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The identification of genes specific to *Prevotella intermedia* and *Prevotella nigrescens* using genomic subtractive hybridization

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ABSTRACT

Prevotella intermedia and *Prevotella nigrescens*, which are often isolated from periodontal sites, were once considered two different genotypes of *P. intermedia*. Although the genomic sequence of *P. intermedia* was determined recently, little is known about the genetic differences between *P. intermedia* and *P. nigrescens*. The subtractive hybridization technique is a powerful method for generating a set of DNA fragments differing between two closely related bacterial strains or species. We used subtractive hybridization to identify the DNA regions specific to *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC 25261. Using this method, four *P. intermedia* ATCC 25611-specific and three *P. nigrescens* ATCC 25261-specific regions were determined. From the species-specific regions, insertion sequence (IS) elements were isolated for *P. intermedia*. IS elements play an important role in the pathogenicity of bacteria. For the *P. intermedia*-specific regions, the genes adenine-specific DNA-methyltransferase and 8-amino-7-oxononanoate synthase were isolated. The *P. nigrescens*-specific region contained a *Flavobacterium psychrophilum* SprA homologue, a cell-surface protein involved in gliding motility, *Prevotella melaninogenica* ATCC 25845 glutathione peroxide, and *Porphyromonas gingivalis* ATCC 33277 leucyl-tRNA synthetase. The results demonstrate that the subtractive hybridization technique was useful for distinguishing between the two closely related species. Furthermore, this technique will contribute to our understanding of the virulence of these species.

1. Introduction

Prevotella species, which are black-pigmented, saccharolytic, anaerobic rods, constitute a *portion* of the microflora found in human gingival crevices in patients with periodontal disease [1,2]. Within the genus, the two previously recognized genotypes I and II of *Prevotella intermedia* were elevated to species rank as *P. intermedia* and *Prevotella nigrescens*, respectively [3–5]. *P. intermedia* is more commonly isolated from sites of periodontal disease, whereas *P. nigrescens* predominates in healthy conditions [2,6–8]. However, not all studies have confirmed these findings, and some indicate that the situation is more complex, in that *P. nigrescens* may be more common in children, in sites of active periodontal disease, and in endodontic infections [9,10].

To understand the role of these organisms in periodontal sites, it is essential to identify the genes specific to each organism. The genome sequence of *P. intermedia* is available in the Oralgen Database (http://www.oralgen.lanl.gov/), whereas that of *P. nigrescens* is not yet available. Therefore, we used a genomic subtractive hybridization technique to identify species-specific regions that distinguish between *P. intermedia* and *P. nigrescens*. This technique has been used successfully to identify genomic differences between closely related strains [11–13]. The identification of genes specific to *P. intermedia* and *P. nigrescens* may help to clarify the etiology of these organisms in periodontal disease.

2. Materials and methods

Bacterial strains and culture conditions. *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC 25261 were grown at 37 °C under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) in GAM broth (Nissui Medical, Tokyo, Japan) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) [14].

Preparation of chromosomal DNA. Standard molecular biology techniques were performed, as described by Sambrook et al. [15]. Chromosomal DNA was isolated and purified from the bacteria using an IsoQuick nucleic acid extraction kit (ORCA Research, Bothell, WA), according to the manufacturer's instructions.

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Subtractive hybridization technique. The subtractive hybridization technique was performed as previously described [16]. The principles of the subtractive hybridization technique are shown in Fig. 1. Briefly, the chromosomal DNA from *P. intermedia* ATCC 25611 (tester) was digested completely with Sau3AI and purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The Sau3AI-digested DNA fragments of *P. intermedia* ATCC 25611 were ligated with the oligonucleotide adaptors RBam12 and RBam24 (Table 1) for 18 h at 11 °C. The ligated DNA fragments were purified using a QIAquick PCR purification kit. To prepare the driver DNA, chromosomal DNA from *P. nigrescens* ATCC 25261 (driver) was digested with HindIII, EcoRI, and MspI. After digestion, the DNA was precipitated with ethanol/sodium acetate and dissolved in distilled water. The first subtractive hybridization was performed in a 10-μl reaction mixture containing 2 μg of the driver DNA from *P. nigrescens* ATCC 25261, 20 ng of the R-adaptor-linked Sau3AI fragments from *P. intermedia* ATCC 25611, 10 mM 3-[4-(2-hydroxyethyl)-1-piperazinyl] propanesulfonic acid (EPPS), and 1 mM ethylenediaminetetraacetic acid (EDTA). The DNA mixture was denatured at 100 °C for 2 min, 2 μl of 5 M NaCl was added, and the mixture was left to hybridize at 55 °C for 24 h. A 2-μl aliquot of the reaction mixture was diluted to 40 μl with a polymerase chain reaction (PCR) mixture containing 100 pmol of RBam24, 0.25 mM of each deoxynucleoside triphosphate, 5 U of Go*Taq*, and 5× Go*Taq* buffer (Promega, USA) to fill in the ends corresponding to the RBam24 adaptor. After denaturation for 5 min at 94 °C, the DNA was amplified by PCR with 30 cycles of 1 min at 70 °C, 3 min at 72 °C, and 1 min at 94 °C, followed by 1 min at 94 °C and 10 min at 72 °C. The PCR products were purified using a QIAquick PCR purification kit. The RBam24 adaptor was removed from the PCR products by digestion with *Sau*3AI, and the DNA fragments were purified using a QIAquick PCR purification kit. In a 40-μl volume, 2 nmol of the second adaptors, JBam12 and JBam24 (Table 1), were ligated for 18 h at 11 °C, and the DNA was purified using a QIAquick PCR purification kit. The second-round subtractive hybridization was performed with 2 ng of DNA from the first-round PCR products and 2 μg of the driver DNA from *P. nigrescens* ATCC 25261, prepared as described above. To identify *P. nigrescens*-specific regions, the tester and driver strains were exchanged, and the same procedures were performed.

