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The Tla Protein of *Porphyromonas gingivalis* W50: a Homolog of the RI Protease Precursor (PrpRI) Is an Outer Membrane Receptor Required for Growth on Low Levels of Hemin

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The *prpR1* gene of *Porphyromonas gingivalis* W50 encodes the polyprotein precursor (PrpRI) of an extracellular arginine-specific protease. PrpRI is organized into four distinct domains (pro, α, β, and γ) and is processed to a heterodimeric protease (RI) which comprises the α and β components in a noncovalent association. The α component contains the protease active site, whereas the β component appears to have a role in adherence and hemagglutination processes. DNA sequences homologous to the coding region for the RI β component are present at multiple loci on the *P. gingivalis* chromosome and may represent a family of related genes. In this report, we describe the cloning, sequence analysis, and characterization of one of these homologous loci isolated in plasmid pJM7. The 6,041-bp *P. gingivalis* DNA fragment in pJM7 contains a major open reading frame of 3,291 bp with coding potential for a protein with an M_r 118,700. An internal region of the deduced sequence (V304 to N768) shows 98% identity to the β domain of PrpRI, and the recombinant product of pJM7 is immunoreactive with an antibody specific to the RI β component. The N terminus of the deduced sequence has regional similarity to TonB-linked receptors which are frequently involved in periplasmic translocation of hemin, iron, colicins, or vitamin B_{12} in other bacteria. We have therefore designated this gene *tla* (TonB-linked adhesin). In contrast to the parent strain, an isogenic mutant of *P. gingivalis* W50 in which the *tla* was insertionally inactivated was unable to grow in medium containing low concentrations of hemin (<2.5 mg liter^{-1}), and hemin-depleted cells of this mutant failed to respond to hemin in an agar diffusion plate assay. These data suggest a role for this gene product in hemin acquisition and utilization. Furthermore, the mutant produced significantly less arginine- and lysine-specific protease activities than the parent strain, indicating that there may be a regulatory relationship between *tla* and other members of this gene family.

Porphyromonas gingivalis, an anaerobic gram-negative rod bacterium, is regarded as an important etiological agent in chronic adult periodontal disease (15, 49). This highly prevalent disease is one of a group of inflammatory conditions which undermine the attachment apparatus of the teeth by destroying the soft connective tissues of the periodontium and the alveolar bone. These destructive processes may be a consequence of direct action of microbial products derived from the subgingival plaque on the adjacent tissues of the periodontium and/or caused by the deregulation of the host's inflammatory response. The extracellular proteases of *P. gingivalis* have the potential to contribute to both of these mechanisms of tissue destruction via the degradation of a wide range of host macromolecules which are fundamental not only to tissue integrity but also to the control of inflammation (22, 47, 57).

Biochemical characterization of *P. gingivalis* proteases has demonstrated a complex pattern of enzymes with different molecular masses and peptide bond specificity. However, because of their relative abundance and ability to degrade and inactivate host proteins central to tissue homeostasis, two types of enzyme, specific for peptide bonds with either arginine or lysine in the P_1 position, have received most attention (43). A common finding in studies of these enzymes has been the association of hemagglutinating activity with the purified protease (35, 40, 41). A molecular explanation for this association has emerged recently following the purification and characterization of the major arginine-specific and lysine-specific protease activities and the cloning and sequence analysis of the corresponding genes from *P. gingivalis* W50 (1, 23), HG66 (39), and 381 (36, 37).

Our studies with *P. gingivalis* W50 have demonstrated that the extracellular Arg-x activity comprises three closely related forms, RI, RIA, and RIB, which are all derived from *prpR1* (44, 45). While both RIA and RIB are monomeric species, RI is a heterodimeric enzyme composed of an α component (M_r, 54,000), which bears the protease active site, and a β component (M_r, 58,000), which, at the primary sequence level, is similar to hemagglutinins from other microorganisms and adhesins which bind to mammalian extracellular matrix proteins. Analysis of the deduced protein sequence of the gene for RI (*prpR1*) demonstrates that the α and β components are contiguous on the initial translation product and are flanked by long N- and C-terminal extensions.

Confirmation of the genomic organization of *prpR1* was established by Southern hybridization using probes to individual regions. These studies also demonstrated, however, that elements of the coding region for the β component of RI were present at multiple loci on the *P. gingivalis* chromosome, suggesting that a number of gene products may share the adhesin-like domain of the RI protease (1). A similar conclusion has been derived from immunochemical studies with a monoclonal antibody (MAb 1A1) which reacts with an epitope on the β

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component of RI. Western blots of *P. gingivalis* whole-cell sonicates demonstrate that this MAb recognizes numerous cellular proteins in the molecular weight range of 20,000 to 120,000 (12). Furthermore, the ability of periodontal patient serum antibody to block the binding of MAb 1A1 to *P. gingivalis* whole cells suggests that proteins bearing this epitope are expressed by the organism in vivo and are targets for the specific immune response of diseased individuals (11).

These observations led us to determine the identities of the other members of this family of gene products which are sequence and antigenically related to RI. During the initial cloning of *prpR1*, we obtained a related clone which corresponds to one of the homologous loci; in this report, we describe the characterization of this gene. Our initial expectation was that it may correspond to another protease of this highly lytic organism, perhaps with different peptide bond specificity to RI. However, on the basis of the deduced sequence, the product of this RI homolog is a TonB-linked protein which contains a region of the adhesin domain of the PrpRI polyprotein. Furthermore, functional studies using a *tla* mutant suggest that this gene product is involved in the acquisition or utilization of low levels of environmental hemin and may therefore represent an outer membrane receptor for this physiologically important iron source and virulence regulator (30, 31).

MATERIALS AND METHODS

Bacteria and batch culture conditions. *P. gingivalis* W50, W50Be1, W50Br1, W83, LB13D-3, A7436, 381, ATCC 33277, and NCTC 11834 and *Porphyromonas asaccharolytica* ATCC 25260 have been described previously (1, 32). *Porphyromonas* species were grown on blood agar plates or in brain heart infusion (BHI) broth (Oxoid, Basingstoke, England) supplemented with hemin (7.7 μM) in an anaerobic cabinet (Don Whitley, Shipley, England) in an environment of N_2-H_2-CO_2 (80:10:10) at 37°C.

The growth of *P. gingivalis* and an isogenic mutant on different iron and hemin sources was examined by using a 2.25% (wt/vol) mycoplasma broth base (BBL, Becton Dickinson, Cockeysville, Md.) as described by Bramanti and Holt (5).

Escherichia coli XL-1 Blue (Stratagene) was grown on LB (tryptone, 1%; yeast extract, 0.5%; NaCl, 0.5%) with 20 μg of tetracycline ml^{-1}. For plasmid selection, ampicillin was added to 50 μg ml^{-1}.

DNA manipulations. Preparation of *P. gingivalis* chromosomal DNA and the initial cloning and screening for the JM7 clone have been described in detail elsewhere (1, 46). *P. gingivalis* W50 was used for all genomic cloning into the pUC18 vector (58).

For DNA sequencing, directional derivatives of pJM7 were constructed following an initial digestion of pJM7 with *Sph*I and *Xba*I, both of which restrict only the DNA within the residual multiple cloning site of pUC18. A nested deletion kit from Pharmacia was used. Plasmids were subjected to *Taq* cycle sequencing reactions using the dideoxy dye termination chemistry of Applied Biosystems and universal forward or reverse primer. The products were analyzed on an model 373A automated DNA sequencer (Applied Biosystems). Complete coverage of both strands and linkage of contigs were achieved by primer walking using pJM7 as a template. DNA sequence data were analyzed by using the Wisconsin Genetics Computer Group software (14) and the Staden DNA and protein analysis programs (National Institute for Medical Research, London, England).

Sequencing-grade plasmid DNA was prepared by ion-exchange chromatography on Qiagen columns. Oligonucleotide primers were synthesized either on a Gene Assembler 4 Plus (Pharmacia) or a model 392 DNA/RNA synthesizer (Applied Biosystems) followed by purification over NAP-10 columns (Pharmacia).

DNA hybridization. Cells were lysed, and the DNA was fixed onto Hybond N^+ (Amersham) with 0.4 M NaOH as instructed by the manufacturer. After rinsing, membranes were prehybridized for 1 h at 65°C in Rapid Hyb (Amersham) and hybridized for 2 h in the same solution with heat-denatured ^{32}P-labelled probes. Membranes were then washed thoroughly under stringent conditions in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS) at 65°C. Autoradiography was performed by using Kodak X-Omat-LS films at −70°C, with intensifying screens, for 1 to 18 h. Southern hybridization was performed in a similar manner except that DNA was electrophoresed in agarose gels, depurinated in 0.25 M HCl, and then transferred onto Hybond N^+ by vacuum blotting (Vacu-aid; Hybaid) under alkaline conditions (0.4 M NaOH). The probe DNA was labelled by the random primer method with the Ready To Go labelling system (Pharmacia) and Redivue [α-^{32}P]dCTP (3,000 mCi/mmol; Amersham).

Construction of isogenic *tla* negative *P. gingivalis* by allelic exchange. A 2.1-kb *ermF-ermAM* macrolide-lincosamide tandem cassette (Erm cassette) was excised from pVA2198 (16) by using restriction enzymes *Sst*I and *Pst*I, blunted with Klenow DNA polymerase, and ligated into the *Sst*II site of pJM7, which had also been blunted. The mixture was transformed into *E. coli* XL-1 Blue, and transformants were selected on LB agar containing ampicillin (50 μg ml^{-1}) and erythromycin (300 μg ml^{-1}) for *ermAM* expression. A representative plasmid, pJE71, was subsequently linearized with *Eco*RI, purified with a Wizard cleanup kit (Promega), and then transformed into *P. gingivalis* by electroporation.

