An Iron-Regulated Autolysin Remodels the Cell Wall To Facilitate Heme Acquisition in *Staphylococcus lugdunensis*

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Bacteria alter their cell surface in response to changing environments, including those encountered upon invasion of a host during infection. One alteration that occurs in several Gram-positive pathogens is the presentation of cell wall-anchored components of the iron-regulated surface determinant (Isd) system, which extracts heme from host hemoglobin to fulfill the bacterial requirement for iron. *Staphylococcus lugdunensis*, an opportunistic pathogen that causes infective endocarditis, encodes an Isd system. Unique among the known Isd systems, *S. lugdunensis* contains a gene encoding a putative autolysin located adjacent to the Isd operon. To elucidate the function of this putative autolysin, here named IsdP, we investigated its contribution to Isd protein localization and hemoglobin-dependent iron acquisition. *S. lugdunensis* IsdP was found to be iron regulated and cotranscribed with the Isd operon. IsdP is a specialized peptidoglycan hydrolase that cleaves the stem peptide and pentaglycine cross-bridge of the cell wall and alters processing and anchoring of a major Isd system component, IsdC. Perturbation of IsdC localization due to *isdP* inactivation results in a hemoglobin utilization growth defect. These studies establish IsdP as an autolysin that functions in heme acquisition and describe a role for IsdP in cell wall reorganization to accommodate nutrient uptake systems during infection.

Invading pathogens must undergo considerable cell surface remodeling to adapt to changing conditions, such as the harsh environment encountered during infection of the vertebrate host. Upon sensing the host environment, pathogens initiate an alteration in gene expression to facilitate this reorganization. The cell wall of Gram-positive pathogens provides an ideal framework from which to elaborate mechanisms that allow the bacterium to interact with the external milieu. Included among the resulting surface changes are the exposure of surface receptors, production and/or secretion of virulence factors and defense molecules, expression of motility apparatuses, and the deployment of nutrient acquisition systems.

One critical system expressed by many Gram-positive pathogens during infection is the iron-regulated surface determinant (Isd) system, a multiprotein transport pathway that captures host hemoglobin to satisfy the bacterial requirement for nutrient iron (1). Iron acquisition is essential for the survival and propagation of invading pathogens during infection. Indeed, Isd system function is critical to the pathogenesis of the important human pathogen *Staphylococcus aureus* (2, 3). To fulfill this requirement, pathogens can exploit host hemoglobin, the abundant oxygen carrier protein that contains four iron-coordinating heme molecules. The Isd system imports heme by first capturing hemoglobin through surface receptors, extracting heme from the hemoglobin polypeptide, and shuttling it through the cell wall to a membrane transporter (4–6). Once heme is internalized, it is either utilized intact as a cofactor for various cellular processes or degraded by cytoplasmic heme oxygenases to release free iron (7). The Isd system is widely conserved among Gram-positive bacteria and is required for hemoglobin-dependent heme acquisition in several human pathogens, including *Staphylococcus aureus* and *Bacillus anthracis* (1).

Significant alterations occur in the peptidoglycan layer to ac-

commodate expression of large amounts of cell wall-attached proteins, including IsdA, IsdB, and IsdC. In *S. aureus*, IsdA and IsdB are affixed to the cell wall by the transpeptidase sortase A (SrtA) (8). SrtA is responsible for anchoring more than 20 proteins to the staphylococcal cell wall through recognition of a conserved LPXTG sorting motif (9). SrtA cleaves between the threonine and glycine in the sorting sequence of IsdA and IsdB and attaches the protein to free amine groups on the pentaglycine crossbridge of the peptidoglycan precursor molecule lipid II through a transpeptidation reaction (10). The Isd-lipid II molecule then is incorporated into the growing peptidoglycan layer such that IsdA and IsdB are displayed on the cell surface (11–13). Surface exposure of these proteins facilitates hemoglobin binding and heme extraction (IsdB), as well as heme transfer to additional components of the Isd system (IsdA) (1, 14). In contrast, *S. aureus* IsdC is attached to

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TABLE 1 Strains used in this study

Bacterial strain	Description	Reference
Wild type	<i>Staphylococcus lugdunensis</i> strain N920143, human breast isolate	23
Δ isdP	Deletion of Isd autolysin gene (<i>SLUG_01010</i>) in N920143 background	45
Δ isdC	Deletion of <i>isdC</i> gene in N920143 background	21
WT pOS1	Wild-type <i>S. lugdunensis</i> transformed with an empty plasmid	This study
Δ isdP pOS1	<i>isdP</i> deletion transformed with an empty plasmid	This study
Δ isdP pisdP	Complementation strain expressing <i>isdP</i> under a constitutive promoter	This study
WT pisdP	IsdP overexpression strain expressing <i>isdP</i> under a constitutive promoter	This study
Newman pOS1	<i>S. aureus</i> strain Newman transformed with an empty plasmid	14
Newman pisdP	<i>S. aureus</i> strain Newman expressing <i>isdP</i> under a constitutive promoter	This study
Δ isdP pisdP ^{E146A}	<i>isdP</i> deletion expressing <i>isdP</i> containing the E146A mutation in the FlgJ domain	This study
Δ isdP pisdP ^{D171A}	<i>isdP</i> deletion expressing <i>isdP</i> containing the D171A mutation in the FlgJ domain	This study
Δ isdP pisdP ^{C393A}	<i>isdP</i> deletion expressing <i>isdP</i> containing the C393A mutation in the CHAP domain	This study
Δ isdP pisdP ^{H454A}	<i>isdP</i> deletion expressing <i>isdP</i> containing the H454A mutation in the CHAP domain	This study
Δ isdP pisdP ^{N473A}	<i>isdP</i> deletion expressing <i>isdP</i> containing the N473A mutation in the CHAP domain	This study
Δ isdP pisdP ^{FLAG}	<i>isdP</i> deletion expressing <i>isdP</i> with an N-terminal FLAG tag	This study
Δ isdP pisdP ^{E146A FLAG}	<i>isdP</i> deletion expressing <i>isdP</i> with an N-terminal FLAG tag and containing the E146A mutation in the FlgJ domain	This study
Δ isdP pisdP ^{D171A FLAG}	<i>isdP</i> deletion expressing <i>isdP</i> with an N-terminal FLAG tag and containing the D171A mutation in the FlgJ domain	This study
Δ isdP pisdP ^{C393A FLAG}	<i>isdP</i> deletion expressing <i>isdP</i> with an N-terminal FLAG tag and containing the C393A mutation in the CHAP domain	This study
Δ isdP pisdP ^{H454A FLAG}	<i>isdP</i> deletion expressing <i>isdP</i> with an N-terminal FLAG tag and containing the H454A mutation in the CHAP domain	This study
Δ isdP pisdP ^{N473A FLAG}	<i>isdP</i> deletion expressing <i>isdP</i> with an N-terminal FLAG tag and containing the N473A mutation in the CHAP domain	This study
Δ srtB	Deletion of <i>srtB</i> gene in N920143 background	45
Δ isdC pOS1	<i>isdC</i> deletion transformed with an empty plasmid	This study
Δ isdC pisdC ^{ΔNPQTS}	<i>isdC</i> deletion expressing <i>isdC</i> with a mutated SrtB sequence	This study
Δ isdP pisdP ^{ΔNPQTS}	<i>isdP</i> deletion expressing <i>isdP</i> with a mutated SrtB sequence	This study
WT pisdP ^{C-FLAG}	WT expressing <i>isdP</i> with a C-terminal FLAG tag	This study
Δ isdP pisdP ^{C-FLAG}	<i>isdP</i> deletion expressing <i>isdP</i> with a C-terminal FLAG tag	This study
Δ srtB pisdP ^{C-FLAG}	<i>srtB</i> deletion expressing <i>isdP</i> with a C-terminal FLAG tag	This study

