Treponema denticola PrCB Is Required for Expression and Activity of the PrcA-PrtP (Dentilisin) Complex

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Received 11 March 2010/Accepted 19 April 2010

The Treponema denticola surface protease complex, consisting of PrtP protease (dentilisin) and two auxiliary polypeptides (PrcA1 and PrcA2), is believed to contribute to periodontal disease by degrading extracellular matrix components and disrupting host intercellular signaling. Previously, we showed that transcription of the protease operon initiates upstream of TDE0760 (herein designated prcB), the open reading frame immediately 5' of prcA-prtP. The prcB gene is conserved in T. denticola strains. PrcB localizes to the detergent phase of Triton X-114 cell surface extracts and migrates as a 22-kDa polypeptide, in contrast to the predicted 17-kDa cytoplasmic protein encoded in the annotated T. denticola genome. Consistent with this observation, the PrcB N terminus is unavailable for Edman sequencing, suggesting that it is acylated. Nonpolar deletion of prcB in T. denticola showed that PrcB is required for production of PrtP protease activity, including native PrtP cleavage of PrcB to PrcA1 and PrcA2. A 6×His-tagged PrcB protein communoprecipitates with native PrtP, using either anti-PrtP or anti-His-tag antibodies, and recombinant PrtP copurifies with PrcB-6×His in nickel affinity chromatography. Taken together, these data are consistent with identification of PrcB as a PrtP-binding lipoprotein that likely stabilizes the PrtP polypeptide during localization to the outer membrane.

Treponema denticola is an oral spirochete strongly associated with periodontal diseases. The predominance of spirochetes in subgingival plaque in severe periodontal diseases and their spatial location within periodontal lesions are highly suggestive of an important role in periodontal pathogenesis (13). Several T. denticola secreted proteases and peptidases have been identified that likely contribute to periodontal pathogenesis as a consequence of their roles in processing host tissue proteins and peptides to fulfill the nutritional requirements of these highly motile and invasive organisms (reviewed in references 16 and 32). Among these, the outer membrane serine protease complex (variously designated CTLP [40], dentilisin [26], and the PrtP complex [5]) has several activities consistent with its identification as a virulence determinant in periodontal disease. The PrtP protease complex degrades extracellular matrix proteins (3, 23) as well as serum components involved in tissue homeostasis (22, 40), regulation of the complement pathway (33, 41), and other innate immune responses (1, 12, 38). PrtP protease activity also contributes to T. denticola coaggregation with Porphyromonas gingivalis (25) and penetration of host tissue (8, 23).

The prcA-prtP locus is conserved in oral Treponema species, and the encoded proteins can be divided into two paralogous families based on phylogenetic analysis and substrate specificity of the protease (9). PrtP is one of only two known acylated members of the subtilisin family, the other being SphB1, a surface-anchored subtilisin autotransporter that catalyzes maturation of the virulence factor FhaB (filamentous hemagglutinin) at the surface of Bordetella pertussis (10). PrcA has no homologues outside the oral Treponema spp. Other than its likely contribution to anchoring the protease complex in the outer membrane, the exact function of PrcA is not known. The native protease consists of a complex comprised of PrtP and the PrcA1 and PrcA2 polypeptides that, if unheated, does not dissociate into its constituents in SDS-PAGE analyses (26, 29, 40), suggesting that protein-protein interactions stabilize the protease complex in the T. denticola outer membrane. Our prior analysis of the T. denticola protease operon demonstrated that transcription of prtP initiates $\geq$580 bp upstream of prcA and includes all of the TDE0760 open reading frame (ORF), immediately 5' of prcA (5). While our group and others continue to characterize interactions between PrtP and PrcA, we chose to further investigate the role of this conserved, genetically linked open reading frame encoding a hypothetical protein in expression and activity of the protease complex. Here we present initial characterization of the TDE0760 (herein designated prcB) gene product and provide evidence supporting a role of PrcB in expression of the PrtP protease complex.

