THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 35, pp. 25542–25550, August 30, 2013

Silkworm Apolipophorin Protein Inhibits Hemolysin Gene Expression of *Staphylococcus aureus* via Binding to Cell Surface Lipoteichoic Acids*

Received for publication, June 19, 2013 Published, JBC Papers in Press, July 19, 2013, DOI 10.1074/jbc.M113.495051

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Background: Silkworm ApoLp protein binds *S. aureus* cell surfaces and inhibits hemolysin gene expression.
Results: ApoLp protein binds LTA. The ApoLp inhibitory effect was attenuated in an LTA synthetase knockdown mutant.
Conclusion: ApoLp protein suppressed *S. aureus* virulence via LTA binding.
Significance: A non-protein macromolecule on the bacterial cell surface functions as a receptor in the bacterial signal transduction pathway.

We previously reported that a silkworm hemolymph protein, apolipophorin (ApoLp), binds to the cell surface of *Staphylococcus aureus* and inhibits expression of the *saePQRS* operon encoding a two-component system, SaeRS, and hemolysin genes. In this study, we investigated the inhibitory mechanism of ApoLp on *S. aureus* hemolysin gene expression. ApoLp bound to lipoteichoic acids (LTA), an *S. aureus* cell surface component. The addition of purified LTA to liquid medium abolished the inhibitory effect of ApoLp against *S. aureus* hemolysin production. In an *S. aureus* knockdown mutant of *ltaS* encoding LTA synthetase, the inhibitory effects of ApoLp on *saeQ* expression and hemolysin production were attenuated. Furthermore, the addition of anti-LTA monoclonal antibody to liquid medium decreased the expression of *S. aureus saeQ* and hemolysin genes. In *S. aureus* strains expressing SaeS mutant proteins with a shortened extracellular domain, ApoLp did not decrease *saeQ* expression. These findings suggest that ApoLp binds to LTA on the *S. aureus* cell surface and inhibits *S. aureus* hemolysin gene expression via a two-component regulatory system, SaeRS.

Understanding the molecular interactions between host and bacteria is important toward elucidating the mechanisms of infectious diseases. Bacteria express various virulence genes according to the host microenvironment (1). To recognize environmental signals, bacteria possess a two-component system comprising a receptor with histidine kinase activity and a transcription factor activated by phosphorylation. Host animals, on the other hand, recognize invading bacteria by diverse receptors and induce immune responses to kill bacteria.

Staphylococcus aureus is a human pathogenic bacterium present in the nares of 30% of healthy individuals that causes many diseases including sepsis and meningitis. *S. aureus* secretes several hemolysins and damages host cells. *S. aureus* hemolysin gene deletion mutants exhibit attenuated virulence against mammals (2, 3), indicating that *S. aureus* hemolysins are essential for its virulence. *S. aureus* has 16 two-component systems, which are assumed to recognize the environment and express adequate virulence genes, including hemolysin genes (4). Within the two-component systems, *agr*, *arlRS*, and *saeRS* are required for the expression of hemolysin genes (5–11). The sensor protein, SaeS, of the two-component system SaeRS is a transmembrane protein with two transmembrane helixes and a short extracellular region of nine amino acids (12). Such sensor proteins are called intramembrane-sensing kinases, most of which are involved in resistance against cell wall stress and antibiotics (12). The *saeRS* locus contains two promoters; *saeP-saeQ-saeR-saeS* is transcribed from P1 and *saeR-saeS* is transcribed from P3 (13, 14). *saeP* and *saeQ* has an essential role in *saeRS* function (14, 15). SaeQ protein is assumed to stabilize SaeS protein (16). The P1 promoter is activated by hydrogen peroxide and α-defensin (17, 18), whereas it is inactivated at low pH and high salt concentrations (17). How these environmental signals alter the P1 activity of *saeRS*, however, is not clear.

S. aureus produces lipoteichoic acids (LTA)[2] and wall teichoic acids (WTA) that have an important role in *S. aureus* virulence (19). *S. aureus* teichoic acids promote bacterial adherence to host cells (20, 21). An *S. aureus* gene-disrupted mutant of the *tagO* gene that encodes WTA synthetase decreases adherence activity to human epithelial cells (22, 23), biofilm forming ability (24), colony spreading ability (25), and virulence in mammals (22, 23). Both LTA and WTA are D-alanylated by enzymes encoded by the *dlt* operon. The gene-disrupted mutant of the *dlt* operon decreases resistance ability to host antimicrobial peptides (26, 27), biofilm-forming ability (24), colony-spreading ability (25), and virulence in mice (28). Whether teichoic acids are involved in bacterial gene expression, however, is unknown.

We investigated the interaction between *S. aureus* and host animals using silkworms, which are larvae of the moth, *Bombyx*

* This work was supported by grants-in-aid for scientific research. This work was supported in part by the Naito Foundation and the Genome Pharmaceuticals Institute.

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[2] The abbreviations used are: LTA, lipoteichoic acid(s); WTA, wall teichoic acid(s); IPTG, isopropyl 1-thio-β-D-galactopyranoside.

