J. Microbiol. Biotechnol. (2003), **13**(2), 282–286

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cloning of a Potentially Strain-Specific DNA Probe of *Prevotella intermedia* ATCC 25611 by Inverted Dot Blot Hybridization Screening Method

KOOK, JOONG-KI[1,2]* JIN JU HAN[1], HWA-SOOK KIM[1], JIN-HYO SEONG[2], DONG-KIE KIM[2], DONG-HEON BAEK[3], AND SON-JIN CHOE[4]

[1]*Department of Oral Biochemistry,* [2]*Oral Biology Research Institute, College of Dentistry, Chosun University, Gwang-ju 501-759, Korea*
[3]*Department of Oral Microbiology and Immunology, College of Dentistry, Dankook University, Cheon-an 330-716, Korea*
[4]*Department of Oral Microbiology and Immunology, College of Dentistry, Seoul National University, Seoul 110-749, Korea*

Received: October 29, 2002
Accepted: December 13, 2002

Abstract The purpose of this study was to isolate a specific DNA probe for the strain ATTC 25611 of the species *Prevotella intermedia* by using a new rapid screening method. The whole-genomic DNA of *P. intermedia* ATCC 25611 was isolated and purified. The *Hin*dIII-digested genomic DNAs from the strain were cloned by the random cloning method. To screen the strain-specific DNA probe, inverted dot blot hybridization tests were performed. In this assay, 20 ng of recombinant plasmids containing the *Hin*dIII-digested genomic DNA fragment were boiled and blotted onto a nylon membrane, and hybridized with digoxigenin-dUTP labeled genomic DNAs in a concentration of 100 ng/ml. Southern blot analysis was performed in order to confirm the results of the inverted dot blot hybridization tests. The data showed that a Pi34 probe (2.1 kbp; 1 out of 32 probes) was specific for *P. intermedia* strain ATCC 25611 and could be useful for the detection and identification of the strain, particularly in epidemiological studies of periodontal disease.

Key words: Inverted dot blot hybridization, DNA probes, *Prevotella intermedia* ATCC 25611

The human oral cavity harbors a variety of moderately saccharolytic species of the bacterial genus *Prevotella*, some of which may have clinical significance. The two most prevalent species of the group are *Prevotella intermedia* and *Prevotella nigrescens*. Formerly considered a single species, they were separated 10 years ago [5, 19], after multiple DNA homology studies [6, 22], serological experiments [8, 16], isoenzyme screenings [5], and whole-protein analyses [19] had provided evidence for at least two distinct subgroups.

*Corresponding author
Phone: 82-62-230-6877; Fax: 82-62-224-3706;
E-mail: jkkook@chosun.ac.kr

Several investigations reported significant differences in the prevalence and/or abundance of the two species in samples collected from various oral sites. It is thought that *P. intermedia* is associated with sites of periodontal disease whilst *P. nigrescens* has been isolated from healthy sites within the mouth, as well as endodontic infections [7] and oral abscesses [15]. Phenotypically, the two species are very similar. Biochemical or serological differentiation is difficult, laborious, and sometimes unreliable [2, 3, 7]. The presently available commercial identification kits, Rapid ID32 A (BioMerieux, Marcy l'Etoile, France) or Rapid ANA II system (Innovative Diagnostic Systems, Norcross, GA, U.S.A.) will identify *P. intermedia* but will not distinguish it from *P. nigrescens*.

To enable definitive identifications to be made for the purposes of research, diagnosis, and epidemiological studies, further techniques must be developed. Therefore, investigations now employ PCR-based assays or DNA probe assays to differentiate between bacterial species. PCR methods utilizing the 16S rDNA as a target are known to have many advantages [9, 20, 21]. However, the DNA sequences of 16S rDNA among the same species are too homogeneous for the PCR assays to identify bacteria at the subspecies or strain level.

DNA probe methods have advantages such as high sensitivity, specificity, and capability of microbial identification at a wide range of levels, from a family to a strain; therefore, it has been widely used for bacterial identification at the species level [4, 10]. As the homology of genomic DNA sequences in the same species is not the same among strains, the DNA probe method can be used for the detection and identification of bacteria at the subspecies or strain level [12].

