Identification and molecular characterization of tryptophanase encoded by *tnaA* in *Porphyromonas gingivalis*

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Indole produced via the β -elimination reaction of L-tryptophan by pyridoxal 5'-phosphate-dependent tryptophanase (EC 4.1.99.1) has recently been shown to be an extracellular and intercellular signalling molecule in bacteria, and controls bacterial biofilm formation and virulence factors. In the present study, we determined the molecular basis of indole production in the periodontopathogenic bacterium *Porphyromonas gingivalis*. A database search showed that the amino acid sequence deduced from *pg1401* of *P. gingivalis* W83 is 45 % identical with that from *tnaA* of *Escherichia coli* K-12, which encodes tryptophanase. Replacement of the *pg1401* gene in the chromosomal DNA with the chloramphenicol-resistance gene abolished indole production. The production of indole was restored by the introduction of *pg1401*, demonstrating that the gene is functionally equivalent to *tnaA*. However, RT-PCR and RNA ligase-mediated rapid amplification of cDNA ends analyses showed that, unlike *E. coli tnaA*, *pg1401* is expressed alone in *P. gingivalis* and that the nucleotide sequence of the transcription start site is different, suggesting that the expression of *P. gingivalis tnaA* is controlled by a unique mechanism. Purified recombinant *P. gingivalis* tryptophanase exhibited the Michaelis–Menten kinetics values $K_m=0.20\pm0.01$ mM and $k_{cat}=1.37\pm0.06$ s⁻¹ in potassium phosphate buffer, but in sodium phosphate buffer, the enzyme showed lower activity. However, the cation in the buffer, K⁺ or Na⁺, did not appear to affect the quaternary structure of the enzyme or the binding of pyridoxal 5'-phosphate to the enzyme. The enzyme also degraded S-ethyl-L-cysteine and S-methyl-L-cysteine, but not L-alanine, L-serine or L-cysteine.

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INTRODUCTION

Tryptophanase (tryptophan indole-lyase, EC 4.1.99.1) occurs in a wide variety of bacteria. The protein encoded by *tnaA* is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyses the β -elimination reaction of L-tryptophan to yield indole, ammonium and pyruvate (Snell, 1975). Tryptophanases from *Escherichia coli* (Phillips, 1987; Watanabe & Snell, 1977) and *Proteus vulgaris* (Zakomirdina *et al.*, 2002) have also been reported to be associated with the *in vitro* β -elimination reactions of a wide range of natural and synthetic amino acids. *E. coli* tryptophanase functions as a tetramer of four 52.8 kDa 471-residue subunits (Deeley & Yanofsky, 1981; Kagamiyama *et al.*, 1972), and although the dimeric form of the enzyme can be isolated (Raibaud & Goldberg, 1976) or induced by cold (Erez *et al.*, 1998), it is inactive. In the

active form of *E. coli* tryptophanase, each subunit contains one molecule of PLP that forms a Schiff base with the side chain of Lys-270, but can be removed by dialysis to produce the apoform of the enzyme (Metzler *et al.*, 1991). K⁺ plays a role in stabilizing the oligomeric structure of tryptophanase, as cold dissociation of the enzyme has been shown to occur slowly in the presence of this cation (Erez *et al.*, 1998), whereas Na⁺ has been shown to inhibit the activity of the enzyme (Snell, 1975).

Indole is one of the malodorous oral volatile products, which include methyl mercaptan, hydrogen sulphide, skatole and cadaverine (Fosdick & Piez, 1953; Kostelc *et al.*, 1981; Claesson *et al.*, 1990; Goldberg *et al.*, 1994). Interestingly, the molecule was recently reported to decrease biofilm formation by *E. coli*, but to increase that by *Pseudomonas* spp. (Lee *et al.*, 2007). The molecule also controls multidrug exporters (Hirakawa *et al.*, 2005) and regulates the pathogenicity island in *E. coli* (Anyanful *et al.*, 2005). Moreover, a relationship between the ability of *Haemophilus influenzae* strains to produce indole and cause

Abbreviations: CRP, cAMP receptor protein; GST, glutathione S-transferase; PLP, pyridoxal 5'-phosphate; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends.

certain infectious diseases, including meningitis, has been reported (Martin *et al.*, 1998). Thus, indole is not only a malodorous product, but also an extracellular and inter-cellular signalling molecule.

Porphyromonas gingivalis is a Gram-negative, black-pigmented, asaccharolytic obligate anaerobe that has been implicated in the initiation and progression of periodontal disease (Lamont & Jenkinson, 2000). This micro-organism is regarded as a secondary colonizer of oral biofilm (Kolenbrander *et al.*, 2002), and its primary niche is the anaerobic environment of subgingival pockets (Lamont & Jenkinson, 1998; Yoshida *et al.*, 2003b). The production of methyl mercaptan from methionine by this micro-organism has been demonstrated (Yoshimura *et al.*, 2000), but most of the enzymes associated with the degradation of amino acids are unknown. As a first step in investigating the relationship between indole production and virulence in *P. gingivalis*, we characterized a gene homologue of *tnaA* and its purified recombinant product.