TABLE 1
List of bacterial strains and plasmids used

Strain or plasmid	Genotypes or characteristics	Source or Ref.
Strains		
S. aureus		
RN4220	NCTC8325-4, restriction mutant	Ref. 41
NCTC8325–4	NCTC8325 cured of φ11, φ12, and φ13	Ref. 42
M0661	RN4220 Δ*saePQRS*::Kan^r	This work
M0661N	NCTC8325-4 Δ*saePQRS*::Kan^r	This work
M0674	RN4220 Δ*ltaS*::phleo^r	Ref. 36
M0674N	NCTC8325-4 Δ*ltaS*::phleo^r (transduction from M0674)	This work
M0674Nspac	NCTC8325-4 Pspac::*ltaS*; Erm^r	This work
M0702	RN4220 Δ*tagO*::Erm^r	Ref. 25
M0793	RN4220 Δ*dltABCD*::Erm^r	Ref. 25
M0702N	NCTC8325-4 Δ*tagO*::Erm^r (transduction from M0702)	This work
M0793N	NCTC8325-4 Δ*dltABCD*::Erm^r (transduction from M0793)	This work
Plasmids		
pMutinT3	Suicidal vector for Gram-positive bacteria; Amp^r, Erm^r	Ref. 43
pMutinT3-ltaS	pMutinT3 carrying 5′-partial region of *ltaS*	This work
pUC-Int	pUC19 carrying integration region for NCTC8325-4	This work
pUC-Int-erm	pUC-Int carrying erythromycin-resistant gene	This work
pInt-sae	pUC-Int-erm carrying *saePQRS* gene	This work
pInt-I9Q-SaeS	pInt-sae carrying SaeS with I9Q substitution	This work
pInt-I9Q,L63Q-SaeS	pInt-sae carrying SaeS with I9Q and L63Q substitution	This work
pInt-Δ34–36-SaeS	pInt-sae carrying SaeS deleted with 34 to 36th amino acids	This work
pInt-Δ35–37-SaeS	pInt-sae carrying SaeS deleted with 35 to 37th amino acids	This work
pInt-Δ34–37-SaeS	pInt-sae carrying SaeS deleted with 34 to 37th amino acids	This work
pKOR3a	Vector for allelic replacement in *S. aureus*, Cm^r	Ref. 38
pluc	pND50 with luc + with a ribosomal binding site	Ref. 44
pluc-hla	pluc with *hla* promoter from RN4220	Ref. 44
pluc-saeP1	pluc with *sae* P1 promoter from RN4220	Ref. 35

mori (29–34). We previously reported that the silkworm hemolymph protein ApoLp inhibits the P1 activity of *saeRS* and decreased the expression of *saePQRS* and hemolysin genes (35). Administration of anti-ApoLp antibody sensitized silkworms to *S. aureus*, indicating that inhibition of hemolysin production by ApoLp contributes to silkworm resistance to *S. aureus* (35). How ApoLp inhibits the expression of *S. aureus* hemolysin genes was, however, not revealed. In the present study, we demonstrated that ApoLp binds LTA, a cell surface component of *S. aureus*, and LTA binding inhibits the expression of *saePQRS* and hemolysin genes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*S. aureus* strains were aerobically cultured in tryptic soy broth at 37 °C, and 10 μg/ml erythromycin, 100 μg/ml kanamycin, or 20 μg/ml phleomycin/ml was added to the medium if required. The JM109 strain of *Escherichia coli* was used as a host for plasmids. Details of the bacterial strains and plasmids used in this study are shown in Table 1. The *ltaS* deletion mutant (M0674N) and the conditional knockdown mutant of *ltaS* (M0674Nspac) were cultured at 30 °C in tryptic soy broth supplemented with 0.5 M NaCl to keep out damaging cells. M0674Nspac was cultured in the presence of 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) or in the absence of IPTG.

Purification of ApoLp—We purified ApoLp from the silkworm hemolymph according to our previously published method (35). We measured the activity required to inhibit *S. aureus* hemolysin production in each purification step. In this study, we used an ApoLp protein fraction purified by phosphocellulose chromatography using buffer A (50 mM MES (pH 6.9), 100 mM NaCl, 2 mM dithiothreitol, 5% glycerol).

Measurement of S. aureus Hemolysin Production—*S. aureus* culture supernatants were collected by centrifugation, and 2-fold serial dilutions were mixed with an equal volume of 5% sheep erythrocytes. Incubation was performed at 37 °C for 1 h and at 4 °C overnight. Hemolytic activity was defined as the reciprocal of dilutions that yielded 50% erythrocyte lysis.

Binding Assay of ApoLp to LTA—Lipoteichoic acids from *S. aureus* (Sigma) were poured into Immulon 1B ELISA plates (Dynatech Laboratories, Chantilly, VA) and incubated at 4 °C overnight. The plates were washed three times with 200 μl of PBS containing 0.5% Tween 20 (PBST) and treated with 200 μl of PBST containing 3% bovine serum albumin for 1 h at room temperature. The plates were washed three times with PBST and incubated with 100 μl of ApoLp solution at room temperature for 30 min. The plates were washed three times with PBST and incubated with anti-ApoLp IgG (35) at room temperature for 2 h. The plates were washed five times with PBST and incubated with anti-mouse IgG AP conjugate (Promega) at room temperature for 2 h. The plates were washed seven times with PBST and incubated with 200 μl of substrate buffer (50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 1 mg/ml *p*-nitrophenylphosphate (Sigma)). Absorbance of 450 nm was measured using a microplate reader (MTP-300, COLONA ELECTRONIC, Ibaraki, Japan). Binding of ApoLp to LTA was determined by subtracting the value without LTA.

Competitive Assay of ApoLp with LTA against S. aureus Hemolysin Production—Overnight culture of *S. aureus* NCTC8325–4 was inoculated into a 200-fold amount of fresh tryptic soy broth and cultured at 37 °C for 1 h. A 600-μl aliquot of the cultures was supplemented with 200 μl of ApoLp solution or a mixed solution of ApoLp and LTA and was cultured at 37 °C for 4 h. The culture supernatant was collected by centrifugation at 10,000 × *g* for 2 min. Hemolytic activity of the supernatant was measured using the hot-cold method described above.

