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Expression of the *tpr* Protease Gene of *Porphyromonas gingivalis* Is Regulated by Peptide Nutrients

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The Tpr protease of *Porphyromonas gingivalis* W83 is a membrane-associated enzyme capable of hydrolyzing chromogenic substrates for trypsin and bacterial collagenases. A previous study by us indicated that Tpr expression was increased under conditions of nutrient limitation. In the present study, we further characterized expression of the *tpr* gene using a *tpr*::*lacZ* reporter gene construct under a range of nutrient conditions. In *P. gingivalis*, transcription of *tpr* was initiated 215 bp upstream of the coding region and regulation of *tpr* expression was at the level of transcription. Deletion mutations in the *tpr* upstream region identified the promoter region immediately upstream of the transcription start site, determined by primer extension analysis. Three identical 17-bp direct repeats identified within the 5′ end of *tpr* mRNA were involved in *tpr* regulation. In an *Escherichia coli* background, *tpr* transcription was initiated after an AT-rich region upstream of *tpr* but not at the *P. gingivalis* start site. Tpr expression in *P. gingivalis* was suppressed by the addition of peptide and protein nutrients to a peptide-limited growth medium but was only slightly affected by addition of free amino acids. Low-molecular-weight fractions of brain heart infusion rich in phenylalanine, proline, and alanine had the greatest inhibitory effects on expression of the *tpr*::*lacZ* construct. Addition of the dipeptide phenylalanyl-phenylalanine to the growth medium resulted in a 10-fold decrease in *tpr* expression. This suggests that specific phenylalanine-containing peptides are a major factor controlling Tpr expression. Neither hemin starvation, heat shock, nor pH change had significant effects on Tpr expression.

Bacterial growth in nature is affected by environmental conditions such as the availability of essential nutrients and cofactors, the accumulation of toxic metabolites, and changes in pH, E_h, or temperature. *Porphyromonas gingivalis* responds to environmental changes by modifying its physiology and expression of potential virulence factors. These responses include induction of expression of DnaK and GroEL homologues by heat stress (21), as well as changes in growth rate and specific enzymatic activities in response to changes in pH (28) and the availability of hemin (25, 42) and collagen (30, 38).

P. gingivalis possesses numerous distinct proteases and peptidases, many of which are membrane associated or secreted (2, 9, 12, 22, 32). This finding is consistent with its peptide- and amino-acid-dependent metabolism (39). This proteolytic activity is recognized as an important virulence factor in periodontal diseases (15). Characterization of one of these proteases, the membrane-associated Tpr protease, indicated that it was a thiol-dependent protease whose proteolytic activity is activated by reducing conditions (5, 33). Tpr activity was significantly increased in cells cultured under nutrient-limited conditions, suggesting that expression of Tpr was regulated (34). In an analysis of the collagenase-like Pz-peptidase activity of Tpr, the membrane fraction of *P. gingivalis* W83 cells grown in Trypticase-yeast extract (TYE) medium in which the Tryticase Peptone content was reduced from 17 to 5 mg/ml (34) (0.5TYE) had twice the Pz-peptidase activity of cells grown in TYE and five times the activity of cells grown in brain heart infusion (BHI). Northern blot analysis suggested that the regulation of *tpr* expression occurred primarily at the transcriptional level (34).

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The mechanisms of gene regulation and expression in this organism are not well understood, and little is known about the promoter structures of *P. gingivalis* genes. One study suggested that the RNA polymerase of *P. gingivalis* was structurally different from that of *Escherichia coli* (19). Putative promoter sequences of a number of cloned *P. gingivalis* genes have been identified, based only on their limited homology with the consensus sequences of *E. coli* promoters and without evidence of their promoter activity in *P. gingivalis*. To better understand the structures of *P. gingivalis* genes and their regulation, it is necessary to analyze native regulatory sequences.

Our previous study showing that Tpr peptidolytic activity (Pz-peptidase) and *tpr* mRNA expression were influenced by nutrient conditions (34) prompted us to analyze the noncoding region directly upstream of the *tpr* locus. The gene encoding the Tpr protease has been cloned and sequenced and consists of an open reading frame of 1,446 nucleotides (5, 33). The finding that the *tpr* protease gene was regulated by growth conditions provided a model for studying gene regulation in this important periodontopathogen. This model is especially relevant to the study of *P. gingivalis* virulence genes, since membrane-associated and extracellular proteases of this organism are recognized as key to its role in periodontitis (15). In the present study, we determine the transcriptional start site of the *tpr* gene, analyze the *tpr* promoter, and using a *tpr*::*lacZ* reporter gene, characterize *tpr* regulation by quantitating β-galactosidase activity in *P. gingivalis* transconjugants.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. gingivalis* W83 was grown anaerobically in BHI broth (33) or TYE broth (11). Nutrient-limited medium was 0.5TYE (34). Cultures were incubated in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) in a 5% CO_2–10% H_2–85% N_2 atmosphere at 37°C. *P. gingivalis* transconjugants were selected on BHI blood agar plates containing gentamicin (200 μg/ml) and erythromycin (10 μg/ml) or tetracycline (10 μg/ml) as described previously (34). *E. coli* was grown in Luria-Bertani (LB) broth (3)

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
P. gingivalis strains		
W83	Wild type, Gm^r	Lab collection
W83/PM	*P. gingivalis* W83 *tpr* mutant, Em^r	32
W83/*lacZ*	*P. gingivalis* W83 with *tpr*::*lacZ* fusion, Em^r	This study
Plasmids		
pTZ19R	Ap^r	43
pBluescript II SK(−)	Ap^r	Stratagene
pYS307	Ap^r, *tpr* cloned in pTZ18R	33
pXCA601	Promoterless *lacZ* gene, Tc^r	1
pBLU-1	*Bam*HI-*Pst*I *tpr* fragment of pBY307 cloned into pBluescript II SK(−)	This study
pBX-1	*Bam*HI-*Hin*dIII 3.5-kb *lacZ* fragment of pXCA601 ligated to *Eco*RI- and *Hin*dIII-digested pBLU-1	This study
R751	Tp^r Tra^+ IncP plasmid mobilizes vectors from *E. coli* to *Bacteroides* recipients	40
pBY2-IN	Km^r Em^r, *tpr* insertional mutation	32
pBYZ	*Bam*HI-*Hin*dIII 4.3-kb *tpr*::*lacZ* fragment of pBX-1 ligated to pBY2-IN	This study
pNJR12	Km^r Sm^r Mob^+ Tc^r, replicable in *P. gingivalis*	23
pNTX	*Pst*I-*Hin*dIII 3.5-kb *lacZ* fragment of pTXZ19R cloned in pNJR12	This study
pNTX-400	*Bam*HI-*Hin*dIII 4.3-kb *tpr*::*lacZ* fragment of pBX-1 cloned in pNJR12	This study
pNTX-100	*Bam*HI-*Hin*dIII 4.0-kb *tpr*::*lacZ* fragment of pTXZ19R-100 cloned in pNJR12	This study
pNTX-30	*Kpn*I-*Hin*dIII 3.93-kb *tpr*::*lacZ* fragment of pTXZ19R-30 cloned in pNJR12	This study
pNTX20	*Bam*HI-*Hin*dIII 3.88-kb *tpr*::*lacZ* fragment of pTXZ19R20 cloned in pNJR12	This study
pNTX52	*Kpn*I-*Hin*dIII 3.85-kb *tpr*::*lacZ* fragment of pTXZ19R52 cloned in pNJR12	This study
pNTX137	*Bam*HI-*Hin*dIII 3.76-kb *tpr*::*lacZ* fragment of pTXZ19R137 cloned in pNJR12	This study
pNTX-100Δ	*Eco*RI-*Hin*dIII 3.9-kb *tpr*::*lacZ* fragment of pTXZ19R-100Δ cloned in pNJR12	This study
pNTX-30Δ	*Kpn*I-*Hin*dIII 3.81-kb *tpr*::*lacZ* fragment of pTXZ19R-30Δ cloned in pNJR12	This study
pTXZ19R	*Pst*I-*Hin*dIII 3.5-kb *lacZ* of pBX-1 cloned in pTZ19R	This study
pTXZ19R-400	*Bam*HI-*Hin*dIII 4.3-kb *tpr*::*lacZ* fragment of pBX-1 cloned in pTZ19R	This study
pTXZ19R-100	*Bam*HI-*Pst*I 500-bp PCR product XZ-100 cloned in pTXZ19R	This study
pTXZ19R-30	*Kpn*I-*Pst*I 430-bp PCR product XZ-30 cloned in pTXZ19R	This study
pTXZ19R20	*Bam*HI-*Pst*I 380-bp PCR product XZ20 cloned in pTXZ19R	This study
pTXZ19R52	*Kpn*I-*Pst*I 348-bp PCR product XZ52 cloned in pTXZ19R	This study
pTXZ19R137	*Bam*HI-*Pst*I 263-bp PCR product XZ137 cloned in pTXZ19R	This study
pTXZ19R-100Δ	*Eco*RI-*Bam*HI 120-bp PCR product XZ-100Δ cloned in pTXZ19R137	This study
pTXZ19R-30Δ	*Kpn*I-*Bam*HI 50-bp PCR product XZ-30Δ cloned in pTXZ19R137	This study

