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MOLECULAR PATHOGENESIS

Identification, Cloning, and Characterization of *Staphylococcus pseudintermedius* Coagulase

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ABSTRACT Coagulase activation of prothrombin by staphylococcus induces the formation of fibrin deposition that facilitates the establishment of infection by *Staphylococcus* species. Coagulase activity is a key characteristic of *Staphylococcus pseudintermedius*; however, no coagulase gene or associated protein has been studied to characterize this activity. We report a recombinant protein sharing 40% similarity to *Staphylococcus aureus* coagulase produced from a putative *S. pseudintermedius* coagulase gene. Prothrombin activation by the protein was measured with a chromogenic assay using thrombin tripeptide substrate. Stronger interaction with bovine prothrombin than with human prothrombin was observed. The *S. pseudintermedius* coagulase protein also bound complement C3 and immunoglobulin. Recombinant coagulase facilitated the escape of *S. pseudintermedius* from phagocytosis, presumably by forming a bridge between opsonizing antibody, complement, and fibrinogen. Evidence from this work suggests that *S. pseudintermedius* coagulase has multifunctional properties that contribute to immune evasion that likely plays an important role in virulence.

KEYWORDS coagulase, immunoglobulin binding, *Staphylococcus*, *Staphylococcus pseudintermedius*, complement C3

Staphylococcus pseudintermedius is a Gram-positive, coagulase-positive opportunistic bacterial pathogen commonly found on the skin and in the nares, mouth, pharynx, and anus of dogs, cats, and horses (1). Bacterial pyoderma caused by *S. pseudintermedius* is one of the most common diseases seen in small-animal veterinary practices worldwide, and it occasionally causes other diseases, including otitis and urinary tract infections (2).

The coagulation system prevents dissemination of microbial organisms by fibrin deposition, with subsequent removal by phagocytes (3). However, some bacterial pathogens, such as *Staphylococcus aureus*, employ a virulence strategy that utilizes coagulation proteins, providing protection from immune defenses and escape from phagocytic killing. Staphylocoagulase activates a host trypsinogen-like enzyme precursor, prothrombin, without proteolytic cleavage to form staphylothrombin and directly initiate blood clotting and subsequent conversion of fibrinogen to fibrinopeptides and self-polymerizing fibrin (4–6). These products mediate adhesion to plasma and extracellular matrix proteins (5, 7). Thus, coagulase embeds bacteria within a network of fibrin, protecting them from immune recognition, and enables staphylococci to multiply as a bacterial community at the center of lesions, forming bacterial microcolonies (8).

Coagulase activity observed in *S. aureus*, *S. pseudintermedius*, *Staphylococcus intermedius*, *Staphylococcus delphini*, and *Staphylococcus schleiferi* subsp. *coagulans* and in a coagulase-variable species, *Staphylococcus hyicus* subsp. *hyicus*, is associated with virulence (9–11). Most of these species, including *S. pseudintermedius*, have been classified as coagulase

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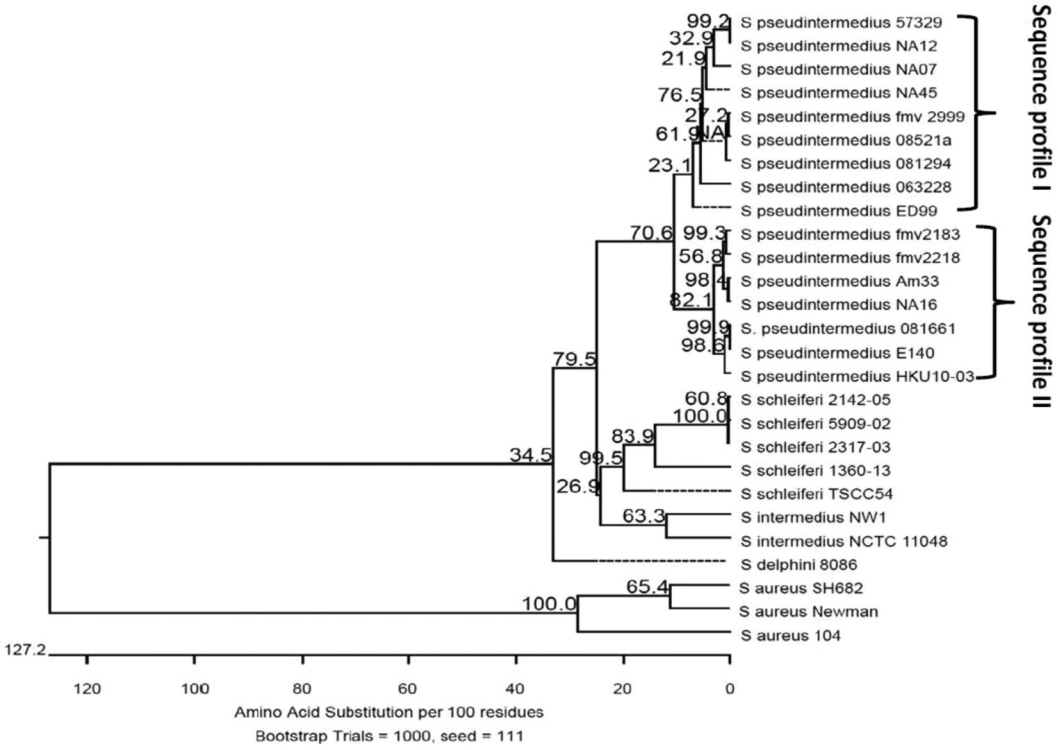

