Induction of Virulence Gene Expression in *Staphylococcus aureus* by Pulmonary Surfactant

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We performed a genomewide analysis using a next-generation sequencer to investigate the effect of pulmonary surfactant on gene expression in *Staphylococcus aureus*, a clinically important opportunistic pathogen. RNA sequence (RNA-seq) analysis of bacterial transcripts at late log phase revealed 142 genes that were upregulated >2-fold following the addition of pulmonary surfactant to the culture medium. Among these genes, we confirmed by quantitative reverse transcription-PCR analysis that mRNA amounts for genes encoding ESAT-6 secretion system C (EssC), an unknown hypothetical protein (NWMN_0246; also called pulmonary surfactant-inducible factor A [PsiA] in this study), and hemolysin gamma subunit B (HlgB) were increased 3- to 10-fold by the surfactant treatment. Among the major constituents of pulmonary surfactant, i.e., phospholipids and palmitate, only palmitate, which is the most abundant fatty acid in the pulmonary surfactant and a known antibacterial substance, stimulated the expression of these three genes. Moreover, these genes were also induced by supplementing the culture with detergents. The induction of gene expression by surfactant or palmitate was not observed in a disruption mutant of the *sigB* gene, which encodes an alternative sigma factor involved in bacterial stress responses. Furthermore, each disruption mutant of the *essC*, *psiA*, and *hlgB* genes showed attenuation of both survival in the lung and host-killing ability in a murine pneumonia model. These findings suggest that *S. aureus* resists membrane stress caused by free fatty acids present in the pulmonary surfactant through the regulation of virulence gene expression, which contributes to its pathogenesis within the lungs of the host animal.

Pathogenic bacteria infecting host animals sense environmental changes and alter the expression of various genes to exert their virulence. Recently, the virulence genes required for *Staphylococcus aureus* to cause sepsis in infected hosts were identified by studying the global alterations of the gene expression patterns induced by serum and blood (1). Pathogens that induce pneumonia, however, are inhaled through the nasal cavities and transferred through the bronchus to the lung, followed by colonization on alveolar lumens and alveolar epithelial cells (2). Bacteria infecting the lungs are then exposed to pulmonary surfactant, which covers the alveolar surfaces. Pulmonary surfactant is a secretion comprising mostly lipids (90% of dry weight) that is produced by alveolar epithelial cells to maintain the surface tension of the alveoli (3). The major lipid constituents of surfactant are phosphatidylcholine (80% of the dry weight of total lipids), phosphatidylglycerol (10%), and free fatty acids (10%) (3). Approximately 60% of the phosphatidylcholine is dipalmitoyl-phosphatidylcholine (DPPC), and the other 40% is phosphatidylcholines containing unsaturated fatty acids, such as 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC). Palmitate is the most abundant free fatty acid in the pulmonary surfactant. When bacteria are exposed to such a lipid-rich environment, distinctive responses different from those in bacteria circulating in the bloodstream or infecting other tissues are likely to be induced, which may contribute to virulence in host lungs. Clarification of the mechanisms underlying the bacterial responses to pulmonary surfactant is important for enhancing our understanding of lung infections and overcoming pneumonia. The effects of pulmonary surfactant on gene expression in pathogenic bacteria have not yet been studied at the molecular level.

Staphylococcus aureus is an opportunistic pathogen that causes sepsis and pneumonia. The recent emergence of *S. aureus* strains resistant to several antibiotics, including methicillin, has led to serious clinical issues. Moreover, some of these resistant strains, community-associated methicillin-resistant *S. aureus*

(CA-MRSA), have acquired higher virulence potentials than those of hospital-associated methicillin-resistant *S. aureus* (HA-MRSA) (4, 5). Our research group has investigated various aspects of the virulence mechanisms of *S. aureus*, e.g., identification and characterization of novel virulence genes (6); investigation of the involvement of the staphylococcal cassette chromosome *mec* element (SCC*mec*), a mobile genetic element that provides methicillin resistance, in pathogenesis (7, 8); analysis of activation mechanisms of innate immunity by use of insects as animal models (9–13); and searching for new antibiotics with novel chemical structures (14, 15). These studies have provided some insights into sepsis and have contributed to the establishment of some drug therapies. Here we performed a genomewide analysis of gene expression in *S. aureus* exposed to pulmonary surfactant to determine the virulence mechanism of lung-infecting *S. aureus*.

MATERIALS AND METHODS

Ethics statement. This study was performed in strict accordance with the recommendations of the *Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions* (16) under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. All mouse protocols followed the regulations for animal care and use of the University of Tokyo and were approved by the Animal Use Committee at the Graduate School of

Pharmaceutical Science at the University of Tokyo (approval number 24-51).

Bacteria, animals, and reagents. *S. aureus* strains Newman, RN4220, MW2, and FRP3757 were aerobically cultured in tryptic soy broth (TSB) (Becton, Dickinson and Co.) at 37°C for 18 to 24 h with vigorous shaking. *Escherichia coli* JM109 was used as a host for plasmids in DNA manipulations. C57BL/6JJc1 mice were obtained from CLEA Japan, Inc. Surfacten, a pulmonary surfactant extracted from cow lungs, was purchased from the Mitsubishi Tanabe Pharma Corporation and suspended in saline. Bovine calf serum was purchased from SAFC Biosciences and was heat inactivated at 50°C for 30 min before use. DPPC and POPC were purchased from Wako Pure Chemical Industries and the NOF Corporation, respectively. 1-Palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) and sodium palmitate were purchased from Sigma-Aldrich. Oleic acid sodium salt and detergents, such as sodium dodecyl sulfate (SDS), Triton X-100, and polyoxyethylene sorbitan monolaurate (Tween 20), were purchased from Nacalai Tesque.

RNA sequence (RNA-seq) analysis by use of a next-generation sequencer. TSB medium (5 ml) supplied with 0.3 mg/ml of Surfacten or 10% calf serum was inoculated with 50 μl of an overnight culture of *S. aureus* Newman. The culture was incubated at 37°C to an optical density at 600 nm (OD_{600}) of 1.0 ± 0.1, and then RNAprotect bacterial reagent (Qiagen) was added at twice the culture volume. After 5 min of incubation at room temperature, the cells were washed in 0.5 ml of phosphate-buffered saline (PBS). Each pellet was suspended in 180 μl Tris-EDTA (TE) buffer (pH 8.0) supplied with 20 μl of 10-mg/ml lysostaphin, and samples were incubated for 30 min at room temperature. RNA extraction was performed with an RNeasy minikit (Qiagen) according to the manufacturer's instructions.

RNA-seq analysis was conducted basically following the manufacturer's instructions. Briefly, the RNA-seq template was prepared using a TruSeq RNA sample preparation kit (version 5; Illumina), omitting the poly(A) selection procedure. The double-stranded PCR products were purified and size fractionated using a bead-mediated method with AMPure (Ambion). Sequencing was conducted on a HiSeq2000 platform (Illumina), using a TruSeq RNA sample prep kit, version 2, and a TruSeq SBS kit, version 3-HS. At least 20 million sequences of 36-base single-end reads were generated per sample. The obtained data were analyzed by CLC Genomics Workbench software, version 6.0.2 (CLC Bio, Aarhus, Denmark) (for details, see Table S1 in the supplemental material). The number of reads per kilobase of exon per million mapped reads (rpkm) for each gene was compared between surfactant- or serum-treated groups and the nontreated group.

