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# Regulatory Mechanism for Exfoliative Toxin Production in *Staphylococcus aureus*[∇]

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**The exfoliative toxin (ET) is a major virulence factor of *Staphylococcus aureus* that causes bullous impetigo and its disseminated form, staphylococcal scalded-skin syndrome (SSSS). ET selectively digests one of the intracellular adhesion molecules, desmoglein 1, of epidermal keratinocytes and causes blisters due to intraepidermal cell-cell dissociation. Most *S. aureus* strains that cause blistering disease produce either ETA or ETB. They are serologically distinct molecules, where ETA is encoded on a phage genome and ETB is enocded on a large plasmid. ETA-producing *S. aureus* strains are frequently isolated from impetigo patients, and ETB-producing *S. aureus* strains are isolated from SSSS. ET-induced blister formation can be reproduced with the neonatal mouse. To determine the regulatory mechanism of ET production, we investigated the role of the two-component systems and global regulators for *eta* or *etb* expression *in vitro* and *in vivo* with the mouse model. Western blot and transcription analyses using a series of mutants demonstrate ETA production was downregulated by *sigB*, *sarS*, and *sarA*, while ETB production was downregulated by *sigB* and *sarA* but not by *sarS*. Production of both toxins is upregulated by *saeRS*, *arlRS*, and *agrCA*. Furthermore, by the *in vivo* neonatal mouse model, *sigB* and *sarS* but not *sarA* negatively regulate the exfoliation activity of the ETA-producing strain, while *sarA* negatively regulates the ETB-producing strain. In both strains, *saeRS*, *arlRS*, and *agrCA* positively regulate the exfoliation activity *in vivo*. The data illustrate similar but distinct regulatory mechanisms for ETA and ETB production *in S. aureus in vitro* as well as *in vivo*.**

*Staphylococcus aureus* is a Gram-positive pathogen that causes a wide variety of diseases. It produces a large number of virulence determinants, including proteases, enterotoxins, cytolytic toxins, protein A, clumping factor, and others that may play important roles in establishing and maintaining infections with the bacterium.

Exfoliative toxin (ET) is one of these extracellular proteins and causes blisters in bullous impetigo and, in the disseminated form, staphylococcal scalded-skin syndrome (SSSS) (22). Neonates and young children are primarily affected. Recently, we and others demonstrated three isoforms of ETs (ETA, ETB, and ETD), which are glutamate-specific serine proteases that specifically cleave a single peptide bond in the extracellular region of human and mouse desmoglein 1 (Dsg 1), a desmosomal cadherin-type cell-cell adhesion molecule (1, 2, 40). The exfoliative activity can be assayed monitoring the elicitation of Nikolsky's sign when neonatal mice are injected with the toxin protein or *S. aureus* strains carrying the ET gene (*et*) (26, 39). Previous studies show the ETA gene (*eta*) is carried on the genome of a temperate phage integrated into the *S. aureus* chromosome (38), whereas the ETB gene (*etb*) is carried on a large plasmid, pETB (12, 39). *Staphylococcus aureus* strains carrying *eta* are frequently isolated from patients with bullous impetigo, whereas those carrying *etb* are obtained from patients with SSSS (41). The ETD (*etd*) gene is located on a pathogenicity island where *etd*-positive strains are primarily isolated from patients with deep pyoderma and not bullous impetigo or SSSS (40).

The production of staphylococcal virulence factors is coordinately modulated by the two-component regulatory systems (TCSs) (e.g., the accessory gene regulator [*agr*], *S. aureus* exoprotein expression [*saeRS*] gene, and autolysis-related locus [*arlRS*]) and a global regulator (e.g., the staphylococcal accessory regulator family [*sarA*, *sarS*, *rot*, and others] and the alternative sigma factor, sigma B [*sigB*]) (6, 28). The *agr* locus has two divergent transcripts, RNA II and RNA III. The RNA II transcript encodes four proteins (AgrB, -D, -C, and -A) that are related to generating a quorum-sensing molecule (AIP) and a two-component regulatory system. The RNA III transcript acts as the effector molecule for *agr*-specific regulation (29). The SaeRS system was identified as the positive transcriptional regulator of exoprotein independent of *agr* and SarA (14, 15, 42). Furthermore, *saeRS* was shown to be an important element for the expression of virulence genes *in vivo* (17, 31). The ArlRS system was first identified as a regulator involved in biofilm formation, autolysis, and extracellular proteolytic activity (11). In addition, ArlR positively regulates the accessory gene regulator (*agr*) (23). The *sarA* and *sarS* loci are recognized as transcription factors and mediate their effect both directly by binding to the target gene promoters and indirectly via the downstream effect on other regulons (7, 37). Sigma B is one of the three alternative σ factors ($\sigma^B$, $\sigma^H$, and $\sigma^S$ RNA polymerases) of *S. aureus* and has been characterized as a regulator in the general stress response (5, 18, 27, 35). Additionally, regulation of the virulence determinants is shown to be mediated either directly by a $\sigma^B$-dependent promoter or

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indirectly by additional global regulators, including SarA and SarS, which possess a $\sigma^B$-dependent promoter (3, 7, 10, 21, 37). Furthermore, Oscarsso et al. noted SigB may suppress *hla* transcription via mechanisms not involving SarA and SarS, suggesting another SigB-dependent factor(s) suppresses *hla* transcription (30).

Thus, the pathogenicity factors are shown to be modulated by complex regulatory mechanisms in *S. aureus*, where many other regulatory elements have been identified for other genes (4, 9); however, the regulatory mechanism for ET (*et*) production has not been extensively studied. Here we investigate the role of the transcription factors *sigB*, *sarS*, *sarA*, *saeRS*, *arlRS*, and *agrCA* involved in the expression of *eta* and *etb in vitro* and *in vivo* by using the neonatal mice model.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are described in Table 1. *Staphylococcus aureus* and *Escherichia coli* were grown at 37°C with shaking in Trypticase soy broth (TSB; Becton Dickinson Microbiology Systems, Cockeysville, MD) or Luria-Bertani broth (5 g yeast extract, 10 g polypeptone, 10 g NaCl per liter, pH 7.2), respectively. When necessary, xylose (1%), ampicillin (100 $\mu$g/ml), chloramphenicol (10 $\mu$g/ml), or tetracycline (3 $\mu$g/ml) was added to the medium. The antibiotics were purchased from Sigma Chemical Co., St. Louis, MO. Anti-ETA rabbit serum and anti-ETB serum were prepared as described previously (36, 40). Anti-Hla was a kind gift from T. Tomita (Tohoku University).

**DNA procedures.** Routine DNA procedures such as DNA digestion with restriction enzymes, DNA ligations, and gel electrophoresis were performed essentially as described previously (33). The oligonucleotides used in this study are described in Table 2. PCR was performed using the Ex *Taq* polymerase (TaKaRa Bio) with the appropriate cycling conditions.

**Isolation of RNA and quantitative RT-PCR analysis of mRNA.** Strains were grown for 6 h in 3 ml TSB medium supplemented with the appropriate antibiotics at 37°C with shaking and subcultured in 30 ml fresh TSB medium adjusted to an initial optical density at 660 nm ($OD_{660}$) of 0.02. Cultures were then incubated at 37°C with shaking and harvested by centrifugation 2, 3, 4, 5, and 6 h after inoculation. The cells were disrupted with the FastPrep instrument (Qbiogene, Carlsbad, CA) with glass beads. Total RNA was extracted with the FastRNA Pro Blue kit (Qbiogene) according to the manufacturer's protocol. After precipitation with ethanol, RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water, and the precipitates were washed in 70% ethanol and dried. The resultant RNA preparations were dissolved in DEPC-treated water. The RNA concentrations of the extracts were measured with a Nano Drop (Scrum, Inc.). Ten micrograms of total RNA was treated with reverse transcription (RT)-grade DNase (WaKo) for 30 min at 37°C to digest the remaining DNA, and cDNA synthesis was performed with a Transcriptor first strand cDNA synthesis kit (Roche) in a final volume of 20 $\mu$l. The resultant cDNA was diluted 5-fold with Tris-EDTA (TE) buffer and used as the template DNA for the subsequent quantitative PCR. Quantitative RT-PCR analysis was performed with the LightCycler instrument (Roche) using LightCycler FastStart DNA master SYBR green I (Roche) according to the instructions provided by the manufacturer. Gyrase B subunit mRNA (*gyrB*) was used as an internal standard. The oligonucleotides used in this study are listed in Table 2. The number of copies of each sample transcript was then quantified relative to the internal control, *gyrB*.

