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Staphylococcus aureus Transcriptome during Infection and Antibiotic Treatment with Moxifloxacin in a Guinea Pig Biofilm Infection Model

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Abstract

Staphylococcus aureus adapts to the host environment during infection, but it is challenging to study its global *in vivo* response. In a previous study (Xu., *et al.* BMC Microbiol 16:80, 2016, doi: 10.1186/s12866-016-0695-6), we reported the transcriptome of *S. aureus* in a prosthetic joint infection. In this study, transcriptome of the same *S. aureus* strain was investigated in a guinea pig implant infection model. Four Teflon® cages were placed subcutaneously in each of the 12 animals before *S. aureus* inoculation. Six animals were treated with moxifloxacin for four days starting three days after infection initiation (day 3), while the other six remained untreated. Cage fluid and cages were collected for RNA sequencing and metabolomics. We found clear distinction of the expression profiles between *in vivo* and *in vitro* samples. In the untreated group, early infection (day 3) samples were characterized with upregulation of many virulence genes, amino acids catabolism and several fermentation pathways compared with the *in vitro* samples, while from early (day 3) to late infection (day 7 and 9) increased ammonia production was indicated by upregulation of urea degradation, arginine deiminase and acetoin biosynthesis pathways. The transcriptome at late stage (seven and nine days after infection initiation) were found not differentiable. Increasing concentrations of several amino acids and fermentation products were detected by metabolites analysis. The transcriptome seven days after termination of moxifloxacin treatment resembled early infection (day 3). Overall, the *S. aureus* gene expression profiles showed its dynamic adaptation to a changing environment during infection development.

Keywords: *Staphylococcus aureus; Moxifloxacin; Biofilm; infection; gene expression; in vivo*

Introduction

Staphylococcus aureus is a major cause of community- and hospital-acquired infections worldwide. *S. aureus* has a remarkable ability to adapt to a biofilm mode of growth in response to the host environment, and this is crucial for its leading role in device-related infections [1,2].

The pathogenesis of *S. aureus* infections has been studied extensively both *in vitro* and *in vivo*. Multiple virulence factors have been revealed such as toxins and cell surface proteins which facilitate adhesion, invasion and evasion in order to survive and hide from the host's immune response [3,4]. However, few studies investigated infection progress and related changes in virulence expression [5]. In

addition to virulence determinants, adaption to the host environment requires adjusting metabolic activities to the changing environment in the host during infection development. Rohmer and co-workers [6] addressed this question in an opinion manuscript arguing that a pathogenic microorganism has to invade a niche that is already occupied either by human cells or by a perfectly adapted microbial resident.

Therefore, the ability to survive depends on the ability to adapt to changes in a hostile environment. Consequently, looking at the ability of pathogenic bacteria to adjust metabolic pathways might give an extra dimension in our understanding of bacterial pathogenicity.

Nonetheless, knowledge of *S. aureus* metabolic changes *in vivo* is scarce.

To describe the complicated interaction between the microorganism and the host, gene expression has been investigated both *in vitro* and *in vivo* at the bacterial level. It has become clear that the *in vivo* setting is far more complex and *in vitro* data cannot be translated to *in vivo* infections. *S. aureus* gene expression has been studied *in vivo* in rabbit [7], guinea pig [8], rat [9] and mice [10,11], as well as in humans [12,13]. Most of these studies focused on a few specific virulence determinants. To our knowledge only Date., *et al*. and Xu., *et al*. have studied *S. aureus* global gene expression in deep infections in humans [10,14].

The primary aim of this study was to study the *in vivo* transcription response of *S. aureus* during infection progression in a guinea pig implant-infection model and changes induced by treatment with moxifloxacin using the RNA-sequencing approach (RNA-Seq). Although virulence is interest of many studies, we chose to focus on the regulation of metabolic pathways, not individual virulence determinants. Moreover, nuclear magnetic resonance (NMR) spectroscopy was used to analyse the metabolites in fluid aspirated from the hollow implants at different time points to determine the biochemical compositions of the investigated environment. To do this, we chose the pan susceptible and biofilm-forming strain (SAU060112) from our previous study [14] to enable comparison of the *in vivo* gene expression profiles between the human prosthetic joint infection (PJI) and the guinea pig implant model. We decided to use this animal implant model because it is well-established [15-19] and guinea pig is highly susceptible to staphylococcal infection and its symptoms and immune response are similar to humans [20]. Moxifloxacin, a broad-spectrum fluoroquinolone antibiotic, was selected for treatment because of the toxic effect of common antibiotics such as penicillin on the gut microbiome of guinea pigs [21,22]. Moxifloxacin binds to and inhibits the bacterial enzymes DNA gyrase (topoisomerase II) and topoisomerase IV, resulting in inhibition of DNA replication and repair and cell death in sensitive bacterial species [23].

