Oral Microbiology Immunology 2009: 24: 133–140
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ORAL MICROBIOLOGY AND IMMUNOLOGY

Detection of a single bacterial cell using a 16S ribosomal RNA-specific oligonucleotide probe designed to investigate periodontal pathogens

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Tsuruda K, Shimazu A, Sugai M. Detection of a single bacterial cell using a 16S ribosomal RNA-specific oligonucleotide probe designed to investigate periodontal pathogens.
Oral Microbiol Immunol 2009: 24: 133–140. © 2009 John Wiley & Sons A/S.

Introduction: The current detection methods for periodontopathogens mainly use polymerase chain reactions. However, there are few methods available for visualizing the bacteria that impact on patients with periodontal disease for use in health education. The purpose of this study was to develop a specific detection method to visualize periodontopathogenic bacteria.
Methods: Fluorescently-labeled oligonucleotide probes directed to specific 16S ribosomal RNA (rRNA) sequences of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were synthesized. Cultured individual bacterial species were fixed with 4% paraformaldehyde and smeared on glass slides. Fluorescein isothiocyanate-labeled oligonucleotide probes were hybridized under stringent conditions with smeared whole cells, and then probe specificity was investigated by epifluorescence microscopy.
Results: Comparatively long (50-mer) oligonucleotide probes for *P. gingivalis* and *A. actinomycetemcomitans* were designed. These probes clearly hybridized with 16S rRNA of the target species *in situ* and single bacterial cells were detectable visually. The probes exhibited no cross-hybridization against the additional organisms that were closely related to the target species.
Conclusions: The fluorescence *in situ* hybridization technique is a specific and reliable method by which to visually identify the target organisms. The oligonucleotide probes designed in this study will be useful for detecting *P. gingivalis* and *A. actinomycetemcomitans* populations.

Key words: 16S ribosomal RNA; *Aggregatibacter actinomycetemcomitans;* fluorescent *in situ* hybridization; *Porphyromonas gingivalis*

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Accepted for publication September 4, 2008

Periodontitis is well recognized as an infectious disease, and several pathogens have been identified on the basis of their association with this disease (9). *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are considered the major periodontal pathogens. *P. gingivalis*, an anaerobic gram-negative bacterium, is associated with adult periodontitis (11) and the development and progression of periodontitis (13, 16). *A. actinomycetemcomitans*, a gram-negative facultatively anaerobic short rod, has been associated with the etiology of periodontal disease, mainly aggressive periodontitis (27, 30). These bacteria are predominantly and frequently detected in periodontal pockets and are capable of colonizing and invading periodontal tissues (17, 20, 23). These bacteria have also been detected on buccal epithelial cells in periodontitis patients

(22) so they are ubiquitously located in the oral cavity.

Microbiological diagnosis is important in the rationale for treatment planning and gaining informed consent from patients with periodontitis. Many studies have shown that individual maintenance and self-care are important to achieve and keep a satisfactory oral health status (4, 5). The success of the treatment and prevention of periodontitis requires the participation and cooperation of patients. For the motivation of patients with periodontal disease, it is useful to visualize their own bacterial pathogens as well as the presentation of clinical parameters. Not only demonstration of the high diversity and numerous bacteria in the plaque sample but also visualization of *P.* and *A. actinomycetemcomitans* can be an effective form of motivation for the patient with periodontitis. There are various methods for the detection of periodontal pathogens such as anaerobic cultures using selective media, molecular biological techniques, and immunoassays (28). Polymerase chain reaction (PCR) techniques based on target DNA sequences are the most frequently used methods (12, 18, 29). However, PCR techniques are not qualitative and record only the presence of the bacteria in the sample.

Molecular biological methods using 16S ribosomal RNA (rRNA) gene sequences are now commonly used for the identification and classification of bacteria. Ribosomal RNA sequences are widely used to rank biological phylogenetic taxonomy including that of microorganisms (10, 15). Since the 16S rRNA contains some very well-conserved regions among biological species, the comparison of 16S rRNA sequences is possible in studies of molecular evolution. Such 16S rRNA sequences also enable the identification microorganisms because the 16S rRNA contains other more variable sequences that change according to species or families (19, 21). The 16S rRNA is therefore used not only for phylogenetic analysis but also for the species-specific identification of bacteria. Fluorescence *in situ* hybridization (FISH) using 16S rRNA is one of the most powerful tools in visualizing microorganisms and enables the detection and enumeration of populations in spontaneous conditions without cultivation (3, 31). Sunde et al. (25) and Thurnheer et al. (26) reported visual detection of target bacteria using FISH *in vivo* and *in vitro*, indicating that FISH is effective for the visual detection of target bacteria from the microflora. In most studies using FISH, approximately 20-mer oligonucleotides were used as 16S rRNA probes because the short-oligonucleotide probes can permeate fixed whole cells, although these shorter probes may frequently and nonspecifically hybridize to sequences similar to the target. Homology search analysis using a DNA sequence databank indicates that the 24-mer oligonucleotide probe shows more than 80% identity to DNA sequences of 33 bacterial species (25).

