A *murC* gene in *Porphyromonas gingivalis* 381

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The gene encoding a 51 kDa polypeptide of *Porphyromonas gingivalis* 381 was isolated by immunoblotting using an antiserum raised against *P. gingivalis* alkaline phosphatase. DNA sequence analysis of a 2.5 kb DNA fragment containing a gene encoding the 51 kDa protein revealed one complete and two incomplete ORFs. Database searches using the FASTA program revealed significant homology between the *P. gingivalis* 51 kDa protein and the MurC protein of *Escherichia coli*, which functions in peptidoglycan synthesis. The cloned 51 kDa protein encoded a functional product that complemented an *E. coli murC* mutant. Moreover, the ORF just upstream of *murC* coded for a protein that was 31% homologous with the *E. coli* MurG protein. The ORF just downstream of *murC* coded for a protein that was 17% homologous with the *Streptococcus pneumoniae* penicillin-binding protein 2B (PBP2B), which functions in peptidoglycan synthesis and is responsible for antibiotic resistance. These results suggest that *P. gingivalis* contains a homologue of the *E. coli* peptidoglycan synthesis gene *murC* and indicate the possibility of a cluster of genes responsible for cell division and cell growth, as in the *E. coli mra* region.

Keywords: *Porphyromonas gingivalis*, *murC*, peptidoglycan synthesis, alkaline phosphatase

INTRODUCTION

Porphyromonas gingivalis, a Gram-negative, anaerobic, rod-shaped bacterium, has been isolated from lesions of advanced adult periodontitis (Slots, 1977; Tanner *et al.*, 1979) and implicated as a periodontal pathogen. The detailed pathogenic mechanism of *P. gingivalis* infections is not yet known. This organism produces a variety of potential virulence factors, including alkaline phosphatase (ALPase) (Slot & Genco, 1984). A previous study showed that ALPase in the cell-associated form of *P. gingivalis* strains exists in the periplasmic space and is released into the culture medium (Minhas & Greenman, 1989). We have purified the ALPase of *P. gingivalis* 381 to homogeneity, characterized it and raised a polyclonal antibody against it (Yamashita *et al.*, 1990).

During attempts to clone the ALPase gene, we discovered a cluster of genes which appear to be involved in peptidoglycan synthesis. To our knowledge, nothing is

known about the cell wall synthetic machinery in oral bacteria. No genetic study of the enzymes for peptidoglycan synthesis of an oral bacterium has been reported. Here, we report the complete sequence of the *P. gingivalis murC* gene.

METHODS

Bacterial strain and plasmids. *P. gingivalis* strain 381 was grown anaerobically [5% (v/v) CO₂, 10% (v/v) H₂, 85% (v/v) N₂] at 37 °C for 2 d in brain heart infusion broth (Difco) supplemented with yeast extract (0.5%), haemin (5 µg ml⁻¹) and menadione (0.5 µg ml⁻¹). *Escherichia coli* JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiD Δ(lac-proAB)* (F' *traD36 proAB⁺lacI⁺lacZ M15*)] was used as the host strain for pBluescript II SK(+) and KS(+) sub-clones. *E. coli* strains were grown aerobically in LB broth at 30 °C with shaking (200 r.p.m.). The selective medium contained 50 µg ampicillin ml⁻¹. The *E. coli murC* mutant, ST 222 (F⁻ *thr trp his thy thi lac gal xyl mtl str A azf*) (Miyakawa *et al.*, 1972) was used for complementation tests. This strain was grown in modified Lennox broth containing 20 mg thymine l⁻¹, as described by Ishino *et al.* (1989).

Preparation and screening of gene library. Chromosomal DNA was isolated from *P. gingivalis* by a modification of the method of Marmur (1961). Briefly, bacterial cells were grown to late exponential phase were lysed in 1.0% (w/v) SDS containing

Abbreviations: ALPase, alkaline phosphatase; MurNAc, *N*-acetylmuramic acid; PBP, penicillin-binding protein.

The GenBank accession number for the nucleotide sequence reported in this paper is D28916.

