SHORT COMMUNICATION

Alterations in the *Staphylococcus epidermidis* biofilm transcriptome following interaction with whole human blood

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To characterize the transcriptional response of *Staphylococcus epidermidis* biofilms to human blood, the authors determined gene expression levels in an *ex vivo* model, using RNA sequencing technology. The expression of many genes involved in general metabolism and biosynthesis of essential molecules was upregulated upon contact with blood. Genes involved in iron utilization were also upregulated indicating that iron utilization is important for survival of *S. epidermidis* biofilms, and this process may be an attractive target for novel treatment strategies.

Abstract

Staphylococcus epidermidis biofilm formation on the surface of intravenous catheters is responsible for 22% of the cases of bloodstream infections, in patients in intensive care units in the USA. The ability of *S. epidermidis* to withstand the high bactericidal activity of human blood is therefore crucial for systemic dissemination. To identify the genes involved in the bacterium's survival, the transcriptome of *S. epidermidis* biofilms, upon contact with human blood, was assessed using an *ex vivo* model. Our results showed an increased transcription of genes involved in biosynthesis and metabolism of amino acids, small molecules, carboxylic and organic acids, and cellular ketones. One of the striking changes observed 4 h of *S. epidermidis* exposure to human blood was an increased expression of genes involved in iron utilization. This finding suggests that iron acquisition is an important event for *S. epidermidis* survival in human blood.

One of the most important virulence factors for *Staphylococcus epidermidis* is its ability to form biofilms on the surface of indwelling medical devices (Vuong & Otto, 2002). It is estimated that 22% of bloodstream infections, detected in intensive care units in the USA, are due to *S. epidermidis* emanating from biofilms on the surface of peripheral or central venous catheters (Otto, 2009). Human blood is a complex mixture of immune circulating cells and soluble factors, which are usually very active at eliminating invading organisms. However, pathogens causing systemic infections develop mechanisms to circumvent the high microbicidal properties of blood (Fradin *et al.*, 2003). Consequently, *S. epidermidis'* ability to withstand the bactericidal activity of human blood is indispensable for biofilm maintenance and pathogenesis. Despite its importance in human health, the changes in the transcriptome of *S. epidermidis* biofilms when in contact with human blood have not been described. This information is essential to understand the strategies used by this bacterium to survive and cause systemic infections. Employing a widely used *ex vivo* system (Fradin *et al.*, 2005; Graham *et al.*, 2005; Mereghetti *et al.*, 2008; Vebo *et al.*, 2009; Malachowa *et al.*, 2011), *S. epidermidis* biofilms were incubated with human blood to analyze the transcriptomic changes induced. Peripheral blood was collected from healthy adult volunteers by venipuncture into BD Vacutainer® sodium heparin tubes (Becton Dickinson, NJ) under a human subjects protocol approved by the Partner's Health Care System Institutional Review Board (Boston, MA). Heparin was selected over EDTA or sodium citrate due to its action on antithrombin III rather than ions that may be essential for
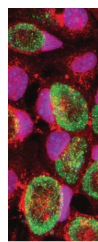
bacterial growth (Nolte *et al.*, 2002). However, the use of high concentrations of heparin may have implications in the inactivation of the complement system (Makrides, 1998), as well in the phagocytic ability of polymorphonuclear cells (Mollnes *et al.*, 2002). Biofilms of *S. epidermidis* strain RP62A (Reference Sequence: NC_002976.3) were grown as described (Franca *et al.*, 2011). Twenty-four hour biofilms were incubated with either 1 mL of tryptic soy broth or human blood for 2 or 4 h at 37 °C in 5% CO_2 with slight agitation. After incubation, biofilms were suspended in 1 mL of either PBS (Boston BioProdutcts, MA) (to assess biofilm cell viability) or RNA protect™ for RNA extraction (QIAGEN, Hilden, Germany). Biofilm cell viability was assessed by flow cytometry using the SYBR Green I (Invitrogen, CA)/propidium iodide (Sigma, MO) staining as described previously (Cerca *et al.*, 2011). For the construction of libraries for sequencing, total RNA was isolated using the RNeasy mini kit (QIAGEN) as we previously optimized (Franca *et al.*, 2012). Genomic DNA was degraded with one cycle of TURBO DNase (Ambion, NY) and 1 : 5 acid-phenol:chloroform treatment (Ambion). Eukaryotic RNA was removed using MICROB*Enrich*™ kit (Ambion). Total RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent, CA). RNA integrity numbers were all above 8. Prokaryotic mRNA was enriched by depleting the ribosomal RNA using Ribo-Zero™ rRNA removal kit for Gram-positive bacteria (Epicentre, WI). Libraries were then prepared using the TruSeq™ RNA Sample Prep kit version 2 (Illumina, CA). All these procedures were performed following the manufacturer's instructions. Sequencing data were generated from paired-end reads (2 × 150 bp) obtained on a MiSeq® system (Illumina). Two independent libraries were constructed, each using three independent and different blood donors. The alignments of the nucleic acid sequencing reads, the normalization of the reads *per* kilobase *per* million mapped reads (Mortazavi *et al.*, 2008), and the analysis of differential