Quantitative RT-PCR analysis. RNAs were extracted from 1- to 5-ml cultures supplied with Surfacten, serum, lipids, or detergents as described above. The genomic DNA was degraded with RQ1 RNase-free DNase (Promega). cDNAs were synthesized by TaqMan reverse transcription (RT) reagents (Applied Biosystems) according to the manufacturer's instructions. Sequences of primers used in this study are described in Table S2 in the supplemental material. cDNAs were mixed with primers and FastStart Universal SYBR green master (ROX) mix (Roche), and the analysis was performed using a Step One Plus real-time PCR system (Applied Biosystems). The expression level of each gene was normalized to that of 16S rRNA, which is widely used as an internal control (7, 17). We confirmed that the amount of 16S rRNA did not vary significantly under our experimental conditions. The induction ratio relative to that of the nontreated group was also calculated.

Lipid extraction. Lipids were extracted from cow lungs by the Bligh-Dyer method, as follows. Cow lungs (90 g) in PBS were homogenized by use of a food processor. Approximately 100 ml of the tissue homogenate was mixed with 250 ml of methanol and 125 ml of chloroform (volume ratio of 0.8:2:1), followed by stirring for 30 min. The solution was filtered through filter paper (Toyo Roshi Kaisha, Ltd.), and 125 ml each of chloroform and water was added with rigorous mixing. The sample was centrifuged at $1,000 \times g$ for 15 min, and the lower (organic) and upper

(water) phases were collected and evaporated. The obtained solid materials were suspended in saline before use.

Measurement of promoter activity. Construction of strains harboring luciferase-encoding plasmids and measurement of luciferase activity were performed essentially as described previously (7). The predicted promoter regions of *essC* and *hlgB* were cloned from the genomic DNA of the Newman strain. Because the *hlgB* gene is suggested to be transcribed as part of an operon with the *hlgC* gene (18), the promoter region of *hlgC* was cloned for analysis of the *hlgB* gene. The PCR primers used for cloning of the *essC* and *hlgC* promoters are listed in Table S2 in the supplemental material. The amplified fragments (550 bp and 441 bp) were digested with both EcoRI and KpnI and then ligated with the pluc vector, a modified pND50 plasmid (19) that contains a functional ribosomal binding site and the translational start codon of *luc* in all reading frames. The constructed plasmids (pluc-*hlgBC*pro and pluc-*essC*pro) were first introduced into the *S. aureus* RN4220 laboratory strain by electroporation (20) and then transduced into the *S. aureus* Newman strain by using phage 80α (21).

Transductants of strain Newman harboring either the pluc-*hlgBC*pro or pluc-*essC*pro plasmid were grown in TSB medium supplied with 12.5 μg/ml chloramphenicol. Each overnight culture (10 μl) was inoculated into 1 ml of TSB medium in the presence of palmitate (0.1 mg/ml) or Surfacten (0.3 mg/ml). The culture was incubated at 37°C to an OD_{600} of 0.7, and then the cells were collected by centrifugation. Immediately after discarding the supernatant, pellets were frozen in liquid nitrogen. Cells were suspended in 50 μl PBS and mixed with 0.5 ml lysis buffer (25 mmol/liter KH_2PO_4 [pH 7.8], 10 μg/ml lysostaphin, 0.04% Triton X-100, 0.1 mmol/liter dithiothreitol, and Complete proteinase inhibitor cocktail [Roche]). After incubation at room temperature for 30 min, the samples were centrifuged. The supernatant was diluted 10-fold with the lysis buffer, and then 100 μl of each sample was mixed with 100 μl substrate solution from a luciferase assay system (Promega). The luciferase activities were measured with a luminometer (Lumat LB 9507; EG&G Berthold). Protein concentrations were determined using the Coomassie Plus protein assay reagent (Thermo) with bovine serum albumin as a standard, and the luminescence units were normalized to the protein amounts.

Construction of disruption mutants of *S. aureus*. Disruption mutants of the two-component system regulators (Δ*agr*, Δ*arlS*, and Δ*saeS*), with Newman as the parent strain, were constructed in a previous study (22). Disruptions of the *sigB* (NWMN_1970), *essC* (NWMN_0223), *psiA* (NWMN_0246), and *hlgB* (NWMN_2320) genes in the Newman strain were performed essentially as described previously (6, 7, 22). Briefly, internal regions within open reading frames of the *sigB* (+33 to +538; the first nucleotide of the start codon is numbered +1, and the length of the target region is 506 bp), *essC* (+109 to +1044; 936 bp), and *hlgB* (+25 to +862; 838 bp) genes were amplified by PCR and cloned into an *S. aureus* integration vector, pSF151 (23). The constructed plasmids were transferred into *S. aureus* RN4220 by electroporation, and the integrated regions containing the Km^r gene were transduced into Newman by use of phage 80α as described above, resulting in disrupted mutants of the *sigB*, *essC*, and *hlgB* genes (designated Δ*sigB*, Δ*essC*, and Δ*hlgB* mutants). For the *psiA* gene, with a relatively short coding region (399 bp), the upstream (−1126 to −241; 898 bp) and downstream (+584 to +1403; 820 bp) regions of the start codon were amplified by a PCR using degenerative primers to create linker sequences at the ends. The Km^r gene of the pSF151 vector with linker sequences was amplified separately. The DNA fragments of the upstream region, the Km^r gene, and the downstream region were linked in this order by a PCR using these fragments as templates, the forward primer of the upstream region, and the reverse primer of the downstream region. The obtained DNA (3 kb) was ligated with SmaI-digested pKOR3A, a vector (containing the Cm^r gene as a marker) for allelic replacement in *S. aureus* (24). The constructed plasmid was introduced into *S. aureus* RN4220 by electroporation, and transformants were grown overnight in TSB medium containing 50 μg/ml kanamycin. The cultures were diluted and spread on TSB agar plates containing 50 μg/ml

kanamycin, and the plates were incubated overnight at 43°C. The temperature-resistant cells were cultured in TSB medium, and the overnight cultures were diluted and spread on TSB agar containing 50 μg/ml kanamycin and 1.5 μg/ml anhydrotetracycline. Among colonies of a variety of sizes, relatively large colonies were selected and spread on two plates, containing either 50 μg/ml kanamycin or 12.5 μg/ml chloramphenicol. Cells resistant to kanamycin and susceptible to chloramphenicol were selected as disrupted mutants of the *psiA* gene in an RN4220 background (RNΔ*psiA* mutant). The integrated region was transferred from the RNΔ*psiA* strain to Newman by phage transduction, resulting in a disrupted mutant of the *psiA* gene in the Newman background (NMΔ*psiA* mutant). In the following sections, the NMΔ*psiA* strain is referred to as the Δ*psiA* strain. The nucleotide sequences of primers used for gene disruption are listed in Table S2 in the supplemental material. The disruption of each target gene in the mutants was confirmed by Southern blotting.

