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BACTERIAL INFECTIONS

The Intracellular Cyclophilin PpiB Contributes to the Virulence of *Staphylococcus aureus* Independently of Its Peptidyl-Prolyl *cis/trans* Isomerase Activity

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ABSTRACT The *Staphylococcus aureus* cyclophilin PpiB is an intracellular peptidyl prolyl *cis/trans* isomerase (PPIase) that has previously been shown to contribute to secreted nuclease and hemolytic activity. In this study, we investigated the contribution of PpiB to *S. aureus* virulence. Using a murine abscess model of infection, we demonstrated that a *ppiB* mutant is attenuated for virulence. We went on to investigate the mechanism through which PpiB protein contributes to virulence, in particular the contribution of PpiB PPIase activity. We determined the amino acid residues that are important for PpiB PPIase activity and showed that a single amino acid substitution (F64A) completely abrogates PPIase activity. Using purified PpiB F64A protein *in vitro*, we showed that PPIase activity only partially contributes to Nuc refolding and that PpiB also possesses PPIase-independent activity. Using allelic exchange, we introduced the F64A substitution onto the *S. aureus* chromosome, generating a strain that produces enzymatically inactive PpiB. Analysis of the PpiB F64A strain revealed that PPIase activity is not required for hemolysis of human blood or virulence in a mouse. Together, these results demonstrate that PpiB contributes to *S. aureus* virulence via a mechanism unrelated to prolyl isomerase activity.

KEYWORDS PPIase, *S. aureus*, cyclophilin, nuclease, PpiB, *Staphylococcus aureus*, chaperone, micrococcal nuclease

Staphylococcus aureus is a Gram-positive bacterium that colonizes the anterior nares of approximately 30% of the population. In addition, *S. aureus* is considered an opportunistic pathogen, causing diseases that range in severity from minor skin and soft tissue infections to life-threatening sepsis, endocarditis, and necrotizing fasciitis (1). One factor contributing to the diversity of diseases caused by *S. aureus* is the myriad of virulence factors produced by the organism. These include immune evasion proteins, microbial surface components recognizing adhering matrix molecules (MSCRAMMs), toxins, superantigens, and exoenzymes. One such exoenzyme secreted by *S. aureus* is staphylococcal nuclease (Nuc), which plays important roles in immune evasion and biofilm growth (2–7).

Previous work in our lab investigating the process of Nuc secretion identified an intracellular peptidyl-prolyl *cis/trans* isomerase (PPIase), PpiB, that contributes to Nuc activity (8). PPIase enzymes (also known as foldases) catalyze the *cis*-to-*trans* isomerization of proline peptide bonds, which is often the rate-limiting step in protein folding (9). PpiB is a functional PPIase belonging to the cyclophilin family, and it assists in the refolding of Nuc *in vitro*. Culture supernatants from a *ppiB* mutant strain had decreased Nuc activity and also decreased hemolytic activity, suggesting that there are additional cellular targets for PpiB within the *S. aureus* cell. While that work clearly demonstrated a role for PpiB in the secretion of *S. aureus* virulence factors, it remains unclear if PpiB contributes to nuclease/hemolytic activity through its PPIase enzymatic activity or via some other function. Furthermore, it is unknown if the reduction in nuclease/hemolytic

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activity in a *ppiB* mutant manifests as decreased virulence, although a recent transposon sequencing (Tn-seq) study suggests that a *ppiB* mutant has decreased fitness in an abscess model of infection (10). In this study, we set out to determine the role of PpiB during infection. Specifically, we investigated if PpiB contributes to virulence via its PPIase activity. Previous work by Alonzo et al., studying the PPIase PrsA2 in *Listeria monocytogenes*, showed that while PrsA2 demonstrates PPIase enzymatic activity, its role in virulence is not entirely dependent on this activity (11). PrsA2 catalytic activity is required for full virulence *in vivo* but is not required to restore defects in hemolytic activity, phospholipase activity, or L2 plaque formation (12). These results show that PPIase enzymes can have additional functions not limited to PPIase activity that contribute to virulence.

In this study, we investigated the role of PpiB in *S. aureus* virulence and showed that a Δ*ppiB* mutant is attenuated in a murine abscess model of infection. We investigated the contribution of PpiB PPIase activity by determining the amino acid residues within PpiB that are necessary for PPIase activity and used this information to construct a strain containing a single substitution (F64A) that abolishes this activity. This strain (which produces an enzymatically inactive form of PpiB) was tested for known PpiB functions, including nuclease refolding, hemolytic activity, and virulence, to determine the contribution of PPIase activity to PpiB function. Together, the results demonstrate that the PPIase activity of PpiB partially contributes to Nuc refolding; however, it is not required for hemolytic activity or virulence. These data suggest that PpiB has additional roles in the cell that contribute to the virulence of *S. aureus*.

RESULTS

PpiB is required for virulence in a murine abscess model of infection. Previous work by our group has demonstrated that PpiB, an intracellular cyclophilin family member, contributes to the activity of secreted virulence factors in *S. aureus* (8). Specifically, reduced nuclease and hemolytic activities were observed in culture supernatants from a *ppiB* mutant strain. We hypothesized that this reduction in activity would manifest as an attenuation of virulence during infection. Due to the previously demonstrated role for Nuc in avoiding clearance by neutrophil extracellular traps (NETs) (4), we postulated that removal of *ppiB* (and the subsequent reduction in Nuc activity [8]) would manifest as increased clearance of the bacteria by the immune system. To test this hypothesis, we employed a murine abscess model of infection to compare the numbers of wild-type and Δ*ppiB* mutant bacteria present in abscesses following a 7-day infection. BALB/c mice were injected subcutaneously in the lower right flank with 10^6 CFU of either the wild type or a Δ*ppiB* strain, and following a 7-day infection period, mice were sacrificed, abscesses were excised, and the number of bacteria present in abscesses was determined. The *ppiB* mutant was significantly attenuated for virulence in the abscess model, with approximately 14-fold fewer bacteria recovered from abscesses (Fig. 1). This result supports our hypothesis that reduced virulence factor activity in the *ppiB* mutant leads to attenuation of virulence *in vivo*.