gene expression were carried out using CLC Genomics Workbench version 5.5.1. Gene expression in tryptic soy broth and human blood was determined using biofilms harvested at the start of the culture as control. Baggerly's statistical test (Baggerly *et al.*, 2003) with false discovery rate (Pawitan *et al.*, 2005) was applied to identify statistically significant changes in gene expression between the tested conditions. Genes that were not detected (reads *per* kilobase *per* million mapped reads = 0) and those with *P* values above 0.05 were discarded. Using Venn diagrams (Oliveros, 2007), the genes that were expressed in both biological replicates and uniquely expressed in the presence of human blood were identified. Of note, transcripts that were equally affected by TSB or blood, as well as TSB only, were discarded from our analysis. A list of the fold change values obtained in each run was created and the values averaged. For further analyses, only genes with fold changes above two were selected for inclusion. Gene ontology enrichment of the up- and down-regulated genes was determined using the Search Tool for the Retrieval of Interacting Genes/Proteins version 9.05 (Franceschini *et al.*, 2013). Only gene sets passing significance thresholds ($P < 0.05$ with false discovery rate) were selected for display. Quantitative PCR was performed, as described before (Franca *et al.*, 2012), to confirm the data obtained by RNA sequencing.

To determine to what extent the microbicidal properties of human blood (Fradin *et al.*, 2005) could affect the cells within the biofilm, we first determined their viability upon incubation with this bodily fluid through SYBR green/propidium iodide staining. Despite some loss of viability, we observed survival of 59% ± 11.4% and 54% ± 5.6% upon 2 or 4 h incubation with whole human blood, respectively (when compared with the viability of the biofilm cells harvested at the start of the culture). These results showed that a majority of cells within *S. epidermidis* biofilms can survive in human blood. The analysis of the transcriptomic libraries of the biofilm cells after
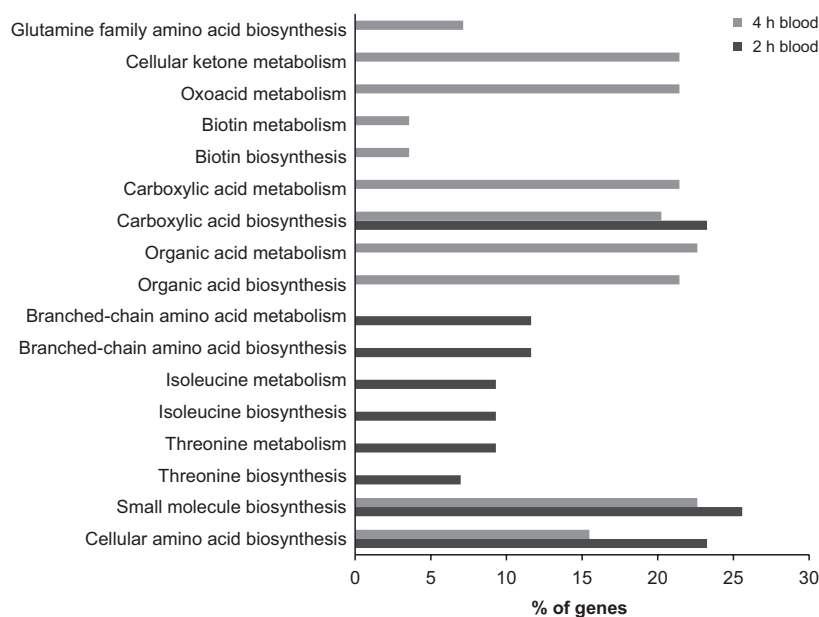

Fig. 1 Percentage of transcripts for genes increased within the different biological classes, after 2 or 4 h of exposure to human blood. Only transcripts with $P < 0.05$ are depicted.