**Construction of the temperature-sensitive shuttle vector for gene manipulation of *S. aureus*.** The temperature-sensitive plasmid in *S. aureus*, pKFT, was constructed as follows: the tetracycline resistance gene (*tetL*) was amplified from pHY300PLK with primers TetLF and TetLR and inserted into the SspI site of pUC18. A fragment carrying the p194Ets replicon was amplified from pCL52.1 (34) with the primers TsoriF and TsoriR. The resultant PCR product was digested with AatII and cloned into the AatII site of the resulting plasmid to obtain pKFT.

The temperature-sensitive plasmid in *S. aureus* pKFC was constructed as follows. A fragment carrying the p194Ets replicon was amplified from pCL52.1 (34) with primers TsoriF and TsoriR. The resultant PCR product was digested with AatII and cloned into the AatII site of pUC18. The chloramphenicol-resistant gene (*cat*) cassette was amplified from pCL15 (24) with the primers CMF and CMR and inserted into the NdeI site of the resulting plasmid to obtain pKFC.

**Construction of mutant *S. aureus* strains. (i) Construction of the *sarA* deletion mutant.** A 2,171-bp DNA fragment containing the *sarA* locus was amplified from TY34 genome DNA with primers sarAF and sarAR. The resultant PCR product was digested with HindIII and subcloned into the same site in pUC19 to obtain pUC19sarA. A 654-bp EcoRV and Eco81I fragment in *sarA* was replaced by the *cat* gene cassette, and the resultant 2,332-bp HindIII fragment containing *sarA*::*cat* was transferred into the HindIII site of pKFT. The resulting plasmid, pFK3, was first transformed into *S. aureus* RN4220 (20), and then the modified plasmid was isolated and electroporated into strain TY34. Transformants were selected at 30°C on TSB plates containing chloramphenicol and tetracycline. A double-crossover disrupted mutant was generated by incubation at 42°C, a nonpermissive temperature for the replication of pKFT. The *sarA* mutant is shown to increase protease production (8); therefore, the mutant can be screened for high protease producers on TSB-containing chloramphenicol agar plates supplemented with 4% skim milk. The transformants were further selected as chloramphenicol-resistant and tetracycline-sensitive colonies. Deletion of the *sarA* gene was confirmed by PCR. The resultant *sarA* deletion mutant was designated FK128.

**(ii) Construction of *sigB*, *sarS*, and TCS mutants by using Campbell-type integration.** Disruption mutants were constructed using a Campbell-type integration as described previously (19). Briefly, DNA fragments containing internal regions of each open reading frame (ORF) were amplified and cloned into the pKFT or pKFC vector. The primers used in this study are described in Table 2. In this step, we cloned the preceding gene of the two-component system (TCS) operon to construct the mutant in which TCS was destroyed. The resulting plasmids were then electroporated into *S. aureus* RN4220, and then each plasmid was extracted and electroporated into TY34. Transformants were selected at 30°C on TSB plates containing tetracycline or chloramphenicol. Each mutant was generated at 42°C and selected as a tetracycline- or chloramphenicol-resistant colony. The disruption of the target genes was confirmed by PCR. In the same way, *sarA sarS* (FK129), *sarA saeR* (FK132), and *sarA agrC* (FK133) double mutants were constructed from the *sarA* deletion mutant strain (FK128) (Table 1). Furthermore, *sarS saeR* (FK134) and *sarS agrC* (FK135) double mutants were constructed from the *sarS* mutant strain (FK130) with the pKFT construct.

**(iii) Construction of the *etb* gene null mutant.** The *etb* gene is carried on a large plasmid, pETB (12, 39); therefore, we constructed the *etb* null mutant strain in TY825 through plasmid curing. *S. aureus* strain TY825 was grown in 10 ml TSB medium supplemented with 3 mg/ml ethidium bromide at 37°C with shaking and subcultured into 10 ml of the same fresh medium. The cultures were then plated on a TSB agar plate at 37°C. The pETB contains the *etb* gene and also a cadmium resistance gene; therefore, the pETB-cured strains were screened for cadmium-sensitive colonies and confirmed by PCR using *etb* primers. The resultant *etb* deletion mutant was designated FK200.

**(iv) Complementation of isogenic *sigB*, *sarA*, and *sarS* mutants.** To complement the *sigB* (FK131), *sarA* (FK128), and *sarS* (FK130) isogenic mutant strains, we introduced the wild-type *sigB*, *sarA*, or *sarS* gene into the xylose-inducible expression vector pWH1520 vector (32). The plasmid pWH1520 was purchased from MoBiTec (Göttingen, Germany). The *sigB*, *sarA*, and *sarS* genes were amplified by PCR with the following primers: sigBFS and sigBRB2, sarAFS and sarARB2, sarSFS and sarSRB2, respectively (Table 2). These amplified DNA fragments were cut with SpeI and BglII and inserted into the same sites of pWH1520. These resulting plasmids, pFK22, pFK23, and pFK24, containing the *sigB*, *sarA*, and *sarS* genes, respectively, were first transformed into *S. aureus* RN4220 and selected as a tetracycline-resistant colony, and then modified plasmids were isolated and electroporated into the *sigB* (FK131), *sarA* (FK128), and *sarS* (FK130) isogenic mutant strains, respectively.

**Western blot analysis.** Strains were grown with shaking at 37°C for 6 h in 3 ml TSB supplemented with the appropriate antibiotics and subcultured into 3 ml fresh TSB adjusted to an initial $OD_{660}$ of 0.02. The cultures were then incubated with shaking at 37°C for 15 h, and the culture supernatants were harvested by centrifugation. Equal aliquots from each supernatant sample were electrophoresed with a 12% polyacrylamide gel. SDS-PAGE and Western blotting were performed as described previously (36, 40). Immunodetection of protein was performed with the ECL (enhanced chemiluminescence) Western blot analysis system (Amersham Pharmacia). The intensity of each band was measured with NIH Image 1.59 (National Institutes of Health).

**ET bioassay.** ET activity was assayed with 2-day-old ICR newborn mice as described previously (40). *Staphylococcus aureus* was grown with shaking at 37°C for 6 h in TSB and washed twice with phosphate-buffered saline (PBS). The cells were suspended in PBS, and 100 $\mu$l containing $10^8$ CFU of *S. aureus* was subcutaneously injected into the back of 2-day-old ICR neonatal mice (5/group). At intervals, the appearance of Nikolsky's sign (peeling of the skin upon slight rubbing) was monitored. These experiments were independently performed