The purpose of the present study was to develop a method for the species-specific detection and visualization of periodontopathogenic bacteria for use in a clinical setting. Fifty-mer oligonucleotide probes, which were designed to enhance specificity, were constructed for species-specific 16S rRNA sequences of *P. gingivalis* and *A. actinomycetemcomitans*, and these FISH probes were examined to determine whether they were able to specifically detect *P. gingivalis* and *A. actinomycetemcomitans*.

Materials and methods

Bacterial strains and culture conditions

The strains and sources of bacteria investigated in this study are listed in Table 1. All bacteria, except *A. actinomycetemcomitans* species, were cultured in brain–heart infusion broth supplemented with 0.001% hemin and 0.0001% vitamin K at 37°C under anaerobic condition (N_2: 80%, H_2: 10%, CO_2: 10%). The cells were grown to mid-logarithmic phase and harvested by centrifugation to optimize the rRNA content. The growing medium was decanted, and the cells were resuspended in phosphate-buffered saline (PBS; pH 7.2).

Cell fixation and storage

Cells were fixed with fresh, cold paraformaldehyde solution (4% in PBS). One volume of cell suspension was mixed with 3 volumes of fixative, and the mixture was incubated at room temperature for at least 3 h. The cells were pelleted by centrifugation (7,000 ***g*** for 5 min), washed with PBS solution to remove residual fixative, pelleted again, and resuspended at a concentration of 10^7 to 10^8 cells per ml in PBS. One volume of fixed cell suspension was added to 1 volume of cold absolute ethanol, and the mixture was stored at −20°C for up to 3 months without apparent influence on the hybridization (21).

Preparation of cell smears

The fixed-cell suspensions were spotted onto cleaned glass slides and allowed to air dry for at least 2 h. After dehydration in 50, 80, and 99% (volume/volume) ethanol (3 min each), the slides were stored dry at room temperature.

Design and synthesis of oligodeoxynucleotide probes

A database of bacterial 16S rRNA sequences was collected and analysed to identify probe targets of appropriate

Table 1. Summary of the bacterial strains used in this study and hybridization results for the oligonucleotide probes

Bacteria	Source	Hybridization signal with probe	
		PG177	AA191
Porphyromonas gingivalis	FDC381	+	−
	ATCC33277	+	−
Prevotella intermedia	ATCC25611	−	−
Prevotella nigrescens	ATCC33563	−	−
Prevotella loescheii	ATCC15930	−	−
Prevotella melaninogenica	ATCC25845	−	−
Prevotella denticola	ATCC33184	−	−
Aggregatibacter actinomycetemcomitans	Y4	−	+
	ATCC29523	−	+
	NCTC9710	−	+
Fusobacterium nucleatum	ATCC25586	−	−
Haemophilus aphrophilus	ATCC15416	ND	−
	ATCC5908	ND	−

specificity. Two oligonucleotides specific for *P. gingivalis* and *A. actinomycetemcomitans* were designed. Probe sequences and target positions corresponding to positions in the *Escherichia coli* 16S rRNA (7) are listed in (Fig. 2A,B). The PG177 probe sequence for *P. gingivalis* was as follows; 5′-ACAGCTATAAATA TTTCCTTGTAATATCATGCAATAATAC AAGTGTATGC-3′. The AA191 probe sequence for *A. actinomycetemcomitans* was as follows; 5′-CACTTGGGTTCAT CTCATGGCATGCGGCCATAAAGTCC CGCACTTTCGTC-3′. An alignment of these probes with the complementary regions of the 16S rRNAs from representative organisms of the five main subgroups of target species and representative non-target organisms characteristic of other phyla that are prevalent in the oral cavity is given in Fig. 1. The 50-mer oligonucleotide probes were labeled with digoxigenin (DIG) synthesized by Nihon Gene Research Laboratories (Sendai, Japan).