Construction of complementation strains of *S. aureus*. Complementation of the *sigB*, *essC*, *psiA*, and *hlgB* genes in disruption mutants of each gene was performed essentially as described previously (6, 7, 22). For *sigB*, the promoter region of the upstream *rsbV* gene (NWMN_1972) (241 bp) and the coding region of the *sigB* gene (851 bp) were amplified separately. Two PCR products were sequentially ligated with a pHY300E vector, resulting in pHY*sigB*. Similarly, for *essC*, the promoter region of the upstream *essB* gene (NWMN_0222) (457 bp) and the coding region of the *essC* gene (4,506 bp) were amplified separately and sequentially cloned, resulting in pHY*essC*. For *psiA*, the promoter and coding regions (638 bp) were amplified together and cloned into the vector, resulting in pHY*psiA*. For *hlgB*, the promoter of the upstream *hlgC* gene (NWMN_2319) and the coding regions of both *hlgC* and *hlgB* (2,318 bp) were amplified together and cloned, resulting in pHY*hlgB*. Constructed plasmids were first introduced into *S. aureus* RN4220 by electroporation and then transferred to each mutant with the *S. aureus* Newman background by phage transduction as described above. Transductants showing resistance to both 50 μg/ml kanamycin (12.5 μg/ml chloramphenicol for the Δ*sigB*/pHY*sigB* strain) and 10 μg/ml erythromycin were selected as complementation strains. The nucleotide sequences of the primers used for complementation are listed in Table S2 in the supplemental material.

Mouse infection experiments. Female mice (6 to 8 weeks of age) were anesthetized by intraperitoneal injection of pentobarbital (Nembutal). TSB medium (50 ml) was added to 0.5 ml of overnight cultures, and bacteria were grown to log phase (OD_{600} = 0.5 to 0.7). Bacteria suspended in PBS (30 μl; 1×10^8 CFU) were spotted into the nostrils of the mice. Survival rates of the mice were monitored. To measure amounts of viable bacteria in the infected lungs, sublethal doses (3×10^7 CFU) of bacterial suspensions were administered to mice. After 2 days, the mice were anesthetized and the lungs were dissected. The diluted homogenates were spread on mannitol salt agar, and yellow colonies were counted. Survival data were plotted using Prism 5 (GraphPad Software, Inc.), and statistically significant differences between the survival curves were analyzed by the log rank test.

RESULTS

Global analysis of gene expression profiles of *S. aureus* altered by pulmonary surfactant. First, we analyzed the gene expression alterations of *S. aureus* induced by pulmonary surfactant and serum. *S. aureus* Newman was grown in TSB supplied with either calf-derived pulmonary surfactant or calf serum, and total RNAs were extracted from cells collected at late log phase (OD_{600} = 1). The growth curves for *S. aureus* treated with either serum or surfactant were similar (see Fig. S1 in the supplemental material). RNA-seq analysis by use of a next-generation analyzer revealed 142 and 259 genes whose transcript levels were increased by surfactant and serum, respectively (Fig. 1). Among them, 49 genes were upregulated by both surfactant and serum (Fig. 1). Recent studies demonstrated that genes involved in iron acquisition, such
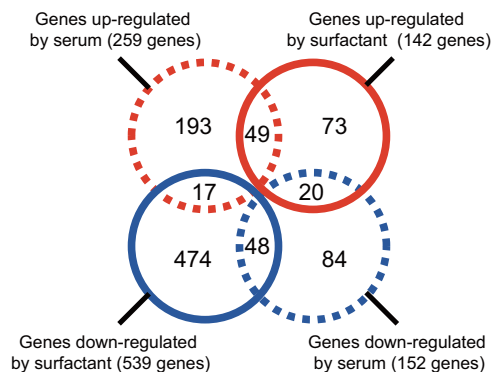
FIG 1 Numbers of *S. aureus* genes with altered expression in cultures with serum or pulmonary surfactant. *S. aureus* Newman was aerobically cultured in TSB medium containing 10% calf serum or 0.3 mg/ml Surfacten, and total RNAs extracted from bacteria at an OD_{600} of 1 were subjected to RNA-seq analysis using a next-generation sequencer. Circles written with dashed and solid lines indicate the results for samples treated with serum and surfactant, respectively. Numbers in blue circles show downregulated genes, and those in red circles indicate upregulated genes for each treatment.

as the *isd*, *sir*, and *sbn* families, were upregulated in *S. aureus* grown in human or calf serum (1, 25). Under our experimental conditions, the expression levels of *isdABEFG*, *sirAB*, and *sbn ABCDEFHI* increased from 4- to 34-fold in the presence of calf serum (see Table S3 in the supplemental material). On the other hand, more than half of the genes upregulated by serum were not induced by surfactant, and vice versa. Therefore, the patterns of gene induction by both host factors differed markedly. The RNA-seq data obtained by the next-generation sequencer are shown in Table S4 in the supplemental material.

We then performed another genomewide analysis by using a next-generation sequencer on RNAs extracted at the early log phase (OD_{600} = 0.3) from bacteria grown in the presence of surfactant or serum. Among the 43 genes whose transcript levels were increased >4-fold at an OD_{600} of 1 in the presence of surfactant, half of them showed a surfactant-dependent increase at an OD_{600} of 0.3 (Table 1). Next, we performed quantitative RT-PCR analysis of the genes that were upregulated by the pulmonary surfactant. We confirmed that mRNA levels of genes encoding ESAT-6 secretion system C (EssC), the unknown factor NWMN_0246, and hemolysin gamma subunit B (HlgB) were increased 3- to 10-fold when *S. aureus* cells were incubated in TSB medium supplied with the pulmonary surfactant (Fig. 2A). On the other hand, serum treatment failed to increase the mRNA amounts for these three genes in *S. aureus* (Fig. 2A). The upregulation of these genes depended on the concentration of the surfactant added to the medium (Fig. 2B). Among these genes, we named the *NWMN_0246* gene, which had not been characterized in *S. aureus*, pulmonary surfactant-inducible factor A (*psiA*). The predicted sequence of the PsiA protein contained an NfeD-like C-terminal partner-binding domain (see Fig. S2 in the supplemental material). Next, we examined whether induction of the abovementioned genes was observed in CA-MRSA strains, clinically isolated MRSA strains that are highly virulent, as well as in strain Newman. Two CA-MRSA strains, MW2 (USA400) (26) and FRP3757 (USA300) (27), were cultured in TSB medium containing the pulmonary surfactant, and RNAs extracted at an OD_{600} of 1 were subjected to quantitative RT-PCR analysis. As observed

TABLE 1 Genes whose expression levels were altered by serum or pulmonary surfactant[a]

