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Hiroshi Hamamoto  
Teikyo University  https://orcid.org/0000-0001-9315-7442

Suresh Panthee  
RIKEN  https://orcid.org/0000-0003-4021-7936

Atmika Paudel  
Hokkaido University

Ohgi Suguru  
The University of Tokyo

Yutaka Suzuki  
The University of Tokyo

Kazuhisa Sekimizu  sekimizu@main.teikyo-u.ac.jp  
Teikyo University  https://orcid.org/0000-0002-2849-8432

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Transcriptome change in *Staphylococcus aureus* in infecting mice

Hiroshi Hamamoto¹,², Suresh Panthee²,³, Atmika Paudel³, Suguru Ohgi⁴,⁶, Yutaka Suzuki⁵, Kazuhisa Sekimizu²,*

1 Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachio-ji shi, Tokyo, 192-0395, Japan
2 Drug Discoveries by Silkworm Models, Faculty of Pharma-Science, Teikyo University
3 International Institute for Zoonosis Control, Hokkaido University, North 20, West 10, Kita-ku, Sapporo Hokkaido 001-0020, Japan
4 Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 111-0033, Japan
5 Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa shi, Chiba, 277-8562, Japan

Present address
6 Kyowa Kirin Co., Ltd., 1-9-2 Otemachi, Chiyoda-ku, Tokyo 100-0004, Japan

Corresponding Author:
* To whom correspondence should be addressed (sekimizu@main.teikyo-u.ac.jp)

# These authors equally contributed to this study
Summary

We performed in vivo RNA-sequencing analysis of *Staphylococcus aureus* in infected mouse liver using the 2-step cell-crush method. We compared the transcriptome of *S. aureus* at 6, 24, and 48 h post-infection (h.p.i) in mice and in culture medium. Genes related to anaerobic respiration were highly upregulated at 24 and 48 h.p.i. The gene expression patterns of virulence factors differed depending on the type of toxin. For example, hemolysins, but not leukotoxins and serine proteases, were highly upregulated at 6 h.p.i. Gene expression of metal transporters, such as iron transporters, gradually increased at 24 and 48 h.p.i. We also analyzed the transcriptome of mouse liver infected with *S. aureus*. Hypoxia response genes were upregulated at 24 and 48 h.p.i., and immune response genes were upregulated from 6 h.p.i. These findings suggest that gene expression of *S. aureus* in the host changes in response to changes in the host environment, such as oxygenation status or immune system attacks during infection.

Introduction

The rapid emergence of multi-antimicrobial resistant strains has created an urgent need for the development of novel therapeutic agents. The number of recent discoveries of therapeutically active antimicrobials with novel mechanisms such as texobactin\(^1\) and lysocin E\(^2\) is limited, however, suggesting a depletion of excellent target molecules to develop as novel antimicrobials. Antimicrobials are typically screened on the basis of their antimicrobial activity *in vitro*. As recently pointed out\(^3,4\), however, pathogen behavior exhibited during *in vitro* culture is much different from that *in vivo* in the host; thus, it is important to identify antimicrobial targets expected to be more efficient in the host.

*Staphylococcus aureus* has successfully adapted to the environmental conditions of the human body. It can survive and respond to various conditions in the human body, and causes a wide range of diseases such as acne, pneumonia, and bacteremia\(^5\) despite its relatively small genome size\(^6\). In addition, *S. aureus* easily acquires multidrug resistance\(^7\). It is estimated that 10,000 people in the United States and 4000 people in Japan die annually from infections caused by methicillin-resistant *S. aureus*\(^8\). *S. aureus* secretes various kinds of toxins such as hemolysins, leukotoxins, and proteases\(^9\), and grows under both aerobic and anaerobic conditions\(^10\). Furthermore, *S. aureus* has at least 5 iron acquisition systems and many metal transporters, which are
essential for its colonization and pathogenesis under host conditions. Many genes have been identified as pathogenic factors of *S. aureus*, and prompt analysis of their transcriptomes under host infection conditions is crucial. By performing RNA sequencing (RNA-Seq) analysis, we revealed the *in vivo* transcriptome of *S. aureus*, which in previous studies had only been analyzed at one specific time-point after systemic infection. We recently established an improved *in vivo* RNA-Seq analysis applicable to a smaller *S. aureus* population size in infected organs by taking advantage of the fact that gram-positive bacteria can be separated from host cells by mechanical disruption due to the presence of a strong cell wall. This technique has been successfully applied to *in vivo* RNA-Seq for *Streptococcus pyogenes*, and in the present study, we applied the method to *S. aureus* in systemic mouse infection.