Whole-cell hybridization with DIG-labeled oligonucleotide probes and detection using fluorescent dye anti-DIG Fab fragment

An aliquot of 8 μl hybridization solution (0.9 M sodium chloride, 20 mM Tris–HCl, 0.01% sodium dodecyl sulfate, pH 7.2) containing 50 ng oligonucleotide probe was applied to the prepared slide and incubated for 2 h at 45°C in an isotonically equilibrated humid chamber. Fluorescein isothiocyanate-labeled or rhodamine-labeled anti-DIG Fab fragments (Roche Diagnostics, Basel, Switzerland) were diluted 1 : 4 in a solution containing 150 mM sodium chloride, 100 mM Tris–HCl (pH 7.5), 0.5% bovine serum albumin and 0.5% blocking reagent (Roche Diagnostics); 10-μl aliquots of these dilutions were added to each smear and incubated with the labeled probe for 1 h at 27°C in the humid chamber. The slide was removed from the humid chamber and immersed in 40 ml washing solution (150 mM sodium chloride, 100 mM Tris–HCl, 0.01% sodium dodecyl sulfate, pH 7.4) at 29°C for 10 min. Slides were rinsed briefly with sterile water, air-dried, and mounted using a Prolonged Antifade kit (Molecular Probe Inc., Eugene, OR). The cell smears were viewed with a U plan Fl × 100 objective (oil immersion) lens on an Olympus BX51 microscope fitted for epifluorescence microscopy (Olympus Optical Co., Tokyo, Japan). Photographs were taken using Kodak Ektachrome P1600 film (Kodak Japan, Tokyo, Japan).

Results

Construction of species-specific probes

Phylogenetic analysis was carried out to design the species-specific DNA probes. 16S rRNA sequences of prevalent oral species were collected from the GenBank database (Table 2). Figure 1 shows a phylogenetic tree based on sequence homology of the 16S rRNA sequence. Comparative analysis of 16S rRNA sequences revealed relatively close relationships between *Porphyromonas* and *Prevotella* strains, but *A. actinomycetemcomitans* differed significantly from these two species (Fig. 1). *P. gingivalis* showed a high sequence similarity to *Tannerella forsythensis*. *A. actinomycetemcomitans* was close to *Haemophilus aphrophilus* (Fig. 1).

Figure 2 shows the sequence homology of the 16S rRNA region in the predominant species of periodontopathogenic bacteria. The target site of the probe specific for the *P. gingivalis* ranged from positions 177 to 226 (Fig. 2A). This probe had 26 mismatches to the corresponding region in *T. forsythensis*, which showed 93% 16S rRNA sequence similarity to *P. gingivalis* 381 in the phylogenetic tree. The target site of the AA191 probe for *A. actinomycetemcomitans* ranged from positions 191 to 240. The AA191 probe has eight mismatches with the comparable region in *H. aphrophilus*. Furthermore, the sequence homology of the designed probe to the overall 16S rRNA in reference organisms was confirmed using the sequence homology tool of the program DNASIS.

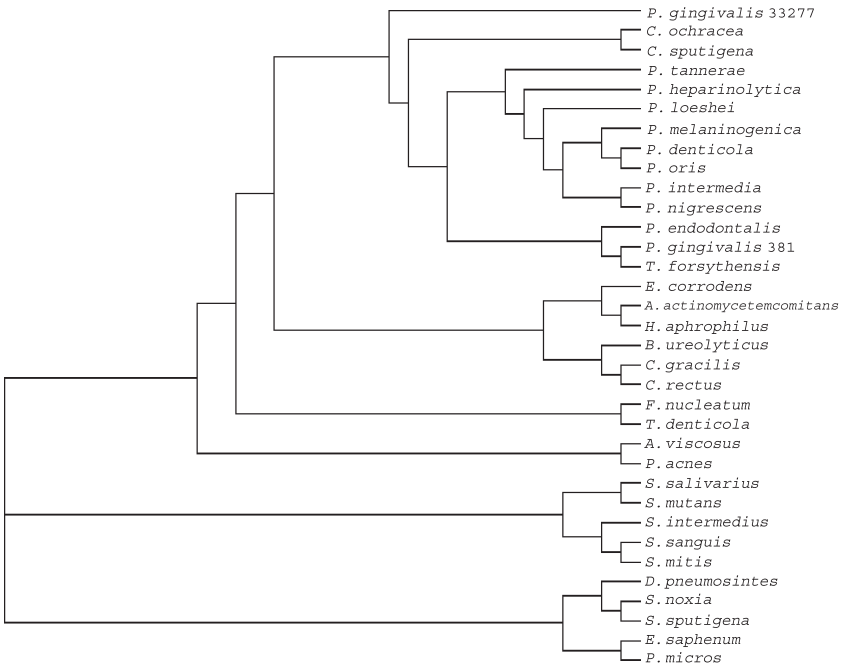

Fig. 1. Phylogenetic tree based on 16S ribosomal RNA sequence comparisons of *Porphyromonas*, *Prevotella*, and representatives of principal oral bacteria. The branch lengths correlate with the significance of the separation of the respective internal and terminal nodes. Multiple branchings indicate that the relative order of the corresponding branches could not be resolved or was not supported using different methods of tree construction.