METHODS

Bacterial strains and culture conditions. *P. gingivalis* W83 and its derivatives were grown anaerobically at 37 °C in enriched BHI broth, containing 37 mg BHI (Difco) ml⁻¹, 5 mg yeast extract (Difco) ml⁻¹, 1 mg L-cysteine ml⁻¹, 5 µg haemin ml⁻¹ and 1 µg menadione ml⁻¹. When necessary, 20 µg chloramphenicol ml⁻¹ or 10 µg erythromycin ml⁻¹ was added to the medium. *E. coli* BL21 (Promega), used for protein purification, was grown aerobically in 2 × TY broth (Difco) at 37 °C with 100 µg ampicillin ml⁻¹.

Construction of mutant strains of *P. gingivalis*. A *tnaA*-deficient mutant strain designated KO100 was constructed by transformation of *P. gingivalis* W83 with a DNA construct containing the chloramphenicol-resistance gene (*cat*) flanked by targeting sequences for *pg1401*. The upstream targeting sequence, the *cat* gene and the downstream targeting sequence were individually amplified from pCM18 (Hansen *et al.*, 2001) or genomic DNA prepared using a Wizard Genomic DNA purification kit (Promega). The *cat* gene was amplified with the primers 090507-cm-F7 and 090507-cm-R8 (Table 1). The flanking sequence upstream of *tnaA* was amplified using the primers 090507-tn-F1 and 090507-tn-R3, which are homologous to the 5' end of the *cat* fragment. Amplification of the flanking sequence downstream of *tnaA* was carried out with the primers 090507-tn-F4 and 090507-tn-R6, which have homology with the 3' end of the *cat* fragment. Finally, the amplicons were linked using the PCR-based overlap extension method (Horton *et al.*, 1989).

Likewise, restoration of *tnaA* in the deficient mutant strain KO100 was performed by replacement of *cat* with *tnaA* from strain W83 linked to a cassette of the erythromycin-resistance genes *ermF-ermAM*. The DNA fragment used for transformation was prepared using the PCR-based overlap extension method as described above. The *ermF-ermAM* cassette was amplified from pVA2198 with the primers 021208-ermAF-f9 and 021208-ermAF-r10. The plasmid was provided by Drs Hasegawa and Yoshimura of Aichi-Gakuin University (Nagoya, Japan). The flanking sequence upstream of *tnaA* was amplified using the primers 021208-tn-f3 and 021208-tn-r4, which are homologous to the 5' end of the *ermF-ermAM* cassette. The DNA fragment containing *tnaA* and the flanking sequence downstream of *tnaA* were amplified with the primers 021208-tn-f6 and 090507-tn-R5, which are homologous to the 3' end of the *ermF-*

ermAM cassette. The three amplified DNA fragments were then linked using overlap extension PCR.

Electroporation of *P. gingivalis* cells was performed using the method of Nakayama *et al.* (1995) with minor modifications. Briefly, an overnight enriched BHI culture was diluted 1:5 in 5 ml fresh enriched BHI and incubated for 8 h at 37 °C to obtain competent cells in the exponential phase. The cells were collected and washed five times with electroporation solution (0.3 M sucrose), and then resuspended in 100 µl of the solution. The PCR constructs for transformation (approx. 5 µg) were added to 80 µl of the cell suspension. The mixtures were pulsed with a Gene Pulser apparatus (Bio-Rad) at 2.0 kV with a time constant of 5 ms. The cell suspensions were then diluted with 10 ml prewarmed enriched BHI broth and incubated anaerobically at 37 °C for 24 h. Aliquots of the cell suspension were plated on enriched tryptic soy agar (containing, per litre, 40 g tryptic soy broth, 5 g BHI, 1 g L-cysteine, 5 mg haemin, 1 mg menadione and 20 g agar) plus 20 µg chloramphenicol ml⁻¹ or 10 µg erythromycin ml⁻¹ and incubated anaerobically for 5 days at 37 °C.

Southern hybridization experiments. Southern hybridization analysis was performed using non-radioactive DIG-labelled PCR probes that were PCR amplified with a DIG DNA labelling kit (Roche Diagnostics). Genomic DNA digested with *SmaI* was separated on a 0.8% agarose gel by electrophoresis and transferred to a nylon membrane. The probes for *tnaA*, *cat* and *ermF* were made by PCR amplification with PCR DIG Labelling Mix (Roche Diagnostics). The PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega). The membranes were hybridized for 6 h under high-stringency conditions with one of the probes, as previously described (Church & Gilbert, 1984).

RT-PCR and RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). Total RNA was extracted from a *P. gingivalis* W83 culture brought to exponential growth using FastPrep Blue tubes (Bio 101) as previously described (Yoshida *et al.*, 2003a). Contaminating DNA in the samples was eliminated by digestion with RNase-free DNase (Takara Bio). The total RNA obtained was used for the following two analyses.

RT-PCR was performed as described by Yoshida *et al.* (2003a). Briefly, RNA was reverse-transcribed into single-stranded cDNA using PrimeScript Reverse Transcriptase (Takara Bio) according to the manufacturer's instructions. The gene-specific primers used in RT-PCR are listed in Table 1. Each reverse primer for RT-PCR analysis was also used to synthesize a cDNA from a specific mRNA in the total RNA. The reaction mixtures used as negative controls contained no reverse transcriptase, so that the existence of contaminating genomic DNA in the samples could be evaluated.

