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Role of Polyphosphate Kinase in Biofilm Formation by *Porphyromonas gingivalis*

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In order to assess the role of polyphosphate kinase (PPK) in the physiology of *Porphyromonas gingivalis*, a *ppk* gene mutant, CW120, was constructed and characterized. *P. gingivalis* was demonstrated to synthesize short-chain polyphosphate (polyP) but not long-chain polyP. CW120 failed to survive in the stationary phase as well as the parental cell did, and it was attenuated in biofilm formation on polyvinylchloride and glass surfaces. Furthermore, the complementation by insertion of an intact copy of the *ppk* gene into the mutant CW120 restored its biofilm formation and stationary-phase survival. These results suggest that PPK may be important for incorporation of these organisms into subgingival plaque in the human oral cavity.

Periodontitis is one of the most common infectious diseases afflicting mankind (35). These infections appear to result from the inflammatory response of the host to mixed anaerobic bacterial infections of the gingival margin. Among the organisms implicated in these diseases, *Porphyromonas gingivalis* has attracted much attention (22). These organisms appear to express a variety of virulence factors that may be important in the etiology of periodontitis (15). Since *P. gingivalis*, along with other pathogenic bacteria in the oral cavity, appears to reside primarily in biofilm structures commonly termed dental plaque, it is important to delineate the molecular basis for biofilm formation by these organisms.

Investigations with several gram-negative bacteria have suggested that the polyphosphate kinase (PPK) gene *ppk* may be a potentially important virulence factor in these organisms (29, 30). This gene is highly conserved among both gram-positive and gram-negative bacteria and appears to play a role in adaptation to nutritional and other environmental stresses as well as stationary-phase survival (26, 34). In addition, *ppk* mutations in *Escherichia coli*, *Salmonella enterica* serovar Dublin, and *Pseudomonas aeruginosa* attenuate swimming, swarming, and twitching motility in these organisms (20, 28–30). Likewise, recent results have suggested an important role for PPK activity in biofilm formation and virulence in *P. aeruginosa* (30). It was therefore of interest to examine the potential role of the *ppk* gene in the properties of *P. gingivalis*. The construction of a specific *ppk* mutant of *P. gingivalis* 381 in the present study demonstrated that this gene plays an important role in stationary-phase survival as well as in biofilm formation in vitro.

Construction and identification of a *ppk*-deficient mutant of *P. gingivalis* 381 (CW120). *P. gingivalis* strains were maintained anaerobically on blood agar plates containing tryptic soy broth (TSB; Difco Laboratory, Detroit, Mich.) supplemented with 10% sheep blood, hemin (5.0 μg/ml), menadione (1.0 μg/ml), and gentamicin (25 μg/ml). *E. coli* strains MG1655 and CF5802 were kindly provided by A. Kornberg, Stanford University School of Medicine (Stanford, Calif.). Plasmid prtT:Em was constructed in our laboratory previously (unpublished results). Plasmids pUC19, Topo/PCR vector, and prtT:Em were maintained in *E. coli* DH5α in the presence of 50 μg of ampicillin per ml. A *ppk* homologous sequence of *P. gingivalis* W83 was identified by searching The Institute for Genomic Research database (http://www.ncbi.nlm.nih.gov) with the amino acid sequence of *E. coli*. A pair of primers, 5′-AAC GAT CAG TAG CAC TGT GG-3′ and 5′-TTA TTT TGC AGC AGG AGT GGC-3′, were designed based upon the sequence of the *ppk* gene of *P. gingivalis* W83 and used for amplifying and cloning a 2.1-kb fragment of the *ppk* gene from strain 381. Inactivation of the *P. gingivalis* 381 *ppk* gene was accomplished following electroporation (36) with an *Em* cassette inserted into the gene following homologous recombination (Fig. 1). The *Em* cassette was introduced into the *Bgl*II sites, which are 703 and 871 bp downstream from the ATG initiation codon and are present within the conserved regions of *ppk*. Since the plasmid pCW111 was linearized, erythromycin-resistant transformants would grow only as a result of a double-crossover event between the regions flanking the *Em* cassette and the *ppk* gene on the chromosome. In order to confirm that the *Em* cassette was inserted into the predicted sites within the *ppk* gene on the chromosome of strain 381, Southern blot analysis (6) was carried out (Fig. 1). Chromosomal DNA from *P. gingivalis* strains was prepared with a Puregene isolation kit (Gentra System, Inc., Minneapolis, Minn.) by following the supplier's protocol. DNA was digested with the restriction enzymes indicated and loaded onto 1% agarose gels for electrophoresis, and the DNA fragments were transferred to nylon membranes (Amersham Corp., Arlington Heights, Ill.) after alkaline denaturation. The labeling of the probes, hybridization, and detection with an enhanced chemiluminescence system were performed as recommended by the supplier (Amersham). Thirty-three erythromycin-resistant colonies were obtained. One of the *ppk* mutants with erythromycin resistance was chosen for further study and designated CW120. The growth rate of CW120 was similar to that of wild-type 381 in TSB medium.

