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Expression of Virulence Factors by *Staphylococcus aureus* Grown in Serum▽†

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Staphylococcus aureus **produces many virulence factors, including toxins, immune-modulatory factors, and exoenzymes. Previous studies involving the analysis of virulence expression were mainly performed by *in vitro* experiments using bacterial medium. However, when *S. aureus* infects a host, the bacterial growth conditions are quite different from those in a medium, which may be related to the different expression of virulence factors in the host. In this study, we investigated the expression of virulence factors in *S. aureus* grown in calf serum. The expression of many virulence factors, including hemolysins, enterotoxins, proteases, and iron acquisition factors, was significantly increased compared with that in bacterial medium. In addition, the expression of RNA III, a global regulon for virulence expression, was significantly increased. This effect was partially restored by the addition of 300 μM $FeCl_3$ into serum, suggesting that iron depletion is associated with the increased expression of virulence factors in serum. In chemically defined medium without iron, a similar effect was observed. In a mutant with *agr* inactivated grown in serum, the expression of RNA III, *psm*, and *sec4* was not increased, while other factors were still induced in the mutant, suggesting that another regulatory factor(s) is involved. In addition, we found that serum albumin is a major factor for the capture of free iron to prevent the supply of iron to bacteria grown in serum. These results indicate that *S. aureus* expresses virulence factors in adaptation to the host environment.**

Staphylococcus aureus is one of the major pathogens of humans; it causes various suppurative diseases, food poisoning, pneumonia, and toxic shock syndrome (11, 20). In addition, *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), often causes serious problems via nosocomial infection in hospitals (10, 16). Furthermore, community-acquired MRSA has recently emerged and has been reported to cause serious infectious diseases, sepsis, and pneumonia (3, 9).

It is well known that *S. aureus* produces many virulence factors, such as hemolysins, leukocidins, proteases, enterotoxins, exfoliative toxins, and immune-modulatory factors (11, 12, 21, 31). The expression of these factors is tightly regulated during growth. The *agr* system, known as the quorum-sensing system, is known to play a central role in the regulation of virulence factors (5, 26, 27). AgrAC is a two-component system (TCS) that consists of a histidine kinase and a response regulator. The Agr system regulates the expression of the gene coding for small RNA, known as RNA III, which is localized divergently at the *agr* operon and regulates the expression of many virulence factors, such as hemolysins, leukocidins, and protein A (26, 27). In addition, RNA III, known as *hld*, encodes delta-hemolysin. The mechanisms of RNA III regulation for the expression of several virulence factors and regulatory factors, including alpha-hemolysin, coagulase, protein A, Map, and Rot, have been demonstrated to occur at the posttranscriptional level by RNA-RNA interaction (7, 13, 17, 19, 25). However, in other virulence factors, the precise mechanism of regulation by RNA III remains unknown. Besides *agr*, many factors, including other TCSs (*arl* and *sae*) and transcriptional regulators (the *sar* family, *rot*, and *mgr*), have been demonstrated to be involved in the expression of virulence factors (5, 26, 33). To date, in regard to research on the expression of virulence factors, bacterial media, such as Trypticase soy broth (TSB), brain heart infusion (BHI) broth, and Luria-Bertani (LB) broth, have been commonly used for *S. aureus* cultivation. However, when *S. aureus* infects a host, the circumstances around bacterial cells are quite different from those in a medium, with the expression pattern of virulence factors in the host suggested to be quite different from that in culture media. Several investigations using animal models have characterized the regulation of virulence factor expression *in vivo* (4, 5, 24, 28). However, from the findings of *in vivo* experiments, it is considered that many factors, including cellular immune factors and nutrient conditions, affect the expression of virulence factors, suggesting that the mechanisms of regulation of virulence factors *in vivo* are very complicated. Here, we used serum for analysis of the expression of virulence factors. Serum contains many biochemical agents, such as serum proteins, carbohydrates, and ions, which are quite different from those in *in vitro* bacterial medium. *S. aureus* infection sometimes causes sepsis, so the organism has the ability to survive and spread in the bloodstream. In this study, we used *S. aureus* MW2 isolated from a patient who suffered fatal septicemia (1) and investigated the expression of virulence factors in serum.

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TABLE 1. Strains used in the study

Strain	Characteristics	Reference
MW2	Clinical strain, sepsis, methicillin resistant (*mec*$^+$)	1
FK73	*agrC*::pCL52.1 in MW2; TC$^{r a}$	22
MS23002	Clinically isolated strain; sepsis	This study
MS23143	Clinically isolated strain; sepsis	This study
MS23218	Clinically isolated strain; sepsis	This study
MS23543	Clinically isolated strain; sepsis	This study
TF3453	Clinically isolated strain; sepsis	This study
TF3483	Clinically isolated strain; sepsis	This study
TY526	Clinically isolated strain; impetigo	This study

[a] TC^r, tetracycline resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *S. aureus* was grown in TSB (Becton Dickinson Microbiology Systems, Cockeysville, MD). Tetracycline (TC; 10 μg/ml) was added when necessary for *S. aureus*. Previously constructed mutants with *agr* inactivated were used in the study (22). Chemically defined medium (CDM) was prepared as described elsewhere (18). Before adding metal ions, the medium was treated with Chelex-100 (Bio-Rad, Tokyo, Japan) to remove the residual metal ions completely. Then, metal ions without iron were added to the medium. We named CDM without iron CDM(−) in this study.

Culturing *S. aureus* in serum and chemically defined medium. *S. aureus* cells grown overnight were harvested and washed with distilled water (DW) treated with Chelex-100. Then, *S. aureus* cells were suspended in serum or CDM(−), and a small portion of bacterial suspension (10^8 cells) was inoculated into 10 ml of serum or CDM(−). *S. aureus* cells were grown at 37°C with shaking, and bacterial cells at various phases were collected. The origin of the serum used in this study was calf, rabbit, or human, and the serum was heated at 56°C for 30 min prior to use. Calf and rabbit sera were obtained from Gibco Life Technologies Japan (Tokyo, Japan). Human serum was prepared from the blood extracted from a volunteer. When necessary, $FeCl_3$, holotransferrin (Sigma-Aldrich, Tokyo, Japan), and hemin (Sigma-Aldrich) were added to the medium.