50 mM NaCl, 50 mM Tris/HCl and 100 mM EDTA (pH 7.5). RNA and protein were removed by successive treatment with RNase A ($1 \mu\text{g ml}^{-1}$) and pronase (0.5 mg ml^{-1}), and then the high-molecular-mass DNA was further purified by repeated phenol extraction. Chromosomal DNA of *P. gingivalis* 381 was partially digested with *Sau*3AI and size-fractionated on a 5–20% (w/v) sucrose gradient in 1 M NaCl/20 mM Tris (pH 8.0)/5 mM EDTA by centrifugation at $100\,000 \text{ g}$ for 21 h at 4°C . DNA in the range 5–10 kb was ligated to pBluescript II SK(+) which had been cut with *Bam*HI and treated with bacterial alkaline phosphatase (Takara Shuzo). The ligated DNA was transformed into competent *E. coli* JM109 cells by electroporation (*E. coli* Pulser; Bio-Rad), and the colonies selected by plating on LB agar plates containing $50 \mu\text{g}$ ampicillin ml^{-1} . A single bacterial colony was grown in 3 ml of LB broth containing $50 \mu\text{g}$ ampicillin ml^{-1} until the OD_{550} reached 0.5. IPTG was then added to a final concentration of 0.4 mM, and the cultures were incubated for an additional 3 h to induce protein synthesis. Restriction endonucleases (Takara Shuzo) were used as recommended by the suppliers.

Antibody preparation. Specific polyclonal antibodies against purified *P. gingivalis* ALPase were raised in a rabbit as previously described (Yamashita *et al.*, 1990). The IgG fraction was purified using an Affi-gel protein A MAPS-II kit (Bio-Rad) according to the method of Yamashita *et al.* (1990). The antibodies were used to identify immunoreactive clones in colony blotting and to perform immunoblot analysis. The antiserum was absorbed with sonicated *E. coli* JM109 cells containing the vector grown in LB broth (Maniatis *et al.*, 1982).

Colony blotting. Transformed bacterial colonies were transferred directly from agar plates onto dry nitrocellulose filters and lysed as described by Meyer *et al.* (1982). Blotting was done according to the methods of Hawkes *et al.* (1982).

Immunoblot analysis. Antigens were electrophoretically transferred onto a nitrocellulose filter immediately after SDS-PAGE by the method of Laemmli (1970). Kaleidoscope-prestained standards (Bio-Rad) were run on each gel for estimation of molecular size. Nitrocellulose sheets were blocked in TBS (0.05 M Tris/0.2 M NaCl, pH 7.5) containing 3% gelatin for 1 h at room temperature and then incubated for 2 h at room temperature with rabbit antiserum against purified *P. gingivalis* ALPase diluted 1:500 in TTBS (TBS containing 0.05% Tween 20) containing 1% gelatin. The nitrocellulose sheets were incubated for an additional 1 h with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:3000 in TTBS containing 1% gelatin. Bound IgG was visualized in TBS containing 0.05% 1-chloro-4-naphthol, 16% (v/v) methanol, and 0.015% hydrogen peroxide.

Purification of recombinant protein. Cells from a 1 l overnight culture of transformed *E. coli* were harvested by centrifuging at $10\,000 \text{ g}$ for 20 min. The cells were washed twice with TBS buffer, suspended in 50 mM Tris/HCl, pH 8.2, and disrupted by sonication. The resulting sonicate was centrifuged at $100\,000 \text{ g}$ for 40 min and the supernatant fraction was collected as the crude enzyme extract. The crude enzyme extract was applied to a DEAE-cellulose column ($6 \times 20 \text{ cm}$) that had been equilibrated with 50 mM Tris/HCl buffer, pH 8.2. The column was washed with the equilibration buffer until no protein was detected in the effluent by A_{280} , and then the column was eluted with a linear 0–1.0 M NaCl gradient in 1 l of the same buffer. Fractions containing the 51 kDa protein were confirmed by the dot-blotting method, and were then pooled and concentrated by filtration through a Centricon-30 ultrafilter (Amicon). The concentrated solution was applied to a hydroxyapatite column

($1.6 \times 16 \text{ cm}$) that was equilibrated with 5 mM potassium phosphate buffer, pH 8.2. The column was eluted with a linear 5–200 mM potassium phosphate buffer gradient. Fractions containing the 51 kDa protein were confirmed by the dot-blotting method with immunodetection and were pooled and concentrated by ultrafiltration. The enzyme was next applied to a Superdex 200 HR 10/30 gel filtration column (Pharmacia). Fractions containing the 51 kDa protein were confirmed by the dot-blotting method, pooled and concentrated by ultrafiltration, and used as the purified enzyme.