Determination of the PPIase active-site residues in PpiB. PpiB is a member of the cyclophilin family of PPIases. In this family of enzymes, six amino acid residues are known to be important for PPIase activity (13). When we aligned the sequence of PpiB to that of the prototypical cyclophilin family member, human cyclophilin A (14), we observed that all six of these residues (H58, R59, F64, Q114, F116, and W152) are conserved in PpiB (Fig. 2A). To investigate if these six residues make up an enzymatic pocket similar to that in cyclophilin A, we used the SWISS-MODEL automated protein structure homology modeling server (15–17) to predict the three-dimensional structure of PpiB and compare it to the previously published structure of cyclophilin A (Fig. 2B and C). The position and coordination of all six amino acids are highly conserved in the PpiB predicted structure. Based on this analysis, we hypothesized that these six residues comprise the PPIase active site in PpiB.

Substitution of PPIase active sites eliminates PPIase activity in PpiB. We have previously shown that purified PpiB protein (i) is functional as a PPIase and (ii) assists

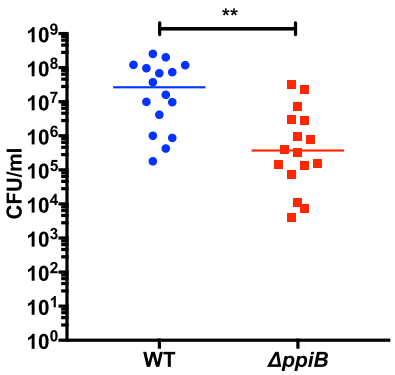

FIG 1 PpiB is required for virulence in a murine abscess model of infection. Female, 6-week-old BALB/c mice were injected subcutaneously with wild-type (WT) *S. aureus* or a Δ*ppiB* mutant strain. The infection was allowed to proceed for 7 days. Mice were then sacrificed before abscesses were excised, homogenized, and diluted and plated to enumerate bacteria present in the abscesses. In mice infected with the Δ*ppiB* mutant strain, a significant (14-fold) reduction in bacterial numbers was detected. Experiments were performed twice with 16 mice for each strain. The median value for each sample is indicated. Significance was determined by Student's *t* test. **, $P < 0.01$.

in the folding of staphylococcal nuclease. Based on these data and the results from the mouse infection assay (Fig. 1), we hypothesized that the PPIase activity of PpiB contributes to the virulence of *S. aureus* by assisting the folding (and hence activation) of secreted virulence factors. To test this hypothesis, we set out to construct a strain of *S. aureus* that expresses an enzymatically inactive form of PpiB and determine if the loss of PPIase activity leads to an attenuation of virulence similar to that observed in the Δ*ppiB* mutant.

The first step in this process was to experimentally determine if the six amino acid residues outlined above (H58, R59, F64, Q114, F116, and W152) comprise the PpiB PPIase active site (18). Site-directed mutagenesis was performed whereby each of the six amino acids was replaced with alanine (H58A, R59A, F64A, Q114A, F116A, and W152A). The resulting recombinant proteins were purified and used in a chymotrypsin-coupled PPIase activity assay previously used by our group to study PpiB (8). As expected, wild-type PpiB protein exhibited PPIase activity in the oligopeptide cleavage assay (Fig. 3). Analysis of the PpiB substitution proteins revealed that 4 of the 6 substitutions abrogated PPIase activity. Specifically, the R59A, F64A, Q114A, and F116A substitution forms of PpiB did not display PPIase activity. The H58A and W152A substitution proteins displayed modest reductions in PPIase activity. The F64A substitution form of PpiB consistently showed no PPIase activity above negative controls (Fig. 3); therefore, we elected to proceed with and further characterize this enzymatically inactive form of PpiB.

PpiB F64A demonstrates reduced nuclease refolding ability. *In vitro* studies by our group have demonstrated that PpiB is required for the optimal folding of Nuc (12). To determine if the PPIase activity of PpiB is necessary for Nuc refolding, we performed a Nuc refolding assay whereby we measured the rate of Nuc refolding in the presence of native PpiB and compared it to the enzymatically inactive PpiB F64A. Recombinant Nuc protein was purified and denatured in 8 M urea. Denatured Nuc was allowed to refold by dilution into urea-free buffer in the absence of a PPIase or in the presence of PpiB or PpiB F64A. The activity of refolded Nuc in each sample was determined by adding an oligonucleotide probe. The probe contains a quencher at the 3′ end and a fluorophore at the 5′ end. When active Nuc is present, it digests the probe and a fluorescent signal is detected. As previously shown (8), when native PpiB is added to the reaction, there is increased fluorescence, indicative of active refolded Nuc (Fig. 4). In the absence of PpiB, low levels of fluorescence are detected, indicating minimal Nuc activity. Reactions including PpiB F64A show an intermediate phenotype with fluorescence levels higher than the negative control (no PpiB) but less than in reactions

A

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Cyclophilin A       1   MVNPTVFFDIAVDGE-------PLGRVSFELFADKVPKTAENFRALSTGE     43
                        |.|..........||       ..|.::|:||.:..|||.|||  ::..:
PpiB                1   MANYPQLNKEVQQGEIKVVMHTNKGDMTFKLFPNIAPKTVENF--VTHAK     48

Cyclophilin A       44  KGFGYKGSCFHRIIPGFMCQGGDFTRHNGTGGKSIYGEKFEDENFILKHT     93
                        .|: |.|..|||:|..||.||||.|. .|.||:||||..||||..:....
PpiB                49  NGY-YDGITFHRVINDFMIQGGDPTA-TGMGGESIYGGAFEDEFSLNAFN     96

