Transcriptome change in *Staphylococcus aureus* in infecting mice

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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 texobactin¹ and lysocin E² 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 bacteremia⁵ despite its relatively small genome size⁶. In addition, *S. aureus* easily acquires multidrug resistance⁷. 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 proteases⁹, and grows under both aerobic and anaerobic conditions¹⁰. 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^{11,12}. 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^{13,14}. 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*¹⁵, 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 fasciitis¹⁵. 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 10⁷ colony forming unit (CFU)/mg in the liver at 6 h.p.i. and kept more than 10⁶ 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.

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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	88,463,592	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%)			

96

97 **Supplementary Table 1** | Summary of *in vivo* RNA-Seq results of *S. aureus* in infected mouse liver

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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 (5.6×10^7 CFU) were injected into the tail vein (n=5).

b, Number of *S. aureus* cells in each organ after infection with (5.3×10^7 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	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	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
	sae00230	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

gene	category	function	6 hr		24 hr		48 hr	
			Fold	FDR-p-val.	Fold	FDR-p-val.	Fold	FDR-p-val.
Trf	Metal sequestration	transferrin	1.4	1.000	-1.0	1.000	3.6	0.017
Ltf		lactotransferrin	23.7	0.029	32.7	0.002	407.1	<0.001
Lcn2		lipocalin 2	162.2	<0.001	349.7	<0.001	861.9	<0.001
Hamp		hepcidin antimicrobial peptide	-1.6	0.802	-2.1	0.713	1.3	1.000
Tlr2	Innate immunity	toll-like receptor 2	140	<0.001	8.4	<0.001	8.0	<0.001
Myd88		myeloid differentiation primary response gene 88	7.1	<0.001	2.3	0.037	3.6	0.001
Saa3		serum amyloid A 3	370	<0.001	840	<0.001	3300	<0.001
Nos2		nitric oxide synthase 2, inducible	234	<0.001	200	<0.001	610	<0.001
mt-Cytb	Respiratory electron transport	mitochondrially encoded cytochrome b	-17	<0.001	-10	<0.001	-21	<0.001
Uqcrcq		ubiquinol-cytochrome c reductase, complex III subunit VII	-1.8	0.661	-3.9	0.001	-16	<0.001
mt-ND4l		mitochondrially encoded NADH dehydrogenase 4L	-15	<0.001	-5.5	<0.001	-18	<0.001
Hilpda	Hypoxia	hypoxia inducible lipid droplet associated	2.3	0.440	5.3	0.002	11.7	<0.001
Hif1a		hypoxia inducible factor 1, alpha subunit	1.3	1.000	1.4	0.835	4.1	<0.001
Hif3a		hypoxia inducible factor 3, alpha subunit	-4.1	0.175	8.1	0.001	4.0	0.157

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)¹⁶, 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 4.3×10^7 CFU and 4.6×10^7 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

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180

181 **Supplementary Figure 3** | Mouse-killing ability of a disruption mutant of the *pflB* gene involved in
 182 anaerobic metabolism

183 Parent and $\Delta pflB$ strains were intravenously injected at doses of 3.8×10^7 CFU and 3.2×10^7 CFU,
 184 respectively. No significant difference was detected by log-rank test ($p=0.6717$, chi square=0.1797,
 185 $df=1$).

186

187 **2.3 Lipid metabolism**

188 KEGG pathway analysis suggested the upregulation of genes regulating fatty acid
 189 degradation. The genes *fadABDE* are involved in beta oxidation of lipids and required for
 190 acetyl-CoA production utilized in the TCA cycle. These genes were upregulated in the
 191 host liver from the initial stage of infection compared with culture medium conditions. In
 192 contrast, expression of the *fabIH* genes, required for type II fatty acid synthetase, a target
 193 for antimicrobial agents development¹⁷, were significantly downregulated in the later
 194 stage of infection. These findings suggest that *S. aureus* infected in mouse liver obtained
 195 a part of its energy from fatty acid degradation.

196

gene	KEGG pathway	6hr Fold change	FDR p-value	24hr Fold change	FDR p-value	48hr Fold change	FDR p-value
<i>fadA</i>	Fatty acid degradation*	12.0	<0.001	11.5	<0.001	6.7	<0.001
<i>fadB</i>		26.3	<0.001	16.8	<0.001	7.3	<0.001
<i>fadD</i>	degradation*	84.7	<0.001	61.9	<0.001	9.7	<0.001
<i>fadE</i>		23.4	<0.001	30.0	<0.001	7.7	<0.001
<i>fab</i>	Fatty acid elongation**	-1.8	0.050	-2.3	0.006	-5.3	<0.001
<i>fabD</i>		-1.9	0.030	-2.3	0.005	-3.7	<0.001
<i>fabG</i>	elongation**	-1.8	0.024	-2.8	<0.001	-3.2	<0.001
<i>fabH</i>		-5.3	<0.001	-4.7	<0.001	-4.3	<0.001
<i>fabI</i>		-1.1	0.959	-1.5	0.214	-3.2	<0.001
<i>fabZ</i>		-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?sac00071

**https://www.kegg.jp/kegg-bin/show_pathway?sac00061

2.4 Metal acquisition system

Iron acquisition is essential for pathogen growth in the host¹⁸. *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 *S. aureus* obtained iron from lysed hemocytes by hemolysins, which were highly upregulated at the initial infection stage in the host, as described below.