Materials and Methods

Infecting organism

S. aureus strain SAU060112 [14], a clinical isolate from a PJI patient, was used.

The bacterium was isolated directly from the primary culture plate from the prosthesis component of a PJI patient and stored in 20% glycerol at -80°C. This strain (*spa* type t908, Clonal Complex 45) is susceptible to penicillin, methicillin and 5 antibiotic classes other than β-lactam [14]. The inoculum was freshly prepared from an overnight LB medium agar by resuspending colonies in 0.9% sterile saline (NaCl). The OD_{600} of the inoculum was adjusted to reach a final concentration of 2 x 10^4 CFU/mL. In addition, a CFU count was made for the inoculum.

Animal model

The research protocol was approved by the Danish Animal Ethics Council (2015-15-0201-00620), and the animals were kept and studied at animal facility maintained by the Department of Biomedical Research's at Aalborg University Hospital, Aalborg, Denmark.

A modified version (changes in sex of the guinea pigs) of the established foreign-body infection model in guinea pigs was used [15,16]. The animals (albino female Dunkin-Hartley guinea pig, Lidköping Kaninfarm, Sweden) were acclimatised for minimum 14 days before subcutaneous implantation of four polytetrafluoroethylene (Teflon) cages (32 mm X Ø 10 mm and perforated with 130 spaced holes, Ø 1 mm) (Angst-Pfister AG, Zürich, Switzerland) in the flanks and closure of the incision by intracutaneous stitches under aseptic conditions. The weight of animals was 350-510g at time of implantation. The first animals were anesthetized with Hypnorm-Dormicum

(fentanyl/fluanisone-midazolam, 1.0 mL/kg + 2.5 mg/kg, intramuscular), but we switched to Zoletil (tiletamin + zolazepam, 0,1 mL/kg intramuscular) due to shorter recovery time after the anaesthesia. For 3 days post-surgery 0.1 mL Metacam (meloxicam, 0.5 mg/mL) was administered orally in commercial baby apple puree. Two weeks after surgery and healing of the wounds, the cage fluid was cultured to rule out contamination before infection. The cage fluid was cultured on 5% horse blood agar (SSI Diagnostica, Denmark) aerobically (5% CO_2) and anaerobically on chocolate agar enforced with vitamin K and cysteine (SSI Diagnostica, Denmark) up to 14 days. None of the cages were contaminated and all animals were included in the study.

The experiment was designed with six animals being treated with moxifloxacin and six animals not receiving antibiotics. On day 0 the tissue cages were infected with 0.1 mL culture containing 2 x 10^3 CFU *S. aureus* SAU060112 by injection directly into each cage. On day 3, cage fluid was aspirated from a subset of cages for assessment of infection by culture as mentioned above, and remaining fluid was saved in RNA later (Ambion, USA) for transcriptome study. Moxifloxacin 2 x 90 mg/kg for 4 days per os was initiated in the selected animals on day 3 (after the aspiration). The target for the dose was a concentration of 10 µg/mL moxifloxacin at the infection site one hour after administration [35]. On day 7 one or two animals in each group were anesthetized, cage fluid and the cages were removed for analysis, and the animals were sacrificed. For the remaining animals, the cage fluid was extracted for analysis. On day 9 cage fluid and cages were obtained from the animals not treated with antibiotic, and they were sacrificed. This endpoint was chosen for ethical reasons. The procedure was the same on day 14 for the remaining animals receiving antibiotic treatment. The RNA later samples were stored at 4°C overnight and kept at -80°C until preparation for RNA-Seq.

Transcriptomics

All commercial kits mentioned were used according to the manufacturer's instructions. RNA was extracted by PowerLyser® Ultraclean® Tissue and Cells RNA Isolations Kit (MO BIO Laboratories, USA) including On-Spin Column DNase I Kit (RNase-Free). Followed by another DNase treatment in the liquid phase of the extracted product as described in the On-Spin Column DNase protocol. The products were cleaned up by RNeasy MinElute Cleanup kit (Qiagen, USA) followed by MicroEnrich™ (Ambion®, Life Technologies) treatment, then another cleanup step by RNeasy MinElute Cleanup kit. The resulting products were treated with RiboZero rRNA Removal Kit (Bacteria) (Illumina, USA) followed by a modified RNeasy MinElute Cleanup according to the RiboZero rRNA Removal protocol before Illumina® TruSeq® Stranded mRNA Library Prep Kit. Libraries were paired end sequenced (2 x 141 bp) using Illumina® HiSeq 2500.

RNA data analysis

The data was analysed using the RNA-Seq tool in CLC genomic workbench (vs. 9.1, Qiagen). Reads were aligned to the annotated genome of SAU060112 (accession number: CCXN00000000) allowing a minimum fraction of 0.8 and a minimum similarity fraction of 0.8. The biological replicates were grouped in the 'RNA experiments' and the total gene reads were normalised to neutralize the inter-individual variations, before performing the edge R application in CLC workbench (Benjamini and Hochberg 1995; Robinson, McCarthy and Smyth 2010). Genes with a false discovery rate p-value < 0.05 (FDR p-value) were classified as differentially expressed; the FDR p-value includes the variation in the depth of sequencing between the replicates in the groups.