RLM-RACE was used to determine the transcription start site of *tnaA* with total RNA. Rapid amplification of 5' cDNA ends was carried out using a FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's instructions. In brief, a 45 base RNA adaptor was ligated to the RNA population using T4 RNA ligase. This RNA population was then used as a template for a random-primed reverse transcription reaction. The cDNA product of this reaction was used as a template for PCR using a *tnaA*-specific outer primer (RACE outer *tnaA* primer) and a reverse primer to the adaptor (RACE outer adaptor primer). The PCR conditions were as follows: 94 °C for 2 min; 35 cycles of 94 °C for 15 s, 60 °C for 20 s and 72 °C for 30 s; and 72 °C for 5 min. The identity of the product was confirmed using inner gene-specific primers (RACE inner *tnaA* primer and RACE inner adaptor primer). After the amplicon was purified and then ligated into pMCL210 (Nakano *et al.*, 1995), *E. coli* was transformed with the ligated mixture. The plasmids isolated from ten different colonies were sequenced with an ABI 3730 Genetic Analyzer (Applied

Table 1. Oligonucleotide primers used in this study

Designation	Sequence (5'–3')*
Construction of mutants	
090507 cm-F7	ATGGAGAAAAAATCACTGGA
090507 cm-R8	TTACGCCCCGCCCTGCCACTC
090507-tn-F1	CGAAAAGTCCTTGTCGTGA
090507-tn-R3	CCAGTGATTTTTTCTCCAT CATTTTAATGCGGTAGGATTC
090507-tn-F4	GCAGGGCGGGGCGTAAT CACTTCACTGTTGAGCTGG
090507-tn-R6	ATCGGTATTCTTGATTGGTA
021208-ermAF-f9	TTTATTGCTTATTATCCGCACCCAAA
021208-ermAF-r10	CGACTCATAGAATTATTTCTCTCCGTT
021208-tn-f3	GGCAGATGGGACGGAATATCT
021208-tn-r4	TTTGGGTGCGGATAATAAGCAATAAA TTTACCGGAGGAGGTTCTTT
021208-tn-f6	AACGGGAGGAAATAATTCTATGAGTCGACT AACTTGCCGCCCAATTAA
090507-tn-R5	GAATTAACAAACAAAATATACGCC
RT-PCR	
032508-pg03-f1	GGTGGTATGATAGGAGGTATTA
040308-pg03-r1	CCACTCCGAAGACAATG
032508-pg03-f2	GGATTGGTCGTTCTTCTTA
033108-pg02-r2	TGAGAATAGGATTGGGATTC
032508-pg02-f3	AATCAGGGAGCTTTCAAG
032508-pg01-r3	TGGCATTCTTCATATTATAGTATG
032508-pg01-f4	ACTTCACTGTTGAGCTGGA
033108-pg01-r4	ACTCTGTCAGTGCCGAAC
032598 pg-f5	AAACAGGTTGTCCGTCAG
032598 pg-r5	TCCTGTATAAAATTAAAGGCGA
060908-Pgtn-f6	AGAATATACGTGAAGTGAGCG
060908-Pgtn-r6	AAAGTCTGAGCGATGAAGTC
RLM-RACE	
RACE outer adaptor primer	GCGAGCACAGAATTAATACGACT
RACE inner adaptor primer	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
RACE outer tnaA primer	CCGGAATCAGTGAGCAA
RACE inner tnaA primer	ATACATAGTCGCTCTTGAGG
Purification of TnaA	
090407-F1	AAGGATCCGAATTACCTTTTTCAGAATCCTACC
090407-R1	AAGTCGACTTACTTGGCTTTTTCAGC

*The nucleotides underlined in each primer sequence are the positions of restriction endonuclease sites incorporated to facilitate cloning. Bold letters show overlapping regions of the 5' or 3' ends of the *cat* gene or the *ermF-ermAM* cassette.

Biosystems). The sequences were analysed using Vector NTI software (Invitrogen).

Preparation of crude enzyme extracts. Crude enzyme extracts of *P. gingivalis* were prepared as described by Yoshida *et al.* (2002). Briefly, cells grown to an OD₆₀₀ of about 0.8 were harvested from 50 ml of culture and then washed with PBS. The cells were sonicated five times for 30 s at 1 min intervals. The supernatant was collected after centrifugation at 15 000 g for 30 min at 4 °C. The concentration of proteins in the crude enzyme extract was determined using a protein assay reagent (Bio-Rad) with BSA as a standard. The samples were stored at –20 °C after adding the same volume of 80 % (v/v) glycerol.

Purification of recombinant TnaA. Recombinant TnaA protein of *P. gingivalis* was purified using the expression vector pGEX-6P-1 (GE Healthcare) as described by Yoshida *et al.* (2002). The coding sequence of *tnaA* was amplified by PCR with KOD DNA polymerase (Toyobo) from genomic DNA of *P. gingivalis* W83 using the primers 090407-F1 and 090407-R1 and ligated into the pGEX-6P-1 vector via

the *Bam*HI and *Xho*I restriction sites, juxtaposing the *tnaA* gene downstream of the coding sequence for glutathione S-transferase (GST) and a PreScission protease cleavage site. The resulting plasmid pGEW101-1 was sequenced to verify the correctness of the PCR amplification of the 1523 bp insert and transformed into *E. coli* BL21 cells. Production cultures of 500 ml were inoculated 1 : 1000 with an overnight culture and grown to an OD₆₀₀ of 0.7 at 37 °C. After induction of protein expression with 0.5 mM IPTG, the cells were incubated at 37 °C for another 2 h. The cells were then harvested by centrifugation at 4 °C, resuspended in 12 ml PBS, and lysed by ultrasonication. Cell debris was sedimented by centrifugation, and the portion of GST fusion protein that remained in the supernatant was adsorbed onto affinity matrix glutathione-Sepharose 4B and cleaved with PreScission protease according to the manufacturer's protocol (GE Healthcare). The concentration of proteins was determined as described by Pace *et al.* (1995). The purity of the samples was analysed by SDS-PAGE.

