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RESEARCH ARTICLE

EssH Peptidoglycan Hydrolase Enables *Staphylococcus aureus* Type VII Secretion across the Bacterial Cell Wall Envelope

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ABSTRACT The ESAT-6-like secretion system (ESS) of *Staphylococcus aureus* is assembled in the bacterial membrane from core components that promote the secretion of WXG-like proteins (EsxA, EsxB, EsxC, and EsxD) and the EssD effector. Genes encoding the ESS secretion machinery components, effector, and WXG-like proteins are located in the *ess* locus. Here, we identify *essH*, a heretofore uncharacterized gene of the *ess* locus, whose product is secreted via an N-terminal signal peptide into the extracellular medium of staphylococcal cultures. EssH exhibits two peptidoglycan hydrolase activities, cleaving the pentaglycine cross bridge and the amide bond of *N*-acetylmuramyl-L-alanine, thereby separating glycan chains and wall peptides with cleaved cross bridges. Unlike other peptidoglycan hydrolases, EssH does not promote the lysis of staphylococci. EssH residues Cys^{199} and His^{254}, which are conserved in other CHAP domain enzymes, are required for peptidoglycan hydrolase activity and for *S. aureus* ESS secretion. These data suggest that EssH and its murein hydrolase activity are required for protein secretion by the ESS pathway.

IMPORTANCE Gene clusters encoding WXG-like proteins and FtsK/SpoIIIE-like P loop ATPases in *Firmicutes* encode type 7b secretion systems (T7bSS) for the transport of select protein substrates. The *Staphylococcus aureus* T7bSS assembles in the bacterial membrane and promotes the secretion of WXG-like proteins and effectors. The mechanisms whereby staphylococci extend the T7SS across the bacterial cell wall envelope are not known. Here, we show that staphylococci secrete EssH to cleave their peptidoglycan, thereby enabling T7bSS transport of proteins across the bacterial cell wall envelope.

KEYWORDS Esx, *Staphylococcus aureus*, cell wall hydrolase, secretion systems

Bacterial pathogens rely on the secretion of toxins and other effector proteins to implement their virulence strategies during host infection (1). In Gram-negative bacteria, precursors with N-terminal signal peptides are translocated into the periplasmic space, a compartment between the bacterial inner and outer membranes, but not into the extracellular milieu (2). Gram-negative bacteria evolved pathways for the secretion of select proteins across the bacterial envelope (types I, II, and V secretion), the bacterial envelope and the plasma membrane of host cells (type III secretion), or the bacterial envelope and phagosomal membranes for pathogens residing within host cells (type IV secretion) (3). In Gram-positive bacteria, the Sec pathway directs signal peptide-bearing precursors across the bacterial envelope into the extracellular milieu (4). Nonetheless, *Actinobacteria* and *Firmicutes* evolved specialized type VIIa and -b secretion systems (T7aSS and T7bSS) to implement specific virulence strategies (5). In mycobacteria, the T7aSS supports the persistent infection of mammalian hosts and a facultative intracellular lifestyle (6–8). T7aSS and T7bSS are defined by two types of genes. All T7SS clusters harbor a gene for the FtsK/SpoIIIE-like P loop ATPase, which fuels the translocation of select protein substrates across the bacterial plasma membrane (9, 10). Another hallmark of the T7SS is the genes coding for WXG-like proteins,

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small α-helical polypeptides that are secreted and assemble into homo- or heteropolymeric structures (11). WXG-like proteins represent mobile components that extend the T7SS pathway beyond the bacterial membrane and contribute to the secretion of effectors, thereby implementing the specific virulence strategies of various bacterial species (12–14).

Staphylococcus aureus is a commensal of the human nasopharynx and gastrointestinal tract (15–18). Colonization represents the key risk factor for invasive disease, most frequently skin and soft tissue infections but also skeletal, lung, and bloodstream infections, which occur in healthy and immunocompromised individuals (19, 20). When analyzed in a mouse model of bloodstream challenge, *S. aureus* establishes chronically persistent infections with abscess lesions and lethal outcomes (21). *S. aureus* mutants with a defective T7bSS, designated ESS (ESAT-6-like secretion system), exhibit diminished persistence, smaller and less abundant abscess lesions, and a reduced bacterial load in host tissues (12, 22). Earlier work characterized five secretion substrates of the *S. aureus* ESS pathway: EsxA and EsxB with the canonical WXG motif, their pairing partners EsxC and EsxD, and the EssD effector (12, 22–25). For simplicity, EsxA, EsxB, EsxC, and EsxD are referred as WXG-like proteins. A complex formed from EsaA, EssA, and EssB engages WXG-like proteins and the effector for translocation across the bacterial membrane with the help of EssC, the FtsK/SpoIIIE-like P loop ATPase (26). However, the mechanism whereby the ESS pathway secretes protein substrates across the bacterial cell wall has heretofore not been investigated.

The cell wall envelope of *S. aureus* is comprised of peptidoglycan, a macromolecule with attached wall teichoic acid, capsular polysaccharide, and polypeptides that protects staphylococci from osmotic lysis (27). Peptidoglycan is assembled by the polymerization of its biosynthetic precursor, lipid II [C_{55}-$(PO_4)_2$-MurNAc-(L-Ala-D-iGln-(NH_2-Gly_5)L-Lys-D-Ala-D-Ala)-GlcNAc], thereby generating glycan strands with the repeating disaccharide [→4)-β-MurNAc-(1→4)-β-GlcNAc-(1→]$_n$ (28, 29). Wall peptides that are attached to glycan strands are subsequently cross-linked via the cleavage of the amide bond of D-Ala-D-Ala and the generation of a new amide bond between the carboxyl group of D-Ala at position four and the amino group of pentaglycine cross bridges (NH_2-Gly_5) of neighboring peptidoglycan strands (30, 31). The high degree of peptidoglycan cross-linking and the large (40 to 50 nm) diameter of the staphylococcal cell wall represent a barrier for the secretion of proteins into the extracellular medium (32). To reveal the mechanisms whereby the *S. aureus* T7bSS translocates its substrates across the cell wall envelope, we studied a heretofore uncharacterized gene, *essH*, which encodes a murein hydrolase with cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain (33).

RESULTS

***essH* is associated with the staphylococcal *ess* locus.** Holden and colleagues analyzed *ess* loci in genome sequences from 153 clinical *S. aureus* isolates, revealing two conserved and two variable modules (34). Module 1, encompassing secretion machinery genes (*esxA-esaA-essA-esaB-essB*), and module 4, containing genes that code for two uncharacterized products, are found in the genomes of all isolates; modules 1 and 4 flank the *ess* locus, whose genes are encrypted in the plus strand of the *S. aureus* genome (Fig. 1A). Module 2 encompasses *essC* with conserved 5′- and variable 3′-coding sequences along with other variable genes that encode secretion substrates (*esxC*, *esxB*, *esxD*, and *essD* in *S. aureus* USA300 LAC) (Fig. 1A). Module 3 contains genes whose products encompass DUF600 domains (EssI) that associate with the EssD effector in the bacterial cytoplasm and block its nuclease activity (25). Genes encoding membrane proteins with unknown function are also found in module 3. Immediately adjacent to module 1 on the minus strand of the *S. aureus* genome is an uncharacterized gene (SAUSA300_0277; GenBank accession number ABD20657) that is here designated *essH* (Fig. 1A). The *essH* gene is associated with module 1 of *ess* loci in other *Staphylococcus* species, including *Staphylococcus argenteus* BN75, *Staphylococcus lugdunensis* HKU09-01, and *Staphylococcus epidermidis* 14.1.R1 (Fig. 1A). Indeed, *essH* and