TABLE 2
Primers used in this study

Target	Sequence (5′-3′)	Ref.
***saePQRS* (upstream)**		
Forward	ATAACGAACAGGAGTCCATCA	This work
Reverse	ATCACCTCAAATGGTTCGCTTCAAAATAAGAGGAGGGCATTT	
***saePQRS* (downstream)**		
Forward	GTTCGCTAGATAGGGGTCCCATTACACAAATTAGACATTA	This work
Reverse	TGATTTGCAACATCCACCTG	
***ltaS* (5′-region)**		
Forward	AAGAAGCTTCTAAATAACGGGGGAAAGAATCATGAGTTC	Ref. 37
Reverse	GGAGGATCCGACAGGAACAAATTTCTTACTAAATGCTTTTG	
***saePQRS* (complementation)**		
Forward	GGAGGATCCTTATTGTGGCAAAAGGTTT	This work
Reverse	AAGAAGCTTATTATTAGGCGGCATACAG	
Genome integration		
Forward	GGAGGATCCTTACGCATCCAAACACTCC	Ref. 45
Reverse	GGTGGTACCAACACAACACTGACACGTCATTTA	
Erythromycin-resistant gene		
Forward	GAAGAATTCAACCTATAAAAATAGGCGTAT	Ref. 46
Reverse	GGTGGTACCAACGTTCTTGCCATTGCTGCA	
SaeS		
R-7S	ACTTCTAATTGATAACACCATTATCG	This work
F-I9Q	CAACAAATTATTGGCGTCGTTTCGAG	
R-62P	TGGATTAATAAAAATACTACATATTAATAAGG	
F-L63Q	CAAATACAAAAAATTAAGCAGTTTAATATAAAAACTAAGC	
R-33F	AAACCACATTAAAATATATGCAATTGC	
F-37M	ATGACACTAACTTTGACCTTAACG	
R-34N	GTTAAACCACATTAAAATATATGCAATTGC	
F-38T	ACACTAACTTTGACCTTAACG	

Construction of the ltaS Deletion Mutant—Deletion of the *ltaS* gene in the *S. aureus* RN4220 strain was performed by Oku *et al.* (36). The deletion was transferred to NCTC8325-4 by phage transduction using phage 80α and confirmed by Southern blot analysis.

Construction of the ltaS Conditional Knockdown Mutant—The *ltaS* conditional knockdown mutant was constructed according to the previously published method (37). Briefly, the 5′ region of the *ltaS* gene was amplified by PCR and inserted into pMutinT3, resulting in pMutinT3-ltaS. The RN4220 strain was transformed with pMutinT3-ltaS, and the transformed strain was resistant against erythromycin. The mutation was transferred to the NCTC8325-4 strain by phage transduction using phage 80α. The desired chromosomal DNA mutation was confirmed by Southern blot analysis.

Construction of the saePQRS Deletion Mutant—Upstream and downstream regions of the *saePQRS* were amplified by PCR using the oligonucleotide primers listed in Table 2 and NCTC8325-4 genomic DNA as the template. The *aph* gene, conferring kanamycin resistance, was amplified by PCR using pSF151 as the template (38). Three DNA fragments of the upstream region, downstream region, and *aph* gene were spliced together using splicing by overlap extension PCR, resulting in an saePQRS-cassette. The saePQRS cassette was inserted into the SmaI site of pKOR3a, resulting in pKOR3a-saePQRS. *S. aureus* RN4220 was transformed with pKOR3a-saePQRS. The transformant was cultured in tryptic soy broth, and 10^3 cells were spread onto tryptic soy agar plates containing 12.5 μg/ml chloramphenicol. The plates were incubated at 43 °C overnight. The resulting colonies were cultured in tryptic soy broth at 37 °C and spread onto tryptic soy agar plates containing 1 μg/ml anhydrotetracycline and 50 μg/ml kanamycin. The resulting colonies were examined for sensitivity to chloramphenicol. The *saePQRS* deletion was confirmed by Southern blot analysis.

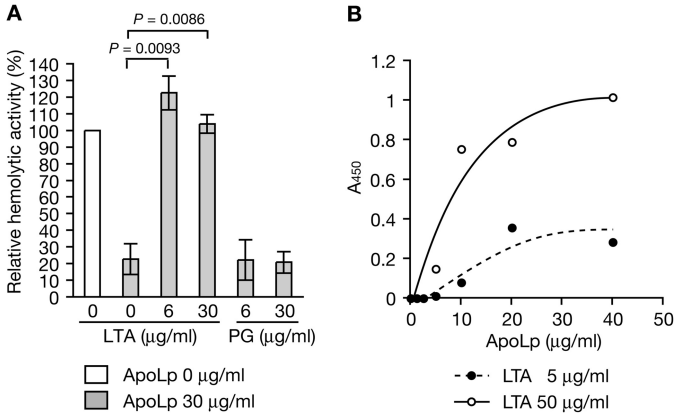


FIGURE 1. **ApoLp binds LTA.** *A*, ApoLp, ApoLp, and LTA (from *S. aureus*, Sigma), or ApoLp and peptidoglycan (*PG*, from *S. aureus*, Sigma) was added to *S. aureus* NCTC8325-4 culture (A_{600} of 0.1), and the mixtures were cultured further for 4 h. Hemolytic activity of the culture supernatant against sheep erythrocytes was measured. Means ± S.D. from two independent experiments are presented. Student's *t* test *p* values are presented. *B*, LTA (5 μg/ml or 50 μg/ml) was immobilized onto a microplate. ApoLp was added to the plate. The amount of ApoLp bound to the plate was measured using an ELISA with anti-ApoLp IgG. The vertical axis represents A_{450}, which reflects the amount of ApoLp bound to LTA.

