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Mutagenesis of a Novel Gene in the *prcA-prtP* Protease Locus Affects Expression of *Treponema denticola* Membrane Complexes

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A novel gene was identified in the *Treponema denticola prcA-prtP* protease operon. Strains with mutations in either the *prcA-prtP* or the *msp* region showed altered expression of a product(s) of the other locus. Together, these results provide information on the assembly of outer membrane complexes involved in *T. denticola* interaction with host cells and tissue.

Two *Treponema denticola* surface components with a wide range of cytopathic activities have been reported: the oligomeric major surface protein (Msp) (7, 13) and a protease complex (20) encoded by the *prcA-prtP* locus (17, 18). The Msp has functional characteristics of an outer membrane porin (6, 7). The protease complex consists of the PrtP protease (dentilisin) and two proteins (PrcA1 and PrcA2) that result from posttranslational processing of the PrcA polypeptide (18). Both Msp and PrcA-PrtP exhibit adhesin-like binding activity and are cytotoxic to epithelial cells (7). Recent reports implicate Msp in the disruption of fibroblast calcium responses and cytoskeletal assembly (1, 3). PrtP activity contributes to *T. denticola* tissue penetration (4, 12) and modulation of inflammatory cytokine responses (2, 5). PrtP is reported to either contribute to (15) or interfere with (21) *T. denticola* binding to *Porphyromonas gingivalis*. Using isogenic mutants, we showed that the production of native Msp and PrtP are related (10, 18) and that PrtP is required for PrcA processing (18). An *msp* mutant producing a C-terminally truncated Msp makes no detectable protease proteins, while an Msp-deficient mutant produces wild-type PrcA-PrtP protease. Strains mutated in the protease locus demonstrate reduced expression and oligomerization of Msp. The complex phenotypes of mutant strains must be taken into account when using mutants to study *T. denticola* interaction with host cells and tissue. The present study was undertaken to extend understanding of the relationship between these membrane complexes. Here, we further characterize the expression of the protease activity and Msp in isogenic mutant strains and identify a novel open reading frame in the protease operon whose expression is required for outer membrane complex formation.

Growth behavior of *T. denticola* strains. To investigate the role of PrtP activity in anaerobic growth in NOS medium (14), parent strain 35405 and isogenic strains with mutations in either the protease locus or the *msp* locus (Table 1) were monitored for 7 days following 1:20 dilution of actively growing cultures (optical density at 600 nm [OD_{600}] of 0.35; triplicate samples; four replicates). The parent strain reached stationary phase within 100 h. All of the mutant strains tested had a slightly slower growth rate, reaching stationary phase at 120 to 140 h (data not shown). Final optical densities of protease-deficient strains, including strain MPE, were consistently lower than that of strain 35405, but differences were statistically insignificant. High-molecular-weight media constituents were not degraded by the protease-deficient strains (data not shown), consistent with the hypothesis that PrtP activity contributes to nutrient processing or acquisition. Interestingly, strain MHE, which produces no Msp but has wild-type levels of protease activity, also grew at a slower rate than 35405. This result supports the hypothesis that Msp is essential for proper outer membrane function and is suggestive of functional interactions between PrtP protease activity and Msp porin activity in nutrient acquisition and uptake.

Gene expression and protease activity. PrtP protease activity and transcription of *prcA* and *prtP* in parent and isogenic mutant strains were monitored throughout growth by quantitative reverse transcription-PCR (QRT-PCR). Protease activity in 35405, as measured by cleavage of succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPFNA) (8), increased

TABLE 1. *Treponema denticola* strains used in this study

Strain[a]	Phenotype[b]	Source or reference
Parent 35405	SAAPFNA$^+$, PrcA$^+$, PrtP$^+$, oligomeric Msp	ATCC
MHE (*msp*)	SAAPFNA$^+$, PrcA$^+$, PrtP$^+$, Msp$^-$	10
MPE (*msp*)	SAAPFNA$^-$, PrcA$^-$, PrtP$^-$, truncated monomeric Msp	10
CKE (*prcA-prtP*)	SAAPFNA$^-$, truncated PrcA, PrtP$^-$, reduced Msp, mostly monomeric	10
CCE (*prtP*)	SAAPFNA$^-$, unprocessed PrcA, PrtP$^-$, reduced Msp, mostly monomeric	18
PNE (*prcA*)	SAAPFNA$^-$, PrcA$^-$, PrtP$^-$, reduced Msp, mostly monomeric	18
P0760 (TDE0760)	SAAPFNA$^-$, PrcA$^-$, PrtP$^-$, reduced Msp, mostly monomeric	This study

[a] The gene disrupted by *ermF-ermB* insertion is given in parentheses.
[b] PrtP protease activity was assayed by hydrolysis of SAAPFNA; PrcA, PrtP, and Msp production and forms are listed.