Cyclophilin A       94  GPGILSMANAGPNTNGSQFFICTAK-------------------------   118
                        ..|.|||||:|||||||||||...|
PpiB                97  LYGALSMANSGPNTNGSQFFIVQMKEVPQNMLSQLADGGWPQPIVDAYGE   146

Cyclophilin A       119 ---TEWLDGKHVVFGKVKEGMNIVE--AMERFGSRNGKTSKKITIADCGQ   163
                            |.|||.||.|||::.:|...:|   |..:.|.:: |....:.|.....
PpiB                147 KGGTPWLDQKHTVFGQIIDGETTLEDIANTKVGPQD-KPLHDVVIESIDV   195

Cyclophilin A       164 LE    165
                        .|
PpiB                196 EE    197
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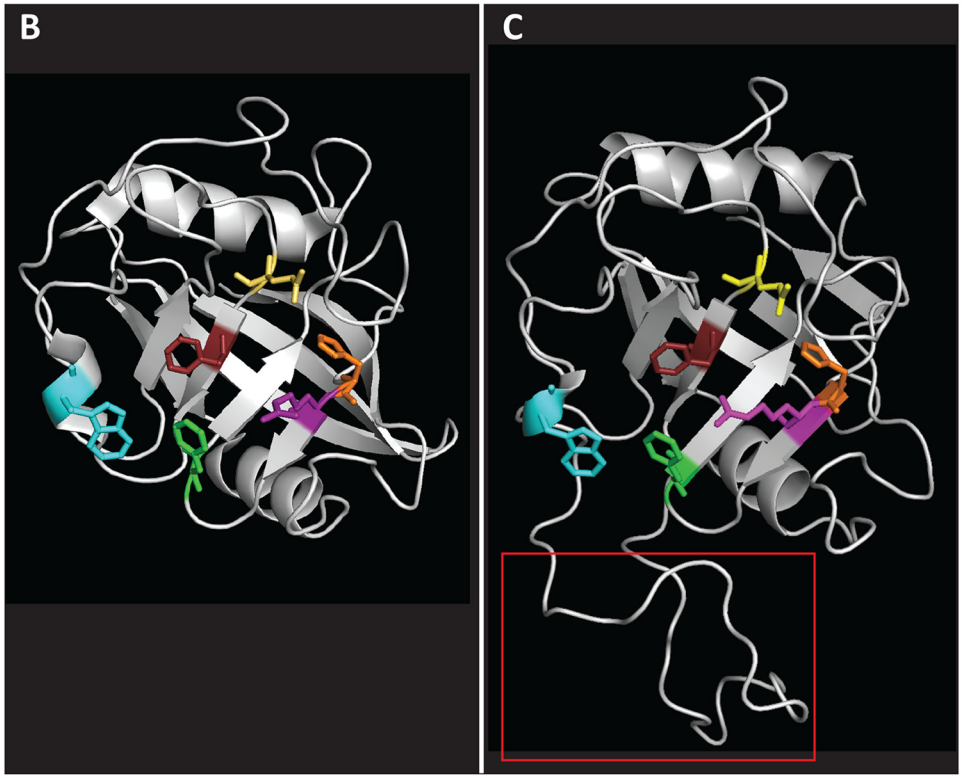

FIG 2 Identification of PPIase active-site residues in PpiB. (A) Sequence alignment of PpiB and human cyclophilin A. The six active-site residues in human cyclophilin A are colored along with their corresponding amino acids in PpiB. (B) Structure of human cyclophilin A (PDB code 2A2N). The six active-site residues are color coded the same as in panel A. (C) Predicted structure of PpiB. The three-dimensional structure of PpiB was predicted using the SWISS-MODEL automated protein structure homology modeling server. The six predicted active-site residues are color coded the same as in panels A and B. The 28-amino-acid internal loop found in PpiB that is absent in cyclophilin A is indicated (red box).

including native PpiB. While these data demonstrate that the PPIase activity of PpiB plays a role in Nuc refolding (compare native PpiB to PpiB F64A), they also show that in the absence of PPIase enzymatic activity, PpiB still contributes to Nuc refolding (compare PpiB F64A to no PpiB). Together, these results suggest that PpiB possesses some additional PPIase-independent activity that contributes to Nuc refolding.

PpiB is expressed in the F64A strain. Having identified an amino acid substitution (F64A) that abrogates PpiB PPIase activity, we next wanted to construct a strain of *S.*

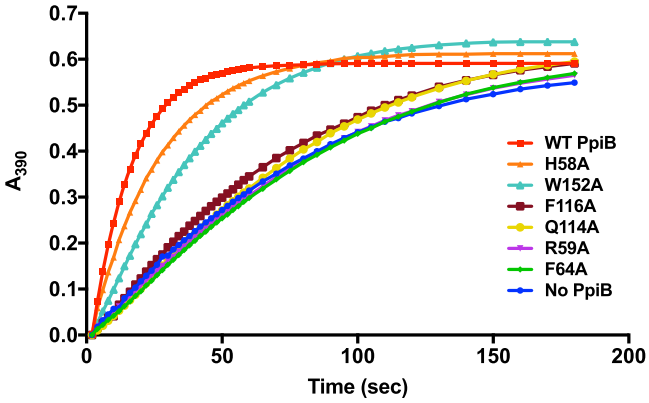

FIG 3 Substitution of predicted active-site residues eliminates PpiB PPIase activity. Purified recombinant PpiB (5 nM) was used in a chymotrypsin-coupled PPIase activity assay. Chymotrypsin cleaves the substrate peptide only when the prolyl peptide bond is in the *trans* conformation; therefore, in the absence of a PPIase (blue line), the reaction proceeds to completion based on the slow endogenous rate of *cis*-to-*trans* isomerization of the peptide bond. Addition of PpiB to the reaction results in an increased rate of cleavage consistent with PpiB functioning as a PPIase (red line). The addition of PpiB containing any of the substitutions F116A, Q114A, R59A, and F64A did not increase the rate of reaction above the negative control. Addition of PpiB containing substitution H58A or W152A resulted in an increase in the rate of cleavage but not to the same extent as wild-type PpiB. Experiments were performed a minimum of three times. Representative data sets are shown.