Electroporation. All procedures were performed at 4°C. A 6-h culture of *P. gingivalis* W50 (30 ml) was harvested by centrifugation at 10,000 × *g* for 10 min, washed in 10% glycerol–1 mM $MgCl_2$ (EPB), and resuspended in 600 μl of EPB. An aliquot of 200 μl was mixed with approximately 200 ng of DNA in 5 μl of Tris-EDTA in a 0.2-cm-path-length cuvette and then electroporated in a Bio-Rad Gene Pulser with the parameters set at 2.5 kV for potential difference, 200 Ω for resistance, and 25 μF for capacitance; these parameters gave time constants between 2.9 and 4.3 ms. *P. gingivalis* cells were then diluted in 1 ml of BHI-hemin broth and allowed to recover for 16 h in an anaerobic cabinet before being plated out on blood agar plates containing 5 μg of clindamycin hydrochloride ml^{-1} (for *ermF* expression). The *P. gingivalis* transformants were initially selected by colony lifts and subsequent probing with the Erm cassette. Allelic exchange in the mutants was then confirmed by Southern hybridization. Mutants were additionally typed as described by Moncla et al. (33). The procedures worked equally well for *P. gingivalis* W50, W83, and 381.

The transforming DNA was also prepared by PCR using pJE71 as the template in a 100-μl reaction volume containing the following: deoxynucleoside triphosphates, 250 nM each; pJE71, 20 ng; primer I (JM7F1; 5′-ATACTTTCG GAGAGAGGTATCTCC-3′; nucleotides [nt] 773 to 796 of pJM7 insert), 0.5 μg; primer II (JM7R1; 5′GTCAGGTTCTGTACAGGAGCA-3′; nt 1783 to 1763 of pJM7 insert), 0.5 μg; $MgCl_2$, 1.5 mM; and *Thermus icelandicus* DNA polymerase, 2.0 U in polymerase buffer IV (Advanced Biotechnologies Ltd., London, England). The reactants were subjected to 25 cycles of denaturation at 95°C for 1.0 min, annealing at 55°C for 1.0 min, and extension at 72°C for 4 min, using an Omnigene thermal cycler (Hybaid). The 3.1-kb amplicon was purified with Wizard cleanup kit (Promega) prior to electroporation. The two methods gave rise to the same predicted marker-exchanged mutants. A typical *P. gingivalis* mutant, W50T1, which is a derivative of strain W50 and defective in *tla*, was selected for further analysis.

Phenotypic characterization of the *tla* mutant. (i) Growth properties and protease activities. The growth rates and final cell yields of *P. gingivalis* W50 and W50T1 were compared in BHI–7.7 μM hemin. Cultures (200 ml) were inoculated with a 10% inoculum of each strain in late exponential phase. Samples were then withdrawn throughout the course of growth up to 24 h, optical densities were measured at 540 nm, and aliquots (2 ml) were added to an equal volume of pH 6.0 buffer (25 mM morpholinepropanesulfonic acid, 200 mM NaCl, 10 mM $CaCl_2$). These samples were then chilled on ice and sonicated for twice for 10 s each time on full power, using an MSE Soniprep 150 (Fisons Instruments, Crawley, England) to produce a whole-culture sonicate. A further aliquot of culture was centrifuged at 13,000 × *g* for 5 min, and the culture supernatant was harvested. The arginine- and lysine-specific protease activities were monitored throughout the growth of *P. gingivalis* by assaying the hydrolysis of benzoyl arginyl *para*-nitroanilide (BApNA) and acetyl-lysyl *para*-nitronanilide, respectively, by the whole-culture sonicate and in the supernatant (45).

(ii) Growth on hemin and organic sources of iron. Growth of the parent and *tla* strains on different iron and hemin sources was examined by using a 2.25% (wt/vol) mycoplasma broth base as the basal medium (5). The following organic supplements were added to give final iron and hemin concentrations in the range of 7 to 20 μM: human hemoglobin, horse myoglobin, bovine lactoperoxidase, bovine cytochrome *c*, and bovine holotransferrin (Sigma Chemical, Poole, England). Growth in hemin-supplemented mycoplasma broth was examined in the concentration range of 0 to 5 mg of hemin $liter^{-1}$ (0 to 7.7 μM). Following inoculation from an overnight BHI broth culture, each organism was serially transferred every 24 h over eight passages, using a 10% inoculum on each occasion, and the final optical density at 540 nm of each culture was recorded.

In some experiments, growth of the wild type and that of the mutant strain were also compared in a plate assay. *P. gingivalis* W50 and W50T1 were initially depleted of endogenous reserves of hemin by subculturing (three times) in the basal medium with no exogenous iron source followed by spread plating onto mycoplasma basal medium agar, again with no additional iron source. Wells were then made in the agar, into which different amounts of hemin (0 to 0.12 μg in 40 μl) or organic sources of iron or hemin in the same concentration range were added. After 4 days, zones of growth of the parent and mutant strain around wells were compared.

SDS-PAGE and Western blotting. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (25) on 6 or 10% separating gels. Western blotting was carried out in a bicarbonate transfer buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$ [pH 9.9], 20% [wt/vol] methanol) at a constant current of 400 mA for 2.5 h. Nitrocellulose membranes were subsequently blocked in 5% bovine serum albumin prior to overnight incubation in primary antibody (1:100 dilution). The production and specificity of MAb 1A1, which recognizes a determinant within the RI β component and sequence-related gene products, are described elsewhere (11, 12). The antibody-antigen reaction was

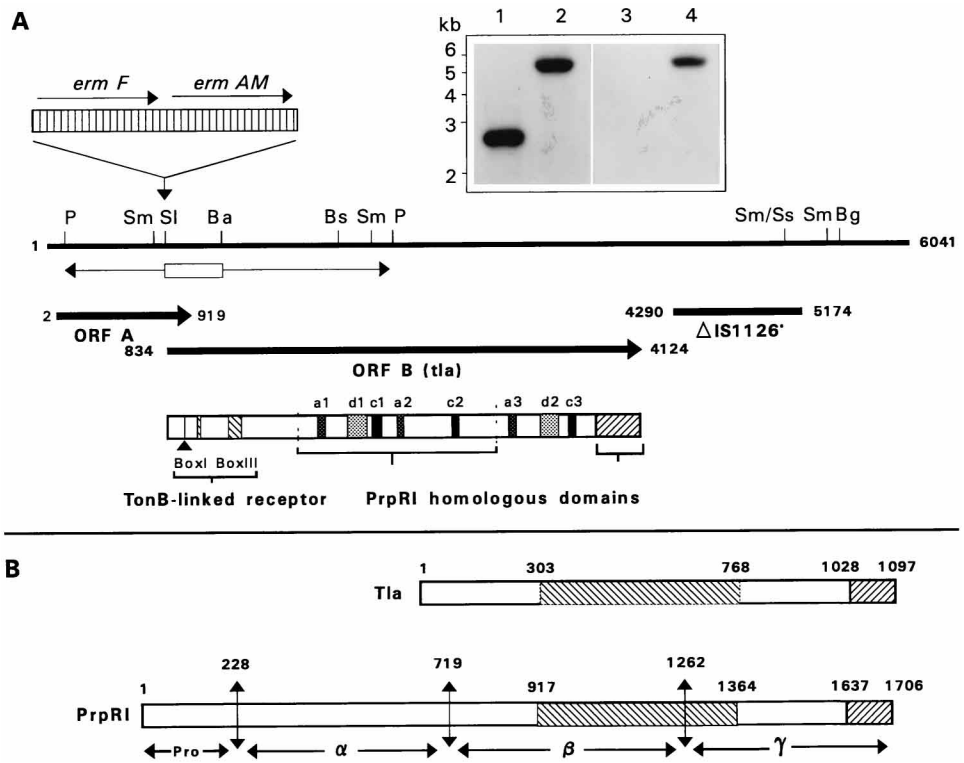

FIG. 1. Partial restriction map and organization of the *tla* locus on the *P. gingivalis* W50 chromosome. (A) The top thick line represents the 6.0-kb *P. gingivalis* W50 insert in pJM7. The vertical lines mark the positions of unique restriction enzyme sites. Bold lines with a single terminal arrow indicate the locations and directions of ORFs deduced from the nucleotide sequence. The deduced sequence of *tla* (ORFB) is shown as a rectangular box under the gene, with peptide repeats (a to d) shaded within it. The putative signal peptidase site is indicated with a closed triangle. Regions of Tla which are homologous to domains of PrpRI (accession no. X82680) and the positions of the TonB boxes are also shown. Δ*IS1126** corresponds to a vestigial *IS1126* (accession no. X77924) in which an essential region of the transposase gene is deleted. (B) Schematic comparison of Tla and PrpRI. Hatched areas correspond to regions of identity. Vertical lines (with terminal arrows) through the PrpRI box represent processing sites between the pro, α, β, and γ domains. The numbers in panel B refer to amino acids, and those on the bold-arrowed lines in panel A are nucleotide base numbers. In constructing *P. gingivalis tla* mutant W50T1, an Erm cassette consisting of *Bacteroides* (*ermF*) and *Enterobacteriaceae* (*ermAM*) genes encoding macrolide resistance was inserted at the unique *Sst*II (SI) site of *tla*. Southern analyses of the *Pst*I-restricted chromosomal DNA of W50 (lanes 1 and 3) and W50T1 (lanes 2 and 4) are shown in panel A. Lanes 1 and 2 were probed with a 526-bp *Sst*II-*Bam*HI fragment of pJM7 (small box under pJM7 insert), and lanes 3 and 4 were probed with the Erm cassette (large hatched box). Abbreviations for restriction enzymes: Ba, *Bam*HI; Bg, *Bgl*II; Bs, *Bss*HII; P, *Pst*I; SI, *Sst*II; Sm, *Sma*I; Ss, *Sst*I. Note that there are no sites within pJM7 for *Eco*RV, *Sph*I, or *Bst*EII.

detected with horseradish peroxidase-conjugated antispecies immunoglobulins, H_2O_2, and diaminobenzidine (0.05%). Protein microsequencing, following SDS-PAGE and subsequent transfer onto polyvinylidene difluoride membranes, has been previously described (1).