Construction of Mutated saeS Genes—A DNA fragment containing *saePQRS* was amplified by PCR using the oligonucleotide primers listed in Table 2 and inserted into pUC-Int-erm, resulting in pInt-sae. Point mutations or deletions were introduced into pInt-sae by PCR using oligonucleotide primers. The mutations were confirmed by sequencing. The *saePQRS*-deleted mutant (M0661) was transformed with plasmids and the desired integration of the plasmids into chromosomal DNA was confirmed by Southern blot analysis. The integrated plas-

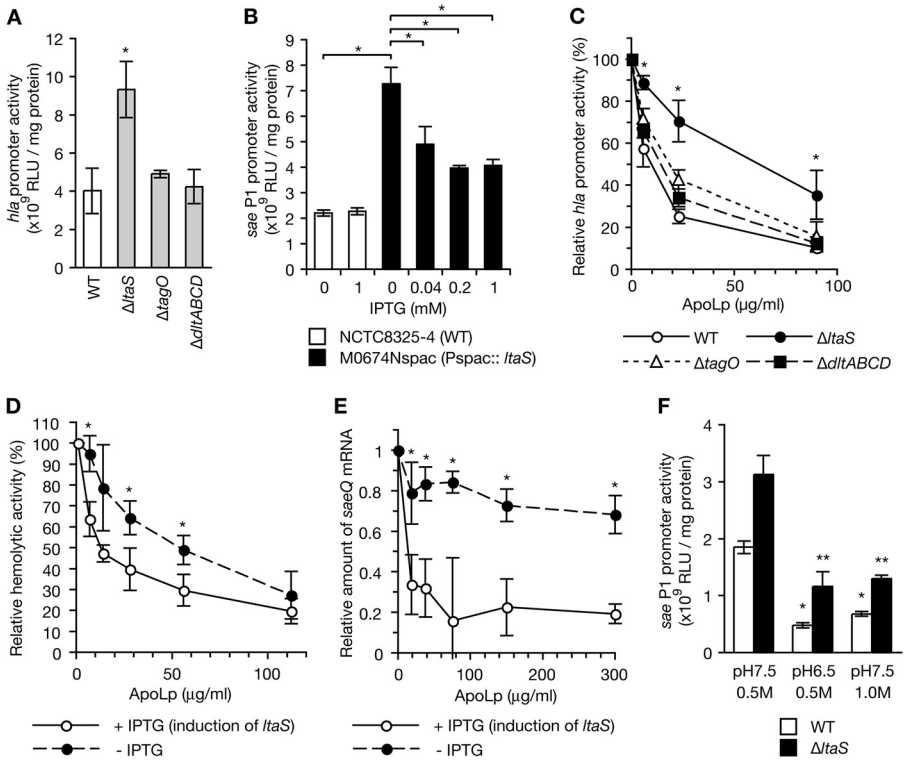


FIGURE 2. **The inhibitory effect of ApoLp against hemolysin production was attenuated in the *ltaS* mutant encoding LTA synthetase.** *A*, *S. aureus* NCTC8325-4 strain (WT), the *ltaS* mutant (Δ*ltaS*), the *tagO* mutant (Δ*tagO*), and the *dltABCD* mutant (Δ*dltABCD*) were transformed with a reporter plasmid carrying the *hla* promoter and cultured at 30 °C for 6 h. Luciferase activities (relative light units (*RLU*)) of the cell lysates were measured. Means ± S.D. of three independent experiments are shown. The *asterisk* indicates Student's *t* test *p* value of <0.05 between the wild-type strain and the *ltaS* mutant. *B*, *S. aureus* NCTC8325–4 strain (WT) and the *ltaS* conditional knockdown mutant (Pspac::*ltaS*) were transformed with a reporter plasmid carrying *sae* P1 and cultured in the absence or presence of IPTG at 30 °C for 6 h. Luciferase activities of the cell lysates were measured. Means ± S.D. of three independent experiments are shown. The asterisk indicates Student's *t* test *p* value of <0.05. *C*, various amounts of ApoLp were added to *S. aureus* cultures (A_{600} of 0.1) of NCTC8325-4 (WT), M0674N (Δ*ltaS*), M0702N (Δ*tagO*), and M0793N (Δ*dltABCD*) that were transformed with a reporter plasmid carrying the *hla* promoter, and the mixtures were cultured further at 30 °C for 6 h. Luciferase activity of the cell lysate was measured. The vertical axis represents relative luciferase activity against that without ApoLp. *Asterisks* indicate a Student's *t* test *p* value of <0.05 between NCTC8325-4 and the *ltaS* mutant. *D*, various amounts of ApoLp were added to *S. aureus* culture of M0674spac carrying IPTG-inducible *ltaS* (A_{600} of 0.1) in the presence or absence of IPTG, and the mixtures were cultured further at 30 °C for 6 h. Hemolytic activity of the culture supernatant against sheep erythrocytes was measured. The *vertical axis* represents relative hemolytic activity against that without ApoLp. Means ± S.D. from two independent experiments are shown. *Asterisks* indicate a Student's *t* test *p* value of <0.05 between +IPTG and −IPTG. *E*, various amounts of ApoLp were added to *S. aureus* culture of M0674spac carrying IPTG-inducible *ltaS* (A_{600} of 0.1) in the presence or absence of IPTG and further cultured at 30 °C for 6 h. Total RNA was extracted from the cultured cells. The amount of *saeQ* mRNA was measured by quantitative RT-PCR. Means ± S.D. from three independent experiments are shown. *Asterisks* indicate a Student's *t* test *p* value of <0.05 between +IPTG and −IPTG. *F*, *S. aureus* NCTC8325–4 strain (WT) and the *ltaS* mutant (Δ*ltaS*) that were transformed with a reporter plasmid carrying *sae* P1 were cultured under normal conditions (pH 7.5, 0.5 M NaCl), a low pH condition (pH 6.5, 0.5 M NaCl), or a high salt condition (pH 7.5, 1.0 M NaCl). Luciferase activities of the cell lysates were measured. Means ± S.D. of three independent experiments are shown. The *asterisks* indicate Student's *t* test *p* value of <0.05 between the normal condition and the low pH condition or between the normal condition and the high salt condition.

mids were transferred to M0661N by phage 80α. These strains were used as *S. aureus* strains expressing wild-type SaeS (WT) or mutated SaeS.