Quantitative analysis of gene expression. Twenty-seven major virulence factors, including toxins, immune-modulatory factors, exoenzymes, and iron acquisition factors were selected, and their expression was analyzed by quantitative PCR. Total RNA was extracted from bacterial cells with a FastRNA Pro Blue kit (MP Biomedicals, OH) in accordance with the manufacturer's protocol. One microgram of total RNA was reverse transcribed to cDNA using a first-strand cDNA synthesis kit (Roche, Tokyo, Japan). Using cDNA as template DNA, quantitative PCR was performed with a LightCycler system (Roche, Tokyo, Japan). Primers were constructed and used to determine the optimal conditions for analysis of expression. The amount of *gyrA* was used as an internal control. The primers are shown in Table S1 in the supplemental material.

Fractionation of calf serum. Fifteen milliliters of calf serum (CS) was applied to a CentriprepYM-50 centrifugal filter unit (Millipore, Tokyo, Japan) to remove molecules larger than 50 kDa. After centrifugation at 1,500 rpm, 15 ml of the flowthrough fraction was applied to a CentriprepYM-3 centrifugal filter unit (Millipore, Tokyo, Japan) to remove molecules larger than 3 kDa. After centrifugation, 1 ml of the flowthrough fraction was applied to a SepPack cartridge (Waters, Tokyo, Japan) to remove lipids and peptides. Finally, 3 kinds of solutions, (i) CS with molecules larger than 50 kDa removed; (ii) CS with molecules larger than 3 kDa removed; and (iii) CS with molecules larger than 3 kDa, lipids, and peptides removed, were prepared.

Purification of albumin from calf serum. Five milliliters of CS was applied to ToyoPearl AF-Blue HC-650 (Tosoh, Tokyo, Japan; 15 by 200 mm) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) (buffer 1). The column was washed with 50 mM potassium phosphate buffer (pH 7.5) (buffer 2) until unbound proteins passed through. Bound proteins were then eluted with a linear gradient from buffer 1 to 50 mM potassium phosphate buffer (pH 7.5) containing 1.5 M KCl (buffer 3) at a flow rate of 0.5 ml/min. Each collected fraction (5 ml each) was resolved by SDS-polyacrylamide gel electrophoresis in a 7.5% gel and then stained with Coomassie brilliant blue. The fractions containing albumin were collected and desalted with a CentriprepYM-50 centrifugal filter unit to remove KCl. Finally, the protein concentration was determined.

RESULTS

Expression of virulence factors in serum. To compare the expression of virulence factors in *S. aureus* cells grown in TSB and the cells in CS, we first checked the growth curve using both media and found similar rates of growth. Then, we collected the cells at various phases, using the same optical density for the culture with TSB or CS. The expression levels of most virulence factors, such as hemolysins, leukocidins, cell surface adhesins, and exoenzymes, were increased significantly in CS, while that of protein A was reduced in CS (Fig. 1 and

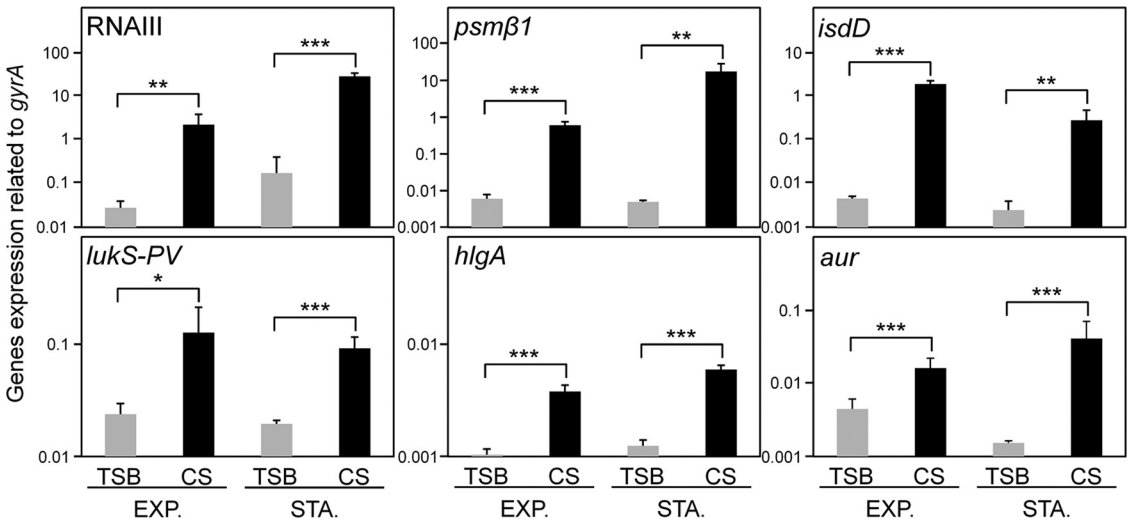

FIG. 1. Expression of virulence factors of *S. aureus* grown in serum. *S. aureus* MW2 cells cultured in TSB and CS were collected at exponential (EXP.) and stationary (STA.) phases. Then, total RNA was extracted by the method described in Materials and Methods. The expression of virulence factors was determined by quantitative PCR. The data shown represent means and standard deviations of the means (SD) of measurements performed in triplicate. The asterisks indicate significant increases compared with TSB as determined by Student's *t* test: ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

TABLE 2. Expression of virulence factors of *S. aureus* grown in TSB, CS and CS with 300 μM $FeCl_3$