FIG 1 Coagulase phylogenetic relationships. Phylogenetic tree showing the inferred evolutionary relationships among coagulase amino acid sequences from various staphylococcus species. The scale bar represents a genetic distance that is equivalent to the number of amino acid substitutions per 100 residues. Bootstrap probability is indicated at diverging points of branches. Coagulase sequences and their GenBank accession numbers are as follows: *S. aureus* strain SH682, coagulase type III (ABW83067.1), *S. aureus* subsp. *aureus* strain Newman, coagulase type III (WP_000744074), *S. aureus* strain 104, coagulase type I (BAD98736.1), *S. intermedius* NW1 (WP_086428747.1), *S. intermedius* NCTC 11048 (WP_019169028.1), *S. schleiferi* TSCC54 (WP_060829724.1), *S. schleiferi* 1360-13 (WP_050345467.1), *S. schleiferi* 2142-05 (AKS71128.1), *S. schleiferi* 2317-03 (AKS73300.1), *S. schleiferi* 5909-02 (WP_050337525.1), *S. delphini* 8086 (WP_019166910.1), *S. pseudintermedius* HKU-103 (WP_015728558.1), and *S. pseudintermedius* ED99 (WP_014614858.1). Brackets indicate the two coagulase sequence profiles; sequence profile I is similar to the coagulase sequence of strain 063228, and sequence profile II is similar to the coagulase sequence of strain 081661.

positive based on plasma clotting tests. However, the protein responsible for this activity is not well defined except in *S. aureus*. In 2001, Komori et al. isolated a protein with coagulase activity from *S. intermedius* that differs physically from other coagulases (12). Since that time strains once identified as *S. intermedius* have been reclassified as *S. intermedius* and *S. pseudintermedius* strains (1). The latter species contains a gene often annotated as encoding coagulase protein (13). The predicted protein, however, is not the same as the previously reported *S. intermedius* protein in size or amino acid composition (12). Preliminary analysis revealed that only one *S. pseudintermedius* protein contains the domains associated with bacterial coagulase activity and that it is present in all available genomes. This candidate *S. pseudintermedius* coagulase protein was studied to determine its biological properties and potential role in the virulence of *S. pseudintermedius*.

RESULTS

Amplification and sequencing of the *coa* gene from *S. pseudintermedius*. The putative *S. pseudintermedius* coagulase gene (*coa*) was amplified by PCR from all 15 strains of *S. pseudintermedius* tested and yielded a product of the expected size of approximately 1,500 bp. DNA sequencing among the 15 isolates showed that two coagulase sequence profiles were observed; sequence profile I was homologous to that of strain 063228, and sequence profile II was homologous to that of strain 081661, as shown in Fig. 1. The complete *coa* gene encodes a protein with a predicted molecular mass of 56,490 Da. It corresponds to bases 2612742 to 2614241 in the genome of *S. pseudintermedius* strain 081661 (GenBank accession number CP016073.1). The pre-

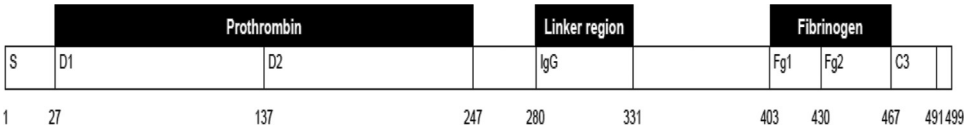

FIG 2 Coagulase protein structure. The predicted structure of *S. pseudintermedius* coagulase protein from *S. pseudintermedius* strain 081661, including the predicted signal peptide (S), the D1 and D2 domains for prothrombin binding, the linker (L) domain containing an IgG binding site, the fibrinogen binding domain of coagulase, and complement binding domain, is shown. Numbers indicate amino acid positions.

dicted protein has a signal sequence in residues 1 to 26 and contains an N-terminal prothrombin binding site in residues 27 to 247, with 34% identity to the D1 and D2 domains of *S. aureus* coagulase. At residues 280 to 331 in the linker region between the prothrombin binding domain and fibrinogen binding domains, it contains a site similar to the two IgG binding domains of surface immunoglobulin-binding protein (Sbi) of *S. aureus* (GenBank accession number WP_061740244.1) with 19% and 21% identity, respectively. At fibrinogen binding regions, residues 403 to 430 and residues 431 to 467, it shares 38% and 50% amino acid identity, respectively, to the corresponding regions in *S. aureus* extracellular fibrinogen binding protein domain A (Efb-A) (GenBank accession number SAZ21881.1). Residues 467 to 491 have 44% identity to complement binding domains of the Sbi protein of *S. aureus* (GenBank accession number WP_061740244.1) (Fig. 2; see also Fig. S1 to S4 in the supplemental material). Whereas 25% identity is generally considered a good predictor of conserved function, only phenotypic testing can provide definitive results (14).

Coagulase tests. The clotting activity of *S. pseudintermedius* strains ($n = 15$) was determined by the plasma tube clotting method, and results showed coagulase-positive reactions in 40% (6/15) of the isolates, with only strain 081661 producing a positive result at 4 h of incubation while strains NA12, 063228, 08521a, 081294, and E140 produced positive results after a 48-h incubation.

Tested with a chromogenic assay, coagulase activity was detected in supernatants from all *S. pseudintermedius* strains within 1 h (Fig. 3). Greater hydrolysis of the chromogenic substrate occurred with bovine prothrombin than with human prothrombin. The reactivity of *S. pseudintermedius* strains with bovine prothrombin was higher than that of the *S. aureus* positive control. Coagulase activity of isolate E140 was higher