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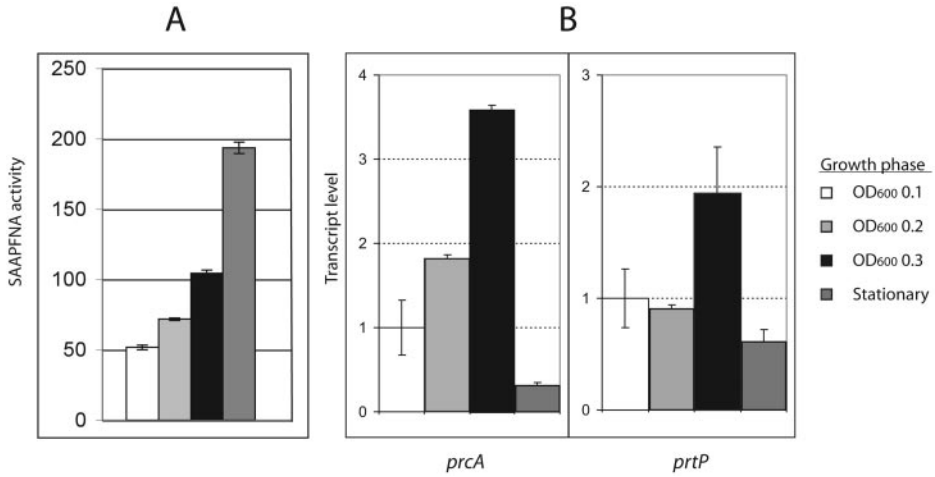

FIG. 1. Protease activity and transcription of *prcA-prtP* in *T. denticola* 35405. (A) SAAPFNA activity (8) expressed in A_{405}/h/ml/OD_{600}. Error bars represent the range of values obtained with triplicate samples of a representative experiment. (B) QRT-PCR of *prcA* and *prtP*. Total RNA was extracted from cultures harvested at an OD_{600} of 0.1, 0.2, and 0.3 and at stationary phase (fifth day). DNase-treated RNA samples were reversed transcribed with random hexamer primers by using the SuperScript First-Strand synthesis system for RT-PCR (Invitrogen). One microliter of the resulting first-strand cDNA was amplified by using QuantiTect SYBR Green PCR (QIAGEN) in 25 μl of reaction buffer, with 16S rRNA serving as an internal control for normalization between samples. Gene-specific primers separated by approximately 80 to 100 bp were designed with Primer Express software (Perkin-Elmer Applied Biosystems). Thermal cycling was performed in an iCycler iQ Multi-Color Real Time PCR detection system (Bio-Rad) at 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Threshold values were calculated by using baseline cycles 2 to 10. Data were analyzed by using software supplied by the manufacturer. The data are shown with the gene expression level defined as 1.0 at an OD_{600} of 0.1. Error bars represent standard deviations of the means of results from triplicate samples in two independent experiments.

during active growth and persisted at high levels in stationary phase (Fig. 1A). Transcription of *prcA* and *prtP* increased during active growth but was greatly reduced at stationary phase (Fig. 1B). We detected no significant differences in *prcA* or *prtP* transcription during active growth between the parent strain and the *msp* mutants MHE and MPE (data not shown). This result is of particular interest due to the distinctly different Msp and PrcA-PrtP phenotypes of these strains (Table 1). The lack of PrcA-PrtP proteins and activity in strain MPE, but not MHE, is therefore due to posttranscriptional events and may be related to the presence of a defective, truncated Msp in MPE.

Expression of *msp* in *prcA* and *prtP* mutants. Total Msp and especially Msp oligomers were greatly reduced in strains with defined mutations in either *prcA* or *prtP* or both (10, 18). To determine whether this result was reflected in *msp* transcription, QRT-PCR assays were performed on parent and mutant strains. As shown in Fig. 2, *msp* transcription in isogenic *prcA* and *prtP* mutants was reduced by greater that 50% during active growth. This result is consistent with Msp protein expression results (10, 18) and suggests that protease gene expression might contribute to the regulation of *msp* transcription. However, reduced *msp* transcription does not account for the absence of Msp oligomers in protease mutants.