Recently, a new method was introduced for rapid screening of bacterial species- or subspecies-specific DNA probes, named the inverted dot blot hybridization

screening method [11]. In that study, 4 putative subspecies-specific DNA probes for *F. nucleatum* were cloned by only 5 inverted dot blot hybridizations and 4 Southern blots in the screening of 96 candidate DNA probes. In the previous study, a probe, Pig3, was reported, which is potentially specific for *P. intermedia* strain ATCC 49046 [1]. The aim of this study was to develop, by employing the new method, *P. intermedia* ATCC 25611 strain-specific DNA probes useful for diagnostics and epidemiological studies of periodontal diseases, particularly in intraindividual or interindividual transmission studies.

MATERIALS AND METHODS

Bacterial Strains and Growth Condition

The bacterial strains used were as follows: *Prevotella intermedia* ATCC 25611, *P. intermedia* ATCC 49046, *P. nigrescens* ATCC 33563, *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586, *F. nucleatum* subsp. *nucleatum* ATCC 23726, *F. nucleatum* subsp. *fusiforme* ATCC 51190, *F. nucleatum* subsp. *polymorphum* ATCC 10953, *F. nucleatum* subsp. *vincentii* ATCC 49256, *Porphyromonas gingivalis* ATCC 53978, *P. gingivalis* ATCC 33277, *P. gingivalis* ATCC 49417, *Actinobacillus actinomycetemcomitans* ATCC 43717, *A. actinomycetemcomitans* ATCC 43718, *A. actinomycetemcomitans* ATCC 33384, *Porphyromonas endodontalis* ATCC 35406, *Camphylobacter rectus* ATCC 33238. All strains were obtained from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). *P. intermedia*, *P. nigrescens*, and *P. gingivalis* were cultured in trypic soy broth (TSB) supplemented with 0.5% yeast extract, 0.05% cysteine HCl-H_2O, 0.5 mg/ml hemin, and 2 μg/ml of vitamin K_1. *F. nucleatum* strains were grown in Schaedler broth (Difco Laboratories, Detroit, MI, U.S.A.). *C. rectus* strain was grown in brain heart infusion broth (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 0.5% yeast extract, 2% sodium formate, 3% sodium fumarate, 0.5 mg/ml of hemin, and 2 μg/ml of vitamin K_1. All of the above species were grown at 37°C in an anaerobic chamber under 10% H_2, 5% CO_2, and 80% N_2. *A. actinomycetemcomitans* was grown in a medium composed of TSB (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 0.6% yeast extract, 5% horse serum, 75 μg/ml bacitracin, and 5 μg/ml vancomycin (Sigma Chemical Co., St. Louis, MO, U.S.A.) in a 37°C incubator containing air and 10% CO_2.

Bacterial Genomic DNA Preparation

Whole genomic DNA was isolated by the method of Lippke *et al.* [13] with a modification; bacterial genomic DNAs were purified by the extraction method of phenol/chloroform instead of cesium chloride [18].

Random Cloning of Bacterial Genomic DNA Digested by *Hin*dIII

The *Hin*dIII-digested bacterial genomic DNA fragments were randomly cloned by the shotgun method [4]. Briefly, whole genomic DNA was digested by *Hin*dIII and dephosphorylated by bacterial alkaline phosphatase. The ligation mixture was used to transform competent *E. coli* DH5α [17]. The transformation mixture was plated onto ampicillin (100 μg/ml)-LB agar pre-plated with 7 μl of 0.8 M IPTG and 40 μl of 20 μg/ml X-gal, and the plates were incubated overnight at 37°C. The white colonies were selected and cultured in ampicillin-LB broth. The plasmid DNAs were prepared by the *Accu*Prep™ Plasmid Extraction kit (Bioneer Corp., Daejeon, Korea) according to the manufacturer's manual.

DNA Labeling

Purified bacterial genomic DNAs or insert DNAs of recombinants were labeled with Digoxigenin-11-dUTP using the DIG-High Prime (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's manual. The DIG-labeled DNAs were purified using an *Accu*Prep™ PCR Purification kit (Bioneer Corp., Daejeon, Korea) according to the manufacturer's instruction.