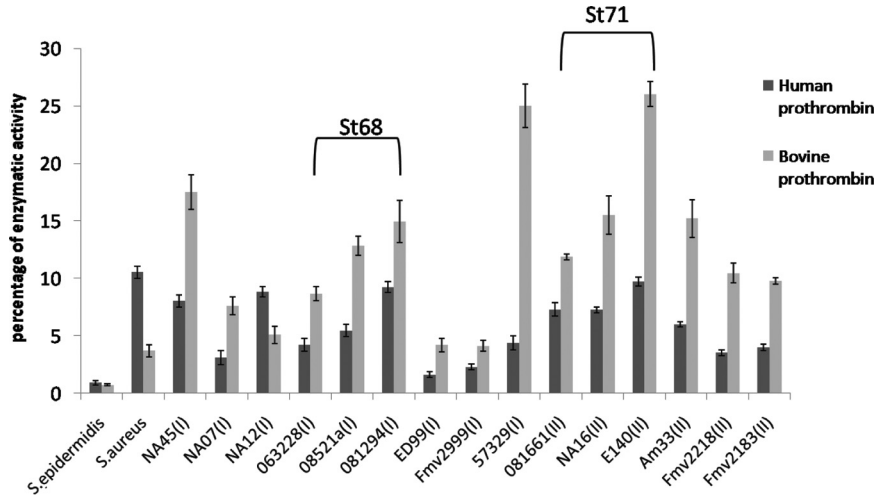

FIG 3 Coagulase activity of *S. pseudintermedius*. Coagulase activity in concentrated bacterial supernatants was determined using a chromogenic assay. The percentage of coagulase-prothrombin complex activity was inferred by the change in the rate of chromogenic substrate S-2238 hydrolysis (Δ absorbance) over time (Δ time). The parenthetical I and II with the designations refer to coagulase sequence profiles. ST68 isolates (063228, 08521a, and 081294) had significantly lower levels of human and bovine prothrombin activation than ST71 isolates (081661, E140, and NA16) ($P < 0.001$).

than that of all other strains, followed by that of NA45 and 081661, with strain 063228 exhibiting the lowest activity. Significantly higher reactivity occurred with both human and bovine prothrombins among strains with sequence profile II (including all sequence type 71 [ST71] isolates) than in isolates with sequence profile I (including all ST68 isolates) ($P < 0.001$). *S. aureus* hydrolysis of the chromogenic substrate was significantly greater than that of all *S. pseudintermedius* strains in its reactivity with human prothrombin, followed, in order, by the reactivity of *S. pseudintermedius* strains 081294, NA45, 08521a, and 063228.

Recombinant coagulase binding to prothrombin, IgG, and complement C3. Recombinant *S. pseudintermedius* coagulase protein (prCoa) bound whole IgG and both Fc and Fab fragments of canine IgG, as determined by enzyme-linked immunosorbent assay (ELISA) (Fig. 4A), with the greatest binding to IgG, followed by that to the Fc fragment and less binding to Fab fragments. prCoa bound canine C3 captured from serum in a concentration-dependent manner (Fig. 4B). There was no significant difference in the binding levels of prCoa to bovine and human prothrombins (Fig. 4C).

Recombinant coagulase activity. prCoa (50 and 100 μg/ml) yielded positive coagulase activity within 5 h after inoculation by the plasma tube clotting method and remained positive at 24 h. It produced detectable hydrolysis by 1 h in the chromogenic coagulase assay, and this hydrolysis was greater with bovine prothrombin than with human prothrombin (Fig. 5).

Coagulase-mediated binding of fibrinogen to *S. pseudintermedius* and *Staphylococcus epidermidis*. Using flow cytometry it was observed that coagulase facilitated fibrinogen binding to opsonized bacteria (Fig. 6 and S5). To confirm the function of the putative coagulase, we analyzed the ability of purified prCoa to facilitate fibrinogen deposition on *S. pseudintermedius* and the coagulase-negative, non-biofilm producer *S. epidermidis* ATCC 12228. The amount of fibrinogen deposited on *S. epidermidis* was equivalent to that found on *S. pseudintermedius*.

Coagulase inhibition of phagocytosis in the presence of rabbit plasma. prCoa reduced phagocytosis when incubated with rabbit plasma and dog blood. The reduction of phagocytosis was dependent on the concentration of coagulase (Fig. 7 and S6).

DISCUSSION

The goal of this study was to identify the *S. pseudintermedius* coagulase gene and to characterize the protein it encodes. Coagulase induces fibrin deposition and enables the establishment of staphylococcal disease (8). *S. pseudintermedius* is a major etiological agent of skin and soft tissue infections in canines (1, 2), and coagulase activity is considered a key virulence factor for this organism. The protein responsible for this activity and its associated gene have not been previously definitively identified and characterized. A protein with coagulase activity was isolated from *S. intermedius* in 2001 (12) prior to the delineation of *S. pseudintermedius* as a separate species in 2005 (1). Neither the sequence of the *S. intermedius* protein nor the gene encoding it was determined, and, therefore, a direct comparison with our findings is not possible. However, the *S. intermedius* coagulase protein differs in size and amino acid composition from the one described in the current study. The *S. intermedius* protein has a molecular weight of 64,500 Da and consists of 615 amino acids. The predicted mature *S. pseudintermedius* coagulase is considerably smaller, consisting of 499 amino acids with a molecular mass of 53,760 Da without its signal sequence and a mass of 56,490 Da with the signal sequence (varying slightly between strains). It migrates on SDS-PAGE gels at approximately 62 kDa (see Fig. S7 in the supplemental material). Thus, these appear to be two distinctly different proteins or variants.

S. aureus produces a family of well-characterized proteins with overlapping functions called zymogen activator and adhesion proteins (ZAAPs) that serve as a basis for comparison with other species of staphylococci (7). These proteins include staphylocoagulase, von Willebrand factor binding protein (vWBP), and fibrinogen binding protein. The *S. pseudintermedius* coagulase in the current study has a domain structure similar to the structures of ZAAPs and is 40% similar to the closest corresponding *S.*