Samples with low coverage was removed from the sample set, which included samples with a total gene counts below 100 reads for the highest expressed functional genes in the gene expression profile.

Raw data from the original *S. aureus* human PJI and *in vitro* cultures (LB-medium) were retrieved from GEO Series accession number GSE62091 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62091).The data was analyzed in parallel as described above.

The data have been deposited in NCBI's Gene Expression Omnibus [36] and are accessible through GEO Series accession number GSE118015 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118015).

NMR spectroscopy analysis

Prior to NMR measurements, samples were centrifuged at 4°C for 5 minutes at 12100g.

Aliquots of 500 µL of supernatants were mixed with 100 µL 0.2 M phosphate buffer (pH 7.4, 99% D_2O, containing 0.42 mM TSP-d4) and 10 mM NaN_3. The mixture was transferred to a 5-mm NMR tube and samples were kept at 278.1 K prior to analysis, no longer than 24h, using a Bruker AVIII-600MHz NMR spectrometer (Bruker BioSpin, Germany) equipped with a cryogenic CPP-TCI probe at 298.1 K. For the analysis, aT_2 relaxation-edited Carr-Purcell-Meiboom-Gill (CPMG) experiment was used ("cpmgpr1d" in Bruker library, spectral width 20 ppm, 64 K datapoints, relaxation delay of 4s, acquisition time 2.73s, total spin-echo time 80.4 ms, 256 scans). The FID was multiplied with an exponential function corresponding to a line broadening of 0.3 Hz, Fourier transformed, phase- and baseline-corrected. Subsequently spectra were imported into ChenomX 8.2, which was used for quantification of metabolites.). For metabolite identification, we used Chenomx, the Human Metabolome Database [37-40] and the BRUKER BBIOREFCODE database (v. 2.7.0-4).

Results

S. aureus infections *in vivo*

The study design is shown in figure 1 and detailed description of the experimental procedure is given in Material and methods. Fourteen days after subcutaneous cage implantation and healing of wounds, *S. aureus* was injected directly into each cage. Prior to the injection all aspirates of cage fluid were confirmed culture negative. After injection, all aspirates were culture positive only for *S. aureus.* On day 3, moxifloxacin was initiated in the treatment group and lasted for four days. On day 7, one or two animals in both groups were sacrificed.

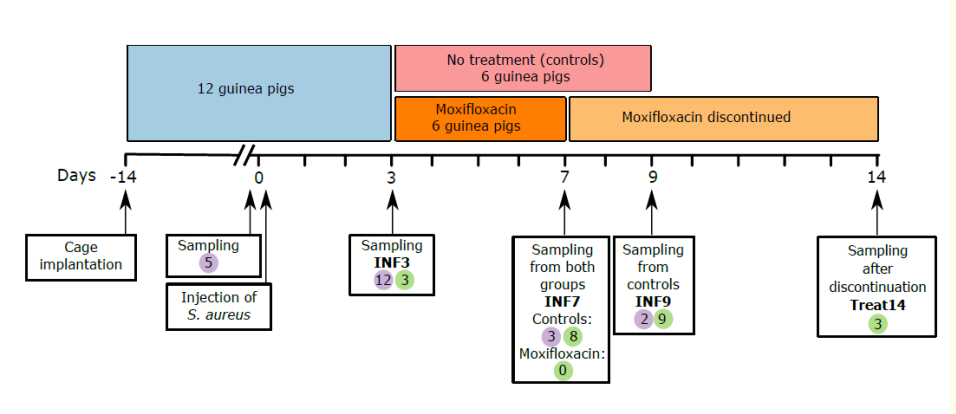

Figure 1: *Flow diagram showing the experimental setup. The experiment was planned to last for 14 days, but for untreated animals the experiment was terminated on day 9 for ethical reasons. Abbreviations: Infection day 3: INF3; Infection day 7: INF7; Infection day 9: INF9; Moxifloxacin treated day 14: Treat14. Numbers in purple indicate numbers of samples analysed by NMR. Numbers in green indicate number of samples with sufficient reads for RNA-seq analysis.*

The remaining animals in the control group were sacrificed on day 9 due to a more aggressive infection than expected, while the animals in the treatment group were sacrificed on day 14 as planned and none of them were cured for the infection.

RNA sequencing qualities

A total of 43 samples including cage fluids and cages were collected for RNA sequencing.

However, due to the difficulties of extracting mRNA, only 23 samples contained sufficient transcripts from *S. aureus* and were included in the transcriptomics analysis (for further detail see table S1). Except samples taken at the end of moxifloxacin treatment (day 7), three to nine samples at each time point comprising of both cage fluid and cage were used for further analysis. As shown in table S1, between four to 438 million reads were obtained for each sample, but most of them were host RNA or bacterial ribosomal RNA despite of application of MicroEnrich™ (Ambion, Life Technologies) and RiboZero rRNA Removal Kit (Bacteria) (Illumina). Only 0.03 - 1.57% of reads were mapped to protein coding sequences of the genome of the strain.