Measurement of Gene Expression by Quantitative Real-time PCR—The protocol and oligonucleotide primers used were essentially the same as those used previously (35). Briefly, *S. aureus* cells were collected by centrifugation, treated with RNAprotect bacteria reagent (Qiagen, Gaithersburg, MD) and lysed in a buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mg/ml lysostaphin). RNA was extracted using an RNeasy Mini kit (Qiagen). RNA was reverse transcribed to cDNA. Quantitative real-time PCR was performed using cDNA as a template and primers for target mRNAs. The signals were detected using a StepOnePlus real-time PCR System (Applied Biosystems). The data were normalized to 16S ribosomal RNA.

Reporter Assay—The RN4220 strain was transformed with reporter plasmids (Table 1). Plasmids were transferred into NCTC8325-4 and mutant strains by phage transduction using phage 80α. The strains were aerobically cultured at 30 °C in tryptic soy broth supplemented with 0.5 M NaCl. The *S. aureus* cultured solutions (600 μl, A_{600} = 0.1) were supplemented with ApoLp solution and cultured for 6 h at 30 °C. The cells were collected by centrifugation and lysed in a buffer (20 mM KH_2PO_4 (pH 7.8), 0.04% Triton X-100, 0.1 mM dithiothreitol, 10 μg/ml lysostaphin, and one tablet of protease inhibitor (Roche Applied Science)). Cell lysate supernatant was incubated with luciferase substrate, and luminescence was measured using a luminometer (Berthold Technologies, Bad Wildbad, Germany). The promoter activity was calculated as luminescence units per milligram of protein.

RESULTS

ApoLp Binds S. aureus Lipoteichoic Acids—We previously reported that ApoLp binds *S. aureus* cell surfaces (35). To identify the *S. aureus* cell surface molecule that ApoLp binds, we examined whether LTA and peptidoglycan, major cell surface

components, abolished the ApoLp-induced inhibition of *S. aureus* hemolysin production. Addition of LTA to *S. aureus* culture medium blocked the inhibitory effect of ApoLp on *S. aureus* hemolysin production (Fig. 1*A*). In contrast, the addition of peptidoglycan did not block the inhibitory effect of ApoLp (Fig. 1*A*). These findings indicate that ApoLp binds LTA of *S. aureus* cell surfaces. We then examined whether ApoLp directly binds LTA. We measured the amount of ApoLp bound to immobilized LTA by enzyme-linked immunosorbent assay. The amount of ApoLp bound to LTA was increased with increasing amounts of added ApoLp (Fig. 1*B*). The amount of ApoLp bound to LTA increased with increasing amounts of immobilized LTA (Fig. 1*B*). These findings indicate that ApoLp directly binds LTA.

The Inhibitory Effect of ApoLp on S. aureus Hemolysin Production Is Attenuated in LTA Synthetase S. aureus Mutants—To determine whether ApoLp binding to LTA is required for the inhibitory effect of ApoLp on *S. aureus* hemolysin production and *saeQ* expression, we examined the inhibitory effect of ApoLp using gene deletion mutants of the *ltaS* gene, which encodes LTA synthetase, and an *ltaS* conditional knockdown mutant. Furthermore, we examined the effect of ApoLp in the deletion mutant of *tagO* encoding WTA synthetase or *dltABCD*, which functions in the D-alanylation of teichoic acids. First, we examined the activity of the *hla* promoter encoding α-hemolysin in the absence of ApoLp in these mutants. The promoter activity of *hla* was higher in the *ltaS*-deleted mutant than in the wild-type strain (Fig. 2*A*). The promoter activity of *hla* did not differ between the wild-type strain, the *tagO*-deleted mutant, and the *dltABCD*-deleted mutant (Fig. 2*A*). Thus, LTA has a role in decreasing *hla* expression, whereas WTA and D-alanylation of teichoic acids have no role in *hla* expression in the absence of ApoLp. In addition, in the *ltaS* conditional knockdown mutant that contains the IPTG-inducible promoter Pspac upstream of the *ltaS* gene, the activity of *sae* P1, which is the promoter for the *saeQ* gene, was higher in the absence of IPTG than in the wild-type strain (Fig. 2*B*). The addition of IPTG to the *ltaS*-conditional mutant decreased the activity of *sae* P1 (Fig. 2*B*). Thus, LTA has a negative role in *sae* P1 expression in the steady state.

We then examined the effect of ApoLp in the *ltaS*, *tagO*, and *dltABCD* mutants. In the wild-type strain, the *tagO*-deleted mutant, and the *dltABCD*-deleted mutant, the addition of 90 μg/ml ApoLp decreased the promoter activity of *hla* to <20% that of the non-treated sample, whereas in the *ltaS*-deleted strain, the addition of ApoLp decreased the promoter activity only to ~40% that of the non-treated sample (Fig. 2*C*). In addition, in the *ltaS* conditional knockdown mutant, the inhibitory effect of ApoLp on hemolysin production was attenuated in the absence of IPTG compared with that in the presence of IPTG, which induces *ltaS* expression (Fig. 2*D*). Furthermore, in the presence of IPTG, the addition of ApoLp decreased *saeQ* expression to <20% that of the non-treated sample, whereas in the absence of IPTG, the addition of ApoLp decreased *saeQ* expression only to ~70% that of the non-treated sample (Fig. 2*E*). These findings indicate that repression of *ltaS* expression reduced the inhibitory effects of ApoLp on hemolysin production and *saeQ* expression and thus indicated that ApoLp binding to LTA was required for the subsequent inhibition of hemolysin production and *saeQ* expression.