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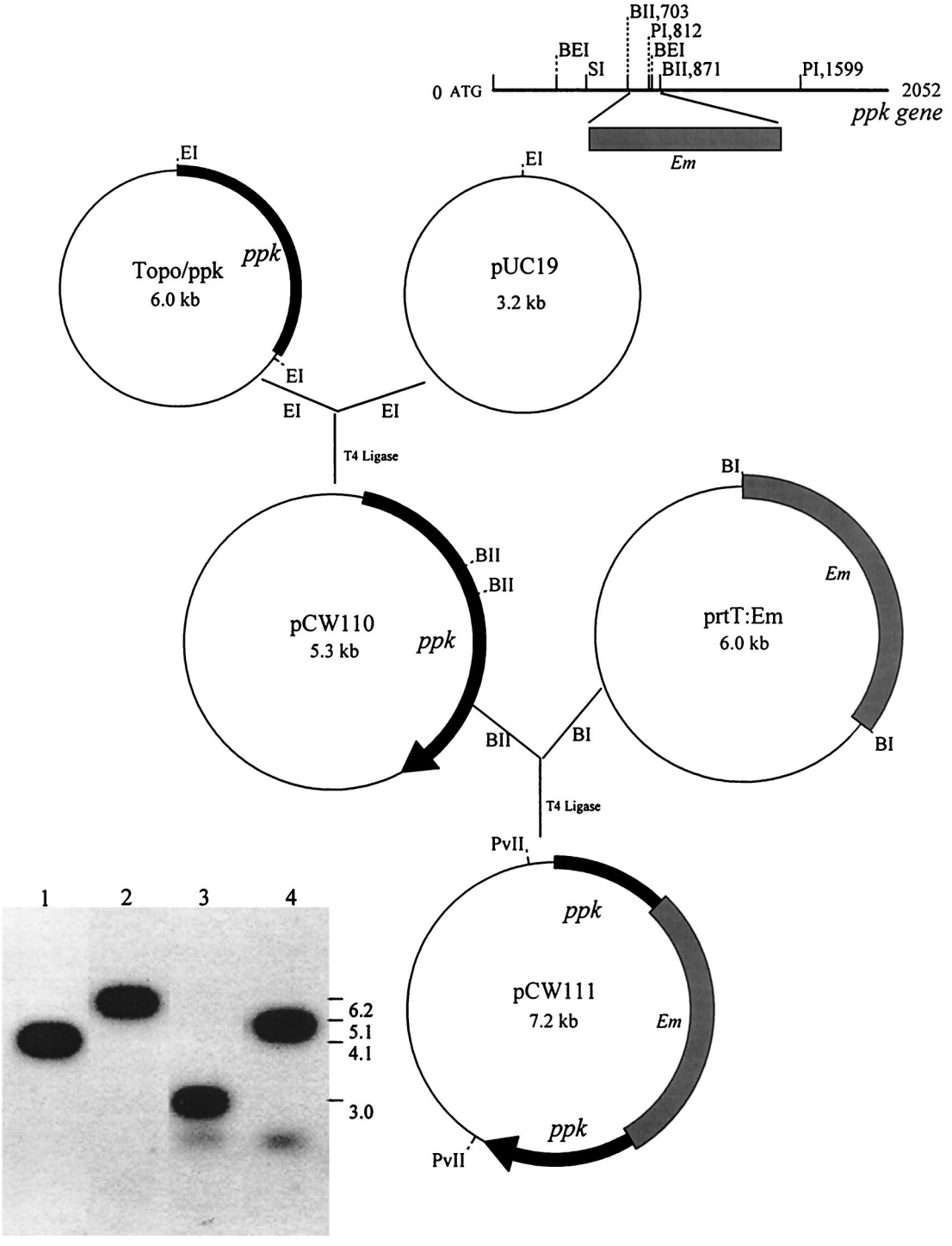

FIG. 1. Construction of the *ppk*-deficient mutant CW120. A 2.1-kb *ppk* fragment amplified by PCR from *P. gingivalis* 381 was ligated into the pCR2.1-TOPO vector. The 2.1-kb fragment was then isolated and inserted into pUC19 following cleavage of plasmid TOPO/*ppk* and pUC19 with *Eco*RI, producing plasmid pCW110. A 2.1-kb *ermF-ermAM Bam*HI-digested cassette from plasmid prtT:Em was next inserted into the *ppk* gene at the *Bgl*II site of pCW110. The resulting plasmid, pCW111, was linearized with *Pvu*II and electroporated into *P. gingivalis* 381. The resulting mutant, CW120, was identified by Southern blot analysis of the genomic DNA of *P. gingivalis* (bottom left). The chromosomal DNA of strains 381 (lanes 1 and 3) and CW120 (lanes 2 and 4) was digested with *Bsp*EI (lanes 1 and 2) or *Stu*I (lanes 3 and 4), respectively, and probed with a 787-bp fragment of the *ppk* gene digested with *Pst*I. BEI, *Bsp*EI; BI, *Bam*HI; EI, *Eco*RI; BII, *Bgl*II; PI, *Pst*I; PvII, *Pvu*II; SI, *Stu*I; *Em*, erythromycin cassette.