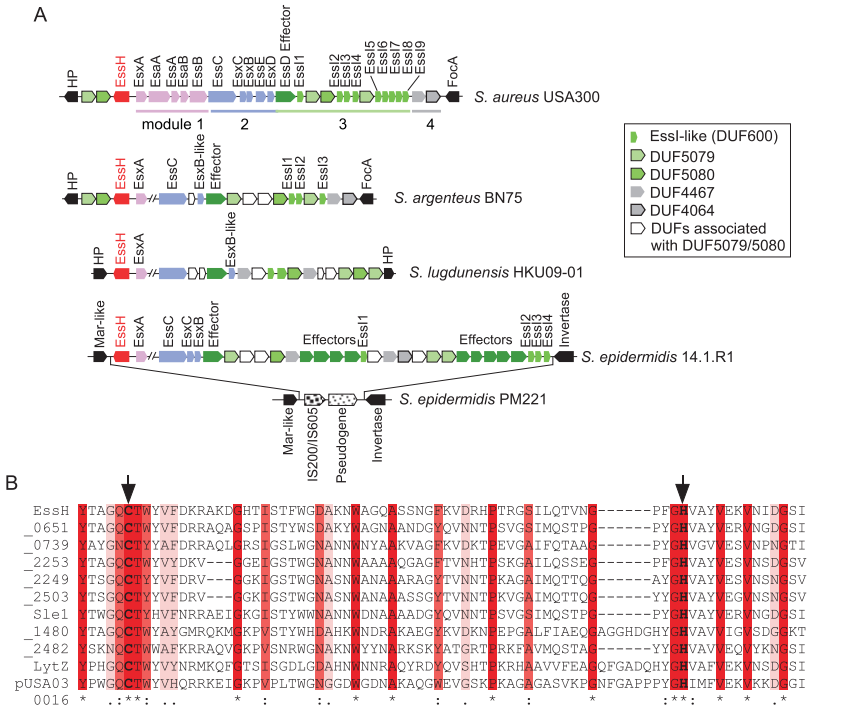

FIG 1 Association of *essH* with the *Staphylococcus aureus* ESS cluster. (A) Genomic organization of ESS clusters of *S. aureus* USA300 LAC, *Staphylococcus argenteus* BN75, *Staphylococcus lugdunensis* HKU09-01, *Staphylococcus epidermidis* 14.1.R1, and *S. epidermidis* PM221. Genes in modules 1, 2, 3, and 4 are shown in pink, blue, shades of green, and gray, respectively. Genes flanking the cluster are shown in black; *essH* is shown in red. DUFs (domains of unknown function) encoded by module 4 genes are listed in the inset. In some strains, module 4 genes are also found upstream of *essH*. The complete set of modules 1 and 2 genes is only shown for *S. aureus* USA300 LAC; these genes are conserved in the other species. FocA, formate channel A; HP, hypothetical protein; MarR, multiple antibiotic resistance regulator. (B) CHAP domains of 11 proteins predicted from the genome sequence of *S. aureus* subsp. *aureus* USA300_FPR3757 (NCBI RefSeq accession number NC_007793.1) were aligned with Clustal W. The locus tag for *essH* is SAUSA300_0277. Proteins are aligned with EssH in order of declining sequence conservation. Genes whose products encompass CHAP domains are identified with the last four digits of their locus tag; SAUSA300_0438 and _2579 are listed as Sle1 and LytZ. TraG (pUSA300016) is encoded on a plasmid. Identical, conserved, and similar residues are denoted by asterisks, colons, and periods, respectively, and highlighted in shades of red. Arrows identify the predicted active site residues, Cys^{199} and His^{254}.

module 1 represent the most conserved genes of ESS clusters, whereas modules 2, 3, and 4 are more variable (Fig. 1A). *essH* is absent in strains that lack the *ess* locus, such as *S. epidermidis* PM221, which carries an insertion sequence (IS) element replacing *essH* and modules 1 to 4 (Fig. 1A). The predicted product of *essH* is 297 amino acids in length, with an N-terminal signal peptide (amino acids 1 to 24), an intervening region without conserved features (amino acids 25 to 124), and a C-terminal CHAP domain (amino acids 125 to 297). The CHAP domain of EssH bears similarity to the MurNAc-L-Ala amidase domain, PRK08581 (NCBI). Both CHAP and PRK08581 belong to the COG3942 superfamily of proteins involved in cell wall biogenesis and the peptidoglycan hydrolase activities of bacteria, archaea, and trypanosomes. In bacteria, CHAP domains are found in murein hydrolases with *N*-acetylmuramoyl-L-alanine amidase activity and the papain-like fold of the cysteine protease CA peptidase clan CL0125 (35). In *S. aureus* USA300 LAC, EssH is 1 of 11 proteins with C-terminal CHAP domains (COG3942 superfamily) (Fig. 1B). Two additional proteins, SAUSA300_1923 and SAUSA300_(LytN) (36), harbor CHAP domains (Pfam05257) with reduced sequence conservation and are not shown in the alignment.

***essH* is required for *S. aureus* ESS secretion.** Bacteriophage ϕ85 was used to generate a lysate from strain $\Phi N\Xi$07490, a variant of *S. aureus* Newman with *Bursa aurealis* transposon insertion at nucleotide 602 of *essH* (37). The bacteriophage ϕ85 lysate was used to transduce the *ermB* marked mutation into *S. aureus* USA300 LAC* via

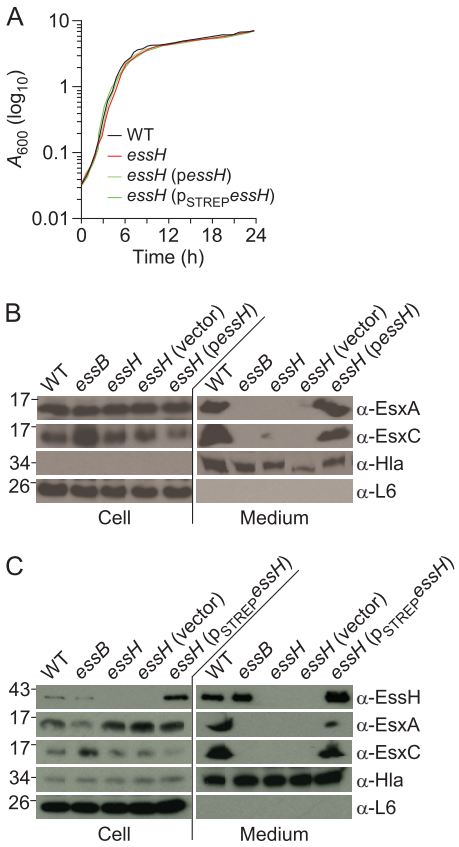

FIG 2 *essH* is dispensable for *S. aureus* growth and required for Esx protein secretion. (A) Overnight cultures of bacteria were normalized to an A_{600} of 5, diluted 1:100 in fresh medium, and grown at 37°C. Growth was monitored as increased absorbance (A_{600}) over 24 h. (B) To assess protein secretion, cultures of *S. aureus* USA300 LAC* (wild type [WT]) or its isogenic *essB*, *essH*, *essH*(vector), and *essH*(p*essH*) variants, were grown to an A_{600} of 3.0 and centrifuged to separate proteins in the extracellular medium fraction (medium) from staphylococci in the sediment. Bacteria were suspended in PBS and lysed with lysostaphin to release their cellular proteins (cell). Proteins were precipitated with trichloroacetic acid (TCA) and resuspended in buffer such that medium fractions were 25 times more concentrated than cell fractions. Extracts were separated by SDS-PAGE and electrotransferred to PVDF membranes for immunoblot analyses with rabbit polyclonal antibodies specific for EsxA (α-EsxA), EsxC (α-EsxC), α-hemolysin (α-Hla), and ribosomal protein L6 (α-L6). Numbers (in kilodaltons) indicate the migratory positions of molecular weight markers by SDS-PAGE. (C) Cultures of *S. aureus* USA300 LAC* (WT) or its isogenic *essB*, *essH*, *essH*(vector), and *essH*(p*essH*$_{STREP}$) variants were grown to an A_{600} of 3.0, fractionated, and analyzed as described above with the addition of rabbit polyclonal antibodies specific for EssH (α-EssH).