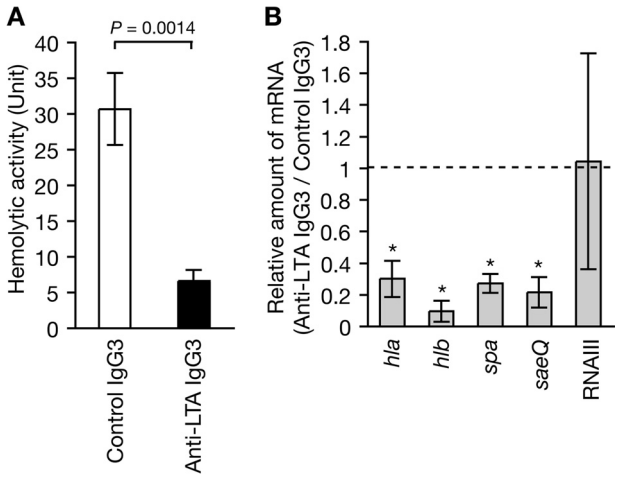


FIGURE 3. **Addition of anti-LTA antibody decreases *S. aureus* hemolysin production.** *A*, anti-LTA mouse IgG3 monoclonal antibody (mAb 55, Hycult Biotech, The Netherlands) or control mouse IgG3 antibody (Bethyl Laboratories, Montgomery, TX) was added to *S. aureus* NCTC8325-4 culture (A_{600} = 0.1) at a final concentration of 250 μg/ml, and the mixture was cultured further for 4 h. Hemolytic activities of the culture supernatants against sheep erythrocytes was measured. Means ± S.D. from three independent experiments are shown. Student's *t* test *p* value is presented. *B*, anti-LTA mouse IgG3 monoclonal antibody or control mouse IgG3 antibody was added to *S. aureus* NCTC8325-4 culture (A_{600} = 0.1) at a final concentration of 250 μg/ml, and the mixture was cultured further for 4 h. Total RNA was extracted from the cultured cells. RNA amounts of *hla*, *hlb*, *spa*, *saeQ*, and RNAIII were measured by quantitative RT-PCR. The vertical axis shows the relative values against the amount of RNAs in the presence of control IgG3. Means ± S.D. of three independent experiments are shown. *Asterisks* indicate Student's *t* test *p* value of <0.05 between anti-LTA IgG3 and control IgG3.

Low pH and high salt conditions decrease the activity of *sae* P1 (17). We examined whether LTA is required for the inactivation of *sae* P1 activity by a low pH or high salt condition. The *sae* P1 activity was lower at pH 6.5 than at pH 7.5 in both the wild-type and the *ltaS*-deleted mutant (Fig. 2*F*). In addition, the *sae* P1 activity was lower in 1.0 M NaCl than in 0.5 M NaCl in both the wild-type and the *ltaS*-deleted mutant (Fig. 2*F*). Thus, LTA is not involved in the regulation of *sae* P1 activity by low pH and high salt conditions.

Addition of Anti-LTA Monoclonal Antibody Decreases S. aureus Hemolysin Production and saeQ Expression—Based on the findings that the ApoLp binding to LTA is necessary to inhibit the expression of the hemolysin genes and *saeQ*, we hypothesized that ApoLp binding to LTA triggers signals to inhibit the expression of hemolysin genes and *saeQ*. Studies of the signal transduction mechanism in mammals revealed that binding of an antibody to the receptor sometimes activates the signaling pathway in the similar way as the binding of true ligands (39, 40). We examined whether binding of the monoclonal antibody to LTA causes similar effects as ApoLp. The addition of anti-LTA IgG3 decreased *S. aureus* hemolysin production compared with the addition of control IgG3 (Fig. 3*A*). Furthermore, the addition of anti-LTA IgG3 decreased the expression of *hla* encoding α-hemolysin, *hlb* encoding β-hemolysin, *spa* encoding protein A, and *saeQ* compared with the addition of control IgG3 (Fig. 3*B*). The addition of anti-LTA IgG3 did not decrease the expression of RNAIII,

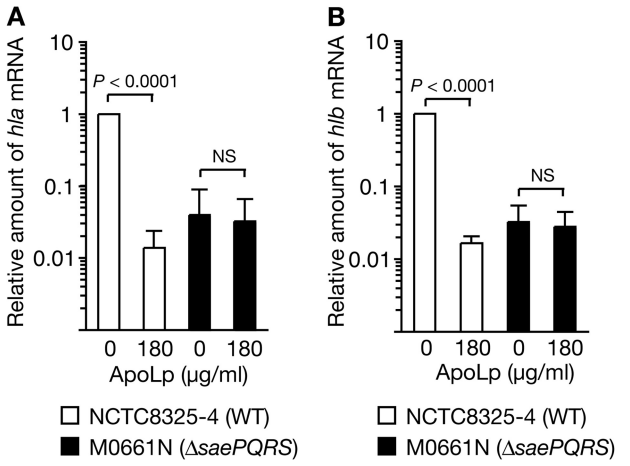
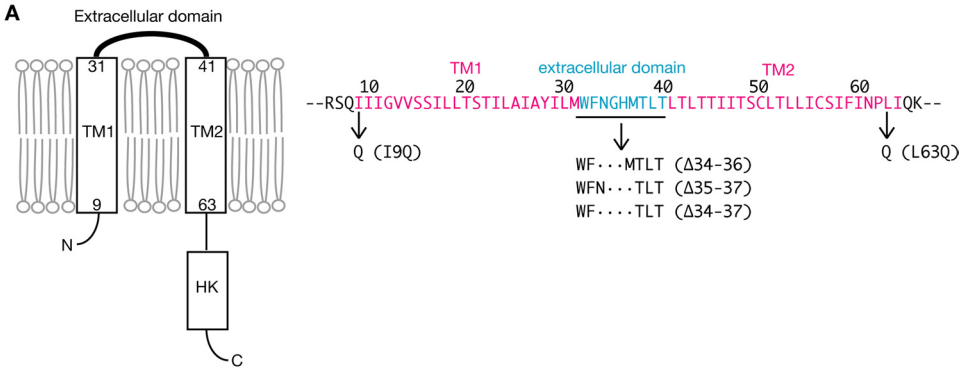