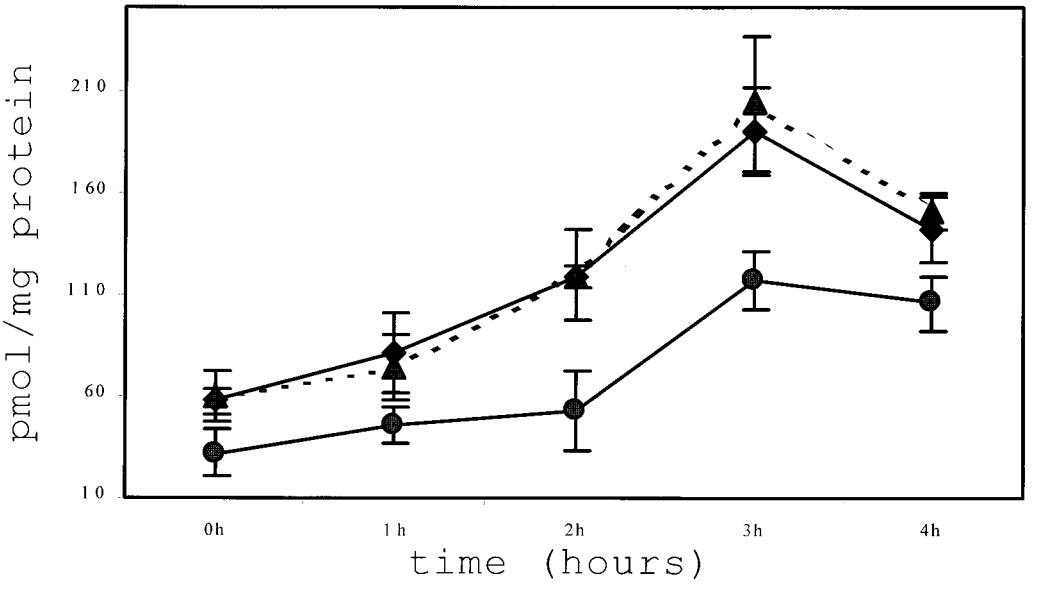

FIG. 2. Short-chain polyP assay of *P. gingivalis* 381, *ppk*-deficient mutant CW120, and complemented strain CW120C. *P. gingivalis* 381 (♦), CW120 (●), and CW120C (▲) were grown to mid-log phase in TSB medium with hemin and menadione. The cells were pelleted, resuspended, and incubated anaerobically in MOPS defined medium containing 0.1 mM P_i, 0.01% BSA, and 4 mg of glucose per ml. The samples were collected at 0, 1, 2, 3, and 4 h. Short-chain polyP was extracted and analyzed as described in the text. The results are averages of quadruple samples and their standard deviations are shown.

Estimation of polyP in *P. gingivalis*. A radioactive assay confirmed the expression of the *ppk* gene in *P. gingivalis* 381, although PPK activity was low (data not shown). In order to compare the PPK activity between the wild-type 381 and mutant CW120, polyphosphate (polyP), the product of PPK, was examined. Bacterial cells (10^7 and $\approx 10^8$ CFU) were pelleted for long-chain and short-chain polyP extraction, respectively. PolyP was assayed with the nonradioactive two-enzymes method described by Ault-Riche at al. (4). Bioluminescence was measured by using a 1450 MicroBeta TriLux counter (Wallac Oy, Turku, Finland). Long-chain polyP (with 60 to several hundred P_i residues) extraction was achieved with milk-glass as described by Ault-Riche et al. (4). As positive and negative controls, *E. coli* strains MG1655 and CF5802 (*ppk* and *ppx* deficient), respectively, were cultured to mid-log phase in Luria-Bertani medium and then transferred into morpholinepropanesulfonic acid (MOPS) medium with 4% glucose and limiting phosphate (0.1 mM P_i) without amino acids. *P. gingivalis* 381 and CW120 were cultured to mid-log phase in TSB medium, transferred to the same prereduced MOPS medium supplemented with hemin and menadione and with or without 0.01% bovine serum albumin (BSA), and incubated anaerobically at 37°C. PolyP was isolated from the samples at different intervals (0 to ≈4 h) and measured (4). PolyP accumulation was observed in MG1655 (350 nmol/mg of protein at 3 h) but not in CF5802. *P. gingivalis* 381 and CW120 were incubated anaerobically in MOPS medium for prolonged time periods to collect samples for up to 24 h, since *P. gingivalis* grew much slower than *E. coli*. However, long-chain polyP was not detectable in *P. gingivalis* 381 or CW120 at any time points (data not shown). The *P. gingivalis* strains were also stimulated with osmotic shock, changes in pH, temperature upshifts, and oxidative stress in TSB. None of these stress conditions could induce detectable accumulation of long-chain polyP. Since bacteria can produce polyP of various chain lengths (7, 17, 32), an assay to detect short-chain polyP (<60 P_i residues) was then carried out. The isolation of short-chain polyP was performed as described by Ruiz et al. (32). Picomolar levels of short-chain polyP were detected from the same bacterial samples. The levels of short-chain polyP from mutant CW120 were decreased to about 50% of that from WT381. The peak of short-chain accumulation (200 pmol/mg of protein) was at 3 h for strain 381. Mutant CW120 also exhibited lower detectable short-chain polyP accumulation (100 pmol/mg of protein). Furthermore, the *ppk*-deficient complemented strain, CW120C (described below), displayed a normal level of short-chain polyP relative to that of the wild-type 381 (Fig. 2).