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype or characteristic(s) | Source or reference |
|---|---|---|
| Strains | | |
| *S. aureus* | | |
| RN4220 | NCTC8325-4 r$^-$ m$^+$ | Kreiswirth et al. (20) |
| TY34 | Clinical isolate (*eta*$^+$ *agr* type III *mecA*$^+$) | |
| TY825 | Clinical isolate (*etb*$^+$ *agr* type IV) | |
| TF5367 | TY34 Δ*eta*::*cat* | |
| FK101 | TY34 TCS2 *MW0199*::pFK5 | This work |
| FK102 | TY34 *lytSR MW0236*::pFK6 | This work |
| FK103 | TY34 *graRS MW0621*::pFK7 | This work |
| FK104 | TY34 *saeRS MW0668*::pFK8 | This work |
| FK105 | TY34 *TCS6 MW1208*::pFK9 | This work |
| FK106 | TY34 *arlRS MW1305*::pFK10 | This work |
| FK107 | TY34 *srrAB MW1446*::pFK11 | This work |
| FK108 | TY34 *phoPR MW1637*::pFK12 | This work |
| FK109 | TY34 *yhcSR MW1790*::pFK13 | This work |
| FK110 | TY34 *vraSR MW1825*::pFK14 | This work |
| FK111 | TY34 *agrCA MW1962*::pFK15 | This work |
| FK112 | TY34 *kdpDE MW2002*::pFK16 | This work |
| FK113 | TY34 *hssRS MW2282*::pFK17 | This work |
| FK114 | TY34 *nreCB MW2314*::pFK18 | This work |
| FK115 | TY34 *TCS16 MW2545*::pFK19 | This work |
| FK128 | TY34 Δ*sarA*::*cat* | This work |
| FK129 | TY34 *sarA*::*cat sarS*::pFK2 | This work |
| FK130 | TY34 *sarS*::pFK21 | This work |
| FK131 | TY34 *sigB*::pFK20 | This work |
| FK132 | TY34 *sarA*::*cat saeRS MW0668*::pFK8 | This work |
| FK133 | TY34 *sarA*::*cat agrCA MW1962*::pFK15 | This work |
| FK134 | TY34 *sarS*::pFK21 *saeRS MW0668*::pFK8 | This work |
| FK135 | TY34 *sarS*::pFK21 *agrCA MW1962*::pFK15 | This work |
| FK136 | TY34 *sigB*::pFK20 complemented with pFK22 | This work |
| FK137 | TY34 Δ*sarA*::*cat* complemented with pFK23 | This work |
| FK138 | TY34 *sarS*::pFK21 complemented with pFK24 | This work |
| FK200 | TY825 *etb* mutant pETB cured | This work |
| FK204 | TY825 *saeRS MW0668*::pFK8 | This work |
| FK206 | TY825 *arlRS MW1305*::pFK10 | This work |
| FK211 | TY825 *agrCA MW1962*::pFK15 | This work |
| FK216 | TY825 *sigB*::pFK1 | This work |
| FK217 | TY825 *sarS*::pFK2 | This work |
| FK218 | TY825 Δ*sarA*::*cat* | This work |
| *E. coli* | | |
| DH5α | F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*($r_K^-$ $m_K^+$) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1* | TaKaRa |
| Plasmids | | |
| pUC19 | *E. coli* cloning vector | TaKaRa |
| pHY300PLK | Shuttle vector between *E. coli* and *S. aureus* | TaKaRa |
| pWH1520 | 7.9-kbp xylose-inducible vector; $P_{xylA'}$ *xylR* Amp$^r$ in *E. coli*, Tet$^r$ in *S. aureus* | Rygus and Hillen (32) |
| pCL52.1 | 8.0-kbp temp-sensitive shuttle vector; Spc$^r$ in *E. coli*, Tet$^r$ in *S. aureus* | Sau et al. (34) |
| pCL15 | 7.0-kbp IPTG-inducible vector; $P_{spac}$, Amp$^r$ in *E. coli*, Cm$^r$ in *S. aureus* | Luong and Lee (24) |
| pKFT | 5.7-kbp temp-sensitive shuttle vector; Amp$^r$ Tet$^r$ in *E. coli*, Tet$^r$ in *S aureus* | This work |
| pKFC | 5.1-kbp temp-sensitive shuttle vector; Amp$^r$ in *E. coli*, Cm$^r$ in *S aureus* | This work |
| pFK2 | pKFT containing 554-bp fragment of *sarS* | This work |
| pFK3 | pKFT containing *sarA*::*cat* for deletion of the *sarA* gene | This work |
| pFK4 | pKFT containing 547-bp fragment of *MW0018*[a] | This work |
| pFK5 | pKFT containing 658-bp fragment of *MW0199*[a] | This work |
| pFK6 | pKFT containing 775-bp fragment of *MW0236*[a] | This work |
| pFK7 | pKFT containing 640-bp fragment of *MW0621*[a] | This work |
| pFK8 | pKFT containing 545-bp fragment of *MW0668*[a] | This work |
| pFK9 | pKFT containing 486-bp fragment of *MW1208*[a] | This work |
| pFK10 | pKFT containing 517-bp fragment of *MW1305*[a] | This work |
| pFK11 | pKFT containing 552-bp fragment of *MW1446*[a] | This work |
| pFK12 | pKFT containing 533-bp fragment of *MW1637*[a] | This work |
| pFK13 | pKFT containing 456-bp fragment of *MW1790*[a] | This work |
| pFK14 | pKFT containing 510-bp fragment of *MW1825*[a] | This work |
| pFK15 | pKFT containing 543-bp fragment of *MW1962*[a] | This work |
| pFK16 | pKFT containing 595-bp fragment of *MW2002*[a] | This work |
| pFK17 | pKFT containing 534-bp fragment of *MW2282*[a] | This work |
| pFK18 | pKFT containing 472-bp fragment of *MW2314*[a] | This work |
| pFK19 | pKFT containing 529-bp fragment of *MW2545*[a] | This work |
| pFK20 | pKFC containing 503-bp fragment of *sigB* | This work |
| pFK21 | pKFC containing 554-bp fragment of *sarS* | This work |
| pFK22 | pWH1520 containing *sigB* gene | This work |
| pFK23 | pWH1520 containing *sarA* gene | This work |
| pFK24 | pWH1520 containing *sarS* gene | This work |

[a] Locus numbers are based on *S. aureus* strain MW2 (http://www.bio.nite.go.jp). Gene names are based on *S. aureus* MW2 and according to the first reference where the TCS is described in the text.

TABLE 2. Oligonucleotide primers used in this study

| Primer | Sequence (5′ to 3′)[a] |
|---|---|
| Standard sequencing | |
| TsoriF | TACGAT**GACGTC**TTTTGCGCAGTCGGC |
| TsoriR | ATA**GACGTC**GTGAGAAACAGCGTACAG |
| TetLF | TTATTGCAATGTG**GAATT**CGGAACGG |
| TetLR | CCGG**GAATT**CCTGTTATAAAAAAAGG |
| CMF | TT**CATATG**CCGGCAATAGTTACCCTT |
| CMR | TT**CATATG**GATCTGGAGCTGTAATAT |
| MW0018F | AAAAGAAGGATACGATGTGTACTGTGC |
| MW0018R | GTAAACGACGAATCGTTACATCGACCG |
| MW0199F | GTAAAATTAAACCATGCTGACCATCGTCTC |
| MW0199R | ATAGCATTTTCTATGAGTGGCTGAAGC |
| MW0236F | GGCCCTTTTGTAGGTCTATTTGTTGGCG |
| MW0236R | CCAATCCTTCTGCAAGTTGACGTTCCAC |
| MW0621F | GACAATACTTTGTTTCAAGAATTGAAA |
| MW0621R | GGTAATAAGAATTTCTAATATAATCATTT |
| MW0668F | TTTGTCAAACCTATTTTGAATATGAAGG |
| MW0668R | GTATATGGACATTCACGGTATTAGCATC |
| MW1208F | TTTCTTTTATAGTGCTTTTGCCGTTCC |
| MW1208R | TATACTATCAATCTCTTCAATAAATGATGG |
| MW1305F | AGC**AAGCTT**TCTTGAATTGGAACTCAC |
| MW1305R | TTGTTT**AAGCTT**CACTATTATAACCCC |
| MW1446F | CCATGAAGCAAGTAATGGCCAAGAGGC |
| MW1446R | CACACGATTTAACTTTTCTCTAAGTCG |
| MW1637F | T**GGATCC**AGTAGATGACGAACATTCAA |
| MW1637R | T**AAGCTT**ATGCTCTCGTAATGACTC |
| MW1790F | GCGTCGAATATCGTCAATTGTTCTCGTA |
| MW1790R | GTCCAACTGGCTTAAGTTGCC |
| MW1825F | GTGAAACGTTAGATTTATACCATACACTCG |
| MW1825R | TTTGTACCGTTTGAATGACGC |
| MW1962F | CACCCTTAAAGAGATGAAATACAAACG |
| MW1962R | TTCTTGGAACAATTCATGAATGCGTGG |
| MW2002F | GAACAAATCACCCTATCGTCCTAAAGGC |
| MW2002R | CCCAGCTTAATGCTTGTTCATGCTGTTG |
| MW2282F | ATTGATGCATACACACAACCAAGTGG |
| MW2282R | ATTTTTTTAATCTTTGGCGTAGTCGC |
| MW2314F | ACATATCAGTTGATTGATCAAGACAGGGG |
| MW2314R | GTACTACTCGATAAACAACCG |
| MW2545F | AAAATGGAATTACGGTGTTATTGTCG |
| MW2545R | AGGCGCGTCATGTTAACAGCTAATGTG |
| sigBF | CAAG**AAGCTT**AGAATACAGATGCACAGG |
| sigBR | GTTA**AAGCTT**AATGGTCATCTTGTTGCC |
| sigBFS | TATG**ACTAGT**TATATAAAAAAAGAGCAG |
| sigBRB2 | AATAA**AGATCT**TCTATTGATGTGCTGC |
| sarAF | ATTTGGGTAGT**AAGCTT**TGACACAAC |
| sarAR | GGATTTG**AAGCTT**TGCAACATCAACTAGC |
| sarAFS | ATCGA**ACTAGT**TGCATCAAATAGGGAG |
| sarARB2 | TCAT**AGATCT**CCAAATTGCGCTAAAC |
| sarSF | TATAATCATTGAAGCATATATGTTTCG |
| sarSR | TTATTGAGAGCTCTAACAGTTTGAGGG |
| sarSFS | ATTAAAA**ACTAGT**GCATATACAAGGAG |
| sarSRB2 | CACTTT**AGATCT**CAGCACACTTGCGT |
| Quantitative RT-PCR | |
| gyrBF | AGGTCTTGGAGAAATGAATG |
| gyrBR | CAAATGTTTGGTCCGCTT |
| etaF | TACAGTTCCGGGAAATTCT |
| etaR | CCCAATACCAACACCATAA |
| etbF | GTGGTAAAGGCGGACAACAT |
| etbR | TCAAATCGTTCCCCAAAGTG |
| saeRF | CTGTAAATGGTCACGAAGT |
| saeRR | GACATTCACGGTATTAGCA |
| agrAF | GCAGTGAAATTCGTAAGCAT |
| agrAR | CGAGTTCTTAATTCTGCTGGA |
| sarAF | CGTAATGAGCATGATGAAAG |
| sarAR | ATTTCGTTGTTTGCTTCAG |
| sarSF | CCACCATAAATACCCTCAAACT |
| sarSR | GTCTTGCTGCGCGTCAT |

[a] Sequence letters in boldface represent restriction sites.

twice, and the data are shown as the total value. All of the animal experiments were approved by the Committee of the Institute of Laboratory Animal Science in Hiroshima University (A08-28).