MATERIALS AND METHODS

Bacterial strains and growth conditions. T. denticola ATCC 35405, ATCC 33520, and OTK and isogenic mutants of 35405 (Table 1) were grown in NOS broth or NOS/GN semisolid medium as previously described (7, 24), with erythromycin (Em) (40 μg ml$^{-1}$) added as appropriate. Cultures were examined by phase-contrast microscopy for purity and typical strain morphology before use. Escherichia coli NovaBlue (Novagen, Inc., Madison, WI) and JM109 (42) were used as hosts for cloning. E. coli was grown in LB agar or broth medium with ampicillin (50 μg ml$^{-1}$), kanamycin (30 μg ml$^{-1}$), and Em (200 μg ml$^{-1}$) as appropriate. Plasmid vector pSTBlue-1 (Novagen) was used for direct cloning of PCR products, and 6×His-tagged constructs were made in pET28b (Novagen).

Construction of plasmids for expression and mutagenesis studies. A 1,070-bp fragment of T. denticola genomic DNA containing the entire prcB ORF and approximately 500 bp 5' of prcB was amplified by a PCR using oligonucleotide

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§ Published ahead of print on 30 April 2010.
primers CX516 and CX529. (Details of all plasmids and oligonucleotide primers used in this study are contained in Tables 2 and 3.) The PCR product, digested with NheI and XhoI, was ligated to similarly digested pET30b, yielding pCF480. This plasmid was used as the source of linear DNA to construct construction of T. denticola CF499, containing the native promoter region, prcA-ermF-ermAM, in pSTBlue-1; PrcB-6His, an open reading frame beginning at Val 38, expressed in pET30b. This study

All genetic manipulations were performed by allelic replacement mutagenesis.

**Transcription analysis.** Total RNA was isolated from T. denticola cells harvested during logarithmic growth (3-day cultures) with TrisZ reagent (Invitrogen, Carlsbad, CA), using the manufacturer’s protocol. DNase-treated RNA samples were reversed transcribed with random hexamer primers, using the SuperScript first-strand synthesis system for reverse transcriptase PCR (RT-PCR) (Invitrogen). One microliter of the resulting first-strand cDNA was amplified for 30 cycles by using Taq DNA polymerase (Invitrogen) and oligonucleotide primer sets specific for the target gene. RT-PCR products, including positive controls (genomic DNA) and negative controls (no RT enzyme), were analyzed on agarose gel electrophoresis.

**Protein electrophoresis and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting were done as described previously (17). SDS-PAGE gels were stained with Coomassie brilliant blue or silver nitrate. Cells were harvested by centrifugation at 10,000 × g for 10 min at 4°C. Whole-cell lysates of T. denticola and E. coli were prepared by suspending cells (1-ml cultures at an optical density of 600 nm [OD600] of 0.2) in standard SDS-PAGE sample buffer containing β-mercaptoethanol and 2 mM phenylmethanesulfonyl fluoride (PMSF) or by sonication of phosphate-buffered saline (PBS)-washed cells prior to suspension in sample buffer. For some experiments, T. denticola cells were treated with Triton X-114 to extract outer membrane and periplasmic components as described for Treponema pallidum (11), with slight modifications. Treponema cells were harvested by centrifugation at 10,000 × g for 10 min at 4°C. Washed in PBS, suspended in a 1:40 volume of 20 mM Tris (pH 7.5)–2 mM EDTA–1 mM diithiothreitol containing 1% Triton X-114, and stirred gently overnight at 4°C. Detergent-extracted cells were centrifuged at 17,000 × g for 10 min at 4°C. The supernatant, enriched for outer membrane and periplasmic components, was loaded onto 10% SDS-PAGE gels for electrophoresis and Western immunoblotting as previously described.

**Semiquantitative RT-PCR.** Total RNA was isolated from T. denticola and E. coli cultures as described above. Total RNA (5 μg) was reverse transcribed using the TaqMan reverse transcription reagents (Applied Biosystems) and primers specific for the target gene. RT-PCR products were loaded onto an agarose gel and probed as described below. The expected size of the amplified product was 288 bp for PrcB and 129 bp for PrcB-6His. The treated samples were probed with an internal control for 16S rRNA (Takara Bio, Inc.).

**Protein gel analysis.** Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% or 10% acrylamide gels, stained with Coomassie blue, and analyzed by Western blotting.