The *ppk* gene of *P. gingivalis* 381 is essential for stationary-phase survival. There was no significant difference in the growth rate between strain 381 and mutant CW120 when cultured anaerobically in TSB medium at 37°C. In a long-term survival assay (8), strains 381 and CW120 were grown to the early stationary phase and further incubated in TSB medium anaerobically at 37°C for several days. Viable cell counts were then determined in triplicate for each day. After plating, the plates were routinely incubated anaerobically at 37°C for 7 days before the viable colonies were counted. The results at days 1 to ≈3 indicated an indistinguishable loss in viability between strains 381 and CW120. However, after the third day, the viability of CW120 was significantly reduced. The viable cells of CW120 were approximately 10, 1, 0.1, and 0.001% of that of wild-type 381 at days 4, 5, 6, and 7, respectively. After 7 days, a number of small-colony variants of CW120 arose, and only a few normal-size colonies could be observed on the plates, as with other bacteria (8, 12, 26). These small colonies from CW120 were lighter in color and could not be subse-

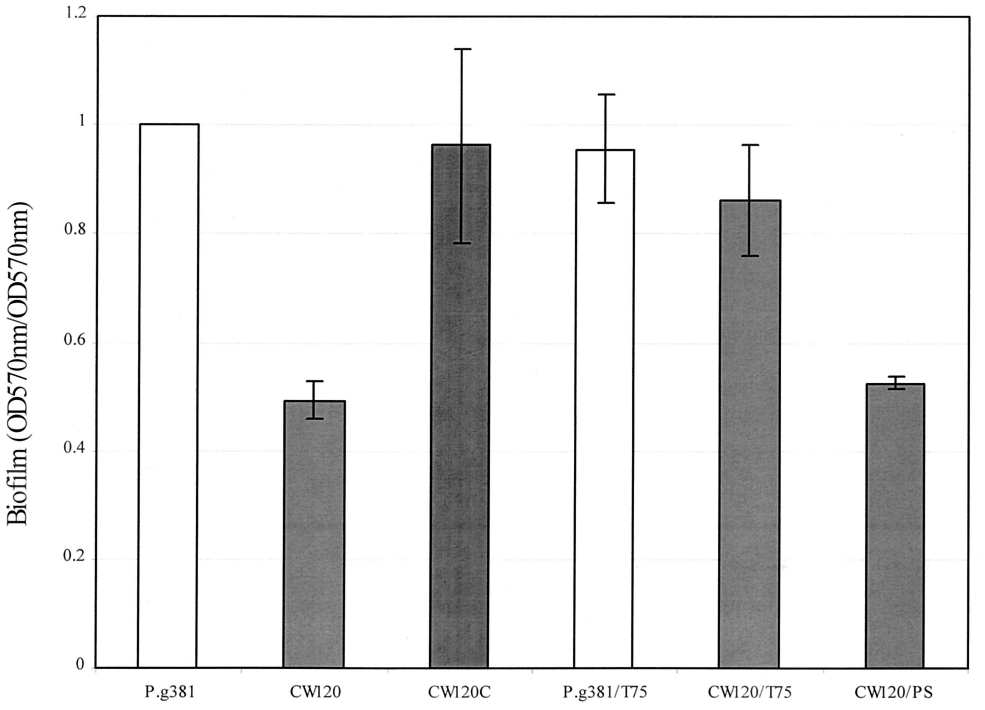

FIG. 3. Biofilm formation assay in PVC plates. *P. gingivalis* strains were incubated overnight in TSB medium diluted with PBS (TSB/PBS ratio, 1:2) with supplementation of hemin, vitamin K in the wells of 96-well PVC microtiter dishes (100 μl/well). The resulting biofilms were analyzed as described previously (24). Biofilm formation was calculated as follows: (OD_{570} for the biofilm)/(OD_{570} of total cell growth). The data are averages of triplicate assays with the standard errors of the means. T75, 75 phosphate polymer of polyP (100 μg/ml); PS, polyvinyl sulfate (100 μg/ml).