selection for erythromycin-resistant variants, which were validated by DNA sequencing. When analyzed for growth under T7bSS-inducing conditions, wild-type USA300 LAC* and its *essH*::*ermB* variant replicated at a similar rate (Fig. 2A). Cultures of wild-type and *essB* and *essH* mutant staphylococci were centrifuged and separated into bacterial cell (pellet) and culture medium (supernatant) fractions. Proteins in each fraction were analyzed by immunoblotting. Antibodies against α-hemolysin (Hla), a secreted protein, and ribosomal protein L6, a cytoplasmic protein, were used as fractionation controls (Fig. 2B and C). Compared to those in wild-type and *essB* mutant *S. aureus*, similar amounts of EsxA and EsxC were detected in bacterial cells from *essH* mutant cultures (Fig. 2B). Unlike the wild-type *S. aureus*, the *essH* mutant did not secrete EsxA and EsxC into the culture supernatant (Fig. 2B). This defect in ESS secretion was restored when the *essH* mutant strain was transformed with p*essH*, a plasmid for the expression of wild-type *essH*, but not following transformation with an empty vector control (Fig. 2B). Of note, plasmid-borne expression of *essH* did not affect staphylococcal growth (Fig. 2A). Plasmid-borne expression of $_{STREP}$*essH*, with an in-frame insertion of eight codons (for amino acids WSHPQFEK = STREP tag) after codon 2 of the predicted mature EssH, also

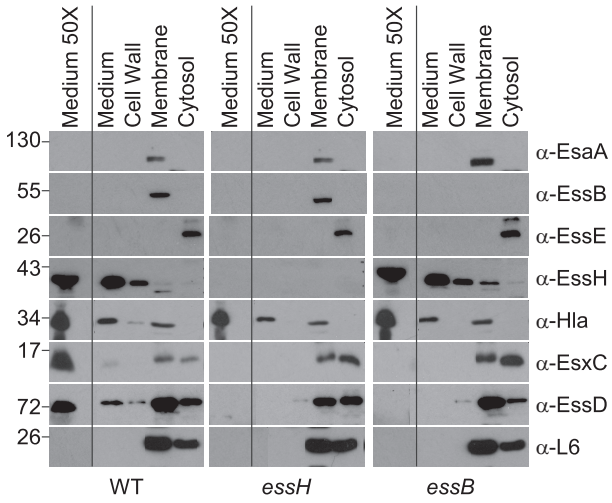

FIG 3 Mutations in *essH* and *essB* do not affect Sec protein secretion. Cultures of *S. aureus* USA300 LAC* WT and *essH* and *essB* mutants were grown to an A_{600} of 3.0 and centrifuged, and proteins in the extracellular medium (medium) were separated from the bacterial sediment. Sediments of 1-ml culture samples were fractionated by suspending staphylococci in sucrose buffer containing lysostaphin. The resulting protoplasts were sedimented by centrifugation. Proteins released by lysostaphin from the bacterial envelope (cell wall) were removed with the supernatant. Protoplasts were lysed and samples ultracentrifuged to sediment membrane proteins (membrane) and separate them from soluble proteins in the supernatant (cytosol). Proteins in all fractions were precipitated with TCA, separated by SDS-PAGE, electro-transferred to PVDF membrane, and analyzed by immunoblotting with rabbit polyclonal antibodies specific for Ess secretion machine components EsaA (α-EsaA), EssB (α-EssB), EssE (α-EssE), murein hydrolase EssH (α-EssH), α-hemolysin (α-Hla), ESS secretion substrates EsxC (α-EsxC), and EssD (α-EssD) as well as ribosomal protein L6 (α-L6). Samples of 50-ml cultures were centrifuged to sediment bacteria, and proteins in the culture medium were precipitated with TCA, washed with acetone, and suspended in sample buffer to generate a concentrated sample of proteins in the culture medium (medium 50×). Numbers on the left indicated kilodaltons.

did not affect *S. aureus* growth and restored the secretion of EsxA and EsxC in the *essH* mutant strain (Fig. 2A and C).

EssH is secreted into the extracellular medium of *S. aureus* cultures. The translational product of *essH* encompasses a predicted 24-residue signal peptide with a type I signal peptidase cleavage site (AQA). To analyze EssH in bacterial extracts, rabbit polyclonal antibodies were raised. DNA corresponding to the coding sequence for the mature portion of $_{STREP}$EssH was PCR amplified, cloned into pET15b, and expressed in *Escherichia coli* BL21(DE3). $_{STREP}$EssH was purified by affinity chromatography on Strep-Tactin–Sepharose and injected into rabbits, and the $_{STREP}$EssH-specific antibody titer in immune serum (αEssH) was determined via an enzyme-linked immunosorbent assay (ELISA). Immunoblotting experiments with αEssH identified EssH in fractionated cultures from wild-type and *essB* mutant *S. aureus* strains: small amounts of EssH were detected within bacterial cells and large amounts in the culture medium (Fig. 2C). EssH could not be detected with αEssH immune serum in fractionated cultures from the *essH* mutant strain (Fig. 2C). EssH production and secretion into the culture medium were restored following the transformation of *essH* mutant staphylococci with p_{STREP}*essH* but not with the empty vector control (Fig. 2C).

Mutations in *essH* and *essB* do not affect Sec-dependent protein secretion. Some *S. aureus* murein hydrolases are required for Sec-mediated secretion into the extracellular medium (32). To test whether *essH* mutation causes a general defect in protein secretion into the medium, 1-ml aliquots of staphylococcal cultures were fractioned by centrifugation, separating the proteins in the extracellular medium (medium) from the proteins that sedimented with bacterial cells (Fig. 3). Staphylococci were suspended in sucrose buffer, and the cell wall peptidoglycans were digested with lysostaphin. After centrifugation, the lysostaphin-digested cell walls were removed as supernatant (cell wall) from the protoplast sediment (Fig. 3). Protoplasts were lysed in

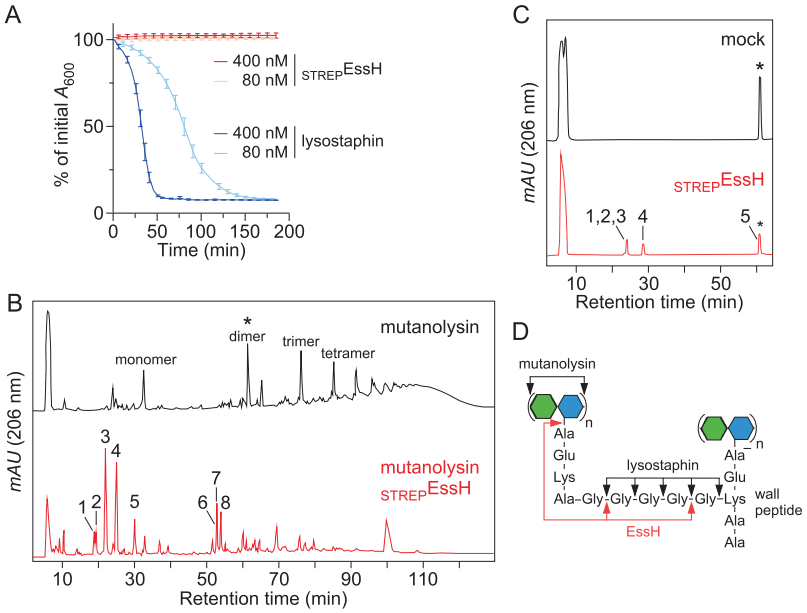

FIG 4 EssH exhibits amidase and endopeptidase activities. (A) Overnight cultures of *S. aureus* USA300 LAC* were treated with lysostaphin or $_{STREP}$EssH, and lysis was measured as a decline in A_{600} over time. (B) Purified *S. aureus* peptidoglycans were digested with mutanolysin and split into two samples that were either left untreated (black trace) or were treated with purified $_{STREP}$EssH (red trace); the carbohydrates in both samples were reduced and analyzed by reversed-phase HPLC. Peaks labeled were desalted and analyzed by MALDI-TOF mass spectrometry; observed *m/z* are reported in Table 1 alongside their structural interpretation. The asterisk identifies the cross-linked muropeptide dimer of *S. aureus* (*m/z* 2,438.59). (C) A sample of purified muropeptide dimer (*) was split into two parts, either left untreated (mock) or treated with $_{STREP}$EssH (red trace), and analyzed by reversed-phase HPLC. Individual peaks were desalted and analyzed by MALDI-TOF mass spectrometry; observed *m/z* are reported in Table 2 alongside their structural interpretations. (D) Diagram of cross-linked *S. aureus* peptidoglycan with arrows identifying the bonds that are cleaved by mutanolysin, lysostaphin, and EssH. mAU, milli-absorbance units; green and blue hexagons, *N*-acetylglucosamine and *N*-acetylmuramic acid, respectively.

hypotonic buffer by using a freeze-thaw protocol, and the sample was subjected to ultracentrifugation (100,000 × *g*), thereby separating the soluble proteins in the supernatant (cytosol) from the proteins that sedimented with staphylococcal membranes (Fig. 3). To detect proteins that are less abundantly secreted, we included a 50× concentrated sample of staphylococcal culture medium in immunoblotting experiments with fractioned USA300 LAC* wild-type and *essH* and *essB* mutant cultures. As a control, ribosomal protein L6 was found to be soluble in the cytosol and sedimented with assembled ribosomes (membrane) but was not in the extracellular medium or the cell wall fraction. Mature Hla was found in the extracellular medium and in the membrane fraction. EssE, a component of the T7bSS, was located in the cytosol, whereas EsaA and EssB were located in staphylococcal membranes. Although ESS secretion of EsxC and EssD was abolished in cultures of the *essH* mutant, neither Sec-mediated secretion of Hla nor production or membrane localization of EsaA and EssB were affected by the *essH* mutation (Fig. 3). The deletion of the *essB* gene did not affect Sec-secretion of EssH and Hla but abolished ESS secretion of EsxC and EssD.