[a] Sm^r, Tp^r, and Ap^r, resistance to streptomycin, trimethoprim, and ampicillin, respectively. Mob^+, capable of being mobilized; Tra^+, capable of self-transfer.

or minimal A medium (3). *E. coli* XL-1 Blue (Stratagene, La Jolla, Calif.) was used as the host for plasmid construction and for some expression studies. For selection purposes, ampicillin (50 μg/ml), kanamycin (50 μg/ml), trimethoprim (200 μg/ml), and tetracycline (10 μg/ml), as appropriate, were used unless stated otherwise. Stocks of bacteria were stored at −70°C in 15% glycerol. Bacterial strains and plasmids used in this study are listed in Table 1.

Primer extension analysis and DNA sequencing. Total RNA was isolated from *P. gingivalis* and *E. coli* strains with TRIzol Reagent (Gibco BRL, Gaithersburg, Md.) by the manufacturer's protocol. To map the 5′ terminus of *tpr* mRNA in *P. gingivalis* and *E. coli*, primer extension analysis was conducted as described previously (3). Three primers, tpr293, tpr170, and tpr64 (Table 2), were used for primer extension analysis. The primers were labeled with [γ-^{32}P]ATP as described previously (3). Hybridization and primer extension were carried out as described previously (3) with Avian myoblastosis virus reverse transcriptase (Gibco BRL). Primer extension products were heated for 2 min at 95°C before being loaded on a sequencing gel. Dideoxy sequencing reaction mixtures with the same primer were run alongside the primer extension products. DNA sequencing reaction experiments were conducted with Sequenase version 2.0 DNA polymerase by following the protocols provided by the manufacturer (United States Biochemicals, Cleveland, Ohio).

Recombinant DNA methods. Plasmid DNA was isolated by the alkaline lysis method (3). Chromosomal DNA was extracted from bacterial cells grown to early stationary phase by a miniprep method (3). Restriction enzyme digestion of DNA samples was carried out according to the manufacturer's recommendations. Subcloning of DNA fragments and PCR products was done by restriction digestion and electrophoresis of agarose gels which were prepared with and run in a Tris-acetate-EDTA buffer (40 mM Tris · acetate, 2 mM $Na_2 \cdot EDTA \cdot 2H_2O$ [pH 8.5]). The desired DNA fragments were excised from the gel and recovered by Glass milk purification as described by the manufacturer (GeneClean kit; Bio 101, Inc., La Jolla, Calif.). Transformation of *E. coli* was done by electroporation (3), except that mobilization plasmid R751 was introduced into *E. coli* strains by conjugation, as described previously (32). Both replicating and nonreplicating plasmids were introduced into *P. gingivalis* from *E. coli* by conjugation (32). Transconjugants were grown on BHI-blood agar containing antibiotics. Gentamicin was used to inhibit growth of *E. coli* donor cells, and erythromycin or tetracycline was used to select for *P. gingivalis* transconjugants containing a chromosomally integrated erythromycin resistance (Em^r) gene or a plasmid-borne tetracycline resistance (Tc^r) gene. Transconjugants were passaged twice on BHI-blood agar containing antibiotics before analysis.

PCR and primers. To analyze the effects of deletion mutations on the expression of *tpr*, fragments containing the *tpr* 5′ end and upstream regions were generated by PCR with pYS307 DNA as a template (33). Primers used for PCR amplification are shown in Table 2. DNA fragment XZ-100 was generated with primers Bm300 and tprPst, fragment XZ-30 was generated with primers Kpn365 and tprPst, fragment XZ20 was generated with primers Bm200 and tprPst, fragment XZ52 was generated with primers Kpn452 and tprPst, and fragment XZ137 was generated with primers Bm100 and tprPst. Fragment XZ-100Δ was generated with primers Eco300 and Bm424, and fragment XZ-30Δ was generated with primers Kpn452 and Bm424. PCR amplification was carried out with *Taq*I polymerase (Gibco BRL). Twenty-five cycles were carried out at a denaturing temperature of 95°C for 1 min, an annealing temperature of 55°C for 1 min, and an extension temperature of 72°C for 1.5 min in a model 480 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.).

Construction of *tpr*::*lacZ* fusion plasmids. Plasmids pNJR12 (23) and pTZ19R (43) were used as vectors for construction of *tpr*::*lacZ* reporter plasmids for analysis in *P. gingivalis* and *E. coli*, respectively. To construct a *tpr*::*lacZ* reporter cassette, the 0.8-kb *Bam*HI-*Pst*I fragment of pYS307 (33) containing DNA upstream of *tpr* and the first 183 bp of the *tpr* coding region was first ligated to pBluescript II SK(−) (Stratagene) which had been digested with the same enzymes, yielding pBLU-1. Then, as shown in Fig. 1, the 3.5-kb *Bam*HI-*Hin*dIII *lacZ* fragment of pXCA601 (1) was ligated to pBLU-1, yielding pBX-1, in which the promoterless *lacZ* gene was immediately preceded by the 5′ end of *tpr* and its upstream region. The 4.3-kb *Bam*HI-*Hin*dIII fragment of pBX-1 was cloned into the corresponding sites of both pTX19R and pNJR12, yielding pTXZ19R-400 and pNTX-400, respectively.

To construct a series of deletions in the region upstream of *tpr* for analysis in the *lacZ* reporter system, a *Pst*I-*Hin*dIII fragment of pBX-1 containing the promoterless *lacZ* gene was first ligated to pTZ19R (43), yielding pTXZ19R.

TABLE 2. Oligonucleotide primers used to amplify *tpr* upstream sequences

Primer	Nucleotide sequence[a]	Location (nt)[b]
Bm300	5′-ATTCGGATCCTCGGGTCTCGTCTG-3′ *Bam*HI	291–314
Kpn365	5′-TTCAGGTACCAATTGTCAATT-3′ *Kpn*I	360–380
Bm200	5′-TAATGGATCCTAACGGTTTTTCATGC-3′ *Bam*HI	410–435
Kpn452	5′-AAATGGTACCTTAATTCG-3′ *Kpn*I	447–464
Bm100	5′-CCTTAATGGATCCCTTCATTTGTG-3′ *Bam*HI	529–552
Eco300	5′-CACGAATTCGGCTGTTCG-3′ *Eco*RI	286–303
Bm424	5′-CGTTAGGATCCATTATTTCAA-3′ *Bam*HI	424–404
tprPst	5′-CATCCCTGCAGGGCTGC-3′ *Pst*I	801–785
tpr293	5′-GCTTTCGCTTCCTCTTGTTGAGGA-3′	710–687
tpr170	5′-AACTGTGACTTTAGGCTCTTAC-3′	587–566
tpr64	5′-TGTGTACAAAAAAACTAACGAATTA-3′	482–458

[a] New restriction sites engineered into the sequence are indicated.
[b] Numbers correspond to the DNA sequence shown in Fig. 2B. nt, nucleotides.