Gene no.	Gene product	Gene expression			
		Surfactant		Serum	
		OD = 1	OD = 0.3	OD = 1	OD = 0.3
NWMN_0093	Truncated IS*200* transposase family	++	++	+/−	+/−
NWMN_0095	CapA (capsular polysaccharide synthesis enzyme)	++	+	+	+/−
NWMN_0112	Hypothetical protein	++	−	++	+/−
NWMN_0207	Hypothetical protein	++	+/−	++	+
NWMN_0221	EssA (ESAT-6 secretion system)	++	++	+	+/−
NWMN_0223	EssC (ESAT-6 secretion system)	++	+	+/−	+/−
NWMN_0224	EsaC (ESAT-6 secretion system)	++	+	+/−	+/−
NWMN_0225	EsxB (ESAT-6-like protein)	++	+	+/−	+/−
NWMN_0232	Hypothetical protein	++	+	+/−	+/−
NWMN_0233	Hypothetical protein	++	+/−	++	−
NWMN_0245	Hypothetical protein	++	++	−	−
NWMN_0246	Hypothetical protein	++	+/−	−	−
NWMN_0360	Hypothetical protein	++	−	+/−	+/−
NWMN_0429	Sle1 (*N*-acetylmuramoyl-L-alanine amidase)	++	+	+/−	−
NWMN_0405	Truncated staphylococcal tandem lipoprotein	++	+	++	+/−
NWMN_0658	Hypothetical protein	++	+/−	++	+/−
NWMN_0765	Hypothetical protein	++	+/−	++	+/−
NWMN_0902	Hypothetical protein	++	+/−	++	+/−
NWMN_0909	Hypothetical protein	++	+/−	ND	−
NWMN_1059	Hypothetical protein	++	+/−	ND	+/−
NWMN_1106	Putative transposase	++	+/−	ND	−
NWMN_1196	Hypothetical protein	++	+/−	++	−
NWMN_1275	4-Oxalocrotonate tautomerase	++	−	++	−
NWMN_1554	Hypothetical protein	++	+/−	+/−	−
NWMN_1716	BsaA2 (lantibiotic precursor)	++	−	ND	ND
NWMN_1745	Hypothetical protein	++	−	+/−	−
NWMN_1776	Hypothetical protein	++	+	++	+/−
NWMN_1861	Hypothetical protein	++	++	++	+
NWMN_1898	Phage portal protein	++	+/−	−	+
NWMN_2074	Hypothetical protein	++	+/−	++	−
NWMN_2188	UreA (urease subunit)	++	+	ND	−
NWMN_2189	UreB (urease subunit)	++	+	+	−
NWMN_2320	HlgB (gamma hemolysin component)	++	+	+/−	+/−
NWMN_2328	ABC transporter ATP-binding protein	++	+/−	+	+/−
NWMN_2417	Hypothetical protein	++	+/−	++	+/−
NWMN_2492	Transcriptional regulator, TetR family	++	−	+/−	+/−
NWMN_2549	Hypothetical protein	++	++	+/−	+/−
NWMN_2550	Hypothetical protein	++	+	−	−
NWMN_2551	Asp1 (accessory secretory protein)	++	+	+/−	−
NWMN_2552	SecY (preprotein translocase subunit)	++	+	+/−	−

[a] *S. aureus* Newman was cultured in TSB medium supplied with 0.3 mg/ml Surfacten to an OD_{600} of 1. The 43 genes listed were identified by RNA-seq analysis to be upregulated >4-fold following surfactant treatment. Alterations in transcript levels observed in RNA-seq data for *S. aureus* incubated with either Surfacten or calf serum to an OD_{600} of 1 or 0.3 are also indicated. ++, upregulated >4-fold; +, upregulated 2- to 4-fold; +/−, altered 0.5- to 2-fold; −, downregulated >2-fold; ND, not detected.

in the Newman strain, the mRNA quantities for *essC* and *psiA* were increased by the surfactant treatment (Fig. 2C). On the other hand, the *hlgB* mRNA levels in MW2 and FRP3757 were <1/10-fold that in Newman, and the ratios of surfactant-dependent induction of *hlgB* mRNA were 1.3- and 1.4-fold, respectively. Recent studies demonstrated that expression of the *hlgB* gene is dependent on the SaeRS system (28) and that the Newman strain possesses a point mutation that leads to constitutive expression of the *saeS* gene (29). Based on these reports, the *hlgB* gene is assumed to be highly expressed in *S. aureus* Newman, which would account for our data showing that the expression level of the *hlgB* gene in *S. aureus* Newman was higher than those in clinical isolates.

Molecular mechanisms of gene induction in *S. aureus* by pulmonary surfactant. Pulmonary surfactant is a lipid-rich secreted material; 90% of its weight comprises lipids, and the remaining 10% contains proteins (3). We thus hypothesized that lipids, the major constituents of pulmonary surfactant, had gene expression-inducing activities in *S. aureus*. Lipid fractions from either the pulmonary surfactant (see Fig. S3 in the supplemental material) or cow lung homogenates (Fig. 3A) induced the upregulation of *essC*, *psiA*, and *hlgB* in *S. aureus* >2-fold. In contrast, the activity in the water fraction of lung extracts was much lower than that in the organic solvent fraction (Fig. 3A). We next examined the effects of the major phospholipids and fatty acids in the pulmonary surfactant, such as DPPC, POPC, POPG, and palmitate, on gene induc-
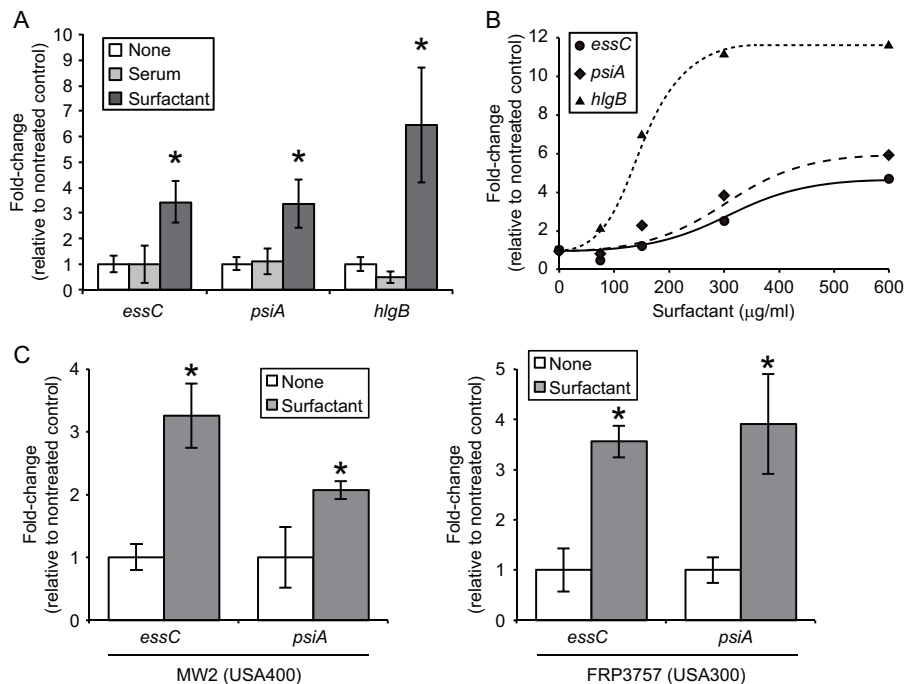
FIG 2 Pulmonary surfactant-dependent induction of *S. aureus* *essC*, *psiA*, and *hlgB* genes. (A) *S. aureus* Newman was cultured in TSB medium containing either 10% serum or 0.3 mg/ml Surfacten to an OD_{600} of 1. Amounts of *essC*, *psiA*, and *hlgB* mRNAs were determined by quantitative RT-PCR. Each value was normalized to 16S rRNA, and the ratio of the surfactant-treated to the nontreated group was calculated. Data represent means ± standard deviations (SD) for 4 experiments. For each gene, statistical differences compared with the nontreated group were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test (*, $P < 0.01$). (B) Dose dependency of pulmonary surfactant for the induction of *S. aureus* gene expression. RNAs were collected from bacteria at an OD_{600} of 1 and subjected to quantitative RT-PCR analysis. The expression level of each gene was normalized to that of 16S rRNA, and the induction ratio relative to the nontreated group is indicated. (C) Induction of gene expression by pulmonary surfactant in CA-MRSA. MW2 (USA400) and FRP3757 (USA300) were cultured in the presence of 0.3 mg/ml Surfacten to an OD_{600} of 1, and quantitative RT-PCR analysis of each gene was performed. Data represent means ± SD for 3 or 4 experiments. Statistical differences for each gene compared with nontreated groups were analyzed by Student's *t* test (*, $P < 0.05$).