**Results and Discussion**

1. Result of *in vivo* RNA-Seq analysis for *S. aureus* infection in mouse liver

We previously reported that the 2-step cell crush method was applicable to *in vivo* RNA-Seq analysis of *S. pyogenes*, which have a rigid cell wall like *S. aureus*, in necrotizing fasciitis. In this method, the first step was to use large beads to crush and lyse the host tissue in lysis buffer, followed by the use of small beads to crush enriched *S. aureus* cells in mouse organs to prepare enriched bacterial RNA for RNA-Seq analysis (Figure 1). In the present study, we performed *in vivo* RNA-Seq analysis of *S. aureus* grown in organs of mice that were systemically infected with *S. aureus*. Injection of *S. aureus* Newman strain into the mouse tail vein killed half of the mice within 48 h post-infection (h.p.i.), and all the mice within 72 h (Supplementary Fig. 1A). Under this condition, the number of bacterial cells per organ increased exponentially in the kidney and heart within 24 h.p.i., and reached $10^7$ colony forming unit (CFU)/mg in the liver at 6 h.p.i. and kept more than $10^6$ CFU/mg level in most of individuals until 48 h.p.i. (Supplementary Fig. 1B). Thus, we performed in vivo RNA-Seq analysis of *S. aureus* in the liver at 6, 24, and 48 h.p.i., and obtained approximately 160 to 700 thousand uniquely mapped reads on the *S. aureus* genome (Supplementary table 1). The number of genes with no mapped reads was 97 (3% of all genes) for RNA extracted from *in vitro* culture and 374 (12% of all genes) in *S. aureus* Newman strain isolated from liver 6 h.p.i. Therefore, most of the genes in the *S. aureus* genome were successfully analyzed by this method (Supplementary Dataset 1).
Figure 1 | The 2-step cell-crush method for in vivo RNA-Seq analysis of S. aureus in mouse liver

C57BL/6J mice were infected with S. aureus Newman strain injected through the tail vein, and organs were harvested at 6, 24, and 48 h.p.i. Tissue and bacterial cells were separately crushed and lysed in lysis buffer using beads of 2 different sizes.
| Sample Origin | in liver | in vitro |
|---------------|---------|---------|
| Harvested time/Medium | 6 h.p.i. | 24 h.p.i. | 48 h.p.i. | TSB |
| Sample name | No.3 | No.23 | No.33 | No.3 | No.13 | No.83 | L10-2 | L10-3 | D2-3 | NMWT-1 | NMWT-2 | MMWT-3 | NMWT-4 |
| cells in the sample (x10^3) | 1,484 | 1,300 | 1,162 | 503 | 143 | 753 | 3,580 | 5,001 | 3,561 |
| Total reads | 178,491,141 | 98,324,305 | 179,892,351 | 105,370,467 | 88,463,582 | 106,305,171 | 104,816,965 | 93,625,792 | 89,721,859 | 19,256,012 | 19,700,338 | 25,971,088 | 44,444,034 |
| Mapped reads | 328,107 | 526,721 | 237,715 | 534,170 | 404,538 | 576,442 | 980,823 | 1,149,746 | 249,904 | 16,519,511 | 16,578,709 | 22,522,546 | 33,925,903 |
| Uniquely mapped reads | 300,156 | 485,379 | 215,196 | 423,322 | 315,772 | 489,315 | 707,532 | 533,073 | 161,085 | 587,376 | 5,596,383 | 7,368,820 | 4,030,565 |
| Non-specifically mapped reads | 27,951 | 41,342 | 22,519 | 110,848 | 88,766 | 87,127 | 273,291 | 616,673 | 88,819 | 10,629,133 | 10,982,326 | 15,153,726 | 29,895,338 |
| The genes not read mapped | 374 (12%) | 269 (9.0%) | 14 (0.47%) | 97 (3.2%) |

**Supplementary Table 1** | Summary of *in vivo* RNA-Seq results of *S. aureus* in infected mouse liver
Supplementary Figure 1 | Conditions of systemic *S. aureus* infection model in this analysis

a, Survival of mice infected with *S. aureus* Newman strain. Bacterial cells (5.6x10^7 CFU) were injected into the tail vein (n=5).

b, Number of *S. aureus* cells in each organ after infection with (5.3x10^7 CFU) *S. aureus* Newman strain.
2.1 Pathway analysis of the genes altered after infection with S. aureus