the cell wall by sortase B (SrtB), which cleaves between the threonine and asparagine residues in the canonical NPQTN sorting sequence and anchors the protein to the crossbridge of mature peptidoglycan through a transpeptidation reaction similar to that of SrtA (1, 15). It has been proposed that SrtB utilizes non-cross-linked, mature peptidoglycan as a substrate for IsdC attachment (15). Due to its anchoring to assembled peptidoglycan and not lipid II, *S. aureus* IsdC is confined within the cell wall envelope and not exposed on the surface of the bacterium (15). The internal localization of IsdC within the peptidoglycan layer mediates the transfer of heme through the thick cell wall to the Isd membrane transporter (16).

Staphylococcus lugdunensis is a Gram-positive bacterium and coagulase-negative staphylococcal species (CoNS). Like other CoNS species, *S. lugdunensis* is a common member of the human skin microbiota but can cause severe disease, including skin and soft-tissue infections, pneumonia, and osteomyelitis (17–19). *S. lugdunensis* is best known for causing devastating infective endocarditis that is associated with significant mortality (20). *S. lugdunensis* is distinguished among the CoNS species due to its ability to utilize host hemoglobin as an iron source through the expression of Isd system genes (21–23). The *S. lugdunensis* Isd system contains the core components of the canonical Isd pathway, including cell wall proteins IsdB and IsdC, a membrane-localized ABC transporter, a heme oxygenase, and SrtB. In addition, the *S. lugdunensis* Isd system contains several unique features, such as

two uncharacterized Isd proteins, IsdK and IsdJ, which have some identity to *S. aureus* IsdA, and a gene encoding a putative autolysin located downstream of the heme oxygenase *isdG* (21, 23).

Bacteria exploit the peptidoglycan hydrolyzing function of autolysins for cell wall remodeling. Autolysins play a crucial role in the maintenance of cell wall growth and peptidoglycan turnover associated with cell division and daughter cell separation (24–26). In addition, some specialized autolysins coordinate assembly of extracytoplasmic protein complexes (i.e., pili and flagella), fratricide, cellular adherence, and virulence factor release (27–29). The studies presented here describe a specialized, iron-regulated autolysin, IsdP, which alters cell wall localization of a core Isd protein, IsdC, and contributes to hemoglobin-dependent heme acquisition in *S. lugdunensis*.

MATERIALS AND METHODS

Bacterial strains and media. *S. lugdunensis* strain N920143 was utilized as the wild-type strain and mutants were generated in this background by allelic replacement (21). Strains used in these studies are listed in Table 1. To express *S. lugdunensis* genes *in trans*, the gene of interest was PCR amplified from genomic DNA and cloned into pOS1 behind the constitutive *lgt* promoter. Transformation of competent *S. lugdunensis* was achieved using plasmid isolated from *E. coli* SLO1B by maxi prep (Qiagen) as described previously (30). Strains harboring plasmids were grown in the appropriate medium with 10 μ g/ml chloramphenicol. Tryptic soy broth (TSB) (Difco) was used as an iron-rich medium. Iron-restricted media were generated by adding the iron chelator 2,2-dipyridyl (DIP)

to TSB or ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) to Roswell Park Memorial Institute culture medium 1640 (RPMI; Invitrogen) supplemented with 1% Casamino Acids (RPMI plus CAS). Metal-devoid NRPMI was generated by treating RPMI plus CAS with 7%, wt/vol, Chelex 100 beads overnight.

RNA purification. Bacterial RNA was harvested as previously described (31). In short, overnight cultures of *S. lugdunensis* strains were subcultured (1:100) into TSB alone or with 350 μ M DIP. Cultures were harvested at late-exponential phase, mixed with ice-cold ethanol-acetone, and frozen at -80°C . Pellets were resuspended in LETS buffer (1 M LiCl, 0.5 M EDTA, 1 M Tris HCl, pH 7.4, 10% SDS) and lysed. RNA was extracted using TRIzol (TRI Reagent; Sigma) and chloroform precipitated with isopropyl alcohol, washed with 70% ethanol, and dissolved in distilled water. Contaminating DNA was removed with DNase I (Amersham Biosciences) treatment. RNA then was purified using the RNeasy minikit according to the manufacturer's protocol (Qiagen). RNA concentration and purity were measured by optical density at 260 nm and 280 nm, respectively.

Real-time RT-PCR. For cDNA synthesis, 2 μ g of RNA was left untreated or was treated with Moloney-murine leukemia virus reverse transcriptase (RT) according to the manufacturer's recommendations (Promega). cDNA was amplified in triplicate using iQ SYBR green supermix (Bio-Rad) in an iCycler iQ (Bio-Rad). Primers (5' to 3') were *S. lugdunensis* 16S-for (AACGATACGTAGCCGACCTG), 16S-rev (TTGC GGAAGATTCCTACTG), *isdP*-for (GTGACGAAGCAGGGAGATTC), *isdP*-rev (CGCGTGTCACTACTCTGAGC), *isdC*-for (TACAGGCTCAG CAGCAAGT), *isdC*-rev (GGTGTACCAGCACTCGTTT), *isdG*-for (GAAGGCTTTAAAGGCATGTTT), and *isdG*-rev (TGCTTTGCGAAA AACATCTG).