N-terminal amino acid sequence determination was carried out to confirm the homogeneity of the recombinant protein. Briefly, the

protein separated by SDS-PAGE was transferred electrophoretically to a PVDF membrane. The band visualized by Coomassie brilliant blue was excised and analysed with a Procise 49X cLC protein sequencer (Applied Biosystems).

TnaA apoenzyme was prepared by dialysing against 20 mM potassium phosphate or sodium phosphate buffer (pH 7.5) for 24 h. Reconstitution of the holoenzyme was achieved by incubation of the apoenzyme with 0.1 mM PLP at 37 °C for 30 min in darkness as described by Raibaud & Goldberg (1976).

Gel-filtration chromatography. A prepacked gel-filtration column (Hiroad 16/60 Superdex; GE Healthcare) was used. The mobile phase used for apoenzyme analysis was identical to that used for dialysis to prepare each apoenzyme (20 mM potassium phosphate or sodium phosphate buffer, pH 7.5) to remove the effect of the other cation. For holoenzyme analysis, each phosphate buffer containing 0.1 mM PLP was used to avoid the release of PLP from the holoenzyme. The column was run at 0.5 ml min⁻¹ at 4 °C. The enzyme elution was monitored at 280 nm. The size of each protein was estimated using a standard curve made with five commercially available proteins (Gel Filtration Calibration kit HMW, GE Healthcare).

HPLC analysis. The indole production in an enzymic reaction was evaluated using reversed-phase HPLC as described by Krstulovic & Matzura (1979). The reaction mixture contained the following reagents in a final volume of 100 µl: 167 mM potassium phosphate buffer (pH 7.5), 0.165 mM PLP, 0.2 mM reduced glutathione, 0.25 mg BSA ml⁻¹, 2 mM L-tryptophan and 50 µg ml⁻¹ crude extract of *P. gingivalis* strain W83, KO100 or RS101. After layering with 100 µl toluene, the mixture was incubated for 2 h at 37 °C. An aliquot (20 µl) of toluene from the sample tube was injected onto a Capcell Pak C18 UG120 column (4.6 mm × 150 mm; Shiseido) with 50 % (v/v) methanol/water as the mobile phase at a flow rate of 0.5 ml min⁻¹ at 40 °C. Excitation and emission wavelengths of 285 and 320 nm, respectively, were used.

Enzyme assay. The degradation of L-tryptophan by purified tryptophanase was examined by measuring the formation of indole as described by Morino & Snell (1970) with minor modifications. Briefly, the reaction mixture contained 200 mM potassium or sodium phosphate buffer (pH 7.5), 0.165 mM PLP, 0.2 mM reduced glutathione, 0.25 mg BSA ml⁻¹, 10 µg purified tryptophanase ml⁻¹, and varying concentrations of L-tryptophan. After being layered with 100 µl toluene, the reaction mixture was prewarmed for 5 min at 37 °C. The reaction was initiated by the addition of substrate solution. After 10 min incubation, the reaction was terminated by the addition of 1 ml Ehrlich's reagent, which was prepared daily by mixing 5 vols 5 % (w/v) *p*-dimethylaminobenzaldehyde in 95 % (v/v) ethanol with 12 vols 5 % (v/v) H₂SO₄ in 1-butanol. After 20 min at room temperature, the mixture was centrifuged at 15 000 *g* for 2 min to remove insoluble material. The supernatant was examined spectrophotometrically at 568 nm. The amount of indole was calculated from a standard curve. The kinetic parameters were computed from a Lineweaver-Burk transformation (v^{-1} versus S^{-1}) of the Michaelis-Menten equation, where v (mmol min⁻¹ g⁻¹) represented the formation of indole and S (mM) was the concentration of L-tryptophan. The k_{cat} value was calculated from the V_{max} value and the molecular mass of *P. gingivalis* tryptophanase. Data were obtained from three independent experiments.

The degradation of S-ethyl-L-cysteine, S-methyl-L-cysteine, L-cysteine, L-alanine and L-serine was measured by assaying pyruvate formation, as previously described (Yoshida *et al.*, 2002). The assays were carried out in 100 µl reaction mixtures containing 200 mM potassium phosphate buffer (pH 7.6), 0.165 mM PLP, 1 µg purified enzyme, and varying concentrations of each substrate. After 5 min incubation at 37 °C, the reactions were terminated by the addition of 50 µl of

4.5 % (v/v) trichloroacetic acid. The reaction mixtures were then centrifuged, and 100 µl aliquots of the supernatants were added to 300 µl 0.67 M sodium acetate (pH 5.2) containing 0.017 % (w/v) 3-methyl-2-benzothiazolinone hydrazone. After incubation at 50 °C for 30 min, the absorbance at 335 nm was determined. The amounts of pyruvate were calculated from a standard curve prepared using crystalline sodium pyruvate. Data were obtained from three independent experiments.