EssH exhibits peptidoglycan hydrolase activity. Bacteria from overnight cultures of *S. aureus* USA300 LAC* were sedimented by centrifugation, washed and suspended in buffer, divided into four aliquots that were treated with 80 nM lysostaphin, 400 nM lysostaphin, and 80 nM $_{STREP}$EssH or 400 nM $_{STREP}$EssH, and analyzed for lytic activity by measuring declines in adsorption at 600 nm light (A_{600}) (Fig. 4A). As expected, the addition of 80 nM and 400 nM lysostaphin to staphylococci caused a rapid decline in A_{600}, as the glycyl-glycine endopeptidase binds to and cleaves the peptidoglycans of intact staphylococci (Fig. 4D). In contrast, the treatment with 80 nM or 400 nM $_{STREP}$EssH did not cause a decline in A_{600}, suggesting that the EssH murein hydrolase is unable to

TABLE 1 MALDI-TOF mass spectrometry of mutanolysin- and $EssH_{STREP}$-treated peptidoglycan fragments

Compound	*m/z*		Δ*m/z* (calculated − observed)	Predicted structure
	Observed	Calculated		
1	1,001.44	1,001.12	0.32	MurNAc-Ala-Gln-(Gly)Lys-Ala-Gly_4
2	796.75	796.94	0.19	Ala-Gln-(Gly_5)Lys-Ala-Ala
3	1,204.54	1,204.33	0.21	MurNAc-(Ala-Gln-(Gly)Lys-Ala-Gly_4)-GlcNAc[a]
4	1,432.45	1,432.6	0.15	MurNAc-(Ala-Gln-(Gly_5)Lys-Ala-Gly_4)-GlcNAc
5	1,047.47	1,048.14	0.33	MurNAc-(Ala-Gln-(Gly)Lys-Ala-Ala)-GlcNAc
6	1,980.69	1,982.20	1.51	MurNAc-(Ala-Gln-(Gly)Lys-Ala-Ala)-GlcNAc-MurNAc-(Ala-Gln-(Gly)Lys-Ala-Gly)-GlcNAc
7	2,368.82	2,367.67	1.15	MurNAc-(Ala-Gln-(Gly_5)Lys-Ala)-GlcNAc-MurNAc-(Ala-Gln-(Gly)Lys-Ala-Gly_4)-GlcNAc
8	1,960.67	1,961.25	0.58	MurNAc-Ala-Gln-(MurNAc-Ala-Gln-(Gly)Lys-Ala-Gly_5)Lys-Ala-Gly_4

[a]$[M+Na]^+$ sodiated ion.

cause bacterial lysis (Fig. 4A). Next, murein sacculi of *S. aureus* were isolated, extracted with acid, and treated with enzymes to remove secondary cell wall polymers, including teichoic acids, proteins, and polysaccharides. The glycan strands of purified peptidoglycan were then cleaved with mutanolysin, an *N*-acetylmuramidase, and the sample was split into two equal aliquots. One aliquot was incubated with purified $_{STREP}$EssH, whereas the other was incubated without enzyme (mock treatment). The samples were analyzed via reversed-phase high-performance liquid chromatography (HPLC). Mock-treated peptidoglycans exhibited the characteristic absorption spectrum of the staphylococcal cell wall: a monomeric wall peptide attached to disaccharide [MurNAc-(Ala-Gln-(Gly_5)Lys-Ala-Ala)-GlcNAc] as well as cross-linked dimeric, trimeric, tetrameric, and multimeric species {[MurNAc-(Ala-Gln-(Gly_5)Lys-Ala)-GlcNAc]$_n$} (Fig. 4B, black). $_{STREP}$EssH treatment collapsed the cross-linked peptidoglycan species of mutanolysin-treated peptidoglycans into monomeric or dimeric wall peptides with or without attached mono- or disaccharides (Fig. 4B, red). Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry experiments identified the *m/z* of peptidoglycan fragments, which were then interpreted to predict the structures of compounds 1 [MurNAc-Ala-Gln-(Gly)Lys-Ala-Gly_4], 2 [Ala-Gln-(Gly_5)Lys-Ala-Ala], 3 [MurNAc-(Ala-Gln-(Gly)Lys-Ala-Gly_4)-GlcNAc], 4 [MurNAc-(Ala-Gln-(Gly_5)Lys-Ala-Gly_4)-GlcNAc], 5 [MurNAc-(Ala-Gln-(Gly)Lys-Ala-Ala)-GlcNAc], 6 [MurNAc-(Ala-Gln-(Gly)Lys-Ala-Ala)-GlcNAc-MurNAc-(Ala-Gln-(Gly)Lys-Ala-Gly)-GlcNAc], 7 [MurNAc-(Ala-Gln-(Gly_5)Lys-Ala)-GlcNAc-MurNAc-(Ala-Gln-(Gly)Lys-Ala-Gly_4)-GlcNAc], and 8 [MurNAc-Ala-Gln-(MurNAc-Ala-Gln-(Gly)Lys-Ala-Gly_5)Lys-Ala-Gly_4] (Table 1). These data suggest that $_{STREP}$EssH cleaves the peptidoglycan of *S. aureus* at two positions: the amide bond between *N*-acetylmuramic acid and the L-Ala moiety of the wall peptide and the amide bonds within the pentaglycine cross bridge.

To test the hypothesis that $_{STREP}$EssH cleaves the peptidoglycan, we purified the mutanolysin-derived monosodiated ion of cross-linked muropeptide with the structure MurNAc-(Ala-Gln-[MurNAc-(Ala-Gln-(Gly_5)Lys-Ala-Gly_5)-GlcNAc]Lys-Ala-Ala)-GlcNAc (observed *m/z* 2,438.59, calculated *m/z* 2,438.03) (Fig. 4B, asterisk). The muropeptide sample was split into two aliquots. One aliquot was incubated with $_{STREP}$EssH and the other was incubated without enzyme (mock treatment). The samples were subjected to reversed-phase HPLC, and the absorption peaks were analyzed via MALDI-TOF mass spectrometry (Fig. 4C). $_{STREP}$EssH cleaved the peptidoglycan substrate with *m/z* 2,438.59 to generate multiple products that were characterized by MALDI-TOF mass spectrometry to encompass compounds 1 [Ala-Gln-(Gly_5)Lys-Ala-Gly], 2 [MurNAc-(Ala-Gln-(Gly_5)Lys-Ala-Gly_4)-GlcNAc], 3 [Ala-Gln-[MurNAc-(Ala-Gln-(Gly_5)Lys-Ala-Gly)-GlcNAc]Lys-Ala-Ala], and 4 [MurNAc-(Ala-Gln-(Gly)Lys-Ala-Ala)-GlcNAc] (Table 2). These data demonstrate that $_{STREP}$EssH indeed cuts the cross-linked disaccharide wall peptide at the amide bond between *N*-acetylmuramic acid and the L-Ala moiety of the wall peptide (Fig. 4D). Of note, $_{STREP}$EssH preferentially cuts the pentaglycine cross bridge after the first and the fourth glycyl residues (Table 2 and Fig. 4D).