PCR. PCR (GoTaq polymerase; Promega) was performed using cDNA isolated from *S. lugdunensis* wild-type or Δ *isdP* mutant cells grown in iron-rich or iron-poor media. Primers within the coding sequences of *isdG* (5' GAAGGCTTTAAAGGCATGTTT 3') and *isdP* (5' CGCGTGT CACTACTCTGAGC 3') amplify only if the genes are cotranscribed.

Protein purification. A vector expressing N-terminal His-tagged *S. lugdunensis* IsdP was created in pET15b (Novagen). Clones were sequenced (GenHunter) and transformed into *Escherichia coli* BL21(DE3). Protein was purified as described previously (7).

Peptidoglycan purification. To purify peptidoglycan from iron-depleted *S. lugdunensis*, cells were grown to log phase in 1 liter of TSB with 350 μ M DIP, boiled for 30 min, and harvested by centrifugation at $8,000 \times g$ for 10 min. Cells were washed twice with 50 ml saline, once with 50 ml water, twice with 50 ml acetone, and then dried at 37°C overnight. Dried cells were weighed and resuspended in 10 ml ice-cold water per gram of cells. To shear off the cell wall, silica beads equaling the volume of water were added and the suspension was vortexed at maximum speed for 20 min. The beads were removed by centrifugation at $1,500 \times g$ for 10 min and washed twice with 20 ml ice-cold water, collecting the supernatant containing peptidoglycan each time. Total supernatant was centrifuged as described above to remove unbroken cells and then again at $6,500 \times g$ for 30 min to sediment peptidoglycan. Peptidoglycan was washed three times with water (40 ml/g starting material), resuspended in phosphate-buffered saline (PBS) (80 ml/g starting material) with 100 μ g/ml RNase, 50 μ g/ml DNase, and 2 μ l toluene per ml PBS, and incubated overnight at 37°C . Peptidoglycan was centrifuged at $6,500 \times g$ for 30 min, washed four times with 25 ml water, and then mixed with 50 ml 5% trichloroacetic acid (TCA) per gram of starting material and incubated at room temperature overnight. Cell walls were centrifuged at $6,500 \times g$ for 30 min, washed with 50 ml 5% TCA, resuspended in 50 ml 5% TCA, and boiled for 15 min. The sample was centrifuged at $6,500 \times g$ for 30 min, washed three times in water and three times in acetone, and then dried at 37°C . After purification, peptidoglycan was stored at -80°C .

Peptidoglycan degradation. Peptidoglycan hydrolysis was determined by zymogram assay as previously described (32). Briefly, purified IsdP or lysostaphin were separated by SDS-PAGE using 15% gels impreg-

nated with 0.2% (wt/vol) *Micrococcus lysodeikticus* ATCC 4698 lyophilized cells (Sigma) or iron-starved, heat-killed *S. lugdunensis* cells. Proteins were renatured in 25 mM Tris-HCl (pH 8.0) with 1% (vol/vol) Triton X-100 overnight at 37°C . To visualize lysis, gels were stained with 1% methylene blue in 0.01% KOH and destained with water. To quantitatively measure autolysis, purified IsdP was incubated with lyophilized *M. lysodeikticus* cells in 0.1 M sodium phosphate buffer, pH 7.4, at 37°C . The absorbance at 578 nm was monitored over 5 h. A decrease in absorbance is indicative of degradation.

Ion mobility-mass spectrometry (IM-MS) of cleaved peptidoglycan. Purified *S. lugdunensis* peptidoglycan was incubated overnight with 200 μ g IsdP in 500 μ l 0.1 M sodium phosphate, pH 7.4. The sample was split into two tubes, each containing 250 μ l, and one sample was incubated with 4 mg lysozyme overnight. Both samples were boiled for 5 min and centrifuged at $14,000 \times g$ for 15 min to remove insoluble material. Soluble peptidoglycan fragments were reduced by the addition of an equal volume of NaBH₄ (10 mg/ml) in 0.5 M borate buffer (pH 8.0) and incubated at room temperature for 20 min. The reaction was stopped with the addition of phosphoric acid.

Ultraperformance liquid chromatography-ion mobility-mass spectrometry (UPLC-IM-MS) experiments were carried out on an Acquity UPLC system (Waters Corp.) coupled to an electrospray ionization (ESI) source on a Synapt G2 HDMS IM-MS instrument (Waters). UPLC separations were performed using an HSS T3 C₁₈ column (1.8- μ m volume; 1.0 by 100 mm) held at 65°C . Samples were dried, resuspended in LC-grade water with 0.1% formic acid, and subsequently stored at 4°C in the autosampler during the course of the experiments. The 5- μ l sample injections were eluted at a flow rate of 50 μ l/min with a linear gradient from 99% solvent B (water with 0.1% formic acid) and 1% solvent A (acetonitrile with 0.1% formic acid) to 5% B and 95% A for a total run time of 25 min. IM-MS data were acquired in positive resolution mode over a mass range of 50 to 5,000 Da with interleaved fragmentation (MS^E) using collision-induced dissociation (IM-MS/MS) collected in the transfer region after the IM with a collision energy ramp from 10 to 50 eV. Source conditions were the following: 3.5 kV capillary, 120°C source temperature, 35-V sampling cone, 5-V extraction cone, 325°C desolvation temperature, and an IM traveling wave with a 650-ms^{-1} velocity and 40-V height. Data were processed off line using Driftscope software (Waters) to select mobility and UPLC-specific regions of the UPLC-IM-MS data. These regions then were exported to MassLynx software (Waters) for manual interpretation of cleavage sites. Structural fragmentation prediction was manually annotated using ChemBioDraw v12 (CambridgeSoft). Theoretical isotopic distributions were calculated with the molecular weight (MW) calculator isotopic distribution tool, v6.49 (M. Monroe, Pacific Northwest National Laboratories).

Immunoblotting. Bacteria were grown in TSB with 350 μ M DIP and incubated at 37°C with agitation for approximately 18 h. Cultures were normalized by the optical density at 600 nm (OD₆₀₀) and centrifuged at $3,000 \times g$ for 10 min. Culture supernatant was concentrated with phenylmethylsulfonyl fluoride (PMSF; 100 μ M) using 3,000-MW-cutoff filters (Amicon) and centrifuging at $3,000 \times g$ for 1.5 h at 4°C . Cell pellets were digested with lysostaphin (12.5 μ g/ml) in TSM (50 mM Tris, 0.5 M sucrose, 10 mM MgCl₂, pH 7.5) for 1 h at 37°C and centrifuged at $6,000 \times g$ for 15 min, and the soluble fraction was retained as the cell wall. Protoplasts were lysed by resuspending pellets in PBS and incubating with lysostaphin (0.2 μ g/ μ l) for 1 h at 37°C , followed by sonication. Protein was separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with polyclonal rabbit anti-IsdC serum or rabbit anti-FLAG (Sigma) and goat anti-rabbit conjugated to Alexa Fluor-680 (Invitrogen). Imaging and densitometric analyses were achieved using the Odyssey infrared imaging system (LI-COR) at 700 nm.