Inverted Dot Blot Hybridization

To screen the strain-specificity of DNA fragment cloned from *P. intermedia* ATCC 25611 genomic DNA, inverted dot blot hybridization was performed. Twenty ng of recombinant plasmid DNAs cloned from *P. intermedia* ATCC 25611 genomic DNA were boiled at 95°C for 10 min and immediately chilled in ice for 5 min. The denatured DNA fragments were spotted onto the positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). In this step, three dot blot membranes were prepared. The membranes were baked at 120°C for 30 min. The inverted dot blot hybridization was analyzed by the DIG hybridization system (Roche Diagnostics, Mannheim, Germany). The three membranes were prehybridized at 42°C for 2 h in hybridization buffer [50% deionized formamide, 5× SSC (0.75 M NaCl plus 0.075 M sodium citrate), 2% blocking reagent (Roche Diagnostics, Mannheim, Germany), 0.1% *N*-lauroylsarcosine, 0.02% SDS]. They were hybridized overnight at 42°C in hybridization solution plus the DIG-labeled *P. intermedia* ATCC 25611, ATCC 49046, and *P. nigrescens* ATCC 33563 genomic DNA (200 ng/ml of hybridization solution), respectively. After hybridization, the membranes were washed twice in 2× wash buffer (2× SSC, 0.1% SDS) at room temperature and twice in 0.5× wash buffer (0.5× SSC) at 68°C.

Southern Blot Hybridization

To confirm the specificity of the recombinant plasmids that were screened by the new method, Southern blot analysis

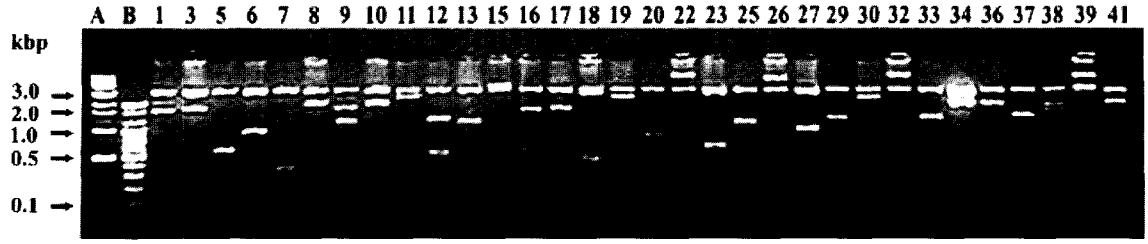

Fig. 1. Agarose gel electrophoresis of *Hin*dIII-digested recombinant plasmids containing genomic DNA fragments of *P. intermedia* ATCC 25611.
Lane A, 1 kilobase pair DNA ladder; lane B, 100 base pair DNA ladder; lanes 1 to 41, *Hin*dIII-digested Pi1 to Pi41, respectively. The arrow pointing in lane 34 indicates position of the 2.1 kbp insert DNA fragment of Pi34.

was performed. The purified bacterial genomic DNAs were digested with *Hin*dIII (Bioneer Corp., Daejeon, Korea) overnight at 37°C, electrophoresed on 0.8% agarose gels in TAE buffer at 30 V for 4 h, and transferred to nylon membranes by vacuum transfer (Bio-Rad Laboratories, St Louis, MO, U.S.A.) in accordance with the manufacturer's instructions. The membranes were baked for 30 min at 120°C. The prehybridization and hybridization were performed with the insert DNA of the Pi34 screened by the inverted dot blot hybridization as being putatively strain-specific, after being labeled with DIG.

Chemiluminescence Detection

Briefly, the membranes were washed twice for 5 min with 2× wash solution (2× SSC, 0.1% SDS) at room temperature for 15 min and twice with 0.5× wash solution (0.5× SSC, 0.1% SDS) at 65°C for 15 min. Detection was performed as follows: the membranes were equilibrated in washing buffer (100 mM maleic acid, 150 mM sodium chloride; pH 7.5; 0.3% Tween 20) for 1 min. The membranes were blocked in blocking solution (1% blocking reagent in 100 mM maleic acid, 150 mM NaCl; pH 7.5) for 1 h, and incubated in DIG-alkaline phosphatase conjugate (150 mU/ml in blocking buffer) for 30 min. The membranes were washed twice in washing buffer for 15 min, equilibrated in detection buffer (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl) for 2 min, and incubated in CSPD substrate solution diluted 1:100 in detection buffer for 5 min. Excess liquid was allowed to drip off the membrane before they were sealed in a hybridization bag and incubated at 37°C for 15 min. The membranes were left at room temperature for 30 to 60 min, and exposed to Lumi-film chemiluminescence (Roche Diagnostics, Mannheim, Germany) at room temperature for 2 h.