EssH residues Cys^{199} and His^{254} contribute to peptidoglycan hydrolase activity. An alignment of EssH CHAP domains with the *S. aureus* USA300 CHAP domain-containing proteins identified the conserved residues Cys^{199} and His^{254}, which are thought to be

TABLE 2 MALDI-TOF mass spectrometry of $EssH_{STREP}$-treated peptidoglycan dimers

Compound	*m/z*		Δ*m/z* (calculated − observed)	Predicted structure
	Observed	Calculated		
1	757.60	759.93	2.33	Ala-Gln-(Gly_5)Lys-Ala-Gly[b]
2	1,432.71	1,432.61	0.10	MurNAc-(Ala-Gln-(Gly_5)Lys-Ala-Gly_4)-GlcNAc[a]
3	1,648.71	1,650.93	2.22	Ala-Gln-[MurNAc-(Ala-Gln-(Gly_5)Lys-Ala-Gly)-GlcNAc]Lys-Ala-Ala[b]
4	1,047.53	1,047.14	0.39	MurNAc-(Ala-Gln-(Gly)Lys-Ala-Ala)-GlcNAc
5	2,438.59	2,438.76	0.17	MurNAc-(Ala-Gln-[MurNac-(Ala-Gln-(Gly_5)Lys-Ala-Gly_5)-GlcNAc]Lys-Ala-Ala)-GlcNAc[a]

[a]$[M+Na]^+$ sodiated ion.
[b]$[M+H]^+$ protonated ion.

involved in catalysis (Fig. 1B). We generated the variant $_{STREP}EssH^{C199A/H254A}$ and purified this protein alongside $_{STREP}EssH$ via Strep-Tactin chromatography (Fig. 5A). Purified murein sacculi of *S. aureus* were cleaved with mutanolysin, and the samples were split into three equal aliquots. Two aliquots were treated with equal amounts of either $_{STREP}EssH$ or $_{STREP}EssH^{C199A/H254A}$. As a control, the third aliquot was mock treated. The samples were analyzed via reversed-phase HPLC (Fig. 5B). As expected, mock-treated peptidoglycans exhibited the characteristic absorption spectrum of the mutanolysin-digested cell wall, i.e., a monomeric wall peptide attached to disaccharide and cross-linked multimeric species (Fig. 5B, black), whereas $_{STREP}EssH$ treatment collapsed the cross-linked peptidoglycan species into monomeric or dimeric wall peptides with or without attached mono- or disaccharides (Fig. 5B, red). Importantly, treatment with $_{STREP}EssH^{C199A/H254A}$ had no effect on cross-linked peptidoglycans (Fig. 5B, green). These data suggest that the conserved residues of the CHAP domain, i.e., EssH residues Cys^{199} and His^{254}, contribute to the peptidoglycan hydrolase activity of this enzyme. The $_{STREP}EssH^{C199A/H254A}$ variant, with substitutions at both conserved residues, cannot cleave the staphylococcal peptidoglycan.

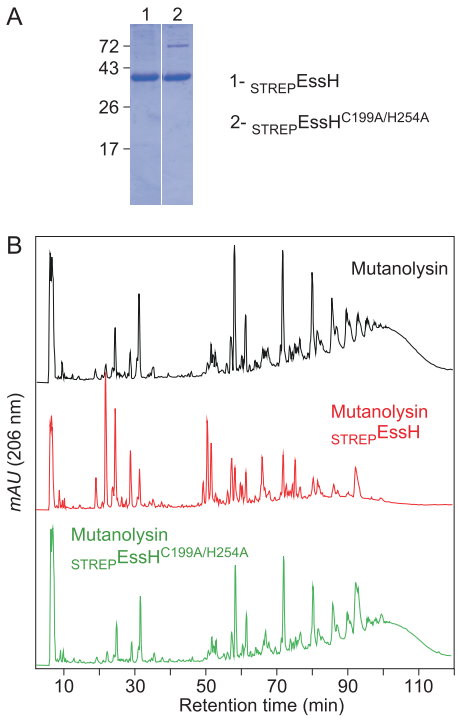

FIG 5 Cys^{199} and His^{254} are required for EssH murein hydrolase activity. (A) Coomassie brilliant blue-stained SDS-PAGE gel separating purified $_{STREP}EssH$ (1) and $_{STREP}EssH^{C199A/H254A}$ (2). The other numbers indicate the migratory positions of molecular weight markers (in kilodaltons). (B) Purified *S. aureus* peptidoglycans were digested with mutanolysin and split into three samples that were either left untreated (black trace) or were treated with $_{STREP}EssH$ (red trace) or $_{STREP}EssH^{C199A/H254A}$ (green trace); the carbohydrates in all samples were reduced and analyzed by reversed-phase HPLC.

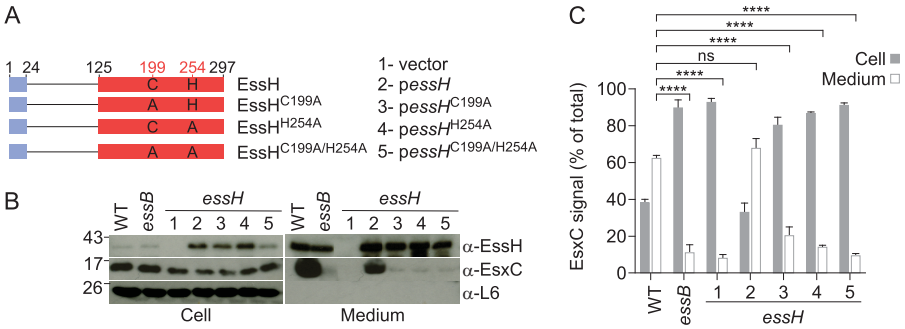

FIG 6 EssH murein hydrolase activity is required for *S. aureus* ESS secretion. (A) Diagram of plasmid (vector)-encoded wild-type EssH(p*essH*) and variants carrying amino acid substitutions C199A, H254A, and C199A/H254A. Blue and red boxes depict the signal peptides and CHAP domains, respectively. Numbers depict lengths of polypeptides in amino acids. (B) Cultures of *S. aureus* USA300 LAC* (WT) and its *essB*, *essH*(vector) (lane 1), *essH*(p*essH*) (lane 2), *essH*(p*essH*^{C199A}) (lane 3), *essH*(p*essH*^{H254A}) (lane 4), and *essH*(p*essH*$^{C199A/H254A}$) (lane 5) variants were grown and fractionated as described in the legend for Fig. 2B. Samples were subjected to immunoblot analyses using rabbit polyclonal antibodies specific for EssH, EsxC, and L6. (C) Densitometry quantification of the abundance of EsxC immune-reactive signal in cell and medium fractions from three independent experiments. Data are analyzed as percentages of total EsxC, i.e., the added densities of protein in cell and medium fractions. Statistical analysis was performed using a two-way ANOVA followed by Tukey's multiple-comparison test.

EssH peptidoglycan hydrolase activity is required for ESS secretion. Plasmids p*essH*^{C199A}, p*essH*^{H254A}, and p*essH*$^{C199A/H254A}$ enable the gene expression and synthesis of the EssH^{C199A}, EssH^{H254A}, and EssH$^{C199A/H254A}$ variants, respectively, in the *S. aureus essH* mutant strain (Fig. 6A). Immunoblotting experiments revealed that *S. aureus essH* mutants secreted similar amounts of wild-type EssH (encoded by p*essH*), EssH^{C199A}, EssH^{H254A}, and EssH$^{C199A/H254A}$ (Fig. 6B). As expected, the vector plasmid did not enable the synthesis or secretion of EssH in the *S. aureus essH* mutant (Fig. 6B). When analyzed for the activity of the ESS pathway in a pairwise comparison, the p*essH* plasmid restored EsxC secretion from the *S. aureus essH* mutant, whereas the vector alone, p*essH*^{C199A}, p*essH*^{H254A}, and p*essH*$^{C199A/H254A}$ did not (Fig. 6B). Immunoblot experiments were performed in triplicates to carry out densitometric quantification of immunoreactive signals (Fig. 6C). The amounts of EsxC in cell and medium fractions were calculated relative to the total amount of signal obtained for each strain (the sum of signals in the cell and medium fractions was set at 100%). As a control, the *essB* mutant was defective in the secretion of EsxC (Fig. 6BC). Further, EssH peptidoglycan hydrolase activity, specifically, the catalytic residues Cys^{199} and His^{254} of the CHAP domain, is required for EsxC secretion via the ESS pathway in *S. aureus* USA300 LAC.