## RESULTS

**Effect of the two-component signal transduction systems on ETA production in TY34.** *Staphylococcus aureus* strain TY34 was isolated from a skin lesion of a patient with impetigo: this strain was *eta* positive, methicillin-resistant *S. aureus* (MRSA), and *agr* type III. To determine the regulation mechanism for ETA production, we constructed isogenic mutants of the two-component systems. *Staphylococcus aureus* encodes at least 16 two-component signal transduction systems (TCSs) (http://www.bio.nite.go.jp/dogan/project/view/MW2). We hypothesized any of the 16 TCSs identified may be the regulatory element for the ETs production. In particular, *saeRS*, *arlRS*, and *agrCA* were previously identified as global regulatory elements for a number of pathogenic factors in *S. aureus* (6, 28). In all TCSs, pairs of sensor genes and response regulator genes are arranged on the operon and are tandemly localized; therefore, we constructed gene disruption mutants of TY34 by using integration of the pKFT vector into the preceding gene in the TCS operon. The disrupted genes were *TCS2R*, *lytS*, *graR*, *saeR*, *TCS6S*, *arlR*, *srrA*, *phoP*, *yhcS*, *vraS*, *agrC*, *kdpD*, *hssR*, *nreC*, and *TCS16R*. The resulting isogenic mutants contained insertions in either the sensor or regulator gene, where the downstream gene in the TCS operon is not transcribed. In each mutant, inhibition of downstream gene transcription was confirmed by quantitative PCR. Insertion mutants were obtained for all of the TCSs with the exception of the *walK*/*walR* (*vicRK*) gene, which has been shown to be an essential gene for cell viability (25). The growth rates of the 15 TCS mutants were similar to that of TY34 (not shown). ETA production was not detectible in the *saeRS* mutant and greatly decreased in *arlRS* and *agrCA* mutants (reduced to 1/5 and 1/25 times that of the wild-type, TY34, respectively) (Fig. 1A and D). In the *graRS* mutant, the production of ETA was 1/2 of that of the wild type (Fig. 1A). The *nreCB* mutant showed a 1.4-fold increase in ETA production. The other 10 mutants did not show significant effects for ETA production.

**Effect of *sigB*, *sarA*, and *sarS* mutations on ETA production in TY34.** *Staphylococcus aureus* expression of various pathogenic factors is known to be coordinately controlled by global regulatory elements: e.g., *sigB*, *sarA*, and *sarS* (4, 9, 28). To study the role of these global regulators affecting ETA production, we constructed a series of *sigB*, *sarA*, and *sarS* single mutants in TY34. The mutant cells showed similar growth rates to the wild type (Fig. 2D). The intensity of the 27-kDa protein corresponding to ETA was the major product in the extracellular protein that greatly increased in the *sigB*, *sarA*, and *sarS* single mutants compared to that in the wild type (Fig. 1D). Furthermore, Western blot analysis demonstrated the amounts of ETA production in the *sigB*, *sarA*, and *sarS* single mutants were approximately 15, 12, and 9 times higher, respectively, than that in the wild-type strain, TY34 (Fig. 1B).

To complement the *sigB*, *sarA*, or *sarS* mutant strain, we cloned each gene under the control of an xylose-inducible promoter *xylA* in plasmid pWH1520 and transferred into a *sigB*, *sarA*, or *sarS* mutant strain. When the *sigB*, *sarA*, or *sarS* gene was induced by addition of xylose, ETA production greatly decreased in the *sigB*, *sarA*, or *sarS* mutant strain containing expression vector pFK22, pFK23, or pFK24, respectively, (Fig. 1C). These data further support the conclusion that SigB, SarA, and SarS negatively regulate ETA production.

The *sigB* mutant showed the highest increase in ETA production comparing these mutants ($P < 0.05$, *sigB* mutant versus *sarS* or *sarA* mutant). Since *sarA* and *sarS* possess a $\sigma^B$-dependent promoter in *S. aureus*, the negative effect of *sigB* may be