quently passaged. Characterization of these variants was not further explored. The heat shock survival and sensitivity to H_2O_2 (8) were also evaluated but no differences were observed between 381 and mutant CW120 (data not shown).

The *ppk* gene of *P. gingivalis* 381 is involved in biofilm formation. Static biofilm formation of *P. gingivalis* strains was first examined on 96-well polyvinyl chloride (PVC) plates as described previously (24). Briefly, the stationary-phase cultures of *P. gingivalis* were inoculated into the diluted TSB medium (TSB/phosphate-buffered saline (PBS) ratio, 1:2). The cells were added to 96-well PVC plates (100 μl/well) and incubated anaerobically at 37°C for 12 h. Four wells of each sample were used for measuring total growth while another four identical wells were assayed for biofilms. Following incubation, the biofilm wells were stained with crystal violet (CV) and quantitated (24). Biofilm formation was scored as the absorbance of CV-stained biofilms at an optical density at 570 nm (OD_{570}) divided by the absorbance of total growth (including biofilm cells and planktonic cells) at OD_{570}. Attachment, as an initial step of biofilm formation, of 381 and CW120 to KB cells (9, 10, 38) and PVC abiotic surfaces (24) was also evaluated. There was no significant difference detected in attachment between the wild-type 381 and mutant CW120 (data not shown). However, with static continued incubation in the diluted TSB medium (TSB/PBS ratio, 1:2), the *ppk* mutant was shown to be attenuated in biofilm formation (Fig. 3).

Continuous biofilm formation in the flow-cell system was also performed as described previously (13), except that the experiment was performed in an anaerobic glove box. Briefly, two-track flow cells were constructed using a microscope slide as the bottom and a no. 1.5 coverglass as the top. The flow cells were washed overnight with 0.1 M HCl, rinsed with several changes of distilled water over a period of 3 h, and then autoclaved. Overnight cultures (0.5 ml of each strain) of *P. gingivalis* 381 and mutant CW120 grown on porphyromonas broth (PB; Todd Hewitt broth [Difco] supplemented with 1 g of yeast extract, 5 mg of hemin, and 1 mg of vitamin K per liter) or PB plus 5 μg of erythromycin/ml (CW120) were spun down, washed with fresh PB, and resuspended in 8 ml of fresh PB without any antibiotic. After 3 h of growth, the cultures were adjusted to 10 Klett units and the suspensions of strain 381 and CW120 were injected (0.5 ml) into separate flow-cell tracks. The flow cells were inverted and bacteria were permitted to attach for 20 min. After that, the flow cells were returned to their original orientations and a flow of PB diluted 1:10 at 0.0125 mm/s (200 μl/min) was begun. After 4 h of flow, one flow cell for each strain was removed from the incubator, stained with BacLight Live/Dead (Molecular Probes, Eugene, Oreg.) (0.5 ml of a 1:1 mixture of the two dyes diluted 1,000-fold in PBS), and observed with a Leica TCS 4D confocal microscope (Leica Laser Technik, Heidelberg, Germany). Three randomly selected fields were imaged in each track. After an overnight (18-h) period of flow, the remaining flow cells were removed from the incubator, stained, and imaged. This experiment was repeated twice. In this continuous flow-cell system (25, 31), it was demonstrated that the attached monolayer of the *ppk* mutant CW120 is similar to that of wild-type 381 on glass surfaces at 4 h (Fig. 4). A few cell clusters on the glass surfaces were seen for strain 381 but not for CW120. After 18 h, characteristic mound-shaped cell clus-