GeneID[a]	Gene name	Function	TSB expression[b]	CS		CS + 300 μM $FeCl_3$	
				Expression[b]	Fold change (TSB)[c]	Expression[b]	Fold change (CS)[d]
	RNA III	Global regulon	1.41	135.30	95.96*	4.75	0.04+
MW1044	*hla*	Hemolysin	1.72	8.66	5.03	2.55	0.29
MW2342	*hlgA*	Hemolysin	0.25	18.76	75.84*	5.08	0.27+
MW1942	*lukM*	Leukocidin	4.63	13.02	2.82	5.70	0.44
MW1767	*lukD*	Leukocidin	0.21	2.19	10.48*	0.90	0.41++
MW1379	*lukS-PV*	Leukocidin	1.73	4.60	2.65*	1.83	0.40
	psmα	Phenol-soluble modulin	0.88	7.93	9.00**	2.52	0.32+
MW1056	*psmβ1*	Phenol-soluble modulin	1.44	73.03	50.84*	11.01	0.15+
MW0759	*sec4*	Enterotoxin	5.16	173.97	33.72*	26.58	0.15+
MW1889	*sea*	Enterotoxin	2.41	6.58	2.73*	2.59	0.39+
MW2558	*aur*	Protease	0.87	7.29	8.40*	2.73	0.37+
MW0932	*sspA*	Protease	0.69	5.75	8.39*	1.81	0.31+
MW1755	*splA*	Protease	0.25	7.32	29.17*	1.96	0.27+
MW0297	*geh*	Lipase	1.21	9.66	7.96*	2.43	0.25+
MW0206	*coa*	Coagulase	3.51	10.67	3.04*	3.63	0.34+
MW1885	*sak*	Staphylokinase	5.16	25.47	4.93*	10.25	0.40+
MW2341	*sbi*	IgG binding	46.74	79.02	1.69	26.37	0.33+
MW0084	*spa*	Protein A	2347.95	370.07	0.16**	193.49	0.52
MW0130	*capG*	Capsule	0.18	12.84	72.84*	2.24	0.17+
MW2586	*icaA*	Intercellular adhesin	0.14	4.20	30.88*	1.75	0.42+
MW0516	*sdrC*	Fibrinogen binding	0.22	1.19	5.33	0.63	0.53
MW0764	*clfA*	Clumping factor	9.15	95.86	10.48*	29.36	0.31+
MW2421	*fnb*	Fibronectin binding	3.10	13.42	4.33	5.86	0.44
MW1884	SCIN	Complement inhibitor	9.86	12.29	1.25	9.33	0.76
MW0088	*sirA*	Iron acquisition	0.78	26.91	34.59*	5.28	0.20+
MW0094	*sbnF*	Iron acquisition	0.05	1.41	28.75*	0.33	0.23+
MW1014	*isdD*	Iron acquisition	0.32	10.36	32.66*	1.60	0.15+

[a] GeneID in *S. aureus* strain MW2.
[b] Expression ratio to *gyrA* (×100) at exponential phase.
[c] Significant changes of expression of *S. aureus* grown in CS compared with TSB as determined by Student's *t* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[d] Significant changes of expression of *S. aureus* grown in CS containing 300 μM $FeCl_3$ compared with CS as determined by Student's *t* test; +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$.

Table 2). In addition, factors responsible for iron acquisition, such as *isdD*, *sbnF*, and *sirA*, were expressed significantly in CS. The higher expression levels of most virulence factors in CS were found to be significant at the stationary and exponential phases (Fig. 1). Since RNA III, comprising the gene (*hld*) encoding delta-hemolysin, is considered to be a global regulon responsible for many virulence factors, we investigated its expression during growth in TSB and CS. RNA III expression in CS was significantly higher than that upon growth in TSB from early exponential phase to stationary phase (Fig. 2). During growth, RNA III expression in CS was almost 10- to 100-fold higher than that in TSB. The expression pattern of RNA III in CS during growth had a tendency similar to that in TSB, showing expression that gradually increased until the stationary phase.

Next, we investigated the expression levels of RNA III in sera from calf, rabbit, and human to determine whether our observation was specific for CS. We found that the expression patterns in rabbit and human sera were quite similar to those in CS, showing higher expression of virulence factors, including RNA III, than in TSB (Table 3).

Identification of the factor responsible for the expression of virulence factors in serum. Since the expression levels of many virulence factors were increased in CS, we tried to identify the factor(s) responsible for these increased expression levels. At first, we used three solutions prepared from CS, (i) CS with proteins larger than 50 kDa removed (solution 1); (ii) CS with proteins larger than 3 kDa removed (solution 2); and (iii) CS with proteins, peptides, and lipids removed (solution 3). The expression of RNA III in all solutions tested was higher than that in TSB, indicating that the factor(s) was not among proteins, peptides, or lipids (Fig. 3).

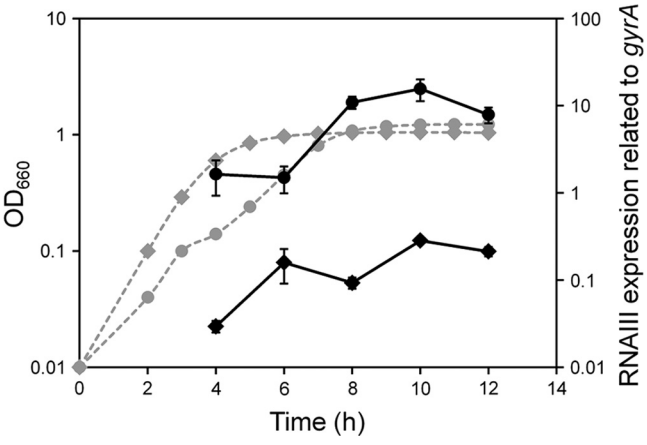

FIG. 2. Expression of RNA III during growth. *S. aureus* MW2 cells cultured in TSB (diamonds) and CS (circles) were collected at various growth phases. The expression of RNA III was determined by quantitative PCR by the method described in Materials and Methods. The data shown represent means ± SD of measurements performed in triplicate. The expression of RNA III and bacterial growth are represented by solid lines and dashed lines, respectively.

TABLE 3. RNA III, *hla*, and *isdD* expression of *S. aureus* grown in human and rabbit sera

Gene name	TSB expression[a]	HS[d]		HS + 300 μM $FeCl_3$		RS[e]		RS + 300 μM $FeCl_3$	
		Expression[a]	Fold change (TSB)[b]	Expression[a]	Fold change (HS)[c]	Expression[a]	Fold change (TSB)[b]	Expression[a]	Fold change (RS)[c]
RNAIII	6.1	260.0	42.5***	66.5	0.3+	143.8	23.5***	18.0	0.1+++
hla	0.4	7.0	18.1***	2.6	0.4+++	1.3	3.3***	0.6	0.4+++
isdD	0.5	85.4	176.2***	8.6	0.1+++	5.0	10.3***	0.8	1.7+++

[a] Expression ratio to *gyrA* (×100) at exponential phase.
[b] Significant changes of expressions of *S. aureus* grown in HS or RS compared with TSB as determined by Student's *t* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[c] Significant changes of expressions of *S. aureus* grown in HS or RS containing 300 μM $FeCl_3$ compared with HS or RS as determined by Student's *t* test; +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$.
[d] HS, human serum.
[e] RS, rabbit serum.