FIGURE 4. **The inhibitory effect of ApoLp against *S. aureus* hemolysin expression was diminished in the *saePQRS*-deleted mutant.** ApoLp (180 μg/ml) was added to *S. aureus* cultures (A_{600} of 0.1) of NCTC8325-4 (WT) and M0661N (ΔsaePQRS), and the mixture was cultured further for 4 h. Total RNA was extracted from the cultured cells. RNA amounts of *hla* (*A*) and *hlb* (*B*) were measured by quantitative RT-PCR. The *vertical axis* shows the relative value against the amount of RNA of NCTC8325-4 in the absence of ApoLp. Means ± S.D. of three independent experiments are shown. Student's *t* test *p* values are presented. *NS,* not significant.

which is a regulatory RNA on various *S. aureus* virulence genes. These findings indicate that IgG3 binding to LTA triggers signaling that leads to inhibition of the expression of hemolysin genes, *spa*, and *saeQ,* as observed for ApoLp, and that LTA acts as a receptor to transmit the signaling pathway that leads to down-regulation of the expression of virulence genes.

Inhibitory Effect of ApoLp on Hemolysin Expression Is Diminished in the saePQRS-deleted Mutant and the saeS Mutant with a Shortened Extracellular Domain—Based on our findings that ApoLp or IgG3 binding to LTA inhibits the expression of *saeQ* and hemolysin genes, together with the reports that the two-component SaeRS is required for the expression of hemolysin genes (10, 11), we hypothesized that ApoLp binding to LTA inactivates SaeRS and thus inhibits the expression of hemolysin genes. We examined whether *saePQRS* is required for the inhibitory effect of ApoLp on hemolysin genes. In a wild-type strain of *S. aureus*, as we reported previously, the addition of 180 μg/ml of ApoLp decreased the expression of *hla* and *hlb* (Fig. 4, *A* and *B*). In contrast, in the *saePQRS*-deleted mutant, the addition of 180 μg/ml of ApoLp did not decrease the expression of *hla* and *hlb* (Fig. 4, *A* and *B*). These findings indi-



B

	WT	I9Q	I9Q/L63Q	Δ34-36	Δ35-37	Δ34-37
TM1	9-31	10-32	10-32	12-34	9-31	12-34
TM2	41-63	39-61	39-61	39-61	35-37	38-60
Extracellular domain (Number of amino acids)	32-40 (9)	33-38 (6)	33-38 (6)	35-38 (4)	32-34 (3)	35-37 (3)

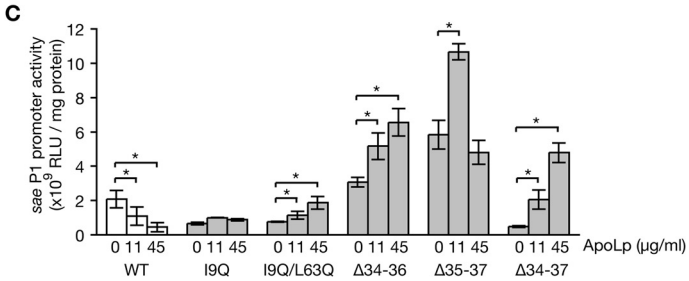


FIGURE 5. **The inhibitory effect of ApoLp against *S. aureus sae* P1 activity was abolished in *S. aureus* strains expressing mutated SaeS with a shortened extracellular domain.** *A*, schematic representation of SaeS. TM1 and TM2 are two-transmembrane domains. *HK*, histidine kinase domain. Amino acid sequences of TM1, TM2, and the extracellular domain are presented in the *right panel*. Amino acid substitutions and deletions in mutated SaeS proteins are shown. *B*, regions of TM1, TM2, and the extracellular domain in wild-type SaeS (WT) and mutated SaeS were predicted by the *in silico* program TMHMM (version 2.0). The number of extracellular amino acids is presented in parentheses. *C*, *S. aureus* strains expressing wild-type SaeS (WT) or mutated SaeS were transformed with a reporter plasmid carrying *sae* P1 and cultured in the presence of ApoLp. *S. aureus* cells were collected at A_{600} of 1.5, and luciferase activities of the cell lysates were measured. Means ± S.D. of three independent experiments are shown. The *asterisks* indicate Student's *t* test *p* value of <0.05.

cate that *saePQRS* is required for the inhibitory effect of ApoLp on the expression of hemolysin genes.