Table 2. 16S ribosomal RNA sequences for a phylogenetic tree

Accession number	Definition	Source
AB035455	16S rRNA gene	*Porphyromonas gingivalis* FDC381
AY546475	16S rRNA gene	*Porphyromonas gingivalis* ATCC33277
L16491	16S rRNA gene	*Porphyromonas endodontalis*
L16468	16S rRNA gene	*Prevotella intermedia* ATCC25611
L16481	16S rRNA gene	*Prevotella loeschei*
L16467	16S rRNA gene	*Prevotella denticola*
L16469	16S rRNA gene	*Prevotella melaninogenica*
L16474	16S rRNA gene	*Prevotella oris*
L16487	16S rRNA gene	*Prevotella heparinolytica*
AY689230	16S rRNA gene	*Prevotella nigrescens*
AJ005634	16S rRNA gene	*Prevotella tannerae*
L04321	16S rRNA gene	*Bacteroides ureolytica*
M22512	16S rRNA gene	*Eikenella corrodens*
U65987	16S rRNA gene	*Eubacterium saphenum*
AJ133496	16S rRNA gene	*Fusobacterium nucleatum* ATCC25586
AF287793	16S rRNA gene	*Selenomonas sputigena*
AF287799	16S rRNA gene	*Selenomonas noxia*
AB035460	16S rRNA gene	*Tannerella forsythensis*
AF133536	16S rRNA gene	*Capnocytophaga sputigena*
L14635	16S rRNA gene	*Capnocytophaga ochracea*
AY119692	16S rRNA gene	*Treponema denticola*
DQ174168	16S rRNA gene	*Campylobacter gracilis*
L04317	16S rRNA gene	*Campylobacter rectus*
X82500	16S rRNA gene	*Dialister pneumosintes*
AY323523	16S rRNA gene	*Peptostreptococcus micros*
AB097215	16S rRNA gene	*Propionibacterium acnes*
X82435	16S rRNA gene	*Actinomyces viscosus*
AF104671	16S rRNA gene	*Streptococcus intermedius*
AY281076	16S rRNA gene	*Streptococcus mitis*
DQ303189	16S rRNA gene	*Streptococcus mutans*
AY188352	16S rRNA gene	*Streptococcus salivarius*
DQ303192	16S rRNA gene	*Streptococcus sanguis*
AY362906	16S rRNA gene	*Haemophilus aphrophilus*
M75035	16S rRNA gene	*Aggregatibacter actinomycetemcomitans* Y4

Specificities of oligonucleotides probe

Five strains belonging to the *Prevotella* group, two strains belonging to the *Porphyromonas* group, and three strains of *A. actinomycetemcomitans* were used to test probe specificity (Table 1). *In situ* hybridizations with either probe PG177 or probe AA191 were performed routinely at 45°C. Amann et al. reported that although hybridization at high stringency resulted in an overall reduction in fluorescence intensity, it was possible to distinguish between strains that differed by a single mismatch within the target site (20-mer) at 45°C hybridization (21). In this study, five strains of the *Prevotella* group showed from 23 to 30 mismatches to the target region of probe PG177 (50-mer). Therefore, it was assumed that this hybridization temperature was sufficient to distinguish more than one mismatch hybrid and to offer high specificity for both the PG177 and AA191 probes.

Representative photomicrographs for whole-cell hybridization are shown in Figs 3 and 4. The PG177 probe is complementary to *P. gingivalis* strain FDC 381 and shows 100% similarity to the target sequence of strain ATCC33277. This oligonucleotide probe labeled with DIG at the 5′-end hybridized equally well with rRNA molecules in whole fixed cells, and the reporter molecule could be detected *in situ* with fluorescently labeled anti-DIG Fab fragments. *P. gingivalis* FDC381 and ATCC33277 were strongly fluorescent (Fig. 3A,B), whereas four strains of the *Prevotella* group showed no hybridization to probe PG177 (Fig. 3C–F). In fluorescence micrographs, each cell of strains FDC381 and ATCC33277 was clearly identified using probe PG177 and showed the distinct shape of short rods. In the same way, probe AA191 showed strong fluorescence when hybridized with *A. actinomycetemcomitans* (strains ATCC29523, NCTC9710, and Y4) cells, whereas two strains of *H. aphrophilus* showed no hybridization to the AA191 probe (Fig. 4). These results showed that the 50-mer length probe in this study was able to permeate the fixed cells and to hybridize with rRNA.

Detection and enumeration of specific cells in mixtures

Defined mixtures of either *P. gingivalis* and *Fusobacterium nucleatum* or *A. actinomycetemcomitans* and *F. nucleatum* were hybridized with two probes to investigate the utility of the probes for enumerating specific bacteria against a background of non-target cells. Fixed *P. gingivalis* (1.0×10^7/ml) and *F. nucleatum* (1.0×10^7/ml) cell suspensions were mixed. In a mixture of *P. gingivalis* (short rods) and *F. nucleatum* (spindle-shaped), the use of the PG177 probe detected only *P. gingivalis* (Fig. 5). The *P. gingivalis* was strongly fluorescent whereas *F. nucleatum* showed no hybridization to the PG177 probe. Similarly, probe AA191 detected only the target bacterial cells in a mixture of *A. actinomycetemcomitans* (coccoid) and *F. nucleatum*. These results suggest that the species-specific probes used in this study can correctly and specifically detect target bacteria.