Subsequently, PCRs were carried out with the primers shown in Table 2, yielding products containing several different *tpr* upstream regions. The PCR products were digested with restriction enzymes whose sites were engineered into their termini, and they were cloned into the corresponding sites on pTXZ19R upstream of the *lacZ* gene (Table 1). For analysis in *P. gingivalis*, the resulting *tpr*::*lacZ* fusions were subsequently cloned in pNJR12 (Table 1).

To construct a suicide plasmid carrying the TX-400 *tpr*::*lacZ* fusion construct, the 4.3-kb *Bam*HI-*Hin*dIII fragment of pBX-1 was ligated to pBY2-IN (32) directly upstream of the *ermF* gene, in the same orientation as and replacing the 5′ end of *tpr* carried on pBY2-IN. The resulting plasmid, pBYZ, carries a promoterless *lacZ* gene and *ermF* in place of a 0.6-kb internal fragment of *tpr*.

Southern blotting. Southern hybridizations were carried out as described previously (32). Hybridization bands were detected by the BluGENE Nonradioactive Nucleic Acid Detection System (Gibco BRL).

Characterization of nutrient factors. Powdered BHI (Difco Laboratories, Detroit, Mich.) was made up as a 20% solution in H_2O, autoclaved, and passaged sequentially through membranes (Amicon Inc., Beverly, Mass.) having molecular weight cutoffs of 50, 10, 5, and 3 kDa. The flowthrough was then fractionated over a Sephadex G-10 column. The amino acid compositions of low-molecular-weight fractions of interest were determined with a model 420 amino acid hydrolyzer and derivatizer (Applied Biosystems, Inc., Foster City, Calif.) at the Nucleic Acids and Protein Sequencing Unit, Biotechnology Laboratory, University of British Columbia. For assays, *P. gingivalis* was grown in 0.5TYE supplemented with individual BHI fractions at a final concentration of 20 mg/ml. The effects of L amino acids (Sigma Chemical Co., St. Louis, Mo.) and di- and tripeptides (BACHEM California, Torrance, Calif.) were determined similarly at concentrations of 1 to 5 mM.

Stress conditions and hemin limitation. For assays of responses to heat shock and pH change, *P. gingivalis* cells were first grown in 0.5TYE to mid-log phase and the cells were transferred to 42°C or were incubated in 0.5TYE at pH 5.5 or 8.5 for 4 h before being assayed. For the hemin starvation response assay, *P. gingivalis* cells at a 1:10 inoculum were grown in 0.5TYE without the supplementation of hemin for two passages.

β-Galactosidase assays. To analyze *P. gingivalis* β-galactosidase activity, *P. gingivalis* strains were grown in various nutritional media to logarithmic-growth phase (optical density at 660 nm = 0.5), unless stated otherwise. Cells were harvested, washed twice with phosphate-buffered saline, and incubated on ice for 10 min in 20 mM Na-*p*-tosyl-L-lysine chloromethyl ketone. Cells were then resuspended in the reporter lysis buffer and analyzed for β-galactosidase activity by a β-galactosidase enzyme assay system (Promega Co., Madison, Wis.) as described by the manufacturer. Assays were done in 96-well microplates, and β-galactosidase activity was measured at 405 nm in a microplate reader (model 3550; Bio-Rad Laboratories, Richmond, Calif.). A standard curve for purified β-galactosidase was prepared for each assay. Protein concentration was measured by using the Bradford reagent supplied by Bio-Rad, with bovine serum albumin (BSA) as the standard. For *P. gingivalis*, 1 U of β-galactosidase activity was equivalent to hydrolysis of 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) min^{-1} mg of total cellular $protein^{-1}$. Assay of β-galactosidase activity in *E. coli* was done as described previously, and β-galactosidase activity was expressed in Miller units (29). At least four independent experiments using triplicate samples were performed for each β-galactosidase assay, and the results were averaged for display as bar graphs.

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence shown in Fig. 2 is AF022499.

RESULTS

Primer extension and sequence analysis of the *tpr* promoter region. The 5′ end of *tpr* mRNA was mapped by primer extension analysis of total RNA isolated from *P. gingivalis* W83. The primer extension product obtained with tpr293 was much larger than expected, suggesting that the transcription initiation site was more than 200 bp upstream of the *tpr* coding region (data not shown). Subsequently, using primer tpr64 (Table 2), we identified the transcription initiation site at two A residues 215 bp upstream of the coding region of *tpr* (Fig. 2). The primer extension product could be detected in *P. gingivalis* cells grown in 0.5TYE but not in cells grown in BHI. This result was consistent with the results of our previous study showing that *P. gingivalis* grown in BHI had no detectable *tpr* mRNA (34).

The nucleotide sequence of the region upstream of *tpr* was determined and is shown in Fig. 2B. The transcription start site determined by primer extension is labeled. Analysis of the region upstream of the transcription start site found no sequences closely resembling the −35 and −10 regions of *E. coli* consensus promoters. Interestingly, the region between the transcription initiation site and the *tpr* coding region contained three identical direct repeats of 17 bp (Fig. 2B).

Analysis of Tpr expression with a *tpr*::*lacZ* reporter gene. To facilitate analysis of *tpr* expression in *P. gingivalis*, we constructed the *tpr*::*lacZ* reporter plasmid pNTX-400, a pNJR12 derivative that replicates in *P. gingivalis*. This plasmid carried the 612-bp upstream region and the first 183 bp of the *tpr* coding sequence fused to a promoterless *lacZ* gene (Fig. 1). The *lacZ* gene lacked the first eight codons of the complete gene and could not be expressed by itself. The *lacZ* gene was fused in frame to the 5′ end of *tpr* so that expression of β-galactosidase was controlled by the *tpr* promoter. Plasmid pNTX-400 was introduced into *P. gingivalis* W83 by conjugation from an *E. coli* donor, and β-galactosidase activity was expressed in W83/pNTX-400 (Fig. 3). β-Galactosidase activity could also be detected in W83/pNTX-400 grown on agar plates containing the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (data not shown). These results indicated that the 612-bp upstream fragment carried sufficient information for initiation of *tpr* expression.