A previously described protocol was used to analyze IsdC surface exposure by immunoblotting (1). Three sets of cultures per strain were grown overnight in TSB with 350 μ M DIP, and 10 ml of each culture was normalized by optical density and centrifuged at $3,000 \times g$ for 10 min.

FIG 1 *isdP* is iron regulated and cotranscribed with the *S. lugdunensis* Isd operon. (A) Schematic of the *S. lugdunensis* Isd operon. *SLUG_00900* is predicted to encode an energy-coupling transporter. *SLUG_00910*, *SLUG_00920*, and *SLUG_00980* are predicted to encode ABC transporter components. (B) Expression of *isdP* (*SLUG_01010*) in iron-replete (TSB) or iron-depleted (TSB + DIP) conditions was determined by qRT-PCR and compared to that of the known iron-regulated genes *isdC* and *isdG* (34). (C) PCR was performed on cDNA from iron-rich (–DIP) or iron-starved *S. lugdunensis* (+DIP) using primers that amplify across the coding regions of *isdP* and *isdG* (arrows in panel A). (D) The PCR shown in panel C was performed using cDNA from iron-starved wild-type or Δ *isdP* mutant cells. RT, reverse transcriptase.

Pellets were resuspended in 500 μ l TSM, and the following enzymes were added: set 1, 50 μ g/ml proteinase K (New England BioLabs); set 2, 50 μ g lysostaphin; set 3, 50 μ g lysostaphin. Samples were incubated at 37°C for 30 min and centrifuged at $6,000 \times g$ for 15 min. The supernatants from set 1 samples were discarded, and the pellets were washed three times with 500 μ l TSM and then resuspended in fresh 500 μ l TSM with 50 μ g lysostaphin. Set 1 samples were incubated at 37°C for an additional 30 min and centrifuged at $6,000 \times g$ for 15 min. Supernatants from set 2 samples (containing lysostaphin-digested cell walls) were collected and incubated with 50 μ g/ml proteinase K for 30 min at 37°C. Supernatants from set 3 samples were collected and kept on ice. The soluble fraction was collected, and all samples were separated by 15% SDS-PAGE and transferred to nitrocellulose. Blots were probed with polyclonal rabbit serum against IsdC and secondary goat anti-rabbit conjugated to Alexa Fluor-680 (Invitrogen). The imaging of blots and densitometric analyses were achieved using the Odyssey infrared imaging system (LI-COR) at 700 nm. Statistical significance was determined using Student's *t* test.

Immunofluorescence. Fluorescent labeling of surface-exposed IsdC was performed as described previously (31). Briefly, overnight cultures grown in iron-rich media, iron-poor media, or iron-poor media with proteinase K for 1 h at 37°C were washed three times with cold phosphate-buffered saline and bound to poly-L-lysine-coated coverslips. Samples were fixed with 2% formaldehyde, washed with PBS, and blocked in 3% bovine serum albumin (BSA) for 1 h. Coverslips were washed, incubated with polyclonal rabbit anti-IsdC for 2 h, washed again, and incubated with goat-anti-rabbit Alexa Fluor-488 for 1 h. Coverslips were washed three final times and mounted on glass slides. Images were captured with a fluorescence microscope. ImageJ was used to quantify the fluorescent signal (33).

Hemoglobin utilization. *S. lugdunensis* strains were grown overnight in RPMI plus CAS with 250 μ M EDDHA. Strains harboring plasmids were grown in the same medium with the addition of 10 μ g/ml chloramphenicol. Overnight cultures were subcultured 1:50 into NRPMI supplemented with non-iron metals (25 μ M ZnCl₂, 25 μ M MnCl₂, 100 μ M CaCl₂, and 1 mM MgCl₂) with or without the addition of 10 μ g/ml chloramphenicol, 100 μ M EDDHA, and 2.5 μ g/ml purified human hemoglobin. Bacterial growth was monitored by measuring the absorbance at 600 nm.

Statistical analyses. Statistical significance was determined with the Student *t* test (GraphPad Prism). Results were considered significant if the *P* value was less than 0.05.

RESULTS

isdP is iron regulated and cotranscribed with the Isd operon.

The genomic location of a putative autolysin gene (*SLUG_01010*) in close proximity to the *S. lugdunensis* Isd locus suggested that this gene is cotranscribed with the Isd operon (Fig. 1A). Expression of the Isd system in *S. aureus* is repressed by Fur in high iron and derepressed in low iron, suggesting that *SLUG_01010* is regulated in response to iron levels (1). Transcriptional analyses demonstrated that *SLUG_01010* is not expressed when *S. lugdunensis* is grown in high iron but is upregulated when iron is chelated by 2,2-dipyridyl (DIP) (Fig. 1B). Expression levels of *SLUG_01010* in low iron were comparable to those of other Isd genes that increase expression under these conditions (34). To determine whether *SLUG_01010* was cotranscribed with the Isd operon, primers were designed to amplify across the coding regions of *isdG* and *SLUG_01010* (Fig. 1A, arrows). PCR analysis resulted in robust amplification across *isdG* and *SLUG_01010* from cDNA generated from iron-limited *S. lugdunensis* cells but not from cDNA from cells grown in iron-rich media (Fig. 1C). Furthermore, amplification was dependent on reverse transcriptase and was absent from a strain inactivated for *SLUG_01010* (Fig. 1D). These results demonstrate that *SLUG_01010* is iron regulated and is contained within the *S. lugdunensis* Isd operon. Based on these data and results described below, this gene will be referred to here as *isdP* (for iron-regulated surface determinant peptidoglycan hydrolase).