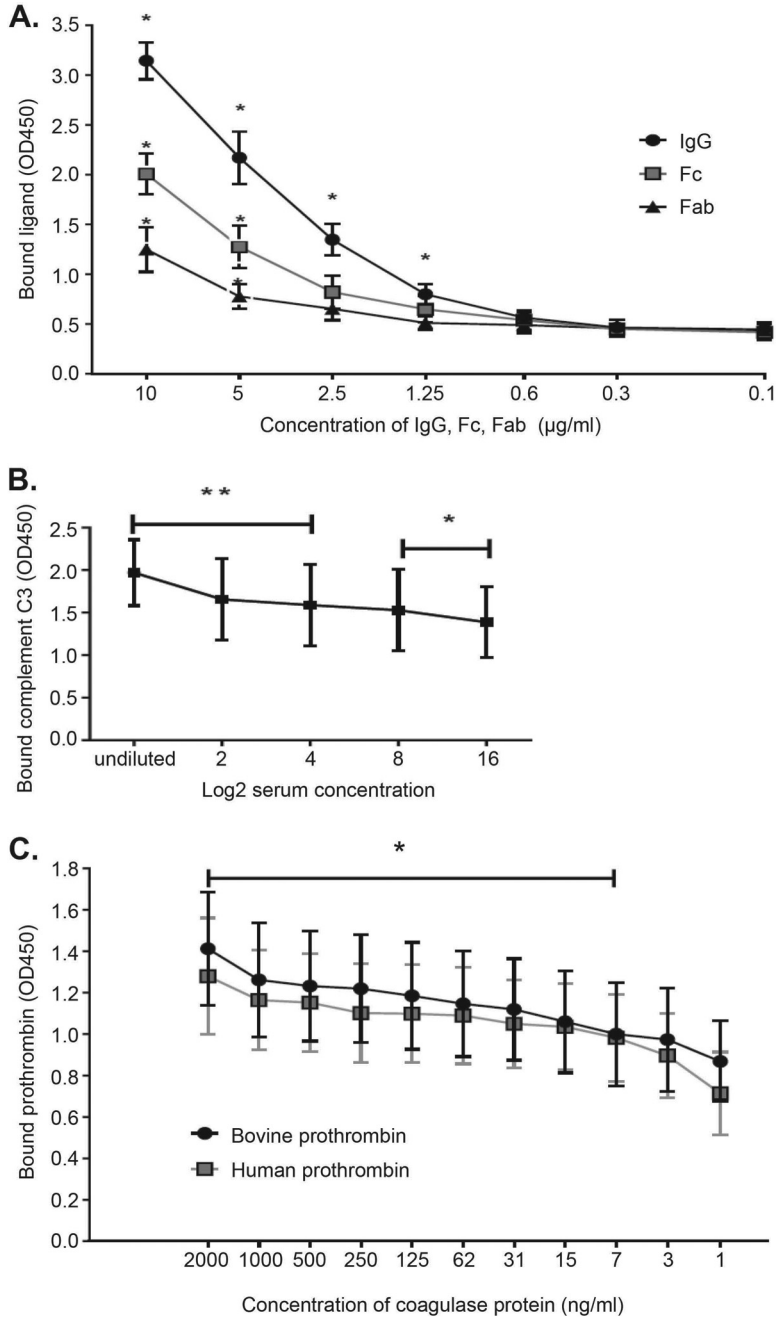

FIG 4 Full-length coagulase protein binding to prothrombin, canine IgG, and complement C3. Data were obtained from three independent experiments. (A) ELISA results showing coagulase binding of canine IgG, Fc, and Fab. There was a significant interaction of treatment (IgG, Fc, and Fab) with the binding of coagulase protein ($P = 0.010$). All three treatments had significant differences between results at different concentrations ($P < 0.001$). For IgG concentrations of 10, 5, 2.5, and 1.25 μg/ml, binding was significantly different from that of the negative control (*, $P < 0.001$). For Fc and Fab concentrations of 5 and 10 μg/ml, binding was significantly different from that of the negative control (*, $P < 0.001$). The coagulase protein bound more IgG and Fc than Fab. At 5 and 10 μg/ml, IgG binding was significantly higher than that of Fab ($P = 0.008$), and binding of IgG and Fc was marginally different ($P = 0.054$). (B) Binding of coagulase to immobilized complement C3. Results for all concentrations were significantly different from the result for the negative control. The first three concentrations differed at a *P* value of <0.01 (**) (0.000, 0.001, and 0.003, respectively), and the last two concentrations differed at a *P* value of <0.05 (*) (0.011 and 0.045). (C) Binding of recombinant coagulase protein to immobilized human and bovine prothrombins. There was no significant difference ($P = 0.785$) in coagulase protein binding levels to human and bovine prothrombin. Binding at all concentrations significantly differed from that of the negative control (*, $P < 0.01$) except at coagulase concentrations of 3.9 ng/ml ($P = 0.053$) and 1.9 ng/ml ($P = 0.739$). OD450, optical density at 450 nm.

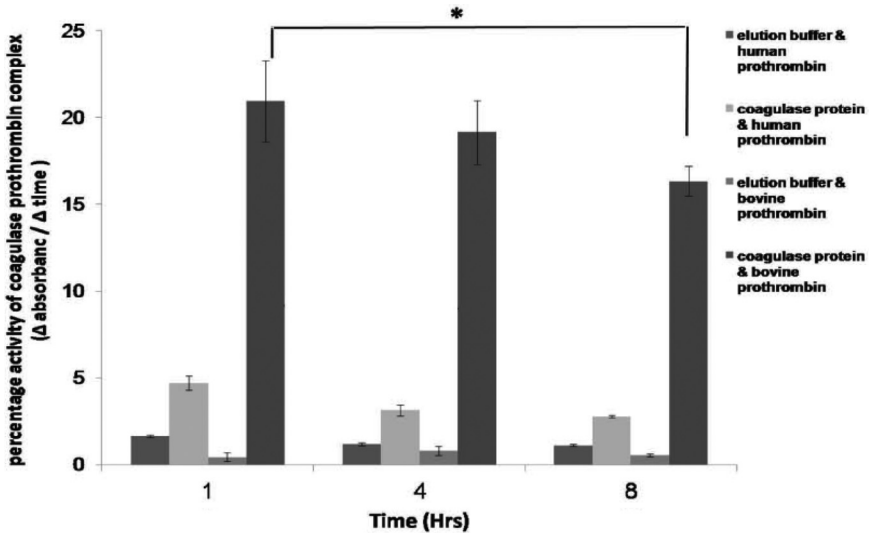

FIG 5 Activity of *Staphylococcus pseudintermedius* coagulase recombinant protein by chromogenic assay. The percentage of coagulase-prothrombin complex activity was inferred by the change in the rate of chromogen S-2238 hydrolysis (Δ absorbance) over time (Δ time). The coagulase-bovine prothrombin complex percentage activity was significantly higher than the activities of the coagulase-human prothrombin complex and of elution buffer with bovine prothrombin (*, $P < 0.001$). There was no significant difference between coagulase and elution buffer interactions with human prothrombin ($P = 0.522$). The percentage of activity did not significantly decrease over time.