Nucleotide sequence accession number. The sequence shown in Fig. 3 will appear in EMBL, GenBank, and DDBJ databases under accession no. Y07618.

RESULTS

Cloning of JM7 and detection of a locus homologous to the *prpR1* gene. JM7 was originally isolated from a pUC18 library of partial *Sau*3AI fragments of *P. gingivalis* W50 genomic DNA in *E. coli* XL-1 Blue during the cloning of the *prpR1* gene. Rabbit antiserum (Rb3158) to the β component of the dimeric RI protease was used to screen *E. coli* clones expressing recombinant proteins (1). Three clones, all immunoreactive with this antiserum, were isolated: JM2, JM7, and JM11, with insert sizes of ~5.2, ~6.0, and ~1.1 kb, respectively. Southern blotting demonstrated that all three plasmids cross-hybridized (1). Restriction endonuclease mapping of the plasmids indicated that pJM11 contained an internal fragment of the insert in pJM2. After nucleotide sequence analysis, the insert in pJM2 was found to correspond to the gene for RI (*prpR1*), and pJM11 contained the coding region for D784 to V1130 within the RI β component. However, on the basis of these restriction maps, pJM7 represented a different locus on the *P. gingivalis* chromosome (Fig. 1A). The relative protein yield in JM7 was unaffected by the inclusion of isopropyl thiogalactopyranoside to 1 mM during logarithmic growth (data not shown), suggesting that expression of the *P. gingivalis* protein in JM7 may be under its own promoter sequences, which are recognized by the *E. coli* host strain.

The antigenic relatedness of the RI protease precursor and the recombinant product of JM7, which had been suggested by the cross-reactivity with the screening antiserum, was further examined by using MAb 1A1. We have previously shown that this antibody recognizes an epitope within the RI β component at G907 to T931 of PrpRI (12). Western blot analysis demonstrated that, in addition to recognizing *prpR1*-derived clones and the *P. gingivalis* RI protease β component, MAb 1A1 was immunoreactive with the recombinant product of the homologous gene in pJM7, which had a maximum size of ≈116 kDa (Fig. 2).

These data indicate that the product of pJM7 and PrpRI are sequence-related and antigenically related proteins. On the basis of the Southern hybridization studies using pJM11 and the immunoreactivities of the proteins with MAb 1A1, the region of similarity includes elements of the β component of the RI. Consequently, the functional properties of this putative adhesin component may be shared by the two proteins.

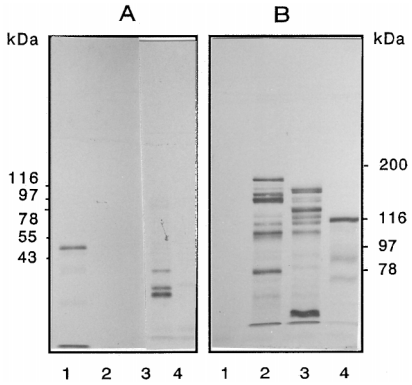

FIG. 2. Antigenic relatedness of *P. gingivalis* RI protease precursor and the recombinant product in JM7. (A) Western blot from an SDS–10% polyacrylamide gel of RI ($\alpha\beta$ heterodimer; lane 1), RIA (α monomer; lane 2), and an *E. coli* clone expressing an internal region of the RI β component (JM11; lane 3) or pUC18 control (lane 4). (B) Western blot from an SDS–6% polyacrylamide gel of *E. coli* containing pUC18 (control, lane 1), JMS harboring the complete *prpR1* (lane 2), or 3′ truncation JM2 (lane 3) and the homologous clone JM7 (lane 4). In both cases, MAb 1A1 was the primary antibody. Similar data were obtained in assays using Rb3158 antiserum to the RI β component.

DNA sequence and analysis of pJM7. The nucleotide sequence of 6,041 bp of the *P. gingivalis* insert in pJM7 (Fig. 3) has a G-C composition of 48.5%, which is consistent with previously reported values for this organism (1). The locus can be divided into three discrete regions: two overlapping open reading frames (ORFs) and a long noncoding sequence at the 3′ end (Fig. 1A). The major ORF, ORFB, begins with an ATG methionine at nt 834 and terminates with a TAA stop codon at nt 4125. This represents a complete ORF of 3,291 bp with coding potential for 1,097 amino acids (aa) and a calculated molecular size of 118,731 Da, which is in agreement with the experimentally determined size of the recombinant protein (Fig. 2). A sequence resembling the Shine-Dalgarno (48) *E. coli* consensus ribosome binding site was located at nt 797 to 804 (RBS_{orfB} in Fig. 3). The termination codon is augmented by a structural loop (68.8% G-C content). This dyad symmetry is a typical feature of rho-independent transcription termination and is found immediately downstream of ORFB at nt 4201.

Analysis of the deduced sequence of ORFB by using a Kyte-Doolittle hydrophobicity plot revealed a predominantly hydrophilic protein with hydrophobic regions at the amino and carboxy termini. The structural and physical characteristics of the N terminus satisfy the criteria for a signal peptide (20, 54), although the two typical lysine residues, in this instance, are replaced by arginines. The putative signal peptidase site is probably between A41 and L42 or A53 and K54; the latter is the most likely since it conforms to the -3 and -1 rule as well as having a helix breaker (Q50) between -4 and -6. The recombinant protein in *E. coli* was found in both the cytosolic and membrane fractions (not shown), which suggests that this signal peptide sequence may be operative in the heterologous *E. coli* host.

At the nucleotide level, bases 1874 to 3150 of pJM7 are 98% identical to nt 3519 to 4795 of the *prpR1* gene of *P. gingivalis* W50 (1). Allowing for a 3-aa gap, NPG at P346 to G347, the common sequence corresponds to 465 aa of the deduced sequence of ORFB (V304 to N768) and residues V918 to N1384 within PrpRI. Interestingly, the last 70 aa of the two proteins are 95.7% identical. The relationship between the deduced sequence of ORFB of pJM7 and that of PrpRI is shown in Fig. 1B. The longest shared region of identity thus includes a dipeptide proline-asparagine repeat, $(PN)_5$, in the deduced sequence of pJM7 ORFB and $(PN)_6$ in PrpRI. Similar repeats are found in the hemagglutinin of *Mycoplasma gallisepticum* (28) and circumsporozoite CS protein of *Plasmodium* spp. (13) and are suggested to have a role in adherence. This region also contains an adenylation motif, (ExKxNGxRA), at E514 to A522 in ORFB which is characteristic of eukaryotic ATP-dependent DNA ligases (53). Four major peptide repeats are found within the ORFB deduced sequence (Fig. 3 and 4). Peptide repeats a1, a2, and a3 are rich in charged amino acids (25 to 30% Asp and Glu) and are a feature of proteins which bind to mammalian extracellular matrix (55, 56).

At the 5′ end of ORFB, an incomplete ORFA encoding the C-terminal 306 residues of an unidentified protein overlaps with ORFB. This organization, which resembles that of an operon (38), suggests that transcription of these two genes may be coupled. The carboxy terminus of ORFA does not bear any significant similarity to known sequences at either the nucleotide or amino acid level.

A recent report has described the presence of numerous copies of an insertion sequence in *P. gingivalis* W83 (PGW83TG [27]), and it was of interest to find that the noncoding 3′ end of the pJM7 sequence, nt 4290 to 5174, is nearly identical to the corresponding region of this transposase gene with the exception of the coding region residues 572 to 1,021 of PGW83TG, which are not present in pJM7. In the deduced sequence of PGW83TG, this sequence includes the helix-turn-helix motif and other regions essential for DNA binding and transposase activity. It is therefore possible that the sequence immediately distal to ORFB at the 3′ end is a noncoding remnant of a transposition event. Hence, we have used the designation ΔIS*1126** for this putative vestigial transposase on pJM7 (Fig. 1A).