S. aureus is known to have 2 manganese transporters; the *mntABC* genes encoding the ABC transporter, and the *mntH* gene encoding a proton-ion coupled transporter. Disruption mutants of both genes show reduced virulence against mice¹⁹. In this analysis, we found that *mntABC* genes and not the *mntH* gene were highly upregulated in the host compared with the culture medium condition (Figure 3B). In addition, we revealed that disruption of the *mntABC* gene operon in *S. aureus* reduced virulence against mice (Figure 3C), indicating that the MntABC transporter significantly contributes to the virulence of *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 reported¹². The expression level of the *cntABCDF* operon encoding the ABC transporter increased from 24 h.p.i. to 48 h.p.i., and the expression level of the *cntKLM* gene, which is involved in staphylopine synthesis, increased several-fold as the infection progressed. Disruption of the *cntK* gene, which is involved in staphylopine synthesis, and the *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 *S. aureus* (Figure 3D, E).

A gradual increase in the copper, molybdenum, and cobalt metal transporters, *copA*, *modABC*, and *cobI*, respectively, was observed after infection. We observed no difference in the expression of the *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 3.8×10^7 CFU, (**c** and **e**) and 4.3×10^7 CFU (**d**) for wild type strain, and 3.8×10^7 CFU, 4.3×10^7 CFU and 3.8×10^7 CFU for $\Delta mntA$ strain (**c**), $\Delta cntE$ strain (**d**) and $\Delta cntK$ 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 models^{3,22}. Several transcription factors are considered to influence *agr* gene regulation, which regulates toxin production^{21,23,24}. The expression levels of *sarHI* 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, *sarHI* 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 genes²⁵, 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	Fold increment					
			6h	FDR p-value	24hr	FDR p-value	48hr	FDR p-value
Two compo nent factors ²⁶	<i>agrC</i>	Quorum sensing control of adhesion and	1	1	-1.2	0.765	-2.3	0.013
	<i>agrA</i>	virulence factors	1.2	0.696	1.2	0.672	-1.9	0.026
	<i>saeS</i>	Virulence factors regulation (toxins,	1.1	0.743	1.1	0.848	-4.7	<0.001
	<i>saeR</i>	enzymes)	1	0.996	-1.1	0.918	-4.2	<0.001
	<i>vraR</i>	Cell wall -affecting antibiotic resistance, cell	2.3	<0.001	1.7	0.024	-2	0.009
	<i>vraS</i>	wall biosynthesis	2.1	0.001	1.3	0.279	-2.6	0.005
	<i>graX</i>		1.9	0.007	1.1	0.951	1	0.996
	<i>graR</i>	AMP resistance, growth at low pH	2.7	<0.001	-1	0.998	-1.3	0.545
	<i>graS</i>		1.9	0.007	1.3	0.464	-1.2	0.694
	<i>braS</i>	Antimicrobial peptide resistance	1.2	0.588	1.3	0.365	-1.2	0.581
	<i>braR</i>		-1.4	0.468	-1.4	0.54	1.1	0.885
	<i>arlS</i>	Pathogenesis mechanisms: autolysis,	1.4	0.199	-1.2	0.576	-2	0.016
	<i>arlR</i>	adhesion, biofilm	-1.5	0.18	-2.1	0.022	-1.9	0.023
	<i>WalR</i>	cell wall maintenance, cell viability	-1.2	0.612	-1.2	0.583	-1.3	0.613
	<i>WalK</i>		1	1	-1.2	0.738	-1.4	0.166
	<i>hptS</i>	intercellular survival, uptake of hexose	-2.2	0.115	1.1	0.922	1.6	0.21
	<i>hptR</i>	phosphate	-3.3	0.012	-2.8	0.029	1.9	0.04
	<i>tcs7R</i>	Uncharacterized function	4.9	<0.001	3	0.001	1.9	0.085
	<i>tcs7S</i>		3.2	<0.001	2.7	0.005	1.4	0.371
	<i>srrB</i>	Anaerobic respiration, metabolism, growth	-1.8	0.027	-1.5	0.141	1.3	0.443
	<i>srrA</i>	at low temperature	-1.4	0.303	-1.4	0.316	2.1	0.03
	<i>phoR</i>	Phosphate uptake and homeostasis	5.3	<0.001	3.4	<0.001	1.2	0.486