To elucidate the trend of S. aureus gene expression in the host environment, we performed KEGG pathway enrichment analysis to compare with gene expression analysis in the culture medium (Table 1). We found that expression of genes involved in carbon metabolism (glycolysis) and TCA cycle pathways was significantly upregulated at 6 h.p.i (Table 1), but not at 48 h.p.i. Expression of genes involved in beta-oxidation, responsible for the production of acetyl-CoA from fatty acids, and the PTS system, required for incorporation of phosphorylated saccharide, was significantly upregulated starting at 24 h.p.i. Expression of genes required for iron acquisition, such as biosynthesis of various secondary metabolites- staphyloferrin A and B, was not upregulated at 6 h.p.i., but was upregulated after 24 h.p.i. In addition, expression of ABC transporters, required for the acquisition of nickel and manganese, was upregulated after 24 h.p.i. On the other hand, expression of genes involved in terpenoid backbone biosynthesis, required for cell wall synthesis, pigment, menaquinone, and peptidoglycan biosynthesis, was downregulated from 24 h.p.i. As for the host side, we performed RNA-Seq analysis using RNA of liver organs mixed in the sample that mapped uniquely on the mice genome (Supplementary Dataset 2) and selected genes with a significant 5-fold difference (false discovery rate [FDR] p-value <0.05) in liver infected with S. aureus compared with liver injected with PBS to perform the GO term enrichment analysis (Reactome, Supplementary Dataset 3). The results suggested that genes involved in the induction of innate immunity and inflammation, and those related to metal sequestration were significantly upregulated (Supplementary Table 3). On the other hand, reactomes related to ATP synthesis, such as respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins, were downregulated at all time-points (Supplementary Table 3). In addition, hif1a and hif3a, which are involved in the hypoxia response, were upregulated after 24 h.p.i., suggesting that the host environment was low in oxygen at the late stage of infection. These results well correlated with the trend of the S. aureus gene expression changes in the host and suggested that carbon metabolism, metal metabolism, and cell wall synthesis of S. aureus were highly influenced by the host condition.
| Hours | Pathway ID | Description | GeneRatio | BgRatio | p.adjust |
|-------|------------|-------------|-----------|---------|----------|
| Up regulated |
| 6hr   | sae00020   | Citrate cycle (TCA cycle) | 15/157    | 22/963  | 0.0000   |
|       | sae01120   | Microbial metabolism in diverse environments | 43/157    | 132/963 | 0.0000   |
|       | sae01200   | Carbon metabolism | 29/157    | 80/963  | 0.0001   |
| 24hr  | sae00997   | Biosynthesis of various secondary metabolites - part 3 | 9/208     | 10/963  | 0.0004   |
|       | sae01120   | Microbial metabolism in diverse environments | 48/208    | 132/963 | 0.0006   |
|       | sae00290   | Valine, leucine and isoleucine biosynthesis | 9/208     | 11/963  | 0.0006   |
|       | sae00020   | Citrate cycle (TCA cycle) | 13/208    | 22/963  | 0.0018   |
|       | sae01210   | 2-Oxocarboxylic acid metabolism | 11/208    | 18/963  | 0.0033   |
|       | sae00650   | Butanoate metabolism | 10/208    | 16/963  | 0.0041   |
|       | sae05150   | Staphylococcus aureus infection | 17/208    | 38/963  | 0.0077   |
|       | sae02060   | Phosphotransferase system (PTS) | 12/208    | 23/963  | 0.0077   |
|       | sae00071   | Fatty acid degradation | 7/208     | 10/963  | 0.0085   |
|       | sae01100   | Metabolic pathways | 125/208   | 497/963 | 0.0204   |
|       | sae02010   | ABC transporters | 28/208    | 85/963  | 0.0396   |
| 48hr  | sae00220   | Arginine biosynthesis | 15/253    | 18/963  | 0.0000   |
|       | sae02010   | ABC transporters | 39/253    | 85/963  | 0.0008   |
|       | sae00997   | Biosynthesis of various secondary metabolites - part 3 | 9/253     | 10/963  | 0.0008   |
|       | sae00340   | Histidine metabolism | 12/253    | 16/963  | 0.0008   |
|       | sae00290   | Valine, leucine and isoleucine biosynthesis | 9/253     | 11/963  | 0.0020   |
|       | sae01230   | Biosynthesis of amino acids | 42/253    | 100/963 | 0.0021   |
|       | sae01210   | 2-Oxocarboxylic acid metabolism | 12/253    | 18/963  | 0.0027   |
|       | sae02060   | Phosphotransferase system (PTS) | 14/253    | 23/963  | 0.0030   |
|       | sae02024   | Quorum sensing | 28/253    | 61/963  | 0.0032   |
|       | sae05150   | Staphylococcus aureus infection | 19/253    | 38/963  | 0.0068   |
|       | sae00052   | Galactose metabolism | 12/253    | 20/963  | 0.0069   |
|       | sae00071   | Fatty acid degradation | 7/253     | 10/963  | 0.0216   |
|       | sae01100   | Metabolic pathways | 148/253   | 497/963 | 0.0284   |
|       | sae02020   | Two-component system | 29/253    | 75/963  | 0.0398   |
| Down regulated |
| 6hr   | sae03010   | Ribosome | 47/297    | 73/963  | 0.0000   |
|       | sae003030  | Purine metabolism | 25/297    | 43/963  | 0.0041   |
| 24hr  | sae03010   | Ribosome | 46/311    | 73/963  | 0.0000   |
|       | sae00240   | Pyrimidine metabolism | 18/311    | 28/963  | 0.0125   |
|       | sae00900   | Terpenoid backbone biosynthesis | 9/311     | 11/963  | 0.0195   |
| 48hr  | sae03010   | Ribosome | 56/460    | 73/963  | 0.0000   |
|       | sae00900   | Terpenoid backbone biosynthesis | 11/460    | 11/963  | 0.0065   |
|       | sae00550   | Peptidoglycan biosynthesis | 20/460    | 24/963  | 0.0065   |
|       | sae00240   | Pyrimidine metabolism | 22/460    | 28/963  | 0.0115   |

**Table 1** KEGG pathway enrichment analysis for upregulated and downregulated genes of *S. aureus*-infected liver compared with culture medium
**Supplementary Table 2** | Gene expression change in representative genes in *S. aureus*-infected mice compared with PBS-injected mice.