IsdP exhibits peptidoglycan hydrolase activity. Sequence analyses predicted that IsdP is an N-acetylmuramoyl-L-alanine amidase, containing an N-terminal signal sequence followed by a disordered domain, an FlgJ (flagellar rod assembly protein of *Salmonella* Typhimurium; lysozyme-like superfamily) domain, and a C-terminal cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain (Fig. 2A). The presence of a secretion signal suggests that IsdP is exported via the Sec system to the extracytoplasmic space, where it would have the potential to interact with the cell wall. FlgJ and CHAP domains typically confer peptidoglycan-hydrolyzing activity. The FlgJ domain exhibits β -N-acetyl-

FIG 2 IsdP is a peptidoglycan hydrolase. (A) Schematic of IsdP protein domains. SEC, secretion signal; DD, disordered domain; FlgJ, flagellar muramidase domain; CHAP, amidase domain. (B) Purified IsdP and lysozyme (lyso) were separated by 15% SDS-PAGE (left) or 15% SDS-PAGE containing lyophilized *Micrococcus lysodeikticus* cells (ATCC 4698; Sigma) (middle) or heat-killed, iron-starved *S. lugdunensis* cells (right). A zone of clearing indicates degradation. (C) IsdP was incubated with lyophilized *M. lysodeikticus* cells, and lysis was measured by monitoring the OD₅₇₈. Data from a representative experiment are shown. (D) An IM-MS conformational space map of a peptidoglycan treated with IsdP and lysozyme. The IM-MS plot represents data within the LC peak at 8.5 min. The peptidoglycan is annotated by the yellow box. (E) A mass spectrum selected from the area marked with the box in panel D. A theoretical isotopic distribution (dotted lines) for the proposed peptidoglycan structure (inset) is overlaid with the experimental data (solid line). Cleavage sites are indicated by arrows. Note the peak at 970.93 is the doubly charged peptidoglycan $[M-CO_2 + 2H]^{2+}$ with cleavage at the lysine side chain/glycine bridge.

lucosaminidase function, cleaving the β -(1-4)-glycosidic linkages connecting *N*-acetylmuramic acid (NAM) and *N*-acetyl-D-glucosamine (NAG) residues that make up the peptidoglycan sugar backbone (35). CHAP domains provide the amidase activity responsible for cleaving the amide bond between the stem peptide and the pentapeptide crossbridge or within the crossbridge itself (36, 37). To determine the hydrolytic function of IsdP, the peptidoglycan-degrading ability of purified protein was tested against *Micrococcus lysodeikticus* lyophilized cells and iron-starved, heat-killed *S. lugdunensis* cells using a gel-based zymogram assay as well as a quantitative analysis of *M. lysodeikticus* lysis (Fig. 2B and C, respectively). In both experiments, IsdP degraded peptidoglycan and lysed *M. lysodeikticus* and *S. lugdunensis* cells. These results confirm that IsdP is an autolysin with peptidoglycan hydrolyzing activity.

To identify the cleavage site of IsdP, purified *S. lugdunensis* peptidoglycan was incubated with IsdP, and the resulting mure-

peptides were analyzed using UPLC-IM-MS. The UPLC-IM-MS analysis yielded a putative structural assignment of cleavage sites based on exact mass with high mass accuracy, which was further supported by strong agreement with the theoretical isotopic distribution and MS/MS data. Reduction of background signal interferences results from the high degree of separation provided by combined liquid chromatography and ion mobility prior to MS, as illustrated in Fig. 2D. The spectra observed in Fig. 2E depict LC and IM selected MS data. MS and MS/MS data were simultaneously collected and manually interpreted. These spectral data support the assignment of a cleavage site at the side chain of the stem peptide lysine that interfaces with the pentaglycine crossbridge.

IsdP impacts release of IsdC into the supernatant. While our results indicate that IsdP is an iron-regulated autolysin that is coexpressed with the Isd system, the relationship between IsdP and the remainder of the Isd system was unclear. Since IsdP is an autolysin, we hypothesized that it impacts cell wall Isd protein

FIG 3 IsdP is required for release of IsdC. (A) Immunoblot analysis of IsdC in the supernatant of iron-starved *S. lugdunensis* strains. Arrows indicate the size of the ladder in kilodaltons. (B) Immunoblot analysis of the *S. lugdunensis* $\Delta isdP$ mutant complemented with *isdP*. Densitometric analysis is shown below. White bars, top IsdC-reactive band; gray bars, bottom IsdC-reactive band. (C) Immunoblot and densitometric analysis of IsdC in the supernatant of *S. lugdunensis* constitutively expressing *isdP*. (D) Immunoblot and densitometric analysis of IsdC in the supernatant of *S. aureus* expressing *isdP*. Error bars represent standard errors of the means (SEM). Asterisks denote $P < 0.05$ compared to wild-type *S. lugdunensis* pOS1 (B and C) or wild-type *S. aureus* (D) as calculated by Student's t test ($n > 3$).

abundance or localization. To address this hypothesis, a strain inactivated for *isdP* was analyzed for perturbations in IsdB and IsdC by immunoblotting. Cell wall fractions were isolated by digestion with lysostaphin, which hydrolyzes peptidoglycan by cleaving within the pentaglycine crossbridge. The abundance and distribution of the hemoglobin receptor IsdB was not changed in the $\Delta isdP$ mutant (data not shown). Interestingly, two IsdC-reactive bands between 20 and 25 kDa in size were observed in wild-type and $\Delta isdP$ cells following lysostaphin digestion of the cell wall (Fig. 3A). No difference in IsdC was observed in cell wall fractions between the wild type and the $\Delta isdP$ mutant (Fig. 3A). However, analysis of culture supernatants revealed a significant loss of the slower-migrating IsdC-reactive band in the $\Delta isdP$ mutant (Fig. 3A). This phenotype is dependent on IsdP, since expression of *isdP* in *trans* partially restored the presence of the slower-migrating IsdC band in the $\Delta isdP$ strain (Fig. 3B). Furthermore, constitutive expression of *isdP* in wild-type cells significantly increased the abundance of the slower-migrating IsdC band in the supernatant compared to the wild type with an empty plasmid (Fig. 3C). We next sought to determine the consequence of expressing *isdP* in *S. aureus*, which does not encode an Isd-associated autolysin. Interestingly, IsdP significantly enhanced the abundance of a higher-molecular-weight, IsdC-reactive band in the supernatant of *S. au-*

reus cultures (Fig. 3D). This is in contrast to the single *S. aureus* IsdC band typically observed following separation by SDS-PAGE (1). Taken together, these results indicate that IsdP alters the abundance of IsdC in culture supernatant.