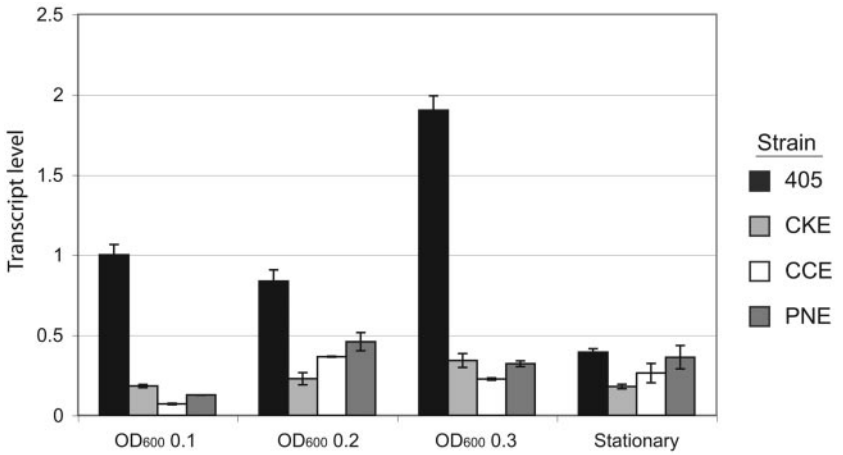

FIG. 2. Transcription of *msp* in protease mutant strains. *T. denticola* strains were harvested at an OD_{600} of 0.1, 0.2, and 0.3 and at stationary phase (fifth day). QRT-PCR was performed as described in the legend to Fig. 1. Data are shown with the gene expression level defined as 1.0 at an OD_{600} of 0.1. Error bars represent standard deviations of the means of results from triplicate samples in two independent experiments.

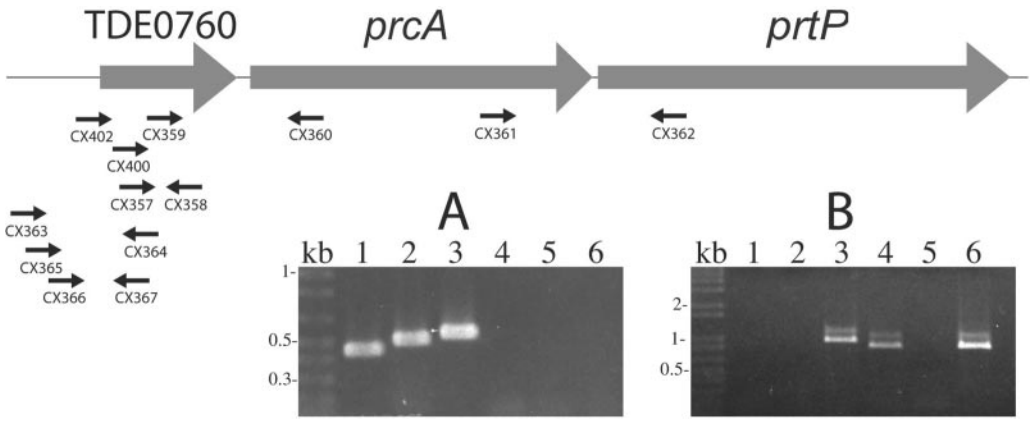

FIG. 3. RT-PCR analysis of *prcA-prtP* operon. DNase-treated RNA samples were reversed transcribed as described in the legend to Fig. 1 by using a gene-specific primer or random hexamer primers supplied with the SuperScript First-Strand synthesis system for RT-PCR (Invitrogen). One microliter of the resulting first-strand cDNA was amplified according to the manufacturer's instructions. The location and orientation of oligonucleotide primers used for RT-PCR are shown relative to TDE0760, *prcA*, and *prtP* in the graphic map. RT-PCR products, including positive (genomic DNA template) and negative (no RT enzyme) controls were analyzed by agarose gel electrophoresis. Panel A demonstrates continuity of transcription between TDE0760 and *prtP.* Lane 1, CX357-CX358; lane 2, CX359-CX360; lane 3, CX361-CX362; lane 4, CX363-CX364; lane 5, CX365-CX364; and lane 6, CX366-CX367. Panel B localizes the 5′ end of the transcript. CX402-CX360, lanes 1 to 3; CX400-CX360, lanes 4 to 6. Lanes 1 and 4, cDNA template; lanes 2 and 5, RNA template; lanes 3 and 6, DNA template.

