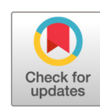
RESEARCH ARTICLE



Evolutionary and Functional Analysis of Coagulase Positivity among the Staphylococci

Amy C. Pickering,[a] Gonzalo Yebra,[a] Xiangyu Gong,[a] Mariya I. Goncheva,[a*] Bryan A. Wee,[a*] Alison C. MacFadyen,[a*] Lukas F. Muehlbauer,[a*] Joana Alves,[a] Robyn A. Cartwright,[a] Gavin K. Paterson,[a] J. Ross Fitzgerald[a]

[a]The Roslin Institute and Edinburgh Infectious Diseases, University of Edinburgh, Easter Bush, Midlothian, Scotland, United Kingdom

ABSTRACT The bacterial genus *Staphylococcus* comprises a large group of pathogenic and nonpathogenic species associated with an array of host species. Staphylococci are differentiated into coagulase-positive or coagulase-negative groups based on the capacity to promote clotting of plasma, a phenotype historically associated with the ability to cause disease. However, the genetic basis of this important diagnostic and pathogenic trait across the genus has not been examined to date. Here, we selected 54 representative staphylococcal species and subspecies to examine coagulation of plasma derived from six representative host species. In total, 13 staphylococcal species mediated coagulation of plasma from at least one host species including one previously identified as coagulase negative (*Staphylococcus condimenti*). Comparative genomic analysis revealed that coagulase activity correlated with the presence of a gene (*vwb*) encoding the von Willebrand binding protein (vWbp) whereas only the *Staphylococcus aureus* complex contained a gene encoding staphylocoagulase (Coa), the classical mediator of coagulation. Importantly, *S. aureus* retained *vwb*-dependent coagulase activity in an *S. aureus* strain deleted for *coa* whereas deletion of *vwb* in *Staphylococcus pseudintermedius* resulted in loss of coagulase activity. Whole-genome-based phylogenetic reconstruction of the *Staphylococcus* genus revealed that the *vwb* gene has been acquired on at least four different occasions during the evolution of the *Staphylococcus* genus followed by allelic diversification via mutation and recombination. Allelic variants of vWbp from selected coagulase-positive staphylococci mediated coagulation in a host-dependent manner indicative of host-adaptive evolution. Taken together, we have determined the genetic and evolutionary basis of staphylococcal coagulation, revealing vWbp to be its archetypal determinant.

IMPORTANCE The ability of some species of staphylococci to promote coagulation of plasma is a key pathogenic and diagnostic trait. Here, we provide a comprehensive analysis of the coagulase positivity of the staphylococci and its evolutionary genetic basis. We demonstrate that the von Willebrand binding protein rather than staphylocoagulase is the archetypal coagulation factor of the staphylococci and that the *vwb* gene has been acquired several times independently during the evolution of the staphylococci. Subsequently, *vwb* has undergone adaptive diversification to facilitate host-specific functionality. Our findings provide important insights into the evolution of pathogenicity among the staphylococci and the genetic basis for a defining diagnostic phenotype.

KEYWORDS *Staphylococcus*, coagulase, coagulase-positive staphylococci, coagulation, diagnostics, evolution, host specificity, phylogeny, von Willebrand binding protein

The *Staphylococcus* genus comprises at least 70 species and subspecies associated with an array of host species and habitats (1). The defining phenotype for the identification of the major human and animal pathogen *Staphylococcus aureus* has traditionally been coagulation of rabbit plasma. However, in addition to *S. aureus* there are

Citation Pickering AC, Yebra G, Gong X, Goncheva MI, Wee BA, MacFadyen AC, Muehlbauer LF, Alves J, Cartwright RA, Paterson GK, Fitzgerald JR. 2021. Evolutionary and functional analysis of coagulase positivity among the staphylococci. mSphere 6:e00381-21. https://doi.org/10.1128/mSphere.00381-21.

Editor Paul D. Fey, University of Nebraska Medical Center

Copyright © 2021 Pickering et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to J. Ross Fitzgerald, ross.fitzgerald@ed.ac.uk.

* Present address: Mariya I. Goncheva, Department of Microbiology and Immunology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada; Bryan A. Wee, Usher Institute, University of Edinburgh, Scotland, United Kingdom; Alison C. MacFadyen, Institute of Biodiversity, Animal Health & Comparative Medicine, University of Glasgow, Glasgow, Scotland, United Kingdom; Lukas F. Muehlbauer, University of Edinburgh, Edinburgh, Scotland, United Kingdom.

Evolutionary and Functional Analysis of Coagulase Positivity among the Staphylococci @jrossfitz

Received 23 April 2021
Accepted 30 June 2021
Published 4 August 2021

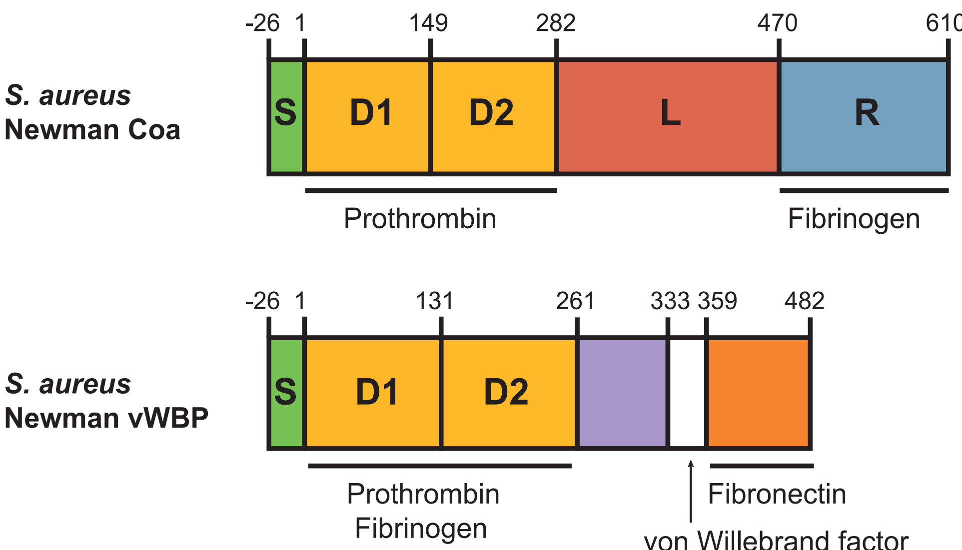


FIG 1 Schematic protein domain structure of Coa and vWbp of *S. aureus* Newman. Both Coa and vWbp contain N-terminal signal peptides (S) and prothrombin-binding D1 and D2 domains. The C-terminal sequence of Coa contains a linker (L) followed by fibrinogen-binding repeats (R). The C-terminal sequence of vWbp contains a von Willebrand factor binding region between two domains. In *S. aureus* Newman, Coa and vWbp share 26.3% amino acid identity.

eight described coagulase-positive staphylococci (CoPS) including *Staphylococcus argenteus*, *Staphylococcus cornubiensis*, *Staphylococcus delphini*, *Staphylococcus intermedius*, *Staphylococcus lutrae*, *Staphylococcus pseudintermedius*, *Staphylococcus coagulans*, and *Staphylococcus schweitzeri*, with *Staphylococcus agnetis*, *Staphylococcus chromogenes*, and *Staphylococcus hyicus* described as coagulase variable (2, 3). To date, the basis for the coagulation phenotype has been examined only for *S. aureus* with staphylocoagulase (Coa) considered to be the classical mediator of coagulation. However, the von Willebrand binding protein (vWbp) also exhibits coagulase activity (4, 5). Of note, a staphylococcal pathogenicity island (SaPI)-encoded vWbp associated with ruminant or equine strains of *S. aureus* confers the ability to coagulate plasma from their cognate host species, demonstrating a role for SaPI-encoded vWbp in host adaptation (6, 7).