***S. epidermidis essH* complements the ESS secretion defect of the *S. aureus essH* mutant.** *S. epidermidis* strain 14.1.R1 harbors a chromosomally encoded *ess* locus, with an *essH*-like gene located upstream of *esxA* on the minus strand (Fig. 1A). *S. epidermidis essH* bears a conserved C-terminal CHAP domain, but its N-terminal domain (amino acids 1 to 142) is not conserved (Fig. 7A). We constructed two new plasmids, namely, p*essH*$_{Se}$ and p*essH*$_{Se(SP)}$ (Fig. 7A). Plasmid p*essH*$_{Se}$ carries the full-length *essH* from *S. epidermidis* 14.1.R1, whereas plasmid p*essH*$_{Se(SP)}$ encodes the *S. aureus* USA300 LAC *essH* signal peptide sequence (codons 1 to 24) fused to *S. epidermidis* 14.1.R1 *essH* codons 27 to 319 (Fig. 7A). The rationale for this experiment is the observation that the signal peptide of EssH$_{Se}$ is significantly longer than that of *S. aureus*. When expressed in the *S. aureus essH* mutant strain, the amino acid differences in the *S. epidermidis* 14.1.R1 EssH sequence hindered the detection of EssH$_{Se}$ and EssH$_{Se(SP)}$ with immune serum against $_{STREP}$EssH (recombinant *S. aureus* EssH) (Fig. 7B). Nevertheless, *S. aureus essH*(p*essH*$_{Se}$) and *essH*(p*essH*$_{Se(SP)}$) strains secreted EsxC at levels similar to those observed with wild-type and *essH*(p*essH*) strains, while ESS-dependent secretion was abrogated in both the *S. aureus essH*(vector) and *essB* mutant controls (Fig. 7B and C). These results suggest functional conservation of EssH orthologues from *S. aureus* USA300 LAC and *S. epidermidis* 14.1.R1 in the staphylococcal ESS pathway.

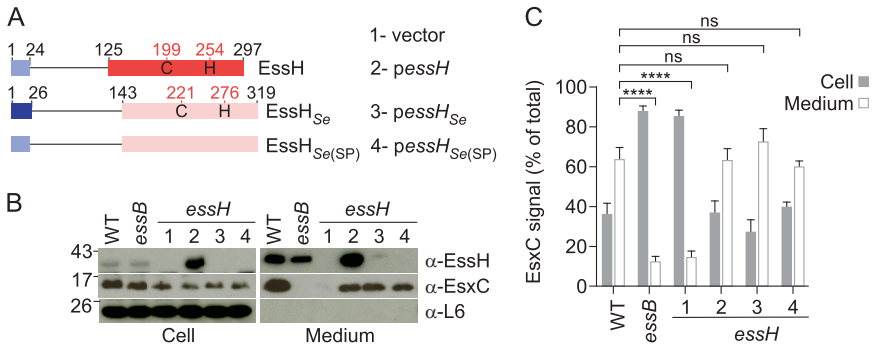

FIG 7 Heterologous complementation by *S. epidermidis* EssH. (A) Diagram of plasmid (vector)-encoded wild-type EssH(p*essH*), *S. epidermidis* EssH$_{Se}$(p*essH*$_{Se}$), and EssH$_{Se(SP)}$, a variant of EssH$_{Se}$ secreted via the signal peptide of *S. aureus* EssH. Blue boxes depict signal peptides, while red and pink boxes depict CHAP domains. Numbers depict lengths of polypeptides in amino acids. (B) Cultures of *S. aureus* USA300 LAC* (WT) and its *essB*, *essH*(vector) (lane 1), *essH*(p*essH*) (lane 2), *essH*(p*essH*$_{Se}$) (lane 3), and *essH*(p*essH* $_{Se(SP)}$) (lane 4) variants were grown and fractionated as described in the legend for Fig. 2B. Samples were subjected to immunoblot analyses using rabbit polyclonal antibodies specific for EssH, EsxC, and L6. (C) Quantification of EsxC in cell and medium fractions was performed by quantifying band densities from three independent experiments as described in the legend for Fig. 6C.

SagB peptidoglycan hydrolase activity is not required for ESS secretion. *S. aureus* mutants defective for *sagB* expression exhibit increased glycan chain lengths within their peptidoglycans, which results in altered protein secretion (32). We wondered whether the loss of *sagB* may also affect the ESS pathway. When analyzed by immunoblotting with specific antibodies and compared to the ESS pathway in wild-type *S. aureus* USA300 LAC*, the overall levels of EssH and EsxC were slightly diminished (Fig. 8A), likely due to the growth defects associated with the absence of SagB. A quantification of the protein in the supernatant indicated that the secretion of EsxC was unchanged between wild-type, *sagB*(vector), and *sagB*(p*sagB*) strains (Fig. 8B). The

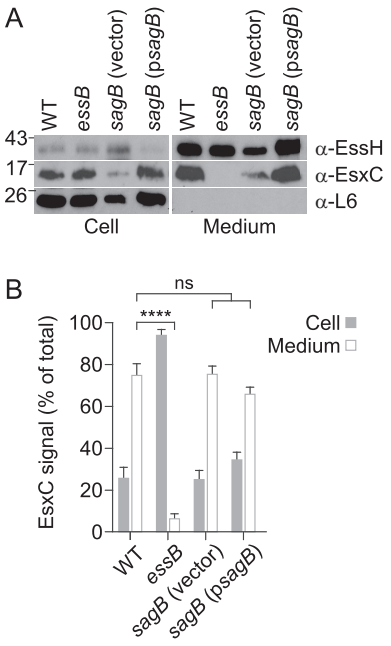

FIG 8 SagB hydrolase is not required for *S. aureus* ESS secretion. (A) Cultures of *S. aureus* USA300 (WT), *essB*, *sagB*(vector), and *sagB*(p*sagB*) variants were grown and fractionated as described in the legend for Fig. 2B. Samples were subjected to immunoblot analyses using rabbit polyclonal antibodies specific for EssH, EsxC, and L6. A representative experiment is shown. (B) Quantification of EsxC in cell and medium fractions was performed by quantifying band densities from three independent experiments as described in the legend for Fig. 6C.

reduced abundance of EssH and EsxC was reversed upon transformation of the *sagB* mutant strain with a plasmid enabling the expression of wild-type *sagB* (p*sagB*) (Fig. 8). Thus, SagB glucosaminidase, which generates the characteristic short-chain glycan strands of *S. aureus* peptidoglycans, is not required for ESS-mediated secretion.

DISCUSSION

To date, three *S. aureus* CHAP domain murein hydrolases have been characterized. Sle1 is secreted into the extracellular medium and binds via its three N-terminal LysM domains to the glycan strands of septal peptidoglycans (38). LytN is secreted via its YSIRK/GXXS signal peptide into the cross wall, i.e., the septal peptidoglycan layer synthesized during staphylococcal cell division (36). Together, Sle1 and LytN cleave the cross wall (septal peptidoglycan) to separate dividing cells, thereby completing the staphylococcal cell cycle (36). ScaH, another CHAP domain hydrolase, is dispensable for the staphylococcal cell cycle (39). In addition to CHAP, ScaH also encompasses an *N*-acetylglucosaminidase domain whose activity is similar to those of Atl, SagA, and SagB *N*-acetylglucosaminidases. A *S. aureus* variant lacking all four glucosaminidases cannot be generated, suggesting that glucosaminidase activity is required for staphylococcal replication (39). Here, we describe the EssH CHAP domain hydrolase of *S. aureus*. Neither the deletion nor the overexpression of *essH* affects bacterial replication; however, the deletion of *essH* abolishes the secretory activity of the *S. aureus* T7bSS. EssH is synthesized as a precursor and secreted via the canonical Sec pathway. Recombinant EssH exhibits *N*-acetylmuramoyl-L-alanine amidase and pentaglycyl endopeptidase activities. Specifically, EssH cleaves the amide bond between *N*-acetylmuramic acid and L-Ala of the wall peptide and the cross bridges of cross-linked wall peptides after the first and fourth glycyl residues. An alignment of EssH with other CHAP domain-containing hydrolases identified the candidate catalytic residues Cys^{199} and His^{254}, which when mutated to Ala, render the variant EssH inactive both *in vitro* and *in vivo*.