Sequence similarity to TonB-linked receptors. A search of the combined databases at the National Centre for Biotechnology Information (NCBI) with the sequences in Fig. 3 revealed that the N terminus of the deduced sequence of ORFB contains sequences with significant similarity to the conserved regions of TonB-dependent proteins. These are a family of outer membrane receptors which are frequently involved in iron, hemin, colicin, and vitamin B_{12} acquisition in many gram-negative bacteria, including *Vibrio cholerae*, *E. coli*, *Bordetella bronchiseptica*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, and *Haemophilus influenzae* (for a review, see reference 7). To acknowledge this similarity as well as the presence of the region of identity with the adhesin domain of the PrpRI, we have given the name TonB-linked adhesin to the product of ORFB and will refer to the gene as the *tla.*

Most TonB-linked receptors characterized to date usually have sequence relatedness in three distinct regions termed the TonB box (box I), box II, and box III. The TonB box region of these receptors is thought to interact directly with TonB. The region of greatest similarity in this family is within the 30-aa sequence of box III. A multiple alignment of the Tla and the TonB box and box III regions of the TonB-linked receptor family with the highest scores is shown in Fig. 5. The Tla box III region demonstrates closest similarity (90%) to the iron-regulated outer membrane virulence protein precursor (IrgA) of *V. cholerae* (accession no. P27772 [17]), the colicin I receptor of *E. coli* (accession no. U00007), and the exogenous ferric siderophore of *B. bronchiseptica* (accession no. V56084).

Southern hybridization. To examine the copy number and distribution of the *tla* in different *P. gingivalis* strains, Southern hybridization experiments were performed under stringent

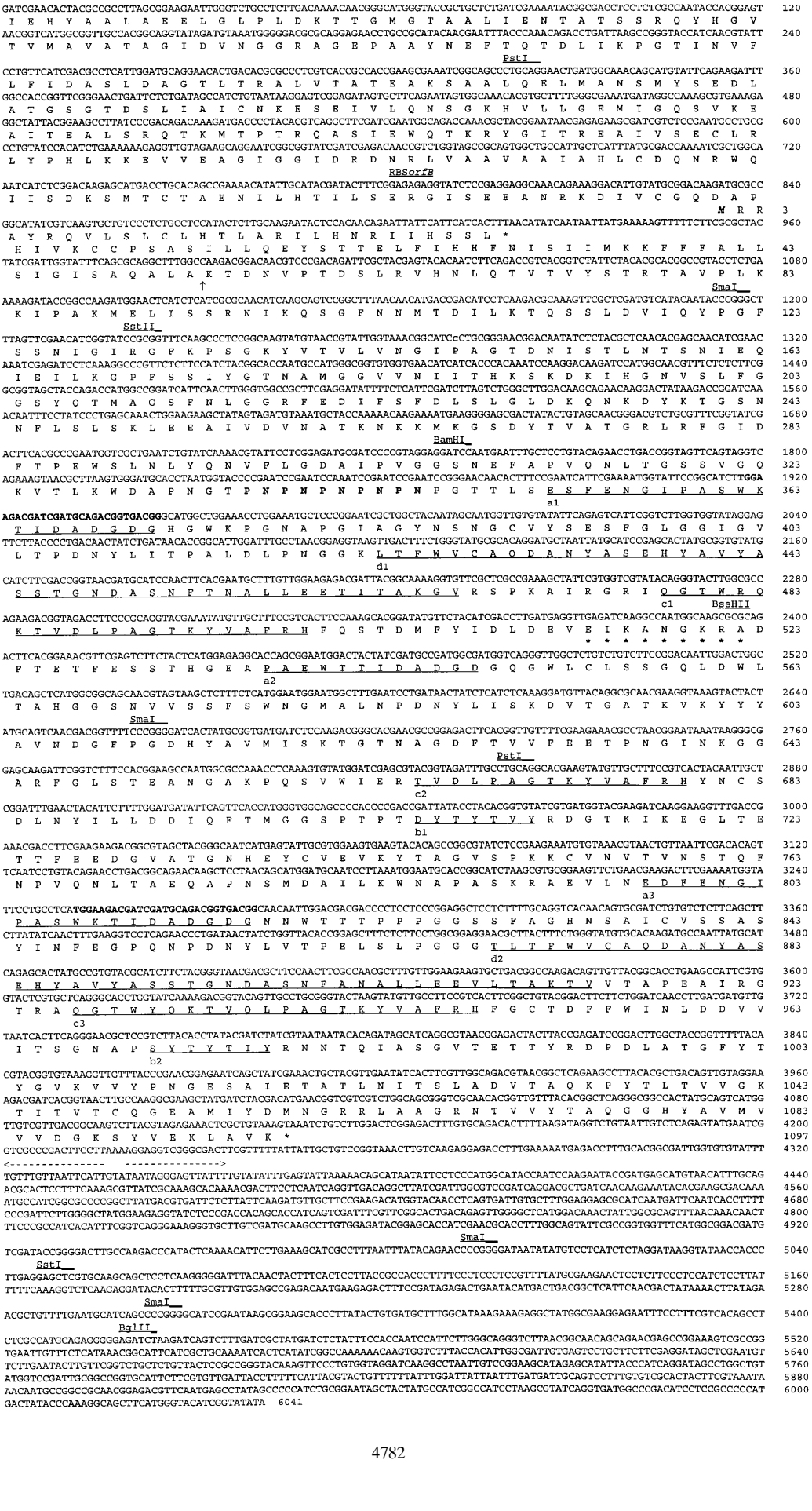
PstI
RBSorfB
SmaI
SstII
BamHI
a1
d1
c1
BssHII
a2
c2
b1
PstI
a3
d2
SmaI
SstI
c3
b2
SmaI
SstI
SmaI
BglII

FIG. 3. Nucleotide sequence of the *P. gingivalis* W50 locus on pJM7 showing the deduced sequence of two overlapping ORFs, ORFA (nt 2 to 919) and ORFB (nt 834 to 4,124), and a vestigial IS*1126* (nt 4290 to 5174). Unique restriction endonuclease sites are shown above the nucleotide sequence, and repeats are in boldface. A putative ribosome binding site for ORFB (RBS_{orfB}) is shown above the sequence at nt 797 to 804, and the first methionine of the deduced sequence of Tla is in boldface italics. The termination codon is denoted with an asterisk below it, and a rho-independent transcription termination sequence is between nt 4201 and 4234 (dashed-arrowed lines). Peptide repeats (a to d) are underlined in the deduced sequence. Proline-asparagine repeats are in boldface; the adenylation motif is shown with boldface asterisks under the deduced sequence. A vertical arrow represents the position of the presumed signal peptidase site.

conditions. Genomic DNA from a variety of laboratory strains of *P. gingivalis*, *P. asaccharolytica* ATCC 25260, and *E. coli* XL-1 Blue were restricted with *Pst*I and probed with a ^{32}P-labelled DNA fragment of ORFB corresponding to the region with TonB-linked protein similarity (*Sst*II-*Bam*HI fragment [Fig. 6]). All *P. gingivalis* strains were positive, whereas DNA from the closely related *P. asaccharolytica* and from *E. coli* were negative under the hybridization conditions used. The *P. gingivalis* strains were grouped into two categories; W50, W50Be1, W50Br1, W83, LB13D-3, and A7436 gave the expected band of approximately 2.5 kb, whereas strains 381, 33277, and 11834 gave a higher-molecular-weight fragment at approximately 3.2 kb. The same two strain groupings were obtained by using endonuclease *Sst*II to digest the chromosomal DNA (not shown). Thus, the coding sequence for *tla* is present on the genomes of all of the *P. gingivalis* strains that we tested, although some polymorphism is present. In contrast, a second probe (*Bam*HI-*Sst*I [Fig. 6]), which contains sequences identical to the adhesin coding domain of *prpR1* as well as the insertion sequence element, hybridized to multiple bands (not shown), thus confirming our previous results (1) and those of Maley and Roberts (27).

Construction of an isogenic *P. gingivalis* W50 *tla* mutant. The Erm cassette constructed by Fletcher et al. (16) is a tandem unit comprising *ermF*, which is expressed in *Bacteroides* spp. and *P. gingivalis*, and *ermAM*, which is expressed in members of the family *Enterobacteriaceae*. The unit encodes macrolide-lincosamide resistance, thus allowing the same antibiotic to be used in both the donor and the recipient.

To examine the function of *tla* in *P. gingivalis*, we inactivated the gene by inserting the *ermF-ermAM* cassette at the *Sst*II site within pJM7 insert (Fig. 1A). The construct, pJE71, was linearized and then electroporated into exponential-phase cells of *P. gingivalis* W50. Homologous recombination, mediated by a double-crossover event in vivo, resulted in gene replacement. All clindamycin-resistant colonies possessed the Erm cassette (not shown). Chromosomal DNA was purified from eight randomly selected mutants and then compared with that of the parent strain W50 following Southern hybridization; all mutants had the desired alteration in their genomes. One representative mutant, W50T1, was selected for further studies. Southern blots of *Pst*I restricted DNA from the parent and mutant strains probed with a fragment from the TonB region of the *tla* gene are shown in Fig. 1A. Instead of the predicted 2.5-kb hybridizing band in the parent, a larger *Pst*I fragment (ca. 5.5 kb) which also incorporates the Erm cassette is found in the mutant W50T1, indicating that the chromosomal integration was specific to the expected locus.

Characterization of *tla* mutant of *P. gingivalis* W50. (i) Growth on different iron sources. Because of the involvement of TonB-linked outer membrane receptors from other bacteria in iron and hemin acquisition, we compared the ability of the *tla* strain W50T1 to grow on different iron sources with that of the parent strain W50. These investigations were performed by examining growth in a mycoplasma broth supplemented with appropriate organic and inorganic iron and hemin compounds, which has previously been shown to be a suitable medium for iron limitation experiments with *P. gingivalis* (5). In the absence of exogenously added iron and hemin, this medium did not support significant growth of either the parent or the *tla* strain beyond four to five subcultures. Supplementation of the medium with hemoglobin, myoglobin, lactoperoxidase, cytochrome *c*, or holotransferrin allowed growth up to the eighth subculturing, and no differences in the final cell yields of the two strains were observed (not shown). Hence, the Tla is apparently not required for iron or hemin acquisition from these organic sources.