aureus coagulase protein. It differs from vWBP in that it lacks a 26-amino-acid motif associated with vWBP activity (15).

Secreted coagulase protein does not interact directly with the bacterial surface but can induce the formation of a fibrinogen shield at some distance from it (16). Coagulase has the ability to associate directly with fibrinogen without the aid of prothrombin (17, 18). This interaction allows coagulase to localize at the cell surface by binding to fibrinogen retained by cell wall-anchored proteins such as clumping factor A (ClfA). The similarity of the fibrinogen binding domains in extracellular fibrinogen binding protein and *S. pseudintermedius* coagulase shown in this study suggests that coagulase may bind fibrinogen to induce a protective fibrinogen-containing barrier. In this study, using flow cytometry, we identified the coagulase-mediated attachment of soluble fibrinogen to the opsonized bacterial surface, creating a potentially protective shield. *S. pseudintermedius* recombinant coagulase-mediated fibrinogen deposition occurred not only with *S. pseudintermedius* but also on non-coagulase-producing *S. epidermidis*. This observation supports the hypothesis that *S. pseudintermedius* coagulase may facilitate the coating of coagulase-negative bacteria with a protective layer of fibrinogen. This finding warrants further investigation.

Phagocytosis by neutrophils is strongly enhanced by opsonization of bacteria with plasma factors such as antibodies and complement activation products (C3b and iC3b) (8). *S. pseudintermedius* coagulase has a novel property in that it binds canine IgG, Fc, and Fab. Based on the similarity of the IgG binding domain of *S. aureus* Sbi and linker domain of *S. pseudintermedius* coagulase, this domain is the most likely region of *S. pseudintermedius* that binds IgG. Moreover, this protein is able to bind canine C3, as shown in binding assays performed in this study. The most likely region for binding with C3 is the coagulase segment of residues 467 to 491 as it has 44% identity to the complement binding domain of the Sbi protein of *S. aureus*, and both contain the same R and N amino acids with 7 linker amino acids in between.

All of the *S. pseudintermedius* coagulase sequences in this study contain the same functional motifs. The predicted *S. pseudintermedius* coagulase proteins diverge into two major groups that correlate with multilocus sequence types (MLSTs). The clinical relevance of higher reactivity among isolates sharing the 081661 genotype, which

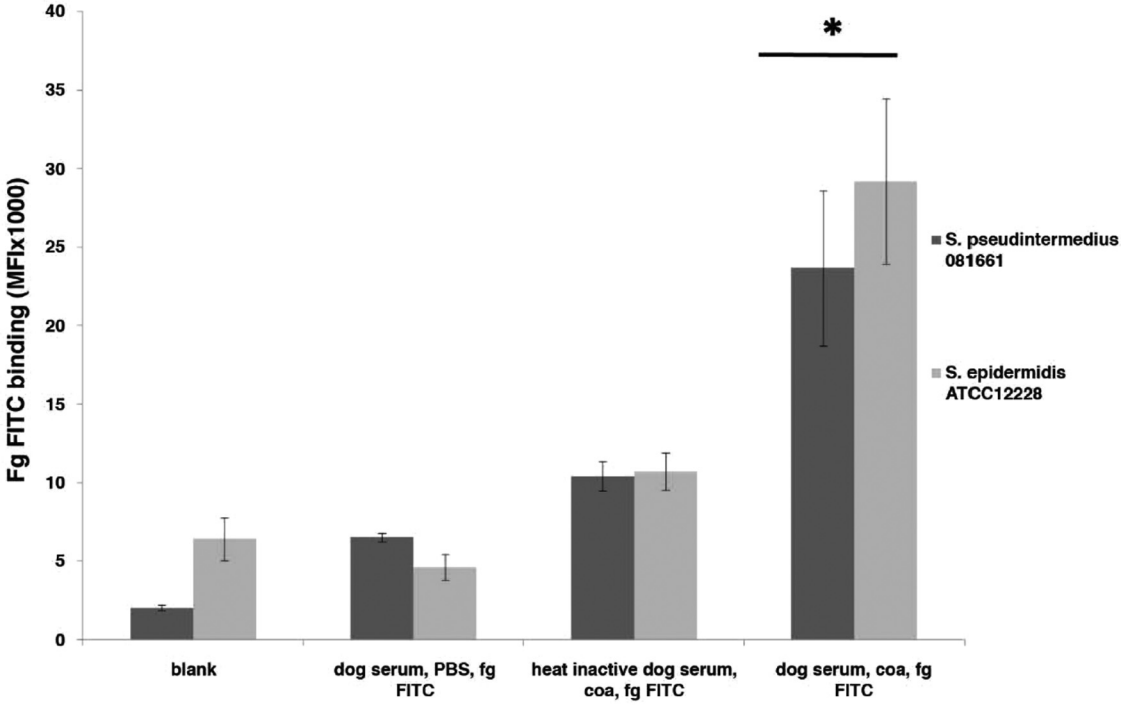

FIG 6 Coagulase-mediated fibrinogen binding. Binding of FITC-labeled fibrinogen (Fg; 50 μg/ml) to serum-opsonized *S. pseudintermedius* and *S. epidermidis* in the presence of coagulase (coa) was determined. The graph represents the mean and standard error of three independent experiments. The difference in FITC-fibrinogen deposition levels between bacterial strains (*S. pseudintermedius* 081661 and *S. epidermidis* ATCC 12228) were not significant ($P = 0.287$). When the result for each serum type was compared to that of the negative control (blank), only the combination of dog serum, coagulase, and fibrinogen-FITC had a significantly high mean fluorescence intensity (MFI) (*, $P < 0.001$). Values for the combinations of dog serum and fibrinogen-FITC ($P = 1.000$) and of heat-inactivated dog serum, coagulase, and FITC-fibrinogen ($P = 0.091$) did not significantly differ from the value for the negative control.