Specialized secretion systems assemble in the bacterial envelope to span its membrane and peptidoglycan layers. The estimated pore diameter of peptidoglycan (~2 nm) represents a physical barrier for the assembly of large macromolecular secretion machines (40), including flagella, type IV pili, and type III (T3SS), type IV (T4SS), and type VI (T6SS) secretion systems (41–43). The genes of peptidoglycan hydrolases supporting the assembly of specialized secretion systems are often clustered with the genes that specify these macromolecular complexes. Most of these enzymes represent lytic transglycosylases that cleave the glycosidic linkages between disaccharide repeats of peptidoglycan (41). For example, the T3SSs of Gram-negative bacteria require the assembly of needle complexes, an element of the secretion machinery that is embedded within the bacterial double membrane envelope and peptidoglycan layer (44). The lytic transglycosidase EtgA forms a 1:1 complex with the EscI inner rod component of the needle complex (45). This interaction triggers murein hydrolase activity and enables the assembly of the T3SS (45, 46). The peptidoglycan layers of Gram-positive bacteria have larger diameters than those of Gram-negative microbes, and in *S. aureus*, peptidoglycan is subject to remodeling by the SagB glucosaminidase (32). The loss of *sagB* leads to peptidoglycans with abnormally long glycan strands that impede the travel of proteins across the bacterial cell wall layer following their membrane translocation via the Sec pathway (32). T7bSS in *S. aureus* is not affected by SagB but requires an active EssH hydrolase. Unlike EtgA, EssH does not display broad lytic activity nor affect glycan strands. Instead, EssH cleaves wall peptides and pentaglycine cross bridges. Interestingly, the transmembrane proteins EsaA and EssB, which have been proposed to form the T7 translocon (26), sediment with the membrane fraction in *essH* mutant bacteria as occurs in the wild type. Perhaps the T7 translocon fails to extend across the cell wall layer in the *essH* mutant strain, thereby restricting the travel of the effector proteins.

While the molecular contribution of EssH to T7 secretion remains to be determined, bioinformatics analyses of *ess* gene clusters clearly reveal a genetic association with *essH*-like genes. This is exemplified by the *S. epidermidis* 14.1.R1 clinical isolate (47).

TABLE 3 Strains and plasmids used in the study

Strain, vector, or plasmid	Description	Reference(s) or source
Strains		
USA300 LAC*	*S. aureus* USA300 LAC lacking pUSA03 (designated as the wild type [WT])	22, 51
RN4220	*S. aureus* restriction deficient, cloning intermediate	56
ΦNΞ07490	*S. aureus* Newman with *Bursa aurealis* insertion at nucleotide 602 of *essH*	37
essH	*S. aureus* USA300 LAC* *essH*::*ermB*	This study
essB	*S. aureus* USA300 LAC* Δ*essB*	24
sagB	*S. aureus* USA300 LAC* Δ*sagB*::*aphA*	32
E. coli DH5α	F$^-$ *ϕ80lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *pho A supE44* λ^- *thi-1 gyrA96 relA1*	Our collection
E. coli BL21	B F$^-$ *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) [*malB*$^+$]$_{K\text{-}12}$(λ^S)	57
Vectors and plasmids		
pOS1	*E. coli/S. aureus* shuttle vector	58
pSEW016	pWWW412 with a modified polylinker herein referred as vector	This study; 52
pET15b	*E. coli* vector for production of recombinant proteins	Addgene
p*essH*	*essH* open reading frame cloned into pSEW016	This study
p*essH*^{C199A}	*essH*^{C199A} cloned into pSEW016	This study
p*essH*^{H254A}	*essH*^{H254A} cloned into pSEW016	This study
p*essH*$^{C199A/H254A}$	*essH*$^{C199A/H254A}$ cloned into pSEW016	This study
p*essH*$_{Se}$	*S. epidermidis* 14.1.R1 *essH* cloned into pSEW016	This study
p*essH*$_{Se(SP)}$	*S. aureus* USA300 LAC *essH* nucleotides 1 to 78 fused to *S. epidermidis* 14.1.R1, *essH* nucleotides 85 to 960 cloned into pSEW016	This study
p$_{STREP}$*essH*	*S. aureus* USA300 LAC *essH* with STREP II tag sequence inserted between nucleotides 72 and 73 and cloned into pSEW016	This study
p*sagB*	*sagB* cloned into pOS1	32
pET15b-$_{STREP}$*essH*	*essH* nucleotides 73 to 894 with N-terminal STREP II tag cloned into pET15b	This study
pET15b-$_{STREP}$*essH*$^{C199A/H254A}$	*essH*$^{C199A/H254A}$ nucleotides 73 to 894 with N-terminal STREP II cloned into pET15b	This study

Most *S. epidermidis* isolates lack a T7SS. However, Bruggemann and colleagues noted the presence of an *ess* gene cluster with aberrant GC content in strain 14.1.R1 (Fig. 1B) (47). The finding that *S. epidermidis* EssH restores T7 secretion in a *S. aureus essH* mutant points to a conserved role for this peptidoglycan hydrolase in facilitating ESS-mediated secretion of substrates.

A cell wall hydrolase has not been reported in association with the T7aSS of mycobacterial species. Mycobacterial genomes harbor up to five different T7SS gene clusters, designated ESX-1 through ESX-5 (48). Each T7SS gene cluster encodes core components that assemble into a membrane complex (49); however, the genetic requirements and mechanisms enabling the assembly of these macromolecular complexes in the mycobacterial envelope are not known. Up to 50% of mycobacterial isolates harbor a conjugative plasmid that encompasses components of a T4SS and an intact T7SS gene cluster. A major difference between the plasmid-encoded T7SS and the chromosomal ESX systems is the presence of two genes at the beginning of the plasmid locus that are predicted to encode peptidoglycan hydrolases (50). In conclusion, we propose that the T7bSS of *S. aureus* is endowed with a conserved *essH* gene that encodes a hydrolase whose activity is required for the secretion of ESS substrates.

MATERIALS AND METHODS

Media and growth conditions. *S. aureus* strains were cultured in tryptic soy broth (TSB) or agar (TSA) at 37°C, unless otherwise stated, and the media were supplemented with 10 μg/ml chloramphenicol for plasmid selection and 0.2% heat-inactivated horse serum (Gibco/Life Technologies) for ESS induction. Bacteriophage ϕ85 transductants of *S. aureus* USA300 LAC* were selected by supplementing TSA with 200 μg/ml of erythromycin. *Escherichia coli* was cultured in lysogeny broth (LB) medium or agar at 37°C, supplemented with 100 μg/ml ampicillin for plasmid selection and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for the production of recombinant proteins.

Bacterial strains and plasmids. Relevant strains and plasmids are listed in Table 3. *S. aureus* USA300 LAC* is a variant of the original clone of the epidemic community-acquired methicillin-resistant *S. aureus* (CA-MRSA USA LAC) strain (51) that has lost plasmid pUSA03 encoding *ermC* (22). The loss of this plasmid does not alter virulence in mice nor T7S and enables the selection of mutants marked with the *ermB* allele. *S. aureus essH*::*ermB* (referred to as *essH* in the text) was generated via ϕ85 bacteriophage-mediated transduction of a transposon-disrupted *essH* allele linked to the erythromycin resistance gene

(*ermB*). Plasmid DNA was passaged through *S. aureus* RN4220 prior to transformation into USA300 LAC* strains. Plasmid pSEW016 was used for complementation studies. pSEW016 is a derivative of pWWW412, a shuttle vector that carries the promoter and Shine-Dalgarno sequences of the *S. aureus hprK* gene (52). In pSEW016, the NdeI cloning site of pWWW412 was replaced with SacI.