However, differences between the parent strain and W50T1 were observed when hemin was the sole iron source. At hemin concentrations of 2.5 mg liter^{-1} and above, the growth of both strains was maintained over all eight subcultures (Fig. 7A). However, in the presence of 1.25 or 0.625 mg of hemin liter^{-1}, the *tla* strain failed to grow beyond the fourth or fifth subculture whereas growth of the parent strain was unaffected, al-

a1 ^{352}E**S**FENGIPASWKTIDADGDG^{371}
a2 ^{537}PAEWTTIDADGDG^{549}
a3 ^{797}E**D**FENGIPASWKTIDADGDG^{818}

b1 ^{705}**D**YTYT**V**Y^{711}
b2 ^{971}**S**YTYT**I**Y^{977}

c1 ^{478}QGTW**R**QKTV**D**LPAGTKYVAFRH^{500}
c2 ^{665}TV**D**LPAGTKYVAFRH^{679}
c3 ^{927}QGTW**Y**QKTV**Q**LPAGTKYVAFRH^{948}

d1 ^{423}LTFWVCAQDANYASEHYAVYASSTGNDASNF**T**NALLEE**TI**TAK**G**V^{467}
d2 ^{870}LTFWVCAQDANYASEHYAVYASSTGNDASNF**A**NALLEE**VL**TAK**T**V^{914}

FIG. 4. Comparison of the peptide repeats in Tla. Four families of peptide repeats, a to d, varying in length from 7 to 45 aa are shown. The a1, a2, and a3 repeats are rich in charged amino acids and may participate in binding to mammalian proteins (55, 56). Differences within each family of repeats are shown in boldface, and the Tla amino acid numbering at the beginning and end of each sequence is shown.

BoxIII

Protein				%Similarity
FepA(ec)	141	**IERIEVLRGP** ARARYGNGA AGGVVNIITK **K**	170	70.0
FepA(pa)	146	**VERIEVIRGP** AAARYGNGA AGGVVNIITK Q	175	66.7
BfeA(bp)	150	**VERIEVIRGP** AAARYGSGA MGGVVNIITK R	179	73.3
BtuB(ec)	124	VQRVEYIRGP RSAVYGSDA IGGVVNIITT R	153	80.0
BtuB(st)	124	VQRIEYIRGP PSAIYGSDA IGGVVNIITT R	153	80.0
BfrA(bb)	163	IDRIEVVRGP MSTLYGSDA MGGVINIITR **K**	192	90.0
IrgA(vc)	134	**IERIEVIRGP** MSTLYGSDA IGGVINIITR **K**	163	90.0
CirA(ec)	130	**IERIEVVRGP** MSSLYGSDA LGGVVNIITK **K**	159	90.0
HemR(pg)	138	**IERIEVLRGA** SSALYGSNA IGGVINIITR S	167	83.3
IrgA(ec)	48	LERVEVVKGP YSVLYGSQA IGGIVNFITK **K**	88	83.3
Tla(pg)	161	**IEQIEILKGP** FSSIYGTNA MGGVVNIITH **K**	190	100

Box I (TonB box)

	1		9
BfeA(bp)	40	MA**T**VQVLGT	48
Tla(pg)	68	LQ**T**VTVYST	76
BtuB(ec)	25	P**DT**L**VVTA**N	32
BtuB(st)	25	P**DT**L**VVTA**N	32
FepA(ec)	51	D**DT**I**VVTA**A	58
IrgA(vc)	32	DE**T**M**VVTA**A	39
CirA(ec)	30	GE**T**M**VVTA**S	37
BfrA(bb)	58	M**DT**V**VVTA**S	65
FepA(pa)	38	EQ**T**V**VATA**Q	45
HemR(pg)	32	T**DT**IVSGNI	40

FIG. 5. Multiple alignments of TonB boxes with the highest similarity scores to *P. gingivalis* Tla. The receptors (box III) are FepA (ferric enterochelin), BfeA (ferrichrome-iron), BtuB (vitamin B_{12}), BfrA (exogenous ferric siderophore), IrgA (iron-regulated outer membrane), CirA (colicin I), HemR (hemin-regulated protein), and Tla (TonB-linked adhesin). Abbreviations for bacteria: ec, *E. coli*; pa, *Pseudomonas aeruginosa*; bp, *Bordetella pertussis*; bb, *B. bronchiseptica*; st, *S. typhimurium*; vc, *V. cholerae*; pg, *P. gingivalis*. Sequences are aligned in descending order of relatedness. Boldface amino acids correspond to residues conserved in more than 50% of the members of the group. Residues which are absolutely conserved are boxed. Amino acid sequence positions are shown at the beginning and end of each sequence. For box III, the degree of similarity is calculated with reference to Tla.

though there was some reduction in cell yield at 0.625 mg liter^{-1} (Fig. 7B). Further reduction of the hemin concentration of the medium compromised the growth of both strains, although low levels of growth of the parent were still observed after eight subculturings in hemin at 0.313 mg liter^{-1} (Fig. 7C). Therefore, these data suggest that the Tla is required for the growth of *P. gingivalis* at low environmental levels of hemin.

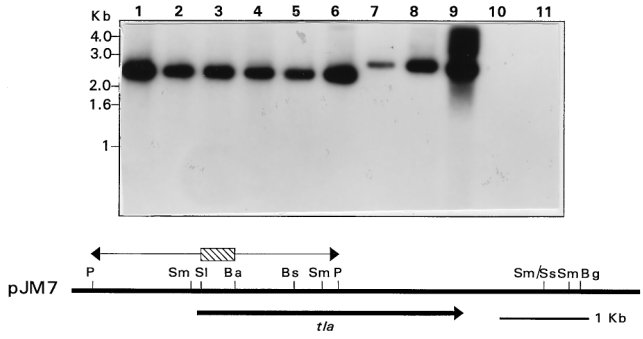

FIG. 6. Southern hybridization analysis of *tla* locus of *P. gingivalis* strains. Bacterial chromosomal DNA was digested with *Pst*I, resolved by agarose gel electrophoresis, and blotted onto Hybond N^+. The membrane was then probed with a ^{32}P-labelled *Sst*II-*Bam*HI fragment of pJM7 (hatched box) which corresponds to the N terminus of Tla. Lanes: 1 to 9, *P. gingivalis* W50, W50Be1, W50Br1, W83, LB13 D-3, A7436, 381, 33277, and 11834, respectively; 10, *P. asaccharolytica* ATCC 25260; 11, *E. coli* XL-1 Blue. Restriction enzymes are abbreviated as in Fig. 1.

Further support for a role for the Tla in hemin utilization was obtained by examining the ability of hemin-starved parent and mutant cells to respond to hemin added to wells in an agar plate assay. No zones of growth were observed for either strain around wells containing no added hemin, confirming that neither strain contained endogenous reserves. In the case of *P. gingivalis* W50, increasing zone sizes of confluent growth were observed around wells to which hemin (0 to 120 ng) had been added (Fig. 8A). However, no growth of *P. gingivalis* W50T1 was observed in the same dose range. Zones of growth of W50T1 were obtained only when ≥200 ng of hemin was added to the wells. In contrast, the parent and mutant strains responded equally to organic forms of iron and hemin in the same plate assay (not shown).

(ii) Effects on protease production. The finding that *tla* is involved in low concentrations of hemin acquisition and utilization and the previously reported effects of hemin on the expression of extracellular enzymes (8) led us to examine the production of proteolytic activity by the *tla* mutant. These experiments were performed with BHI medium (5 mg of hemin liter^{-1}), which we have previously used for the purification and characterization of proteases from *P. gingivalis* W50. The

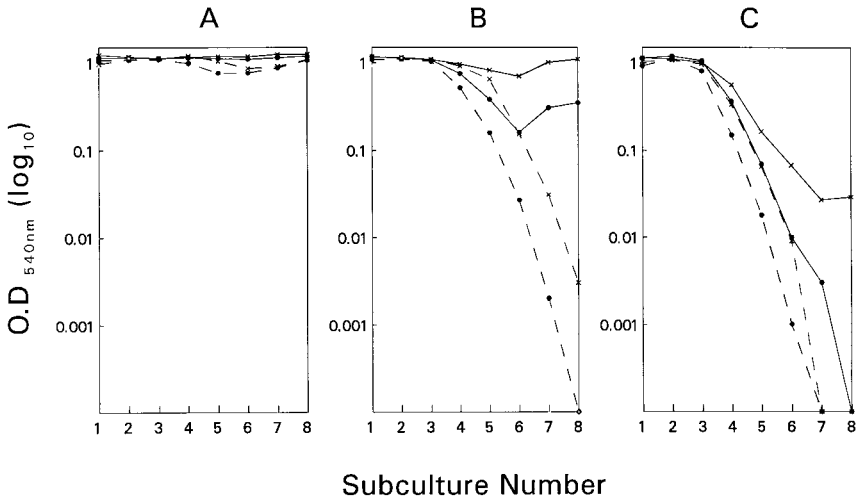

FIG. 7. Growth of *P. gingivalis* W50 and W50T1 in mycoplasma broth supplemented with decreasing concentrations of hemin. Points represent the optical densities after 24 h of serial transfers of each organism. (A) Five (×) and 2.5 (●) mg of hemin liter^{-1}; (B) 1.25 (×) and 0.625 (●) mg liter^{-1}; (C) 0.3125 mg liter^{-1} (×) and no additions (●). Solid lines, *P. gingivalis* W50; dotted lines, *tla* strain W50T1. Note that where the slope of line shows a 10-fold decrease between successive subcultures, growth has ceased and the optical density at 540 nm ($O.D_{540nm}$) is solely a result of the 10% inoculum.

growth rates and final cell yields of the *tla* mutant and parent strain W50 were identical in this complex medium, which probably contains multiple inorganic and organic sources of iron and hemin (Fig. 9, inset). Despite the similar growth characteristics, significant alterations to the arginine- and lysine-specific protease activities were observed in the W50T1 mutant. After 24 h of growth, the levels of both of these activities in the cell-associated fraction and culture supernatant were reduced by approximately 30% (Fig. 9).