RESULTS

Organization of the *tnaA* homologue in *P. gingivalis* W83

The *tnaA* genes that encode tryptophanase in *Pr. vulgaris* (Kamath & Yanofsky, 1992), *Enterobacter aerogenes* (Kawasaki *et al.*, 1993), *H. influenzae* (Martin *et al.*, 1998) and *E. coli* (Deeley & Yanofsky, 1981; Rezwani *et al.*, 2004) have been sequenced. A database search identified the ORF *pg1401*, which was assigned by the J. Craig Venter Institute (<http://www.jcvi.org/>), as a homologue of the *tnaA* gene in the genome of *P. gingivalis* W83 (Nelson *et al.*, 2003). The ORF (1380 bp) is slightly shorter than those of the *tnaA* genes from the bacteria described above (1389–1416 bp). In those organisms, *tnaA* is located between *tnaC*, which encodes a leader peptide, and *tnaB*, which encodes tryptophan permease; the latter consists of an operon with *tnaA* (Deeley & Yanofsky, 1981) (Fig. 1a). By contrast, the gene upstream of *pg1401* in *P. gingivalis*, *pg1402*, encodes a putative endonuclease/exonuclease/phosphatase-family protein. The amino acid sequence encoded by the gene downstream of *pg1401* contains an inosine monophosphate cyclohydrolase domain that is involved in purine biosynthesis. We have designated this gene *orfX*, since the ORF was not identified in the database. Unexpectedly, no genes homologous to *tnaB* or *tnaC* were identified either in the *pg1401* region or in the whole-genome sequence of *P. gingivalis* W83, showing that the gene organization of this region in *P. gingivalis* is different from that in other bacteria. However, the amino acid sequence deduced from *pg1401* shows 48.1 %, 50.9 %, 42.0 % and 44.9 % identity with the deduced sequences from *Pr. vulgaris*, *Ent. aerogenes* SM-18, *H. influenzae* Eagan, and *E. coli* K-12, respectively.

Transcription of the *tnaA* region in *P. gingivalis* W83

RT-PCR was used to characterize the transcripts of the *pg1401* region in *P. gingivalis* W83. The locations of the primer pairs used to detect intragenic and intergenic regions are shown in Fig. 1(a). RT-PCR amplification generated products of the expected size from an oligonucleotide pair complementary to *pg1404/pg1403* (Fig. 1b). In contrast, no PCR products corresponding to the regions spanning the borders of *pg1403/pg1402*, *pg1402/pg1401* or *pg1401/orfX* were amplified, indicating that *pg1401* was transcribed alone. No PCR products were observed from

Fig. 1. Genetic organization and transcriptional analysis of the *tnaA* region of *P. gingivalis* W83. (a) Gene arrangements of the *tnaA* region in *E. coli* K-12 and *P. gingivalis* W83. The DNA sequence of each region was obtained from the database. Large and small arrows indicate the positions of the ORF and the primers used for RT-PCR, respectively. (b) RT-PCR analysis of *tnaA* gene expression. Total RNA was prepared from a culture in the exponential growth phase. The expected size (bp) of each PCR amplicon is shown in parentheses. Lanes marked (+) are standard RT-PCR amplifications. Lanes marked (-) are negative controls containing no cDNA. The positions of DNA size standards (in bp) are indicated on the left. (c) Determination of the transcription start site by RLM-RACE. The coding region starting with ATG (bold and underlined) is shown with the upstream promoter region in italics. The identified transcriptional start site is shaded and boxed. The positions of the *tnaA*-specific outer and inner primers used for RLM-RACE are indicated by arrows.

total RNA preparations that had not been first reverse transcribed, demonstrating that the RT-PCR products were not derived from contaminated chromosomal DNA. The data also showed expression of *orfX*, which has not been recognized as an ORF in the database (Fig. 1b).

The transcription start site of *pg1401* was determined by RLM-RACE using total RNA extracted from *P. gingivalis* W83 grown to the early exponential phase. PCR fragments amplified with inner primers were subjected to DNA sequencing of the upstream region up to the transcription start site of *pg1401*. The junction of the adaptor with *P. gingivalis* DNA is the 5' end of the transcript. As shown in Fig. 1(c), one clear ending sequence corresponding to a position 52 bp upstream of the first nucleotide of the translation initiation codon was obtained by sequencing of the 5' RLM-RACE PCR product in all ten plasmids analysed.