Plasmid p*essH* for complementation studies was constructed by amplifying USA300 genomic DNA by PCR using the primer pairs bearing sequences 5′-CCCCGAGCTCATGAAGAAAACAATTTTACTGACGATGAC AACTC-3′ and 5′-CCCCGGATCCTTAATGGATGTAATTATATGATGAAACTTCTGAAGCAGAG-3′. The amplified product was cloned into vector pSEW016, referred to as vector in the text, using SacI and BamHI restriction sites. The replacement of codons 199 and 254 (C199A and H254A, respectively) was achieved by site-directed mutagenesis of p*essH* using mutation-containing primers 5′-CTTATATACTGCTGGACAA GCAACATGGTATGTCTTTG-3′/5′-CGTTTATCAAAGACATACCATGTTGCTTGTCCAGCAG-3′ and 5′-CAGTAAA TGGTCCATTTGGTGCAGTAGCCTACG-3′/5′-CTTTTTCAACGTAGGCTACTGCACCAAATGGACC-3′, respectively. Plasmid p_{STREP}*essH* was constructed by amplifying the *essH* signal peptide sequence by using primers 5′-CCCCGAGCTCATGAAGAAAACAATTTTACTGACGATGACAACTC-3′ and 5′-TTTTTCGAATTGA GGATGTGACCACGTATATGCTTGAGCCGAGTTAGGCG-3′and the mature *essH* coding sequence with an appended STREP-tag using primers 5′-TGGTCACATCCTCAATTCGAAAAAAATGATAGCAAAACATTAGA AGAAGCAAAGAAAGC-3′ and 5′-CCCCGGATCCTTAATGGATGTAATTATATGATGAAACTTCTGAAGCAGA G-3′. Amplified overlapping DNA fragments were fused by PCR, cloned into pSEW016, and sequenced. For the recombinant production of proteins $_{STREP}$EssH and $_{STREP}$EssH$^{C199A/H254A}$, template DNA was amplified by PCR using the primers 5′-CCCCCCATGGGCTGGTCACATCCTCAATTCGAAAAAT ATACGAATGATAGCAAAACATTAGAAGAAGCAAAG-3′ and 5′-CCCCGGATCCTTAATGGATGTAATTATAT GATGAAACTTCTGAAGCAGAG-3′ and cloned into pET-15b using NcoI and BamHI restriction sites.

Protein purification. Cultures of *E. coli* (2 liters) that had been grown to an absorbance at 600 nm (A_{600}) of 2.0 were centrifuged (10,000 × *g* for 10 min). Sedimented cells were suspended in buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl), and the resulting suspensions were lysed in a French press at 14,000 lb/in^2 (Thermo Spectronic, Rochester, NY). Unbroken cells were removed by centrifugation (5,000 × *g* for 15 min), and the crude lysates subjected to ultracentrifugation (100,000 × *g* for 1 h at 4°C). Soluble recombinant proteins were subjected via gravity flow to chromatography on Strep-Tactin–Sepharose (IBA) with a packed volume of 1 ml preequilibrated with buffer A. The columns were washed with 20 bed volumes of buffer A and eluted with 4 ml of 2.5 mM desthiobiotin in buffer A. Aliquots of the eluted fractions were mixed with equal volumes of sample buffer and separated on 12% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Recombinant proteins were dialyzed against phosphate-buffered saline (PBS) and their concentrations determined with the bicinchoninic acid assay (Pierce).

EssH antiserum. Purified $_{STREP}$EssH (100 μg) was emulsified with complete Freund's adjuvant (Difco), and the emulsion was injected subcutaneously into a New Zealand White rabbit purchased from Harlan Sprague Dawley. At 21-day intervals, the animal received two booster immunizations with 100 μg of $_{STREP}$EssH emulsified with incomplete Freund's adjuvant (Difco). Serum was obtained following the centrifugation of blood samples (10,000 × *g* for 10 min) and mixed with 0.02% sodium azide for storage.

Fractionation of bacterial cultures and immunoblotting. To assess protein secretion, *S. aureus* culture aliquots were centrifuged (10,000 × *g* for 10 min) to separate proteins in the medium and the cells. The cells from bacterial pellets were washed and lysed with lysostaphin (10 μg/ml for 1 h at 37°C). Proteins in these suspensions (medium or cells) were precipitated by the addition of 10% final concentration trichloroacetic acid, washed in cold acetone, and dried. For the subcellular localization of proteins, pelleted cells were suspended in TSM buffer (50 mM Tris-HCl [pH 7.5], 0.5 M sucrose, 10 mM $MgCl_2$) prior to lysostaphin treatment. The samples were centrifuged at 15,000 × *g* for 10 min, and the supernatants (cell wall fraction) were transferred to a new tube and protoplasts were lysed by repeated freeze-thawing (3 cycles). The extracts were centrifuged at 100,000 × *g* for 40 min at 4°C to separate the supernatants containing cytosolic proteins. The pellets containing membrane proteins were suspended in PBS. Proteins in all fractions were precipitated with trichloroacetic acid (TCA), washed with methanol, and dried. All precipitates were solubilized in 100 μl of 0.5 M Tris-HCl (pH 8.0)-4% SDS and heated at 90°C for 10 min. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane for immunoblot analysis. The membranes were blocked by incubating in 0.5% milk for 1 h at room temperature. To prevent the binding of primary antibodies to protein A, 0.8 mg of human IgG was added to 10 ml of blocking buffer for 1 h at room temperature prior to the addition of primary polyclonal antibodies (at a dilution of 1:5,000 for T7 proteins or 1:10,000 for L6 and Hla). Membranes were incubated for an additional hour, washed 3 times for 10 min in TBS-T (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20), and incubated with 1:10,000 anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) for 1 h. The membranes were washed again in TBS-T, and immunoreactive products were revealed by chemiluminescent detection using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). The blots were developed on Amersham Hyperfilm ECL (GE Healthcare Life Sciences).

Biochemical assays. The general lytic activity of enzymes was assayed using washed cells of *S. aureus*. Overnight cultures of staphylococci were washed twice with 50 mM Tris-HCl, pH 8.0, and suspended in 650 μl of the same buffer at an absorbance at 600 nm (A_{600}) of ~1.6. Triplicate sets of 90 μl of cell suspension were aliquoted into 96-well plates. Buffer or enzymes were added to each triplicate set at the indicated concentrations. Changes in A_{600} were monitored every 5 min at 37°C with agitation in a Synergy HT plate reader (BioTek). The change in absorbance expressed as a percentage of the input for each well was normalized to the negative control and plotted over time.

S. aureus peptidoglycans were prepared in 100 mM phosphate buffer, pH 5.5, as previously described (53), the concentrations were adjusted according to the A_{600}, and they were incubated for 18 h at 37°C with 100 μl of enzyme, either mutanolysin (500 units/ml; Sigma) or $_{STREP}$EssH (300 μg/ml). After 18 h of incubation with mutanolysin, peptidoglycan samples were boiled for 10 min to quench the reaction, and the samples were centrifuged at 15,000 $\times$ *g* for 15 min. When indicated, soluble material was further incubated with $_{STREP}$EssH. All samples were neutralized with sodium hydroxide to reach pH 7.0, dried, and reduced via the addition of 250 mM sodium borate and 3 to 5 mg of sodium borohydride. The samples were incubated for 30 min, and the reactions were stopped by the addition of 20% phosphoric acid to reach pH $<$4.0 as described previously (53). Reduced muropeptides were separated by reversed-phase HPLC on a C_{18} column (250 mm by 4.6 mm, ODS-Hypersil; Thermo Scientific) as described previously (54). Individual peaks were desalted using reversed-phase ZipTip C_{18} pipette tips (Millipore) in 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA), and 0.75 μl was cospotted with 0.75 μl of matrix, α-cyano-4-hydroxycinnamic acid, at 10 mg/ml in 50% acetonitrile and 0.1% TFA onto a metal target plate. The samples were subjected to MALDI-TOF mass spectrometry using an Autoflex Speed Bruker MALDI instrument. Ions were detected in reflectron positive mode.

Statistical analyses. Immunoblot quantification was performed using ImageJ software (National Institutes of Health) (55) and analyzed for statistical significance by two-way analyses of variance (ANOVA) followed by Tukey's multiple-comparison tests with Prism (GraphPad Software). Experiments were repeated three times.

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