For both *S. aureus* Coa and vWbp, which contain 26.3% amino acid identity in strain Newman, the N-terminal D1 and D2 domains are sufficient for the coagulation of plasma via the nonproteolytic activation of prothrombin, which in turn cleaves fibrinogen to form insoluble fibrin, thus promoting clot formation (Fig. 1) (8, 9). Allelic variation of the D1 and D2 domains in both Coa and vWbp is associated with attenuated binding efficiencies to prothrombin from different host species, underpinning the host species-dependent coagulation observed (5, 10–12). For *S. aureus*, mediators of coagulation promote bacterial survival in fibrin clots, allowing persistence in a murine subcutaneous infection model and bacterial dissemination in a murine bloodstream infection model (13–15). Both *coa* and *vwb* genes are also required for the development of kidney microabscesses during invasive disease (14, 16, 17). The key role of both Coa and vWbp in the pathogenesis of *S. aureus* disease suggests that the capacity for coagulation of plasma by other staphylococci is likely to be relevant to pathogenicity (8). A recent study used PCR to identify genes more similar to *vwb* than *coa* among four non-*aureus* CoPS (18). Furthermore, a recombinant form of a protein encoded by *S. pseudintermedius* orthologous to vWbp of *S. aureus* was demonstrated to have coagulase activity (19). However, the evolutionary genetic and functional basis of the coagulase phenotype for non-*aureus* staphylococci has not been examined to date.

Here, we perform a comprehensive analysis of the coagulation capacity of 54 representative staphylococcal species and subspecies in plasma from multiple host species. Combining comparative genomic, evolutionary, and functional analyses, we examined

TABLE 1 Coagulase activity of staphylococci in plasma from six host species

		Coagulase activity in plasma type[a]:					
Species	Associated host	Human	Canine	Equine	Porcine	Avian	Rabbit
S. agnetis	Bovine	−	+	+	+++	−	+
S. argenteus	Human	−	+++	++	++	−	++++
S. aureus	Human	++++	+	+++	++	−	++++
S. chromogenes	Bovine	+++	+++	−	−	−	+++
S. coagulans	Canine	−	++	++	−	++	+++
S. condimenti	Unknown	−	−	+	−	−	−
S. cornubiensis	Human	−	+++	++++	++++	+++	++++
S. delphini	Dolphin	−	+++	++	+++	+	+++
S. hyicus	Porcine	−	++	−	++++	−	−
S. intermedius	Avian	−	+++	−	−	−	++++
S. lutrae	Otter	−	+++	++	−	+	++++
S. pseudintermedius	Feline	−	+++	+++	++	−	+++
S. schweitzeri	Monkey	+	+	+	−	−	+

[a]Only species that demonstrated coagulation of any plasma type in all three biological replicates are shown. +, ++, +++, and ++++, coagulating activity observed after 24, 6, 4, or 2 h of incubation, respectively; −, no coagulating activity detected after 24 h.

the molecular basis for coagulation among CoPS. Importantly, we demonstrate that vWbp is the archetypal coagulation factor of CoPS, whereas the classical Coa is limited to members of the *S. aureus* complex only. Acquisition of the *vwb* gene occurred on four independent occasions during the evolutionary history of the staphylococci and was followed by diversification via recombination and host-specific functional specialization. Taken together, these findings reveal the evolutionary history of a key diagnostic and pathogenic trait of staphylococci.

RESULTS

Comprehensive examination of CoPS and host species-associated coagulation. Of the 71 known staphylococcal species and subspecies that comprise the *Staphylococcus* genus (1), 12 are reported to mediate coagulation of rabbit plasma (2). However, our previous findings indicate host-dependent variation in susceptibility to plasma coagulation (6, 7). Here, we investigated the capacity for 51 representative staphylococcal species and 3 subspecies to mediate coagulation of plasma from six host species including human, canine, equine, porcine, avian, and rabbit (see Table S1 in the supplemental material). We also included five *Mammaliicoccus* species previously classified as members of the family *Staphylococcaceae* (20). A coagulation phenotype was observed for 13 staphylococcal species in plasma from at least one host species (Table 1). Unexpectedly, *Staphylococcus condimenti*, previously determined to be coagulase negative (21), exhibited coagulation of equine plasma. Considerable variation was observed in the capacity for different staphylococcal species to coagulate plasma from different host species, with coagulation of human plasma restricted to *S. aureus*, *S. chromogenes*, and *S. schweitzeri* (Table 1). Of note, *S. condimenti* and *S. hyicus* did not coagulate rabbit plasma (traditionally used for identifying the coagulase phenotype in clinical diagnostic labs) but mediated coagulation of plasma from other host species. Furthermore, the time to coagulation mediated by CoPS varied from 2 to 24 h according to host species (Table 1). In summary, this genus-wide analysis of coagulation using plasma from multiple host species reveals considerable variation in the efficiency of plasma coagulation depending on the host species and suggests a revision of the group of CoPS to include *S. condimenti*.

The capacity for coagulase activity was acquired on multiple occasions during the evolution of the staphylococci. To examine the distribution of CoPS species across the staphylococcal phylogeny, we constructed the most comprehensive species phylogeny to date based on 231 concatenated core genes present in 50 staphylococcal species (five of which included two subspecies each) for which the whole-genome sequences were available (Table S2). CoPS are distributed into three distinct clades including the *S. aureus* complex (*S. aureus*, *S. argenteus*, and *S. schweitzeri* [22]), the Hyicus group containing coagulase-

FIG 2 All CoPS species contain the *vwb* gene. Maximum likelihood phylogenetic tree of 50 staphylococcal species and 5 additional subspecies based on a core genome alignment of 231 concatenated genes. A list of the genome sequences used is included in Table S2. Coagulase-positive species are highlighted in bold, and coagulase-variable species are highlighted with an asterisk. Species containing the *vwb* gene are represented with orange branches, with orange circles indicating the branch on which this gene was acquired. Red circles indicate the nodes in which additional *vwb* copies were acquired. The single acquisition event of the *coa* gene by an ancestor of the *S. aureus* complex is indicated by a green circle. Genome locations of the *vwb* and *coa* genes in each CoPS lineage are represented by colored bars on a blue ring depicting the chromosome. Support values are shown on each branch and were calculated using the Shimodaira-Hasegawa test. The *Mammaliicoccus* cluster is the root of the tree.

variable species (*S. hyicus*, *S. agnetis*, and *S. chromogenes* [3, 23, 24]), and the Intermedius group (*S. intermedius*, *S. delphini*, *S. pseudintermedius*, *S. cornubiensis*, *Staphylococcus ursi*, *S. lutrae*, *S. schleiferi*, and *S. coagulans* [25–28]), as previously described (Fig. 2) (28–30).