It is generally known that serum contains little (free) iron, so we hypothesized that iron depletion in serum increased the expression levels of various virulence factors. Indeed, the *isdD* gene was expressed significantly in CS and CS fractionates (solutions 1 to 3) compared with that in TSB (Table 2 and Fig. 1 and 3). In addition, other iron acquisition genes were expressed significantly in solutions 1 to 3 (data not shown). The addition of 300 μM $FeCl_3$ to CS decreased the expression levels of many virulence factors, including RNA III, significantly (Table 2). In particular, the expression of RNA III showed a decrease of about 25-fold after the addition of 300 μM $FeCl_3$ to the CS. Then, we investigated the effects of various concentrations of $FeCl_3$ on the expression of RNA III. $FeCl_3$ at concentrations below 30 μM had no effect on the reduction of RNA III expression in CS, while in CS with proteins larger than 50 kDa removed (solution 1), 0.3 μM $FeCl_3$ decreased the expression of RNA III and concentrations of $FeCl_3$ above 30 μM decreased its expression significantly (Fig. 4). We further investigated the effects of other iron sources, holotransferrin and hemin, on the expression of RNA III in CS (Fig. 5). The addition of holotransferrin or hemin containing 3 μM Fe(III) or Fe(II) had no effect on its expression. Owing to the solubility of these reagents in CS, we could not investigate the effects of high concentrations of holotransferrin and hemin on RNA III expression. In addition, we investigated the effects of various iron sources on the expression of RNA III and iron acquisition factors in solutions 1 to 3 and found that all 3 μM iron sources, including $FeCl_3$, decreased their expression levels significantly (see Fig. S1 in the supplemental material).

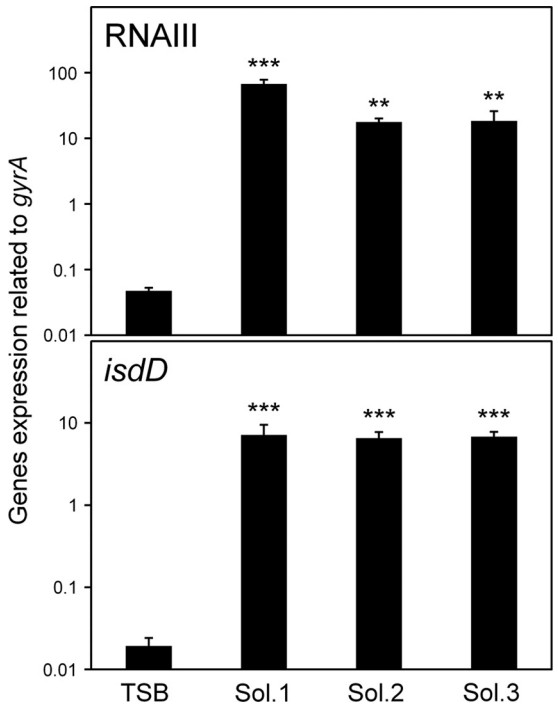

FIG. 3. Expression of RNA III and *isdD* in *S. aureus* grown in various fractions of serum. *S. aureus* MW2 cells cultured in TSB; CS with molecules larger than 50 kDa removed (solution [Sol.] 1); CS with molecules larger than 3 kDa removed (solution 2); and CS with molecules larger than 3 kDa, lipids, and peptides removed (solution 3) were collected at the exponential phase. The expression levels of RNA III and *isdD* were determined by quantitative PCR by the method described in Materials and Methods. The data shown represent means and SD of measurements performed in triplicate. The asterisks indicate significant increases compared with TSB as determined by Dunnett's test: **, $P < 0.01$; ***, $P < 0.001$.

Virulence factor expression of clinical isolates grown in serum. We investigated the expression of RNA III in CS using 7 clinical isolates and found that, with the exception of one strain, TY526, all the strains showed significant increase of RNA III expression in CS (Fig. 6). Furthermore, the addition of 300 μM $FeCl_3$ to CS resulted in decreased RNA III expression. Other factors, such as *psmβ1* and *icaA*, also showed results similar to those of RNA III (data not shown).

Expression of virulence factors in chemically defined medium. Since iron availability is restricted in serum, we also investigated the effects of iron on the expression of virulence factors in chemically defined medium. Table 4 shows the results of the expression of virulence factors grown in the presence or absence of 3 μM $FeCl_3$ in CDM(−). In CDM(−), *S. aureus* could still grow, although the growth rate was decreased slightly compared with that in CDM(−) with iron. This growth in CDM(−) is due to the presence of iron inside bacteria cultured in TSB as precultured cells. Since iron acquisition genes were increased significantly in CDM(−) (Table 4), it is considered that the concentration of iron in bacteria was significantly low. In a result similar to that with CS, the expression of many factors, including RNA III, *hla*, *psm*, *sec4*, *capG*, *isdD*, *sirA*, and *sbnF*, was increased significantly compared with that in TSB, and that of *spa* was decreased. However, the increased levels of expression of virulence factors were different in CS