Sample name	Total reads	Mapped total	(%)	Mapped mRNA	(%)	% af bacterial RNA
Infection day 3						
2,1,2	46,433,080	113,813	0.25	21,887	0.05	19.23
5,4,2*	29,376,771	413,878	1.41	9,522	0.03	2.30
7,2,2*	5,586,272	10,327	0.18	5,887	0.11	57.01
8,3,2*	22,573,063	77,310	0.34	4,359	0.02	5.64
10,3,2	20,570,284	1,315,663	6.40	22,016	0.11	1.67
11,1,2	17,220,133	114,787	0.67	29,372	0.17	25.59
Infection day 7						
7,2,3	9,273,787	5,222,094	56.31	70,114	0.76	1.34
8,3,3	7,618,296	171,430	2.25	119,346	1.57	69.62
10,3,3	4,909,066	491,685	10.02	57,653	1.17	11.73
11,1,3	27,042,687	1,653,048	6.11	12,501	0.05	0.76
12,4,3	29,380,090	20,149,709	68.58	54,366	0.19	0.27
12,2,B	12,329,629	997,574	8.09	83,042	0.67	8.32
12,4,B	6,215,105	861,450	13.86	14,337	0.23	1.66
11,4,3	29,575,316	8,155,006	27.57	18,823	0.06	0.23
Infection day 9						
7,2,4	151,959,995	1,631,833	1.07	628,867	0.41	38.54
8,4,4	35,378,506	866,526	2.45	36,430	0.10	4.20
10,4,4	4,813,939	84,467	1.75	33,600	0.70	39.78
11,1,4	31,300,234	83,472	0.27	15,948	0.05	19.11
11,4,4*	33,036,153	98,242	0.30	6,660	0.02	6.78
7,3,B	36,346,375	24,169,872	66.50	20,805	0.06	0.09
8,4,B	15,200,972	954,277	6.28	27,626	0.18	2.89
10,3,B	20,776,709	5,985,541	28.81	27,050	0.13	0.45
10,4,B	14,866,576	151,140	1.02	25,856	0.17	17.11
11,4,B	23,154,590	2,465,153	10.65	39,765	0.17	1.61
Moxifloxacin day 7						
1,1,3*	253,548	8,899	3.51	94	0.04	1.06
1,1,B*	23,800,560	2,548,189	10.71	6,869	0.03	0.27
1,2,B*	36,494,649	6,379,579	17.48	1,838	0.01	0.03
Moxifloxacin day 14						
2,2,3*	30,539,980	13,372,100	43.79	7,992	0.03	0.06
2,1,3	20,544,346	560,112	2.73	27,540	0.13	4.92
4,3,3*	2,510,429	708,045	28.20	780	0.03	0.11
6,1,3*	16,369,651	1,140,827	6.97	1,189	0.01	0.10
2,1,B*	30,670,230	39,209	0.13	3,261	0.01	8.32
3,1,B	10,136,951	948,890	9.36	45,112	0.45	4.75
3,2,B	438,446,274	796,629	0.18	258,403	0.06	32.44
4,4,B*	518,747	79,201	15.27	87	0.02	0.11
6,1,B*	55,704,931	103,057	0.19	3,788	0.01	3.68
6,3,B*	53,424,072	94,701	0.18	1,674	0.00	1.77

Table S1: *RNA reads. The sample name is given by numbers: animal number, cage number, sampling number. B: cage. *indicate the samples not included in the analysis.*

Comparison of *in vivo* and *in vitro* expression profiles

Principal component analysis (PCA) showed that the *in vivo S. aureus* gene expression profiles were distinct from *in vitro* growth in LB medium (Figure 2), the cage fluid samples and the cage implants at the same time points were grouped together. Note that PC1 explains 79% of the variation and PC2 only 7%, hence the major grouping is along PC1, separating the *in vitro* transcriptomes from the rest of the samples. From *in vitro* cultures to infection day 3, more than 400 genes were differentially expressed indicating adaption of *S. aureus* towards host environment. As expected, many virulence genes had increased transcript levels from the *in vitro* culture to infection day 3 (supplemental file 2). We found the human PJI profile (sampled on the 4th day of admission) [14] to be most related to the infection day 7 and 9 in this study, whereas the transcriptomes seven day after termination of moxifloxacin treatment (Treat14) resembled the infection expression profile on day 3. The output file of differential gene expression analysis can be found in supplemental file 2.