To investigate the genetic basis for the coagulase-positive phenotype, we examined the genus-wide distribution of genes known to encode proteins with coagulase activity (Fig. 2). Unexpectedly, we found that the *coa* gene (encoding the classical staphylocoagulase) was restricted to the *S. aureus* complex, but the *vwb* gene was identified among all CoPS in addition to coagulase-negative *staphylococcus* (CoNS) *S. schleiferi* (Fig. 2). The *vwb* genes were identified at distinct genomic locations in each coagulase-positive clade, suggesting that acquisition occurred independently via horizontal transfer on at least four occasions (Fig. 2). *S. agnetis* and *S. hyicus* each have two *vwb* copies

in tandem, presumably as a result of a gene duplication event that occurred prior to divergence of these two closely related species. *S. lutrae* also carries two copies of *vwb*, one in the same genomic location as the other members of the Hyicus clade and the second copy in a unique genomic location (Fig. 2). Taken together, we infer that *vwb* was acquired on multiple occasions during the evolutionary history of the staphylococci, leading to the emergence of distinct groups of CoPS.

The presence of the *vwb* gene in *S. condimenti* (and the lack of a *vwb* gene in the closely related species *Staphylococcus carnosus*) indicates an independent acquisition during the evolution of this species, which correlates with the capacity to coagulate equine plasma as identified in the current study (Table 1). The presence of the *vwb* gene in both the Hyicus and Intermedius coagulase-positive clades, and the absence of *coa*, suggested that vWbp may be the mediator of coagulation for these species.

vWbp diversification has occurred via mutation, recombination, and duplication. To investigate the diversity and relatedness of the *vwb* and *coa* genes among the CoPS, a phylogenetic tree was constructed based on sequences extracted from genomes representing 41 non-*S. aureus* species (Table S2), and 802 *S. aureus*, *S. schweitzeri*, and *S. argenteus* genomes (31). From those, we selected representative copies of each phylogenetic cluster within the *S. aureus* complex to avoid redundancy, leaving a total of 12 Coa and 129 vWbp protein sequences from 11 species across the three CoPS clades (Fig. 3). vWbp sequences form four distinct clusters that correspond to chromosomal genes from the Hyicus ($n = 8$) and Intermedius ($n = 32$) clades, the *S. aureus* complex ($n = 51$), and SaPI-associated genes from *S. aureus* ($n = 37$), respectively. The only vWbp copy from the only *S. condimenti* genome was most closely related to the Hyicus clade vWbp copies (Fig. 3). The vWbp clades are more closely related to each other (34.7% average pairwise protein similarity between the full-length vWbp alleles of the Hyicus and Intermedius clades) than to the *S. aureus* Coa protein (22.6% and 21.6% average pairwise protein similarity between the full-length Coa and vWbp alleles of the Hyicus and Intermedius clades, respectively), which forms a monophyletic clade indicating a distinct evolutionary history (Fig. 3; also see Table S3). The topology of the vWbp Hyicus and Intermedius clades correlates with the species distribution in the core genome-based *Staphylococcus* genus phylogeny (Fig. 2), consistent with ancient independent acquisition by progenitors of the Hyicus and Intermedius clades, followed by evolution according to species and a low degree of interspecies gene exchange (Fig. 3). The exception to this were the paralogous *vwb* genes in *S. agnetis* and *S. hyicus*, which have distinct evolutionary histories compared to the ancestral chromosomal copy, whereas the *S. lutrae vwb* copies clustered together in the phylogeny, consistent with gene duplication since speciation (Fig. 3).

Previously, it was shown that recombination has contributed to the evolution of the *coa* genes among *S. aureus* isolates (12). In order to examine the impact of recombination on the evolutionary history of the *vwb* gene in the *S. aureus* complex, we employed both RDP4 and FastGEAR analysis using a 100-bp sliding window approach on 39 *vwb* gene sequences representing the breadth of sequence diversity. This analysis revealed high levels of recombination affecting the N-terminal D1 and D2 coagulation domains, explaining the lower pairwise protein similarity observed in the *S. aureus* complex for the D1 and D2 domains, in comparison to full-length vWbp (Fig. 4; Table S3). Of note, *S. aureus* complex chromosomal *vwb* copies exhibited extensive sequence admixture between *S. aureus*, *S. argenteus*, and *S. schweitzeri* consistent with a shared ecology providing opportunities for horizontal gene transfer and recombination (Fig. 4).

Taken together, we identified a complex evolutionary history of the *vwb* gene with evidence for diversification of *vwb* in a species-dependent manner in the Hyicus and Intermedius clades. In contrast, a high degree of recombination between the members of the *S. aureus* complex has generated extensive allelic diversity of *vwb*.

vWbp is the archetypal coagulation factor of the staphylococci. From our phylogenetic analysis of the distribution of genes encoding coagulase activity, and the correlation of the *vwb* gene with coagulase positivity, we hypothesized that *vwb* is the main

FIG 3 Staphylococcal vWbp has an evolutionary history distinct from that of coagulase. (A) Unrooted maximum-likelihood tree constructed using FastTree v2 of the Coa and vWbp protein sequences. Each of the main clusters is highlighted and labeled. Coagulase positivity has evolved on multiple occasions through acquisition of *vwb* followed by evolution according to species. (B) Phylogenetic reconstruction of the non-*aureus* CoPS based on vWbp protein sequences.

mediator of coagulation in the *Staphylococcus* genus. To date, the capacity for vWbp from non-*aureus* CoPS to mediate coagulation has not been tested. Accordingly, we produced recombinant vWbp variants derived from CoPS species including human *S. aureus* Newman, canine *S. pseudintermedius* ED99, avian *S. intermedius* ATCC 29663, equine *S. delphini* 8086, and porcine *S. hyicus* ATCC 11249 in addition to Coa from

FIG 4 The chromosomal *vwb* has undergone extensive recombination among the *S. aureus* complex species. Midpoint-rooted maximum-likelihood tree constructed using FastTree v2 of the *S. aureus* complex *vwb* nucleotide sequences. Colored circles denote each *Staphylococcus* species. Plot of the FastGEAR analysis of the *vwb* nucleotide alignment applied to 100-bp-long windows. For each window (i.e., column in the plot), each color represents different phylogenetic clusters based on the BAPS algorithm. The axis at the top shows, for reference, the location of the coagulase (D1+D2) domain using the amino acid coordinates of the RF122 *S. aureus* isolate vWbp.