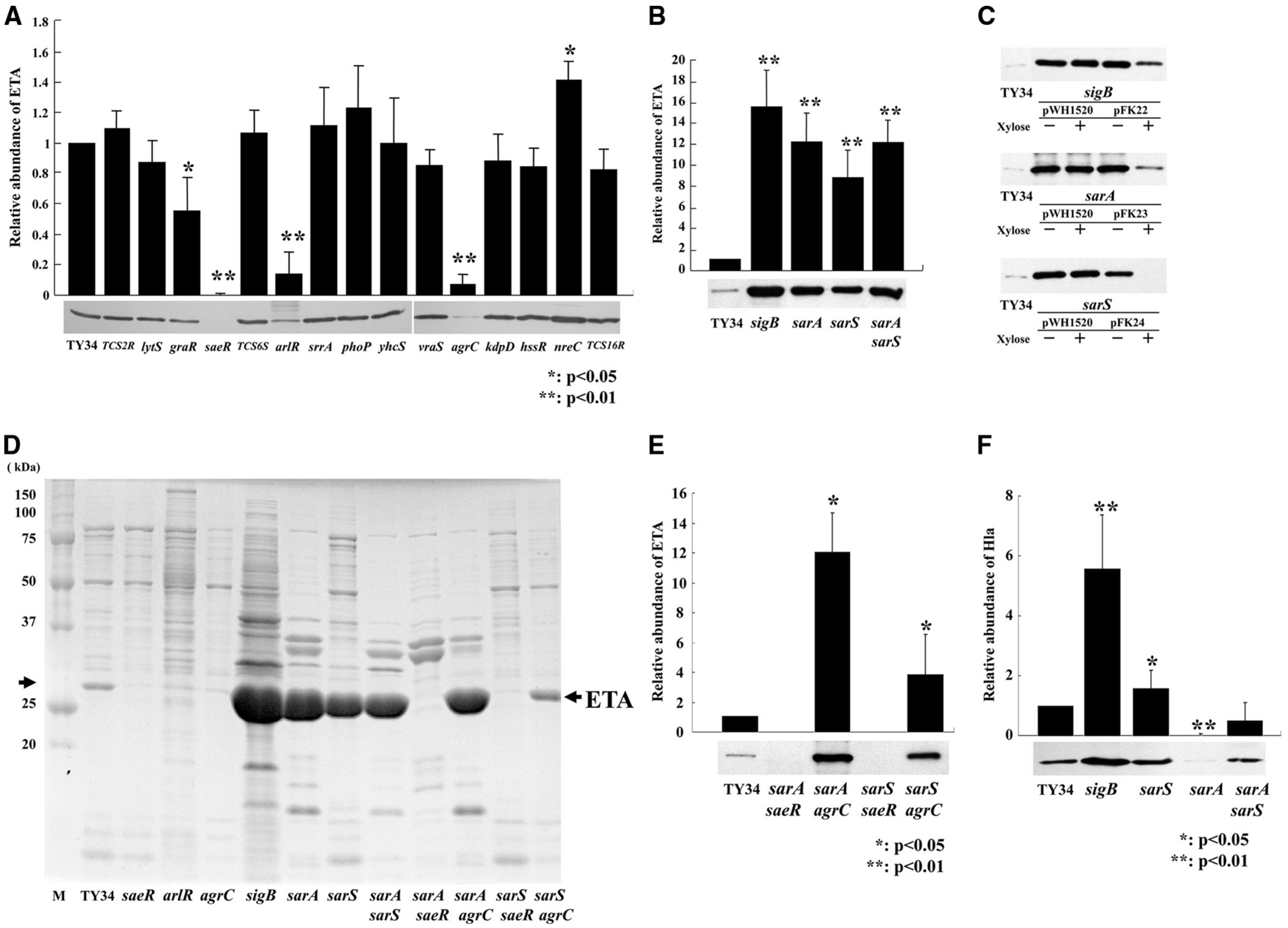

FIG. 1. Comparison of levels of ETA production in wild-type strain TY34 and its regulator mutants. (A) Western immunoblot analysis of ETA production in the wild-type strain, TY34, and its 15 isogenic TCS gene mutants. (B) Western immunoblot analysis of ETA production in the wild-type strain, TY34, and its *sigB* (FK131), *sarA* (FK128), *sarS* (FK130), and *sarA sarS* (FK129) isogenic mutants. (C) Western immunoblot analysis of ETA production in the wild-type strain, TY34, and the *sigB* (FK131) mutant containing pWH1520 or pFK22, the *sarA* (FK128) mutant containing pWH1520 or pFK23, and the *sarS* (FK130) mutant containing pWH1520 or pFK24. Strains were grown in the presence (+) or absence (−) of 1% xylose. (D) SDS-PAGE analyses of the wild-type strain TY34 extracellular proteins and those of its *saeR* (FK104), *arlR* (FK106), *agrC* (FK111), *sigB* (FK131), *sarA* (FK128), *sarS* (FK130), *sarA sarS* (FK129), *sarA saeR* (FK132), *sarA agrC* (FK133), *sarS saeR* (FK134), and *sarS agrC* (FK135) isogenic mutants. M, molecular mass markers. (E) Western immunoblot analysis of ETA production in the wild-type strain, TY34, and its *sarA saeR* (FK132), *sarA agrC* (FK133), *sarS saeR* (FK134), and *sarS agrC* (FK135) isogenic mutants. (F) Western blot analysis of Hla production of the wild-type strain, TY34, and the *sigB* (FK131), *sarA* (FK128), *sarS* (FK130), and *sarA sarS* (FK129) mutants. The cells were grown in TSB with shaking at 37°C for 15 h, and culture supernatants were harvested by centrifugation. The concentration of ETA or Hla was detected by Western immunoblotting with antiserum as described in Materials and Methods. The intensity of each band was measured with the program NIH Image and quantified relative to that of wild-type strain TY34. The data are means from four independent experiments. Error bars denote standard deviations.

partially attributed to inactivation of *sarS* and *sarA* (7, 37). To determine the relationship between these transcription factors, we constructed a *sarA sarS* double mutant and compared its ETA production to the single mutant. The amount of ETA produced by the *sarA sarS* double mutant was almost equal to that of the *sarA* single mutant, suggesting this is not a simple dual-regulation system through two transcriptional regulators, SarA and SarS (Fig. 1B and D).

**The relationship between the positive regulators *agr* and *saeR* and the negative regulators *sarA* and *sarS*.** To evaluate the relationship between positive regulators *agr* and *saeR* and negative regulators *sarA* and *sarS*, we further constructed *saeR sarA*, *saeR sarS*, *agr sarA*, and *agr sarS* double mutants and analyzed the amount of ETA production. ETA production was not detectible in the *saeR sarA* and *saeR sarS* double mutants, as well as the *saeR* single mutant (Fig. 1D and E). Surprisingly, *agr sarA* and *agr sarS* mutants showed the highest increase in ETA production compared with the *agr* mutant. In particular, ETA production in the *agr sarA* mutant was similar level to the *sarA* single mutants (Fig. 1D and E). Thus, the introduction of *sarA* or *sarS* mutation into an *agr* mutant restored the ability to produce ETA.

**Expression of the *eta* gene in TY34 and its isogenic *saeRS*, *arlRS*, *agrCA*, *sigB*, *sarA*, and *sarS* mutants.** To further verify the data from our Western blot experiments, we examined the transcription level of the *eta* gene by using LightCycler RT-

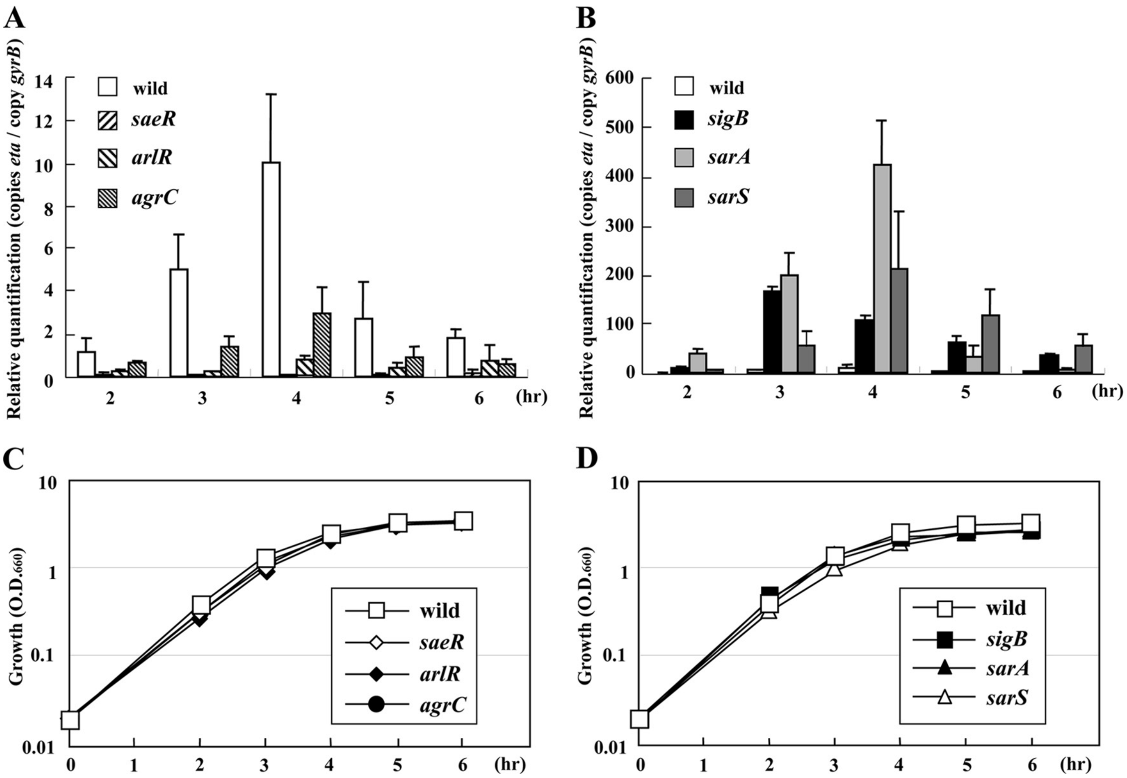

FIG. 2. Quantitative transcript analysis of *eta* gene products from TY34 and its isogenic mutants compared to their growth curves. *Staphylococcus aureus* TY34 and its mutants were grown in TSB with shaking at 37°C. The cell growth was monitored by measuring the turbidity at 660 nm (C and D). The total RNA was extracted from the cultures at the time points shown. The expression level was measured by LightCycler RT-PCR as described in Materials and Methods and quantified relative to the internal control *gyrB* (A and B). The data presented are mean values from four independent RNA isolations. Error bars denote standard deviations. (A and C) Wild-type TY34 and the *saeR* (FK104), *arlR* (FK106), and *agrC* (FK111) mutants; (B and D) wild-type TY34 and the *sigB* (FK131), *sarA* (FK128), and *sarS* (FK130) mutants.

PCR. Total RNA was extracted from the culture at several growth intervals after inoculation for 2, 3, 4, 5, and 6 h. The wild-type expression of the *eta* gene was elevated in the exponential phase (up to 4 h) and decreased during the early stationary phase (Fig. 2A). The *eta* expression was diminished in the *saeR*, *arlR*, or *agrC* mutants at all time points and significantly increased in the *sigB*, *sarA*, or *sarS* mutants (Fig. 2A and B). Using the *sigB* mutant, the expression level of *eta* reached a maximum at 3 h. This was 1 h earlier than the peak expression in the wild type or *sarA* or *sarS* mutant. Changes in *eta* expression were consistent with the Western blot data for ETA production (data not shown). These data suggest ETA production was regulated by these regulators at the transcriptional level.