**2.2 Energy metabolism**

We further analyzed the gene expression changes in each *S. aureus* pathway in mouse liver after infection. The expression of genes involved in the glycolysis pathway was suppressed in mouse liver compared with the culture medium conditions (Supplementary Figure 2) throughout the infection period. Expression of genes involved in the TCA cycle was relatively upregulated until 24 h.p.i. and downregulated at 48 h.p.i. On the other hand, fermentation-related genes such as *pflB*, *ldh*, and *adhE* were highly upregulated at 24 and 48 h.p.i. In addition, the genes involved in nitrate respiration such as *narK*, which is required for nitrate uptake; the *narGHJI* operon encoding respiratory nitrate reductase; and the *nirBD* operon encoding assimilatory nitrite reductase, which produces nitrate (NO$_3^-$), an electron acceptor instead of oxygen in anaerobic conditions in the electron transport chain (Figure 2a)\(^6\), were upregulated at 24 h.p.i. and highly upregulated at 48 h.p.i. (Figure 2b). We established a disruption strain of the *narK* gene, the gene most upregulated in this system at 48 h.p.i. and found that *narK* gene-disrupted mutants had significantly reduced virulence in the mouse systemic infection model (Figure 2c). These results suggest that nitrate respiration was upregulated at the late stage of infection and required for full virulence of *S. aureus* under reduced oxygen pressure caused by progression of the infection. On the other hand, the mouse killing ability of the *pflB* gene-disrupted mutant was not significantly reduced (Supplementary Figure 3). *PflB* is involved in synthesizing acetyl CoA from pyruvate in anaerobic conditions, although other enzymes also cover this enzymatic reaction. Thus, an alternative pathway might compensate its function in the *pflB* gene disruption mutant.
Figure 2 | Gene expression changes regarding nitrate respiration of *S. aureus* infected in liver

a, Metabolic pathway of nitrate in *S. aureus*. b, Expression changes in genes involved in the nitrogen respiration pathway. The values in the box show Transcripts Per Million (TPM) and the boxes which TPM more than 300 filled by red. c, Survival curves of mice treated with wild-type and *narK* gene-disrupted strains by tail vein injection with 4.3x10^7 CFU and 4.6x10^7 CFU, respectively. (n=5 in each group, log-rank test, p=0.0035 chi square=8.544, df=1).
Supplementary Figure 2 | Gene expression changes related to glycolysis and the TCA cycle in infected mouse liver compared with culture medium.
Supplementary Figure 3| Mouse-killing ability of a disruption mutant of the pflB gene involved in anaerobic metabolism

Parent and ∆pflB strains were intravenously injected at doses of 3.8x10^7 CFU and 3.2x10^7 CFU, respectively. No significant difference was detected by log-rank test (p=0.6717, chi square=0.1797, df=1).

2.3 Lipid metabolism

KEGG pathway analysis suggested the upregulation of genes regulating fatty acid degradation. The genes fadABDE are involved in beta oxidation of lipids and required for acetyl-CoA production utilized in the TCA cycle. These genes were upregulated in the host liver from the initial stage of infection compared with culture medium conditions. In contrast, expression of the fabIH genes, required for type II fatty acid synthetase, a target for antimicrobial agents development\(^1\), were significantly downregulated in the later stage of infection. These findings suggest that S. aureus infected in mouse liver obtained a part of its energy from fatty acid degradation.
| gene | KEGG pathway | 6hr Fold change | FDR p-value | 24hr Fold change | FDR p-value | 48hr Fold change | FDR p-value |
|------|--------------|----------------|-------------|----------------|-------------|----------------|-------------|
| fadA | Fatty acid degradation* | 12.0 | <0.001 | 11.5 | <0.001 | 6.7 | <0.001 |
| fadB | Fatty acid | 26.3 | <0.001 | 16.8 | <0.001 | 7.3 | <0.001 |
| fadD | Fatty acid degradation* | 84.7 | <0.001 | 61.9 | <0.001 | 9.7 | <0.001 |
| fadE | Fatty acid | 23.4 | <0.001 | 30.0 | <0.001 | 7.7 | <0.001 |
| fab | Fatty acid elongation** | -1.8 | 0.050 | -2.3 | 0.006 | -5.3 | <0.001 |
| fabD | Fatty acid | -1.9 | 0.030 | -2.3 | 0.005 | -3.7 | <0.001 |
| fabG | Fatty acid elongation** | -1.8 | 0.024 | -2.8 | <0.001 | -3.2 | <0.001 |
| fabH | Fatty acid elongation** | -5.3 | <0.001 | -4.7 | <0.001 | -4.3 | <0.001 |
| fabI | Fatty acid elongation** | -1.1 | 0.959 | -1.5 | 0.214 | -3.2 | <0.001 |
| fabZ | Fatty acid elongation** | -2.7 | <0.001 | -4.1 | <0.001 | -4.5 | <0.001 |

Table 2 | Gene expression change of *S. aureus* lipid metabolism in the host liver compared with culture medium

*https://www.kegg.jp/kegg-bin/show_pathway?sa00071

**https://www.kegg.jp/kegg-bin/show_pathway?sa00061
2.4 Metal acquisition system

Iron acquisition is essential for pathogen growth in the host\textsuperscript{18}. \textit{S. aureus} has at least 5 iron acquisition systems, and the genes involved in these systems are known to be upregulated in the host, since the pathogen-infected host hides iron by increasing metal sequestration proteins, as shown in Supplementary Table 2. Although the expression of iron acquisition system genes was not upregulated at 6 h.p.i., it was highly increased at 24 and 48 h.p.i. (Figure 3A), a pattern that did not correspond to that of the host’s metal sequestration proteins, which were upregulated from 6 h.p.i. It might be that \textit{S. aureus} obtained iron from lysed hemocytes by hemolysins, which were highly upregulated at the initial infection stage in the host, as described below.