	<i>phoP</i>		1.2	0.555	-1.1	0.871	1.6	0.252
	<i>airR</i>	Oxidative stress response	-1.3	0.634	1.6	0.272	-1.8	0.089
	<i>airS</i>		1.1	0.802	1	0.998	-1.1	3831
	<i>kdpD</i>	Potassium homeostasis regulation	1.4	0.545	3.5	<0.0001	3.7	<0.001
	<i>kdpE</i>		2.1	0.123	3.1	0.005	4.4	<0.001
	<i>hssR</i>	Heme metabolism regulation	-1.7	0.317	-5.3	0.018	1.7	0.228
	<i>hssS</i>		1.8	0.058	-1.1	0.888	-1.1	0.826
	<i>nreC</i>	Response to low oxygen, nitrate reduction	1.3	0.337	1.2	0.576	1.2	0.56
	<i>nreB</i>		1.1	0.872	1.2	0.635	1.6	0.177
Transcr iption factor ²⁴	<i>sarA</i>	Positive to agr expression, induction of	-1.6	0.051	1.7	0.118	-5.4	<0.001
	<i>sarH1</i>	exoproteins and repression of spa	-13.6	<0.001	-8.7	<0.001	-4.3	<0.001
	<i>sarU</i>	Repressor of agr, sarA	-5.2	0.925	18.6	0.001	4.3	<0.001
	<i>sarR</i>	Positive to agr expression,	1.1	0.792	1.1	0.801	-5.2	<0.001
	<i>sarT</i>	Negative to agr, sarA	-9.4	0.868	6.9	0.039	5.3	<0.001
	<i>sarX</i>	Negative to agr, hla, sarU expression	-8.6	0.001	-1.2	0.778	-1.7	0.255
	<i>sarZ</i>	Negative to agr	-1.7	0.041	-1.5	0.136	-2.6	0.009
	<i>CcpA</i>	Positive to agr ²⁷	1.8	0.015	-1.1	0.797	-2.3	0.011
	<i>codY</i>	Positive to agr	1.8	0.001	1.4	0.257	-1.1	0.783
	<i>mgrA</i>	Negative to agr	-1.9	0.017	1.1	0.966	-4.6	<0.001
		Cytoplasmic regulator; induction of efflux						
		pumps and capsule expression; repress of						
		surface proteins, Positive to agr, sarZ, sarX						
	<i>rsr</i>	Repressor of agr ²⁸	4.9	<0.001	2.3	0.003	-1.9	0.092
	<i>sigB</i>	Stress response	-2.4	<0.001	-2.6	<0.001	-2.2	<0.001
	<i>yjbH</i>	Virulence factor regulator ²⁵	5.6	<0.001	2.4	<0.001	-1.4	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 staphylopin 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 (5.6×10^7 CFU) were injected into C57BL/6J mice via the tail vein. At 6, 24, and 48 h.p.i, 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 ϕ 5-mm zirconia beads by shaking using a Bead Crusher (μ T-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 μ l of TE containing 0.2 mg/mL lysostaphin and incubated at room temperature for 30 min. After adding 700 μ l of RLT and 200 μ l volume of ϕ 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 MICROBExpressTM 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 analysis²⁹ for a normalized dataset by scaling using the default setting. Genes with an FDR $p < 0.05$ using the Benjamini and Hochberg's algorithm³⁰ 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 pathview³¹ 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 described³². 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 pCK20³². The plasmid was then transformed to *S. aureus* RN4220²³ by electroporation. Double cross-over recombination; gene disruptions were performed as previously described³³. 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' AGCGAACCATTGAGGTGAT 3' and KmR; 5' GGGACCCCT ATCTAGCGAAC 3'). The PCR product was cloned into the pKOR3a vector³³ 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.*

aureus Newman³⁴ by phage transduction using phage 80α as previously described³⁵.

Disrupted gene	Primer name	Sequence (5'→3')
pflB	0162_F	GTGTCCCTTAAGCATAGT
	0162_R	CAGATGGAGGCGTTTAGT
mntA	0603_F	CTACAGTCAGTGCTACTC
	0603_R	TGGTGCTGGTAAATCTTC
nark	2288_F	TTTTTGTCGACCTACGTTCTTGTGTGTCACC
	2288_R	TTTTTGGATCCGTTTAGTGTTGGGTTTATGG
cntE	2359_F	TTTTTGTCGACCGCGATAGCCAAAGAATCT
	2359_R	TTTTTGGATCCCTTGGCCCTTTTGAGAT
cntK	2367_F	ACTAAATACTGCCCCCT
	2367_R	ATGCATCTATCGCCAATC

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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