S. aureus Newman and tested their capacity to promote coagulation of plasma from an array of different host species in dose-dependent assays (Fig. 5; Table 2; Fig. S1 to S3) (23, 32–35). Each variant had the capacity to mediate plasma coagulation from their cognate host species, though there was considerable variation in the capacity to coagulate plasma from other species (Fig. 5; Table 2). Overall, the activity was similar to that observed for the parent bacterial species (Table 1), consistent with vWbp being the key mediator of coagulation in non-*aureus* staphylococci.

To confirm this in a representative CoPS species of clinical relevance, we constructed a *vwb* deletion mutant in *S. pseudintermedius* ED99 (34), reintroduced the *vwb* gene to produce a repaired derivative strain, and also employed single and double mutants of *vwb* and *coa* in *S. aureus* strain Newman (16). Single deletions of the *vwb* and *coa* genes in *S. aureus* Newman did not ablate plasma coagulation, with deletion of both genes required for loss of the coagulation phenotype (Fig. 6A). In contrast, deletion of *vwb* in *S. pseudintermedius* (ED99Δ*vwb*) resulted in loss of ability to coagulate plasma after 24 h whereas the isogenic wild-type and ED99Δ*vwb* repaired strain produced complete clots

FIG 5 Recombinant vWbp proteins exhibit host-specific coagulation of plasma. Coagulation of plasma from human (A), canine (B), equine (C), porcine (D), avian (E), and rabbit (F) origin by 50 μg ml^{-1} recombinant vWbp encoded by *S. pseudintermedius* (pink), *S. intermedius* (orange), *S. delphini* (dark green), *S. hyicus* (light green), and *S. aureus* (blue), in addition to *S. aureus* Coa (purple). Coagulation was scored as 0.5 for trace levels of coagulation, 1.0 to 2.5 for partially coagulating plasma, and 3.0 for a complete clot. Data are shown as the mean from 6 replicates with error bars representing standard deviation.

after 2 h (Fig. 6A). Furthermore, antibodies specific for vWbp^{pseudintermedius} delayed coagulation in a dose-dependent manner, confirming that vWbp mediates plasma coagulation by *S. pseudintermedius* ED99 (Fig. 6B). These data demonstrate that vWbp is required for the coagulase positivity of *S. pseudintermedius* ED99.

Taken together, these evolutionary and functional data support the hypothesis that vWbp is the major determinant of coagulase positivity among staphylococci. For the *S. aureus* complex species only, Coa provides redundancy in coagulase activity. In addition, allelic diversification has resulted in host-adaptive evolution supporting the capacity of CoPS to adapt to different host niches.

DISCUSSION

The ability to coagulate plasma is considered a defining phenotype for the identification of the major human pathogen *S. aureus*. Furthermore, the importance of

TABLE 2 Minimal concentration of recombinant vWbp or Coa protein required to coagulate plasma after 24 h

	Minimal concn (μg ml^{-1}) for plasma type[a]:					
Protein	**Human**	**Canine**	**Equine**	**Porcine**	**Avian**	**Rabbit**
Coa^{aureus}	25		50			25
vWbp^{aureus}	25	25	50	25		25
vWbp^{intermedius}		25	5	25	25	5
vWbp^{pseudintermedius}		25	25	50		25
vWbp^{delphini}		5	5	25	25	5
vWbp^{hyicus}		25		50		

[a]Values are the minimal concentration of recombinant protein required to induce coagulation after 24 h. Recombinant protein was applied to PBS-diluted plasma at 50, 25, 5, or 1 μg ml^{-1}.

coagulases Coa and vWbp in *S. aureus* pathogenesis is well established for catheter-related biofilm formation and bacterial survival during bloodstream infections (13, 15, 36, 37). However, the evolutionary and genetic basis of coagulation for non-*aureus* staphylococci or its role in pathogenesis had not been dissected previously. Here, we have performed a comprehensive phylogenetic and functional analysis of coagulase positivity among the staphylococci. The ability to coagulate plasma has evolved on multiple occasions across the genus via independent acquisitions of the *vwb* gene whereas the *coa* gene was likely acquired on a single occasion by an ancestor of the *S. aureus* complex (Fig. 2). Our functional analyses demonstrated that the *vwb* gene identified among CoPS mediates coagulation of plasma in a host-dependent manner and is solely responsible for the coagulase phenotype in a representative non-*aureus* species, *S. pseudintermedius* (Fig. 5 and 6). Taken together, these data suggest that vWbp is the archetypal coagulation factor of the *Staphylococcus* genus.

We identified extensive allelic diversity and recombination of the *vwb* gene among members of the *S. aureus* complex (Fig. 3), consistent with an overlapping habitat for *S. aureus*, *S. argenteus*, and *S. schweitzeri* (Fig. 4). A previous study has demonstrated gene flow between *S. aureus* and *S. argenteus* via recombination, driven by a possible shared ecological niche in livestock (38). In spite of the evidence for horizontal transfer of *vwb* sequences, SaPI-encoded variants of vWbp were restricted to *S. aureus*, which may reflect its multihost ecology and the role of vWbp in host adaptation (Fig. 3) (6). However, whole-genome sequencing of other species is very limited compared to *S. aureus*, and expanded sequencing of *S. schweitzeri* and *S. argenteus* isolates may reveal novel variants. Of note, extensive variation and evidence for recombination have previously been reported for the *coa* gene of *S. aureus* and *S. argenteus* (12, 39, 40). In contrast, our *vwb* phylogenetic analysis for non-*aureus* staphylococci supported a model of initial *vwb* gene acquisition followed by diversification according to species via mutation rather than interspecies recombination (Fig. 3).

In order to examine the possibility that the allelic variation of *vwb* between staphylococcal species in part reflected the independent evolution within distinct host ecologies, we examined the coagulase activity of recombinant vWbp from representative CoPS species in plasma from six different host species. In each case, recombinant vWbp variants exhibited coagulation of plasma from their cognate host species and various levels of coagulation from other host species (Table 2; Fig. 5). The host species-dependent activity of vWbp presumably reflects variation in the sequence of prothrombin, the main substrate for vWbp (10, 11). Overall, these data indicate that *vwb* has undergone adaptive evolution to facilitate functional activity in its preferred host species, highlighting an important role for vWbp in host-pathogen interactions.

The coagulase test has been used since the 1940s to distinguish the virulent *S. aureus* from less-virulent CoNS (41). Since the 1970s, additional CoPS have been identified, largely associated with infections of animals (33), and the coagulase test has been widely used in human and veterinary medicine. Although the coagulase test is still commonly used, particularly in resource-poor settings around the world, molecular

A

	Plasma Type	
Strain	Human	Canine
ED99	-	++++
ED99Δ*vwb*	-	-
ED99Δ*vwb* Rep	-	++++
Newman	++++	+
NewmanΔ*coa*	++++	+
NewmanΔvwb	++++	+
NewmanΔ*coa*Δ*vwb*	-	-

Coagulating activity observed after 24 h (+) or 2 h (++++) of incubation. No coagulating activity detected after 24 h (-).