\textit{S. aureus} is known to have 2 manganese transporters; the \textit{mntABC} genes encoding the ABC transporter, and the \textit{mntH} gene encoding a proton-ion coupled transporter. Disruption mutants of both genes show reduced virulence against mice\textsuperscript{19}. In this analysis, we found that \textit{mntABC} genes and not the \textit{mntH} gene were highly upregulated in the host compared with the culture medium condition (Figure 3B). In addition, we revealed that disruption of the \textit{mntABC} gene operon in \textit{S. aureus} reduced virulence against mice (Figure 3C), indicating that the MntABC transporter significantly contributes to the virulence of \textit{S. aureus}. In addition, a staphylopine-mediated transport system related to the acquisition of broad metal ions such as iron, zinc, copper, nickel, and cobalt was recently reported\textsuperscript{12}. The expression level of the \textit{cntABCDF} operon encoding the ABC transporter increased from 24 h.p.i. to 48 h.p.i., and the expression level of the \textit{cntKLM} gene, which is involved in staphylopine synthesis, increased several-fold as the infection progressed. Disruption of the \textit{cntK} gene, which is involved in staphylopine synthesis, and the \textit{cntE} gene, which is involved in the secretion of staphylopine from the bacterial cell, significantly reduces virulence, suggesting that this metal transporter is essential for the virulence of \textit{S. aureus} (Figure 3D, E).

A gradual increase in the copper, molybdenum, and cobalt metal transporters, \textit{copA}, \textit{modABC}, and \textit{cobI}, respectively, was observed after infection. We observed no difference in the expression of the \textit{mgtE} gene, a transporter of magnesium, in the host compared with that in the culture medium condition.
Figure 3 Gene expression change in *S. aureus* metal acquisition system in the host liver compared with culture medium.

a. Time course of expression changes in the iron acquisition systems in *S. aureus*-infected liver...
compared with culture medium. The values in the box show TPM and the boxes with TPM more than 200 filled by red. b, Time course of expression changes in the divalent cation acquisition systems in *S. aureus*-infected liver compared with culture medium. The values in the box show TPM and the boxes which TPM more than 100 filled by red. c-e, Mouse-killing ability of a disruption mutant of the *mntA*, *cntE* and *cntK* gene, respectively. Bacterial suspensions were intravenously injected at doses of 3.8x10⁷ CFU, (c and e) and 4.3x10⁷ CFU (d) for wild type strain, and 3.8x10⁷ CFU, 4.3 x10⁷ CFU and 3.8x10⁷ CFU for ∆*mntA* strain (c), ∆*cntE* strain (d) and ∆*cntK* strain (e), respectively. Statistical analyses were performed by Log-rank (Mantel-Cox) test (n=5 in each group c: p=0.0006, chi square=11.74, df=1, d: p=0.0035, chi square=8.544, df=1, e: p=0.0015, chi square=10.04, df=1).

2.5 Virulence factors and their regulators

*S. aureus* possesses a wide variety of toxins, and the expression of these toxins, such as hemolysin, increases after infection¹⁴. In this study, we revealed the time course of these changes in gene expression (Figure 4). Expression of genes encoding hemolysins such as *hla* and *hlgABC* was highly upregulated from the initial stage of infection. Expression of these genes contribute to iron acquisition of *S. aureus* in the host at the early stage of infection. The expression level of leukotoxin genes was not increased at 6 h.p.i, but was increased after 24 h.p.i. Expression of superantigen genes, which are involved in evading the immune system²⁰, increased starting at 6 h.p.i, although significant expression increments were observed from 24 h.p.i. It is uncertain why the expression of genes corresponding to the host's immune response was delayed even though the innate immune system of the mice was already activated at 6 h.p.i.