The IsdP CHAP domain is necessary for IsdC release. Specific glutamic acid and aspartic acid residues have been demonstrated to confer hydrolase activity in FlgJ (35, 38). Likewise, CHAP domains contain highly conserved cysteine, histidine, and asparagine residues within an approximately 150-amino-acid region that are important for amidase function (39, 40). In keeping with this, we hypothesized that these residues in IsdP are required for IsdC release. Residues corresponding to amino acids required for FlgJ or CHAP activity were replaced with alanine in IsdP, expressed in the *S. lugdunensis* $\Delta isdP$ mutant, and tested for IsdC release by immunoblotting (Fig. 4). All three substitutions in the CHAP domain significantly impaired IsdC release into the supernatant (Fig. 4B). In contrast, substitutions in the FlgJ domain did not significantly alter IsdC release. Equivalent protein levels of the IsdP mutants were produced (see Fig. S1 in the supplemental material), suggesting that expression and stability were not impacted by these mutations and that the IsdC phenotype observed in the C393A, H454A, and N473A mutants was the result of the loss of hydrolytic function. These results are consistent with the identi-

FIG 4 IsdP CHAP domain is required for IsdC release. (A) Schematic of IsdP point mutants, indicated by arrows. (B) Immunoblot analysis of IsdC in the supernatant of the *S. lugdunensis* Δ isdP strain expressing IsdP mutants. Densitometric analysis is shown below and compared to that of the strain complemented with wild-type *isdP*. Error bars represent SEM. Asterisks denote a *P* value of <0.04 as calculated by Student's *t* test ($n > 3$).

fied peptidoglycan cleavage site for IsdP (Fig. 2D and E) and indicate that the amidase activity of the IsdP CHAP domain is required for the release of IsdC into culture supernatant.

SrtB sorting of IsdC, but not IsdP, is required for IsdC processing and release. Protein release into the supernatant likely reflects the state or localization of the polypeptide within the peptidoglycan layer as it is being continually shed. As IsdC is expressed during iron limitation, the protein undergoes multiple processing steps to reach maturation as a cell wall-anchored protein. In *S. aureus*, full-length protein (termed P1) is trafficked to the membrane due to the presence of an N-terminal signal sequence, which is cleaved following membrane transport through the Sec system. Passage across the bilayer terminates when the C-terminal hydrophobic domain and positively charged tail remain embedded in the membrane, resulting in the production of the P2 form of IsdC (41). In *S. aureus*, SrtB cleaves IsdC between the threonine and asparagine in the C-terminal NPQTN sorting sequence (this motif is NPQTS in *S. lugdunensis*) and covalently attaches IsdC to the pentapeptide crossbridge in assembled peptidoglycan, generating mature IsdC (M) (15). To determine whether *S. lugdunensis* SrtB is required for IsdC to be released into the culture supernatant, immunoblot analysis was performed on cell wall and supernatant fractions isolated from a strain inactivated for *srtB*. A higher-molecular-mass IsdC band (approximately 26 kDa) was observed in the cell wall fraction of the Δ srtB mutant, consistent with the inability of this mutant to further process the P2 form of IsdC into M (Fig. 5A). The only IsdC-reactive band observed in the supernatant of the Δ srtB mutant was

FIG 5 IsdC, but not IsdP, must be sorted by SrtB for IsdC release. (A) Immunoblot analysis of IsdC in the cell wall or supernatant of wild-type pOS1 or the Δ isdP pOS1, Δ isdC pOS1, or Δ isdC strain expressing *isdC* with an altered SrtB signal. (B) Immunoblot analysis of IsdC in the cell wall or supernatant of wild-type pOS1 or the Δ isdP pOS1, Δ isdP pidP, or Δ isdP strain expressing *isdC* with an altered putative SrtB motif. Densitometric analysis of supernatant immunoblots is shown below. Arrows indicate the size of the ladder in kilodaltons. White bars, top IsdC-reactive band; gray bars, bottom IsdC-reactive band. Error bars represent SEM. Statistical analyses of top IsdC-reactive bands were calculated by Student's *t* test ($n > 3$). (D) Immunoblot analysis of FLAG-tagged IsdP in cellular fractions. Densitometric analysis is shown below. White bars, protoplasts; gray bars, cell wall fraction. Error bars represent SEM.

FIG 6 IsdP alters IsdC localization in the cell wall. (A) Immunofluorescence of IsdC on the surface of *S. lugdunensis* cells. (B) Quantification of immunofluorescence shown in panel A relative to that of the $\Delta isdC$ mutant. Black bars, iron rich; white bars, iron poor; gray bars, iron poor plus proteinase K. (C) IsdC immunoblot and densitometric analyses of samples treated with proteinase K followed by cell wall isolation with lysostaphin. Data are presented as percent IsdC surviving proteinase K surface degradation (P→L) relative to total cell wall IsdC (L only). Gray bars, top IsdC-reactive band; white bars, bottom IsdC-reactive band. Error bars represent SEM. Statistical analysis was calculated using Student's *t* test, with *n* = 3 independent experiments. Asterisks denote *P* < 0.05 compared to the wild type under each condition (B) or to the wild type alone (C).

a band of approximately 15 kDa (Fig. 5A). This band could be a cleavage product of IsdC that is generated due to the nonproductive processing of the protein in this mutant. These results suggest that SrtB must attach IsdC to the cell wall in order for the IsdC species of approximately 21 to 23 kDa to be released into the supernatant.

To determine whether IsdC must be sorted to be released into the supernatant, a vector was constructed expressing the protein with an altered sorting sequence to arrest IsdC at the P2 species, preventing further processing to mature protein. Immunoblot analysis of the strain expressing the nonsorted IsdC (*isdC*^{ANPQTS}) revealed an absence of the 21- to 23-kDa IsdC-reactive bands in the cell wall as well as the supernatant (Fig. 5B). A faint 26-kDa band was observed in the cell wall fraction, and an IsdC-reactive band of lower molecular mass (~15 kDa) was observed in the supernatant of the nonsorted IsdC strain, similar to that found in the $\Delta srtB$ mutant (Fig. 5B). These data suggest that IsdC must be sorted for the 21- to 23-kDa IsdC bands to be released into the supernatant.

Sequence analysis of the IsdP peptide revealed a putative SrtB sorting motif, NPQTS, located within the N-terminal region of the FlgJ domain. Intriguingly, this sequence is found in only two proteins encoded within the *S. lugdunensis* genome, IsdC and IsdP. Based on this information, we hypothesized that IsdP is also a SrtB substrate and that SrtB-dependent processing of IsdP is required for its effect on IsdC. To address this hypothesis, a vector encoding *isdP* with an altered sorting motif was created and transformed into the $\Delta isdP$ mutant. Immunoblot analysis of superna-

nts demonstrated the restoration of the slower-migrating IsdC-reactive band in the strain expressing the nonsorted IsdP (*isdP*^{ANPQTS}) to a level equivalent to that of the $\Delta isdP$ mutant complemented with wild-type *isdP* (Fig. 5C). To determine whether SrtB is required to attach IsdP to the cell wall, localization of IsdP in a $\Delta srtB$ mutant was tracked by engineering a FLAG tag on the C terminus of the autolysin. Equivalent levels of IsdP were observed in protoplasts and cell wall fractions when the tagged protein was expressed in wild-type, $\Delta isdP$, or $\Delta srtB$ cells (Fig. 5D). Taken together, these data suggest that IsdP is not a SrtB substrate and that SrtB-dependent processing of IsdP is not required to release IsdC.