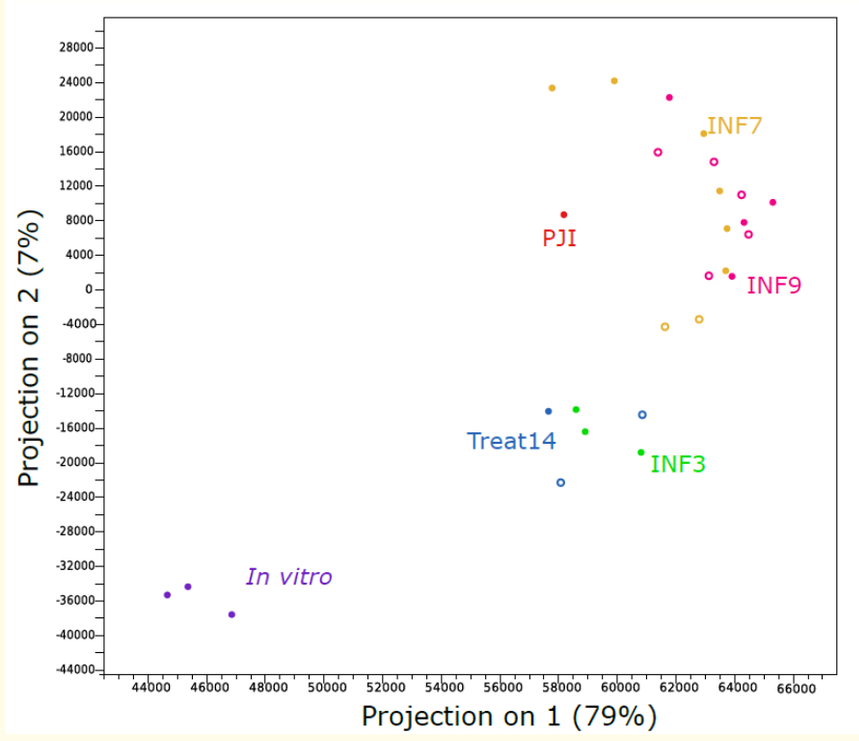

Figure 2: *Principal component analysis of all transcriptome profiles. Green: Infection day 3 (INF3). Yellow: Infection day 7 (INF7). Magenta: Infection day 9 (INF9). Blue: Moxifloxacin treated day 14(Treat14). Red: Human PJI (14). Purple: S. aureus SAU060112 culture in vitro in LB medium. Full circle: cage fluid. Hollow circle: cage. Principal component 1 separates the aerobic in vitro culture from the in vivo infection samples, whereas principal component 2 indicates the difference of the infection states.*

S. aureus gene expression changes during infection development in the guinea pig model

As shown in the PCA plot (Figure 2), among the *in vivo* samples, the expression profiles of infection day 3 grouped together with Treat14 and were different from infection day 7 and 9.

Furthermore, there was no discernible difference between infection day 7 and 9, therefore, the samples from those two days were grouped together for further analyses.

Interestingly, from infection day 3 to infection day 7 and 9, *S. aureus* reduced transcription of 11 known or putative virulence genes (*chp, hly, lip, nuc, plc, rot, sak, sbi, scn, spa* and *entC*), and iron acquisition genes (*isdA, isdB, isdC, isdE, isdF,* and *isdG*). In fact, only a single virulence gene (*clfA*) which codes for clumping factor A had increased transcription on infection day 7 and 9 compared with day 3. Interestingly, this gene was slightly less transcribed on infection day 3 compared with *in vitro* culture (3 fold). Several amino acids were detected by NMR in the cage fluids (Figure 3) and the concentration of these amino acids increased over time.

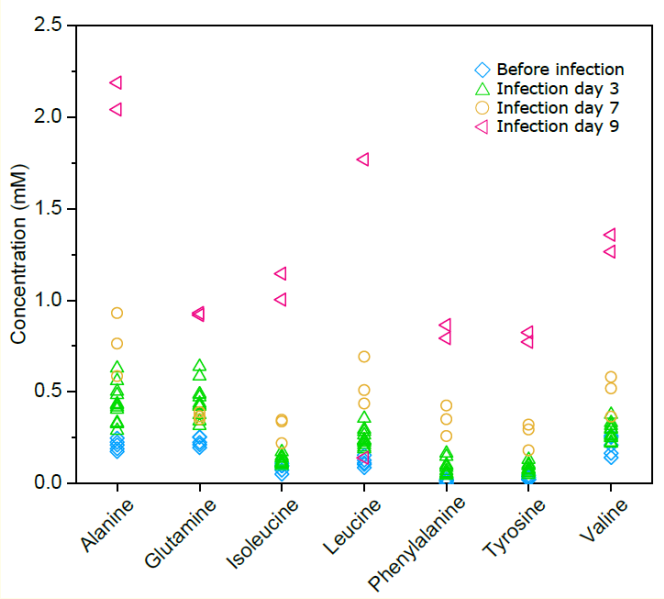

Figure 3: *Concentration of amino acids detected in supernatant by NMR analysis. The concentrations of all detected amino acids increased after infection initiation.*

From infection day 3 to day 7 and 9, a total of 109 genes were found differentially transcribed, of which 61 had reduced and 48 increased transcription on infection day 7 and 9.

Elevated concentrations of fermentation products (lactic acid, ethanol, format and acetate) were detected in the cage fluids after infection by metabolites analysis (Figure 4). As expected, genes coding for the fermentation pathways were more transcribed on infection day 3 compared with *in vitro* cultures (Figure 5). These included ethanol fermentation pathway (*pflB, ADH, adhP*) and pyruvate conversion to lactate (L-lactate dehydrogenase *ldh*). In addition, among the genes with the most increased transcription from *in vitro* to INF3 were *tdcB* and *pflB* coding for enzymes involved in the threonine degradation pathway, and *ald* coding for alanine dehydrogenase. Ammonia was produced from both processes (Figure 5).