tion in *S. aureus*. *S. aureus* Newman was cultured to an OD_{600} of 1 in TSB medium supplied with each lipid at 0.1 or 1 mg/ml, and RNAs were extracted. The amounts of mRNA for the three genes (*essC*, *psiA*, and *hlgB*) induced by surfactant treatment were measured by quantitative RT-PCR. Neither DPPC, POPC, nor POPG, the major phospholipids in surfactant, affected the expression levels of the three genes (Fig. 3B). On the other hand, supplementation with palmitate increased the mRNA levels of the three genes 2- to 8-fold (Fig. 3B). Moreover, like palmitate, oleate promoted the expression of the three genes (Fig. 3C). These free fatty acids have detergent-like activities, and the growth of *S. aureus* is inhibited when these fatty acids are supplied at high concentrations (30). Under our experimental conditions, the viability of *S. aureus* was partly affected by supplementation with palmitate: the numbers of CFU in log phase were 2- to 5-fold lower than those for the nontreated group (see Fig. S4 in the supplemental material). We therefore considered the possibility that reactions of detergent-like substances targeting the cell membrane stimulate gene expression. We examined the effects of various detergents on *S. aureus* gene expression and found that supplementation of the medium with SDS, Triton X-100, and Tween 20 led to increases in the mRNA levels of the three genes (Fig. 3C). Among these detergents, Tween 20 showed the highest induction ability (Fig. 3C).

We further evaluated the molecular mechanisms of gene induction in *S. aureus* by pulmonary surfactant. To test whether pulmonary surfactant induced the upregulation of transcriptional activities of the *essC* and *hlgB* genes at the transcription initiation step, we performed promoter analysis of these genes. The promoter sequences of the *essC* and *hlgB* genes were cloned into a luciferase reporter plasmid, and the promoter activities were then assessed. Supplementation of the culture medium with palmitate or pulmonary surfactant increased the promoter activities of the *essC* and *hlgB* genes in *S. aureus* strains harboring the respective plasmids (Fig. 4). In contrast, serum treatment did not increase the *essC* and *hlgB* promoter activities (see Fig. S5 in the supplemental material), consistent with the gene induction results (Fig. 2A). Induction of some genes induced by damage to the cell membranes depends on the function of the *sigB* gene, which encodes a stress-responsive RNA polymerase subunit (31). We therefore examined the involvement of SigB in the upregulation of the *essC*, *psiA*, and *hlgB* genes. In contrast to the parent *S. aureus* strain, a disruption mutant of the *sigB* gene (ΔsigB mutant) did not show increases in the mRNA levels of the three genes, even in the presence of palmitate or pulmonary surfactant (Fig. 5A). The reduced ability to respond to palmitate and surfactant in the ΔsigB mutant was restored by complementation with the *sigB* gene (Fig. 5B). Moreover, the mRNA level of the *sigB* gene itself increased with *S. aureus* grown in medium supplemented with palmitate or surfactant (Fig. 5C). In addition, genes whose expression is regulated in a SigB-dependent pathway, such as *asp23* (31–36), *clfA* (32, 37, 38), *csb7* (31, 32, 39), and *opuD2* (NWMN_2089; an ortholog of SACOL2176 in *S. aureus* strain COL) (31–34, 37), were also upregulated by treatment with palmitate or surfactant (Fig. 5D). These findings suggest that activation of the SigB pathway is in-
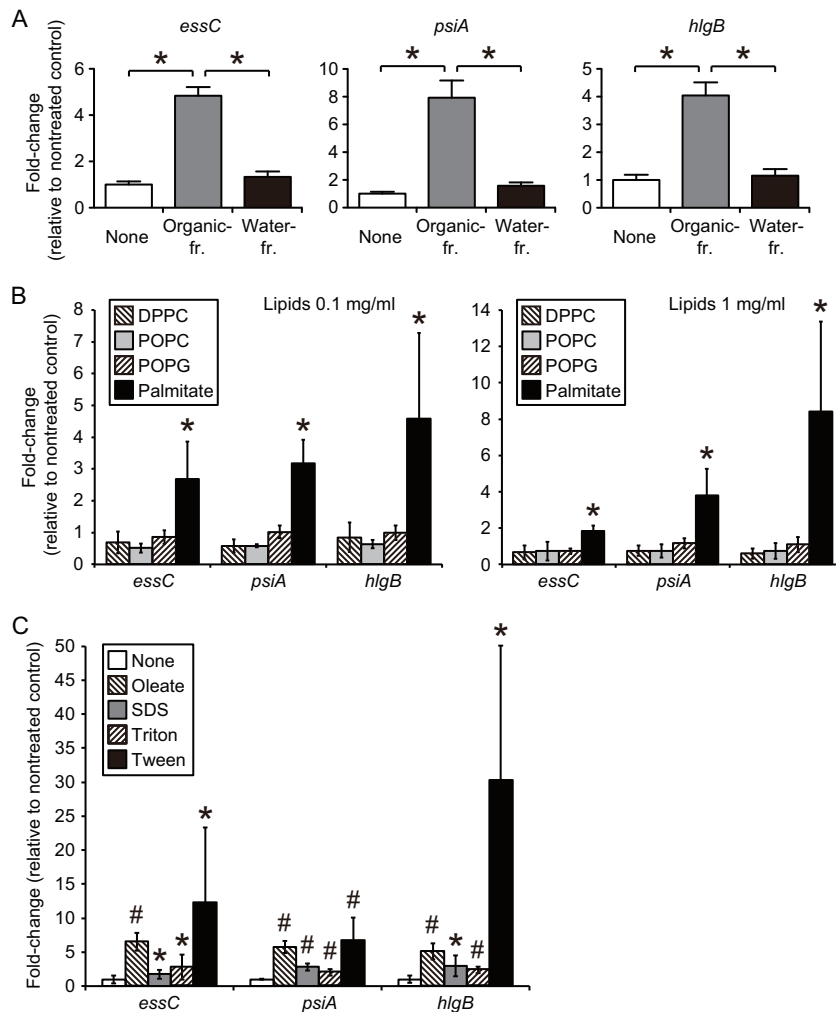
FIG 3 Induction of *essC*, *psiA*, and *hlgB* genes in *S. aureus* by pulmonary surfactant lipid components. (A) Effects of lipid fractions of cow lung homogenates on gene expression of *S. aureus*. Cow lung homogenates were fractionated by the Bligh-Dyer method to an organic phase (Organic-fr.) and a water phase (Water-fr.). *S. aureus* Newman was cultured in TSB medium supplied with either the organic or water phase to an OD_{600} of 1, and the expression levels of the *essC*, *psiA*, and *hlgB* genes relative to those of the nontreated group were determined by quantitative RT-PCR. Data represent means ± SD for 4 experiments. Statistical differences were analyzed by one-way ANOVA with Tukey's multiple-comparison test (*, $P < 0.001$). (B) Effects of lipids contained in pulmonary surfactant on gene expression of *S. aureus*. *S. aureus* Newman was cultured in TSB medium supplied with 0.1 (left) or 1 (right) mg/ml of either DPPC, POPC, POPG, or palmitate to an OD_{600} of 1. mRNA levels of the *essC*, *psiA*, and *hlgB* genes were measured by quantitative RT-PCR. The expression level of each gene was normalized with that of 16S rRNA, and the induction ratio relative to the nontreated group is indicated. Data represent means ± SD for 3 or 4 experiments. Statistical differences were analyzed by one-way ANOVA with Tukey's multiple-comparison test (*, $P < 0.05$). (C) Effects of oleate and detergents on gene expression of *S. aureus*. *S. aureus* Newman was cultured in TSB medium containing either 0.1 mg/ml oleate, 0.0003% SDS, 0.006% Triton X-100, or 0.006% Tween 20 to an OD_{600} of 1. Expression levels of the three genes relative to those in the nontreated group were determined by quantitative RT-PCR as described above. Data represent means ± SD for 7 experiments (*, $P < 0.05$; #, $P < 0.005$).