**The effect of *saeRS*, *arlRS*, *agrCA*, *sigB*, *sarA*, and *sarS* mutations on ETB production in TY825.** There are at least three serologically distinct exfoliative toxins (ETs), and ETB is one of the three major isoforms of ETs encoded on plasmid pETB (12, 39). To determine the regulatory mechanism for ETB production, we constructed a series of TCSs, *sigB*, *sarS*, and *sarA* mutants, in TY825. *Staphylococcus aureus* TY825 was isolated from a patient with impetigo. This strain is *etb* positive, methicillin-susceptible *S. aureus*, and *agr* type IV. Among TCS mutants, changes in the production of ETB were found in the *saeR*, *arlR*, and *agrC* mutants (Fig. 3A and B). The other 12 two-component system gene disruption mutants did not show any significant effect on ETB production (data not shown). The amount of ETB production decreased in the *saeR*, *arlR*, or *agrC* mutant at 1/100-, 1/5-, and 1/6-fold, respectively, compared to that in the wild type. We also investigated ETB production in the *sigB*, *sarA*, or *sarS* mutants of TY825. The 27-kDa protein corresponding to ETB is one of the major extracellular protein products that greatly increased in the *sigB* and *sarA* single mutants compared to the wild type (Fig. 3D). Western blot analysis demonstrated the levels of production of ETB in the *sigB* and *sarA* mutants were 20 times and 16 times, respectively, that of the wild type (Fig. 3C). Of note, unlike with ETA, the mutation in the *sarS* gene had little effect on ETB production (1.9 times that of the wild type) (Fig. 3C). We analyzed at the transcriptional level for the *etb* gene and found the expression of the *etb* gene was consistent with the Western blot data (Fig. 4A and B).

**The exfoliative activity of TY34 and TY825 and their derivative mutant strains using the *in vivo* neonatal mouse model.** To further assess the effect of *saeRS*, *arlRS*, *agrCA*, *sigB*, *sarS*, and *sarA* on ETA or ETB production in a clinical setting, we used the neonatal mouse model. In *S. aureus*, the exfoliative toxin is the only toxin causing exfoliation of neonatal mouse skin. We first assessed the exfoliative activity of purified ETA and ETB by subcutaneous injection of the toxin at various concentrations into neonatal mice (Fig. 5). For purified ETA, concentrations of toxin over 1.4 μg caused exfoliation in all

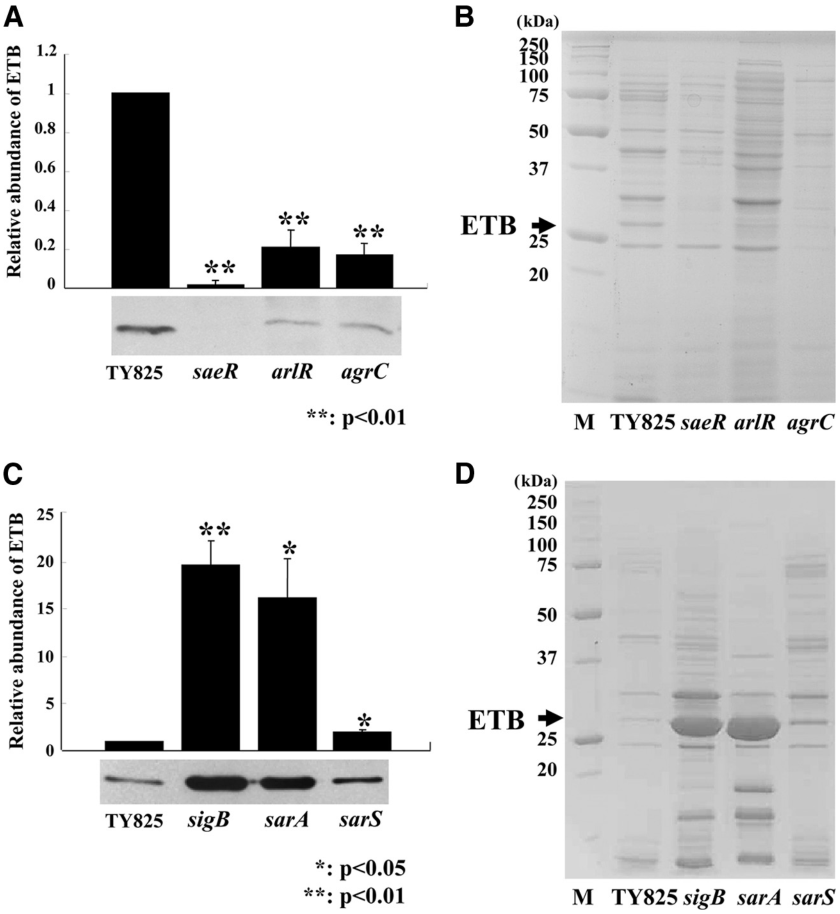

FIG. 3. ETB production in TY825 and in its regulator mutants. (A and B) Western blot (A) and SDS-PAGE (B) analyses of ETB production in wild-type strain and its *saeR* (FK204), *arlR* (FK206), and *agrC* (FK211) isogenic mutants. M, molecular mass markers. (C and D) Western blot (C) and SDS-PAGE (D) analyses of ETB production in the wild-type strain and its *sigB* (FK216), *sarA* (FK218), and *sarS* (FK217) isogenic mutants. Cells were grown in TSB with shaking at 37°C for 15 h, and culture supernatants were harvested by centrifugation. The concentration of ETB was detected by Western immunoblotting with anti-ETB antiserum, as described in Materials and Methods. The intensity of each band was measured by NIH Image and quantified relative to that of the parental strain, TY825. The data presented are mean values from three independent experiments. Error bars denote standard deviations.

tested mice, and the onset of exfoliation was dose dependent (Fig. 5A). It took at least 3 h postinjection for the mice to show exfoliation even at the highest dose (70 μg). In contrast, purified ETB showed significantly lower exfoliation activity (Fig. 5B). At least 23 μg of toxin was necessary to cause exfoliation of neonatal mice skin.

We administered approximately $10^8$ CFU of *S. aureus* TY34 or its derivative mutants by subcutaneous injection into the skin of the back of neonatal mice and monitored exfoliation of the epidermis. All tested mice did not die during the experiment. Using the wild-type strain, TY34, the mice started to show exfoliation 5 h after injection, and all mice were positive after 7 h (Fig. 6A to C). Whereas, mice injected with the *eta* null mutant of TY34 did not show any exfoliation until 18 h postinjection (Fig. 6A). As expected from the *in vitro* experiments, exfoliation started 1 h earlier in mice injected with the *sigB* or *sarS* mutant compared to the wild type (Fig. 6A). Unexpectedly, the timing of exfoliation in mice injected with the *sarA* mutant was identical to that of the wild type, although ETA production in the *sarA* mutant was greatly increased *in vitro*. In mice injected with the *saeR*, *arlR*, or *agrC* mutant, exfoliation of the epidermis was markedly delayed, in good agreement with attenuated ETA production seen in the *in vitro* experiments (Fig. 6B). In particular, mice injected with the *saeR* mutant did not show any exfoliation during the test period. Similarly, mice injected with the *sarA saeR* or *sarS saeR* double mutant also did not show any exfoliation until 18 h. Whereas, mice injected with the *sarA agrC* or *sarS agrC* double mutant were similar to the wild type (Fig. 6C). We concluded ETA production was upregulated by *saeRS*, *arlRS*, and *agrCA* and was downregulated by *sigB* and *sarS* by the *in vivo* mouse model.

We further investigated the exfoliation activity of ETB-producing *S. aureus*. Injection of ETB-producing *S. aureus* TY825 did not induce exfoliation of the skin but caused death. The mice started to die 7 h after inoculation. The pETB-cured *etb* null mutant of TY825 also showed a similar symptom (Table 3). Mice injected with the *sigB* or *sarS* mutant started to die earlier, with symptoms of exfoliation of the skin at 5 to 6 h (Table 3). Of note, the mice injected with the *sarA* mutant of