**Table 1.** T. denticola strains used in this study

<table>
<thead>
<tr>
<th>T. denticola strain</th>
<th>Relevant features</th>
<th>Source or reference</th>
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<tr>
<td>35405</td>
<td>Parent for mutagenesis</td>
<td>ATCC</td>
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<tr>
<td>35320</td>
<td>prcB, polar mutation</td>
<td>ATCC</td>
</tr>
<tr>
<td>20</td>
<td>prcB-6×His</td>
<td>This study</td>
</tr>
<tr>
<td>CF449</td>
<td>prcB-6×His</td>
<td>This study</td>
</tr>
<tr>
<td>CF522</td>
<td>ΔprcB, nonpolar mutation</td>
<td>This study</td>
</tr>
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* ATCC, American Type Culture Collection, Rockville, MD.

**Table 2.** Plasmids used for expression and mutagenesis

<table>
<thead>
<tr>
<th>E. coli plasmid</th>
<th>Characteristics; use</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pSY118</td>
<td>ermF-ermAM cassette</td>
<td>28</td>
</tr>
<tr>
<td>pSY119</td>
<td>3′ end of prcA-prpP</td>
<td>29</td>
</tr>
<tr>
<td>pCF411</td>
<td>ermF-ermAM inserted 5′ of truncated prcA in pSY119; PrpP expression in E. coli</td>
<td>This study</td>
</tr>
<tr>
<td>pCF415</td>
<td>prcB-6×His in pSTBlue-1; PrcB-6×His expression in E. coli</td>
<td>This study</td>
</tr>
<tr>
<td>pCF416</td>
<td>prcB-6×His in pCF411; PrcB-6×His and PrpP expression in E. coli and construction of T. denticola CF417</td>
<td>This study</td>
</tr>
<tr>
<td>pCF483</td>
<td>ermF-ermAM between TDE0758 and TDE0759; prcB-6 × His in “native” protease locus; construction of T. denticola CF499</td>
<td>This study</td>
</tr>
<tr>
<td>pCF521</td>
<td>ermF-ermAM inserted between TDE0758 and TDE0759; ΔprcB; construction of T. denticola CF522</td>
<td>This study</td>
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<tr>
<td>pCF569</td>
<td>prcB-6×His beginning at Met1, expressed in PET30b</td>
<td>This study</td>
</tr>
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<td>pCF570</td>
<td>prcB-6×His beginning at Met1, expressed in PET30b</td>
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<tr>
<td>pCF571</td>
<td>prcB-6×His beginning at Val1, expressed in PET30b</td>
<td>This study</td>
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* Construction of the plasmids, including intermediate steps, is described in Materials and Methods.
membrane components, was partitioned into aqueous and detergent phases at 37°C, and each phase was reextracted in Triton X-114 and partitioned a second time. The extracted material was then precipitated in acetone and resuspended in equal volumes to equal sample loading. Prior to electrophoresis, samples were either heated at 100°C for 5 min or held on ice.

Proteins blotted onto nitrocellulose membranes were detected with rabbit polyclonal antibodies raised against recombinant T. denticola proteins, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Scientific, Rockford, IL). 6×His-tagged proteins were detected with HisProbe HRP reagent (Thermo Scientific) or with mouse monoclonal anti-6×His antibodies (EMD Biosciences, San Diego, CA). Protein bands of interest were visualized using SuperSignal West Pico chemiluminescence substrate (Thermo Scientific). (EMD Biosciences, Rockford, IL). Proteins were expressed as described previously (15). Whole-cell extracts for zymography were prepared similarly to those for SDS-PAGE, except without heating, alkylating agents, or reducing agent. Following electrophoresis, the gel was stained with Coomassie brilliant blue to detect active protease. PrtP-dependent hydrolysis of the chromogenic substrate succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (SAAPFNA) was assayed by the change in absorbance at 405 nm, as described previously (15). Whole-cell extracts for zymography were prepared similarly to those for SDS-PAGE, except without heating, alkylating agents, or reducing agent. Following electrophoresis, the gel was stained with Coomassie brilliant blue to detect active protease. PrtP-dependent hydrolysis of the chromogenic substrate succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (SAAPFNA) was assayed by the change in absorbance at 405 nm, as described previously (15).