volved in the gene induction by free fatty acids present in the pulmonary surfactant. There are a group of regulators of gene expression, called two-component systems, that sense and respond to environmental changes in bacteria. Among them, Agr (40), ArlRS (41), and SaeRS (42) are involved in sensing host environments and regulating virulence in pathogenic bacteria infecting host animals. The *essC*, *psiA*, and *hlgB* genes were still upregulated depending upon the pulmonary surfactant in the deletion mutant of each gene (see Fig. S6 in the supplemental material). These findings suggest that these two-component systems are not involved in the surfactant-dependent induction of those genes.

Involvement of pulmonary surfactant-inducible genes in pneumonia caused by *S. aureus*. Pathogens invading host animals through the airway contact the pulmonary surfactant that covers the alveolar surfaces in the lungs. Responding to pulmonary surfactant and expressing an array of virulence factors in a distinct pattern seem to be important for bacteria to exert their pathogenicity in lung infections. We considered that the above surfactant-inducible genes contribute to virulence exertion of *S. aureus* in lung infection. To test this, we constructed *S. aureus* disruption mutants of each of the three surfactant-inducible genes that we found in the present study (Δ*essC*, Δ*psiA*, and Δ*hlgB* mutants), together with complementation strains of each gene, and examined their virulence potentials in a mouse pneumonia model. The growth curves for these disruption mutants in TSB medium were indistinguishable from that of the parent strain (see
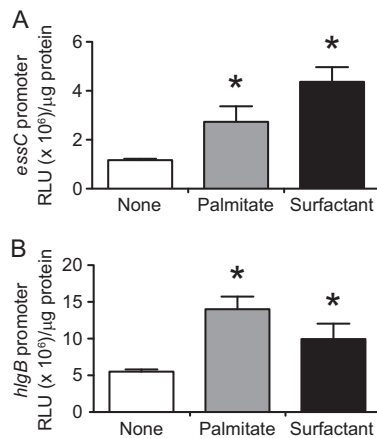
FIG 4 Palmitate and pulmonary surfactant upregulate *essC* and *hlgB* promoter activities. Promoter sequences of either the *essC* (A) or *hlgB* (B) gene were inserted into a reporter plasmid upstream of the luciferase gene. *S. aureus* Newman harboring each plasmid was cultured in TSB medium supplied with 0.1 mg/ml palmitate or 0.3 mg/ml Surfacten to an OD_{600} of 0.7. The luciferase activities were measured with a luminometer, and the luminescence units were normalized to the protein amounts. Data represent means and SD for 3 experiments. Statistical differences compared with the nontreated group were analyzed by one-way ANOVA with Dunnett's multiple-comparison test (*, $P <$ 0.05). RLU, relative light units.

Fig. S7 in the supplemental material). Suspensions of the parent or mutant strains were spotted into the nostrils of anesthetized mice, and the lungs were dissected 2 days later (see Fig. S8 in the supplemental material). Bacterial numbers were much smaller in mutants than in the parent strain (Fig. 6A) and strains complemented with each deleted gene (Fig. 6B). Moreover, all of these mutants showed delayed killing of mice compared to the parent and complementation strains (Fig. 6C). These findings suggest that the three genes induced by pulmonary surfactant are required for *S. aureus* virulence in animal lungs.

DISCUSSION

Pathogens possess complex gene expression regulatory systems that are necessary for rapid adaptation to host environments that have not yet been clarified. Recently, several mammalian infection models, including those of pneumonia, skin abscess formation, bacteremia, and osteomyelitis, were established to investigate the virulence mechanism of *S. aureus* (4). *S. aureus* and other pathogenic bacteria show altered expression patterns of their virulence genes at different infection sites depending on the growth conditions within each tissue. Therefore, analyses of gene expression regulations under conditions that mimic host environments are important for determining bacterial pathogenesis. In the present study, we cultured *S. aureus* in the presence of pulmonary surfactant, a lipid-rich material secreted at infection sites such as lung alveoli, and we observed that an array of virulence genes were induced. Among them, the *essC*, *psiA*, and *hlgB* were upregulated by palmitate, a major fatty acid in surfactant, and were necessary for *S. aureus* virulence in lung infections (Fig. 7). Our results suggest that *in vitro* gene analysis studies following treatment with pulmonary surfactant would be useful for investigating the virulence mechanisms of pathogens that infect host lungs.

Mechanistic insights into the role of the three genes induced by pulmonary surfactant in *S. aureus* virulence. The present

study demonstrated that the *essC*, *psiS*, and *hlgB* gene expression levels were increased by treatment with surfactant. In this section, we discuss the relationships between our results and previous reports concerning the involvement of the three genes in pathogenesis.

The *essC* gene is a member of the *ess* cluster, a genomic region that contains the *esxAB*, *esaABC*, and *essABC* genes and is conserved in Gram-positive bacteria such as *Listeria monocytogenes*, *Bacillus thuringiensis*, and *Mycobacterium tuberculosis* (43). The *ess* genes encode components of the type VII secretion system, a recently identified Sec-independent secretion pathway of bacteria that is involved in virulence via the secretion of various toxins, including EsxA, EsxB, and EsaC (43–45). Interestingly, the amount of EsaC secreted into the blood increases when *S. aureus* infects host animals (43). Based on these reports, it is plausible that *S. aureus* possesses a system to sense the host environment and to thereby upregulate the expression of Ess factors. Validation of this hypothesis at the molecular level, however, has been inadequate. Here we demonstrated that the *essC* gene was induced by fatty acids in pulmonary surfactant and detergents. A previous report showed that the extracellular protein patterns of *S. aureus* are altered in response to detergents (29) and, together with our results, showed that the activation of the Ess secretion system is likely to be involved in membrane damage stress responses.