Analysis of the arginine-specific protease activity throughout the course of growth of the mutant and wild type indicated that the reduction in activity was predominantly a function of differences during the early stages of growth. For example, the rate of BApNA activity production during the first 10 h of growth was 51% lower in the mutant, whereas from 10 to 24 h, the rates differed by only 17% (Fig. 10). Furthermore, in the early stages of growth, the reduction of BApNA activity in the mutant compared with that in the wild type was more pronounced in the cell-associated fraction. At 8 h, the cell-bound

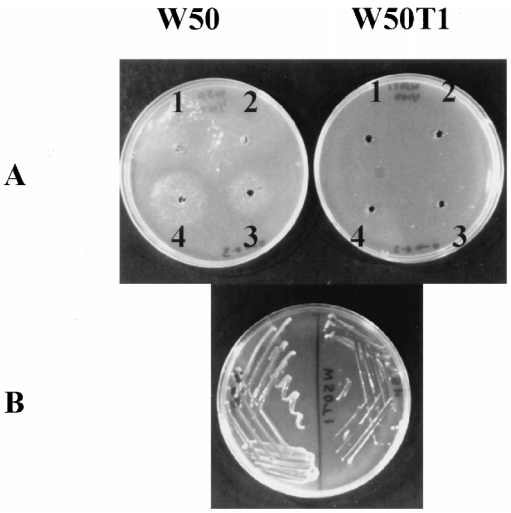

FIG. 8. Comparison of the growth of hemin-depleted *P. gingivalis* W50 and W50T1 (*tla*) in response to different amounts of hemin. *P. gingivalis* W50 and W50T1 were subcultured (three times) in mycoplasma broth without hemin and then plated onto mycoplasma agar plates without hemin (A). Four wells, bored into the agar, acted as reservoirs for hemin solutions containing 0, 40, 80, or 120 ng of hemin (wells 1, 2, 3, and 4, respectively). The inocula from panel A were also plated on mycoplasma agar containing hemin at 5 μg ml^{-1} (B) to confirm the viability.

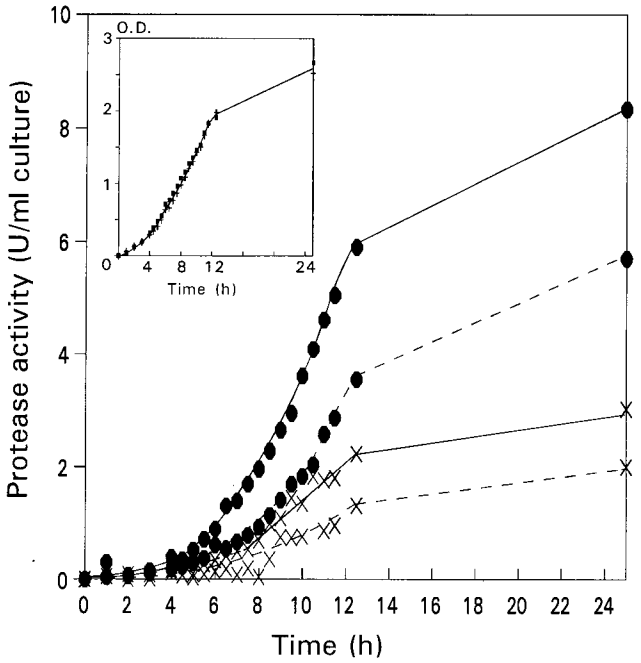

FIG. 9. Total arginine- and lysine-specific protease production by *P. gingivalis* W50 and W50T1 throughout growth. Both strains were grown in BHI broth (5 mg of hemin liter^{-1}), and samples were withdrawn throughout the course of logarithmic growth for Arg-x (●) and Lys-x (×) activity assays of whole-culture sonicates. Solid lines, *P. gingivalis* W50; dotted lines, *tla* strain W50T1. The inset shows optical densities (O.D.) of W50 (■) and W50T1 (+) cultures used for enzyme assays.

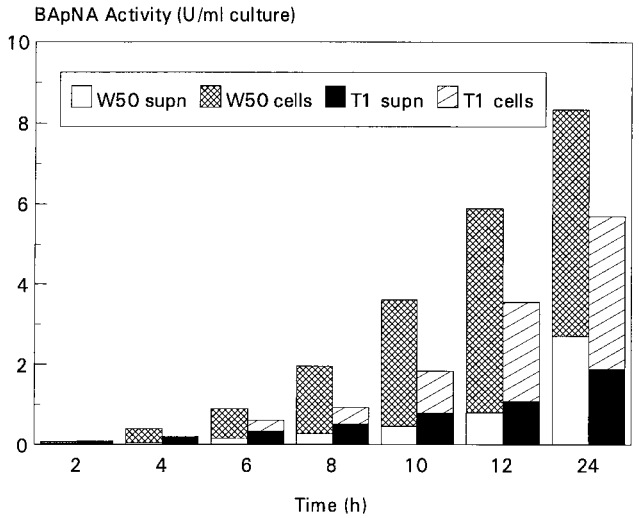

FIG. 10. Distribution of Arg-x enzyme activity between the cell and extracellular compartments throughout the growth of *P. gingivalis* W50 and W50T1. Growth conditions were as for Fig. 9. supn, supernatant.

BApNA activity in the mutant was reduced by 75% compared to the wild-type level, whereas the supernatant activity was about twofold higher. Hence, inactivation of the *tla* locus leads to a reduction in Arg-x protease activity through alteration to enzyme production and/or activation in the cell-associated fraction in the initial stages of exponential growth.

DISCUSSION

Loci homologous to *prpR1*. Data from a number of sources suggest that the gene for the arginine-specific protease of *P. gingivalis*, RI, is a member of a family of sequence-related genes. On Southern blots of *P. gingivalis* genomic DNA, probes designed to the coding region for the α component (catalytic subunit) of RI hybridized to a second locus, additional to *prpR1*, which corresponds to a gene for another arginine-specific protease and which has been designated *prR2* (44) or *rgpB* (34). The Southern hybridization pattern of probes designed to the coding region of the β component, or adhesin subunit, of RI indicated that sequences within this region are represented in at least two other loci (1, 34, 44). Furthermore, database analysis of *P. gingivalis* genes, excluding those for arginine-specific proteases, demonstrates that homologous sequences are also found within *prtP* (2) and *kgp* (accession no. U59691 [36]), both of which may contribute to the Lys-x protease activity of this organism, and *hagA* (accession no. U41807 [18]), which encodes a high-molecular-weight hemagglutinin. In addition to this information at the DNA level, MAb 1A1, which binds to an epitope within the β component of RI, recognizes multiple protein bands on Western blots of *P. gingivalis* whole-cell sonicates, suggesting that the binding determinant of this antibody is present on a number of sequence-related gene products (12).

The ability of MAb 1A1 to block *P. gingivalis* culture supernatant hemagglutination activity suggests that proteins bearing this epitope have an adherence function. Furthermore, blocking experiments using overlapping peptides designed to the RI β component have identified another region of this molecule at PrpRI positions 1083 to 1112 which contains residues which are critical to the culture supernatant hemagglutination process (22a). On the basis of the primary sequence of the β component of RI and studies using purified enzymes (41) and MAbs (12), this polypeptide may have a role in adherence mechanisms of the whole organism or in targeting the action of the catalytic component of proteases to specific host cell surfaces or soluble proteins.

In this report, we describe the cloning and sequence analysis of another member of this family of genes that are linked by their sequence similarity to the RI β component and their immunoreactivity with MAb 1A1. The deduced translation of the *tla* also contains sequences at positions 466 to 495 corresponding to PrpRI positions 1083 to 1112, a region which contain the residues essential for binding to the erythrocyte surfaces. Database comparisons indicate that the N terminus of the product of this gene contains sequence motifs which are found in outer membrane proteins which interact with the cytoplasmic membrane protein, TonB, from other gram-negative bacteria. Hence, we have given the name TonB-linked adhesin to this RI protease homolog.