DNA manipulations. The isolation of plasmid DNA, endonuclease restriction and ligation were carried out as previously described (Yamashita *et al.*, 1993). DNA fragments were routinely analysed on 0.7 or 2.0% (w/v) agarose gels (SeaKem GTG agarose, FMC BioProducts) as described by Maniatis *et al.* (1982). Southern blot analysis was performed as previously described (Yamashita *et al.*, 1993) using digoxigenin-labelled probes according to the instructions of the suppliers (Boehringer Mannheim).

DNA sequence analysis. The plasmids pBluescript II KS(+) and SK(+) (Stratagene) were used as vectors in preparation of unidirectional deletion plasmids for sequencing. Unidirectional deletion subclones were prepared by exonuclease III/mung bean nuclease digestion with appropriate sets of restriction enzymes. A single-stranded template DNA was isolated. The nucleotide sequences were determined using the dideoxy method (Sanger *et al.*, 1977) with a fluorescent primer cycle sequencing kit (Applied Biosystems) and a model 381 automated sequencer (Applied Biosystems). Approximately 300 bases were read from each sample. The nucleotide sequences were analysed with the computer software package 'DNA strider v.1.2' (Marck, 1988). The Protein Identification Resource and the Swiss Protein Source were searched for proteins that share homology with the deduced amino acid sequence of the 51 kDa protein of *P. gingivalis* 381 using the FASTA program (Pearson & Lipman, 1988).

RESULTS AND DISCUSSION

Cloning of the gene encoding a 51 kDa protein and immunoblot analysis

An initial screening was performed by the colony blotting method using a polyclonal antibody raised against the purified ALPase of *P. gingivalis* strain 381. More than

Fig. 1. Restriction endonuclease map of the *P. gingivalis* DNA fragment in pAL1 and pAL13, and in subclones pAL12 and pAL13-2. All clones are derivatives of pBluescript II SK(+). These clones are aligned beneath the restriction sites used for construction. Restriction enzymes: B, *Bam*HI; C, *Nco*I; E, *Eag*I; H, *Hind*III; Hi, *Hinc*II; N, *Nsi*I; P, *Pst*I; R, *Eco*RI; S, *Sac*II; Sa, *Sau*3AI; V, *Eco*RV. Arrows indicate direction of transcription.

Fig. 2. (a) SDS-PAGE analysis of proteins from recombinant *E. coli*. Lanes: 1, crude extract of *E. coli* JM109 harbouring pAL13 (induced with IPTG); 2, purified 51 kDa protein of *E. coli* JM109 harbouring pAL13; M, prestained molecular mass standards. Ten microlitres of sample was loaded in each lane. (b) Immunoblot analysis of the expressed 51 kDa protein cross-reactive with anti-*P. gingivalis* ALPase antiserum. Lanes: 1, purified ALPase from *P. gingivalis* 381 (molecular mass 61 kDa) (Yamashita *et al.*, 1990); 2, purified 51 kDa protein from *E. coli* JM109 harbouring pAL13; M, prestained molecular mass standards. Ten microlitres of sample was loaded in each lane.

10000 transformants were screened but only two clones reacted strongly with the *P. gingivalis* ALPase polyclonal antibody. These clones were designated pAL1 and pAL2. Immunoblot analysis showed that pAL1 and pAL2 expressed proteins of approximately 47 and 40 kDa, respectively. These clones, which exhibited no ALPase activity, did not react at all with preimmunization sera. Initially, clone pAL1, which reacted more strongly with antibody, was selected for further study (Fig. 1). Details of the second clone, pAL2, will be reported elsewhere. Subclone pAL12, which was constructed by deleting the *PstI*-*EcoRI* (one site in the polylinker) DNA fragment, still expressed the 47 kDa protein. Nucleotide sequence analysis of the *Bam*HI/*Sau*3AI-*PstI* fragment in subclone pAL12 revealed a 1278 bp incomplete ORF (bases 432–1709 in Fig. 3) with a calculated molecular mass of 47 394 Da. These results suggested that an in-frame gene fusion under *lac* control had been fortuitously constructed and that this sequence was missing the 5' end of the gene. The 1.5 kb *Bam*HI/*Sau*3AI-*Sac*II fragment within pAL12 was therefore used to synthesize a digoxigenin-labelled probe. This probe was used to identify complete-ORF-containing fragments in genomic digestions of *P. gingivalis*. After the second round of screening by colony blotting, a transformant containing a 1.9 kb *NsiI*/*PstI*-*NsiI* fragment was isolated and designated pAL13 (Fig. 1).