Discussion

Members of the genera *Porphyromonas* and *Prevotella* are anaerobic gram-negative, black-pigmented, oral pathogens regularly isolated from cases of gingivitis and periodontitis. The rapid, specific, and visible identification of obligate anaerobes

A

Position	173				187				212
Probe PG177	3′-CGTA--	-----TGTGA	A-CATA----	------ATAA	CATACTATAA	TGTTC-CTTT	ATAAATATCG	ACA-5′	
Target	GCAT--	-----ACACT	T-GTAT----	------TATT	GCATGATATT	ACAAG-GAAA	TATTTATAGC	TGT	
P. intermedia	TACCCGATGT	TGTCCACATA	TGGCAT----	--C--TGACG	----------	TGGA-CCAAA	GATTCATCGG	TGGAGGATGG	
P. melaninogenica	-N---GA---	---CTA-ATA	TCGCATAGTC	TTCGATGACG	GCATCAGATT	TGAA-GTAAA	GATTTATCGG	TAATGGATGG	
C. ochracea	TACCC----	------CATA	--GTATAGT-	----AAYNYG	GCATCAYNAT	ACTA-TTAAA	GCTTTGGTGG	TGAAAGATGA	
T. denticola	-ACC-----	TGCC------	---------C	T---------	----------	----------	----------	--GAAGATGG	
P. denticola	TACCGCAT--	-----G-TTC	TTCGAT----	------GACG	GCATCAGATT	NGAAG-CAAA	GATCCATCGG	TAACGGAGGG	
P. nigrescens	TACCCGAT--	-----G-TGT	TTCATT----	------GACG	GCATCCGATA	TGAAA-CAAA	GGTNTTCCGG	TAAGGGATGG	
P. loesheii	TACCTGAT--	-----GGTGT	TGTGAT----	-----GA-G	GCATCTTATT	GCAAC-TAAA	GATTTATTGG	TAAAGGATGG	
P. asaccharolytica	TACACCAT--	-----ATACT	CCTTAG----	------ATCC	-CATGAGAAG	AGGAG-GAAA	GATTAATCGC	TAAGAGATGG	
T. forsythensis	TACCTCAT--	-----A-AAA	CAGGGG----	-----TTCC	GCATGGGGCN	NTTTGTTAAA	GATTTATTGG	TTGCGGATGG	
P. gingivalis	AACCGCAT--	-----ACACT	T-GTAT----	-----TATT	GCATGATATT	ACAAG-GAAA	TATTTATAGC	TGTAAGATAG	
***P. gingivalis* 381**	AACCGCAT--	-----ACACT	T-GTAT----	-----TATT	GCATGATATT	ACAAG-GAAA	TATTTATAGC	TGTAAGATAG	
A. a Y4	TACCGCGT--	----------	----------	---AGGGTC	G---GGAGAC	GAAAGTGCGG	GACTT-T---	---ATGGC-C	
H. aphrophilus	TACCGCGT--	----------	----------	---AGAATC	G---GGAGAT	GAAAGTGTGG	GACCT-T---	---CGGGC-C	
E. coli	TACCGCAT--	----------	----------	---AACGTC	G---CAAGAC	CAAAGAGGGG	GACCT-T---	---CGGGC-C	
S. mitis	TACCGCAT--	----------	----------	---AAGAGT	A---GATGTT	GCATGACATT	TGCTTAA---	---AAGGTGC	
A. viscosus	TACGGGAT--	----------	----------	---A---TT	--------CT	G--GGTCTGT	CGC-AT----	-----GGCGG	
C. rectus	TACTCTAT--	----------	----------	----A-CTC	--------CT	TC-TTTACAT	AAGTTA----	----AGTCGG	