Results and Discussion

Cloning of *Hin*dIII-Digested Genomic DNA Fragments of *P. intermedia* ATCC 25611

A number of *E. coli* DH5α clones containing recombinant plasmids larger than pBluescriptII KS(+) were obtained. Among them, 32 clones were chosen and their specificity for *P. intermedia* ATCC 25611 was assessed. The size of the insert DNA fragments on electrophoresis ranged from 0.2 to 8 kbp (Fig. 1).

Screening for Potential *P. intermedia* ATCC 25611-Specific DNA Probes

Figure 2 shows that all of the recombinant plasmids, except Pi32 and Pi34, exhibited strong hybridization signals with DIG-labeled genomic DNA of *P. intermedia* ATCC 25611 (A) and ATCC 49046 (B), and no or weak signals with *P. nigrescens* ATCC 33563 (C), except Pi11 and Pi22. Pi32 showed a stronger signal with *P. intermedia* ATCC 49046 than with *P. intermedia* ATCC 25611. The reason for this difference is not immediately clear, but it may be due to a difference in the copy number of the DNA fragments between the two strains. Pi11 and Pi22 showed strong hybridization signals with both *P. intermedia* and *P. nigrescens* species, indicating that the sequence homology between the two cloned DNA fragments was high. Since the cloning vector pBluescriptII KS(+) alone, which was also used as a negative control in

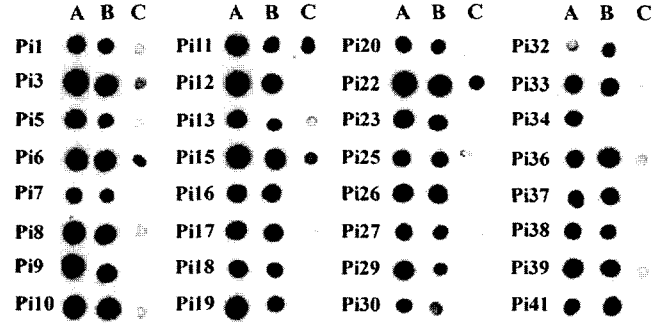

Fig. 2. Composite summary of the inverted dot blot hybridization screening.
The 32 recombinant plasmid DNAs containing genomic DNA fragments derived from *P. intermedia* ATCC 25611 were hybridized with DIG-labeled genomic DNAs. The genomic DNAs were from: (A) *P. intermedia* ATCC 25611, (B) *P. intermedia* ATCC 49046, and (C) *P. nigrescens* ATCC 33563. Pi (Pi1-Pi41) designates the recombinant plasmids containing genomic DNA fragments from *P. intermedia* ATCC 25611.

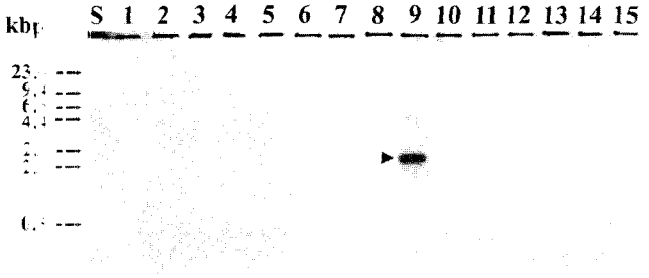

Fig. 3. Southern blot analysis to confirm the specificity of the cloned DNA fragment inserted into Pi34 plasmid.
The *Hin*dIII-digested bacterial genomic DNAs were electrophoresed and transferred to nylon membranes; hybridized with a DIG-labeled insert DNA fragment. *Hin*dIII-digested genomic DNAs (lanes 1-15) were from: 1) *F. nucleatum* subsp. *nucleatum* (ATCC 25586), 2) *F. nucleatum* subsp. *nucleatum* (ATCC 23726), 3) *F. nucleatum* subsp. *polymorphum*, 4) *F. nucleatum* subsp. *vincentii*, 5) *F. nucleatum* subsp. *fusiforme*, 6) *P. gingivalis* (ATCC 53978), 7) *P. gingivalis* (ATCC 33277), 8) *P. gingivalis* (ATCC 49417), 9) *P. intermedia* (ATCC 25611), 10) *P. intermedia* (ATCC 49046), 11) *P. nigrescens* (ATCC 33563), 12) *A. actinomycetemcomitans* (ATCC 43717), 13) *A. actinomycetemcomitans* (ATCC 43718), 14) *A. actinomycetemcomitans* (ATCC 33384), 15) *C. rectus*. Lane S, *Hin*dIII-digested λ DNA size marker. Arrowhead indicates position (2.1 kbp) of hybrids formed.

the previous study, gave no signals with DIG-labeled genomic DNA from *P. intermedia* ATCC 25611 [11], the cross-reaction test of the vector was omitted in the present study.