We further evaluated the expression of 2-component regulatory systems identified in *S. aureus*, required for environmental responses and toxin regulators. The expression of *agr* and *sae* genes, which are necessary for toxin expression²¹, did not increase in the host, but rather tended to decrease at 48 h.p.i. (Table 3). These findings were consistent with findings in other models³,²². Several transcription factors are considered to influence *agr* gene regulation, which regulates toxin production²¹,²³,²⁴. The expression levels of *sarH1* and *sarX*, which negatively regulate *agr* gene expression, were decreased, while that of *rsr*, a repressor of *agr* expression, was increased. On the other hand, the expression levels of *sarA*, *sarZ*, *ccpA*, and *mgrA*, which positively regulate *agr* expression, tended to decrease. Furthermore, *sarH1* and *sarT*, which are negative regulators of *agr* and *sarA* genes, decreased at 6 h.p.i, but increased after 24 h.p.i. These
findings suggest that the expression of transcription factors involved in the regulation of virulence gene expression in the host condition was not consistent with the interpretation of the regulation of toxin production and *agr* gene expression based on *in vitro* analysis. Furthermore, expression of the tcs7RS gene, a 2-component regulatory system with unknown function, and *kdpDE* genes, a 2-component regulatory system for potassium homeostasis, was also upregulated throughout the infection process. In addition, we recently reported a novel virulence regulator, the *yjbH* gene, which regulates the expression of an iron transporter, and several virulence factors such as spa and leukotoxin and oxidative stress response genes, were upregulated until 24 h.p.i., suggesting that *yjbH* contributes to the expression of virulence-related genes in the early stage of infection in mice. These results suggest that transcription of *agr* gene was not positively regulated and other genes such as *yjbH* gene regulate the expression of virulence-related genes.

**Figure 4** | Time course of expression changes in virulence factors in *S. aureus*-infected liver compared with culture medium
| Gene    | Function                                                                 | 6h          | 24hr         | 48hr         | Fold increment |
|---------|---------------------------------------------------------------------------|-------------|--------------|--------------|----------------|
| **Two component factors**<sup>6</sup> |                                                                          |             |              |              |                |
| agrC    | Quorum sensing control of adhesion and virulence factors                 | 1           | 1            | -2.3         | 0.013         |
| agrA    | Virulence factors regulation (toxins, enzymes)                           | 1.2         | 0.769        | 1.2          | 0.266         |
| sasR    | Virulence factors regulation (toxins, enzymes)                           | 1.1         | 0.946        | 1.1          | <0.001        |
| vraR    | Cell wall -affecting antibiotic resistance, cell wall biosynthesis       | 2.3         | <0.001       | 1.7          | 0.009         |
| sarS    | Cell wall -affecting antibiotic resistance, cell wall biosynthesis       | 2.1         | 0.001        | 1.3          | 0.005         |
| graX    | AMP resistance, growth at low pH                                         | 1.9         | 0.007        | 1.1          | 0.981         |
| graR    | AMP resistance, growth at low pH                                         | 2.7         | <0.001       | 1.0          | 0.996         |
| graS    | AMP resistance, growth at low pH                                         | 1.9         | 0.007        | 1.3          | 0.694         |
| braR    | Antimicrobial peptide resistance                                         | 1.2         | 0.088        | 1.3          | 0.885         |
| sarR    | Pathogenicity, metabolism: autolysis, adherence, biofilm                 | -1.4        | 0.468        | -1.4         | 1.1           |
| sarA    | Pathogenicity, metabolism: autolysis, adherence, biofilm                 | 1.4         | 0.019        | -1.2         | 0.016         |
| walK    | Cell wall maintenance, cell viability                                    | 1.2         | 0.612        | -1.2         | 0.613         |
| whiB    | Intercellular survival, uptake of hexose phosphate                       | 1           | 0.115        | 1.1          | 0.216         |
| hptR    | Intercellular survival, uptake of hexose phosphate                       | -3.3        | 0.012        | -2.8         | 0.046         |
| tcsR    | Uncharacterized function                                                 | 4.9         | <0.001       | 3.0          | 0.865         |
| tcsS    | Uncharacterized function                                                 | 3.2         | <0.001       | 2.7          | 0.371         |
| srbB    | Anaerobic respiration, metabolism, growth at low temperature             | -1.8        | 0.027        | -1.5         | 0.443         |
| sarB    | Anaerobic respiration, metabolism, growth at low temperature             | -1.4        | 0.303        | -1.4         | 0.03          |
| phoR    | Phosphate uptake and homeostasis                                         | 5.3         | <0.001       | 3.4          | 0.486         |
| phoP    | Phosphate uptake and homeostasis                                         | 1.2         | 0.555        | -1.1         | 0.252         |
| airR    | Oxidative stress response                                                | -1.3        | 0.634        | 1.6          | 0.089         |
| airS    | Oxidative stress response                                                | 1.1         | 0.802        | 1.6          | 3.831         |
| kdpD    | Potassium homeostasis regulation                                         | 1.4         | 0.545        | 3.5          | <0.001        |
| kdpE    | Potassium homeostasis regulation                                         | 2.1         | 0.123        | 3.1          | <0.001        |
| hisS    | Heme metabolism regulation                                               | -1.7        | 0.317        | -5.3         | 0.228         |
| hisR    | Heme metabolism regulation                                               | 1.8         | 0.058        | -1.1         | 0.826         |
| nireC   | Response to low oxygen, nitrate reduction                                 | 1.3         | 0.337        | 1.2          | 0.56          |
| mefB    | Response to low oxygen, nitrate reduction                                 | 1.1         | 0.872        | 1.2          | 1.77          |
| **Transcription factors**<sup>7</sup> |                                                                          |             |              |              |                |
| sarA    | Positive to agr expression, induction of exoproteins and repression of spa | -1.6        | 0.051        | 1.7          | <0.001        |
| sarB    | Positive to agr expression, repression of spa, sarA                      | -1.3        | <0.001       | 1.8          | <0.001        |
| sarC    | Negative to agr, sarA                                                   | 1.1         | 0.792        | 1.1          | 0.001         |
| sarD    | Negative to agr, sarA                                                   | -1.1        | 0.925        | 1.1          | <0.001        |
| sarT    | Negative to agr, hla, sarU expression                                    | -9.4        | 0.068        | 6.9          | <0.001        |
| sarX    | Negative to agr                                                         | -8.6        | 0.001        | -12          | 0.255         |
| sarY    | Negative to agr                                                         | -1.2        | 0.015        | -1.5         | 0.011         |
| codY    | Negative to agr                                                         | 1.2         | 0.014        | 1.4          | 0.783         |
| mgrA    | Cytoplasmic regulator; induction of efflux pumps and capsule expression; repression of sarZ, sarX | -1.9        | 0.017        | 1.1          | <0.001        |
| mraY    | Repressor of agr<sup>8</sup>                                             | 4.9         | <0.001       | 2.3          | 0.992         |
| sigA    | Stress response                                                         | -2.4        | <0.001       | -2.8         | <0.001        |
| sigB    | Virulence factor regulator<sup>9</sup>                                   | 0.8         | <0.001       | 2.4          | 0.139         |