Protease activity assays. Gelatin zymography to detect the active PrtP protease was performed as described previously (15). Whole-cell extracts for zymography were prepared similarly to those for SDS-PAGE, except without heating, alkylating agents, or reducing agent. Following electrophoresis, the gel was stained with Coomassie brilliant blue to detect active protease. PrtP-dependent hydrolysis of the chromogenic substrate succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (SAAPFNA) was assayed by the change in absorbance at 405 nm, as described previously (15).

Immunoprecipitation assay. Three-day-old 10-ml cultures of T. denticola were harvested by centrifugation at 5,000 × g for 5 min, washed with cold PBS, and incubated for 30 min at 4°C on a roller in 1 ml of Tris-buffered saline (TBS) (pH 7.4) containing 0.5% Triton X-100 and 10 μl 6×His protease inhibitor cocktail (Roche, Indianapolis, IN). The suspension was centrifuged at 13,000 × g for 10 min at 4°C to remove unlysed cells. One-hundred-microliter aliquots of the suspension were diluted 1:4 in TBS, and 5 μl of rabbit polyclonal antiserum raised against PrtP, PrcA, FlaA, or Msp (5, 17, 29) was added to each tube. Following overnight incubation at 4°C on a roller to form the antigen-antibody complexes, proteins were lysed by treatment with BugBuster (EMD Biosciences), and extracted with nickel affinity chromatography (2). Eluted fractions containing PrtP or PrcA were concentrated by acetone precipitation, subjected to SDS-PAGE, transferred to nylon membranes, and stained with amido black or with HisProbe to visualize eluted proteins. Bands of interest were submitted to the Protein Structure Facility at the University of Michigan for N-terminal amino acid sequencing.

Nucleotide sequence accession numbers. The prcB sequences of T. denticola strains ATCC 33520 and OTK have been assigned GenBank accession numbers FJ355200 and FJ355201, respectively.

RESULTS

Conservation of prcB. Prolyl-phenylalanyl protease activity is found in diverse T. denticola strains (35). T. denticola strains 35405, 33520, and OTK are antigenically distinct, consistent with their assignment to distinct serotypes (20), and all exhibit prolyl-phenylalanyl protease activity (not shown). To determine the level of intraspecies prcB conservation, the entire TDE0760 ORF and flanking DNAs were amplified from the genomic DNA of T. denticola strains 33520 and OTK, and the DNA sequences were compared with that of 35405. The three T. denticola prcB DNA sequences were identical over the entire ORF, including 210 bp upstream of the first AUG codon in the TDE060 ORF, resulting in a total of 7 amino acid differences between the three deduced polypeptides (Fig. 1 and data not shown). Counting from the first AUG codon in the ORF, the deduced amino acid sequences are identical for residues 1 to 65, which includes the predicted untranslated region upstream of the GUG translation initiation codon in the annotated T. denticola genome sequence (36). Interstrain homology is also very high for residues 120 to 198, which include a predicted transmembrane helical domain.

Expression and localization of PrcB. To determine whether the hypothetical protein encoded by prcB is expressed in T. denticola, we constructed strain CF417, in which prcB is modified to encode a C-terminal 6×His tag and the erm cassette is inserted at the 5′ end of prcA (Fig. 2). Because the N terminus of PrcB could not be deduced unambiguously from the DNA sequence, the 6×His motif was added to the C terminus of PrcB. The erm cassette contains transcription terminators in both orientations, and native transcription downstream of the insertion site is blocked (5, 6). To determine whether addition of the 6×His tag...
to PrcB affected protease expression and activity, we also constructed CF499, in which the protease operon is wild type except for the 6×His modification of prcB. In CF499, the erm cassette is located between TDE0758 and TDE0759, upstream of the predicted protease operon promoter region upstream of prcB is intact in both CF499 and CF417. As shown in Fig. 3A, PrcB-6×His is a 22-kDa protein in T. denticola strains CF417 and CF499 and in the recombinant E. coli strain carrying pCF415, in which expression of PrcB-6×His is initiated from the native T. denticola promoter in the plasmid.