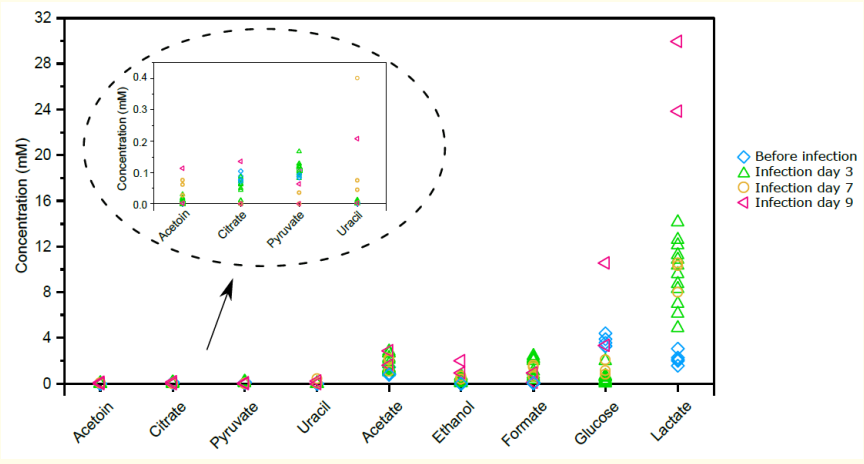

Figure 4: *Concentration of some metabolites detected in supernatant by NMR analysis.*

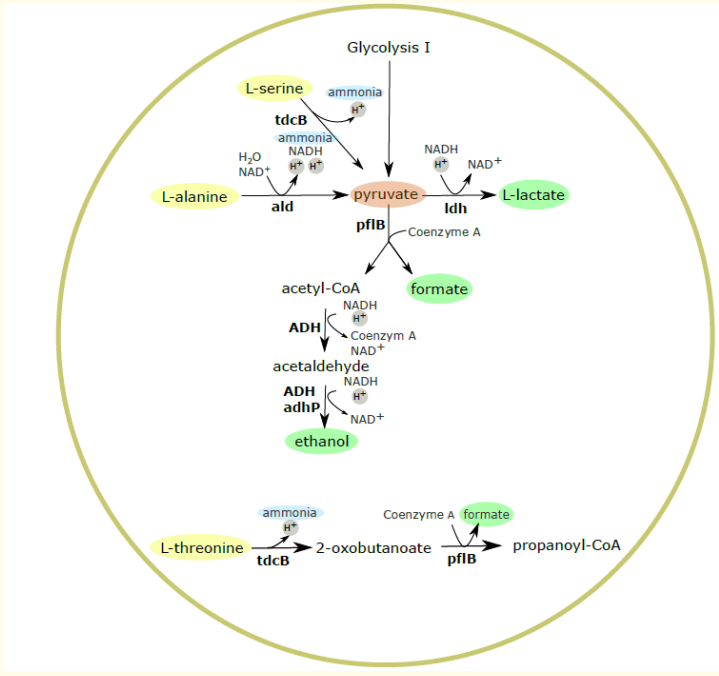

Figure 5: *Metabolic pathways more transcribed on infection day 3 compared with in vitro culture.*

From infection day 3 to day 7 and 9, we found transcripts of α-acetolactate synthase (*alsS*) and α-acetolactate decarboxylase (*aldB*) elevated (Figure 6). Under anaerobic conditions these two enzymes catalyze pyruvate conversion to acetoin (Booth and Kroll 1983). Acetoin concentration was increased by time as detected by NMR (Figure 4). Moreover, there were more L-lactate dehydrogenase (*ldh*) transcripts on INF3, while more D-lactate dehydrogenase gene *ldhD-1* was transcribed on infection day 7 and 9.

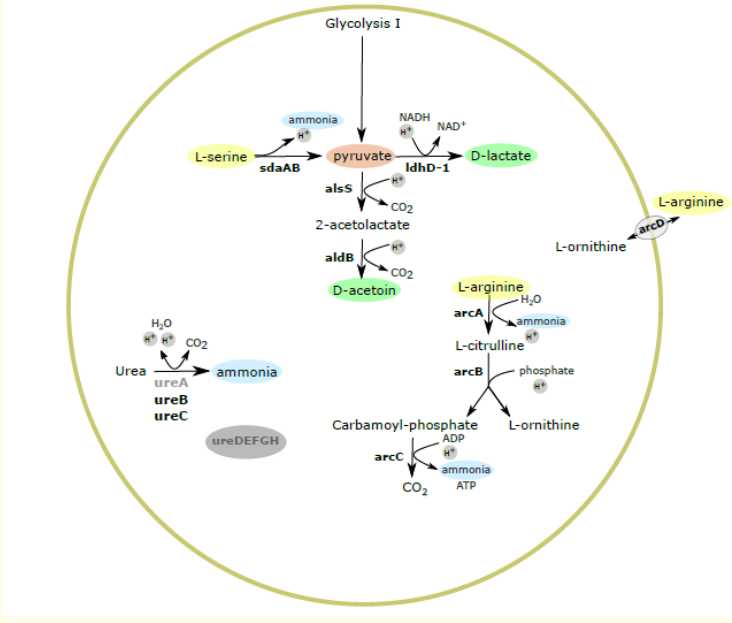

Figure 6: *Metabolic pathways more transcribed on infection day 7 and 9 compared with infection day 3.*