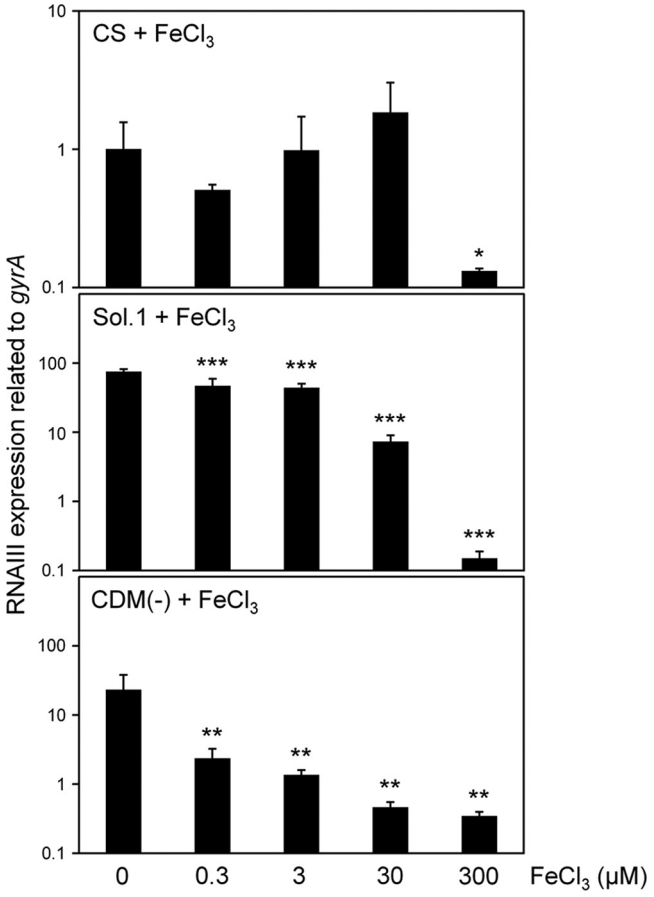

FIG. 4. Effect of $FeCl_3$ on the expression of RNA III. *S. aureus* MW2 cells cultured in CS, CS with molecules larger than 50 kDa removed (solution 1), and CDM(−) with or without various concentrations of $FeCl_3$ were collected at the exponential phase. The expression of RNA III was determined by quantitative PCR by the method described in Materials and Methods. The data shown represent means and SD of measurements performed in triplicate. The asterisks indicate significant decreases compared with the medium with no addition of $FeCl_3$ as determined by Dunnett's test: ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

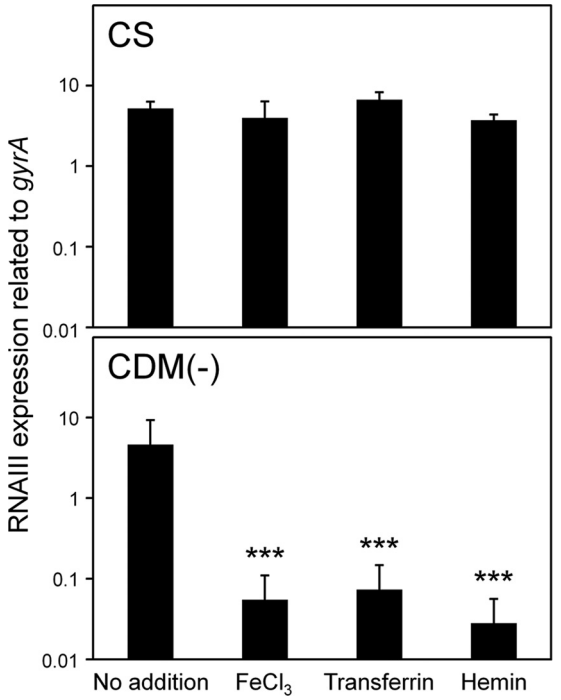

FIG. 5. Effect of hemin and holotransferrin on the expression of RNA III. *S. aureus* MW2 cells cultured in CS and CDM(−) with or without 3 μM $FeCl_3$, hemin, or holotransferrin were collected at the exponential phase. The expression of RNA III was determined by quantitative PCR by the method described in Materials and Methods. The data shown represent means and SD of measurements performed in triplicate. The asterisks indicate significant decreases compared with the medium with no addition of iron sources as determined by Dunnett's test: ∗∗∗, $P < 0.001$.

and CDM(−). The increases in the levels of RNA III, *psm*, *sec4*, and *isdD* in CDM(−) were higher than in CS, while those of others were lower than in CS. When 3 μM $FeCl_3$ was added to CDM(−), the expression of almost all factors was drastically decreased. In addition, the expression of RNA III was significantly reduced when 0.3 μM $FeCl_3$ was added to CDM(−) (Fig. 4). Furthermore, the addition of holotransferrin and hemin containing 3 μM Fe(III) or Fe(II) resulted in a significant decrease of RNA III (Fig. 5).

We also investigated the expression of virulence factors grown in TSB treated with Chelex-100 (see Table S2 in the supplemental material). The expression of many virulence factors, including RNA III, was increased compared with that in TSB without Chelex-100, showing an expression pattern similar to those grown in CS or CDM(−), although some factors showed different expression patterns under the three conditions (see Table S2 in the supplemental material). In Fig. 7, the amounts of expression of RNA III and *isdD* in TSB with Chelex-100, CDM(−), and CS can be seen to be higher than in TSB, CDM(−) with 3 μM $FeCl_3$, and CS with 300 μM $FeCl_3$. The expression patterns of virulence factors were similar under the three iron-depleted conditions [TSB with Chelex-100, CDM(−), and CS] or conditions with iron supplied [TSB, CDM(−) with 3 μM $FeCl_3$, and CS with 300 μM $FeCl_3$]. In addition, the expression patterns were quite different under iron-depleted and iron-supplied conditions, although the expression levels for some factors were different under the three conditions (Fig. 7 and Tables 2 and 4; see Fig. S2 in the supplemental material).

Expression of virulence factors in a mutant with *agr* inactivated. We investigated the expression of virulence factors in a mutant with *agr* inactivated grown in CS or CDM(−) (Fig. 8; see Table S3A and B in the supplemental material). Among the factors that were expressed significantly in CS, the expression of RNA III, *psm*, and *sec4* in the mutant with *agr* inactivated was not increased, while the expression of other factors was still increased almost as much as that of the wild type grown in CS (see Table S3A in the supplemental material). We also investigated the expression of virulence factors in the *agr* mutant grown in CDM(−) (see Table S3B in the supplemental material) and found results similar to those with CS.

Effects of serum albumin on the expression of virulence factors in CDM with iron. Serum albumin was purified from CS by affinity chromatography. The purified albumin showed a single band in a 7.5% polyacrylamide gel (Fig. 9A). In

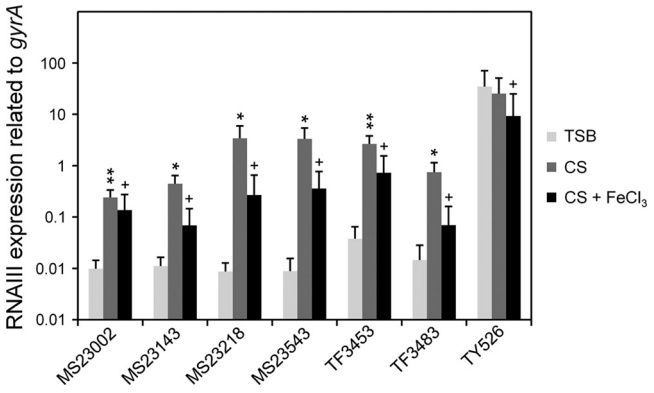

FIG. 6. RNA III expression in clinical isolates grown in serum. *S. aureus* clinical isolates cultured in TSB, CS, and CS with 300 μM $FeCl_3$ were collected at the exponential phase. The expression of RNA III was determined by quantitative PCR by the method described in Materials and Methods. The data shown represent means and SD of measurements performed in triplicate. The asterisks indicate significant increases compared with TSB as determined by Student's *t* test: *, $P < 0.05$; **, $P < 0.01$. The pluses indicate significant decreases compared with the medium with no addition of $FeCl_3$ as determined by Student's *t* test: +, $P < 0.05$.