pAL13 encodes a 51 kDa protein

Fig. 2(a) shows the SDS-PAGE analysis of the purified 51 kDa protein from recombinant *E. coli* harbouring pAL13 induced with IPTG. Fig. 2(b) shows the immunoblot analysis using the polyclonal antibody against purified *P. gingivalis* ALPase. Immunoblotting of the purified protein from transformant pAL13 separated by SDS-PAGE consistently identified a protein band of molecular mass approximately 51 kDa reacting with the anti-ALPase serum. A corresponding band was not present in the extract of *E. coli* JM109 containing pBluescript II SK(+). These findings suggest either that the 51 kDa protein and 61 kDa ALPase share immunologically similar domains

and contain common antigenic epitopes, or that the preparation of ALPase used to raise antiserum contained some of the 51 kDa protein. We cannot at present distinguish between these possibilities.

Nucleotide sequence and identification of ORFs

The *PstI*-*XbaI* (vector) fragment of pAL12 was recloned into pBluescript II KS(+) to construct pAL12 KS, which was used to prepare unidirectional deletion plasmids for sequencing in a reverse direction. A series of nested deletion plasmids were generated from both the *SaI*I and *Eag*I sites in pAL12 and pAL12KS, respectively. For sequencing of approximately 500 bp downstream from the *NsiI*/*PstI* site, the synthetic primers pAL13-2 and pAL13KS were used (Fig. 1). Plasmid pAL13KS was constructed by recloning of the *XbaI* (vector)-*SaI*I (vector) fragment into pBluescript II KS(+). Since the coding region was sequenced on both strands by using overlapping fragments, we had no indication of a sequencing error. This sequence contains one complete and two incomplete ORFs (Fig. 3). A long ORF was identified which contained 1371 bp coding for a putative protein with 457 amino acids (with a calculated molecular mass of 50 801 Da). A potential RBS, ACGAGAA, was identified as starting at base position 320. Two additional ORFs were identified downstream (LORF, initiated at base position 1727) and upstream (UORF) from ORF. A potential RBS for LORF, AGAAGGT, was identified starting at base position 1710, upstream from CTG, the proposed start codon (Stormo, 1986). The ACGAGAA and the AGAAGGT sequences showed homology to the 3' end of *Bacteroides fragilis* 16S rRNA. In particular, the latter sequence was identical to the RBS in the fimbriin gene of *P. gingivalis* 381 (Dickinson *et al.*, 1988).

Amino acid sequence comparison of ORFs

The deduced amino acid sequences of the ORFs were compared with sequences of other proteins in the European Molecular Biology Laboratory database. As

Fig. 3. Nucleotide sequence of the *P. gingivalis* homologue of the *E. coli* *murC* gene. The complete nucleotide sequence determined is presented. The deduced amino acid sequences of the three ORFs located in this region, UORF (bases 1–338), ORF (bases 339–1709) and LORF (bases 1727–2455) are shown. The deduced positions and amino acid sequences of the *P. gingivalis* *murC* gene product are shown. RBSs are underlined. The translation and termination codons are marked with asterisks. Relevant restriction sites are indicated above their corresponding recognition sequences.

shown in Fig. 4, the ORF gene product exhibited significant homology (31 % identical amino acid residues and 72 % conservative changes) with the *E. coli* MurC protein and included the ATP-binding domain GXXGKT (Ikeda *et al.*, 1990) (positions 114–119 of the ORF). The ORF of *murC* in *E. coli* is 1473 bp in length and encodes a peptide of 491 amino acid residues (with a

Fig. 4. Homology between the deduced amino acid sequence of the ORF gene product and the MurC protein from *E. coli*. Amino acids are numbered on the right. Asterisks and dots indicate identical amino acid residues and conservative changes, respectively.