Subcloning. The second-round PCR products were digested with *Sau*3AI, cloned into *Bam*HI-digested pBluescript II SK+ (Stratagene, La Jolla, CA), and then used to transform *Escherichia coli* DH5α (Toyobo, Osaka, Japan). We then determined the nucleotide sequences of the inserts.

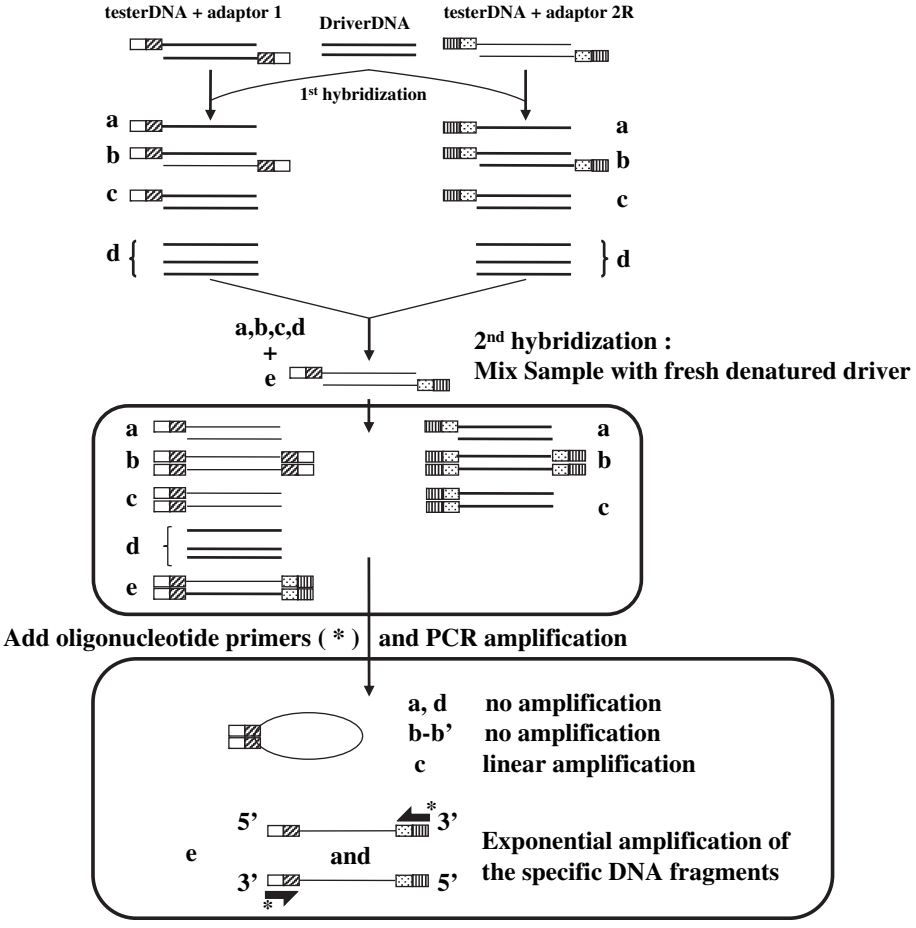

Fig. 1. The principle of subtractive hybridization. Genomic DNA from *P. intermedia* or *P. nigrescens* was subtracted using DNA from *P. nigrescens* or *P. intermedia*, respectively. 1. Adaptor1-binding tester DNA (*P. intermedia* or *P. nigrescens*), 2. Driver DNA (*P. nigrescens* or *P. intermedia*), 3. Adaptor2R-binding tester DNA (*P. intermedia* or *P. nigrescens*). The first hybridization was performed as follows: an excess of driver DNA was added to each adaptor-ligated tester sample. The samples were denatured and allowed to anneal, generating the type a, b, c, and d molecules in each sample. During the second hybridization, the two primary hybridization samples were mixed together without denaturing and formed new type e hybrids. These new hybrids were tester molecules with different ends, which corresponded to the sequences of Adaptors 1 and 2R. After the ends were filled in by DNA polymerase, the type e molecules had different primer annealing sites at their 5′ and 3′ ends. The entire population of molecules was subjected to PCR to amplify the desired tester-specific sequences. During this PCR, only type e molecules, the equalized differentially expressed tester-specific molecules with two different adaptors, could be amplified exponentially.