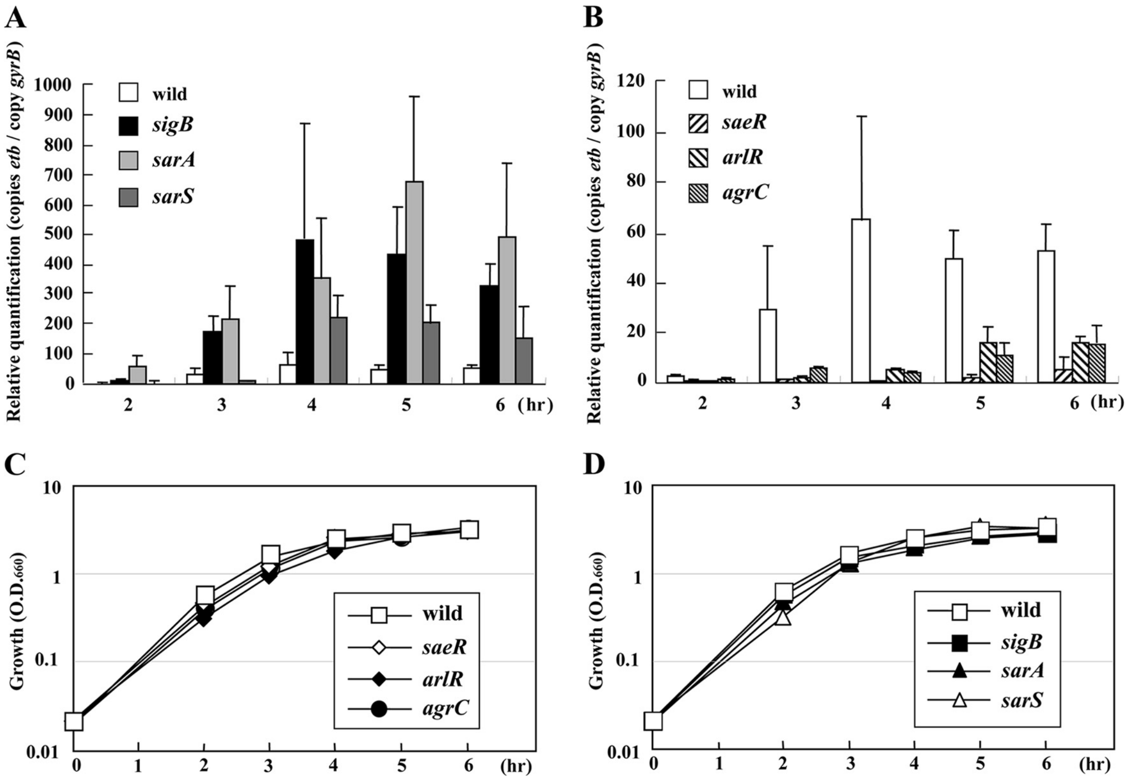

FIG. 4. Quantitative transcript analysis from the *etb* gene of TY825 and its isogenic mutants compared to their growth curves. Cells were grown in TSB medium with shaking at 37°C. Cell growth was monitored by measuring turbidity at 660 nm (C and D). Total RNA was extracted from the cultures at the time points shown. The expression level was measured by LightCycler RT-PCR as described in Materials and Methods and quantified relative to that of the internal control gene *gyrB* (A and B). The data presented are mean values from four independent RNA isolations. Error bars denote standard deviations. (A and C) Wild-type strain TY825 and the *saeR* (FK204), *arlR* (FK206), and *agrC* (FK211) mutants; (B and D) wild-type strain TY825 and the *sigB* (FK216), *sarA* (FK218), and *sarS* (FK217) mutants.

TY825 did not die; furthermore, the mice showed exfoliation of the skin after 4 h. After 6 h, all mice showed exfoliation of the skin (Fig. 6D). Mice injected with the *saeR* or *agrC* mutant did not die, and those injected with the *saeR*, *arlR*, or *agrC* mutant did not show symptoms of exfoliation of the skin (Table 3).

## DISCUSSION

**ETA production was upregulated by the SaeRS, ArlRS, and Agr system.** TCSs sense stimuli and respond to environmental conditions; therefore, the TCSs may be central elements for regulation of ETA production. We demonstrated ETA production was upregulated by the SaeRS, ArlRS, and Agr system by Western blotting and quantitative RT-PCR (Fig. 1A and 2A). Transcription analysis further showed the *eta* transcription was at the maximum level during the exponential phase and diminished in the early stationary phase. This indicates some regulatory elements may be involved in the activation of *eta* transcription to function primarily in the exponential growth phase (2 to 4 h).

The *agr* locus is known to play a central role in the regulation of pathogenic factors (29). We demonstrated that *agr* upregulated *eta* expression (Fig. 1A and 2A). However, *eta* expression started to decline during the early stationary phase (5 to 6 h), even though *agrA* expression was increasing (not shown). Our data suggest the production of ETA requires the *agr* system; however, full expression of *agr* is not necessary for *eta* transcription.

**ETA production was strongly downregulated by SigB, SarA, and SarS.** We demonstrated ETA production was strongly downregulated by SigB, SarA, and SarS in TY34 (Fig. 1B and C and 2B). The *eta* transcript level of the *sigB* mutant was similar to that in the *sarA* or *sarS* mutants (Fig. 2B). Transcription analysis demonstrated the *eta* expression in the *sigB* mutant reached a maximum at 3 h, 1 h earlier than that in the wild type. At 3 h, the expression of *saeR* also reached a maximum where the *sigB* mutation increased the expression of *saeR* (not shown). In the *sigB* mutant, the expression of *sarS* was significantly attenuated at all times, whereas *sarA* expression was not affected until 3 h (not shown). Taken together, the data suggest the negative effect of SigB on *eta* expression was mediated by upregulation of *sarS* and downregulation of *saeRS* that was most apparent at 3 h. Conversely, the *sarS* mutation did not affect the transcription of any of the other regulators (e.g., *saeRS*, *agr*, and *sarA*) (data not shown). Additionally, the mutation in *sarS* did not cause a change in the exoprotein synthesis pattern except for ETA (Fig. 1D). The data suggest SarS directly regulates *eta* transcription.

Our data demonstrate the regulatory network for *eta* transcription by *sigB*, *sarA*, and *sarS* is very similar to *hla* expression

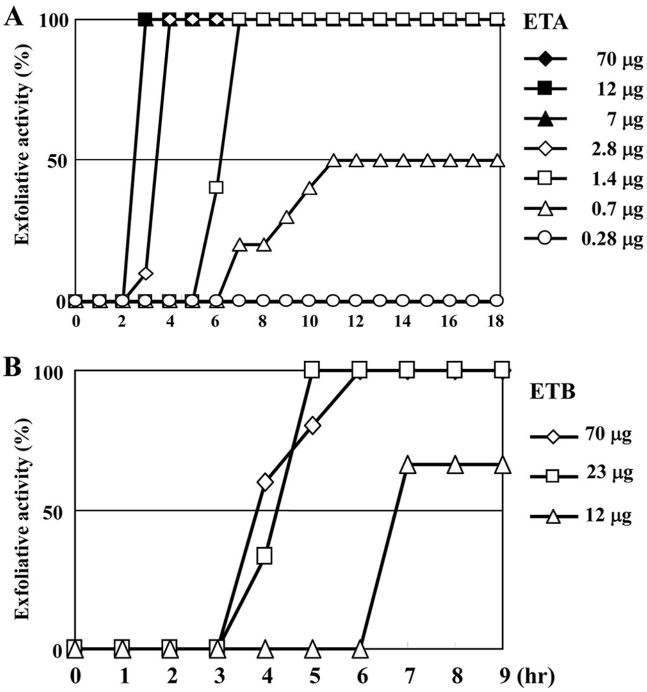

FIG. 5. Exfoliative activity with *in vivo* injection of *S. aureus* purified exfoliative toxin into neonatal mice. Two-day-old ICR neonatal mice (10 mice/group) were subcutaneously injected in the back with 100 µl PBS containing various concentrations of ETA (A) or ETB (B). At the intervals shown, the occurrence of Nikolsky's sign was monitored.