Furthermore, we found several genes involved in ammonia production related metabolic pathways had higher transcript level on day 7 and 9. Urea degradation related genes were more transcribed including urease subunits alpha (*ureC*) and beta (*ureB*) as well as four accessory genes (*ureD, ureE, ureF* and *ureG*) necessary for the functional incorporation of the urease metallocenter. The urease enzyme catalyzes the hydrolysis of urea to form two molecules of ammonia. On day 3 transcription of these genes was negligible. Furthermore, we found increased transcript levels of genes involved in the arginine deiminase pathway on day 7 and 9. It includes the *arcD* gene, which encodes a membrane-bound arginine-ornithine antiporter, and *arcABC,* which codes for the enzymes converting arginine to citrulline, ATP and ammonia anaerobically [24]. Lastly, we found sdaAB (L-serine dehydratase, iron-sulfur-dependent, beta subunit) had increased expression on day 7 and 9. L-serine dehydratase converts L-serine to pyruvate and ammonium.

Effect of antibiotics treatment

Treatment with moxifloxacin shifted the gene expression profile "back to the start", which is indicated by the resemblance of Treat14 and infection day 3 profiles described before.

Comparing Treat14 with infection day 7 and 9, 258 genes were differentially transcribed, while 108 genes were differentially transcribed from infection day 3 to day 7 and 9 (Figure 7). 57 Genes were commonly differentially transcribed including eight virulence genes (*chp, hly, nuc, rot, sak, sbi, scn* and *spa*) and L-lactate dehydrogenase (*ldh*). These nine genes were least transcribed on day 7 and 9, while *clfA* and genes coding for proteins involved in several metabolic processes (urease and its accessary proteins (*ureBCDEFG*), arginine deiminase pathway (*arcABCD*), pyruvate fermentation to R-acetoin (*alsS, aldB*), and D-lactate dehydrogenase (*ldhD-1*)) had highest transcript level on day 7 and 9.

Figure 7: *Venn diagram showing differentially expressed genes. 258 genes were differentially transcribed comparing Treat14 with infection day 7 and 9 (yellow), while 108 genes were differentially transcribed from infection day 3 to day 7 and 9 (blue).*

Additionally, the Treat14 group had increased transcription of the dissimilatory reduction of nitrate to ammonium (DRNA) pathway. The involved genes (*narG, narH, narT* and *nasD)* and the two-component regulatory system *nreA/nreB/nreC* had 2- to 10-fold higher transcript level in the Treat14 group than all the other groups.

Discussion

To understand *S. aureus* transcriptional changes *in vivo* during infection development and antibiotics treatment, we used a guinea pig implant infection model and performed RNA-seq analysis of the samples taken at different time points. *S. aureus* implant infection was successfully established in all 12 animals and none of the six moxifloxacin treated animals recovered. However, we only succeeded in obtaining sufficient mRNA reads from 23 out of 43 sequenced samples. All samples except two had less than 1% reads mapped to protein coding genes. It was most likely due to the combination of factors such as high amount of host RNA and bacterial ribosomal RNA, unavoidable loss of mRNA during RNA extraction, and low bacterial cell number. At the end of four days moxifloxacin treatment three samples were collected but none of them generated enough mRNA reads indicating low transcription activity of the surviving *S. aureus* (Table S1 and figure 1). Obtaining sufficient bacterial mRNA reads is a main technical challenge for studying bacterial transcriptome in human samples.

For example, in a recent study of *Pseudomonas aeruginosa* transcriptome during different human infections, only 15 of 28 sequenced human samples had sufficient reads for analysis [25].

The PCA plot shows the clear separation between the *in vitro* and *in vivo* samples (Figure 2). Despite the difference between the hosts, the human PJI sample clustered near the guinea pig samples. A similar tendency has been observed by Date., *et al.* [10], who found that *S. aureus* gene expression profiles were remarkably similar in abscesses in humans and mice, and distinct from *in vitro* cultures. A set of genes likely important for establishment or maintenance of infection had higher transcription *in vivo* including numerous proteases, toxins, and transporters [10]. Unsurprisingly, we found that compared with *in vitro* samples many virulence genes were transcribed at higher level on infection day 3, but as the infection developed, *S. aureus* reduced transcription for 11 of them on infection day 7 and 9. The PCA plot also shows clustering of infection day 7 and 9 samples indicating stabilization of the infection expression profile. The up- and down-regulation of virulence genes illustrated the fine-tuning of transcriptome by *S. aureus* to adapt to the challenging environment in course of the infection. Clumping factor A was the only virulence gene upregulated on infection day 7 and 9. This protein is known as fibrinogen-binding surface protein and inhibits phagocytosis by human polymorphonuclear leukocytes [26]. In a previous study [27] which also used the guinea pig implant infection model, clfA transcript level was shown to be highest late during growth *in vivo* in agreement with our observation. In addition, the cage and cage fluid samples were clustered together in the PCA plot, which was surprising because the cage samples were expected to contain mostly biofilm whereas planktonic cells were expected to be in the fluid samples. A likely explanation is that the majority of *S. aureus* in the cage fluid formed clumps in the late stage of infection, which could have been verified by microscopic examination of the samples.