To analyze potential *tpr* regulatory sequences, we constructed plasmids pNTX-100, pNTX-30, pNTX20, pNTX52, pNTX137, pNTX-100Δ, and pNTX-30Δ (Table 1), all of which could replicate in *P. gingivalis*. Each plasmid contained a portion of the sequence upstream of *tpr* and the 5′ end of *tpr* fused in frame to the promoterless *lacZ* gene. We then measured β-galactosidase activity in *P. gingivalis* transconjugants carrying these plasmids (Fig. 4). Further deletions downstream, including deletions of the TX20, TX152, and TX137 constructs carried on pNTX20, pNTX52, and pNTX137, respectively, also resulted in loss of β-galactosidase activity. The promoterless *lacZ* gene in pNTX, which carried no *tpr* DNA, expressed minimal levels of β-galactosidase activity that were unaffected by the nutritional conditions tested (Fig. 4). On the other hand, *P. gingivalis* carrying pNTX-400 or pNTX-100, both of which retained the entire *tpr* promoter region, expressed much higher levels of β-galactosidase activity in 0.5TYE medium than in BHI (Fig. 4). These results were in agreement both with the results of primer extension analysis showing that the *tpr* tran-

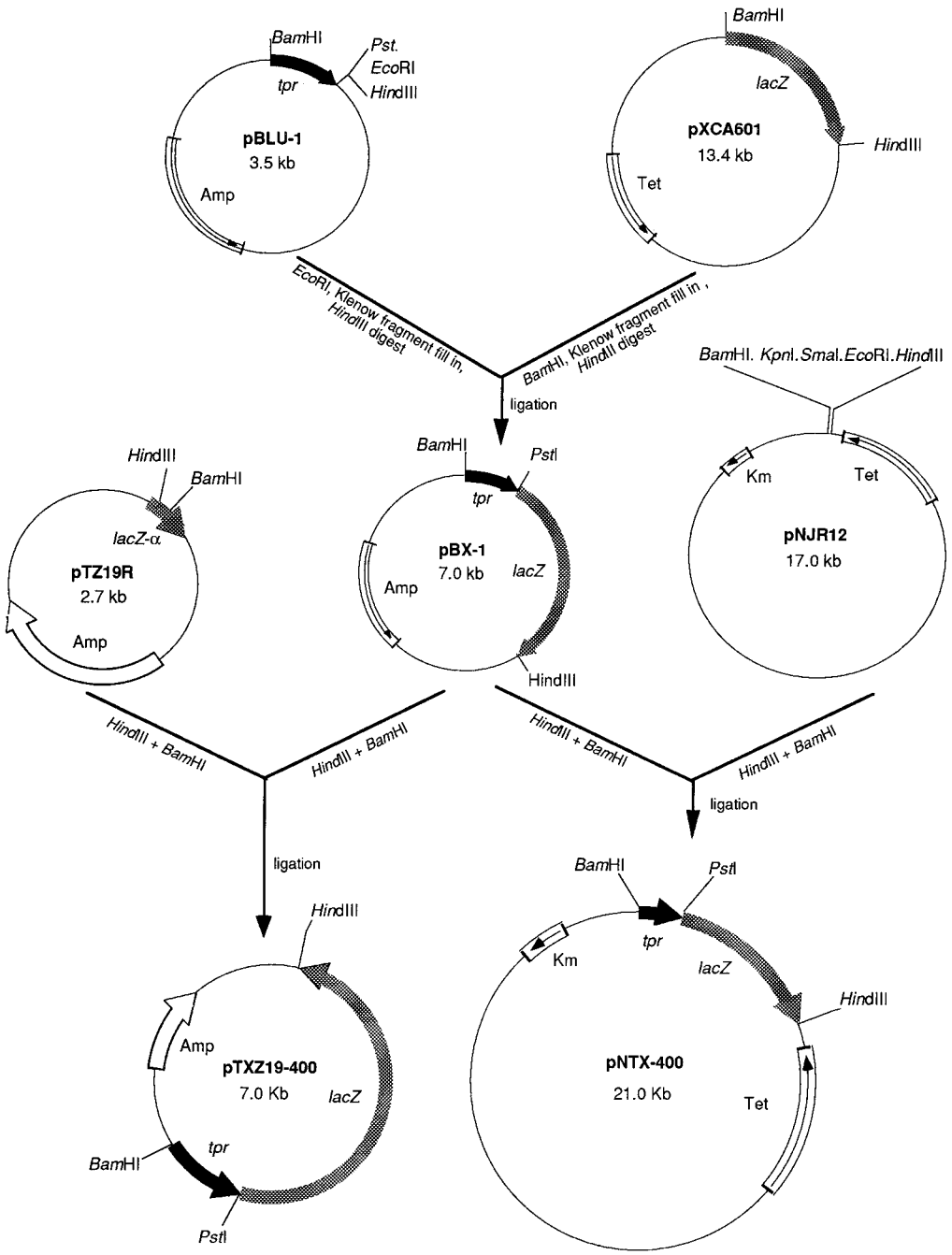

FIG. 1. Construction of *tpr*::*lacZ* fusion plasmids pTXZ19-400 and pNTX-400 for assay of β-galactosidase activity under control of the *tpr* promoter in *E. coli* and *P. gingivalis*, respectively. Relevant restriction enzyme sites are shown. The 0.8-kb fragment containing DNA upstream of *tpr* and the first 183 bp of the *tpr* coding region are shown as a black arrow. The promoterless *lacZ* gene is shown as a gray arrow. Plasmid vector DNAs that encode the alpha fragment of β-galactosidase (*lacZ*-α), ampicillin resistance (Amp), Tc^r (Tet), and kanamicin resistance (Km) are shown by arrows.

script began 215 bp upstream of the coding region and with the regulated expression of *tpr* in the wild-type W83 (34). Deletion of all or part of the putative *P. gingivalis* promoter region (pNTX-30 and pNTX20) abolished β-galactosidase activity in *P. gingivalis* transconjugants. The important role of the three direct repeats in the 5′ region of *tpr* was suggested by pNTX-100Δ, which contained the putative promoter region but lacked the repeat region. In this construct, high levels of β-galactosidase activity were constitutively expressed, with scant evidence of regulation by growth conditions. This result strongly implicated the three direct repeats in control of *tpr* expression.

The effects of the same *tpr*::*lacZ* reporter gene constructs were also analyzed in *E. coli*, with a high-copy-number plasmid vector (Fig. 1). As shown in Fig. 4, β-galactosidase was expressed in *E. coli* cells carrying the TX-400, TX-100, TX-30, TX20, and TX52 constructs on a multicopy plasmid (Table 1). Expression was not enhanced by addition of isopropyl-1-thio-β-D-galactopyranoside, and it was not affected by the orienta-

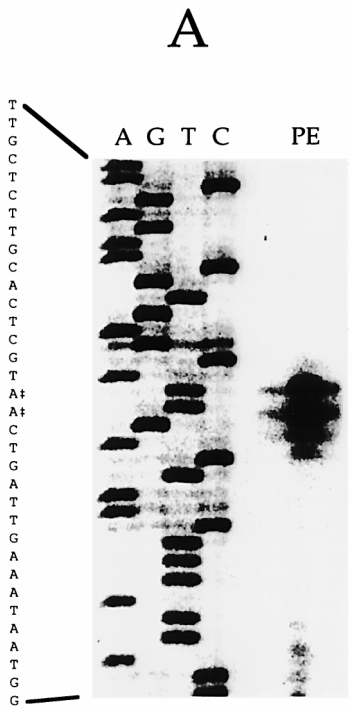

FIG. 2. Identification of the *tpr* promoter and transcription initiation site. (A) Primer extension analysis of *tpr* transcription in *P. gingivalis* W83. A DNA sequence ladder of the promoter region is shown. PE, primer extension reaction product. Oligonucleotide tpr64 was used as the primer for both the DNA sequence and primer extension reactions. Transcription initiation sites (‡) at two A residues are indicated. (B) Nucleotide sequence of the *Bam*HI-*Pst*I fragment containing DNA upstream of the *tpr* gene and the 5′ end of *tpr*. The transcription start site determined by primer extension (‡‡) is shown at nucleotides 398 to 399. Putative −35 and −10 promoter regions are underlined. Three 17-bp direct repeats in the 5′ region of the transcript are indicated by double underlining. An asterisk at nucleotide 545 indicates the *tpr* transcription start site when it is expressed in *E. coli*. The ATG start codon of Tpr at nucleotide 613 is underlined.