B

Position	187				216				220
Probe AA191	3′-CTG	CTTTCACGCC	CTGAA-A---	---TACCG-G	CGTA------	----------	----------	C---------	
Target	GAC	GAAAGTGCGG	GACTT-T---	---ATGGC-C	GCAT------	----------	----------	G---------	
P. intermedia	----------	TGGA-CCAAA	GATTCATCGG	TGGAGGATGG	GGAT------	----------	----------	----------	
P. melaninogenica	GCATCAGATT	TGAA-GTAAA	GATTTATCGG	TAATGGATGG	GGAT------	----------	----------	----------	
C. ochracea	GCATCAYNAT	ACTA-TTAAA	GCTTTGGTGG	TGAAAGATGA	GATT------	----------	----------	----------	
T. denticola	----------	----------	----------	--GAAGATGG	GGATAGCTAG	TAGAAATATT	AGATAATACC	GAATGTGCTC	
P. denticola	GCATCAGATT	NGAAG-CAAA	GATCCATCGG	TAACGGAGGG	GGAT------	----------	----------	----------	
P. nigrescens	GCATCCGATA	TGAAA-CAAA	GGTNTTCCGG	TAAGGGATGG	GGAT------	----------	----------	----------	
P. loesheii	GCATCTTATT	GCAAC-TAAA	GATTTATTGG	TAAAGGATGG	CGAT------	----------	----------	----------	
P. asaccharolytica	-CATGAGAAG	AGGAG-GAAA	GATTAATCGC	TAAGAGATGG	GCCT------	----------	----------	----------	
T. forsythensis	GCATGGGGCN	NTTTGTTAAA	GATTTATTGG	TTGCGGATGG	GTAT------	----------	----------	----------	
P. gingivalis	GCATGATATT	ACAAG-AGGG	TATTTATAGC	TGTAAGATAG	GCAT------	----------	----------	----------	
P. gingivalis 381	GCATGATATT	ACAAG-GAAA	TATTTATAGC	TGTAAGATAG	GCAT------	----------	----------	----------	
***A. a* Y4**	G---GGAGAC	GAAAGTGCGG	GACTT-T---	---ATGGC-C	GCAT------	----------	----------	G---------	
H. aphrophilus	G---GGAGAT	GAAAGTGTGG	GACCT-T---	---CGGGC-C	ACAT------	----------	----------	G---------	
E. coli	G---CAAGAC	CAAAGAGGGG	GACCT-T---	---CGGGC-C	TCTT------	----------	----------	G---------	
S. mitis	A---GATGTT	GCATGACATT	TGCTTAA---	---AAGGTGC	AAAT------	----------	----------	GCAT------	
A. viscosus	--------CT	G--GGTCTGT	CGC--AT---	-----GGCGG	GCCT------	----------	----------	G---------	
C. rectus	--------CT	TC-TTTACAT	AAGTTA----	----AGTCGG	GAAA------	----------	----------	GTNT------	
					231			242	
Probe AA191	----------	----------	-----GGT--	----------	---ACTCTAC	TTGGG-----	----------	---TTCAC-5′	
Target	----------	----------	-----CCA--	----------	---TGAGATG	AACCC-----	----------	---AAGTG	
P. intermedia	----------	----------	----------	----------	----------	----------	----------	----------	
P. melaninogenica	----------	----------	----------	----------	----------	----------	----------	----------	
C. ochracea	----------	----------	----------	----------	----------	----------	----------	----------	
T. denticola	ATTTACATAA	AGGTAAATGA	GGAAAGGAGC	TACGGCTCCG	CTTCAGGATG	GGCCC-----	----------	----------	
P. denticola	----------	----------	----------	----------	----------	----------	----------	----------	
P. nigrescens	----------	----------	----------	----------	----------	----------	----------	----------	
P. loesheii	----------	----------	----------	----------	----------	----------	----------	----------	
P. asaccharolytica	----------	----------	----------	----------	----------	----------	----------	----------	
T. forsythensis	----------	----------	----------	----------	----------	----------	----------	----------	
P. gingivalis	----------	----------	----------	----------	----------	----------	----------	----------	
P. gingivalis 381	----------	----------	----------	----------	----------	----------	----------	----------	
***A. a* Y4**	----------	----------	-----CCA--	----------	---TGAGATG	AACCC-----	----------	---AAGTGTG	
H. aphrophilus	----------	----------	-----CCA--	----------	---TGGGATG	AACCC-----	----------	---AAGTGGG	
E. coli	----------	----------	-----CCA--	----------	---TCGGATG	TGCCC-----	----------	---AGATGTG	
S. mitis	----------	----------	---CACTA--	----------	---CCAGATG	GACCT-----	----------	---GCGTTTG	
A. viscosus	----------	----------	-----GGA--	----------	----AAGATG	CGCCT--GGG	TGTTTTTGGT	GGGGGATGGG	
C. rectus	----------	----------	---T------	----------	---TCGG-TG	TA-----GGA	T----------	--GAGGCTAT	

Fig. 2. Sequence alignment of 16S ribosomal RNAs in the vicinity of the oligonucleotide target regions and sequences of the probes. Target positions for the probes are indicated by boxes. The probe and target site sequence is displayed in the upper row. Positions that differ from the target sequence are shown underlined in bold type; positions numbered according to Brosius et al. (7) (A) The sequence of the oligonucleotide probe for *Porphyromonas gingivalis*, PG177. (B) The sequence of the oligonucleotide probe for *Aggregatibacter actinomycetemcomitans*, AA191.