exposure to human blood showed that among 680 transcribed genes (comparing cells from biofilms exposed to blood with those harvested at the start of the culture) only 139 were uniquely expressed in biofilms exposed to human blood for 2 h. Within these, 42 had increased transcription, 34 had decreased transcription, and 63 genes had small changes in the levels of their transcripts (around twofold change). After 4 h of exposure, there were 1195 gene transcripts detected, where 445 of these were changed only in the presence of human blood, including 94 with increased levels of transcripts, 97 with decreased levels of transcripts, and 254 that presented only small changes in their expression profile. Gene ontology enrichment analysis showed that, in the presence of TSB, there was not a significant enrichment of any biological class, as a very broad range of genes, with very different functions, were found differentially expressed. However, in the case of human blood, the increased transcripts, detected after 2 h of incubation with human blood, included those involved in amino acid, carboxylic acid and small molecules biosynthesis, that is, biosynthetic pathways for vitamins, organic acids, ketones and alcohol (Fig. 1). After 4 h of exposure, it was further found that transcripts for genes involved in the biosynthesis of organic acids, cellular ketones, and glutamine, as well as oxoacid and carboxylic acid metabolism, were also increased. Interestingly, the increased transcription of genes involved in amino acid biosynthesis and metabolism was previously reported for *Staphylococcus aureus* during lung colonization (Chaffin *et al.*, 2012) and incubation in blood (Malachowa *et al.*, 2011). It has additionally been detected for transcripts of other microorganisms such as group A Streptococcus (Graham *et al.*, 2005) and *Candida albicans* (Fradin *et al.*, 2003) during incubation with human blood. Transcripts for genes associated with iron uptake, recognition, and sequestration were also increased 4 h after incubation with human blood (Table 1). Of note, transcripts that were equally affected by blood or TSB, as well in TSB only, were discarded from our analysis. Iron is an essential cofactor in basic metabolic pathways needed by microorganisms (Jordan & Reichard, 1998; Jakubovics & Jenkinson, 2001). Hence, iron sequestration is one of the first lines of host defense against bacterial infections, in a process that was designated nutritional immunity (Skaar, 2010), by leading to

very low levels of free iron in most host tissues (Massonet *et al.*, 2006; Chaffin *et al.*, 2012). Besides the production of siderophores, one of the mechanisms described in *S. aureus* for iron utilization, is the use of the two-component Heme-sensor system (HssRS) (Torres *et al.*, 2007). This sensor responds to the presence of heme, hemin, hemoglobin, or blood, activating the expression of the heme-regulated transporter (HrtAB) efflux pump which controls hemin associated toxicity, and plays an essential role in heme homeostasis (Friedman *et al.*, 2006; Torres *et al.*, 2007). Orthologs of HssRS and HrtAB were also found in *S. epidermidis* (Juarez-Verdayes *et al.*, 2012). As reported in Table 1, the expression of *hssR* transcripts was significantly increased. This suggests that *hssS* was activated, and thus, the bacterial cells were sensing the presence of heme. However, transcripts for *hrtA* were found to be decreased. Heme is excreted via HrtAB complex to avoid heme-related toxicity, and the decrease transcription of *hrtA* observed in the blood suggests that the heme in the cytoplasm was being used for cellular functions (Torres *et al.*, 2007). Besides this two-component sensing system, transcripts for receptors that specifically recognize transferrin in human blood (Modun *et al.*, 1998a, b) were increased (Table 1), further validating the importance of iron acquisition for *S. epidermidis* biofilms survival in human blood. Interestingly, it was shown that catecholamine inotropes, frequently used in patients in intensive care units, interact with both lactoferrin and transferrin, leading to the release of bound iron (Lyte *et al.*, 2003). This interaction facilitates the acquisition of iron by *S. epidermidis*, contributing to the development of catheter-associated infections (Neal *et al.*, 2001; Lyte *et al.*, 2003). The neutralization of the iron-binding activity of these receptors has been proposed as a vaccine strategy for several pathogens including *Neisseria meningitides* (Thompson *et al.*, 2003), *Hemophilus ducreyi* (Afonina *et al.*, 2006), *S. aureus* (Stranger-Jones *et al.*, 2006) and *Escherichia coli* (Alteri *et al.*, 2009). Iron utilization in *S. epidermidis* biofilms in the presence of human blood needs to be further investigated to determine whether this mechanism may be used to target potential vaccine candidates.

In conclusion, these results indicate that survival of bacterial cells within *S. epidermidis* biofilms in human blood is primarily related to the ability to synthesize essential

Table 1 Genes involved in iron uptake and metabolism that were found differentially expressed 4 h after incubation with human blood

Gene	Fold change (4 h)	Function	Localization
SERP0400	19.4	Iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP0401	18.0	Iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP0402	10.0	Iron compound ABC transporter, ATP-binding protein	Cytoplasmic membrane
SERP0949	8.0	Transferrin receptor	Unknown*
SERP0403	7.3	Transferrin receptor	Cytoplasmic membrane
SERP1953	4.16	*hssR*, two-component regulatory system HssRS	Cytoplasmic
SERP1775	3.81	Iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP1776	3.05	Iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP0306	2.75	Iron compound ABC transporter, ATP-binding protein	Cytoplasmic membrane
SERP1951	−2.70	*hrtA*, transporter complex HrtAB involved in hemin import	Cytoplasmic membrane

The localization of proteins of interest within the bacterial cell was predicted using PSORTb version 3.0.2 (Yu *et al.*, 2010).
*This receptor is predicted to be located on the cell wall, on the extracellular, or in the cytoplasmic membrane.

molecules that are not available in this setting. This implies that overall survival in a likely stressful environment is a key factor in pathogenesis of device-related infections. In addition, iron utilization seems to be an important component that cells within *S. epidermidis* biofilms use to respond to the presence of human blood, which may be targeted for the development of preventive or treatment strategies.

Acknowledgements

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