CDM(−) containing purified albumin at 20 mg/ml (almost a 50% concentration of the albumin in serum), the addition of a low concentration of $FeCl_3$ (3 μM) did not decrease the expression of RNA III, showing a result similar to that with CS (Fig. 9B). This effect was also found when holotransferrin or hemin was added to CDM(−) containing serum albumin. Furthermore, other virulence factors, including iron acquisition genes, were not decreased (or the decrease was suppressed) by addition of 3 μM iron sources, including $FeCl_3$ in CDM(−) containing albumin, compared with those with 3 μM iron sources in CDM(−) (see Fig. S2 in the supplemental material). The expression of *isdD* in CDM(−) containing albumin was decreased by the addition of 3 μM $FeCl_3$, but its level of decrease (a 2-fold decrease) was significantly lower than that (a 53-fold decrease) in CDM(−) with 3 μM $FeCl_3$.

DISCUSSION

In this study, we showed that many virulence factors, including hemolysins, leukocidins, exoenzymes, and adhesins, were expressed significantly when *S. aureus* was grown in CS. Since we observed similar results using calf, rabbit, and human sera, it is considered that this effect is common among mammalian sera. Our results in this study indicated that depletion of free iron is strongly associated with high expression of virulence factors in serum. Generally, the iron source in serum is transferrin, although blood contains a high concentration of Fe(II) as a heme in hemoglobin. Therefore, free iron is very rare in serum, with the concentration of free iron in serum estimated to be 10^{-24} M (30). Because iron is an essential element for bacteria, including *S. aureus*, bacteria, especially pathogenic

TABLE 4. Expression of virulence factors of *S. aureus* grown in TSB, CDM(−), and CDM(−) with 3 μM $FeCl_3$

GeneID[a]	Gene name	Function	TSB expression[b]	CDM(−)		CDM(−) + 3 μM $FeCl_3$	
				Expression[b]	Fold change (TSB)[c]	Expression[b]	Fold change [CDM(−)][d]
	RNAIII	Global regulon	1.41	1942.26	1377.53**	31.66	0.02+++
MW1044	*hla*	Hemolysin	1.72	5.76	3.35*	1.80	0.31
MW2342	*hlgA*	Hemolysin	0.25	0.86	3.48*	0.25	0.29+
MW1942	*lukM*	Leukocidin	4.63	9.31	2.01*	2.80	0.30+++
MW1767	*lukD*	Leukocidin	0.21	0.54	2.57*	0.23	0.43++
MW1379	*lukS-PV*	Leukocidin	1.73	2.71	1.56	0.95	0.35+++
	psmα	Phenol-soluble modulin	0.88	111.72	126.74*	2.97	0.03+
MW1056	*psmβ1*	Phenol-soluble modulin	1.44	2380.99	1657.47**	46.09	0.02++
MW0759	*sec4*	Enterotoxin	5.16	177.22	34.35***	21.86	0.12++
MW1889	*sea*	Enterotoxin	2.41	4.67	1.94**	1.56	0.34+
MW2558	*aur*	Protease	0.87	1.24	1.43*	0.40	0.32++
MW0932	*sspA*	Protease	0.69	1.40	2.04**	0.56	0.40++
MW1755	*splA*	Protease	0.25	0.82	3.25*	0.35	0.42++
MW0297	*geh*	Lipase	1.21	5.26	4.33***	1.72	0.33++
MW0206	*coa*	Coagulase	3.51	7.29	2.08**	3.90	0.53+
MW1885	*sak*	Staphylokinase	5.16	5.86	1.14	1.50	0.26+
MW2341	*sbi*	IgG binding	46.74	40.99	0.88	15.80	0.39+
MW0084	*spa*	Protein A	2347.95	143.31	0.06**	93.82	0.65+
MW0130	*capG*	Capsule	0.18	2.25	12.75*	0.58	0.26++
MW2586	*icaA*	Intercellular adhesin	0.14	0.21	1.55	0.13	0.60++
MW0516	*sdrC*	Fibrinogen binding	0.22	0.18	0.79	0.05	0.29+++
MW0764	*clfA*	Clumping factor	9.15	54.36	5.94*	19.21	0.35+++
MW2421	*fnb*	Fibronectin biding	3.10	5.06	1.63**	8.27	1.63
MW1884	SCIN	Complement inhibitor	9.86	53.69	5.45**	13.62	0.25+++
MW0088	*sirA*	Iron acquisition	0.78	13.99	17.98*	0.17	0.01+
MW0094	*sbnF*	Iron acquisition	0.05	8.23	167.55**	0.04	0.00++
MW1014	*isdD*	Iron acquisition	0.32	14.91	46.98*	0.25	0.02+

[a] GeneID in *S. aureus* strain MW2.
[b] Expression ratio to *gyrA* (×100) at exponential phase.
[c] Significant changes of expression of *S. aureus* grown in CDM(−) compared with TSB as determined by Student's *t* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[d] Significant changes of expression of *S. aureus* grown in CDM(−) containing 3 μM $FeCl_3$ compared with CDM(−) as determined by Student's *t* test; +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$.