from a highly complex microbiota in periodontitis is a difficult task. The goal of the present study was to determine whether the new oligonucleotide probes constructed in this study could specifically and visually detect *P. gingivalis* and *A. actinomycetemcomitans* by FISH. The 50-mer oligonucleotide probes constructed showed clear fluorescence with

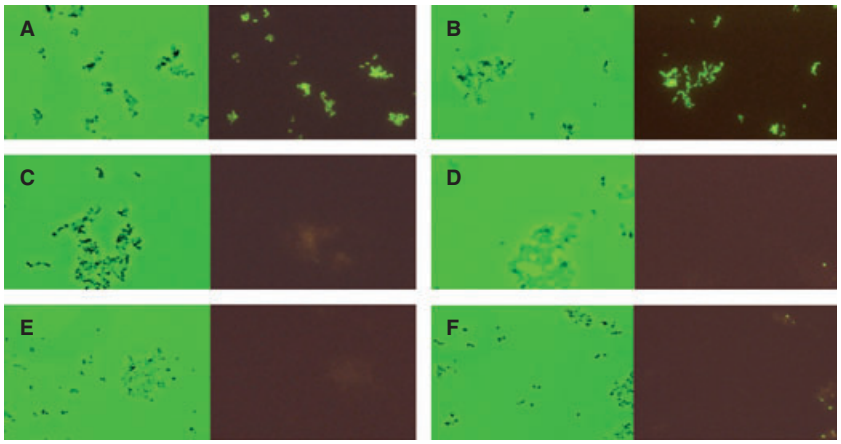

Fig. 3. Microscopic observation of whole-cell hybridization with fluorescein isothiocyanate-labeled PG177 probe. All hybridizations were performed at 45°C and wash steps were performed at 30°C. Phase-contrast (left) and epifluorescence (right) photomicrographs are displayed for each field. (A) *Porphyromonas gingivalis* ATCC33277, (B) *P. gingivalis* FDC381, (C) *Prevotella intermedia* ATCC25611, (D) *Prevotella nigrescens* ATCC33563, (E) *Prevotella melaninogenica* ATCC25845, and (F) *Prevotella denticola* ATCC33184 hybridized with the *P. gingivalis* probe.

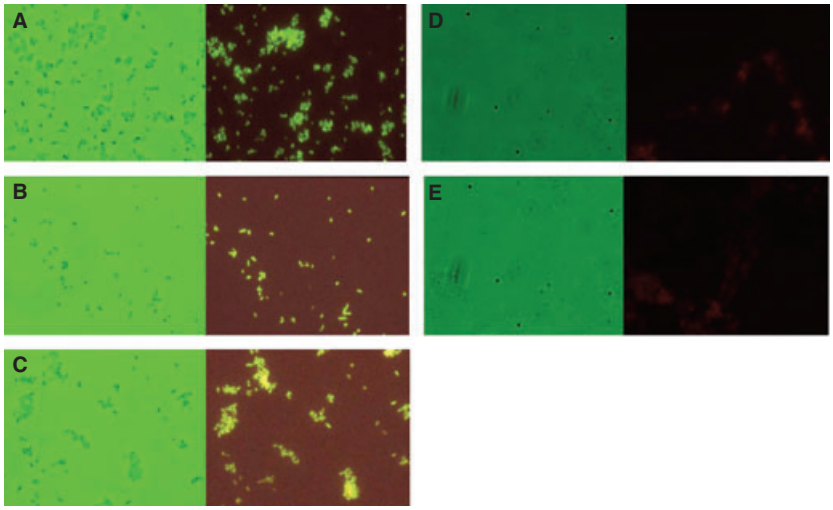

Fig. 4. Microscopic observation of whole-cell hybridization with fluorescein isothiocyanate-labeled or rhodamine-labeled AA191 probe. All hybridizations were performed at 55°C and wash steps were performed at 30°C. Phase-contrast (left) and epifluorescence (right) photomicrographs are displayed for each field. (A) *Aggregatibacter actinomycetemcomitans* Y4, (B) *A. actinomycetemcomitans* ATCC29523, (C) *A. actinomycetemcomitans* NCTC9710, (D) *Haemophilus. aphrophilus* ATCC15416, and (E) *H. aphrophilus* ATCC5908 hybridized with the *A. actinomycetemcomitans* probe.

P. gingivalis and *A. actinomycetemcomitans*, which were used as positive control strains (Figs 3 and 4). These probes were also effective in the specific identification and visualization of bacterial single cell morphology.