PrcB-6×His localized to the detergent phase of Triton X-114 extracts of T. denticola CF499 (Fig. 3B) and was found only in small amounts in the cell pellet fraction of the extract. This suggests that PrcB is very hydrophobic and may localize to the outer membrane of T. denticola. Interestingly, a 15-kDa 6×His-labeled degradation product of PrcB-6×His that appeared upon multiple freeze-thaws of PrcB-6×His samples localized to the aqueous phase of Triton X-114 extracts (Fig. 3C), demonstrating that the PrcB N terminus (not the C-terminal predicted transmembrane domain) is responsible for partitioning of PrcB to the hydrophobic (detergent) phase of the Triton X-114 extract.

As expected, CF417 lacked PrcA, PrtP, and protease activity due to polar effects of erm insertion at the 5′ end of prcA (Fig. 4A). In contrast, CF499 expressed protease activity and PrtP protein at essentially wild-type levels, suggesting that the C-terminal modification of PrcB has only a minimal effect on protease expression. In both CF499 and the parent strain, PrcA was detected as both the full-length form (70 kDa) and the 40-kDa PrcA2 cleavage product resulting from PrtP activity when blots were probed with anti-PrcA2 antibodies (Fig. 4A).

N-terminal analysis of PrcB. Current annotations of TDE0760 (prcB) by the J. Craig Venter Institute (JCVI) (http://cmr.jcvi.org/) and the Los Alamos National Laboratory (LANL) (http://www.oralgen.lanl.gov/) are somewhat ambiguous. Both identify the translation initiation codon as a GUG codon 112 bp downstream of the initial AUG codon in the ORF. However, predicted protein molecular masses are 17 kDa (JCVI; consistent with either the first or second AUG as the start codon, encoding Met1 or Met7) and 22 kDa (LANL; consistent with the GUG start codon, encoding Met1) (data not shown). Analysis of the TDE0760 ORF by the FGENESB algorithm predicted that translation of the TDE0760 ORF initiates at the second AUG codon (Met7) (39). There is no clearly identifiable ribosome binding site (RBS) immediately upstream of either potential AUG start codon, although the JCVI annotation identifies the sequence GGGGA 10 bp upstream of the proposed AUG start codon. It should be noted that the RBS identification algorithm used by JCVI assumes that the start codon has been predicted correctly (39). A potential σ70 class promoter is located 62 bp upstream of the Met7 AUG codon (not shown). Using software designed to identify type I signal peptides, the 17-kDa polypeptide is pre-

**FIG. 1. Conservation of prcB in T. denticola.** The deduced amino acid sequence of the complete ORF, including the PrcB coding region, is shown for T. denticola strains ATCC 35405, ATCC 33520, and OTK, aligned using Clustal 2.0. The N-terminal valine residue predicted by JCVI (Val38) and the two possible N-terminal methionine residues (Met1 and Met7) predicted by the present study are shaded gray. The predicted signal peptide and semiconserved amino acids are shown by colons and dots, respectively.

**FIG. 2.** Protease locus in T. denticola parent and prcB mutant strains. The genes of the protease operon, prcB, prcA, and prtP, are shown as gray arrows. The location of the protease operon promoter region is indicated by “P.” The protease mRNA transcript is shown as a thin arrow above the genes transcribed in each strain. The location and orientation of the erm cassette are shown by a black arrow. The T. denticola strains are 35405 (wild-type parent), P0760 (erm insertion at the 5′ end of prcB), CF417 (prcB modified to encode a C-terminal His tag; erm insertion replaces the 5′ end of prcA), CF499 (prcB modified to encode a C-terminal His tag; erm insertion is upstream of the protease locus), and CF522 (∆prcB; promoter region is intact; erm insertion as in CF499).
dicted to be a cytoplasmic protein lacking a secretion signal, while the 22-kDa polypeptide initiating from either AUG codon is predicted to be an inner membrane protein with a noncleaved signal peptide. Analysis of the deduced 22-kDa PrcB protein by the LipoP algorithm revealed a predicted signal peptidase II cleavage site (VTFLG/H20841CKTLP) after residue 23, counting from the Met1 start codon, or residue 17, counting from the Met7 start codon (log odds, 12.0 and 13.5, respectively).