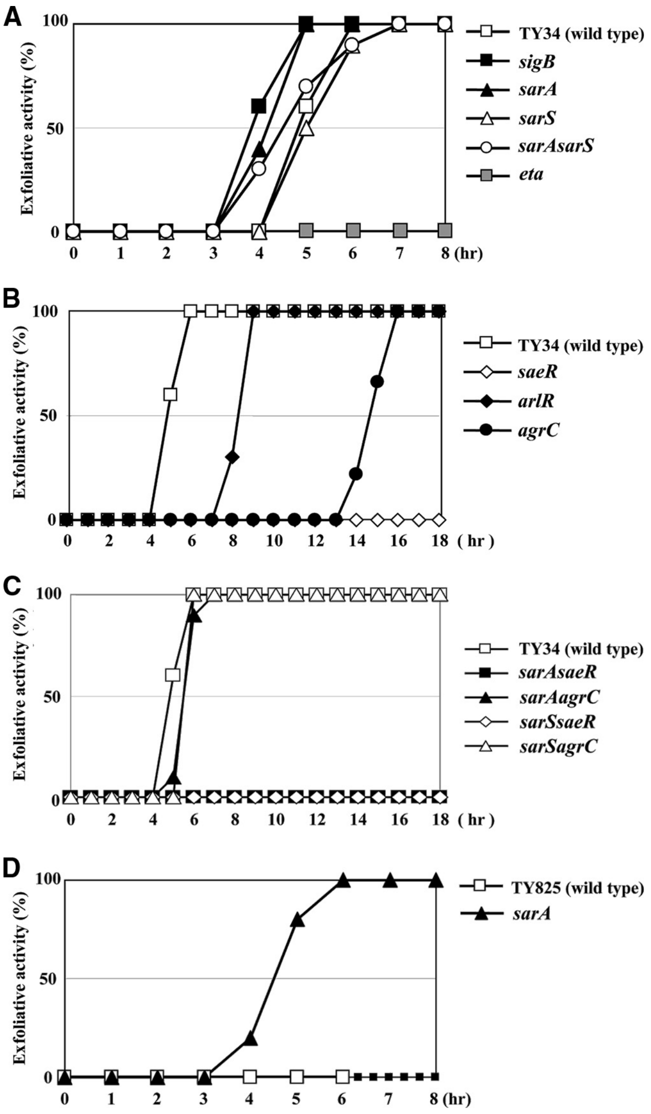

FIG. 6. Exfoliative activity *in vivo* after injection of *S. aureus* mutants into neonatal mice. Two-day-old ICR neonatal mice (10 mice/group) were subcutaneously injected in the back with 100 µl PBS containing $10^8$ CFU of *S. aureus* TY34 or the *saeR* (FK104), *arlR* (FK106), and *agrC* (FK111) isogenic mutants (A); the *eta* (TF5367), *sigB* (FK131), *sarA* (FK128), *sarS* (FK130), and *sarA sarS* (FK129) isogenic mutants (B); or the *sarA saeR* (FK132), *sarA agrC* (FK133), *sarS saeR* (FK134), and *sarS agrC* (FK135) isogenic mutants (C). Alternatively, mice were injected with 100 µl PBS containing $10^8$ CFU of *S. aureus* TY825 and its *sarA* (FK218) isogenic mutant (D). At the intervals shown, the occurrence of Nikolsky's sign was monitored.

(30). However, Western blot analysis demonstrated Hla production increased in the *sigB* and *sarS* mutants but is completely diminished in the *sarA* mutant (Fig. 1F). This suggests SarA controls the production of ETA and Hla through different mechanisms, and SigB, SarA, and SarS preferentially downregulate ETA production more strongly than other pathogenic factors such as Hla in TY34, although the *eta* gene is exogenously acquired on the genome of a temperate phage (38).

**Relationship between the positive regulators *agr* and *saeR* and the negative regulators *sarA* and *sarS*.** We further constructed these *saeR sarA*, *saeR sarS*, *agr sarA*, and *agr sarS* double mutants and analyzed the concentration of ETA produced. ETA production in the *saeR sarA* or *saeR sarS* double mutant was not detectible as well as that in the *saeR* mutant, although the *sarA* or *sarS* mutant produces a large amount of ETA (Fig. 1D and E). These data strongly suggest *saeRS* is an essential TCS for ETA production as a major positive regulator. Conversely, ETA production in the *agr sarA* or *agr sarS* mutant is very similar to that in the *sarA* or *sarS* mutant, respectively, suggesting *agr* may not effectively function in the *sarA* or *sarS* mutant (Fig. 1D and E).

***In vivo* neonatal mouse model.** We investigated the regulation mechanism of *eta* by using the *in vivo* mouse model and demonstrated the difference between the *in vitro* and *in vivo* environments. Using the *in vivo* exfoliation assay, ETA production was suggested to be upregulated by *saeR*, *arlR*, and *agrC* and downregulated by *sigB* and *sarS*. This is in good agreement with the *in vitro* experiments (Fig. 1). In the *in vivo* mouse model, particularly *saeR* and *-S* are necessary TCSs for the production of ETA (Fig. 6B and C). An unknown key signal may induce ETA production via sensing by using these TCSs *in vivo*. The *saeR*S mutant completely lost exfoliative activity even in the *sarA* or *sarS* mutation genetic background. Reports show the regulation by *saeRS* is important in several animal models: e.g., the murine model for hematogenous pyelonephritis (16, 17, 31), where the promoter of *saeRS* is activated by $H_2O_2$ and subinhibitory concentrations of α-defensins (13). However, it is not known what signaling molecule(s) is

TABLE 3. ET bioassay results

| Time (h) | No. of mice ($n = 5$/group) with result after injection with[a]: | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TY825 (wild type) | | *etb* mutant | | *saeR* mutant | | *arlR* mutant | | *agrC* mutant | | *sigB* mutant | | *sarA* mutant | | *sarS* mutant | |
| | Activity | Death | Activity | Death | Activity | Death | Activity | Death | Activity | Death | Activity | Death | Activity | Death | Activity | Death |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 5 | 0 | 0 | 2 |
| 7 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 5 | 0 | 1 | 5 |
| 8 | 0 | 3 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 3 | 5 | 0 | 1 | 5 |

[a] Aliquots of bacterial cell suspension ($10^8$ bacteria ml$^{-1}$) were administered subcutaneously to groups of 2-day-old ICR neonatal mice, and the occurrence of Nikolsky's sign (exfoliation activity) was monitored.

actually involved in the regulation of ETA production *in vivo*. Further investigations are needed to understand the regulatory mechanism of ETA production. Unexpectedly, the *sarA* mutant showed identical exfoliative activity to that of the wild-type strain TY34, although it produced a large amount of ETA in *in vitro* experiments. This suggests *sarA* in TY34 may not work *in vivo* or some additional factors produced in the *sarA* mutant may inhibit the exfoliative activity.

**The regulatory network for *etb in vitro* and *in vivo*.** We suggest the regulatory pathway for ETB production is similar to but distinct from that for ETA production. ETB production was upregulated by *saeRS*, *arlRS*, and *agr* and downregulated by SigB and SarA in TY825 *in vitro* (Fig. 3 and 4). Interestingly, the inactivation of *sarS* had little effect on ETB production, although *etb* expression increased in the *sarS* mutant (Fig. 3C and 4A). Thus, the negative effect of *sigB* on *etb* expression was predicted to be mediated primarily by *sarA* in TY825. We attempted to investigate the regulatory mechanism of *etb* by using the *in vivo* neonatal mouse model; however, administration of TY825 did not show exfoliation of the epidermis, possibly due to the low specific activity of ETB (Fig. 5B). Furthermore, the toxic effect of TY825 made it difficult to interpret the exfoliative activity of the mutant strains *in vivo*. The *sarA* mutant showed a significantly stronger exfoliative activity and didn't show a toxic effect (Fig. 6D and Table 3). Thus, in the *in vivo* environment, *sarA* appears to primarily downregulate ETB production. Conversely, in mice injected with the *saeR*, *arlR*, or *agrC* mutant, the ET did not cause death or exfoliation of the epidermis (Table 3). The data suggest *saeRS*, *arlRS*, and *agr* positively regulate ETB production, and the lethal factor(s) is also positively regulated by *saeRS*, *arlRS*, or *agr* and is not located on plasmid pETB.

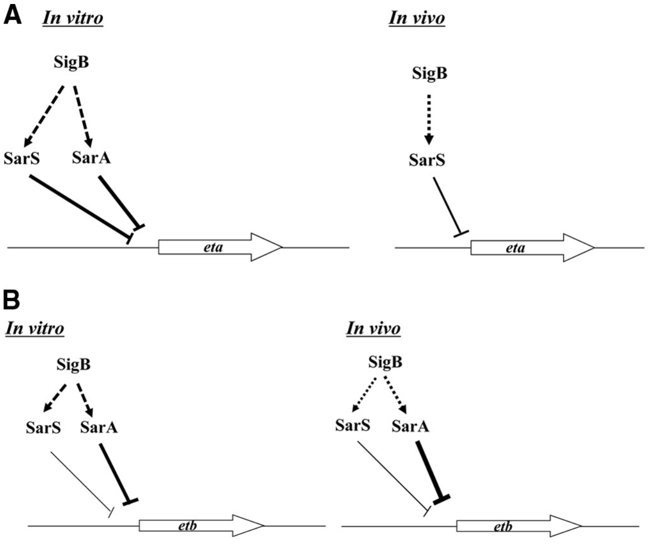

FIG. 7. Regulation model of SarA, SarS, and SigB for *eta* (A) and *etb* (B) expression *in vitro* and *in vivo*. Arrows indicate activation of gene expression. Repression is noted as bars. Dotted arrows and bars indicate presumed regulation.

In conclusion, we examined the regulation pathway of *eta* and *etb* both *in vitro* and *in vivo*, observing the regulators *saeRS*, *arlRS*, *agr*, *sigB*, *sarA*, and *sarS*. The expression of *eta* and *etb* was positively regulated by common regulators *saeR*, *arlR*, and *agrC*, both *in vitro* and *in vivo*. However, they are downregulated differently. The expression of *eta* is negatively regulated by *sarA* and *sarS*, whereas *etb* expression is negatively regulated by *sarA* and slightly negatively regulated by *sarS in vitro* (Fig. 7A and B). *In vivo*, *eta* is negatively regulated by *sarS*, while *etb* is negatively regulated by *sarA*. Whether or not this difference in the regulation mechanisms of these ET genes' expression may affect virulence of *S. aureus* in blistering diseases remains to be determined.

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