B

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BamHI
GGATCCTGCTCCCATAGCATCATCTTGTGATCCCAATGATCTTCAAGCCACTGACATAGCAACTCTTTGATAACACCGAAATCGACTACACGCCCGATCG   100

AATCAAGAGCCGGTGCACTGCAAATAAAGTGGATACGATAGTTGTGTCCATGCAGATGTCTGCACTTGTGATCGTGCCCCACTACGCGGTGTCCCATGCT   200

AATGTCGTGATAACGTTCGGCTGTAATCATATATTGTCAAAGCTAAGTTTCTGCTGTTCAAAGGTAATCAAATAATCCCCTGCTTCACGTATTCGGCTGT   300

                                                    -35                  -10        ‡‡
TCGGGTCTCGTCTGAAAAACATTTTTGTAATTTTTTGAAACACTCTTCAGACACACATTTTCAGGTGAAAATTGTCAATTTTTGCTCTTGCACTCGTAAC   400

TGATTGAAATAATGGCTGTTAACGGTTTTTCATGCGTGATTTGTCCAAATTGCACCTTAATTCGTTAGTTTTTTTGTACACAAATGCGTGATTTGTCCAA   500

                                *
GAAAATGCGTGATTTGTCCAAGTCTTTTCCTTAATATATCCCTTCATTTGTGTGACTCTTAAAGTGTAAGAGCCTAAAGTCACAGTTTTAATCAATCTAA   600

AATTTTTCAATTATGGAAAAGAAATTAGTACCGCAATCCATTTCAAAAGAAAGATTGCAGAAGTTAGAAGCACAAGCAACTCTTACTCCTCAACAAGAGG   700

AAGCGAAAGCCCGTAAAATCGAAAGAGAGAAAGCCAGACTAAAAGAACTGAACATTCCTACCGAATCTAAAGAATCCAAAGATTGCAGCCCTGCAGGGAT   800
                                                                                    PstI
GAT  803
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tion of the insert in the vector (data not shown). Generally, cells grown in minimal A medium had two to three times more β-galactosidase activity than cells grown in LB medium. Control of *tpr* expression appeared to be quite different in *P. gingivalis* than in *E. coli*. The putative *tpr* promoter regions had no apparent effect on expression in an *E. coli* background, but deletion of all three 17-bp repeats abolished β-galactosidase activity in *E. coli*. Primer extension analysis of total RNA from *E. coli*/pTX-400 total RNA with primer tpr170 (data not shown) indicated that transcription started at a C nucleotide 68 bp upstream of the *tpr* translation start codon (Fig. 2B). This site, 147 bp downstream of the *tpr* transcription start site in *P. gingivalis*, is immediately upstream of the −35 promoter region suggested by Bourgeau et al. (5) and is 24 bp downstream of the last direct repeat sequence preceding *tpr*. These data show that *E. coli* RNA polymerase recognized as a transcription promoter a region upstream of *tpr* that was different from that recognized in *P. gingivalis*. This finding accounted for the differences observed between β-galactosidase expression in *P. gingivalis* and that in *E. coli* strains carrying the same *tpr*::*lacZ* fusion construct.

Analysis of a chromosomal *tpr*::*lacZ* fusion. Unlike the multicopy pNTX-400 construct, the *tpr* gene exists in a single copy in *P. gingivalis* W83. To more accurately model native expression of the *tpr* gene, we constructed the suicide vector pBYZ (Table 1) and introduced it into *P. gingivalis* W83 by conjugation. Since this plasmid cannot replicate in *P. gingivalis*, the *tpr*::*lacZ* construct could be maintained only by integrating it into the *P. gingivalis* chromosome. Plasmid pBYZ carried the TX-400 *tpr*::*lacZ* construct described in the previous section inserted in place of the 5′ end of *tpr* carried on pBY2-IN (32). As shown in Fig. 5C, homologous recombination between pBYZ and chromosomal DNA resulted in either duplication of *tpr* (single-crossover event) or allelic exchange of the wild-type *tpr* gene with the *tpr*::*lacZ* reporter gene (double-crossover event). Analysis of a total of 75 Em^r and gentamicin-resistant (Gm^r) transconjugants indicated that all had *lacZ* integrated into their chromosomes (data not shown) and that all but three

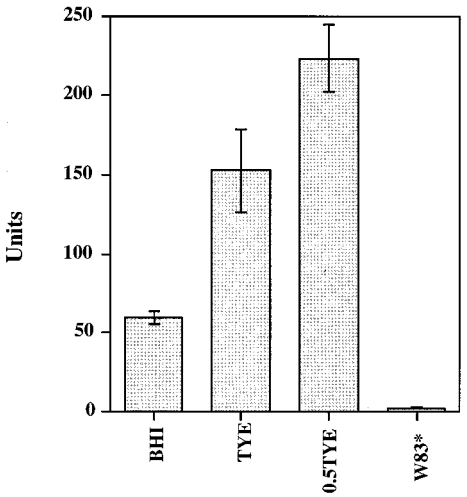

FIG. 3. Effects of growth media on β-galactosidase expression in *P. gingivalis* W83/pNTX-400. Cells were grown in BHI, TYE, or 0.5TYE. W83* is *P. gingivalis* W83 grown in TYE, which served as the negative control. One unit of β-galactosidase activity is equivalent to 1 nmol of ONPG hydrolyzed min^{-1} mg of total cellular $protein^{-1}$.

were the result of single-crossover events. Southern blot analysis of both single- and double-crossover constructs is shown in Fig. 5A and B. When *P. gingivalis* chromosomal DNA was digested with *Bam*HI and probed with a 0.8-kb *Bam*HI-*Pst*I fragment of *tpr*, a single 9.5-kb hybridizing band was detected in clones 29, 55, and 64, indicating an allelic exchange event resulting in the replacement of the *tpr* gene with the *tpr*::*lacZ* reporter gene construct. In clones 17 and 65, the *tpr* probe hybridized with 18.5- and 3.5- or 12.7- and 9.5-kb bands, respectively, indicating single-crossover homologous recombination and duplication of *tpr*.

β-Galactosidase activity assays performed on these mutants showed that both single- and double-crossover mutants carrying *tpr*::*lacZ* had the same pattern of β-galactosidase activity when they were grown in 0.5TYE as when they were grown in BHI (Fig. 6), and this pattern was similar to that of W83/pNTX-400 (Fig. 3). These data suggest that *tpr* regulation in these mutants was the same as in the wild-type strain and that expression of *tpr* does not require the intact *tpr* gene product.

Nutrient conditions regulate *tpr* expression. By using the *tpr*::*lacZ* reporter gene, we analyzed *tpr* expression under various nutrient and growth conditions. Clone 55, a *tpr*::*lacZ* double-crossover allelic exchange mutant, was designated *P. gingivalis* W83/*lacZ*. Results of growth studies and β-galactosidase activity assays with W83/*lacZ* are shown in Fig. 7. Growth rate and final optical density were highest when W83/*lacZ* was grown in BHI. The highest β-galactosidase activity in W83/*lacZ* was found in 0.5TYE at stationary-growth phase. Growth in BHI, TYE, or 0.5TYE supplemented with 1% BSA or 1% gelatin suppressed *lacZ* expression throughout growth. The pattern of β-galactosidase activity in 0.5TYE supplemented with 1% Casamino Acids was of particular interest. Under these conditions, β-galactosidase activity was low for the first 24 h of incubation and then increased rapidly over the next 24 h until it was at nearly the same level as that of cells grown in 0.5TYE.