included all of the ST71 isolates, is of interest. Coagulase tests are routinely used to identify *S. aureus* and other pathogenic staphylococci. Although less than half of the *S. pseudintermedius* isolates in this study tested positive with the rabbit plasma tube coagulation test, the *coa* gene was present in all samples, as determined using PCR and sequencing. Furthermore, all of the isolates tested positive with a chromogenic coagulase assay. The discrepancies between the tests may result from generally weak sensitivity of the rabbit plasma tube coagulase test to *S. pseudintermedius*. Whereas S. *aureus* isolates are typically coagulase positive at 4 h, S. *pseudintermedius* may have a delayed tube coagulase result of over 72 h (19).

In this study, we demonstrated that *S. pseudintermedius* coagulase effectively reduced phagocytosis of bacteria and decreased phagocytosis in a coagulase dose-dependent manner in a reaction that depends on the interaction of *coa* with both prothrombin and fibrinogen to form a protective fibrin shield around bacteria.

In summary, the gene examined in this study encodes a novel *S. pseudintermedius* protein that displays coagulase activity, binds prothrombin, immunoglobulin, and complement C3, facilitates the deposition of fibrinogen on the bacterial surface, and provides protection from phagocytosis.

MATERIALS AND METHODS

Bacterial strains. A total of 15 clinical isolates obtained from the University of Tennessee, College of Veterinary Medicine Clinical Bacteriology Laboratory, as well as from European and North American collaborators through previous studies, as noted in Table 1, were used (20, 21). They were identified as *S. pseudintermedius* and represent nine MLST lineages associated with methicillin resistance (21). Methods for bacterial isolation, identification, and antimicrobial susceptibility testing were those routinely used in the laboratory (22). Coagulase-positive *S. aureus* ATCC 23529 and coagulase-negative *S. epidermidis* ATCC 12228 were used as assay controls.

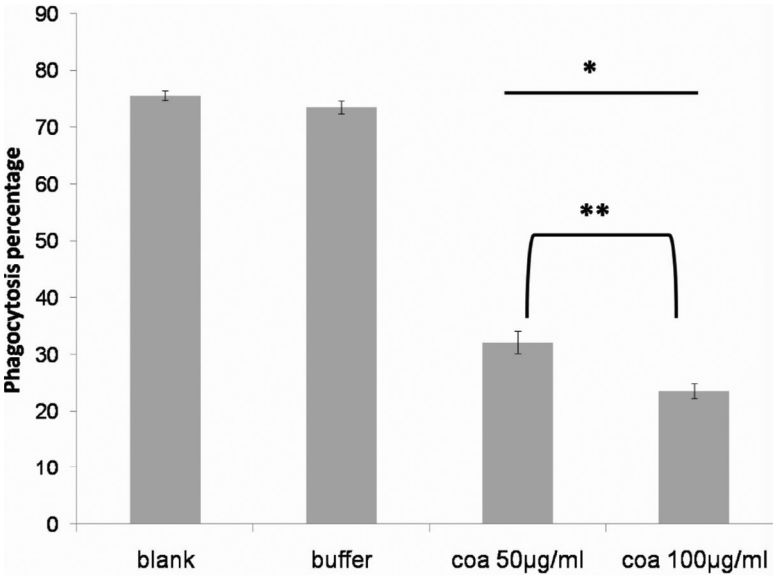

FIG 7 Recombinant coagulase inhibits phagocytosis of *S. pseudintermedius*. Phagocytosis of fluorescein-labeled bacteria incubated in the presence of rabbit plasma, whole dog blood, and coagulase (coa; 50 and 100 μg/ml) is shown. The graph represents the means and standard errors of three independent experiments, and there was a significant treatment difference ($P < 0.001$). Comparing the phagocyte-associated fluorescence for each treatment, the result for the blank did not differ from that for the elution buffer control ($P = 1.000$), and both showed significant increases in phagocyte-associated bacteria ($P = 0.015$ and $P = 0.002$, respectively). Values for the blank and buffer significantly differed from those for both coagulase treatments (*, $P < 0.001$). The two coagulase treatments showed significant decreases in phagocyte-associated bacteria ($P = 0.003$ for 50 μg/ml coagulase and $P = 0.001$ for 100 μg/ml coagulase). There was a small but significant difference between results for 50 μg/ml and 100 μg/ml coagulase (**, $P = 0.012$).

DNA extraction and PCR amplification. Bacterial strains were grown on Trypticase soy agar plates with 5% sheep blood overnight at 37°C. Bacteria derived from a single colony were suspended in 5 ml of Trypticase soy broth (TSB) (Becton, Dickinson, and Co., Sparks, MD) and incubated in a rotary shaker at 225 rpm at 37°C. Bacteria were harvested from 1.8 ml of microbial culture, and DNA was extracted using an UltraClean Microbial DNA isolation kit (Qiagen, Carlsbad, CA).