Table 3: Gene expression change in the *S. aureus* 2-component system and virulence-related transcription factors in the host liver compared with culture medium
Conclusions

In the present study, *S. aureus* gene expression changes in mouse liver after systemic infection were analyzed over time by *in vivo* RNA-Seq. The results suggested that *S. aureus* responds to changes in oxygenation and environmental influences associated with deterioration of the host's cardiovascular status as the infection progresses, and obtains energy through anaerobic respiration. Further, expression of metal transporters did not increase until 6 h.p.i., but increased remarkably after that time. Our results also revealed the contribution of the manganese transporter *mntABC* and staphylopine to the pathogenicity of the broad metal transport system for the first time. As for the expression of pathogenic toxins, the timing of the upregulation differed depending on the toxin. Blood hemolytic toxin significantly increased from the early stage of infection, while the expression levels of leukocyte toxin, superantigens, and serine proteases increased in the late stage of infection. *S. aureus* exhibits a sophisticated response to changes in environmental conditions in the host during infection by regulating gene expression.

Material and Method

Ethics statement

All mouse experiments were performed at the University of Tokyo following the regulations for animal care and use and approved by the Animal Use Committee at the Graduate School of Pharmaceutical Science at the University of Tokyo (P27-4).

*S. aureus* infection and organ collection for RNA isolation

*S. aureus* Newman strain was grown overnight on TSB medium at 37°C. The full growth was diluted 100-fold with 5 ml TSB and regrown, and then the cells were centrifuged and suspended in PBS pH 7.2. The cells (5.6×10⁷ CFU) were injected into C57BL/6J mice via the tail vein. At 6, 24, and 48 h.pi, mice were killed to isolate liver. The organs were immediately placed in liquid nitrogen and maintained at -80°C until RNA extraction. One kidney and a part of the liver were homogenized to calculate viable cell numbers in each organ. Each experiment was conducted with 3 animals and data are represented as an average.

Enrichment of *S. aureus* Newman RNA from infected mouse organs and removal of ribosomal RNA
Mouse organs were homogenized and lysed in buffer RLT using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) with 5-mm zirconia beads by shaking using a Bead Crusher (μT-12, Taitec, Saitama, Japan) at 2500 rpm for 1 min. The samples were centrifuged, and precipitants were washed with an equal volume of PBS. Bacterial cell precipitates were suspended in 200 μl of TE containing 0.2 mg/mL lysostaphin and incubated at room temperature for 30 min. After adding 700 μl of RLT and 200 μl volume of 0.5-mm zirconia beads, the samples were shaken at 2500 rpm for 5 min, and debris was removed by centrifugation. The RNA was purified according to the manufacturer’s instructions. Ribosomal RNA in the above samples was removed using a Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) (Illumina, San Diego, CA) according to the manufacturer’s protocol.

Library preparation and RNA-sequencing

RNA-Seq analysis for differential expression analysis was performed with the HiSeq platform (Illumina) or Ion Proton system (Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, for HiSeq, the RNA-Seq libraries were prepared using the TruSeq RNA sample preparation kit (version 2; Illumina), except the poly(A) selection procedure was omitted. The double-stranded PCR products were purified and size-fractionated using a bead-mediated method with AMPure XP (Beckman Coulter, California, CA). The RNA-Seq libraries were quantified by a bioanalyzer (Agilent, California, CA). Thirty-six base-pair single-end sequencing was conducted on a HiSeq 2000 or 2500 platform, using a TruSeq SR Cluster Kit v3-cBot-HS and a TruSeq SBS kit (version 3-HS). For the Ion Proton system, library preparation for RNA-Seq was performed using an Ion Total RNA-Seq Kit v2 following the manufacturer’s instructions. Briefly, rRNA depletion from the RNA was achieved using a MICROBExpress™ Kit (Thermo Fisher Scientific, Waltham, MA). The RNA was then fragmented by RNase III, reverse transcribed, and amplified. The size distribution and yield of the amplified library was confirmed in the bioanalyzer, and the libraries were enriched in an Ion PI Chip v2 using the Ion Chef (Thermo Fisher Scientific). Subsequent sequencing was performed in the Ion Proton System. The data were deposited in the DNA Data Bank of Japan (DDBJ) BioProject under accession number PRJDB3874.