aureus that expresses this enzymatically inactive form of PpiB on the chromosome. To do this, we utilized the allelic-exchange plasmid pJB38 (19). Once the chromosomal copy of *ppiB* had been exchanged for the *ppiB* F64A allele, we performed growth rate analysis to confirm that there was no difference in growth rate between the wild type, Δ*ppiB* mutant, and F64A strain (see Fig. S1 in the supplemental material). Western blotting was then performed to ensure that the PpiB F64A protein was being expressed at levels comparable to those of wild-type PpiB. Intracellular protein fractions from overnight cultures of wild-type *S. aureus*, the Δ*ppiB* mutant, and PpiB F64A strains were separated by SDS-PAGE (Fig. S2) and probed using anti-PpiB antiserum. Results show comparable levels of PpiB protein in the wild type and F64A strain and no PpiB in the

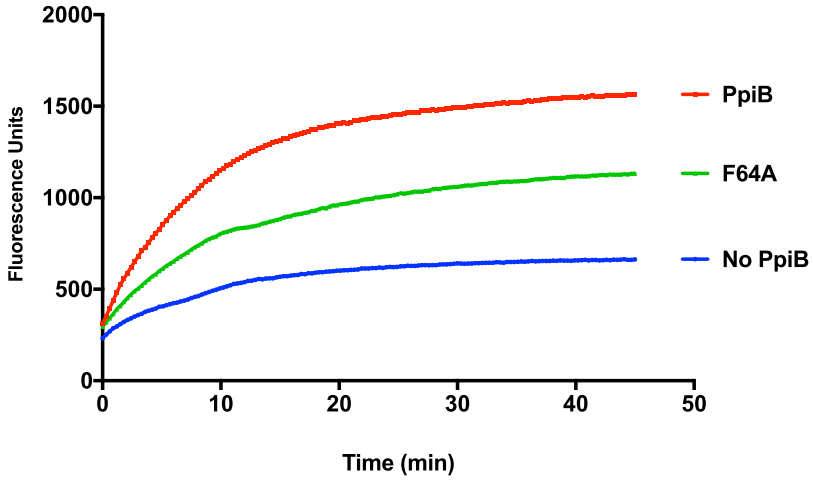

FIG 4 PpiB increases the rate of Nuc refolding in the absence of PPIase activity. Denatured Nuc was diluted 1:40 into denaturant-free buffer containing an oligonucleotide probe. Refolding and subsequent activity of Nuc were visualized by fluorescence resulting from cleavage of the probe. Refolding reactions in the presence of PpiB (red line) demonstrate high levels of nuclease activity, while reactions without PpiB (blue line) demonstrate low levels of nuclease activity. Addition of PpiB F64A to the reaction resulted in an increase in nuclease activity (above the negative control), although the increase was less than that observed with wild-type PpiB protein (green line). Experiments were performed a minimum of three times. Representative data are shown.

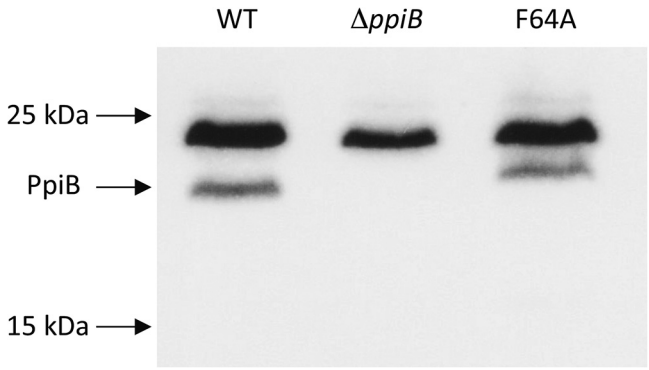

FIG 5 PpiB expression levels are comparable in the wild type and F64A substitution strain. A Western immunoblot was performed using polyclonal anti-PpiB antisera and whole-cell lysates from wild-type *S. aureus*, the Δ*ppiB* mutant, and PpiB F64A substitution strain. No PpiB was detected in the Δ*ppiB* strain, while comparable levels of PpiB protein were detected in the wild-type and PpiB F64A substitution strains. A slight difference in apparent molecular weight of the PpiB F64A substitution protein was observed.

ppiB mutant (Fig. 5). While the amounts of PpiB were similar in the wild type and F64A strain, the F64A substitution form of PpiB appeared to run at a slightly higher molecular weight than wild-type PpiB. This apparent difference in migration was also visible when the purified recombinant forms of each protein were analyzed by SDS-PAGE and stained with Coomassie blue (data not shown). Although the reason for this difference is unclear, previous studies have demonstrated that single amino acid substitutions can lead to differences in SDS-PAGE migration rates (20). It is possible that the variation is due to differences in charge when phenylalanine is changed to alanine. The Western blot analysis also revealed the presence of a cross-reacting band that migrated at a slightly higher apparent molecular weight than PpiB. Interestingly, this band appeared to be reduced in the *ppiB* mutant, although equal quantities of protein were loaded in each lane (Fig. S2). We speculate that this band may represent an intracellular protein whose expression is controlled by PpiB in a PPIase-independent manner (as it is present at similar levels in the wild type and the F64A strain).