To characterize the N terminus of PrcB, we attempted to determine the N-terminal amino acid sequence of PrcB-6×His purified from T. denticola CF499 and from E. coli expressing PrcB-6×His from pCF415. The N terminus of the intact 22-kDa PrcB-6×His protein was blocked, strongly suggesting that PrcB is acylated. As a control, we also examined a 15-kDa 6×His-labeled degradation product of PrcB-6×His that appeared upon multiple freeze-thaws of purified PrcB-6×His samples (Fig. 3C). The N-terminal sequence of the 15-kDa polypeptide was readily obtained and matched the deduced amino acid sequence beginning at residue 73 of the 22-kDa PrcB protein. Taken together with the observation that PrcB localized to the detergent phase of Triton X-114 extracts, while the 15-kDa PrcB fragment localized to the aqueous phase, we propose that PrcB is a lipoprotein, likely localized to the outer membrane. Since it was not possible to definitively identify the N-terminal residue of the PrcB polypeptide by either computational or biochemical methods, we constructed recombinant E. coli strains in which PrcB-6×His translation starts at Met1, Met7, or Val38. When expression of PrcB-6×His was induced, the Met1 and Met7 constructs produced a 22-kDa polypeptide whose migration by SDS-PAGE was not distinguishable from that of PrcB-6×His expressed in T. denticola strains CF499 and CF417, and the Val38 PrcB-6×His construct produced a 17-kDa polypeptide that did not align with PrcB-6×His expressed in T. denticola (data not shown).

**PrcB is required for native expression of PrtP.** To characterize the contribution of PrcB to protease expression and

![FIG. 3. Expression and localization of PrcB. PrcB-6×His and PrtP were detected on blots by use of HisProbe reagent and anti-PrtP antibodies, respectively. Triton X-114 extracts (TX-114) were partitioned into aqueous (Aq) and detergent (Det) phases. Molecular mass standards are shown in panels B and C. (A) Expression of PrcB-6×His detected in lysates of E. coli/pCF415 (E.c.) and T. denticola strains CF499 and CF417. (B) PrcB-6×His localizes to the detergent phase of a T. denticola CF499 Triton X-114 extract. (C) T. denticola CF499 Triton X-114 extract showing differential phase partitioning of full-length and N-terminally truncated PrcB-6×His. Samples were heated (+) or not heated (−) prior to SDS-PAGE.**

![FIG. 4. Protein expression in prcB mutants. Results of Western immunoassays, gelatin zymography, and chromogenic substrate cleavage assays are shown. Samples for immunoblots were heated whole-cell extracts, and samples for protease activity assays were unheated extracts (zymograms) or cells in growth medium. (A) PrcB-6×His was detected in CF417 and CF499 as a 22-kDa protein (molecular size scale not shown). CF417 expressed neither PrcA nor PrtP, while CF499 expressed native protease complex proteins and activity. (B) The ΔprcB mutant CF522 expressed PrcA in uncleaved form (70 kDa), but no PrtP protein or protease activity was detected. (C) PrtP-dependent SAAPFNA hydrolysis, assayed by the change in absorbance at 405 nm. Data shown are mean values for triplicate samples from a representative experiment. T. denticola strains are as in Fig. 2: 35405 (ATCC 35405; parent strain), CF417 (prcB-6×His polar mutation), CF499 (prcB-6×His nonpolar mutation), CF522 (ΔprcB), and P0760 (erm insertion at 5′ end of prcB).
activity, we constructed CF522, a ΔprcB mutant in which the putative protease locus promoter region is intact (Fig. 2). In CF522, as in CF499, erm is located upstream of the protease locus, between TDE0758 and TDE0759. As shown in Fig. 4B, CF522 produced an uncleaved full-length PrcA protein but produced no detectable PrtP protein or gelatinase activity. In both the parent strain 35405 and in CF499, cleavage of PrcA from the full-length form (70 kDa) to a cleaved form (PrcA2; 40 kDa) was evident (Fig. 4A), while CF522 produced only uncleaved full-length PrcA (Fig. 4B), consistent with the lack of PrtP gelatinase activity. In a quantitative assay of SAAPFN degradation, CF522 had a level of activity similar to that of the negative-control strain P0760 (Fig. 4C).