Since β-galactosidase activity in W83/*lacZ* was greatly suppressed in BHI compared with that in 0.5TYE, we size fractionated BHI and analyzed individual fractions for their effects on β-galactosidase activity. Low-molecular-mass (<700 Da) fractions rich in phenylalanine, proline, and alanine had the most suppressive effect on β-galactosidase activity in W83/*lacZ* grown in 0.5TYE supplemented with the individual BHI frac-

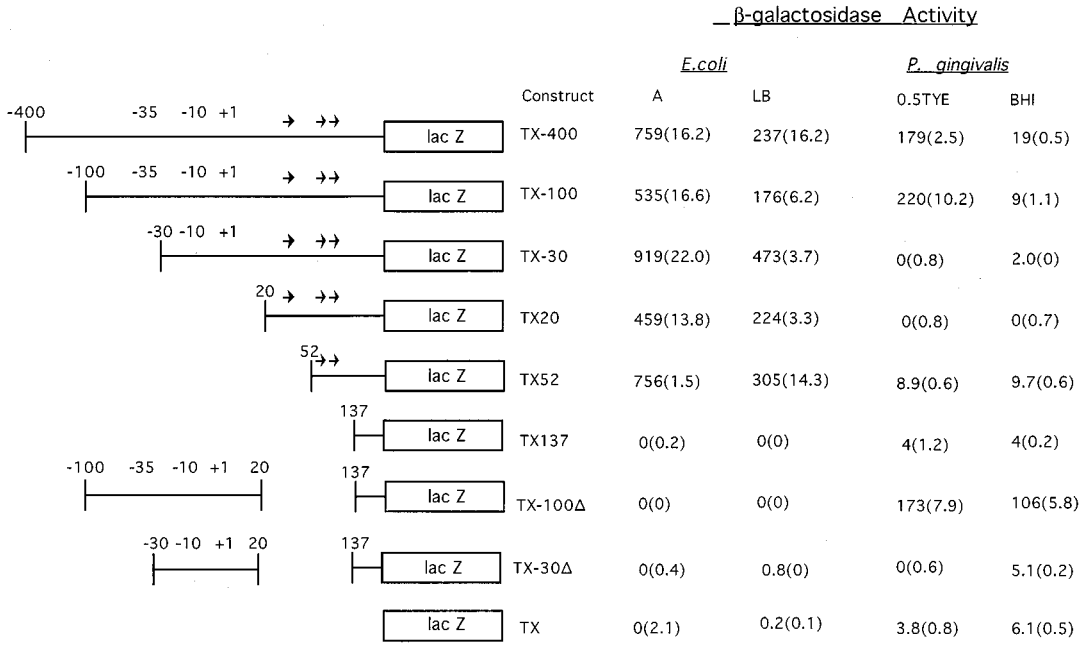

FIG. 4. β-Galactosidase activities in *P. gingivalis* and *E. coli* carrying plasmids with the TX-400 *tpr*::*lacZ* construct and various deletion derivatives. Schematic maps of the *tpr*::*lacZ* constructs show the *tpr* locus DNA present in each construct (single line) and *lacZ* (open box). The locations of the 17-bp direct repeats are indicated by arrows, and putative −35 and −10 regions are shown. β-Galactosidase activities of *E. coli* grown in minimal A medium (A) or LB medium and of *P. gingivalis* grown in 0.5TYE or BHI medium are shown as means (standard deviations). β-Galactosidase activity in *E. coli* is expressed in Miller units. For *P. gingivalis*, 1 U of β-galactosidase activity is equivalent to hydrolysis of 1 nmol of ONPG min^{-1} mg of total cellular $protein^{-1}$.

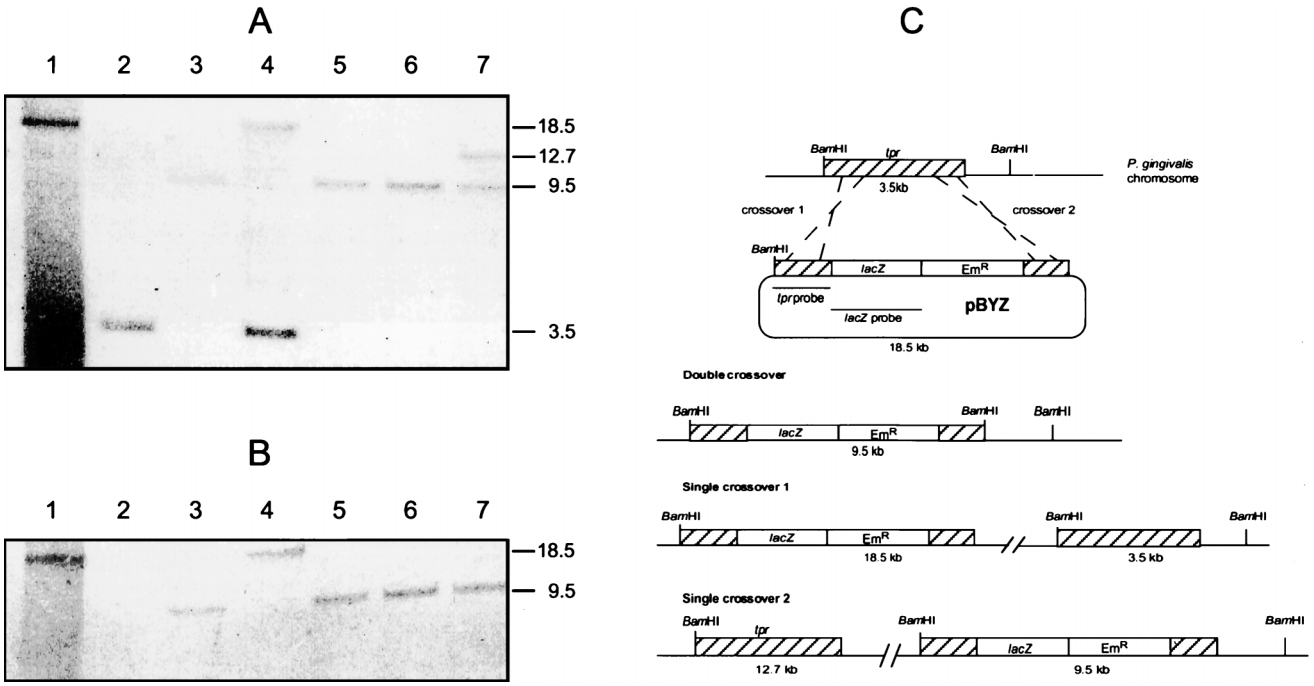

FIG. 5. Southern blot analysis of *Bam*HI-digested chromosomal DNA from *P. gingivalis* W83 strains showing integration of the *tpr*::*lacZ* construct at the *tpr* locus by single- or double-crossover homologous recombination. (A) Southern blot probed with a 0.8-kb *Bam*HI-*Pst*I 5′ fragment of *tpr*. (B) Southern blot probed with a 3.5-kb *lacZ* gene. Lanes: 1, plasmid pBYZ; 2, chromosomal DNA from *P. gingivalis* W83; 3 to 7, chromosomal DNA from *P. gingivalis* transconjugants 29, 17, 55, 64, and 65, respectively. The positions and sizes in kilobases of hybridizing bands are indicated. (C) Possible single- and double-crossover homologous recombination events in the *tpr* locus between pBYZ and the *P. gingivalis* chromosome. The locations of *tpr* DNA (hatched boxes), the promoterless *lacZ* reporter gene (open boxes), and the Em^r determinant (open boxes) are shown. The locations of the *Bam*HI fragments hybridizing with the *tpr* and *lacZ* probes are shown. The numbers below each construct indicate the sizes of the fragments generated by *Bam*HI digestion.

tions (data not shown). To determine whether *tpr* expression was related to the presence of free amino acids in the medium, the β-galactosidase activity of *P. gingivalis* W83/*lacZ* was assayed in 0.5TYE supplemented with individual L amino acids.

