermidis</i>	G+	C+	Co-	esp

Table 2: Co-culturing of staphylococci strains alters ncRNA transcription. Overview of DE ncRNAs in the staphylococci strains. Cut-off: $\log_2\text{foldchange} \geq 1.5$ and adjusted p-value > 0.05 . Adj p-value = adjusted p-value, Ns = not significantly altered in RNAseq data, NA = not applicable. * Annotations referring to genes of reference genomes *S. aureus* USA300 and *S. epidermidis* RP62a. ⁿ no homolog found in reference genome. Where ncRNAs are antisense to more than one gene, all genes are listed.

Differentially expressed <i>S. aureus</i> ncRNAs							
ncRN A	Size (bp)	Fold change	Adj p-value	Type	Flanking/antisense gene(s)*	Fold change ^{Gene}	
au15	457	2.3	1.05E-02	Antisense	SAOUHSC_00846	NA	
Differentially expressed <i>S. epidermidis</i> ncRNAs							
ncRN A	Size (bp)	Fold change	Adj p-value	Type	Flanking/antisense gene(s)*	Fold change ^{Gene}	
epi17	1057	-3.2	1.76E-02	Antisense	SE0302, <i>rplJ</i> , <i>rplL</i>	Ns, Ns, Ns	

	epi34	4669	-3.0	3.83E-02	Antisense	SE0623, <i>dltA</i> , <i>dltB</i> , <i>dltC</i> , <i>dltD</i>	Ns, -2.1, -2.1, -2.0, -2.4
	epi52	638	-2.7	4.57E-02	Intergenic	NA ⁿ	Ns
	epi80	576	-2.7	1.30E-02	Antisense	<i>esp</i>	-2.3
	epi10 5	680	-3.9	1.76E-02	Antisense	<i>SarZ</i>	Ns
	epi11 6	2629	-2.8	3.78E-02	Antisense	SE2154, SE2155, <i>fda</i>	-2.1, Ns, Ns
	epi13 5	365	-2.0	4.91E-02	Intergenic	SE2336, SE2337	Ns