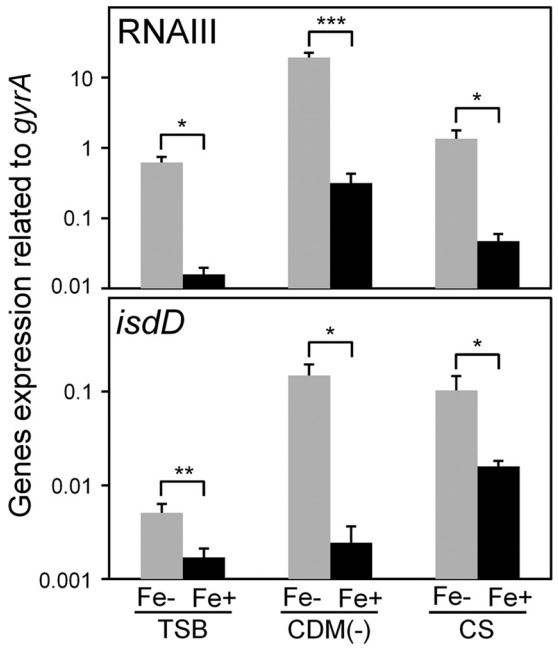

FIG. 7. Expression of RNA III and *isdD* in iron-depleted and -supplied media. *S. aureus* MW2 cells cultured in iron-depleted media [TSB with Chelex-100, CDM(−), and CS] or in iron-supplied media [TSB, CDM(−) with 3 μM $FeCl_3$, and CS with 300 μM $FeCl_3$] were collected at the exponential phase. The expression of RNA III and *isdD* was determined by quantitative PCR by the method described in Materials and Methods. The data shown represent means and SD of measurements performed in triplicate. The asterisks indicate significant decreases compared with iron-limited medium as determined by Student's *t* test: ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

bacteria, need to scavenge free iron or iron-binding proteins, including hemin, lactoferrin, and transferrin, from host tissues or fluids (14). To counter the sequestration of free iron, bacteria produce high-affinity iron chelators called siderophores. *S. aureus* produces several siderophores (staphyloferrins A and B, staphylobactin, and aureochelin) to bind iron and then transports the iron-siderophore complexes to the cytoplasm via ATP-binding cassette (ABC) transporters (2, 6, 8, 14, 32). In *S. aureus*, ABC transporters encoded by *sirABC*, *sitABC*, *fhuCBG*, and *sstABCD* were demonstrated to transport the complexes. Furthermore, *S. aureus* has a system encoded within the *isd* locus to scavenge iron from heme (23). In this study, we found increased expression of the factors involved in iron acquisition in serum compared with that in TSB, implying that the serum used in this study contained little iron. Therefore, it is reasonable that not only factors for iron acquisition, but also factors (toxins and exoenzymes) for the destruction of erythrocytes, leukocytes, and host tissues to capture iron, were upregulated in *S. aureus* under iron-restricted conditions like those in serum.

Among the factors expressed significantly in serum or CDM(−), RNA III expression was markedly enhanced. Since RNA III is thought to be a global regulator for many virulence factors (5, 26), we focused on its regulation. Generally, it has been demonstrated that RNA III expression is tightly associated with *agr*, a quorum-sensing system in *S. aureus* (5, 26). In our study, *agr* expression was increased significantly in serum or CDM(−) (data not shown), and this increase was suppressed by the addition of iron. In addition, in a mutant with *agr* inactivated, the expression of RNA III was not increased in CS. These results strongly suggested that high expression of RNA III in CS was caused by high expression of *agr*. In this study, we showed the expression of virulence factors in CS at the transcriptional level, not the translational level. Since the mechanisms of RNA III regulation for the expression of several virulence factors occur at the posttranscriptional level by RNA-RNA interaction, it is considered that a significant increase of RNA III affects the expression of virulence factors at the protein level. In the mutant with *agr* inactivated, the expression of many virulence factors, except *hld*, *psm*, and *sec4*, was still high in CS and CDM(−) at the transcriptional level, showing the same levels of expression as the wild type. These results indicate that factors other than *agr* and/or RNA III are also associated with the transcriptional regulation of virulence factors in CS.

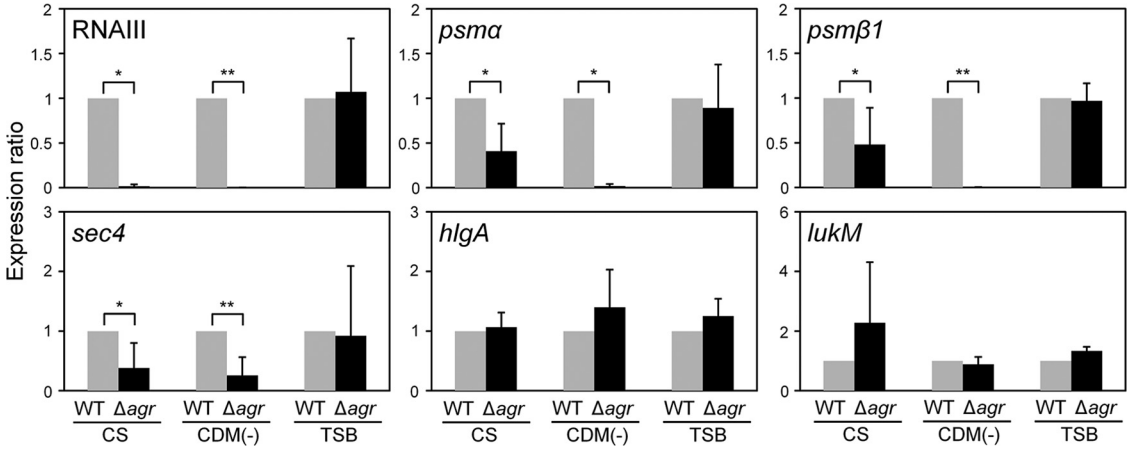

FIG. 8. Effect of *agr* inactivation on the expression of virulence factors. *S. aureus* MW2 and the mutant with *agr* inactivated were cultured in TSB, CS, and CDM(−). These cells were collected at the exponential phase. The expression of virulence factors was determined by quantitative PCR by the method described in Materials and Methods. The data shown represent means and SD of measurements performed in triplicate. The asterisks indicate significant decreases compared with the wild type as determined by Student's *t* test: ∗, $P < 0.05$; ∗∗, $P < 0.01$.