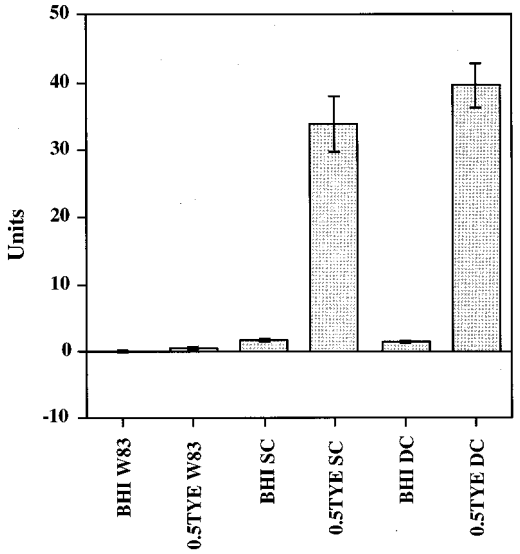

FIG. 6. β-Galactosidase activities in *P. gingivalis tpr*::*lacZ* chromosomal mutants. *P. gingivalis* W83, a single-crossover (SC) transconjugant, and a double-crossover (DC) transconjugant (*P. gingivalis* W83/*lacZ*) were grown in BHI or 0.5TYE. One unit of β-galactosidase activity is equivalent to hydrolysis of 1 nmol of ONPG min^{-1} mg of total cellular protein^{-1}.

The supplementation of 0.5TYE with concentrations of free amino acids up to 5 mM did not significantly reduce β-galactosidase activity, suggesting that *tpr* expression was not induced by depletion of a single free amino acid (data not shown). The effects of various peptides and chemicals on β-galactosidase activity in W83/*lacZ* were then tested, and the results are shown in Fig. 8. The dipeptide phenylalanyl-phenylalanine suppressed β-galactosidase activity by approximately 10-fold compared to the activity of cells grown in 0.5TYE without supplementation. The dipeptides phenylalanyl-alanine and alanyl-proline also suppressed β-galactosidase activity to a lesser degree. The potential nitrogen sources ammonium acetate, ammonium nitrate, and ammonium sulfate had no significant effect on *tpr* expression.

Effects of other environmental conditions on *tpr* expression. To analyze whether *tpr* expression was influenced by heat shock or other stress conditions, β-galactosidase activity was measured in W83/*lacZ* cells which had been heat shocked, incubated at pH 5.5, incubated at pH 8.5, or hemin starved. The results suggested that *tpr* expression was not affected by pH changes or hemin starvation and was suppressed by approximately 25% by heat shock at 42°C (data not shown). Succinate, which can replace hemin as a required growth factor (27), had no significant effect on *tpr* expression.

DISCUSSION

The present study represents an initial attempt to characterize regulation of a potential virulence factor of *P. gingivalis*. There have been several reports stating that *P. gingivalis* proteases are regulated by the growth environment (6, 25, 28, 38), but little is known about how this regulation is achieved at the

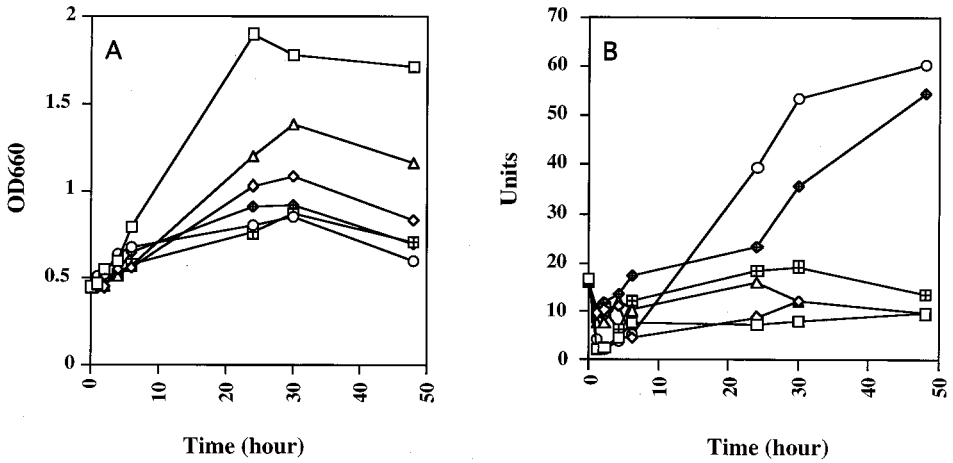

FIG. 7. Growth (A) and β-galactosidase activity (B) of *P. gingivalis* W83/*lacZ*. Cells were first grown in BHI to mid-log phase and resuspended in BHI (□); TYE (◇); 0.5TYE (○); or 0.5TYE supplemented with 1% BSA (△), 1% gelatin (⊞), or 1% Casamino Acids (⊕) and incubated for the time intervals indicated. One unit of β-galactosidase activity is equivalent to hydrolysis of 1 nmol of ONPG min^{-1} mg of total cellular $protein^{-1}$. OD_{600}, optical density at 600 nm.

genetic level. Our previous report that *tpr* proteolytic activity (Pz-peptidase) was influenced by growth conditions made this an inviting model for investigating gene regulation in this organism (34). In that study, Northern blot analysis suggested that *tpr* expression was regulated by nutritional conditions and that this regulation occurred at the transcriptional level.

Primer extension analysis identified the *tpr* transcription initiation site, and DNA sequencing revealed three direct repeats in the 5′ noncoding region of the transcript that appear to act as regulatory elements. Initial primer extension reactions were carried out with primer tpr293 (Table 2). This primer would have been appropriate if the promoter sequence proposed by Bourgeau et al. (5) were correct. The location of the transcription start site 215 bp upstream of the *tpr* coding region may not be unusual for this organism. In the few studies of transcription in *P. gingivalis*, the transcription start sites were located similar distances upstream of the coding regions (18, 22). Further studies are needed for a better understanding of promoter structure in *P. gingivalis* and to determine whether promoters similar to that of *tpr* are a common feature in this organism. There have been a number of reports of expression of cloned *P. gingivalis* genes in *E. coli*. Some of these cloned genes were proposed to be transcribed from their own promoters, and regions homologous to *E. coli* promoters were identified (5, 7, 8, 16, 17, 31, 36). On the other hand, Klimpel and Clark, using antisera to the *E. coli* RNA polymerase core enzyme and σ^{70}, found no proteins in *P. gingivalis* extracts that cross-reacted with either antiserum (19). This result suggested that *E. coli* and *P. gingivalis* RNA polymerases may be significantly different, which is supported by our data showing that *E. coli* and *P. gingivalis* RNA polymerases initiate *tpr* transcription at different sites. Thus, these data underscore the importance of analyzing gene expression in native systems and the limitations of predicting gene structure based on canonical *E. coli* studies.

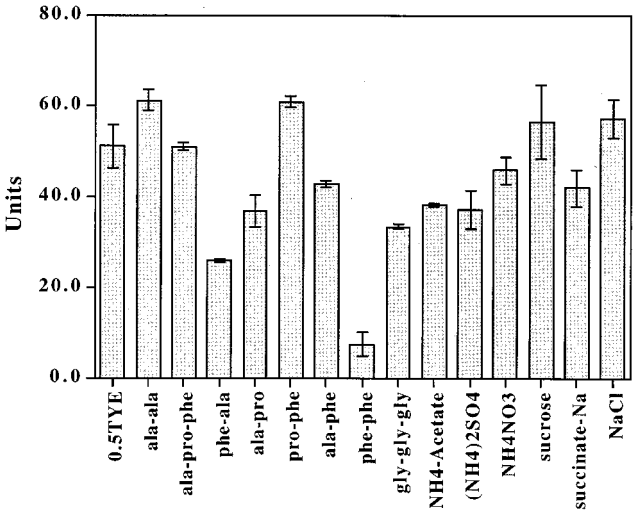

FIG. 8. Effects of peptides and several chemicals on β-galactosidase expression in *P. gingivalis* W83/*lacZ*. Cells were grown in BHI broth to mid-log phase, resuspended in 0.5TYE or 0.5TYE supplemented with 5 mM concentrations of peptides or 40 mM concentrations of other chemicals, and incubated overnight, after which β-galactosidase activity was analyzed. One unit of β-galactosidase activity is equivalent to hydrolysis of 1 nmol of ONPG min^{-1} mg of total cellular $protein^{-1}$. ala-ala, alanine-alanine; ala-pro-phe, alanine-proline-phenylalanine; phe-ala, phenylalanine-alanine; ala-pro, alanine-proline; pro-phe, proline-phenylalanine; ala-phe, alanine-phenylalanine; phe-phe, phenylalanyl-phenylalanine; gly-gly-gly, glycine-glycine-glycine.