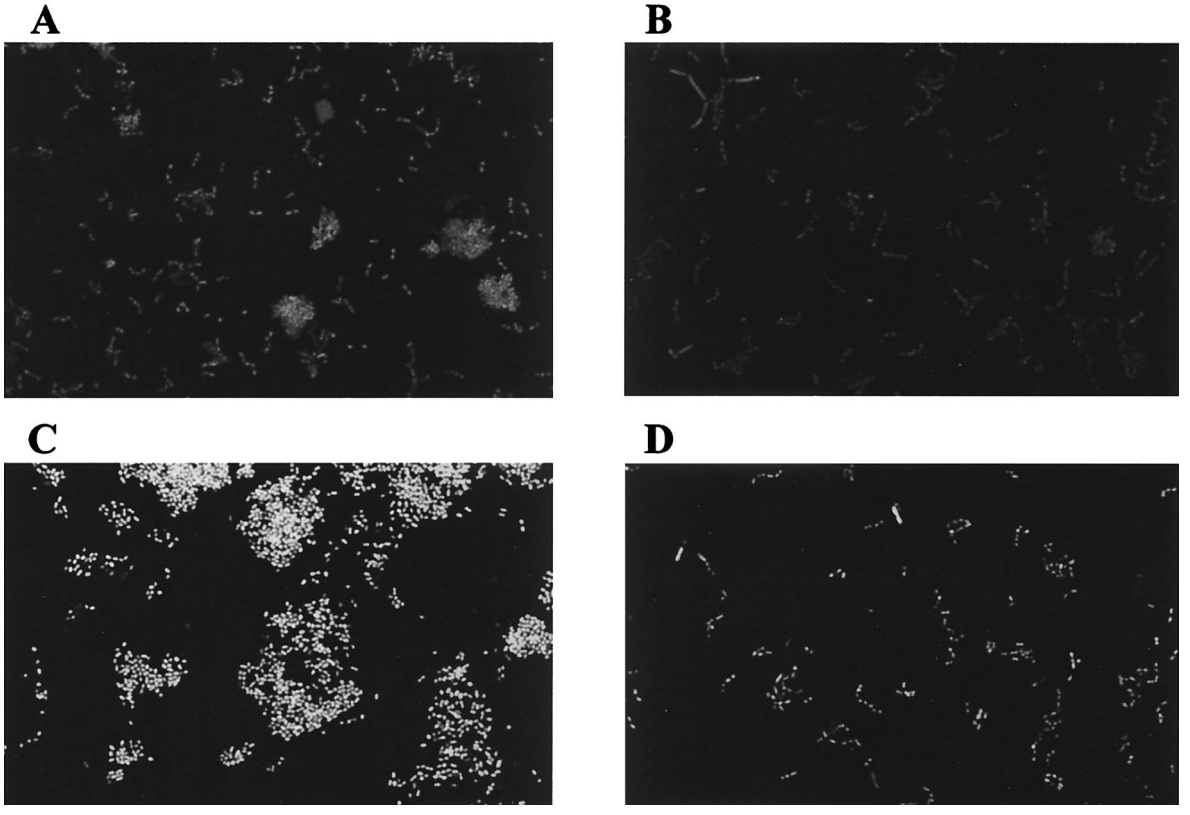

FIG. 4. Confocal images of *P. gingivalis* 381 and CW120 biofilms in a flow cell. (A) Strain 381 at 4 h; (B) CW120 at 4 h; (C) strain 381 at 18 h; (D) CW120 at 18 h.

ters were formed by strain 381. By contrast, no cell clusters could be observed for mutant CW120. Mutant CW120 only formed a monolayer biofilm whose architecture differed dramatically from that of the wild-type biofilm. Thus, biofilm maturation appears to be strongly affected in the *ppk* mutant under these conditions. This defect does not result from attenuated interactions between the mutant cells, since autoaggregation of the wild-type strain 381 and *ppk* mutant are similar (data not shown) (39).

Complementation of the *ppk* gene mutation. Since multiple transposons have been detected in several strains of *P. gingivalis*, it was important to confirm that the altered phenotypes of a constructed *P. gingivalis* mutant resulted from inactivation of the gene and not from a secondary spontaneous mutation (6). Therefore, complementation of the PPK defect was carried out. The *tetA(Q)* gene was chosen for use as a selection marker for the complementation. The *tetA(Q)* cassette was ligated either upstream or downstream of the *ppk* gene in the suicide vectors (pACYC:PPK and pKFT2, respectively), and the suicide plasmids were linearized for recombination into the *P. gingivalis* chromosome by electroporation. Neither approach resulted in generation of tetracycline-resistant transformants in strains 381 and CW120. Therefore, a strategy was devised to add a strain 381 promoter upstream of the *tetA(Q)* gene by inserting a 5′-end fragment of the *ppk* gene, including the 450-bp upstream flanking region (Fig. 5A). Plasmid pKFT2 containing the tetracycline resistance gene [*tetA(Q)*] was kindly provided by M. Curtis (Royal London School of Medicine and Dentistry, London, United Kingdom) and maintained in *E. coli* DH5α in the presence of 50 μg of ampicillin/ml. The resulting plasmid, pCW112, was linearized with *Hin*dIII and electroporated into strain CW120 (36) to restore PPK activity. Following 16 h of incubation, the cell cultures were plated onto TSB agar plates containing tetracycline (1 μg/ml) and incubated anaerobically at 37°C for 7 to 10 days. The transformants that grew only on tetracycline plates and not on erythromycin plates were potential target colonies which were then grown anaerobically in TSB with tetracycline (0.25 μg/ml) for characterization. One of the 12 resulting purified transformants, which were demonstrated to have lost their erythromycin resistance and were tetracycline resistant, was named CW120C. In addition, Southern blot analysis confirmed the integration of the target gene from plasmid CW112 into the CW120 chromosome (Fig. 5B and C). The complemented mutants exhibited normal levels of stationary-phase survival ability, similar to that of parental strain 381 (data not shown). Significantly, the *ppk* gene-complemented strain CW120C produced biofilms at levels similar to those produced by wild-type 381 (Fig. 3). In addition, the complemented strain, CW120C, produced wild-type levels of short-chain polyP that were elevated relative to the levels produced by the CW120 mutant (Fig. 2). Furthermore, when commercial polyP (Type 75; Sigma Chemical Co., St. Louis, Mo.) was added to the cultures of 381 and the *ppk* mutant CW120, CW120 formed biofilms at a level similar to that of 381, but CW120 stationary-phase survival ability could not be rescued. PolyP (Type 75) did not affect biofilm formation of the wild-