The NWMN_0246 gene, which we named *psiA* in this study, encodes a 132-amino-acid protein with an unknown function. The primary sequence contains an NfeD-like C-terminal partner-binding domain. Several NfeD-like proteins are found in prokaryotes, and some of them contribute to lipid metabolism (46). Thus, the *psiA* gene product might be involved in membrane repair responses in cells damaged by fatty acids and detergents. Our genomewide analysis showing that the *psiA* gene was not induced by serum suggests that this gene functions as an *S. aureus* virulence factor acting specifically in lung infections. We expect that further investigation of the potential role of PsiA in lipid metabolism could contribute to novel approaches toward reaching an understanding of pulmonary infections caused by *S. aureus*.

The *hlgB* gene encodes a component of hemolysin gamma (Hlg), a representative extracellular toxin of *S. aureus*. Hlg shows hemolytic and cytolytic activities against human monocytes, polymorphonuclear cells, and macrophages (47). Hlg and other related hemolysins are predicted to form transmembrane channel complexes on target cells, and the tertiary structure of the Hlg protomer is similar to those of hemolysin alpha (Hla) and leukocidin (48). Among these hemolysins, *S. aureus* Hla, Panton-Valentine leukocidin, and hemolysin beta (Hlb) contribute to evoking inflammation and host killing in experimental mammalian pneumonia models (49–51). On the other hand, Malachowa et al. reported only a modest contribution of Hlg to host killing by *S. aureus* in a sepsis model (1), and there has been little analysis of its virulence potential in a pneumonia model. Furthermore, the expression of *hla* and *hlg* genes is differentially regulated by the two-component systems Agr and Sae, respectively (28). Under our experimental conditions for surfactant treatment, the transcript levels of the *hla* and *agr* genes were not significantly affected, whereas the mRNA quantities of *hlgB* and *hlgC* were upregulated. Moreover, disruption of the *hlgB* gene in *S. aureus* attenuated its host-killing ability in a pneumonia model. These results suggest a possible role for Hlg as a major cytotoxin of *S. aureus* in lung infections. Because alveolar macrophages possessing high phago-
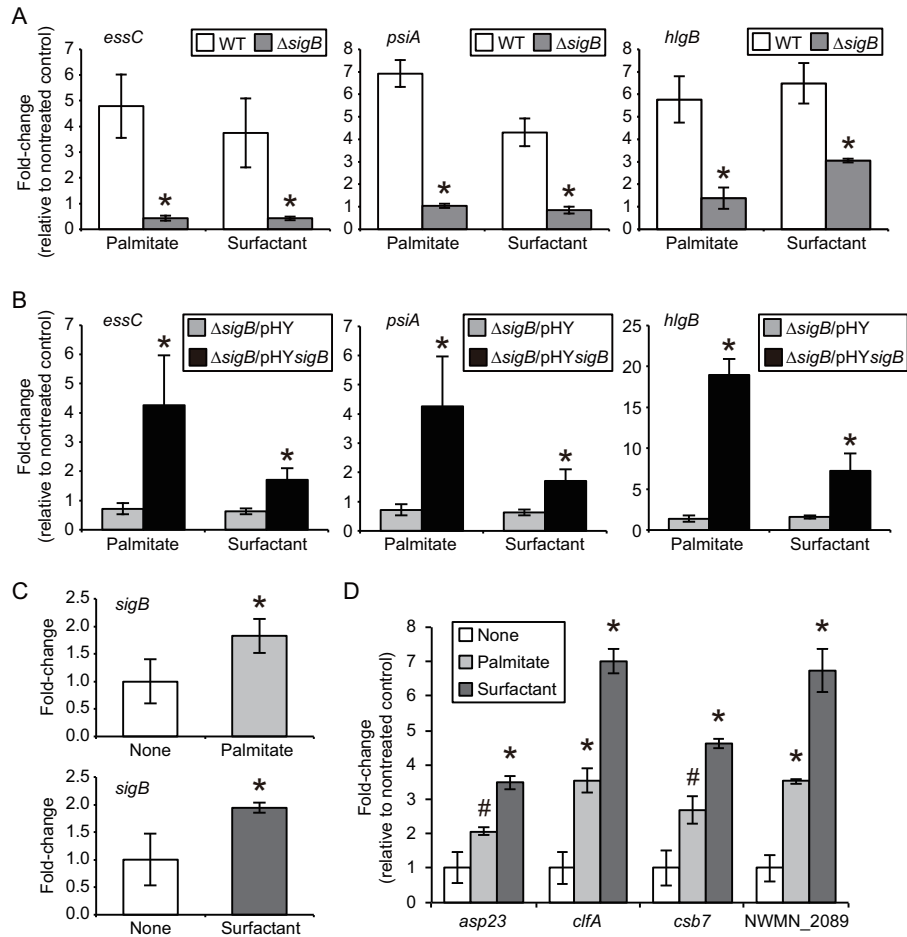
FIG 5 Involvement of SigB in *S. aureus* gene induction by palmitate and pulmonary surfactant. (A) Requirement of the *sigB* gene in palmitate- and surfactant-dependent gene induction. *S. aureus* Newman as the parent strain (wild type [WT]) or a disruption mutant of the *sigB* gene (Δ*sigB*) was cultured in TSB medium containing 0.1 mg/ml palmitate or 0.3 mg/ml Surfacten to an OD_{600} of 1. Expression levels of the three genes relative to those of the nontreated group were determined by quantitative RT-PCR as described in the text. Data represent means ± SD for 3 experiments. Statistical differences between the WT and Δ*sigB* strains were analyzed by Student's *t* test (*, $P < 0.05$). (B) Complementation of phenotypes of the *S. aureus sigB* disruption mutant. Either a control plasmid (pHY300E) or a complementation plasmid containing an open reading frame of the *sigB* gene (pHY*sigB*) was introduced into the Δ*sigB* mutant, resulting in Δ*sigB*/pHY or Δ*sigB*/pHY*sigB*, respectively. Cells were cultured in the presence of 0.1 mg/ml palmitate or 0.3 mg/ml Surfacten to an OD_{600} of 1, and quantitative RT-PCR analysis of the three genes was performed as described in the text. Data represent means ± SD for 3 experiments. Statistical differences between the Δ*sigB*/pHY and Δ*sigB*/pHY*sigB* strains were analyzed by Student's *t* test (*, $P < 0.05$). (C) Induction of *sigB* gene expression in *S. aureus* by palmitate and pulmonary surfactant. *S. aureus* Newman was cultured in TSB medium supplied with either 0.1 mg/ml palmitate or 0.3 mg/ml Surfacten to an OD_{600} of 1. The expression level of the *sigB* gene relative to that for the nontreated group was determined by quantitative RT-PCR. Data represent means ± SD for 3 experiments. Statistical differences compared to the nontreated group were analyzed by Student's *t* test (*, $P < 0.05$). (D) Induction of SigB-regulated genes by palmitate and pulmonary surfactant. *S. aureus* Newman was cultured in TSB medium supplied with either 0.1 mg/ml palmitate or 0.3 mg/ml Surfacten to an OD_{600} of 1. Expression levels of the *asp23*, *clfA*, *csb7*, and NWMN_2089 genes relative to those for the nontreated group were determined by quantitative RT-PCR. Data represent means ± SD for 3 experiments. For each gene, statistical differences compared with the nontreated group were analyzed by one-way ANOVA with Dunnett's multiple-comparison test (#, $P < 0.01$; *, $P < 0.001$).

cytic capacities are likely to be present at or attracted to infected alveoli, promotion of Hlg production in response to pulmonary surfactant seems to be reasonable for *S. aureus* to avoid attacks by host immune cells at infection sites.