The tryptophanase of *P. gingivalis* W83 is encoded by *pg1401*

To determine whether the product of the *pg1401* gene functions as a tryptophanase, a mutant *P. gingivalis* W83 strain, in which the 1.4 kb gene was replaced by the 0.7 kb *cat* gene, was constructed (Fig. 2a). The DNA fragment for transformation was prepared using overlap extension PCR. Integration of the PCR products at the expected location in the chromosome of the resulting mutant strain, designated KO100, was confirmed by Southern blot analysis (Fig. 2b). HPLC analysis demonstrated that incubation of crude extract from KO100 with L-tryptophan showed no indole production (Fig. 3). Similarly, a *pg1401*-restored strain, designated RS101, was constructed from strain KO100, in which the *cat* gene was replaced by *pg1401* linked to the *ermF-ermAM* cassette (Fig. 2a). As expected, the ability of

Fig. 2. Construction of derivatives of *P. gingivalis* W83. (a) Chromosomal gene arrangement of the *tnaA* region in the parental and mutant strains. The size of each fragment in bp is shown. (b) Verification of the derivatives by Southern blot hybridization. Each chromosomal DNA was digested with *SmaI*. Hybridizations with the *tnaA* gene probe (left panel), the *cat* gene probe (centre panel) and the *ermF* probe (right panel) were carried out at 60 °C for 6 h. Lanes: 1, *P. gingivalis* W83; 2, *P. gingivalis* KO100; 3, *P. gingivalis* RS101. The positions of DNA size standards (in kbp) are shown.

crude extracts to produce indole from L-tryptophan was restored by replacement of the *cat* gene with *pgl401*. These results confirm that the *pgl401* gene encodes tryptophanase, the only enzyme associated with indole production in *P. gingivalis*. Based on these results, the *pgl401* gene of *P. gingivalis* W83 was redesignated *tnaA*.

Enzymic properties of recombinant TnaA from *P. gingivalis* W83

To characterize the enzymic properties of tryptophanase from *P. gingivalis* W83, recombinant TnaA was purified using the pGEX-6P-1 vector system. All purification steps were monitored by SDS-PAGE analysis, as shown in Fig. 4. The protein obtained was judged to be homogeneous in an SDS-PAGE gel stained with Coomassie brilliant blue. The molecular mass of the denatured polypeptide agreed well with the predicted molecular mass (52.5 kDa). The quaternary structure of the recombinant protein might be a heterooligomer consisting of subunits derived from both *P. gingivalis* and *E. coli* tryptophanase, since the host *E. coli* BL21 produces its own tryptophanase. However, this possibility was excluded by protein sequence analysis,

Fig. 3. Reversed-phase HPLC profiles of indole production from L-tryptophan. An aliquot (20 µl) of toluene that had been layered on the reaction mixture was injected onto a column with L-tyrosine as an internal standard. The reaction mixtures contained (a) crude extract of *P. gingivalis* W83, (b) crude extract of *P. gingivalis* KO100, (c) crude extract of *P. gingivalis* RS101 or (d) purified recombinant TnaA of *P. gingivalis* W83. Arrows indicate the elution positions of indole and tyrosine.

which showed that the N-terminal amino acid sequence of the protein obtained was GPLGSEL. The first five amino acids (i.e. GPLGS) are derived from the cloning site of pGEX-6P-1, whereas the remaining two correspond to the second and third amino acids of *P. gingivalis* tryptophanase. The forward primer used for amplification of *P. gingivalis* *tnaA* (Table 1) did not contain the start codon so that the GST-TnaA fusion protein was produced. In contrast, no findings suggesting that the protein contained subunits from *E. coli* tryptophanase were obtained, indicating that the recombinant tryptophanase of *P. gingivalis* W83 formed a homooligomer. Indole production by the recombinant enzyme was confirmed by HPLC (Fig. 3d).

The kinetic activity of the recombinant TnaA protein was spectrophotometrically evaluated by determination of its production of indole from L-tryptophan. When potassium

Fig. 4. SDS-PAGE analysis of GST-TnaA protein expression and purification. Recombinant protein was purified by affinity chromatography on glutathione-Sepharose 4B resin and digested with PreScission protease. Lane 1, total proteins from sonicated *E. coli* BL21 cells harbouring pGEW101-1 (no induction). Lane 2, total proteins from sonicated BL21 cells harbouring pGEW101-1 (induction by IPTG). Lane 3, total proteins from sonicated BL21 cells, after loading on affinity resin. Lane 4, flowthrough following PreScission protease digestion of GST-TnaA fusion protein bound to glutathione-Sepharose 4B. Each protein sample (approx. 5 µg) was subjected to SDS-PAGE and stained with Coomassie brilliant blue. Positions of molecular mass markers (in kDa) are shown.

phosphate buffer was used in the enzymic reaction, the K_m and k_{cat} values of the protein were 0.20 ± 0.01 mM and 1.37 ± 0.06 s⁻¹, respectively. When the reaction was performed in sodium phosphate buffer, the values were 0.09 ± 0.01 mM and 0.24 ± 0.01 s⁻¹, respectively. Thus, the enzymic activity of TnaA from *P. gingivalis* W83 is much higher in the presence of K⁺ than Na⁺. On the basis of these results, the following enzymic characterization experiments were performed using potassium phosphate buffer with the active holoform of TnaA.

The effects of pH and temperature on the enzymic activity of TnaA were determined by using L-tryptophan as a substrate (Fig. 5). The activity of the purified enzyme was highest at pH 7.5. The enzyme was stable up to 40 °C, but only 40% of the activity remained at 60 °C. The enzyme was completely inactivated at temperatures above 70 °C.