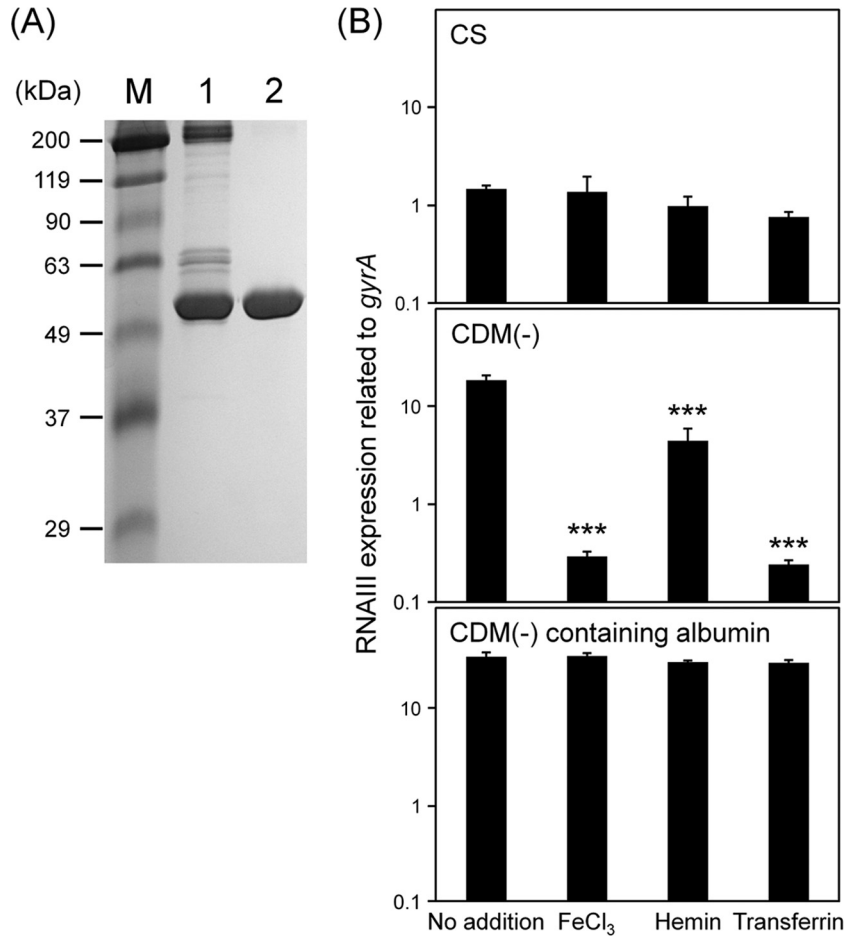

FIG. 9. Effect of serum albumin on the expression of RNA III. (A) Serum albumin from calf serum was purified using ToyoPearl AF-Blue HC-650 and then added to CDM(−). Small portions of CS and CDM(−) containing purified albumin were subjected to SDS-PAGE using a 7.5% polyacrylamide gel and then stained with Coomassie brilliant blue. M, size marker; lane 1, CS (3 μg); lane 2, CDM(−) containing purified albumin from CS (2 μg). (B) *S. aureus* MW2 cells cultured in CS, CDM(−), and CDM(−) containing purified serum albumin from CS, with or without 3 μM iron sources, were collected at the exponential phase. The expression of RNA III was determined by quantitative PCR by the method described in Materials and Methods. The data shown represent means and SD of measurements performed in triplicate. The asterisks indicate significant decreases compared with no addition of an iron source as determined by Dunnett's test: ***, $P < 0.001$.

Recently, Queck et al. reported an *agr*-dependent but RNA III-independent gene regulation mechanism in *S. aureus* (29). They demonstrated that many virulence factors, such as leukocidins, serine proteases, and protein A, were regulated by RNA III through *agr* but that *psm* expression was directly regulated by *agr*, not through RNA III. In our study, the expression of *psm*, *sec4*, and RNA III was drastically increased in CS and CDM(−) compared with that of other virulence factors, and this increase was not observed only in the *agr* mutant, showing that these 3 factors exhibited responses that differed from those of other virulence factors. Therefore, it is considered that the expression of *sec4*, together with *psm* and RNA III, was directly regulated by *agr*, although the mechanism of *agr* upregulation in serum remains unknown. To determine whether other regulators were involved in the expression of virulence factors in CS, we investigated the expression of all TCSs in CS or CDM(−). The expression of many TCSs was increased in CS compared with that in TSB (data not shown), while only *agr* expression was increased in CDM(−). However, we investigated the expression of several virulence factors in all TCSs, except mutants with *vicRK* inactivated, and found no difference between the wild type and each TCS mutant (data not shown). These results indicate that another factor(s) besides TCSs is involved in the increased expression of virulence factors, except *hld*, *psm*, and *sec4* in CS.

A ferric uptake regulator (Fur) is a repressor of iron acquisition system binding to upstream regions of iron acquisition factor genes when iron is abundant (35). In *S. aureus*, Fur regulates the factors, including *isd*, involved in iron acquisition. Torres et al. reported that Fur affected the expression of cytolysins (increased expression with *fur* inactivation) and a subset of immune-modulatory factors (decreased with *fur* inactivation), although the Fur box, which is the consensus sequence for binding Fur, is not present in the upstream regions of the genes coding for their factors (34). To investigate the association of Fur with the expression of virulence factors in CS, we attempted to construct a mutant with *fur* inactivated but were unsuccessful for an unknown reason. Further study is needed to resolve the association of Fur with the expression of virulence factors in CS.

Of 7 clinical isolates tested, 6 strains showed a response similar to that of MW2, with increased expression of RNA III in CS; this increase was suppressed by the addition of iron, although the levels of increase were quite different among strains. However, one strain, TY526, did not increase RNA III expression in CS, although RNA III expression in TSB was quite high compared with those of other strains. These results indicate that the expression of virulence factors in CS is variable among strains.

In addition, we found that serum albumin captured free iron to inhibit bacterial acquisition of free iron. Previously, it was reported that serum albumin had binding affinity to free iron, heme, or transferrin (15, 36). However, this is the first report to demonstrate that iron capture by serum albumin affects the expression of a virulence factor(s) in *S. aureus*. Serum albumin is the most abundant protein in the blood and has a structural domain for heme binding (15). Purified albumin from calf serum suppressed the increase of RNA III expression in spite of the addition of $FeCl_3$, holotransferrin, and hemin, suggesting its affinity for these molecules. Since *S. aureus* causes bacteremia, it can clearly grow and survive in the bloodstream by utilizing several iron-scavenging systems. In addition, in this study, *S. aureus* produced a high level of cytolysins, including hemolysins, to disrupt and sequester iron from host tissues or cells. Therefore, iron scavenging between the host and parasite in blood might be one of the critical factors involved in *S. aureus* infection.

In conclusion, we showed that many virulence factors were expressed significantly upon growth in serum. Increased expression of *agr* was associated with the expression of some virulence factors, but another regulatory factor(s) is also involved. Our findings in this study suggest that the expression pattern of virulence factors of *S. aureus* upon infection of a host is quite different from that upon growth in bacterial media.

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