The PpiB F64A strain does not demonstrate reduced hemolytic activity. Previous work in our lab has shown that culture supernatants from a *ppiB* mutant strain have decreased hemolytic activity (12). *S. aureus* secretes a number of toxins capable of lysing erythrocytes (21). The identity of the PpiB-regulated toxin and the role of PpiB in hemolysis remain unknown. To determine if PPIase activity is required for PpiB-dependent hemolytic activity and to explore the identity of the hemolysin responsible, we performed erythrocyte lysis assays using both human and rabbit blood with culture supernatants from the wild-type, Δ*ppiB* mutant, and PpiB F64A strains. A significant decrease in human erythrocyte lysis was observed in Δ*ppiB* mutant supernatants (as previously reported); however, no decrease in hemolytic activity was observed using supernatants from the F64A strain (Fig. 6A). This result demonstrates that the PPIase activity of PpiB is not required for hemolysis. Interestingly, when the experiments were repeated using rabbit blood, only a small, nonsignificant reduction in hemolysis was observed in *ppiB* mutant culture supernatants (Fig. 6B). This result shows that the PpiB-dependent hemolytic factor is less potent against rabbit erythrocytes than human erythrocytes, indicating that alpha toxin (Hla) is not responsible, as it has greater affinity for rabbit erythrocytes than human erythrocytes (22–25). Together these results show that (i) alpha toxin is not the PpiB-dependent hemolysin and (ii) PpiB-dependent hemolysis is independent of PPIase activity, strongly suggesting that PpiB possesses some additional function.

The PPIase activity of PpiB does not contribute to *S. aureus* virulence. The results outlined in Fig. 1 show that PpiB is required for virulence in a murine abscess model of infection. To test the hypothesis that PpiB contributes to virulence via its

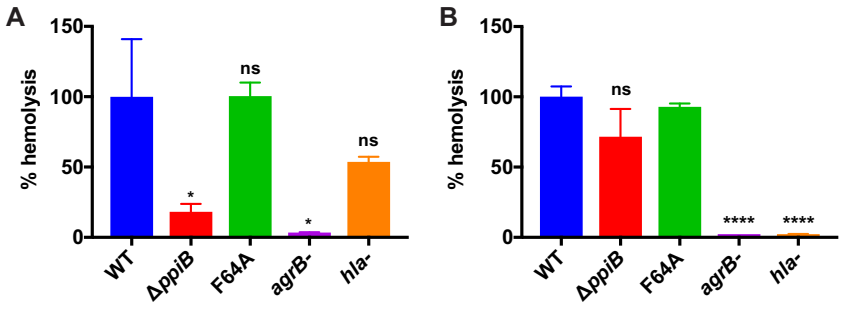

FIG 6 PpiB-dependent hemolytic activity does not require PPIase activity. Erythrocyte lysis assays were performed using *S. aureus* culture supernatants and whole human blood (A) or rabbit blood (B). (A) A significant decrease in hemolytic activity against human erythrocytes was observed using culture supernatants from a *ppiB* mutant strain. Culture supernatants from the PpiB F64A strain did not display any significant decrease in hemolytic activity compared to the wild-type strain. Hemolytic activity was greatly reduced in an *agrB* mutant, while a small, nonsignificant reduction in hemolysis was observed in an *hla* mutant. (B) Using rabbit blood, a small, nonsignificant reduction in hemolytic activity was observed in the *ppiB* mutant, while no decrease was observed in the PpiB F64A strain. Hemolytic activity was greatly reduced in both the *agrB* and *hla* mutant strains. Hemolysis assays were performed a minimum of 3 times. Hemolytic activity in the wild-type strain for each experiment was set to 100%, and the relative hemolytic activity of the other strains is indicated as a percentage. The data presented are the averages of 4 replicates. Significance was determined by Student's *t* test. ****, $P < 0.001$; *, $P < 0.05$; ns, not significant.

PPIase activity, we repeated the murine abscess infection to compare the virulence of wild-type *S. aureus* to the Δ*ppiB* mutant and PPIase-inactivated F64A strain. As previously shown (Fig. 1), a significant reduction in bacterial numbers was observed in the abscesses of mice infected with the Δ*ppiB* mutant. In contrast, there was no reduction in bacterial numbers when the F64A strain was used (Fig. 7). These data clearly show that while PpiB contributes to virulence in *S. aureus*, it does not do so via its PPIase activity. Together, our data show that PpiB has an additional role in the cell not limited to prolyl isomerization.

DISCUSSION

Bacterial PPIase proteins have been extensively studied based on their ability to catalyze the *cis*-to-*trans* isomerization around proline-peptide bonds and assist in the

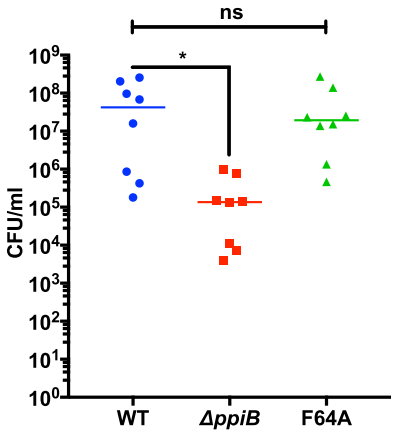

FIG 7 The PPIase activity of PpiB does not contribute to *S. aureus* virulence. Groups of eight BALB/c mice were injected subcutaneously with wild-type *S. aureus*, the Δ*ppiB* mutant, and the PpiB F64A strain. The infection was allowed to proceed for 7 days. Mice were then sacrificed before abscesses were excised, homogenized, and diluted and plated to enumerate bacteria present in the abscesses. As previously observed (Fig. 1), a decrease in the number of bacterial cells was observed in the abscesses of mice infected with the Δ*ppiB* mutant strain. No significant decrease in bacterial numbers was observed in abscesses of mice infected with the PpiB F64A strain. The median value for each sample is indicated. Significance was determined by Student's *t* test. *, $P < 0.01$.

folding of secreted virulence factors (11, 26–29). Previous work by our group on the *S. aureus* cyclophilin PpiB demonstrated that PpiB is a functional PPIase, that it affects the refolding and activity of the virulence factor Nuc, and that it contributes to hemolytic activity (8). Based on these findings, we sought to investigate whether PpiB contributes to virulence in *S. aureus* and whether its demonstrated PPIase activity (which assists in the folding of at least one of these virulence factors) contributes to disease.