To determine whether the absence of PrtP in CF522 was due to a defect in prtP transcription, we performed RT-PCR on parent and mutant strains, using oligonucleotide primer sets targeted to prtP and flaA (Table 3). As expected, RT-PCR showed that prtP was transcribed at comparable levels in 35405, CF499, and CF522 (Fig. 5). To confirm that the lack of PrtP protein and activity in CF522 was not due to inadvertent insertion of a stop codon or to a frameshift during cloning or mutagenesis steps, the sequences of both DNA strands in CF522, from the erm insertion through the 3′ end of prtP, were determined and verified as identical to those in strain 35405 (except for deletion of prcB) (data not shown). The protein expression and protease activity profile of CF522 was further confirmed in an identical, independently constructed prcB mutant (data not shown).

**PrcB interacts with PrtP.** To initiate a study of potential protein interactions in the protease complex, we performed immunoprecipitation of sonicated extracts of *T. denticola* CF499 and CF417, using a commercial anti-His-tag monoclonal antibody as well as polyclonal antibodies raised against specific *T. denticola* proteins. Using anti-PrtP antibodies, PrcB-6×His coimmunoprecipitated with PrtP from CF499 (Fig. 6A). PrcB was present in both CF499 and CF417 but was not immunoprecipitated from CF417 by anti-PrtP antibodies (Fig. 6A). To test the specificity of the PrcB-PrtP interaction, immuno-
m共赢precipitations were also done with antibodies raised against PrtP, PrcA2, FlaA, and Msp (Fig. 6B). PrcB and PrtP were coimmunoprecipitated from strain CF499 with anti-PrtP antibodies, but PrcB was not detected in the eluate fraction of immunoprecipitations done with polyclonal antibodies raised against PrcA2, FlaA, or Msp. These data suggest that PrcB does not interact with PrcA, the other major component of the protease complex. As shown in Fig. 6C, PrC was coimmunoprecipitated with PrcB-6×His by use of anti-His-tag antibody, further demonstrating the PrtP-PrcB interaction. To generate a recombinant system for further study of protein interactions in the protease complex, PrtP and PrcB-6×His were coexpressed in E. coli. Extracts from E. coli strains expressing PrtP, PrcB-6×His, or both proteins were subjected to Ni²⁺ affinity chromatography. As shown in Fig. 6D, PrtP eluted with PrcB-6×His from E. coli/pCF416 (lanes 3), but PrtP alone expressed in E. coli/pCF411 was not retained on the affinity column (lanes 1). These results provide further evidence of a direct interaction between PrtP and PrcB and provide a workable recombinant system for molecular analysis of this interaction.

**DISCUSSION**

Considerable effort has gone into characterization of the role of T. denticola PrtP protease (dentilisin) in periodontal disease, but little is known about how components of the protease complex are secreted, translocated through the periplasmic space, and anchored in the outer membrane. The present study addressed the role of PrcB in expression and activity of the protease complex. The conserved structure of the prcB-prcA-prtP operon is consistent with functional linkage of PrcB with PrtP protease activity. While PrcB has not been reported as a component of the protease complex, our results indicate that PrcB interacts with PrtP and contributes to stability of the native protease complex. The most likely reason that this association has not been noted previously is that the PrtP-PrcB interaction does not appear to be stable under SDS-PAGE conditions.

The addition of a C-terminal 6×His tag facilitated detection of PrcB in T. denticola and did not appear to interfere with its function, as reflected in protease expression and activity in strain CF499 that were comparable to those in the parent strain. As noted, the choice of C- versus N-terminal 6×His labeling of PrcB was due to the ambiguous nature of the N terminus of native PrcB. This is the first use of this technology in T. denticola, and it provides a relatively rapid and economical means of detecting expression of “hypothetical proteins” of interest identified in the genome annotation without generating protein-specific detection reagents.