IsdP alters localization of IsdC within the cell wall. In *S. aureus*, IsdC is buried within the peptidoglycan layer and functions to relay heme from Isd proteins that acquire the molecule at the bacterial surface through the cell wall to the membrane transport system. Thus, IsdC is believed to span the depth of the cell wall but not be exposed on the surface. In contrast, IsdC in *S. lugdunensis* has been reported to be present on the cell surface and also is believed to span the cell wall to perform its relay function (21). Since (i) IsdC is affixed to the cell wall by SrtB, (ii) SrtB attachment of IsdC is required for its release into the supernatant, (iii) IsdP alters release of IsdC, and (iv) IsdP exhibits amidase activity, we hypothesized that IsdP impacts the localization of IsdC within the cell wall of *S. lugdunensis*. To address this hypothesis, surface-exposed IsdC was detected on wild-type, $\Delta isdP$ mutant, or $\Delta isdC$ mutant cells using immunofluorescence (Fig. 6A and B). Very little IsdC was detected on the surface of cells grown in iron-rich

FIG 7 IsdP is required for full growth with hemoglobin as a sole iron source. (A) Representative growth assay of *S. lugdunensis* strains grown in iron-deplete media in the presence of hemoglobin. (B) Graphical representation of data at 48 h from three independent experiments. Error bars represent SEM. (C) Growth complementation of *isdP*. (D) Graphical representation of growth complementation at 24 h. $P < 0.05$ (*) and $P < 0.0001$ (**) compared to the wild type as calculated by Student's *t* test.

TSB, consistent with the iron-dependent regulation of *isdC*, while abundant IsdC was observed under iron-restricted conditions in wild-type cells (Fig. 6A and B). Less IsdC was apparent on the surface of $\Delta isdP$ cells grown under iron-poor conditions compared to the wild type, although the difference was not statistically significant (Fig. 6A and B). Detection of surface-exposed IsdC following a brief proteinase K treatment revealed significantly less fluorescence of IsdC on $\Delta isdP$ cells than on the wild type, further supporting that less IsdC is presented on the cell surface in this mutant (Fig. 6A and B).

While these results indicate a defect in IsdC surface presentation in the *isdP* mutant, immunoblot analysis suggested there was no difference in total cell wall-associated IsdC between the $\Delta isdP$ mutant and the wild type (Fig. 3A). To directly compare surface-exposed IsdC to total cell wall IsdC in each strain, cell wall fractions were digested with proteinase K prior to or following isolation with lysostaphin. Proteinase K treatment before lysostaphin digestion should result in cleavage of proteins exposed on the surface of the cell wall, while proteins buried within the peptidoglycan layer are protected from degradation. In contrast, proteinase K treatment following isolation of the cell wall with lysostaphin will expose all cell wall proteins to degradation. Thus, these conditions allow visualization and quantification of surface-exposed IsdC (susceptible to proteinase K treatment before lysostaphin digestion) compared to IsdC that is buried within the peptidoglycan layer (resistant to proteinase K treatment before lysostaphin digestion). Surface-exposed and buried IsdC then can

be compared to total cell wall IsdC (cell wall fraction isolated by lysostaphin digestion alone). The results of these experiments demonstrated that more IsdC survived proteinase K digestion in the $\Delta isdP$ mutant than in the wild type (Fig. 6C). Interestingly, the top IsdC-reactive band appeared to be more readily degraded by proteinase K before lysostaphin digestion, indicating that this IsdC species is more surface exposed than the lower band. Taken together, these data indicate that less of the total cell wall-associated IsdC in the $\Delta isdP$ mutant is presented on the surface and instead is buried within the peptidoglycan layer.

IsdP is required to support hemoglobin-dependent growth.

As an integral component of the Isd system, IsdC is required for heme uptake. Due to the impact of IsdP on IsdC, we hypothesized that loss of IsdP impacts heme acquisition. Indeed, growth in the presence of hemoglobin as a sole iron source was impaired in a strain inactivated for *isdP* (Fig. 7A and B). Strains inactivated for *isdC* or *srtB* also were decreased for hemoglobin-dependent growth. Hemoglobin-dependent growth was restored upon expression of *isdP* in *trans* (Fig. 7C and D). These results indicate that IsdP contributes to *S. lugdunensis* heme acquisition.

DISCUSSION

Heme acquisition is critical to the survival and virulence of many human pathogens during infection. The initial steps of heme acquisition through the Isd system (hemoglobin binding, heme extraction, and heme shuttling through the cell wall) all require the activity and coordination of cell wall-bound Isd proteins. While

FIG 8 Model of IsdP contribution to Isd-dependent heme acquisition. Under iron-restricted conditions, IsdP is expressed with the *S. lugdunensis* Isd system. IsdP remodels the cell wall by cleaving the stem peptide and pentapeptide crossbridge of mature peptidoglycan, exposing amide groups to which IsdC (blue) is anchored by SrtB. A subpopulation of IsdC also is presented on the cell surface (green). Coordination of IsdP and SrtB populates the cell wall with IsdC such that surface-exposed IsdC can bind extracellular heme and IsdC spanning the peptidoglycan layer shuttles heme from the surface to the membrane.

much is known regarding the mechanism of sortase-mediated cell wall attachment of these proteins, several questions remain, including how SrtB is able to anchor IsdC to mature, assembled peptidoglycan. It has been suggested that *S. aureus* SrtB affixes IsdC to unlinked peptidoglycan strands, thereby exploiting exposed peptide crossbridges that otherwise would be hidden in mature, cross-linked cell wall (15). The data presented here suggest that in *S. lugdunensis*, IsdP cleaves peptidoglycan to expose previously inaccessible regions of the cell wall and contributes to SrtB-mediated anchoring of proteins to the peptidoglycan layer. IsdP is a peptidoglycan hydrolase that influences cell wall attachment and processing of IsdC, a major component of the Isd system required for shuttling heme through the thick peptidoglycan layer. This work demonstrates the heretofore unprecedented role of an autolysin in bacterial heme acquisition. Thus, we propose that, in coordination with SrtB, IsdP facilitates cell wall positioning of IsdC to impact heme uptake through the Isd system (Fig. 8).