We utilized a murine abscess model of infection to show that a Δ*ppiB* mutant is attenuated compared to wild-type *S. aureus*. A 14-fold reduction in bacterial numbers was observed in abscesses of mice infected with the Δ*ppiB* mutant. Our initial hypothesis was that this reduction in virulence was due to the loss of PpiB PPIase activity and its role in the secretion and folding of Nuc, as well as a hemolytic toxin. To test this hypothesis, we created a strain (F64A) in which the PPIase activity of PpiB was eliminated and tested it for known PpiB-related phenotypes. Results from *in vitro* nuclease refolding assays show that PpiB F64A can still assist the refolding of Nuc, although not to the levels observed when using wild-type PpiB protein. This demonstrates that the while PPIase activity contributes to Nuc folding *in vitro*, PpiB also assists refolding of Nuc in a PPIase-independent manner. Many PPIases also possess chaperone activity (30–33); therefore, it is possible that PpiB may also function as a chaperone. Typically, chaperone proteins prevent the aggregation of proteins inside the cell under stressful conditions (34). By binding to Nuc and protecting it under denaturing conditions, PpiB may act as a chaperone as well as assisting in the folding of Nuc via its PPIase activity. The idea of PpiB functioning as both a PPIase and chaperone is consistent with recent work by Skagia et al., who demonstrated similar dual roles for a PpiB homologue in *Escherichia coli* (32, 35). Deletion of *ppiB* in *E. coli* results in increased swimming motility, swarming motility, and biofilm formation. The swarming and biofilm phenotypes could not be complemented by enzymatically inactive PpiB mutants, indicating that these processes require PpiB PPIase activity. However, the swimming phenotype could be restored by an enzymatically inactive form of PpiB, indicating that this process required a PPIase-independent function of the PpiB protein. Together, these data show that *E. coli* PpiB can function as a PPIase but also has PPIase-independent activity.

Interestingly, when we examined the predicted three-dimensional structure of PpiB (Fig. 2C), we observed a 28-amino-acid loop that is present in *S. aureus* PpiB but absent in human cyclophilin. To investigate if this loop could potentially impart PPIase-independent activity, we performed a BLAST search using the 28-amino-acid sequence (i.e., residues 122 to 149 in PpiB). To identify potentially novel functions for this region we excluded hits to members of the *Staphylococceae* and to annotated PPIases. The resulting matches show that residues 122 to 135 in PpiB have a high degree of homology to proteins in the PMIT family [protein-L-isoaspartate(D-aspartate) *O*-methyltransferase]. Proteins in this family are methyltransferases that catalyze the repair of damaged aspartic acid residues. Although widespread in nature, *S. aureus* does not appear to encode any PMIT family members. A multiple-sequence alignment of PpiB homologues shows that the PMIT region is unique to *S. aureus* PpiB (Fig. S3); therefore, it is tempting to speculate that the PPIase-independent function of PpiB may be mediated via methyltransferase activity and repair of damaged aspartic acid residues. Work is under way in our lab to further explore the role of this PMIT homologous region of PpiB.

To further investigate the role of PpiB in the cell, we performed whole-blood hemolysis assays. While our previous work demonstrated a hemolytic defect in a *ppiB* mutant, it was unknown which hemolytic toxin PpiB was regulating. In this study, we performed whole-blood hemolysis assays with both human and rabbit blood. Interestingly, we observed that the F64A strain does not have a hemolytic defect in either source of blood. This shows that the PPIase activity of PpiB does not contribute to hemolytic activity. Furthermore, we demonstrate that while PpiB contributes to hemolysis in human blood, it does not contribute to hemolysis in rabbit blood. As it is well established that the *S. aureus* alpha toxin is the main contributor to erythrocyte lysis in rabbit blood, we conclude that PpiB does not affect alpha toxin production or secretion

(22). The dramatic reduction in hemolysis in whole human blood is suggestive that PpiB may regulate α phenol-soluble modulins (αPSMs), as it has been shown that these toxins strongly contribute to human erythrocyte lysis (23–25). Consequently, we hypothesize that PpiB regulates activity of the αPSMs via a PPIase-independent mechanism. Interestingly, if this hypothesis is correct, and the PSMs are subject to regulation by PpiB, they would represent the second *S. aureus* secreted virulence factors that (i) are PpiB dependent and (ii) are secreted from the cell via noncanonical methods (Nuc being the other). Though Nuc is secreted by the general secretion system, several aspects of Nuc secretion are atypical. First, it has an unusually long (60-amino-acid) secretion signal sequence (7). Second, it is initially secreted from the cell as a longer, less active form (NucB) that is subsequently proteolytically cleaved into a shorter, more active form (NucA) (36). Third, there is evidence of sec-independent secretion of Nuc (37). Finally, the 13-amino-acid section of NucB that is proteolytically removed (to generate NucA) has been shown to aid in the secretion process via an unknown mechanism (38). The unconventional nature of Nuc secretion suggests that additional factors are involved, one of which may be PpiB. In contrast to Nuc, the αPSMs are not secreted via the general secretion pathway. Instead, they are secreted via a dedicated ABC transport system, the Pmt system (39). While very little is known about this system, it is possible that PpiB could also be involved in this process. Work is ongoing in our lab to determine the molecular interaction partners of PpiB in an effort to better understand how it is involved in the secretion and activity of Nuc and (potentially) the αPSMs, and if additional secreted virulence factors are regulated by PpiB.