The *prcA-prtP* transcript includes an additional upstream gene. Expression of PrcA, which is encoded directly upstream of the gene encoding PrtP, is required for PrtP expression and activity (16, 18). A recent study reported that the *prtP* transcript is at least 5 kb, large enough to include both *prcA* and *prtP* (16). RT-PCR using primer pair CX361 and CX362 (Fig. 3A, lane 3) demonstrated that *prcA* and *prtP* are cotranscribed. Transcription of *prtP* was not detected in *prcA* mutant strain PNE (data not shown), and a typical rho-independent transcription termination sequence is found directly downstream of *prtP*. Taken together, these data demonstrate that *prcA* and *prtP* are cotranscribed. Because *prcA* and *prtP* together comprise only 4.2 kb, we examined the sequence upstream of *prcA* by RT-PCR and demonstrated that the *prcA-prtP* transcript extends upstream through the 5′ end of TDE0760 (Fig. 3B, lanes 1 and 4). TDE0760 encodes a conserved hypothetical protein of 18 or 21 kDa, depending on whether the translation initiates at nucleotide 799483 (ATG) or at 799576 (GTG), as indicated by the *T. denticola* genome annotation. Sequence analysis using PSORT (http://psort.nibb.ac.jp/) predicts that the TDE0760 protein, including the additional N-terminal region, is membrane associated and may be acylated.

Construction of a TDE0760 mutant. To characterize the relationship between TDE0760 and the protease complex, we constructed a TDE0760 isogenic mutant by allelic replacement mutagenesis. A 1,475-bp PCR product containing all but the 3′ end of TDE0760 that was generated by using standard PCR conditions (9) with CX412 (GAGCTTTGTCTTCTACATTG) and CX413 (CAAATTGATGATCTTCCCTG) was cloned in pSTBlue-1 (Novagen). The *ermF*/*ermB* cassette (11) was inserted in opposite transcriptional orientation to TDE0760 at a *pshA1* site 43 nucleotides 3′ to the putative ATG initiation codon at nucleotide 799483, yielding pCF353. Plasmid pCF353 was then digested with PvuII, releasing the vector sequences. *T. denticola* was electroporated with the resulting linear DNA, and transformants were selected for erythromycin resistance (10, 19). Genomic DNA from Em^r colonies was screened by PCR for the presence of the *ermF*/*ermB* cassette in TDE0760 by using combinations of oligonucleotide primers specific for TDE0760, *prcA*, and *ermF*/*ermB* (data not shown). A single validated isolate was designated *T. denticola* P0760.

Protein expression in mutant strain P0760. As shown in Fig. 4, mutagenesis of TDE0760 resulted in the loss of expression of both PrcA2 and PrtP. RT-PCR products of *prtP*, *prcA*, and the 3′ region of TDE0760 were present in 35405 but not in P0760 (data not shown). SAAPFNA activity was absent in P0760, essentially identical to that of CKE, PNE, CCE, and

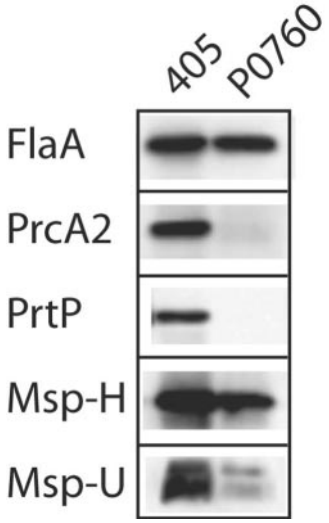

FIG. 4. Western immunoassay of *T. denticola* parent and mutant strains. Equal amounts of whole-cell extracts of *T. denticola* 35405 (405) and P0760 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to membranes, and probed with antibodies raised against recombinant FlaA, PrcA2, PrtP, or Msp polypetides. All samples were heated at 100°C for 5 min prior to electrophoresis, with the exception of Msp-U, which was held at 4°C.

MPE (data not shown). While total Msp was considerably reduced in P0760 (Fig. 4, Msp-H), Msp oligomers (Fig. 4, Msp-U) were barely detectable in P0760 compared with 35405.

While it has not yet been possible to determine the mechanism for the connection between the expression of Msp and the PrcA-PrtP protease complex, the present study further explored this relationship. We have presented new information on growth and transcription in *T. denticola* strains with mutations in genes encoding periodontal virulence factors. Transcriptional analysis supports the hypothesis that the production of active protease complex and Msp oligomers are closely related. While its function is not yet known, initial characterization of TDE0760 contributes to our understanding of protease complex expression. This report should help to provide a context for interpretation of studies using these and similar defined mutant strains to assay biological activities of Msp and PrtP.

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