B

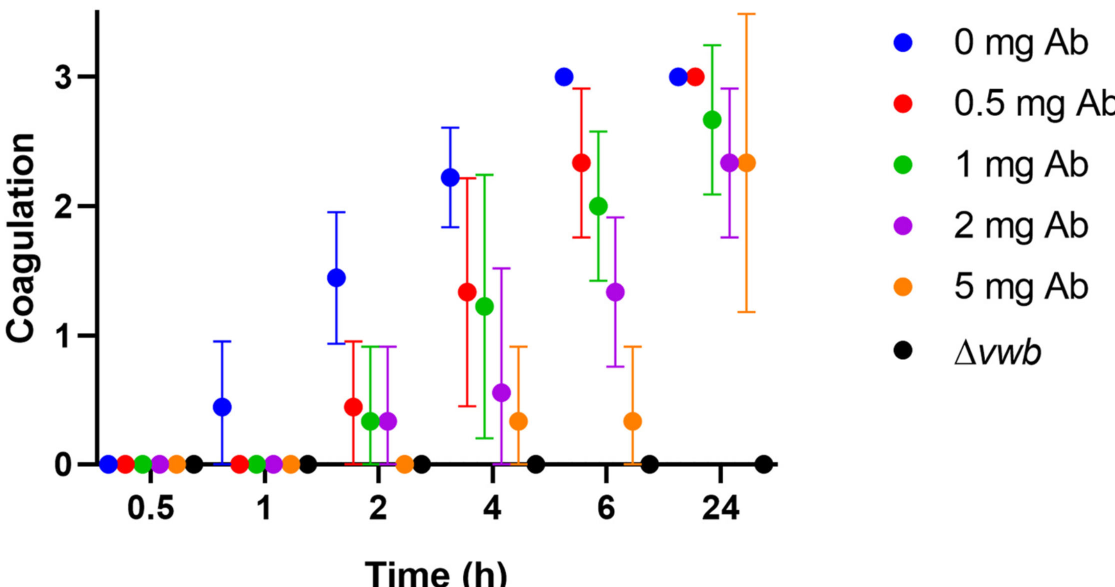


FIG 6 *vwb* is required for the coagulase phenotype of *S. pseudintermedius* ED99. (A) Coagulation assay of wild-type and mutant strains of *S. pseudintermedius* ED99 and *S. aureus* Newman in human and canine plasma. Average data are provided from three biological replicates. (B) Antibody (Ab)-mediated inhibition of coagulation in canine plasma using ED99 wild type in the presence of increasing concentrations of anti-vWbp^{pseudintermedius} antibody. The mean values are given from three experiments with error bars representing standard deviation ($n = 3$).

genetic or proteomic approaches have largely supplanted biochemical testing (42). Our findings suggest that *vwb* sequence analysis to identify alleles associated with specific CoPS would offer a robust and simple diagnostic approach for the identification of CoPS to species level and should be performed when describing novel staphylococcal species. Of 14 species that contained a *vwb* gene, *S. schleiferi* was the only one which

did not exhibit a coagulase phenotype. This may reflect lack of gene expression or a distinct host tropism that was not represented among the plasma samples included, and analysis of additional isolates would be required to address this. Similarly, an understanding of the genetic basis for the variation in coagulase positivity among some staphylococcal species would require genomic analysis of more isolates. Of note, we examined the genome sequences of the newly identified *Staphylococcus durrellii*, *Staphylococcus lloydii*, and *Staphylococcus pseudoxylosus* type strains and did not identify a *vwb*-like gene, consistent with their characterization as CoNS (43, 44). Furthermore, the genome of the recently identified *Staphylococcus ursi* type strain MI 10-1553^{T}, reported as a CoNS of the otherwise coagulase-positive Intermedius clade, contains a *vwb*-like remnant consistent with a deletion event that abrogated the ability to coagulate plasma (26).

In conclusion, the current study provides the first comprehensive genetic and functional analysis of the coagulation capacity of the *Staphylococcus* genus. We propose that vWbp, rather than Coa, is the archetypal coagulation factor of CoPS across the genus with the *vwb* gene acquired independently on multiple occasions, leading to distinct coagulase-positive clades within the *Staphylococcus* phylogeny. Diversification of the *vwb* gene has occurred via mutation or recombination generating allelic diversity, which in part reflects adaptive evolution to enable functionality of vWbp in the preferred host species, suggesting a key role in host-pathogen interactions. Taken together, our findings provide clarity into the evolutionary genetic and functional basis for a defining diagnostic and pathogenic phenotype of the *Staphylococcus* genus.

MATERIALS AND METHODS

Phylogenetic analysis. Whole-genome sequences of the staphylococcal and mammaliicoccal type strains were obtained from NCBI (see Table S2 in the supplemental material). Each genome was annotated with Prokka v1.12 (45), and a core genome alignment was generated using Roary v3.12 (46), based on a minimum percent identity cutoff of 80. The core genome alignment was then used to build a maximum likelihood phylogenetic tree with FastTree v2.1.7 (47). Coagulase and vWbp amino acid sequences were extracted using Protein BLAST (blastp) from the reference whole-genome sequences from a genome sequence data set representative of the global *S. aureus* diversity (31). Sequences were aligned using the ProbCons algorithm (48) implemented in Jalview v2 (49). Phylogenetic trees were constructed using FastTree, and recombination analysis was performed using the programs Recombination Detection Program (RDP4) (50) and FastGEAR (51). Average pairwise protein similarities were calculated using MEGA X v10.1.8 (52) after classifying the D1 and D2 domains using the Conserved Domain Database (53).

Coagulation assay. The bacterial strains used for coagulation assays are detailed in Table S1. All strains were cultured overnight in brain heart infusion (BHI) broth at 37°C with shaking. For bacterial coagulation assays, the cells were diluted in phosphate-buffered saline (PBS) to an optical density at 600 nm (OD_{600}) of 1.0. For recombinant protein coagulation assays, each recombinant protein was diluted in PBS to the required concentration using the Nanodrop ND-1000 spectrophotometer. Plasma from all hosts was sourced from SeraLab, Lampire, or BioIVT in sodium citrate and diluted 1:3 in PBS before use. Coagulation was tested by mixing 190 μl of diluted plasma with 10 μl of recombinant protein or bacterial cells in sterile borosilicate glass tubes. All coagulation assays were performed statically at 37°C in biological triplicate with the level of coagulation observed at 1, 2, 4, 6, and 24 h by tilting the tubes. Antibody blocking of coagulation was performed using IgY antibody generated against $vWbp^{pseudintermedius}$ after lipopolysaccharide (LPS) removal (Gallus Immunotech) at a concentration of 5, 2, 1, 0.5, or 0.1 mg in the 200-μl reaction mixture with concentrated supernatant.