TonB-dependent proteins. The *tonB* gene of *E. coli* codes for a 26-kDa protein with a hydrophobic sequence at the N terminus which is anchored to the cytoplasmic membrane and two proline-rich regions which are suggested to form a rod-like structure which spans the periplasmic space (42). In the cytoplasmic membrane, TonB is thought to form a complex with two other membrane proteins, ExbB and ExbD (3). TonB-like proteins with similar structural features have been found in many other gram-negative organisms, including members of the genera *Salmonella*, *Serratia*, *Yersinia*, *Klebsiella*, *Vibrio*, and *Pseudomonas* (for a recent review, see reference 7). TonB is involved in the active transport of solutes across the outer membrane via a process which is mediated at the surface of the cell by specific transport proteins, known as TonB-linked receptors. In this way, the TonB-ExbB-ExbD complex acts as a conduit for energy flow from the cytoplasm to the cell surface, with concomitant import of a specific ligand.

Substrates for TonB-linked receptors in *E. coli* include ferric siderophores, vitamin B_{12}, and group B colicins (42). In addition, outer membrane receptors for transferrin (9, 10, 26), lactoferrin (4), and hemoglobin (52) with significant primary sequence similarity to TonB-dependent proteins have been found in *Neisseria* species, and the acquisition of protein-bound hemin has been shown to be a TonB-dependent process in *H. influenzae* (21).

Furthermore, it has recently been suggested that in addition to having a role in energy transduction across the periplasmic space, the TonB-ExbB-ExbD complex and associated membrane receptors may provide a mechanism for signal transduction from the outer membrane to the cytoplasm. For example, transcription of the genes for the outer membrane ferric siderophore receptor, PupB, in *Pseudomonas putida* (24) and the outer and cytoplasmic membranes transport proteins of ferric citrate in *E. coli* (19) can be initiated solely by ligand interactions with TonB-dependent receptors at the cell surface.

Role of the TonB-linked adhesin of *P. gingivalis*. The general properties of TonB-dependent proteins suggested that the Tla of *P. gingivalis* may be required for the recognition and transport of an essential nutrient(s) possibly related to iron acquisition and/or as a means of environmental sensing. The iron requirements for the growth of *P. gingivalis* can be provided not only by free hemin but also by the heme binding proteins hemoglobin, hemopexin, lactoperoxidase, and catalase and the iron binding proteins transferrin, lactoferrin, and serum albumin (5). The growth experiments described in the present report demonstrated that insertional inactivation of *tla* compromised the ability of the organism to grow in medium containing low concentrations of hemin but had no effect on

growth in media where organic compounds provided the sole iron and hemin sources. This was shown to be the case both in liquid broth and on solid media. These data therefore strongly suggest that *tla* is involved in the acquisition or utilization of limiting concentrations of hemin, perhaps by functioning as a TonB-linked receptor to facilitate uptake of this important iron source. By analogy, utilization of low concentrations of free hemin by both *H. influenzae* and *Y. enterocolitica* is a TonB-dependent process (21, 51).

A number of *P. gingivalis* outer membrane hemin binding proteins and proteins which are expressed only under hemin-limiting growth conditions have been described elsewhere (6, 50). One of these studies (50) described an outer membrane protein of 118 kDa which was expressed only by cells grown in continuous culture under hemin limitation. In the present work, we repeated these studies by using chemostat-grown *P. gingivalis* W50 cells (provided by P. D. Marsh, CAMR, Porton, Salisbury, England) and were able to confirm the expression of a novel protein of this molecular weight during hemin-limited growth. However this protein is not reactive with MAb 1A1, suggesting that it is not Tla (not shown). Moreover, we have been unable to identify unambiguously the Tla in outer membranes from *P. gingivalis* cells grown continuously in hemin excess conditions or in batch culture because of the expression of multiple proteins immunoreactive with MAb 1A1 in the size range of 20 to 120 kDa under these conditions. Northern analyses of mRNA have so far failed to reveal the presence of a *tla* transcript in cells grown under any growth condition. This may reflect the instability of the mRNA as we have previously reported for the *prpR1* transcript of *P. gingivalis* (44). Alternatively, low levels of mRNA and protein expression may indicate that Tla is a minor constituent of the total protein pool in *P. gingivalis* W50, which would not be inconsistent with a role in signalling and regulation. Reporter gene expression studies with *tla* are in progress to determine the regulatory influences on expression of this gene.

Hemin is recognized as an important virulence regulator of *P. gingivalis*. For example, cells grown in continuous culture under hemin-limiting conditions have been shown to be less virulent in animal models of soft tissue destruction than cells grown at hemin excess (29, 31), and this finding correlates with lower levels of hydrolytic enzyme production by hemin-limited cultures (30). It was therefore of interest to note that the *tla* mutant produced significantly lower levels of both arginine- and lysine-specific protease activity than the parent *P. gingivalis* W50. There may be a number of explanations for this reduction, relating either to the ability of the mutant strain to respond to external hemin levels or to an inability to accumulate and process sufficiently high concentrations of this regulator intracellularly. However, while the mechanism requires further investigation, this observation does suggest a regulatory link between *tla* and other members of this sequence-related family of genes that may play an important role in the virulence potential of the organism.

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REFERENCES

1. **Aduse-Opoku, J., J. Muir, J. M. Slaney, M. Rangarajan, and M. A. Curtis.** 1995. Characterisation, genetic analysis and expression studies of a protease antigen (PrpRI) of *Porphyromonas gingivalis*. Infect. Immun. **63:**4744–4754.
2. **Barkocy-Gallagher, G. A., N. Han, J. M. Patti, J. Whitlock, A. Progulske-Fox, and M. S. Lantz.** 1996. Analysis of the *prtP* gene encoding porphypain, a cysteine proteinase of *Porphyromonas gingivalis*. J. Bacteriol. **178:**2734–2741.
3. **Bell, P. M., C. D. Nau, J. T. Brown, J. Konisky, and R. J. Kadner.** 1990. Genetic suppression demonstrates interaction of TonB protein with outer membrane transport proteins in *Escherichia coli*. J. Bacteriol. **172:**3826–3829.
4. **Biswas, G. D., and P. F. Sparling.** 1995. Characterisation of *lbpA*, the structural gene for a lactoferrin receptor in *Neisseria gonorrhoeae*. Infect. Immun. **63:**2958–2967.
5. **Bramanti, T. E., and S. C. Holt.** 1991. Roles of porphyrins and host iron transport proteins in regulation of growth of *Porphyromonas gingivalis* W50. J. Bacteriol. **173:**7330–7339.
6. **Bramanti, T. E., and S. C. Holt.** 1990. Iron-regulated outer membrane proteins in the periodontopathic bacterium, *Bacteroides gingivalis*. Biochem. Biophys. Res. Commun. **166:**1146–1154.
7. **Braun, V.** 1995. Energy-coupled transport and signal transduction through the Gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. FEMS Microbiol. Rev. **16:**295–307.
8. **Carman, R. J., M. D. Kamakrishnan, F. H. Harper.** 1990. Hemin levels in culture medium of *Porphyromonas gingivalis* regulate both hemin binding and trypsin-like protease production. Infect. Immun. **58:**4016–4019.
9. **Cornelissen, C. N., G. D. Biswas, J. Tsai, D. K. Paruchuri, S. A. Thompson, and P. F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilisation and is homologous to TonB-dependent outer membrane receptors. J. Bacteriol. **174:**5788–5797.
10. **Cornelissen, C. N., and P. F. Sparling.** 1994. Iron piracy: acquisition of transferrin-bound iron by bacterial pathogens. Mol. Microbiol. **14:**843–850.
11. **Cridland, J. C., V. Booth, F. P. Ashley, M. A. Curtis, R. F. Wilson, and P. Shepherd.** 1994. Preliminary characterisation of antigens recognised by monoclonal antibodies raised to *Porphyromonas gingivalis* and by sera of patients with periodontitis. J. Periodontal. Res. **29:**339–347.
12. **Curtis, M. A., J. Aduse-Opoku, J. M. Slaney, M. Rangarajan, V. Booth, J. Cridland, and P. Shepherd.** 1996. Characterization of an adherence and antigenic determinant of the RI protease of *Porphyromonas gingivalis* which is present on multiple gene products. Infect. Immun. **64:**2532–2539.
13. **Dame, J. B., J. L. William, T. F. McCutchan, J. L. Weber, R. A. Wirtz, W. T. Hockmeyer, W. L. Maloy, J. D. Haynes, I. Schneider, D. Roberts, G. S. Sanders, E. P. Reddy, C. L. Diggs, and L. H. Miller.** 1984. Structure of the gene encoding the immunodominant surface-antigen on the sporozoite of the human malarial parasite *Plasmodium falciparum*. Science **225:**593–599.
14. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acid. Res. **12:**387–395.
15. **Ebersole, J. L., M. A. Taubman, D. J. Smith, and D. E. Frey.** 1986. Human immune responses to microorganisms: patterns of systemic antibody levels to *Bacteroides* spp. Infect. Immun. **51:**507–513.
16. **Fletcher, H. M., H. A. Schenkein, R. M. Morgan, K. A. Bailey, C. R. Berry, and F. L. Macrina.** 1995. Virulence of *Porphyromonas gingivalis* W83 mutant defective in the *prtH* gene. Infect. Immun. **63:**1521–1528.
17. **Goldberg, M. B., S. A. Boyko, J. R. Butterton, J. A. Stoebner, S. M. Payne, and S. B. Calderwood.** 1992. Characterisation of a *Vibrio cholerae* virulence factor homologous to the family of TonB-dependent proteins. Mol. Microbiol. **6:**2407–2418.
18. **Han, N., J. Whitlock, and A. Progulske-Fox.** 1996. The hemagglutinin gene (*hagA*) of *Porphyromonas gingivalis* 381 contains four large, contiguous, direct repeats. Infect. Immun. **64:**4000–4007.
19. **Härle, C., I.-S. Kim, A. Angerer, and V. Braun.** 1995. Signal transfer through three compartments: transcription initiation of the *Escherichia coli* ferric citrate transport system from the cell surface. EMBO J. **14:**1430–1438.
20. **Izard, J. W., and A. A. Kendall.** 1994. Signal peptides: exquisitely designed transport promoters. Mol. Microbiol. **13:**765–773.
21. **Jarosik, G. P., J. D. Sanders, L. D. Cope, U. Muller-Eberhard, and E. J. Hansen.** 1994. A functional *tonB* gene is required for both utilisation of heme and virulence expression by *Haemophilus influenzae* type b. Infect. Immun. **62:**2470–2477.
22. **Kadowaki, T., M. Yoneda, K. Okamoto, K. Maeda, and K. Yamamoto.** 1994. Purification and characterization of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal-disease from the culture supernatant of *Porphyromonas gingivalis*. J. Biol. Chem. **269:**21371–21378.