Visualization of bacteria in various clinical samples is important in the diagnosis of periodontal diseases. The FISH technique is a useful method for the visualization, identification, enumeration, and localization of the bacterial species within a variety of biological samples (8, 25, 26). This technique is effective for studying complex microbiota and their relationship with different growth conditions and hosts (2). It is a major advantage of FISH that growing bacteria are detectable, particularly those anaerobic periodontal species that cannot be cultured or that are difficult to culture. *P. gingivalis* is an obligate anaerobe showing nutrition requirements for vitamin K and hemin, and it is difficult to detect this species using culture-dependent techniques. The present results demonstrated that FISH with a single-use probe could simply and easily detect *P. gingivalis* and *A. actinomycetemcomitans* (Figs 3 and 4). It was shown that the clear shape of single bacterial cells could be visualized by FISH with the present oligonucleotide probes.

The length of the probe is important because shorter probes frequently and non-specifically hybridize to similar sequences to the target. Homology search analysis using a DNA sequence databank indicated that only two or three species showed more than 80% identity to our 50-mer oligonucleotide probes whereas this value was 33 species for a 24-mer oligonucleotide probe (25). Probe lengths may be increased to obtain the necessary specificity and single-cell fluorescence. Simultaneous application of several fluorescently mono-labeled oligonucleotide probes directed to different target sites of one rRNA molecule has additively increased the signal intensity (1), but the number of sites on the rRNA with a defined specificity for the probe is limited. It was reported

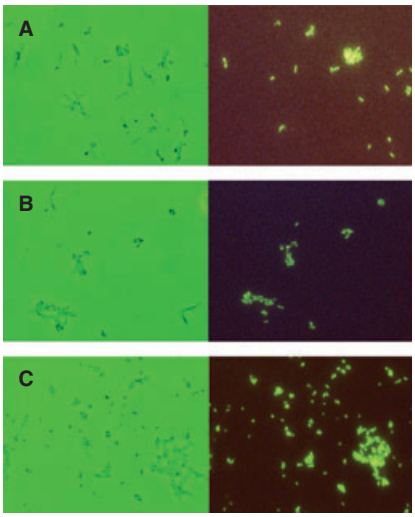

Fig. 5. Specific identification of targeted whole cells in a mixed bacterial sample. A fixed cell suspension of *Fusobacterium nucleatum* (1.0×10^7/ml) was mixed with equal numbers of either *Porphyromonas gingivalis* (1.0×10^7/ml) or *Aggregatibacter actinomycetemcomitans* (1.0×10^7/ml). All hybridizations were performed at 45°C and wash steps were performed at 30°C. Phase-contrast (left) and epifluorescence (right) photomicrographs are displayed for each field. (A) The mixture of *P. gingivalis* ATCC33277 and *F. nucleatum* ATCC25586, (B) the mixture of *P. gingivalis* FDC381 and *F. nucleatum* ATCC25586 hybridized with the *P. gingivalis* probe, and (C) the mixture of *A. actinomycetemcomitans* Y4 and *F. nucleatum* ATCC25586 hybridized with the *A. actinomycetemcomitans* probe.

that DIG significantly amplifies the signal intensity as an effective reporter molecule (21). Therefore, 50-mer oligonucleotides labeled with DIG were used to enhance the signal intensity. Fixed target cells in this study were permeable to oligonucleotides of this length and exhibited species-specific DNA–DNA hybridization *in situ* (Figs 3A,B and 4). This method showed sufficient fluorescence intensity to detect single bacterial cells so FISH using the present probes has proved to be a useful method for the direct detection and identification of *P. gingivalis* and *A. actinomycetemcomitans*.

The results of the direct detection of the target bacterium in the mixed bacterial samples of *F. nucleatum* and *P. gingivalis* or *F. nucleatum* and *A. actinomycetemcomitans*, demonstrated that the present oligonucleotide probes reacted strongly enough for clear microscopic discrimination of the targeted cells (Fig. 5). From the bacterial cell count by fluorescent microscopy, the present probes could exactly detect the targeted bacterial cell numbers that were applied in the bacterial species mixtures (data not shown). It is expected that our 50-mer length oligonucleotide probes exactly and visually detect target bacteria in a more complicated microflora in a host. Our results show that longer oligonucleotides may be applied to various natural samples.

The FISH approach using the present 50-mer oligonucleotide probes can visualize periodontal pathogens and enable the presentation of visual data to a patient with periodontal disease. Many studies have shown the importance of individual-adjusted maintenance and self-care programs to achieve and maintain satisfactory oral health status (6, 24). The success of the treatment depends on the capacity of the patient's for oral self-care (14). Explaining the disease status by showing images of the periodontal pathogenic bacteria to a patient will be a powerful motivation for oral self-care. The present FISH method will enable specific and visible detection of periodontopathogens in a patient in a clinical setting.

Acknowledgment

This study was supported by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science (13672155).

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