P. gingivalis, which produces numerous membrane-associated and secreted proteases, has the ability to degrade proteins to short peptides and transport them into the cell for metabolism (10). Our results suggest that the availability of these peptides regulates expression of at least one of these proteases. A variety of nutrients, including those found in complex growth media as well as BSA, gelatin, and Casamino Acids, could suppress *tpr* expression. Of particular interest were results of a time course growth and expression study of the effects of supplementation with Casamino Acids (Fig. 7). Under these conditions, transcription of *tpr*, represented by β-galactosidase activity, was initially suppressed. However, after 48 h of incubation, *tpr* transcription increased to the level seen in cells grown in 0.5TYE without supplementation. Casamino Acids is an acid hydrolysate of casein in which, according to the manufacturer, free amino acids and small peptides are present in a ratio of 82 to 18%, respectively. This ratio suggests that the initial suppressive effect on *tpr* expression was due to the peptide component of the hydrolysate. Once the peptides were exhausted, transcription of *tpr* (and β-galactosidase activity) increased. While none of the 20 essential amino acids had a significant effect, this fact does not rule out the possibility that

tpr expression might be influenced by combinations of free amino acids. Considered together, these results suggested that peptides, rather than free amino acids, regulate *tpr* expression.

The effects of allelic exchange and *tpr* gene duplication on β-galactosidase expression indicated that the Tpr protein is not involved in its own regulation. Our results also showed that a plasmid-borne *tpr*::*lacZ* fusion and a chromosomally integrated *tpr*::*lacZ* fusion exhibited the same regulation pattern, suggesting that shuttle vectors can be used to analyze the effects of 5′ deletion mutations on *tpr* expression. We observed higher levels of *tpr* expression in *P. gingivalis* cells in stationary-growth phase than in cells in logarithmic-growth phase. This is similar to the *rpoS* regulon in which 30 or more genes are expressed in response to starvation and during the transition to stationary phase (14). Proteins in this regulon can enhance long-term survival in nutrient-deficient medium and have diverse functions, including protection against DNA damage, determination of morphological changes, mediation of virulence, osmoprotection, and thermotolerance. Differential levels of expression of families of genes within this regulon are affected by supplementary regulatory factors, working individually and in combination to modulate activities of different promoters. At present, it is not known whether a similar regulon exists in *P. gingivalis* or whether expression of genes other than *tpr* is also regulated by the availability of peptides.

The identity of the factor(s) that controls *tpr* expression has not yet been determined. However, our results suggest that a short peptide or peptides containing phenylalanine are responsible. Low-molecular-weight fractions of BHI suppressed *tpr* expression to various extents. Analysis of BHI showed that a low-molecular-weight fraction rich in phenylalanine, alanine, and proline had the most suppressive effect on *tpr* expression as indicated by β-galactosidase activity. Attempts to further characterize this fraction in order to identify the specific factor that suppressed *tpr* expression were not successful, probably due to the inability to obtain sufficient quantities of individual peptides. When it was used to supplement 0.5TYE, the dipeptide phenylalanyl-phenylalanine had the most effect on *tpr* expression, as measured by β-galactosidase activity. Supplementation with other peptides and some chemicals and challenge by heat shock, pH change, or hemin limitation had little or no effect on *tpr* expression.

In an analogous system, Marugg et al. showed that the PrtP and PrtM proteases of *Lactococcus lactis* were regulated at the transcriptional level by leucine-containing peptides but not by free amino acids (26). Peptide content of the growth media had no effect on transcription of *prtP* and *prtM* in an Opp$^-$ strain of *L. lactis*, indicating that peptide uptake was required for this regulation to take place. In *P. gingivalis*, acquisition of peptide nutrients is certain to be an extremely important process. At present, the molecular mechanisms of peptide uptake in *P. gingivalis* are not known. Further studies are required to address this issue as well as to determine the specific molecular factors that control *tpr* transcription.

The actual mechanism of *tpr* regulation remains unclear, but our results are suggestive of the involvement of specific DNA binding factors. We identified three identical direct repeats between the transcription start site and the coding region of *tpr*. Deletion of these repeats abolished nutrient-dependent *tpr* regulation in *P. gingivalis*, even though the promoter region remained intact. The potential involvement of direct repeats in gene regulation has been reported in other studies. The regulatory region of the *torCAD* operon of *E. coli* contains four decameric direct repeats. These repeats, designated *tor* boxes, were found to be the targets of TorR, which regulates *torC* expression (41). In *P. gingivalis*, four direct repeats of 41 bp were identified upstream of the *hagB* gene (37). Transcription of *hagB* was greatly reduced when cells were grown in the absence of hemin, suggesting a possible regulatory role for these repeats (20). Three 12-bp direct repeats, tentatively proposed as transcription termination attenuators, were found within the putative transcription termination region of the *prtT* protease gene of *P. gingivalis* (22). The *ospD* genes of various Lyme disease-associated *Borrelia* spp. are preceded by between 1 and 12 copies of a 17-bp direct repeat that contains a potential −35 promoter sequence (24). While the functions of the direct repeats listed above are not known at this time, they may represent binding sites for specific regulatory proteins.

Most DNA binding proteins that act as transcription factors bind to the 5′ region of the promoter to exert effects on RNA transcription initiation. The locations of the three direct repeats within the untranslated region of the *tpr* transcript indicated that they are not involved in the initiation of transcription. However, if the three direct repeats are a regulatory protein binding site, they may influence *tpr* mRNA synthesis at the transcript elongation and termination stages. The genes in the TyrR regulon of *E. coli* are regulated by the TyrR protein, whose binding site is located downstream of the putative RNA polymerase binding site (35). He and Zalkin (13) found that the operator (PurR binding site) of the *purB* gene of *E. coli* was 242 bp downstream of the transcriptional start site and overlapped codons 62 to 67 in the structural gene. PurR-mediated repression of *purB* occurred by a transcriptional "roadblock" mechanism, and they identified a truncated *purB* mRNA species in a Northern blot (13). In our system, such a truncated mRNA would be extremely difficult to detect due to its small size. Furthermore, our Northern blot analysis of W83/PM, a *tpr*-deficient mutant, suggested that truncated *tpr* mRNA was unstable (34).

Another possible role of the 17-bp direct repeat in the *tpr* locus may be in site-specific genetic recombination, which can contribute to both genomic plasticity and antigenic variability. The genes encoding the major cysteine proteases and hemagglutinins of *P. gingivalis* contain large direct repeat regions that appear to contribute to such recombination-based heterogeneity in this gene family (4). The Tpr protease is distinct from this group of enzymes, and there have as yet been no studies of its conservation in different strains. There are 17-bp direct repeats associated with the gene encoding the highly variable surface-expressed VlsE protein of *Borellia burgdorferi*. The *vlsE* gene contains a cassette region flanked by 17-bp direct repeats. Recombination between the cassette and up to 15 silent cassette sequences resulted in antigenic variation of the VlsE protein (44).

The mechanism(s) by which nutrients regulate *tpr* expression in *P. gingivalis* W83 remains to be determined. Our current understanding of *tpr* expression is as follows. Transcription of the *tpr* gene begins 215 bp upstream of the coding region. Regulation appears to be at the transcript elongation and termination stages. A regulatory *trans*-acting factor may directly or indirectly sense the presence of certain short, phenylalanine-containing peptides and act on the three direct repeats to modulate *tpr* expression. Future studies will focus on the overall significance of Tpr among the array of proteolytic enzymes of this organism and on the specific role of Tpr in processing and acquisition of essential peptide nutrients.

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