Molecular mechanisms of gene expression induction by fatty acid and detergents. The two-component systems Agr and Sae, which are major regulators of virulence factors in pathogenic bacteria (17), are induced in *S. aureus* cultured in serum (25) and blood (1), respectively. In contrast to these reports, we found that neither the *agr* gene nor the *sae* gene was upregulated in the culture medium supplied with pulmonary surfactant. In addition, disruption of these two-component system genes did not suppress

the gene expression stimulated by the surfactant. Montgomery and colleagues reported that mutations in the *agr* and *sae* genes attenuated the host-killing ability of *S. aureus* in a mouse pneumonia model (17). Our data do not conflict with the notion, based on this previous report, that Agr and Sae systems are required for *S. aureus* virulence; the robust impact of *agr* and *sae* mutations on the expression of numerous virulence genes likely masked the effects of the surfactant-inducible genes we focused on in the present study, leading to attenuated host killing as observed by Montgomery et al.

We then focused on SigB, an alternative sigma factor that contributes to various stress responses in several bacteria, as a candi-
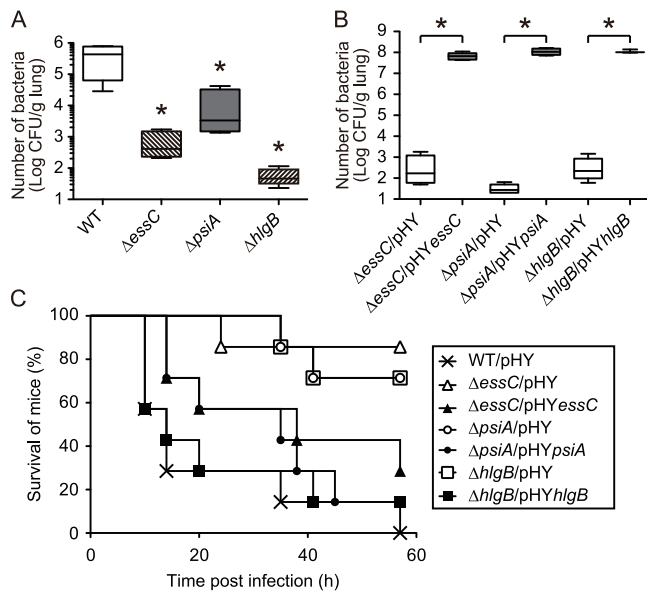
FIG 6 Involvement of *essC*, *psiA*, and *hlgB* genes in *S. aureus* virulence in a mouse pneumonia model. (A) Survival of *S. aureus* mutants in mouse lungs. Sublethal doses of either *S. aureus* Newman (WT) or a mutant strain (Δ*essC*, Δ*psiA*, or Δ*hlgB* strain) were administered into the lungs of anesthetized mice through the nasal cavities. After 2 days, the lungs were dissected and numbers of viable bacteria in the homogenates were determined. Data represent means ± SD for 5 mice. Statistical differences compared with the WT were analyzed by one-way ANOVA with Dunnett's multiple-comparison test (*, $P < 0.05$). (B) Survival of *S. aureus* mutants and complementation strains in mouse lungs. Mice were infected with either *S. aureus* mutants harboring empty vectors (Δ*essC*/pHY, Δ*psiA*/pHY, and Δ*hlgB*/pHY strains) or mutants harboring complementation vectors (Δ*essC*/pHY*essC*, Δ*psiA*/pHY*psiA*, and Δ*hlgB*/pHY*hlgB* strains). After 2 days, the numbers of viable bacteria in lung homogenates were determined. Data represent means ± SD for 3 to 6 mice. Statistical differences of the mutants compared with each complemented strain were analyzed by Student's *t* test (*, $P < 0.001$). (C) Host-killing ability in a mouse pneumonia model by mutant and complementation strains of surfactant-inducible genes. Bacterial cell suspensions of *S. aureus* Newman harboring an empty vector (×; WT/pHY), disruption mutants of each surfactant-inducible gene containing empty vectors (open symbols; Δ*essC*/pHY, Δ*psiA*/pHY, and Δ*hlgB*/pHY strains), or mutants complemented with each gene (closed symbols; Δ*essC*/pHY*essC*, Δ*psiA*/pHY*psiA*, and Δ*hlgB*/pHY*hlgB* strains) were administered into the lungs of anesthetized mice through the nasal cavities (*n* = 7). Statistical differences between the wild type and each mutant or between each mutant and its complementation strain were determined to be significant ($P < 0.05$) by the log rank test.

date regulator of surfactant-dependent gene expression. In *Bacillus subtilis*, activation of SigB-dependent transcription of stress-responsive genes is induced by either heat shock or decline of the intracellular ATP level (52, 53). On the other hand, SigB of *S. aureus* differs from that of *B. subtilis* with regard to triggering stimuli for activation; while *S. aureus* SigB is stimulated by environmental stresses such as alkali, it is not activated by energy stressors (31). Under our experimental conditions, the pH values of the culture medium were not significantly changed (data not shown). Recently, unsaturated long fatty acids with antibacterial effects against *S. aureus* were demonstrated to increase the *sigB* mRNA level (54). Moreover, mutations in the *sigB* gene in *S. aureus* affect the expression profiles of numerous genes (32). These findings together with our results suggest that detergent-like palmitate contained in the pulmonary surfactant damages cell membranes, followed by activation of the stress-responsive regu-
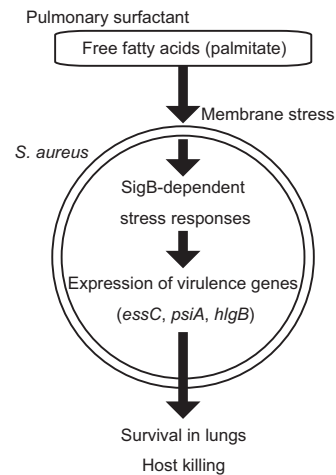
FIG 7 Proposed mechanism of *S. aureus* virulence gene induction by free fatty acids in pulmonary surfactant.

lator SigB and downstream expression of virulence genes (Fig. 7). Previous reports suggest that SigB contributes to *S. aureus* virulence in several infection models (55, 56). To our knowledge, however, there are no reports demonstrating that host-derived materials such as pulmonary surfactant induce SigB-mediated virulence gene expression in pathogenic bacteria. Although the transcriptional activity of *B. subtilis* SigB is controlled through interactions with Rsb family proteins (52), genes encoding these regulatory proteins (*rsbRSTX*) are missing in the genome of *S. aureus* (57, 58). For this reason, the SigB regulatory system of *S. aureus* is considered to differ from that of *B. subtilis* (59), and most of the activation pathways remain unclear. Precise mechanisms of host sensing via SigB in *S. aureus* under conditions of lung infections should be clarified in future studies.

ACKNOWLEDGMENTS

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