Allelic replacement of *S. pseudintermedius* ED99. Mutant and repaired strains of *S. pseudintermedius* ED99 were generated using the allele replacement plasmid pIMAY and the primers detailed in Table S4, as described previously (54, 55). Briefly, the upstream and downstream flanking regions of the *vwb* gene were amplified by PCR and annealed by splicing PCR to generate a deleted form of the gene. For the repaired mutant, primers were designed to amplify the full-length *vwb* gene in addition to upstream and downstream flanking regions with a synonymous mutation introduced into the *vwb* gene to allow identification of the repaired strain in comparison to the wild type. Restriction digestion with EcoRI (NEB) and T4 ligation (NEB) into pIMAY was carried out prior to transformation of the pIMAY::*vwb* and pIMAY::*vwb* Rep constructs into *Escherichia coli* DC10B cells, followed by transformation into *S. pseudintermedius* ED99 or ED99Δ*vwb*. Allele replacement was performed as previously described (55), and genetically manipulated strains were validated by whole-genome sequencing, growth analysis in BHI using a FLUOstar Optima microplate reader (600 nm), and Western blot analysis of vWbp expression using 5 μg ml^{-1} anti-$vWbp^{pseudintermedius}$ IgY and 0.5 μg ml^{-1} F(ab′)$_2$ rabbit anti-chicken IgG-horseradish peroxidase (HRP) (Bethyl Laboratories).

Expression and purification of recombinant proteins. The N-terminal amino acids are important for the function of coagulase, with N-terminal modification of vWbp impairing coagulation efficiency

(56). In order to use the C-terminal His tag expression construct pET21b with the NheI site, all additional plasmid N-terminal amino acids (Met-Ala-Ser-Met) were removed from the plasmid using pET21b-specific primers (Table S4). This modified plasmid was used to clone *vwb*^{hyicus}, *vwb*^{aureus}, and *coa*^{aureus}. All pET21b constructs were produced using standard restriction digestion and NheI, NdeI, and XhoI (NEB) and T4 ligase (NEB) procedures. Expression constructs were expressed in BL21(DE3) *E. coli* cells (NEB) using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. Cell lysates were produced at 20 kpsi using a One Shot cell disruptor (Constant Systems). All recombinant proteins underwent two purification steps using the high-performance liquid chromatography (HPLC) Äkta system (GE Healthcare): first, immobilized metal affinity chromatography with 1 ml Ni-nitrilotriacetic acid (NTA) superflow columns (Qiagen) in either native or denaturing purification buffers, and second, ion-exchange chromatography using Q Sepharose (GE Healthcare) for positively charged proteins or SP Sepharose (GE Healthcare) for negatively charged proteins. All proteins were dialyzed to PBS using Float-A-Lyzers (Spectrum Laboratories) and analyzed by SDS-PAGE.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
FIG S1, DOCX file, 0.6 MB.
FIG S2, DOCX file, 0.6 MB.
FIG S3, DOCX file, 0.6 MB.
TABLE S1, DOCX file, 0.01 MB.
TABLE S2, DOCX file, 0.01 MB.
TABLE S3, DOCX file, 0.01 MB.
TABLE S4, DOCX file, 0.01 MB.

ACKNOWLEDGMENTS

J.R.F. was funded by institute strategic grant funding ISP2: BBS/E/D/20002173 and BBS/E/D/20002174 from the Biotechnology and Biological Sciences Research Council (United Kingdom), SHIELD grant MR/N02995X/1 from the Medical Research Council (United Kingdom), and a Wellcome Trust collaborative award 201531/Z/16/Z. The funders had no role in study design, date collection and interpretation, or the decision to submit the work for publication.

We thank Anthony Chow for the provision of a number of staphylococcal species for this study.

These authors contributed to the design of the study: A.C.P., G.Y., R.A.C., J.R.F.; these authors contributed to the acquisition, analysis, or interpretation of the data: A.C.P., G.Y., X.G., M.I.G., B.A.W., A.C.M., L.F.M., J.A., R.A.C., G.K.P., J.R.F.; these authors contributed to the writing of the manuscript: A.C.P., G.Y., B.A.W., J.R.F.

REFERENCES

1. Parte AC, Sardà Carbasse J, Meier-Kolthoff JP, Reimer LC, Göker M. 2020. List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. Int J Syst Evol Microbiol 70:5607–5612. https://doi.org/10.1099/ijsem.0.004332.
2. González-Martín M, Corbera JA, Suárez-Bonnet A, Tejedor-Junco MT. 2020. Virulence factors in coagulase-positive staphylococci of veterinary interest other than *Staphylococcus aureus*. Vet Q 40:118–131. https://doi.org/10.1080/01652176.2020.1748253.
3. dos Santos DC, Lange CC, Avellar-Costa P, dos Santos KRN, Brito MAVP, Giambiagi-deMarval M. 2016. *Staphylococcus chromogenes*, a coagulase-negative *Staphylococcus* species that can clot plasma. J Clin Microbiol 54:1372–1375. https://doi.org/10.1128/JCM.03139-15.
4. Smith W, Hale JH. 1944. The nature and mode of action of *Staphylococcus* coagulase. Br J Exp Pathol 25:101–110.
5. Bjerketorp J, Jacobsson K, Frykberg L. 2004. The von Willebrand factor-binding protein (vWbp) of *Staphylococcus aureus* is a coagulase. FEMS Microbiol Lett 234:309–314. https://doi.org/10.1016/j.femsle.2004.03.040.
6. Viana D, Blanco J, Tormo-Más MÁ, Selva L, Guinane CM, Baselga R, Corpa JM, Lasa Í, Novick RP, Fitzgerald JR, Penadés JR. 2010. Adaptation of *Staphylococcus aureus* to ruminant and equine hosts involves SaPI-carried variants of von Willebrand factor-binding protein. Mol Microbiol 77:1583–1594. https://doi.org/10.1111/j.1365-2958.2010.07312.x.
7. Guinane CM, Ben Zakour NL, Tormo-Mas MA, Weinert LA, Lowder BV, Cartwright RA, Smyth DS, Smyth CJ, Lindsay JA, Gould KA, Witney A, Hinds J, Bollback JP, Rambaut A, Penadés JR, Fitzgerald JR. 2010. Evolutionary genomics of *Staphylococcus aureus* reveals insights into the origin and molecular basis of ruminant host adaptation. Genome Biol Evol 2:454–466. https://doi.org/10.1093/gbe/evq031.
8. McAdow M, Missiakas DM, Schneewind O. 2012. *Staphylococcus aureus* secretes coagulase and von Willebrand factor binding protein to modify the coagulation cascade and establish host infections. J Innate Immun 4:141–148. https://doi.org/10.1159/000333447.
9. Kroh HK, Panizzi P, Bock PE. 2009. Von Willebrand factor-binding protein is a hysteretic conformational activator of prothrombin. Proc Natl Acad Sci U S A 106:7786–7791. https://doi.org/10.1073/pnas.0811750106.
10. Raus J, Love DN. 1991. Comparison of the affinities to bovine and human prothrombin of the staphylocoagulases from *Staphylococcus intermedius* and *Staphylococcus aureus* of animal origin. J Clin Microbiol 29:570–572. https://doi.org/10.1128/jcm.29.3.570-572.1991.
11. Friedrich R, Panizzi P, Kawabata S-I, Bode W, Bock PE, Fuentes-Prior P. 2006. Structural basis for reduced staphylocoagulase-mediated bovine prothrombin activation. J Biol Chem 281:1188–1195. https://doi.org/10.1074/jbc.M507957200.
12. Watanabe S, Ito T, Sasaki T, Li S, Uchiyama I, Kishii K, Kikuchi K, Skov RL, Hiramatsu K. 2009. Genetic diversity of staphylocoagulase genes (*coa*): insight into the evolution of variable chromosomal virulence factors in *Staphylococcus aureus*. PLoS One 4:e5714. https://doi.org/10.1371/journal.pone.0005714.
13. Loof TG, Goldmann O, Naudin C, Mörgelin M, Neumann Y, Pils MC, Foster SJ, Medina E, Herwald H. 2015. *Staphylococcus aureus*-induced clotting of

plasma is an immune evasion mechanism for persistence within the fibrin network. Microbiology (Reading) 161:621–627. https://doi.org/10.1099/mic.0.000019.