As *S. lugdunensis* enters its host, it senses an iron-deplete environment and induces expression of Isd genes through derepression of the operon by the iron-dependent regulator, Fur. These studies describe an autolysin that is coexpressed with the Isd heme uptake system in iron-restricted conditions. Coregulation of *isdP* with the Isd operon suggests that the low-iron environment signals cell wall remodeling in coordination with expression of the heme binding Isd proteins. This is reminiscent of known specialized autolysins that are encoded adjacent to the systems upon which they act. These autolysins modify peptidoglycan to accommodate the assembly and function of large structures that span the cell wall, including type II, III, and IV secretion systems, pili, and flagella (38, 42, 43).

Our results demonstrate that IsdP exhibits a peptidoglycan-hydrolyzing capability that cleaves the cell wall between the stem peptide and pentaglycine crossbridge. While IsdP is predicted to contain both an FlgJ muramidase and a CHAP amidase domain, only the CHAP domain is required for IsdC release. Nevertheless,

it remains possible that IsdP is a bona fide bifunctional autolysin and may undergo cleavage of the FlgJ and CHAP domains to allow for their separate activities. Such bifunctional hydrolases are common in bacteria, including the main autolysin in *S. lugdunensis*, AtlL (32, 44).

IsdC is currently the only known target of IsdP activity. Moreover, IsdC must be anchored to the cell wall by SrtB to be affected by IsdP. The presence of IsdC-reactive bands in the supernatant is likely due to cell wall shedding and may simply reflect the attachment of IsdC within the peptidoglycan layer. Thus, loss of the slower-migrating IsdC band in the $\Delta isdP$ mutant is indicative of a perturbation in IsdC processing and attachment. UPLC-IM-MS analysis demonstrated that IsdP cleaves between the pentaglycine crossbridge and stem peptide of mature, cross-linked peptidoglycan. In *S. aureus*, IsdC is affixed to the cell wall at this very location by SrtB (15). In keeping with this, it is plausible that IsdP is required to cleave mature peptidoglycan to allow for SrtB-mediated anchoring of IsdC.

The identity of the two IsdC-reactive bands is not known. It is possible that the bands differ in the amount of cell wall fragment attached to them. The difference in size between the two bands is approximately 2 to 4 kDa. This difference is consistent with the addition of the staphylococcal pentaglycine crossbridge and tetrapeptide stem peptide (~2.3 kDa). This cell wall fragment was found to be attached to the threonine in the *S. aureus* IsdC sorting sequence following digestion of the cell wall with $\Phi 11$ hydrolase, which cleaves between L-Ala and the sugar backbone or between D-Ala and the pentaglycine crossbridge (15). Our data suggest that (i) IsdP is an amidase that could cleave the cell wall at similar sites and (ii) expression of IsdP impacts the presence of the top IsdC band in the supernatant. It is possible that the top IsdC-reactive band represents mature IsdC attached to a cell wall fragment while the bottom IsdC-reactive band is mature IsdC that is not attached to the cell wall. This possibility is consistent with an alternative hypothesis for IsdP function in which IsdP cleaves peptidoglycan

to release cell wall-anchored IsdC into the supernatant. Such a hypothesis is supported by the loss of the top IsdC band in the supernatant of the *isdP* mutant; however, this theory does not explain the decrease in surface-exposed IsdC in the absence of IsdP.

In *S. aureus*, the containment of IsdC within the cell wall reflects its role as a heme transfer protein, shuttling the molecule through the thick peptidoglycan layer to the Isd membrane transport system. In contrast, our data are consistent with other reports that IsdC not only spans the cell wall but also is surface exposed in *S. lugdunensis* (45). Furthermore, our results suggest that *S. lugdunensis* SrtB and IsdP coordinate to populate the cell wall and surface with IsdC to facilitate heme shuttling through the peptidoglycan layer (Fig. 8). It is intriguing to note the correlation of the presence of an autolysin with the absence of IsdA within an Isd system. *S. aureus* IsdA is presented on the surface of the cell and is able to bind heme but does not directly participate in heme extraction from hemoglobin. Instead, IsdA passes heme to IsdC to initiate its transport through the cell wall (6, 46). Since *S. lugdunensis* lacks IsdA, it would rely entirely on IsdB for heme binding at the cell surface. However, presentation of IsdC on the exterior of the cell wall also could contribute to heme binding. One possibility is that exposure of IsdC could be achieved through anchoring to lipid II, similar to IsdB and IsdA in *S. aureus*. If this is the case, IsdP may be required for anchoring of IsdC to lipid II, allowing for dual cell wall localization of IsdC (i.e., surface presentation via lipid II anchoring and attachment to sites hidden within the mature peptidoglycan layer). We hypothesize that the dual localization of IsdC functions to enhance the heme uptake capacity at the cell surface in an effort to compensate for the lack of surface-exposed, heme-binding IsdA. Thus, in *S. lugdunensis*, we propose that IsdC performs two functions: heme binding at the cell surface and heme transfer through the peptidoglycan layer to the membrane.

IsdC performs a critical function in hemoglobin-dependent heme uptake, linking heme extraction from hemoglobin at the cell surface to heme import across the bacterial membrane. As such, any perturbation in the localization or function of IsdC would significantly impact activity of the Isd system as a whole. Indeed, growth with hemoglobin as a sole iron source is significantly impaired in a strain inactivated for *isdC*. Moreover, loss of *isdP* also results in decreased growth with hemoglobin and correlates with the impact of IsdP on IsdC. The growth defect of the Δ *isdP* mutant does not phenocopy that of the Δ *isdC* mutant, suggesting that loss of *isdP* does not completely abolish IsdC activity. Considering the contribution of IsdP to heme uptake and the importance of Isd system function in the pathogenesis of other organisms, IsdP is likely to contribute to virulence of *S. lugdunensis* during infection.

In summary, we report the characterization of the Isd autolysin IsdP and describe its role in hemoglobin-dependent heme acquisition in *S. lugdunensis*. Isd systems differ slightly among bacterial pathogens and likely reflect the pathogenic mechanisms of the different species that express them. For example, *Bacillus anthracis* produces secreted Isd hemophores and an S-layer heme binding protein in addition to the core Isd system to enhance heme uptake (47–49). Thus, the inclusion of an autolysin in the *S. lugdunensis* Isd system may signify a unique requirement of this pathogen. Finally, these studies reveal a more in-depth picture of potential mechanisms driving cell wall reorganization that allow for inte-

gration of peptidoglycan-attached proteins in response to environmental changes.

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