By determining metabolites concentrations and analyzing transcription of metabolic pathway genes, several major changes were found during infection development. Several amino acids had increasing concentrations in course of the infection as detected by NMR in the cage fluids (Figure 3), which could be caused by combined effects of exoenzymes, toxins and immune response. In the human PJI [14], we observed even higher concentrations of these amino acids. The free amino acids were likely taken up by *S. aureus* and utilized as indicated by the elevated transcript level of several amino acid catabolic pathways (Figure 5 and 6).

Upregulation of these pathways was also observed in the human PJI sample [14]. *S. aureus* has to grow on other carbon sources when glucose is limited such as in abscesses. Halsey., *et al.* has shown glutamate and amino acids that serve as substrates for glutamate synthesis are major carbon sources during growth, while other amino acids that generate pyruvate are important for ATP synthesis [28].

Oxygen limited condition after infection initiation was indicated by both elevated concentrations of fermentation products compared with before infection (Figure 4) and increased transcript level of several fermentation pathways *in vivo* compared with *in vitro* cultures (Figure 5). This was also observed in human PJI [14]. Both host and the microorganisms consume oxygen during infection, and hypoxia is known to be associated with a number of diseases including infection [29.30]. In human, hypoxia generally promotes the activities of innate immune cells while suppressing the response of the adaptive immune system [30]. However, oxygen concentration is crucial for host healing [31].

Ammonia production related metabolic pathways had higher transcript level on infection day 7 and 9. Production of ammonia is suggested to facilitate pH homeostasis during infection and is known to be a strategy employed by bacteria to neutralize acidic environment [32].

Responses of *S. aureus* towards acid stress was studied previously by Rode., *et al*. [33], who found a high concentration of lactic acid induces a response from *S. aureus* different from other acids at low (pH = 4.5). In the growth medium (pH = 4.5) acidified with acetic acid, HCl or lactic acid, only pH in cultures acidified with lactic acid gradually increased to 7.5 through accumulation of ammonium and the removal of acid groups [33]. The metabolites analysis of cage fluids showed that lactate concentration increased significantly from before infection to infection day 3 and topped on day 9 (Figure 4). This may explain the increased expression of ammonia production pathways over time.

The overall picture of the transcription response of *S. aureus* to the *in vivo* environment revealed increased expression of multiple virulence genes upon infection, amino acids catabolism, and regulation of responses towards hypoxic and acidic environment during infection.

Moxifloxacin treatment did not eradicate infection in any of the six animals in agreement with the general observation that implant-related infection cannot be cured by antibiotics alone. Seven days after termination of moxifloxacin treatment, the overall expression profile of *S. aureus* resembled that of infection day 3 although many genes were differentially expressed (Figure 7). We found it interesting that DRNA pathway was more transcribed in the Treat14 group than any other time. Ammonia produced by this pathway can be used for neutralizing acidic environment. Additionally, this pathway has been shown to be an important fitness factor in hypoxic environments in the presence of nitrate [34].

Our study illustrates that the bacterial gene expression is dynamic during infection development and in response to antibiotic treatment. Both virulence genes and genes involved in metabolic processes were actively regulated in response to changing environment *in vivo*. Therefore, similar to *in vitro* bacterial growth experiment, a single *in vivo* sample is unlikely to give a true picture of how bacteria behave during infection, thus should be interpreted with caution.

Conclusion

In conclusion, our study show that *S. aureus* adjusts its expression profile, both virulence genes and metabolic pathways, in response to environmental changes during infection. The transcriptome and metabolome analyses indicate the environment within the subcutaneous cages to be anaerobic and acidic. The bacteria navigated by regulating several pathways responsible for maintaining pH homeostasis by ammonia production, and the fermentation pathways from pyruvate were active. The resemblance between the human and the guinea pig infections and the distance to the *in vitro* culture expressions profiles underline the need and usefulness of further investigation of *S. aureus* infection *in vivo*.

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Author Contributions

The study was conceived by LHL, HCS and TRT. LHL and YX designed the study, LHL handled the animals and did all the RNA laboratory work. LHL and RW prepared and analyzed the samples for NMR. LHL, YX and KLN analyzed the RNA data. RD an RW analyzed the NMR spectra. LHL, YX, TRT and HCS wrote manuscript. All authors have read and approved the manuscript.

Conflicts of Interest

None of the authors have any conflict of interest to declare.

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