14. McAdow M, Kim HK, DeDent AC, Hendrickx APA, Schneewind O, Missiakas DM. 2011. Preventing *Staphylococcus aureus* sepsis through the inhibition of its agglutination in blood. PLoS Pathog 7:e1002307. https://doi.org/10.1371/journal.ppat.1002307.
15. Thomer L, Emolo C, Thammavongsa V, Kim HK, McAdow ME, Yu W, Kieffer M, Schneewind O, Missiakas D. 2016. Antibodies against a secreted product of *Staphylococcus aureus* trigger phagocytic killing. J Exp Med 213:293–301. https://doi.org/10.1084/jem.20150074.
16. Cheng AG, McAdow M, Kim HK, Bae T, Missiakas DM, Schneewind O. 2010. Contribution of coagulases towards *Staphylococcus aureus* disease and protective immunity. PLoS Pathog 6:e1001036. https://doi.org/10.1371/journal.ppat.1001036.
17. Cheng AG, DeDent AC, Schneewind O, Missiakas D. 2011. A play in four acts: *Staphylococcus aureus* abscess formation. Trends Microbiol 19:225–232. https://doi.org/10.1016/j.tim.2011.01.007.
18. Chmagh AA, Abd Al-Abbas MJ. 2019. Comparison between the coagulase (*coa* and *vwb*) genes in *Staphylococcus aureus* and other staphylococci. Gene Rep 16:100410. https://doi.org/10.1016/j.genrep.2019.100410.
19. Sewid AH, Hassan MN, Ammar AM, Bemis DA, Kania SA. 2018. Identification, cloning and characterization of *Staphylococcus pseudintermedius* coagulase. Infect Immun 86:e00027-18. https://doi.org/10.1128/IAI.00027-18.
20. Madhaiyan M, Wirth JS, Saravanan VS. 2020. Phylogenomic analyses of the *Staphylococcaceae* family suggest the reclassification of five species within the genus *Staphylococcus* as heterotypic synonyms, the promotion of five subspecies to novel species, the taxonomic reassignment of five *Staphylococcus* species to *Mammaliicoccus* gen. nov., and the formal assignment of *Nosocomiicoccus* to the family *Staphylococcaceae*. Int J Syst Evol Microbiol 70:5926–5936. https://doi.org/10.1099/ijsem.0.004498.
21. Probst AJ, Hertel C, Richter L, Wassill L, Ludwig W, Hammes WP. 1998. *Staphylococcus condimenti* sp. nov., from soy sauce mash, and *Staphylococcus carnosus* (Schleifer and Fischer 1982) subsp. *utilis* subsp. nov. Int J Syst Evol Microbiol 48:651–658.
22. Tong SYC, Schaumburg F, Ellington MJ, Corander J, Pichon B, Leendertz F, Bentley SD, Parkhill J, Holt DC, Peters G, Giffard PM. 2015. Novel staphylococcal species that form part of a *Staphylococcus aureus*-related complex: the non-pigmented *Staphylococcus argenteus* sp. nov. and the non-human primate-associated *Staphylococcus schweitzeri* sp. nov. Int J Syst Evol Microbiol 65:15–22. https://doi.org/10.1099/ijs.0.062752-0.
23. Devriese LA, Hajek V, Oeding P, Meyer SA, Schleifer KH. 1978. *Staphylococcus hyicus* (Sompolinsky 1953) comb. nov. and *Staphylococcus hyicus* subsp. *chromogenes* subsp. nov. Int J Syst Evol Microbiol 28:482–490. https://doi.org/10.1099/00207713-28-4-482.
24. Taponen S, Supré K, Piessens V, Van Coillie E, De Vliegher S, Koort JMK. 2012. *Staphylococcus agnetis* sp. nov., a coagulase-variable species from bovine subclinical and mild clinical mastitis. Int J Syst Evol Microbiol 62:61–65. https://doi.org/10.1099/ijs.0.028365-0.
25. Murray AK, Lee J, Bendall R, Zhang L, Sunde M, Schau Slettemeås J, Gaze W, Page AJ, Vos M. 2018. *Staphylococcus cornubiensis* sp. nov., a member of the *Staphylococcus intermedius* Group (SIG). Int J Syst Evol Microbiol 68:3404–3408. https://doi.org/10.1099/ijsem.0.002992.
26. Perreten V, Kania SA, Bemis D. 2020. *Staphylococcus ursi* sp. nov., a new member of the '*Staphylococcus intermedius* group' isolated from healthy black bears. Int J Syst Evol Microbiol 70:4637–4645. https://doi.org/10.1099/ijsem.0.004324.
27. Igimi S, Takahashi E, Mitsuoka T. 1990. *Staphylococcus schleiferi* subsp. *coagulans* subsp. nov., isolated from the external auditory meatus of dogs with external ear otitis. Int J Syst Bacteriol 40:409–411. https://doi.org/10.1099/00207713-40-4-409.
28. Becker K, Heilmann C, Peters G. 2014. Coagulase-negative staphylococci. Clin Microbiol Rev 27:870–926. https://doi.org/10.1128/CMR.00109-13.
29. Lamers RP, Muthukrishnan G, Castoe TA, Tafur S, Cole AM, Parkinson CL. 2012. Phylogenetic relationships among *Staphylococcus* species and refinement of cluster groups based on multilocus data. BMC Evol Biol 12:171. https://doi.org/10.1186/1471-2148-12-171.
30. Naushad S, Barkema HW, Luby C, Condas LAZ, Nobrega DB, Carson DA, De Buck J. 2016. Comprehensive phylogenetic analysis of bovine non-aureus staphylococci species based on whole-genome sequencing. Front Microbiol 7:1990. https://doi.org/10.3389/fmicb.2016.01990.
31. Richardson EJ, Bacigalupe R, Harrison EM, Weinert LA, Lycett S, Vrieling M, Robb K, Hoskisson PA, Holden MTG, Feil EJ, Paterson GK, Tong SYC, Shittu A, van Wamel W, Aanensen DM, Parkhill J, Peacock SJ, Corander J, Holmes M, Fitzgerald JR. 2018. Gene exchange drives the ecological success of a multi-host bacterial pathogen. Nat Ecol Evol 2:1468–1478. https://doi.org/10.1038/s41559-018-0617-0.
32. Simou C, Hill PB, Forsythe PJ, Thoday KL. 2005. Species specificity in the adherence of staphylococci to canine and human corneocytes: a preliminary study. Vet Dermatol 16:156–161. https://doi.org/10.1111/j.1365-3164.2005.00452.x.
33. Hajek V. 1976. *Staphylococcus intermedius*, a new species isolated from animals. Int J Syst Bacteriol 26:401–408. https://doi.org/10.1099/00207713-26-4-401.
34. Bannoehr J, Ben Zakour NL, Waller AS, Guardabassi L, Thoday KL, van den Broek AHM, Fitzgerald JR. 2007. Population genetic structure of the *Staphylococcus intermedius* group: insights into *agr* diversification and the emergence of methicillin-resistant strains. J Bacteriol 189:8685–8692. https://doi.org/10.1128/JB.01150-07.
35. Duthie ES, Lorenz LL. 1952. Staphylococcal coagulase: mode of action and antigenicity. J Gen Microbiol 6:95–107. https://doi.org/10.1099/00221287-6-1-2-95.
36. Zapotoczna M, McCarthy H, Rudkin JK, O'Gara JP, O'Neill E. 2015. An essential role for coagulase in *Staphylococcus aureus* biofilm development reveals new therapeutic possibilities for device-related infections. J Infect Dis 212:1883–1893. https://doi.org/10.1093/infdis/jiv319.
37. Vanassche T, Peetermans M, Van Aelst LNL, Peetermans WE, Verhaegen J, Missiakas DM, Schneewind O, Hoylaerts MF, Verhamme P. 2013. The role of staphylothrombin-mediated fibrin deposition in catheter-related *Staphylococcus aureus* infections. J Infect Dis 208:92–100. https://doi.org/10.1093/infdis/jit130.
38. Moradigaravand D, Jamrozy D, Mostowy R, Anderson A, Nickerson EK, Thaipadungpanit J, Wuthiekanun V, Limmathurotsakul D, Tandhavanant S, Wikraiphat C, Wongsuvan G, Teerawattanasook N, Jutrakul Y, Srisurat N, Chaimanee P, Eoin West T, Blane B, Parkhill J, Chantratita N, Peacock SJ. 2017. Evolution of the *Staphylococcus argenteus* ST2250 clone in northeastern Thailand is linked with the acquisition of livestock-associated staphylococcal genes. mBio 8:e00802-17. https://doi.org/10.1128/mBio.00802-17.
39. Aung MS, San T, San N, Oo WM, Ko PM, Thet KT, Urushibara N, Kawaguchiya M, Sumi A, Kobayashi N. 2019. Molecular characterization of *Staphylococcus argenteus* in Myanmar: identification of novel genotypes/clusters in staphylocoagulase, protein A, alpha-haemolysin and other virulence factors. J Med Microbiol 68:95–104. https://doi.org/10.1099/jmm.0.000869.
40. Aung MS, Urushibara N, Kawaguchiya M, Sumi A, Takahashi S, Ike M, Ito M, Habadera S, Kobayashi N. 2019. Molecular epidemiological characterization of *Staphylococcus argenteus* clinical isolates in Japan: identification of three clones (ST1223, ST2198, and ST2550) and a novel staphylocoagulase genotype XV. Microorganisms 7:389. https://doi.org/10.3390/microorganisms7100389.
41. Lominski I, Grossfeld E. 1944. Coagulase test for rapid detection of *Staph. aureus*. Br Med J 2:854. https://doi.org/10.1136/bmj.2.4382.854.
42. Váradi L, Luo JL, Hibbs DE, Perry JD, Anderson RJ, Orenga S, Groundwater PW. 2017. Methods for the detection and identification of pathogenic bacteria: past, present, and future. Chem Soc Rev 46:4818–4832. https://doi.org/10.1039/c6cs00693k.
43. MacFadyen AC, Leroy S, Harrison EM, Parkhill J, Holmes MA, Paterson GK. 2019. *Staphylococcus pseudoxylosus* sp. nov., isolated from bovine mastitis. Int J Syst Evol Microbiol 69:2208–2213. https://doi.org/10.1099/ijsem.0.003416.
44. Fountain K, Gibbon MJ, Loeffler A, Feil EJ. 2021. Closed genome sequences of *Staphylococcus lloydii* sp. nov. and *Staphylococcus durrellii* sp. nov. isolated from captive fruit bats (*Pteropus livingstonii*). Int J Syst Evol Microbiol 71:004715. https://doi.org/10.1099/ijsem.0.004715.
45. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
46. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, Fookes M, Falush D, Keane JA, Parkhill J. 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31:3691–3693. https://doi.org/10.1093/bioinformatics/btv421.
47. Price MN, Dehal PS, Arkin AP. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol 26:1641–1650. https://doi.org/10.1093/molbev/msp077.
48. Do CB, Mahabhashyam MSP, Brudno M, Batzoglou S. 2005. ProbCons: probabilistic consistency-based multiple sequence alignment. Genome Res 15:330–340. https://doi.org/10.1101/gr.2821705.

49. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009. Jalview version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 25:1189–1191. https://doi.org/10.1093/bioinformatics/btp033.
50. Martin DP, Murrell B, Golden M, Khoosal A, Muhire B. 2015. RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evol 1:vev003. https://doi.org/10.1093/ve/vev003.
51. Mostowy R, Croucher NJ, Andam CP, Corander J, Hanage WP, Marttinen P. 2017. Efficient inference of recent and ancestral recombination within bacterial populations. Mol Biol Evol 34:1167–1182. https://doi.org/10.1093/molbev/msx066.
52. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096.
53. Lu S, Wang J, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Marchler GH, Song JS, Thanki N, Yamashita RA, Yang M, Zhang D, Zheng C, Lanczycki CJ, Marchler-Bauer A. 2020. CDD/SPARCLE: the conserved domain database in 2020. Nucleic Acids Res 48:D265–D268. https://doi.org/10.1093/nar/gkz991.
54. Monk IR, Tree JJ, Howden BP, Stinear TP, Foster TJ. 2015. Complete bypass of restriction systems for major *Staphylococcus aureus* lineages. mBio 6:e00308-15. https://doi.org/10.1128/mBio.00308-15.
55. Pietrocola G, Gianotti V, Richards A, Nobile G, Geoghegan JA, Rindi S, Monk IR, Bordt AS, Foster TJ, Fitzgerald JR, Speziale P. 2015. Fibronectin binding proteins SpsD and SpsL both support invasion of canine epithelial cells by *Staphylococcus pseudintermedius*. Infect Immun 83:4093–4102. https://doi.org/10.1128/IAI.00542-15.
56. Friedrich R, Panizzi P, Fuentes-Prior P, Richter K, Verhamme I, Anderson PJ, Kawabata S-I, Huber R, Bode W, Bock PE. 2003. Staphylocoagulase is a prototype for the mechanism of cofactor-induced zymogen activation. Nature 425:535–539. https://doi.org/10.1038/nature01962.