Conformation of Pi34 Specificity by Southern Blot Analysis

Pi34 hybridized only with DIG-labeled genomic DNA of *P. intermedia* ATCC 25611 (Fig. 2). To verify the specificity of Pi34, Southern blot analysis was performed. In this analysis, *P. intermedia* ATCC 25611 and ATCC 49046 as well as *P. nigrescens* ATCC 33563 were used along with 5 strains of *F. nucleatum*, 3 strains of *A. actinomycetemcomitans*, 3 strains of *P. gingivalis*, and 1 strain of *C. rectus*. Purified insert DNA fragments of Pi34 were used as a DIG-labeled probe in the analysis. The Southern blot data showed that the DIG-labeled insert DNA fragment hybridized strongly only with *Hin*dIII-digested genomic DNA of *P. intermedia* ATCC 25611. Size of the signal band (2.1 kbp) coincided with that of the insert DNA fragment. The data indicate that the insert DNA fragment could be used as a strain-specific DNA probe for detection and identification of *P. intermedia* strain ATCC 25611.

There are few data on the intraspecies genetic diversity of *P. intermedia* and *P. nigrescens*. By applying restriction endonuclease analysis, van Steenbergen *et al.* [23] reported that all isolates of the *P. intermedia* and *P. nigrescens* group obtained from 10 individuals showed digest patterns different from each other. A recent study performed with the ribotyping method by Mättö *et al.* [14] also showed considerable genetic heterogeneity in oral isolates of *P. intermedia* and *P. nigrescens*. Since the clonal distribution of bacteria in the oral cavity, especially in the periodontal pocket, should be explored more extensively in the future, strain-specific DNA probes can be of value in epidemiological studies.

The data also showed that only three dot blot hybridizations and one Southern blot hybridization were enough to screen the thirty-two cloned DNA fragments for the isolation of *P. intermedia* strain ATCC 25611-specific DNA probe. This fact again demonstrates the value of the inverted dot blot hybridization screening method.

The nucleotide sequence of the insert DNA Pi34 has been submitted to the GenBank under the accession number AY195620.

Acknowledgments

This work was supported by grant No. R05-2001-000-00451-0 (Joong-Ki Kook) from the Basic Research Program of the Korea Science & Engineering Foundation from the Republic of Korea.

NOMENCLATURE

CSPD®; 25 mM disodium 3-<-4-methoxyspiro{1.2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl>phenyl phosphate)

REFERENCES

1. Bak, J.-S., S.-H. Kim, D.-K. Kim, J.-H. Seong, B.-O. Kim, M.-K. Kim, and J.-K. Kook. 2002. Study on development of DNA probe for identification of *Prevotella intermedia* G8-9K-3. *J. Kor. Acad. Periodont.* **32:** 281-290.
2. Baumgartner, J. C., K. S. Bae, T. Xia, J. Whitt, and L. L. David. 1999. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and polymerase chain reaction for differentiation of *Prevotella intermedia* and *Prevotella nigrescens*. *J. Endodontics* **25:** 324-328.
3. Dahlén, G. G., J. R. Johnson, and R. Gmür. 1996. *Prevotella intermedia* and *Prevotella nigrescens* serotypes, ribotypes and binding characteristics. *FEMS Microbiol. Lett.* **138:** 89-95.
4. DiRienzo, J. M., S. Cornell, and H. Boehringer. 1991. Use of randomly cloned DNA fragments for the identification of oral spirochetes. *Oral Microbiol. Immunol.* **6:** 88-96.
5. Frandsen, E. V., K. Poulsen, and M. Kilian. 1995. Confirmation of the species *Prevotella intermedia* and *Prevotella nigrescens*. *Int. J. Syst. Bacteriol.* **45:** 429-435.