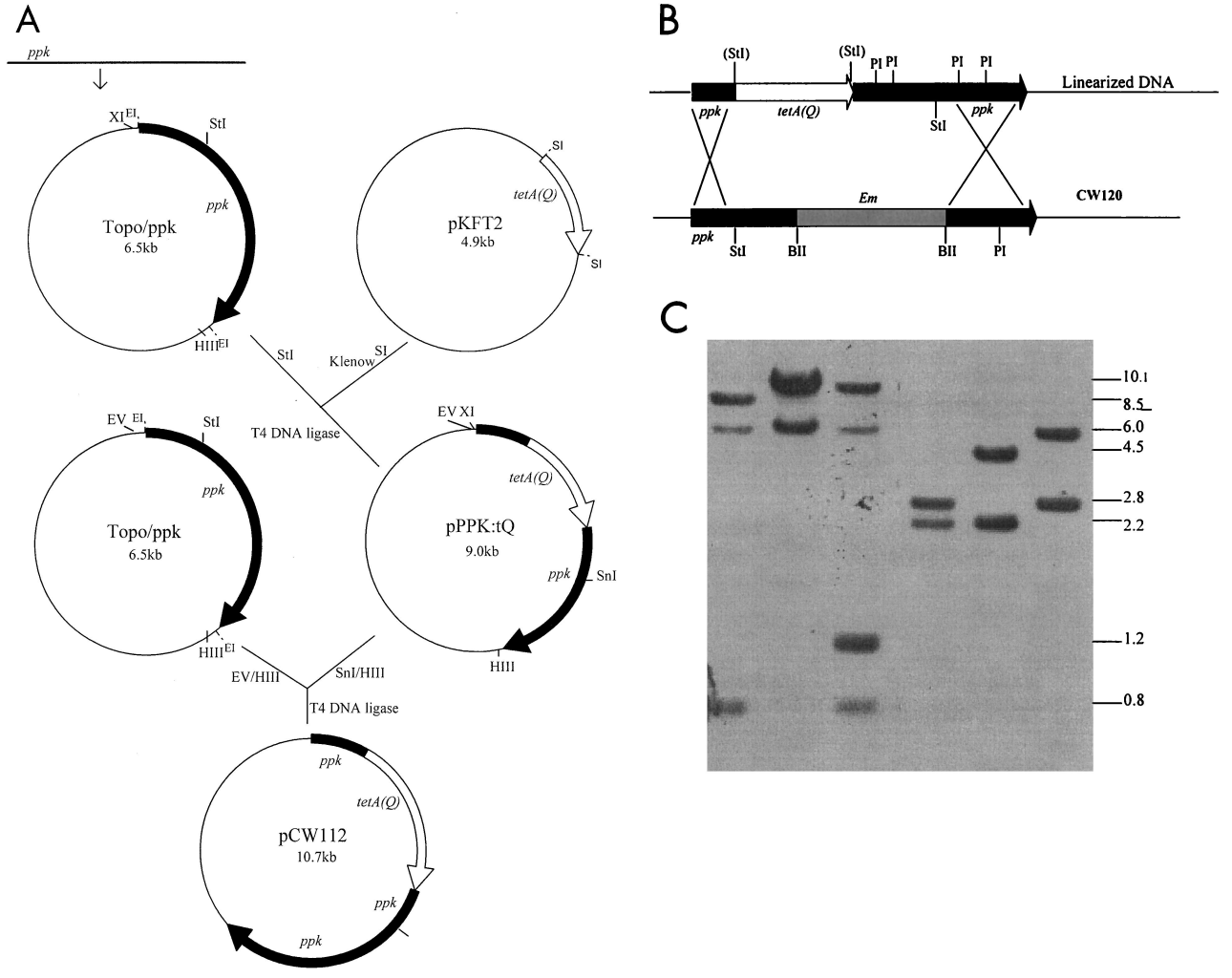

FIG. 5. Strategy for complementation of the *ppk*-deficient mutant CW120. (A) A 2.4-kb *ppk* gene fragment, including the 400-bp upstream flanking region, amplified by PCR from *P. gingivalis* 381 was ligated into the pCR 2.1-TOPO vector. A 2.2-kb *tetA(Q)* cassette digested with *Sst*I from pKFT2 was blunted and inserted into the *ppk* gene at the *Stu*I site in the same transcription direction as the *ppk* gene. The portion of the *ppk* gene downstream from *tetA(Q)* was cut out with *Sna*B I and *Hin*dIII. An intact copy of the *ppk* gene was ligated downstream of *tetA(Q)* at the *Sna*B I and *Hin*dIII sites of pPPK:tQ. The resulting plasmid, designated pCW112, was linearized with *Hin*dIII and electroporated into the *ppk*-null mutant CW120. (B) The predicted integration of the resulting plasmid pCW112 into CW120. (C) Southern blot analysis of the genomic DNA of *P. gingivalis* wild-type 381, *ppk* mutant CW120, and the *ppk*-complemented strain CW120C. The chromosomal DNA of 381(lanes 1 and 4), CW120 (lanes 2 and 5), and CW120C (lanes 3 and 6) was digested with *Stu*I (lanes 1, 2, and 3) and *Pst*I (lanes 4, 5, and 6), respectively, and probed with a 2.4-kb *ppk* fragment.

type strain 381. When another polyanion, polyvinyl sulfate, or orthophosphate was added to cultures of the *ppk* mutant CW120, biofilm formation of CW120 was not augmented (Fig. 3). This suggested that the complementing effects of polyP addition were not due to a nonspecific polyanionic effect or to phosphate limitation. Taken together, these results suggested that the defects exhibited by CW120 resulted from the loss of PPK activity and not from a secondary spontaneous mutation.

In several bacteria species, *ppk* genes have been mutated to determine the role of polyP production in virulence, including some of the major pathogenic species (1, 3, 11, 16, 18, 41). The PPK of *P. gingivalis* exhibited good homology with the PPK of *E. coli* (identity, 37%; similarity, 59%). However, there is no detectable *ppk*/*ppx* (exopolyphosphatase, *ppx*) operon in *P. gingivalis* as observed in *E. coli* or some other microorganisms (2, 5, 8, 19, 33). Therefore, this suggests that polyP metabolism in *P. gingivalis* may be somewhat different from that in *E. coli.