Differential gene expression analysis
All data were analyzed using CLC Genomics Workbench software, version 12 (CLC Bio, Aarhus, Denmark). Reads were aligned to the Newman genome (Accession No. NC_009641) and the mouse genome (Mus_musculus.GRCm38) allowing a minimum length fraction of 0.95 and minimum similarity fraction of 0.95. Differential gene expression analysis was performed using edgeR analysis\(^\text{29}\) for a normalized dataset by scaling using the default setting. Genes with an FDR \(p<0.05\) using the Benjamini and Hochberg’s algorithm\(^\text{30}\) were classified as having significantly different expression.

**Data analysis**

For the KEGG pathway analysis, we selected *S. aureus* genes whose expression levels changed more than 2-fold in mouse liver compared with *in vitro* culture medium conditions and whose FDR p-value was less than 0.05. R ver. 3.6.1, Bioconductor 3.10 package, and pathview\(^\text{31}\) were used. GO term enrichment analysis was performed on the site http://geneontology.org using the Reactome pathway. The genes selected for expression were significantly (FDR p-value < 0.05) upregulated or downregulated 5-fold in *S. aureus*-infected mice compared with PBS-treated mice and analyzed by the Fishers exact test with FDR correction (FDR p-value < 0.05 was considered statistically significant).

**Construction of *S. aureus* mutants and complement strain**

Single cross-over recombination; gene disruptions were performed as previously described\(^\text{32}\). In summary, the internal regions within the open reading frames of the gene were amplified by PCR (Prime Star Max DNA polymerase, Takara, Tokyo, Japan) using the primers listed in **Supplementary Table 2**, and the PCR product was cloned into integration vector pCK20\(^\text{32}\). The plasmid was then transformed to *S. aureus* RN4220\(^\text{23}\) by electroporation. Double cross-over recombination; gene disruptions were performed as previously described\(^\text{33}\). The genome DNA regions upstream and downstream of the target region were amplified by PCR using the listed primers, and then overlap extension-PCR was performed using these 2 DNA fragments together with the *aph* gene amplified from the pSF151 vector (primers: KmF; 5’ AGCGAACCATTTGAGGTGAT 3’ and KmR; 5’ GGGACCCCT ATCTAGCGAAC 3’). The PCR product was cloned into the pKOR3a vector\(^\text{33}\) and introduced into the RN4220 strain by electroporation. Integration of the mutant cassette in the genome was confirmed by PCR and further transformed into *S.
S. aureus Newman\textsuperscript{34} by phage transduction using phage 80α as previously described\textsuperscript{35}.

| Disrupted gene | Primer name | Sequence (5'->3') |
|----------------|-------------|------------------|
| pflB           | 0162\_F    | GTGTCCTTTAAGCATAGT |
|                | 0162\_R    | CAGATGGAGGCGTTTATAGT |
| mntA           | 0603\_F    | CTACAGTCAGTGCTACTC |
|                | 0603\_R    | TGGTGCTGGTAAATCTTC |
| narK           | 2288\_F    | TTTTTTGACCTACGTTTCTTGTGTGCACC |
|                | 2288\_R    | TTTTTGGATCGTTTATTGTTGGGTGTATGG |
| cntE           | 2359\_F    | TTTTTTGACGCAGAGCTAGCCAAAGAATCTC |
|                | 2359\_R    | TTTTTGATCCCTGGCCCTTTTGGAGAT |
| cntK           | 2367\_F    | ACTAAATACTGCCCTCTC |
|                | 2367\_R    | ATGCACTATCAGCCAATC |

**Supplementary Table 2** Primers used in this study

**Mouse survival assay**

S. aureus Newman wild-type and mutant strains were grown overnight on TSB medium supplemented with antibiotics on a rotary shaker maintained at 37˚C to obtain full growth. The full growth was diluted 100-fold with TSB and cultured overnight on the same shaker, and then the cells were centrifuged and resuspended in PBS pH 7.2 to an optical density of 0.7 at 600 nm. From this, 200 µl of the cells was injected intravenously into C57BL/6J mice, and mouse survival was determined. Survival analysis was performed using GraphPad Prism ver 9.0 (GraphPad Software), and statistical analysis was performed using the log-rank test.

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**Author contributions**

H.H. established and performed the *in vivo* RNA-Seq analysis. H.H and S.P. wrote the manuscript. S.P. and S.O. prepared the gene disruption mutants. H.H., S.P., and S.O.
performed the mouse systemic infection assays. Y.S. performed the RNA-Seq by Hi-Seq. K.S. critically revised the article for important intellectual content and provided final approval of the article.

**Competing interest declaration**

The authors declare competing financial interests as follows: Dr. Sekimizu is a consultant for Genome Pharmaceutical Institute Co, Ltd.

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