6. Fukushima, H., H. Moroi, J. Inoue, T. Onoe, T. Ezaki, E. Yabuuchi, K. P. Leung, C. B. Walker, W. B. Clark, and H. Sagawa. 1992. Phenotypic characteristics and DNA relatedness in *Prevotella intermedia* and similar organisms. *Oral Microbiol. Immunol.* **7:** 60-64.
7. Gharbia, S. E., M. Haapasalo, H. N. Shah, A. Kotiranta, K. Lounatmaa, M. A. Pearce, and D. A. Devine. 1994. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. *J. Periodontol.* **65:** 56-61.
8. Gmür, R. and C. Wyss. 1985. Monoclonal antibodies to characterize the antigenic heterogeneity of *Bacteroides intermedius*, pp. 91-119. *In* A. J. L. Macario and E. Conway de Macario (eds.), *Monoclonal Antibodies Against Bacteria*, vol. **I**, Academic Press, New York, U.S.A.
9. Kim, E. K., H. S. Yun, C. E. Chang, T. Lin, S. C. Kim, J. S. So, and S. I. Pavlova. 2002. Molecular identification of vaginal Lactobacillus spp. Isolated from Korean women. *J. Microbiol. Biotechnol.* **12:** 312-317.
10. Komiya, A., T. Kato, T. Nakagawa, A. Saito, J. Takahashi, S. Yamada, and K. Okuda. 2000. A rapid DNA probe method for detection of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. *J. Periodontol.* **71:** 760-767.
11. Kook, J.-K., M.-K. Kim, J.-H. Seong, D.-K. Kim, B.-O. Kim, J.-C. Park, K.-K. Kim, S.-J. Choe, and B.-M. Min. 2003. A new method for rapid screening of bacterial species- or subspecies-specific DNA probes. *FEMS Microbiol. Lett.* **219:** 121-127
12. Krieg, N. R. 2001. Identification of procaryotes, pp. 33-38. *In* G. Garrity (ed.), *Bergey's Manual of Systematic Bacteriology*, Vol. **1**, 2th ed. Springer Verlag, New York, U.S.A.
13. Lippke, J. A., M. N. Strzempko, F. F. Raia, S. L. Simon, and C. K. French. 1987. Isolation of intact high-molecular-weight DNA by using guanidine isothiocyanate. *Appl. Environ. Microbiol.* **53:** 2588-2589.
14. Mättö, J., M. Saarela, B. von Troil-Lindén, E. Könönen, H. Jousimies-Somer, H. Torkko, S. Alaluusua, and S. Asikainen. 1996. Distribution and genetic analysis of oral *Prevotella intermedia* and *Prevotella nigrescens*. *Oral Microbiol. Immunol.* **11:** 96-102.
15. Milsom, S. E., S. V. Sprague, D. Dymock, A. J. Weightman, and W. G. Wade. 1996. Rapid differentiation of *Prevotella intermedia* and *P. nigrescens* by 16S rDNA PCR-RFLP. *J. Med. Microbiol.* **44:** 41-43.
16. Nakazawa, F., J. J. Zambon, H. S. Reynolds, and R. J. Genco. 1988. Serological studies of oral *Bacteroides intermedius*. *Infect. Immun.* **56:** 1647-1651.
17. Park, J.-Y., S.-J. Park, S.-J. Nam, Y.-L. Ha, and J.-H. Kim. 2002. Cloning and characterization of the L-lactate dehydrogenase gene (*ldhL*) from *Lactobacillus reuteri* ATCC55739. *J. Microbiol. Biotechnol.* **12:** 716-721.
18. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed., pp. 1.38-1.39. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, U.S.A.
19. Shah, H. N. and S. E. Gharbia. 1992. Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal of a new pigmented species, *Prevotella nigrescens* sp. nov. *Int. J. Syst. Bacteriol.* **42:** 542-546.
20. Slots, J., A. Ashimoto, M. J. Flynn, G. Li, and C. Chen. 1995. Detection of putative periodontal pathogens in subgingival by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clin. Infect. Dis.* **20 (suppl. 2):** 304-307.
21. Tran, S. D. and J. D. Rudney. 1999. Improved multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, and *Porphyromonas gingivalis*. *J. Clin. Microbiol.* **37:** 3504-3508.
22. van Steenbergen, T. J., C. A. Vlaanderen, and J. de Graaff. 1982. Deoxyribonucleic acid among strains of *Bacteroides melaninogenicus* and related species. *J. Appl. Bacteriol.* **53:** 269-276.
23. van Steenbergen, T. J., U. Van der Velden, F. Abbas, and J. de Graaff. 1991. Microflora and bacterial DNA restriction enzyme analysis in young adults with periodontitis. *J. Periodontol.* **62:** 235-241.