PCR primers were designed, using the IDT SciTools application (Integrated DNA Technologies, Coralville, IA), for amplification and sequencing of the *S. pseudintermedius* putative coagulase gene based on genomic data obtained for strains NA45, 063228, and 081661 (13) with GenBank accession numbers CP016072.1, CP015626.1, and CP016073.1, respectively. PCR amplification of full-length coagulase genes was carried out using the oligonucleotide primers pseud coagulase F (5′-TTTGGCCATGGATGAAAAAGA AATTGCTT-3′) and pseud coagulase R (5′-TTTGGGGATCCTGACCGTTGTAAGCTTTAT-3′) containing restriction enzyme sites for NcoI and BamH1 (underlined). The reaction mixtures consisted of a 25-μl total volume containing 2.5 μl of genomic DNA, 20 pmol of each primer (1 μl), 12.5 μl of rTaq polymerase enzyme, and 8 μl of nuclease-free water. Amplification conditions consisted of an initial denaturation (94°C for 1.5 min), followed by 35 cycles of denaturation (94°C for 60s), annealing (55°C for 2 min), and

TABLE 1 Origins and sequence types of *S. pseudintermedius* strains used in this study

S. pseudintermedius strain	Country of isolation	Multilocus sequence type
NA45	USA	ST84
NA12	USA	ST64
NA07	USA	ST124
NA16	USA	ST71
081661	USA	ST71
063228	USA	ST68
0821a	USA	ST68
081294	USA	ST68
FMV2999-10	Portugal	ST199
FMV2218-10	Portugal	ST198
FMV2183-10	Portugal	ST197
Am33	Thailand	ST111
57395	Israel	ST45
E140 (DK729)	Denmark	ST71
E141 (ED99)	Scotland	ST25

extension (72°C for 60 s), with a single final extension (72°C for 5 min). PCR products were resolved and visualized on 1.4% agarose gels.

Coagulase sequence analysis. PCR products were enzymatically treated to destroy single-stranded DNA (ExoSap-IT; USB Corp., Cleveland, OH) and submitted to the University of Tennessee Genomics Core Facility for DNA sequencing using the dideoxy chain-termination method. PCR primers were used for direct DNA sequencing of PCR amplification products. The BLAST sequence alignment tool (http://www.ncbi.nlm.nih.gov/blast/) and Geneious software (Biomatters, Auckland, New Zealand) were used to determine nucleotide sequence similarities between *S. pseudintermedius* isolates. Protein sequences were predicted from each DNA sequence using an online tool (Expasy translate [http://web.expasy.org/translate/]), and a phylogenetic tree was generated using Lasergene MegAlign (version 14.1.0 using Clustal W) comparing coagulase proteins from *S. aureus*, *S. intermedius*, *S. schleiferi*, and *S. delphini* with sequences obtained from GenBank.

Cloning, expression, and purification of coagulase protein. Cloning of *coa* was carried out using the pETBlue-2 vector (MilliporeSigma, Burlington, MA). PCR amplification of full-length coagulase was done with oligonucleotide primers containing NcoI and BamHI restriction sites. PCR products from *S. pseudintermedius* strain 081661 were digested with NcoI and BamHI and purified (Wizard SV Gel and PCR Clean-Up System; Promega, Madison, WI). They were ligated into the vector, transformed into DH5α *Escherichia Coli*, and plated on LB agar containing 50 μg/ml ampicillin, 0.1 μM isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The presence of the cloned fragment was confirmed by PCR.

The *coa* gene construct was expressed in *E. coli* Tuner(DE3)/pLacI competent cells (MilliporeSigma). They were plated on LB agar containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol (LB amp-cam). Stationary-phase broth cultures were grown from freshly transformed *E. coli* for approximately 16 h. Overnight starter cultures were inoculated into fresh medium at a 1:100 dilution and grown to an optical density (at 600 nm) of 0.6. IPTG (MilliporeSigma) was added to a final concentration of 1 mM, and the cells were incubated at 30°C for an additional 4 h in a shaking incubator at 225 rpm for induction of protein expression.

For the purification of recombinant protein, 100 ml of induced bacterial cultures was centrifuged at 10,000 $\times$ *g* for 15 min. Bacteria were suspended in 5 ml of solubilization buffer (BugBuster master mix; MilliporeSigma) containing 20 μl of protease inhibitor (Cocktail Set III, EDTA-free; MilliporeSigma) and incubated for 30 min at 37°C in a shaking incubator at 225 rpm. Samples were centrifuged at 16,000 $\times$ *g* for 45 min at 4°C to remove insoluble cell debris. Proteins were applied to an immobilized metal ion affinity chromatography column (PrepEase Ni-TED column; Thermo Fisher Scientific, Waltham, MA) and eluted with successively higher concentrations of imidazole up to 250 mM (His-Select elution buffer; MilliporeSigma). Protein concentrations were determined with a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific).

Chromogenic coagulase assay. A chromogenic assay was used to measure the activity of *S. pseudintermedius* coagulase. Single colonies of bacterial isolates were cultured overnight in 2 ml of TSB at 37°C. Bacteria were centrifuged at 12,000 $\times$ *g* for 2 min, and then supernatants were concentrated as described above. Tests were performed in flat-bottom microtiter plates. The molar concentrations of coagulase and prothrombin were calculated using molecular mass estimates of 62,000 Da for coagulase and 72,000 Da for human and bovine prothrombin.

An equimolar amount of purified prCoa was mixed with 1×10^{-16} M human prothrombin or bovine prothrombin, or an 18-μl aliquot of concentrated supernatant was mixed with 1×10^{-16} M human prothrombin or bovine prothrombin and incubated for 30 min at 37°C. Chromogenic thrombin tripeptide substrate H-D-Phe-Pip-Arg-pNA (Molecular Innovations, Novi, MI) was added to a final concentration of 1 mM in a total reaction buffer volume of 100 μl of phosphate-buffered saline (PBS) per well. After an initial reading the reaction was allowed to proceed by incubation in the dark for 1, 4, or 8 h at 37°C. The absorbance was measured at 405 nm. Reagent controls (prothrombin alone without substrate), the *S. aureus* ATCC 25923 positive control, and the *S. epidermidis* ATCC 12228 negative control were included with each batch. The change in absorbance (*A*) was plotted, and the slope of the curve (dA/dt) was interpreted to be the rate of substrate hydrolysis measured as the increase in the absorbance and thus reflective of enzymatic function (percent coagulase prothrombin complex activity) (5).