To identify substrates other than L-tryptophan for the recombinant TnaA of *P. gingivalis*, several other compounds were assayed. The specificity of the purified enzyme to various substrates is shown in Table 2. In the reactions, enzymic degradation of the substrates invariably resulted in the formation of pyruvate. Thus, the breakdown of substrates other than L-tryptophan was determined by assaying the production of pyruvate. Degradation of L-tryptophan was measured as described above, because indole inhibits the assay using 3-methyl-2-benzothiazoli-

Fig. 5. Effect of pH (a) and temperature (b) on indole formation from L-tryptophan by the recombinant TnaA of *P. gingivalis* W83. To examine the effect of pH the enzyme was incubated with 2 mM L-tryptophan at 37 °C for 10 min in 167 mM potassium phosphate. To examine the effect of temperature the same concentration of the enzyme was incubated with 2 mM L-tryptophan for 10 min in 167 mM potassium phosphate (pH 7.5). The results shown were obtained from two independent experiments.

none hydrazone to detect pyruvate formation. The kinetic parameters of *P. gingivalis* TnaA for S-ethyl-L-cysteine were comparable with those for L-tryptophan. In contrast, the k_{cat} and k_{cat}/K_m values of the enzyme for S-methyl-L-cysteine were much lower than those for L-tryptophan. The enzyme had no detectable elimination activity with L-alanine, L-serine or L-cysteine as substrate.

Quaternary structure of recombinant TnaA from *P. gingivalis* W83

We hypothesized that the difference in enzymic activity of *P. gingivalis* TnaA in the presence of different monovalent cations (K⁺ and Na⁺) (Table 2) could be due to a structural change in the enzyme. To evaluate this hypothesis, the quaternary structures of the apo- and holoform of *P. gingivalis* TnaA in the presence of each cation were examined by gel-filtration chromatography (Fig. 6a). In contrast to the results predicted by the hypothesis, no matter which buffer (potassium or sodium

Table 2. Steady-state kinetic parameters for TnaA from *P. gingivalis* W83

The reaction was performed in the presence of 200 mM potassium phosphate buffer (pH 7.5). The values calculated from the reaction mixtures containing 200 mM sodium phosphate buffer (pH 7.5) instead of potassium phosphate buffer are shown in parentheses. Values are given as the means $\pm$ SD of three independent experiments. No detectable elimination activity with L-alanine, L-serine or L-cysteine as substrate was observed.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
L-Tryptophan	0.20 ± 0.01 (0.09 ± 0.01)	1.37 ± 0.06 (0.24 ± 0.01)	6.87 ± 0.1 (2.53 ± 0.09)
S-Methyl-L-cysteine	2.04 ± 0.18	0.50 ± 0.05	0.25 ± 0.004
S-Ethyl-L-cysteine	0.26 ± 0.01	1.87 ± 0.03	7.15 ± 0.17

phosphate) was used for dialysis and the mobile phase, both forms of the enzyme eluted at approximately 205.8 kDa, as estimated using a standard curve made with commercially available proteins. This size corresponds to the tetramer of *P. gingivalis* TnaA. These results showed that the quaternary structure of *P. gingivalis* TnaA was not affected by K^+ or Na^+ .

The protein samples used for gel-filtration chromatography were also scanned by spectrophotometric absorption spectra. The holoenzyme in the potassium phosphate and sodium phosphate buffers displayed a characteristic absorption spectrum common to PLP-dependent enzymes, with band maxima at 337 and 423 nm. These maxima were not observed in the spectra of the apoenzyme. The type of phosphate buffer did not affect the absorption spectra of *P. gingivalis* TnaA, suggesting that the cation (K^+ or Na^+) had no significant effect on the binding of PLP. The difference of phosphate buffer did not have marked effects on the spectra of PLP (data not shown).

DISCUSSION

Tryptophanase degrades tryptophan into pyruvate, ammonia and indole. Of those products, the former two molecules can be used as a carbon or nitrogen source in cells, whereas indole is secreted from the cells. Indole at very high concentrations (5 mM) is toxic to microorganisms, including *E. coli*, possibly by causing membrane changes that result in the generation of superoxide (Garbe *et al.*, 2000). However, the toxic concentration of indole is approximately 15-fold higher than the physiological concentration observed in stationary-phase supernatants of *E. coli*. Interestingly, it has been shown that indole has an effect on physiological and pathological functions in bacteria (Anyanful *et al.*, 2005; Hirakawa *et al.*, 2005). Our objective in this study was to determine the molecular basis of indole production in *P. gingivalis*.

The *tnaA* and *tnaB* genes constitute the *tna* operon in *E. coli* (Deeley & Yanofsky, 1981), *Pr. vulgaris* (Kamath & Yanofsky, 1992), *Ent. aerogenes* (Kawasaki *et al.*, 1993), and *H. influenzae* (Martin *et al.*, 1998). *In vitro* transcription experiments located the presumptive *tna* promoter at more

than 300 nt from the *tnaA* initiation codon in *E. coli*. This promoter requires a cAMP-CAMP receptor protein (CRP) for activity (Deeley & Yanofsky, 1982). The long transcribed leader region between the promoter and *tnaA* includes a coding region for a short leader peptide, designated TnaC. This peptide plays an essential role in the full induction of the *tna* operon, as well as in setting the basal uninduced level of *tna* expression (Stewart & Yanofsky, 1986). In contrast, no nucleotide sequences homologous to *tnaB*, *tnaC* or the CRP-binding region are present around *tnaA* in *P. gingivalis*. In addition, the gene does not consist of an operon with any genes. These findings suggest that the gene arrangement around *tnaA* in *P. gingivalis* is unique and that the expression of *tnaA* is controlled by a mechanism distinct from that in *E. coli* and other bacteria. The tryptophanase of *E. coli* is over-expressed and becomes one of the most abundant proteins when cells experience alkaline stress (Blankenhorn *et al.*, 1999). The transcriptional data presented here would be helpful for further research along these lines.