The requirement of LTA and *saePQRS* for the inhibitory effect of ApoLp on hemolysin expression suggests that the sensor protein SaeS senses the binding of ApoLp to LTA. SaeS is a transmembrane protein with two transmembrane helixes and a short extracellular region of 9 amino acids (Fig. 5*A*) (12). We hypothesized that the short extracellular region contributes to recognize ApoLp. To address this point, we constructed a deletion mutant of the extracellular domain or introduced an amino acid substitution in the transmembrane domain that theoretically shortens the extracellular domain. The lengths of the extracellular domains of the SaeS mutant proteins were predicted to be short by the *in silico* program TMHMM (version 2.0; Fig. 5*B*). The addition of ApoLp to an *S. aureus* strain expressing wild-type SaeS decreased *sae* P1 activity in a dose-dependent manner, whereas the addition of ApoLp to *S. aureus* strains expressing SaeS mutant proteins with a shortened extracellular domain did not decrease *sae* P1 activity (Fig. 5*C*). Rather, some mutants with a shortened extracellular domain increased *sae* P1 activity in the presence of ApoLp. These findings indicate that the extracellular domain of SaeS is required for the recognition of ApoLp binding to LTA and the subsequent down-regulation of *sae* P1 activity.

DISCUSSION

The findings of the present study revealed that ApoLp binds LTA. In addition, the inhibitory effects of ApoLp against the expression of hemolysin genes and *saeQ* were attenuated in LTA synthetase-deficient *S. aureus* mutants. Furthermore, anti-LTA monoclonal antibody decreased the expression of hemolysin genes and *saeQ* in a similar way as ApoLp. Thus, binding of ApoLp or antibody protein to LTA transmits a signal to decrease the expression of hemolysin genes and *saeQ*. This study first revealed that LTA, a non-protein bacterial cell surface component, acts as a receptor to recognize environmental signals.

This study revealed that the two-component system *saeRS* has an essential role in the inhibitory effect of ApoLp against hemolysin expression. Furthermore, the extracellular domain of the sensor protein of *saeRS* was required for the inhibitory effect of ApoLp against *sae* P1 activity. These findings suggest that the extracellular domain of SaeS recognizes ApoLp binding to LTA and transmits signals to decrease the expression of hemolysin genes. Because ApoLp is a large molecule of 294 kDa (35), the binding of ApoLp to LTA will occur in a milieu outside the peptidoglycan layer (Fig. 6). The conformational change of LTA by ApoLp binding might alter the lipid fluidity to transmit signals to SaeS or it might allow direct access to the extracellular region of SaeS (Fig. 6). Furthermore, this study demonstrated that LTA has a negative role in the *sae* P1 expression in the absence of ApoLp. This suggests that the presence of LTA is recognized by SaeS in the steady state. Additional studies are needed to elucidate how the extracellular domain of SaeS recognizes LTA and its binding to ApoLp.

Low pH and high salt conditions inactivate the P1 activity of *saePQRS* (17, 18). The inhibitory effects of low pH and high salt conditions against *sae* P1 activity were not abolished in the *ltaS* mutant (Fig. 2*F*). This finding suggests that LTA does not act as a receptor for SaeS to recognize low pH and high salt conditions and that there is an LTA-independent pathway to inactivate the SaeRS two-component system.

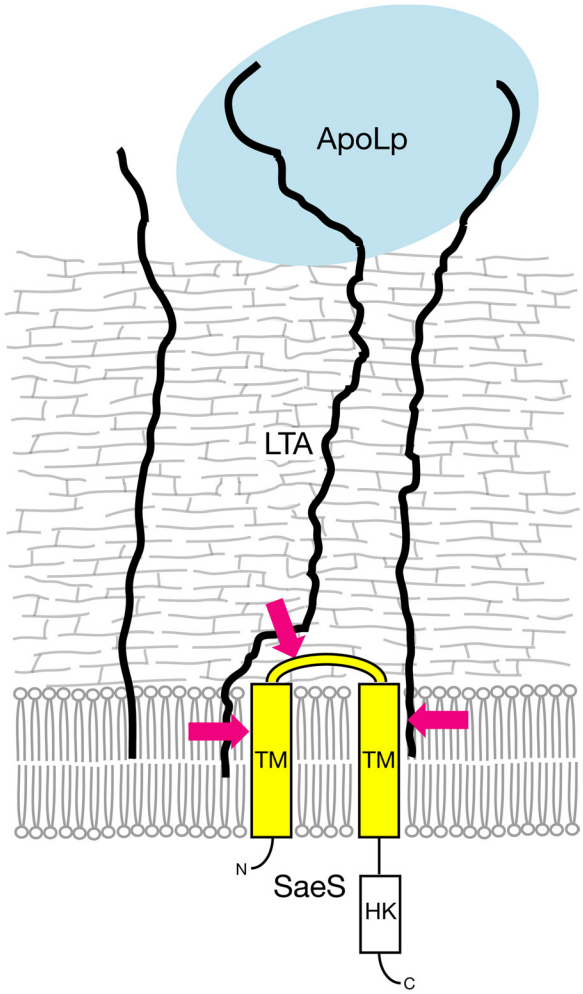


FIGURE 6. **Model of the inhibitory mechanism of ApoLp against SaeS through binding to LTA.** Binding of ApoLp to LTA inactivates the SaeS sensor kinase. The extracellular domain of SaeS is essential for recognizing the binding of ApoLp to LTA. The *magenta arrows* indicate possible pathways for the physical alteration of LTA that leads to inactivation of SaeS.

Teichoic acids have been thought to have an important role in *S. aureus* virulence by promoting *S. aureus* adherence to host cells or as a required component for *S. aureus* resistance against host antimicrobial peptides. The present study revealed a novel function of LTA to bind host proteins and transfer signals to repress the expression of *S. aureus* virulence genes. Thus, LTA acts as a receptor in bacteria to facilitate recognition of the host environment. The biologic meaning of the novel function of LTA in *S. aureus* infectious processes requires further study.

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