Results from the murine abscess infection model suggest that the reductions in nuclease and hemolytic activities in a *ppiB* mutant (and possibly additional defects) manifest as attenuation of virulence. Importantly, the PpiB F64A strain was not attenuated, clearly demonstrating that the PPIase activity of PpiB does not contribute to virulence. Collectively, the data presented in this report suggest that PpiB has an additional role, not limited to PPIase activity, in *S. aureus*. We hypothesize that PpiB may act intracellularly as a chaperone (possibly with methyltransferase activity) or, alternatively, PpiB may act under a novel mechanism not previously described for a bacterial PPIase.

MATERIALS AND METHODS

Strains and strain construction. All bacterial strains and plasmids used are listed in Table 1, and oligonucleotides are listed in Table 2. A Δ*ppiB* mutant strain was constructed by allelic exchange using plasmid pJB38 (19). DNA sequences flanking the *ppiB* gene were amplified using primer pairs 207/208 and 209/210 and cloned into pJB38 to generate plasmid pRKC0212. This plasmid was recombined onto the *S. aureus* chromosome and then excised to generate a Δ*ppiB* deletion strain, according to published protocol (40). The F64A substitution strain was also constructed by allelic exchange using plasmid pJB38. DNA sequences flanking the *ppiB* gene were amplified using primer pairs 311/312 and 313/314 (with genomic DNA as the template), and the *ppiB* gene containing the F64A mutation was amplified using primer pair 315/316 (and plasmid pRKC0378 as the template). The resulting PCR products were ligated together and cloned into pJB38, generating plasmid pRKC0438. This plasmid was recombined onto the *S. aureus* chromosome and then excised to generate the F64A substitution strain. *S. aureus* containing a transposon insertion in the *hla* gene was obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) transposon mutant library (41) and transduced into *S. aureus* USA300 TCH1516 using bacteriophage ϕ11.

Bacterial growth conditions. *S. aureus* cultures were routinely grown at 37°C with shaking in tryptic soy broth (TSB) and *E. coli* cultures at 37°C with shaking in lysogeny broth (LB). Where appropriate, antibiotics were used at the following concentrations: chloramphenicol, 5 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; and kanamycin, 50 μg ml^{-1}. For comparative analysis of supernatants, *S. aureus* cultures were synchronized as follows. Overnight starter cultures (5 ml) of each strain were diluted 1:100 in 10 ml of fresh, prewarmed TSB and grown for 3 h to mid-exponential phase. The 3-h, mid-exponential-phase cultures were subsequently diluted into 25 ml of fresh TSB at a starting optical density at 600 nm (OD_{600}) of 0.05. The cultures were then grown for the time desired, typically 15 h.

Murine abscess model of infection. A subcutaneous abscess model was chosen to closely mimic skin and soft tissue infections, as described by Malachowa et al. (42). Cultures of *S. aureus* were grown in TSB for 2.5 h to an OD_{600} of approximately 0.75. Bacterial cells were pelleted by centrifugation and resuspended in sterile phosphate-buffered saline (PBS) for inoculation. Inocula were prepared at 10^6 CFU/50 μl for injection and confirmed via serial diluting and plating. Six-week-old BALB/c female mice were shaved on the right flank before being treated with Nair to completely remove hair. Mice were injected in the right flank with 50 μl and infections were allowed to persist for 7 days. Over the course

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Reference or source
Strains		
S. aureus		
TCH1516	Community-associated USA300 MRSA isolate	44
RN4220	Restriction-deficient transformation recipient	45
RKC0323	TCH1516 Δ*ppiB*	This work
RKC0505	TCH1516 PpiB F64A	This work
RKC0165	TCH1516 *agrB*	17
RKC0183	TCH1516 *hla*	This work
NE1354	USA300 JE2 *hla*::Bursa; *hla* NARSA transposon mutant	41
E. coli		
DH5α	Cloning strain	Invitrogen
BL21(DE3)/pLysS	Protein expression strain	Promega
NEB Express	Protein expression strain	NEB
Plasmids		
pMK4	Gram-positive shuttle vector (Cm^r)	46
pJB38	Temperature-sensitive allelic exchange plasmid (Cm^r)	19
pET24d	C-terminal His_6 tag expression vector	Novagen
pMALc5X	N-terminal MBP fusion expression vector	NEB
pRKC0276	pET24d *nuc*	8
pRKC0212	pMALc5X *ppiB*	8
pRKC0376	pMALc5X *ppiB* H58A	This work
pRKC0377	pMALc5X *ppiB* R59A	This work
pRKC0378	pMALc5X *ppiB* F64A	This work
pRKC0379	pMALc5X *ppiB* Q114A	This work
pRKC0380	pMALc5X *ppiB* F116A	This work
pRKC0381	pMALc5X *ppiB* W152A	This work
pRKC0295	pJB38 containing DNA flanking *ppiB* gene	This work
pRKC0438	pJB38 containing *ppiB* F64A allele	This work

[a]MRSA, methicillin-resistant *S. aureus*; Cm^r, chloramphenicol resistant.

of infection, mice were monitored for irregular activity or distress. After 7 days, mice were euthanized with CO_2, and abscesses were excised and homogenized. Homogenates were then diluted and plated onto TSB for colony counting.