* PolyP accumulation may vary in different microorganisms (4, 14, 17, 21, 23, 32, 37, 40, 43). Long-chain polyP could not be detected in *P. gingivalis* and may require specific stress conditions (4). The inability to detect polyP accumulation under these stress conditions may also indicate that another factor(s) is involved in these responses in *P. gingivalis* (45) or that *P. gingivalis* might degrade long-chain polyP during isolation (7). PPK may not be the only enzyme capable of catalyzing the production of short-chain polyP. A second gene may be involved in short-chain polyP synthesis, as suggested for other bacteria (45). The *ppk* mutant CW120 displayed lower, but significant, levels of short-chain polyP accumulation at 3 h, providing a mechanism for short-chain polyP accumulation independent of PPK activity.

It appears that the *ppk* gene is essential for stationary-phase long-term survival in *P. gingivalis*, although *ppk* may not be the only enzyme involved in the production of polyP. However, unlike *E. coli*, the *ppk* mutant CW120 of *P. gingivalis* still remained sensitive to heat and oxidants, as did parental strain 381. Some studies have reported that long-chain polyP, even at relatively low levels, is essential for adaptation to various stresses and for survival of bacteria in the stationary phase (4, 26, 27).

As with *P. aeruginosa* (30), the present results suggest that the *ppk* gene does not affect the initial attachment of *P. gingivalis* to abiotic surfaces. Therefore, the *ppk* gene appears to be involved in biofilm maturation of *P. gingivalis*. The molecular mechanisms involved in biofilm maturation still remain to be elucidated. However, since the metabolism of biofilm bacteria is similar to that of stationary-phase cells (44) it is of interest that the *ppk* mutant CW120 was attenuated in both biofilm formation and stationary-phase survival. The present results suggest that the mutant could be altered in colonizing the subgingival margin and subsequently periodontal inflammation. Therefore, the *ppk* gene of *P. gingivalis*, as well as of other periodontopathogens, might be targeted for the development of specific inhibitors of subgingival plaque formation and periodontitis. Such a strategy has been suggested for other virulent bacteria (42).

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