Fig. 5. Homology between the amino acid sequence of the UORF gene product and the *E. coli* MurG protein (a), and between the LORF gene product and PBP2B protein from *S. pneumoniae* (b). Asterisks and dots indicate identical amino acid residues and conservative changes, respectively.

calculated molecular mass of 53625) (Ikeda *et al.*, 1990). Significant homology (31 % identical amino acid residues and 73 % conservative changes) was also found between the UORF gene product and the *E. coli* MurG protein, which functions in lipid II biosynthesis (Fig. 5a). Finally, significant similarity (17 % identical amino acid residues

murC⁻/pBS*murC*Δ *murC*⁻/pBS*murC*⁺

(a)

(b)

Fig. 6. Complementation of the *ts* growth defect of an *E. coli* *murC* mutant with the *P. gingivalis* gene. Eight transformants of the *murC* mutant strain ST222 with pBluescript SK(+) (left) or the *murC*-containing clone pAL13 (right) were streaked on modified Lennox plates supplemented with ampicillin. The plates were incubated at 30 °C (a) or 42 °C (b) for 26 h.

and 70 % conservative changes) was detected between the LORF gene product and PBP2B from *Streptococcus pneumoniae*, which functions in peptidoglycan synthesis and is responsible for antibiotic resistance (Fig. 5b). The final steps of peptidoglycan synthesis are known to be catalysed by PBPs (Nanninga, 1991; Waxman & Strominger, 1983).

Complementation of *E. coli* *murC* mutation by *P. gingivalis* *murC* gene

Fig. 6 shows complementation of the *ts* growth defect of an *E. coli* *murC* mutant with the *P. gingivalis* gene. Strain ST 222 was transformed with pBluescript II SK(+) (left sector) and pAL13 (right sector) and plated at 30 and 42 °C. Plasmid pAL13 was a derivative of pBluescript II SK(+) harbouring the entire *P. gingivalis* *murC* gene. Plasmid pAL13 was able to rescue the *ts* growth defect for ST 222 (right sector at 30 and 42 °C). However, when this strain was transformed with pBluescript II SK(+), complementation of the *ts* growth defect was not observed (Fig. 6, compare left sector at 30 and 42 °C). These data demonstrate that the *P. gingivalis* gene encodes an active product that confers the MurC⁺ phenotype in *E. coli*.

Fig. 7 shows the alignments of the genes of the *murG*-*pbp2b* region of *P. gingivalis* and *mra* region of *E. coli*. The *murG* and *murC* genes are also adjacent in *E. coli*, although the spacing between them is different: the spacing in

Fig. 7. Comparison of clusters of genes in the *mra* region of *E. coli* chromosome with the *murG*-*pbp2b* region of *P. gingivalis*. Open arrows indicate homologous genes. Lengths and distances are arbitrary.

E. coli is 53 bp, but there is no spacing in *P. gingivalis*. The next gene in *E. coli*, *ddlB* (coding for D-alanine:D-alanine ligase), does not resemble the downstream partial sequence in the cloned *P. gingivalis* fragment.

The 51 kDa protein showed a high degree of homology with the *E. coli* MurC protein, which functions in cell wall peptidoglycan synthesis. The biosynthesis of the bacterial cell wall peptidoglycan is a complex process (Rogers *et al.*, 1980) and UDP-MurNAc:L-alanine ligase (MurC protein) catalyses the first step in the biosynthesis of the cell wall peptidoglycan. The region of about 12 kb, *mra* (murein synthesis cluster *a*), at 2 min on the *E. coli* chromosome map, contains genes concerned with peptidoglycan synthesis, which have been shown to be tightly clustered forming an operon (Miyakawa *et al.*, 1972).

These results suggest that *P. gingivalis* contains a homologue of the *E. coli* peptidoglycan synthesis gene *murC* and indicate the possibility of a cluster of genes responsible for cell division and cell growth as in the *E. coli* *mra* region. It is important that the *murC* gene of *P. gingivalis*, a periodontopathic bacterium, is isolated. Peptidoglycans have a number of biological activities in mammalian systems and are known to activate the alternative pathway of complement fixation and cause polyclonal B lymphocyte activation (Hunter, 1984). The inhibition of its biosynthesis has either a bacteriostatic effect or, more often, a bactericidal one. Research on genes responsible for peptidoglycan synthesis may lead to a new therapy for periodontal disease.

In conclusion, we have isolated and sequenced a gene encoding the *P. gingivalis* MurC protein. The MurC protein sequences have been found to be conserved between *E. coli* and *P. gingivalis* and it is suggested that the *murC* gene and neighbouring cell wall synthesis genes may be clustered.

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