22a. **Kelly, C. G., V. Booth, H. Kendal, J. Aduse-Opoku, J. Slaney, M. A. Curtis, and T. Lehner.** Colonization and haemagglutination inhibiting epitopes of *Porphyromonas gingivalis*. Clin. Exp. Immunol., in press.

23. **Kirszbaum, L., C. Sotiropoulos, C. Jackson, S. Cleal, N. Slakeski, and E. Reynolds.** 1995. Complete nucleotide sequence of a gene *prt*R of *Porphyromonas gingivalis* W50 encoding a 132 kDa protein that contains an arginine-specific thiol endopeptidase domain and a haemagglutinin domain. Biochem. Biophys. Res. Commun. **207:**424–431.
24. **Koster, M., W. van Klompenburg, W. Bitter, J. Leong, and P. Weisbeek.** 1994. Role for the outer membrane ferric siderophore receptor PupB in signal transduction across the bacterial cell envelope. EMBO J. **13:**2805–2813.

25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
26. **Legrain, M., V. Marzarin, S. W. Irwin, B. Bouchon, M.-R. Quentin-Millet, E. Jacobs, and A. B. Schryvers.** 1993. Cloning and characterisation of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. Gene **130:**81–90.
27. **Maley, J., and I. S. Roberts.** 1994. Characterisation of IS1126 from *Porphyromonas gingivalis* W83: a new member of the IS4 family of insertion sequence elements. FEMS Microbiol. Lett. **123:**219–224.
28. **Markham, P. F., M. D. Glew, K. G. Whithear, and I. D. Walker.** 1993. Molecular cloning of a member of the gene family that encodes pMGA, a hemagglutinin of *Mycoplasma gallisepticum*. Infect. Immun. **61:**903–909.
29. **Marsh, P. D., A. S. McDermid, A. S. McKee, and A. Baskerville.** 1994. The effect of growth rate and haemin on the virulence and proteolytic activity of *Porphyromonas gingivalis* W50. Microbiology **140:**861–865.
30. **Marsh, P. D., A. S. McKee, and A. S. McDermid.** 1988. Effect of haemin on enzyme activity and cytotoxin production by *Bacteroides gingivalis* W50. FEMS Microbiol. Lett. **55:**87–92.
31. **McKee, A. S., A. S. McDermid, A. Baskerville, A. B. Dowsett, D. C. Ellwood, and P. D. Marsh.** 1986. Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50. Infect. Immun. **52:**349–355.
32. **Millar, D. J., E. E. Scott, J. M. Slaney, S. U, P. Benjamin, and M. A. Curtis.** 1993. Production and characterisation of monoclonal antibodies to the principle sonicate antigens of *Porphyromonas gingivalis* W50. FEMS Immunol. Med. Microbiol. **7:**211–222.
33. **Moncla, B. J., P. Braham, L. K. Rabe, and S. L. Hillier.** 1991. Rapid presumptive identification of black-pigmented gram-negative anaerobic bacteria by using 4-methylumbelliferone derivatives. J. Clin. Microbiol. **29:**1955–1958.
34. **Nakayama, K., T. Kadowaki, K. Okamoto, and K. Yamamoto.** 1995. Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of *Porphyromonas gingivalis*. J. Biol. Chem. **270:** 23619–23626.
35. **Nishikata, M., and F. Yoshimura.** 1991. Characterization of *Porphyromonas* (*Bacteroides*) *gingivalis* hemagglutinin as a protease. Bioch. Biophys. Res. Commun. **178:**336–342.
36. **Okamoto, K., T. Kadowaki, K. Nakayama, and K. Yamamoto.** 1996. Cloning and sequencing of the gene encoding a novel lysine-specific cysteine proteinase (lys-gingipain) in *Porphyromonas gingivalis*: structural relationship with the arginine-specific cysteine proteinase (arg-gingipain). J. Biochem. **120:** 398–406.
37. **Okamoto, K., Y. Misumi, T. Kadowaki, M. Yoneda, K. Yamamoto, and Y. Ikehara.** 1995. Structural characterisation of argingipain, a novel arginine-specific cysteine proteinase as a major periodontal pathogenic factor from *Porphyromonas gingivalis*. Arch. Biochem. Biophys. **316:**917–925.
38. **Oppenheim, D. S., and C. Yanofsky.** 1980. Translational coupling during expression of the tryptophan operon of *Escherichia coli*. Genetics **95:**785–795.
39. **Pavloff, N., J. Potempa, R. N. Pike, V. Prochazka, M. C. Kiefer, J. Travis, and P. J. Barr.** 1995. Molecular cloning and structural characterisation of the Arg-gingipain proteinase of *Porphyromonas gingivalis*. Biosynthesis as a proteinase-adhesin polyprotein. J. Biol. Chem. **270:**1007–1010.
40. **Pike, R., W. McGraw, J. Potempa, and J. Travis.** 1994. Lysine-specific and arginine-specific proteinases from *Porphyromonas gingivalis*: isolation, characterization and evidence for the existence of complexes with hemagglutinins. J. Biol. Chem. **269:**406–411.
41. **Pike, R. N., J. Potempa, W. McGraw, T. H. Coetzev, and J. Travis.** 1996. Characterisation of the binding activities of proteinase-adhesin complexes from *Porphyromonas gingivalis*. J. Bacteriol. **178:**2876–2892.
42. **Postle, K.** 1990. TonB and the Gram-negative dilemma. Mol. Microbiol. **4:**2019–2025.
43. **Potempa, J., R. Pike, and J. Travis.** 1995. The multiple forms of trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of either Arg-gingipain or Lys-gingipain. Infect. Immun. **63:**1176–1182.
44. **Rangarajan, M., J. Aduse-Opoku, J. M. Slaney, K. A. Young, and M. A. Curtis.** 1997. The *prpR1* and *prR2* arginine-specific protease genes of *Porphyromonas gingivalis* W50 produce five biochemically distinct enzymes. Mol. Microbiol. **23:**955–965.
45. **Rangarajan, M., S. J. M. Smith, S. U, and M. A. Curtis.** 1997. Biochemical characterisation of the arginine-specific proteases of *Porphyromonas gingivalis* W50 suggests a common precursor. Biochem. J. **323:**701–709.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Scott, C. F., E. J. Whitaker, B. F. Hammond, and R. W. Colman.** 1993. Purification and characterization of a potent 70-kDa thiol lysyl-proteinase (Lys-gingivain) from *Porphyromonas gingivalis* that cleaves kininogens and fibrinogen. J. Biol. Chem. **268:**7935–7942.
48. **Shine, J., and L. Dalgarno.** 1974. The 3 prime-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosomal binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
49. **Slots, J., L. Bragd, M. Wikström, and G. Dahlén.** 1986. The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in destructive periodontal disease in adults. J. Periodontol. **13:** 576–577.
50. **Smalley, J. W., A. J. Birss, A. S. McKee, and P. D. Marsh.** 1993. Haemin-binding proteins of *Porphyromonas gingivalis* W50 grown in a chemostat under haemin-limitation. J. Gen. Microbiol. **139:**2145–2150.
51. **Stojiljkovic, I., and K. Hantke.** 1992. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in Gram-negative bacteria. EMBO J. **11:**4359–4367.
52. **Stojiljkovic, I., V. Hwa, L. D-S. Martin, P. O'Goara, X. Nassif, F. Heffron, and M. So.** 1995. The *Neisseria meningitidis* haemoglobin receptor: its role in the iron utilisation and virulence. Mol. Microbiol. **15:**531–541.
53. **Tomkinson, A. E., N. F. Totty, M. Ginsburg, and T. Lindahl.** 1991. Location of the active site for enzyme-adenylate formation in DNA ligases. Proc. Natl. Acad. Sci. USA **88:**400–404.
54. **von Heijne, G.** 1985. Structural and thermodynamic aspects of the transfer of proteins into and across membranes. Curr. Top. Membr. Transp. **24:**151–179.
55. **Westerlund, B., and T. K. Korhonen.** 1993. Bacterial proteins binding to mammalian extracellular matrix. Mol. Microbiol. **9:**687–694.
56. **Williamson, M. P.** 1994. The structure and function of proline-rich regions in proteins. Biochem. J. **297:**249–260.
57. **Wingrove, J. A., R. G. DiScipio, Z. Chen, J. Potempa, J. Travis, and T. E. Hugli.** 1992. Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas* (*Bacteroides*) *gingivalis*. J. Biol. Chem. **267:**18902–18907.
58. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.