Site-directed mutagenesis and protein purification. Oligonucleotide primers were designed to replace the six predicted PpiB active-site residues with alanine. The residues were substituted using the

TABLE 2 Oligonucleotides used in this study

Name	Sequence
207	CAAGAGCTCAAACTAGAAAATGACGTTAGCTC
208	CGGACGCGTTATATTCTCCATTCATGTTATGATAC
209	CGGACGCGTTATCTAAACATAATTAACTACCAAC
210	GGGGTACCACAAATCCACATACAATATTG
311	CACCTAACATTGCACCCATAAA
312	GCATGTGTCACAAAATTTTCAA
313	TTCGGTCAAATCATTGATGG
314	AATGGATATGTCACCTTAAACCA
315	ATGGCTAACTATCCACA
316	TTATTCTTCAACATCAATAGATT
234	GGAATCACATTCGCTCGTGTCATTAATG
235	CATTAATGACACGAGCGAATGTGATTCC
236	GAATCACATTCCACGCTGTCATTAATGAC
237	GTCATTAATGACAGCGTGGAATGTGATTC
238	GTCATTAATGACGCTATGATTCAAGGT
239	ACCTTGAATCATAGCGTCATTAATGAC
240	ACTAATGGTTCAGCTTTTTTCATTGTT
241	AACAATGAAAAAAGCTGAACCATTAGT
242	GGTTCACAATTTGCTATTGTTCAAATG
243	CATTTGAACAATAGCAAATTGTGAACC
244	GGTGGTACACCAGCTTTAGATCAAAAA
245	TTTTTGATCTAAAGCTGGTGTACCACC
FRET probe	Cy3-CCCCGGATCCACCCC-BHQ2[a]

[a]BHQ2, black hole quencher 2.

following primer pairs: for H58A, 234/235; for R59A, 236/237; for F64A, 238/239; for Q114A, 240/241; for F116A, 242/243; and for W152A, 244/245. The DNA was cloned into the pMALc5X vector (New England BioLabs [NEB]) for substitutions. The product was then digested with DpnI enzyme to eliminate any methylated DNA from the parental strain. The digested product was transformed into competent cells and grown overnight at 37°C. Products were sequenced to confirm changes to alanine. The resulting plasmids, pRKC0376 to pRKC0381, express an N-terminal maltose binding protein (MBP) fusion to the PpiB substitution proteins. The presence of MBP fused to PpiB does not affect the PPIase activity of PpiB (8). The MBP-PpiB fusion proteins were expressed in *E. coli* as follows. A 100-ml flask of LB was inoculated with 1 ml of an overnight starter culture of NEB Express cells containing the substitution plasmids (pRKC0376 to pRKC0381, depending on the substitution) and grown to an OD_{600} of 0.6. Expression of the MBP-PpiB fusions was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (to a final concentration of 0.3 mM), and the culture was grown for an additional 3 h. The cells were harvested, resuspended in 5 ml of column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA), sonicated, and centrifuged for 20 min at 20,000 $\times$ *g*. Cell lysates were loaded onto 0.5 ml of amylose resin that had been equilibrated with column buffer, washed two times with 10 ml of column buffer, and eluted in six 0.5-ml fractions with column buffer containing 10 mM maltose. Fractions containing MBP-PpiB with substitutions were pooled and stored in 40% glycerol.

Chymotrypsin-coupled PPIase activity assay. The chymotrypsin-coupled PPIase activity assay was performed as described previously for PpiB (9). Reaction mixtures containing assay buffer, chymotrypsin, and purified PpiB, PpiB F64A, or water were prepared and stored briefly on ice. The reaction was initiated by adding the mixture to the oligopeptide Suc-AAFP-pNA (Sigma) in a cuvette, and absorbance was measured at 390 nm using a Genesys 30 spectrophotometer (Thermo Fisher).

Nuclease refolding assay. The nuclease refolding and activity assay was performed as described by Wiemels et al. (8). Recombinant histidine-tagged Nuc protein (Nuc-His_6) was purified and denatured in 8 M urea. Denatured Nuc was diluted 1:40 into a reaction mixture (10 nM final concentration) containing buffer (20 mM Tris [pH 8.0], 10 mM $CaCl_2$), the fluorescence resonance energy transfer (FRET) probe (2 μM), and either purified recombinant PpiB (1 μM), PpiB F64A (1 μM), or water. Dilution of the denatured Nuc (and the corresponding dilution of urea) facilitated refolding of the protein. The activity of refolded Nuc was observed by an increase in nuclease activity and cleavage of the oligonucleotide FRET probe. Variations in the rate of Nuc refolding are manifested as variations in the rate of nuclease activity against the FRET probe over time.

Western blotting. Intracellular protein samples for Western blots were prepared from stationary-phase cultures of the wild type, Δ*ppiB* mutant, and F64A strain as previously described (43). Samples were separated by SDS-PAGE (Fig. S1), transferred to a polyvinylidene difluoride (PVDF) membrane, and probed using rabbit polyclonal anti-PpiB antibody that was raised using MBP-tagged PpiB protein (8).

Hemolysis assay. Cell-free supernatants from 15-h synchronized *S. aureus* cultures were diluted 1:2 in reaction buffer (40 mM $CaCl_2$, 1.7% NaCl) and incubated at 37°C with 25 μl of whole blood (human or rabbit). Following a 10-min incubation, samples were centrifuged at 5,500 $\times$ *g*, and 100 μl of the supernatant was transferred to a 96-well plate. Erythrocyte lysis was determined by measuring absorbance of the samples at 543 nm.

Ethics statement. Human blood was obtained from anonymous donors at Ohio University. All collections, handling, and usage of blood were approved by the Ohio University Institutional Review Board. Rabbit blood was obtained from BioIVT. Six-week-old BALB/c mice were ordered from Envigo and held at the Ohio University Office of Laboratory Animal Resources. All animal work was done under approval of the Institutional Animal Care and Use Committee by trained lab personnel.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00379-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 2, PDF file, 2.6 MB.
SUPPLEMENTAL FILE 3, PDF file, 1.1 MB.

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