Native prtP transcription downstream of the insertion site in T. denticola P0760 and CF417 is blocked because the cem cassette contains transcription terminators that are functional in both orientations (5, 6). However, prtP was transcribed at similar levels in the ΔprcB mutant CF522 and the parent strain 35405. The absence of PrtP protein in CF522 was not due to either a defect in transcription or the introduction of a frameshift during mutagenesis. Taken together with the demonstrated PrcB-PrtP interaction, this suggests that PrtP is translated in CF522 but is subsequently degraded due to misfolding or incorrect localization in the absence of PrcB. Based on the phenotype of CF522 and the current genome annotation of PrtP as a cytoplasmic protein, we initially hypothesized that PrcB serves as a PrtP-specific molecular chaperone to stabilize PrtP prior to or during its secretion across the inner membrane. However, our data demonstrating the molecular weight, cellular localization, and likely acylation of PrcB are not consistent either with this model or with the current genome annotation, which predicts that PrcB translation initiates at Val^18, counting from the first AUG codon. While we were unable to experimentally identify the PrcB translation initiation site, our data are consistent with its being the second AUG codon (Met'), as predicted by the FGENESB algorithm. The perfect homology between deduced amino acid sequences of this N-terminal region of PrcB in diverse T. denticola strains is also supportive of this interpretation. Annotation of prcB is complicated by the absence of an efficient consensus ribosome binding site upstream of any of the three potential start codons in the TDE0760 ORF. This is in stark contrast to the case for prcA and prtP, each of which has a consensus Shine-Dalgalmo sequence (AGGAGG) within 8 bp of the respective AUG start codon. Preliminary data (not shown) suggesting that the PrcB protein is expressed at lower levels than PrcA and PrtP are consistent with the DNA sequence information. This issue is being addressed in ongoing studies that are beyond the scope of the present work.

Prediction of signal peptidase II cleavage and acylation sites remains somewhat problematic for T. denticola and other spirochetes. As noted by Setubal et al. (37), spirochete lipobox sequences are much less clearly defined than those of other bacteria. PrcB, PrcA, and PrtP may or may not be identified as lipoproteins, depending on the algorithm used for analysis. The LipoP algorithm (27) identifies high-probability type II signal peptidase cleavage sites for all three proteins (for PrcB, VSFTLG/CKTLT, with log odds of 12.0/13.5; for PrcA, FLFGS/CPQQK, with log odds of 15.4; and for PrtP, LIVSS/CNFGM, with log odds of 20.8). In contrast, the SpLip algorithm, designed specifically for lipoprotein identification in spirochetes (37), predicts neither PrcB nor PrtP as a lipoprotein. Interestingly, although we have experimentally confirmed its acylation (4, 18), the SpLip algorithm also fails to identify T. denticola OppA as a lipoprotein (data not shown).

Interactions between PrcB and PrtP were assayed both in T. denticola and in an E. coli background, for several reasons. First, as noted above, PrcB appears to be expressed at relatively low levels in T. denticola. Second, the PrtP polypeptide expressed in E. coli does not have proteolytic activity (26), while high levels of PrtP protease activity in T. denticola extracts complicate the possibility of obtaining unambiguous results that are dependent on intact, stable protein. Third, the use of two different ligand-receptor assay systems increases the reliability of results. The ability of PrcB-6×His to bind PrtP in either environment is indicative of a direct interaction between PrtP and PrcB and supports the hypothesis that PrcB-PrtP interaction is a key feature in expression of the PrtP protease complex. The native protease consists of a complex of PrtP, PrcA1, and PrcA2 that, if unheated, does not dissociate into its constituent polypeptides in SDS-PAGE analysis (40). The aeryl moieties of the PrcA and PrtP lipoproteins presumably serve in localization to the outer membrane. However, in their active forms, both proteins are reported to undergo further process-
ing: PrtP is activated by cleavage at residue 158 (26), and the 70-kDa PrcA protein is cleaved to PrcA1 (30 kDa) and PrcA2 (40 kDa) by PrtP activity (29). While PrcB has not been detected as part of the SDB-stable active protease complex, our data suggest that the asp moiety of PrcB serves to anchor it in the outer membrane. It is not yet clear whether PrcB is anchored at the periplasmic face of the outer membrane or is exposed on the cell surface. In either case, PrcB most likely functions in either presentation or stabilization of the protease at the cell surface. The specific protein-protein interactions required for stability and localization of the outer membrane protease complex remain to be studied more fully. We are continuing to investigate the specific contribution of PrcB to expression of the protease complex, including defining the interacting domains of PrtP and PrcB.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DE013565 and DE018221 (National Institute of Dental and Craniofacial Research), by the Office of the Vice President for Research (University of Michigan), and by the Undergraduate Research Opportunity Program (University of Michigan).

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