When potassium phosphate buffer was used in the reaction mixture, the K_m value (0.20 ± 0.01 mM) of TnaA from *P. gingivalis* W83 for L-tryptophan was slightly lower than those from the other bacteria, including *E. coli* B (0.32 mM) (Newton *et al.*, 1965; Watanabe & Snell, 1977) and *Bacillus alvei* (0.27 mM) (Hoch *et al.*, 1966), indicating that the affinity of *P. gingivalis* tryptophanase to L-tryptophan was comparable with those of *E. coli* and *B. alvei*. Its k_{cat} and k_{cat}/K_m values ($1.37 \pm 0.06 s^{-1}$ and $6.87 \pm 0.1 mM^{-1} s^{-1}$) was also less than those in *E. coli* ($6.8 s^{-1}$ and $30 mM^{-1} s^{-1}$) (Phillips & Gollnick, 1989) and *Pr. vulgaris* ($6.3 s^{-1}$ and $23 mM^{-1} s^{-1}$) (Demidkina *et al.*, 2003). Thus, the enzymic activity of tryptophanase from *P. gingivalis* was lower than that from *E. coli* and *Pr. vulgaris*. This investigation also demonstrated that *P. gingivalis* TnaA still has a weak enzymic activity in the presence of Na^+ , although NaCl showed an inhibitory effect on *P. gingivalis* TnaA (data not shown). In contrast, *E. coli* tryptophanase had almost no enzymic activity in the presence of only Na^+ (Honda & Tokushige, 1986). Comparison of the K_m values did not suggest that Na^+ lowers the affinity of the substrate to the enzyme. Since the cation in the buffer, K^+ or Na^+ , did not appear to affect

Fig. 6. Effect of K^+ and Na^+ on the structure of TnaA from *P. gingivalis* W83. (a) Gel-filtration chromatography. The apoform of *P. gingivalis* TnaA was eluted with potassium or sodium phosphate buffer at pH 7.5, the same buffer as used for dialysis to prepare the apoenzyme. The holoform of *P. gingivalis* TnaA was eluted with potassium or sodium phosphate buffer at pH 7.5 with PLP, the same buffer as used for preparation of the holoenzyme. Arrows indicate the predicted elution positions of the tetramer (210 kDa) and dimer (105 kDa). The flow rate was 0.5 ml min⁻¹. (b) Absorption spectra of recombinant TnaA of *P. gingivalis* W83 (1.5 mg ml⁻¹) in 20 mM potassium (left panel) or sodium (right panel) phosphate buffer at pH 7.5. Each protein sample was the same as used for gel-filtration chromatography. ○, Holoenzyme in buffer containing PLP; □, apoenzyme in buffer without PLP.

either the quaternary structure of TnaA from *P. gingivalis* W83 or the binding of PLP to the apoenzyme (Fig. 6), K^+ may function as an activator for the catalysing enzyme. Honda & Tokushige (1986) reported that NH_4^+ is an active cation for *E. coli* tryptophanase in addition to K^+ . Morino & Snell (1967) reported that dissociation of the tetrameric holoenzyme of *E. coli* TnaA into the dimeric apoenzyme was accelerated by K^+ , and that in the absence of the cation the apoenzyme remained tetrameric even at low temperatures. However, Erez *et al.* (1998) concluded that

incubation of the tetrameric form of *E. coli* TnaA in potassium phosphate buffer at 5 °C led to the conversion of approximately 24% of the protein into a dimeric form. Contrary to these conflicting reports, we did not observe dissociation of *P. gingivalis* TnaA into the dimeric form in the presence or absence of K^+ at 4 °C. The findings suggest that the quaternary structure of TnaA from *P. gingivalis* W83 is more stable in the absence of K^+ than that from *E. coli*. X-ray crystal structural analyses demonstrated that the tryptophanases from *E. coli* and *Pr. vulgaris* contain K^+ -binding sites close to the active site, and that K^+ ions interact with the O^ε atom of Glu-72 in one subunit and the backbone carbonyl O atoms of Gly-55 and Pro-275 (Isupov *et al.*, 1998; Ku *et al.*, 2006). *P. gingivalis* TnaA contained Glu-72 and Gly-55, even though Pro-275 was replaced by Asn. Structural studies are necessary to understand the stability of *P. gingivalis* TnaA in the presence or absence of K^+ .

In conclusion, the molecular basis of indole production in *P. gingivalis* was elucidated. Transcripts of *P. gingivalis* *tnaA* and the encoded protein were characterized. However, the expression mechanism of the gene, which appears to be different in *P. gingivalis* and other bacteria, remains to be elucidated. Further studies of the expression of *P. gingivalis* *tnaA* are under way to clarify the relationship between indole production and the physiological and pathological functions of this micro-organism.

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