Tube coagulase test. prCoa at final concentrations of 50 and 100 μg/ml or a single overnight bacterial colony was inoculated into 0.25 ml of EDTA-treated pooled rabbit coagulase plasma (Difco Laboratories, East Molesey, United Kingdom) in a sterile plastic tube, mixed, and then incubated at 37°C. After 4, 24, and 48 h, the mixtures were examined for clotting by tipping the tubes to a 45° angle. Any detectable clotting was interpreted as a positive reaction. *S. aureus* (ATCC 25923) and S. *epidermidis* (ATCC 12228) were included as positive and negative controls, respectively. EDTA-treated plasma alone with buffer was included as an additional negative control.

Phagocytosis assay. For fluorescent labeling of *S. pseudintermedius* strain 081661, bacteria were suspended in PBS, pH 7.2, and incubated with 1 mM pHrodo green dye (Invitrogen, Mulgrave, Australia) in dimethyl sulfoxide (DMSO) for 45 min at room temperature protected from light. This dye is nonfluorescent outside cells but fluorescent in phagosomes (acidic environment). Bacteria were washed twice with PBS, suspended in PBS containing 10% glycerol, and stored at −80°C. Phagocytosis by leukocytes was measured with 10 μl of pHrodo green-labeled *S. pseudintermedius* strain 081661 bacteria (10^9 cells/ml) mixed with 10% rabbit plasma for 30 min at 37°C in the presence of prCoa (50 or 100 μg/ml) or buffer. Then, 200 μl of dog blood freshly collected in EDTA was added and incubated for 30 min at 37°C. The reaction was stopped using red blood cell lysis buffer (Sigma). Cells were washed with PBS and analyzed by flow cytometry (Applied Biosystems Attune flow cytometer; Thermo Fisher

Scientific). Neutrophils were gated based on forward and side scatter, and for each sample, fluorescence intensity of 10,000 neutrophils was determined. Phagocytosis was expressed as the percentage of neutrophils that fluoresced compared to that of neutrophils that did not fluoresce.

Fibrinogen deposition on *S. pseudintermedius* and *S. epidermidis*. Bacteria grown to mid-log phase were incubated with 50 μl of dog serum for 30 min at 37°C, washed with PBS, and incubated with prCoa (50 μg/ml) for 1 h at 37°C with shaking. After another washing step, bacteria were incubated with fluorescein isothiocyanate (FITC)-conjugated fibrinogen from pooled human plasma (50 μg/ml; Zedira, Darmstadt, Germany) for 1 h at 37°C with shaking. Controls included heat-inactivated serum with added prCoa, FITC-fibrinogen, or dog serum alone with FITC-fibrinogen. Washed bacteria were analyzed by flow cytometry as described above.

Prothrombin, complement, and IgG binding assays. To detect the ability of prCoa to bind with complement factor C3, microtiter plates were coated overnight at 4°C with affinity-purified goat anti-dog C3 (Bethyl, Montgomery, TX) (10 μg/ml). After wells were washed with phosphate-buffered saline with Tween 20 (PBST), dog serum (undiluted and 2-fold serially diluted) was added, and the plates were incubated for 1 h at 37°C, washed, and then incubated with prCoa (5 μg/ml) for another hour at 37°C and washed. prCoa binding was detected using a 1:3,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-His monoclonal antibody (Thermo Fisher Scientific) and visualized using TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Thermo Fisher Scientific).

To detect the binding of IgG, IgG-Fc, and IgG-Fab to prCoa, canine IgG was digested with immobilized papain using a Fab preparation kit (Thermo Fisher Scientific) according to the manufacturer's instructions using a 6-h incubation for 1 mg/ml dog IgG whole molecule (Rockland Antibodies and Assays, Limerick, PA) with papain. Digestion was verified by running the digested IgG in 7.5% polyacrylamide gels using sample buffer without reducing agent (Bio-Rad, Hercules, CA). This was followed by purification and separation of Fab fragments from Fc fragments using a protein A spin column (NAb Protein A Plus; Thermo Fisher Scientific). prCoa (2 μg/ml)-coated plates were incubated with canine whole-molecule IgG, Fc, or Fab fragments (beginning concentration of 10 μg/ml and then serially diluted 2-fold) for 1 h at 37°C. After plates were washed, HRP-conjugated sheep anti-dog IgG heavy chain (1:4,000) (Bethyl) was added, and bound antibody was detected with TMB substrate.

To measure the binding of prCoa to prothrombin, human and bovine prothrombins (2 μg/ml) were adsorbed to a microtiter plate well at 4°C for 16 h. After the wells were washed with PBST, prCoa (beginning at 2 μg/ml and then serially diluted 2-fold) was added, and the plates were incubated for 1 h. Following incubation, HRP-conjugated mouse anti-His monoclonal antibody (Thermo Fisher Scientific), diluted 1:3,000, was added and incubated for 1 h. Plates were washed, and TMB substrate was added. Bound proteins were quantified by measuring the absorbance at 450 nm in a microplate reader.

Statistical analysis. Repeated-measures mixed-effects analyses of variance were used to test within and between subject effects for binding of prCoa to human and bovine prothrombins, complement C3, and IgG and the reactivity of bacterial supernatant and coagulase recombinant protein to prothrombin in the chromogenic and phagocytosis assays. A 2-by-4 analysis of variance was used to test between-subject effects for fibrinogen-FITC deposition. *Post hoc* analysis included simple contrasts in comparison to a control or pairwise comparisons. All *post hoc* tests used a Bonferroni adjustment. All analyses were run in SPSS, version 24.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00027-18.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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