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Multiple displacement amplification as an aid in checkerboard DNA–DNA hybridization

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Objective: The study aimed to determine if multiple displacement amplification could be used to provide abundant target DNA and DNA probes for checkerboard DNA–DNA hybridization.
Methods: Multiple displacement amplification was used to amplify 1 and 10 ng DNA from 16 individual bacterial species, DNA from single colonies, from a mixture of 20 bacterial species and oral biofilm samples, such as supragingival plaque, subgingival plaque, buccal swab and root canal samples. Samples in reaction buffer were heat-denatured at 95°C for 3 min and cooled to 4°C. Φ29 DNA polymerase was added and the mixture was incubated at 30°C for 16–18 h. The quantity of the product was evaluated by the Picogreen assay. The amplified material was labeled with digoxigenin. The probes were compared with probes obtained from unamplified DNA using checkerboard DNA–DNA hybridization. Both amplified DNA and unamplified DNA were used as targets on the membrane. Amplified oral biofilm samples were compared to unamplified samples using checkerboard DNA–DNA hybridization.
Results: The DNA yield ranged from 4 to 11 μg. DNA–DNA hybridization showed that the amplified genome of each species used either as target or as probe provided signals equivalent to controls and that amplification of a mixture of species provided signals comparable to those provided by the unamplified source mixture. Amplified oral biofilm samples exhibited comparable proportions of bacterial DNA when compared to the original unamplified samples.
Conclusions: The multiple displacement amplification technique is a simple and reliable method to uniformly amplify DNA for use in checkerboard DNA–DNA hybridization. It is also a useful tool in the amplification of clinical samples.

Key words: bacteria; DNA probes; multiple displacement amplification; oral biofilm

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The major pathologies that affect the oral cavity are infectious in nature and comprise some of the most prevalent infections found in humans. Studies of the complex microbial ecosystem that initiates and maintains the progression of periodontal lesions have been facilitated in recent years by molecular techniques, including sequencing of 16S rDNA directly from oral biofilm samples (13) and checkerboard DNA–DNA hybridization (20). A major advantage of the latter technique is its high throughput that allows, in its original format, the quantification of the levels of 40 bacterial species in up to 28 samples, simultaneously.

The checkerboard technique requires the extraction and purification of high-quality DNA from a wide range of microbial species. In addition, many species of interest are difficult to grow and/or difficult to lyse, impeding the recovery of highly purified DNA. Thus, many laboratories may be reluctant to implement this technique because of these constraints.

The purpose of the present investigation was to overcome this barrier by examining

the use of multiple displacement amplification (MDA) (2) for its ability to produce large amounts of DNA from test species to be used for the preparation of DNA standard and DNA probes. This method would permit the implementation of the checkerboard technique in laboratories that are unwilling to invest the considerable resources needed to obtain and maintain pure cultures of fastidious species as well as to undertake the isolation and purification of DNA for the preparation of standards and DNA probes. In addition, the applicability of multiple displacement amplification in enhancing the amount of DNA obtained from clinical samples from periodontal pockets, soft tissues and endodontic lesions was explored.

Materials and methods

Bacterial strains and growth conditions

The reference strains used for the preparation of DNA probes and standards are listed in Table 1. The majority of strains were grown on Trypticase soy agar supplemented with 5% defibrinated sheep blood [Baltimore Biological Laboratories (BBL), Cockeysville, MD] with the following exceptions. *Tannerella forsythia* was grown on Trypticase soy agar supplemented with 5% sheep blood and 10 μg/ml *N*-acetylmuramic acid (Sigma Chemical Co., St Louis, MO). *Porphyromonas gingivalis* was grown on Trypticase soy agar supplemented with 5% sheep blood, 0.3 μg/ml menadione (Sigma) and 5 μg/ml hemin (Sigma). *Eubacterium* and *Neisseria* species were grown on Fastidious Anaerobic Agar (BBL) with 5% defibrinated sheep blood. *Treponema denticola* and *Treponema socranskii* were grown in Mycoplasma broth (Difco Laboratories, Detroit, MI) supplemented with 1 mg/ml glucose, 400 μg/ml niacinamide, 150 μg/ml spermine tetrahydrochloride, 20 μg/ml sodium isobutyrate, 1 mg/ml L-cysteine, 5 μg/ml thiamine pyrophosphate and 0.5% bovine serum. All strains were grown at 35°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2).

DNA isolation and preparation of DNA probes

After 3–7 days of growth, cells were harvested and placed in 1.5-ml microcentrifuge tubes containing 1 ml TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6). Cells were washed twice by centrifugation in TE buffer at 1300 ***g*** for 10 min. The cells were resuspended and lysed with either 10% sodium dodecyl sulfate and Proteinase K (20 mg/ml) for gram-negative strains or in 150 μl of an enzyme mixture containing 15 mg/ml lysozyme (Sigma) and 5 mg/ml achromopeptidase (Sigma) in TE buffer (pH 8.0) for gram-positive strains. The pelleted cells were resuspended by 15 s of sonication in a Kontes micro-ultrasonic cell disrupter (Kontes, Vineland, NJ) at room temperature and incubated at 37°C for 1 h. DNA was isolated and purified using the method of Smith et al. (17). The concentration of the purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity was assessed by the ratio of the absorbance at 260 and 280 nm. Whole genomic probes were prepared for each test strain by labeling 1–3 μg DNA with digoxigenin (Boehringer Mannheim, Indianapolis, IN) using a random primer technique (5).

Multiple displacement amplification

Genomiphi™ (Amersham Biosciences, Arlington Heights, IL) was used for whole genomic amplification as described by the manufacturer. In brief, 1 μl of each of the DNA templates containing 1–100 ng of DNA was added to 9 μl of sample buffer (50 mM Tris–HCl pH 8.2, 0.5 mM EDTA containing random hexamer primers) in 200-μl microcentrifuge tubes (Stratagene, La Jolla, CA). Templates in sample buffer were heat denatured at 95°C for 3 min in a Perkin-Elmer Thermocycler and cooled to 4°C. Then, 1 μl of *Φ*29 DNA polymerase mix including additional random hexamers was mixed on ice with 9 μl reaction buffer containing dNTPs. The mixture was then added to the denatured sample to make a final volume of 20 μl and was incubated at 30°C for 16–18 h. 10 ng of *λ* DNA (contained in 1 μl) was used as a control. The amplification reaction was terminated by incubation of the samples at 65°C for 10 min. The amplified material was either immediately used, stored short-term at 4°C or at −20°C for longer storage.

Quantification of amplified DNA isolated from pure cultures or single colonies

Stock solutions of 10 ng/μl whole genomic DNA were prepared for the following species: *Actinomyces naeslundii* genospecies 1 and 2, *Actinomyces israelii*, *Actinomyces gerencseriae*, *Actinomyces odontolyticus*, *T. denticola*, *T. socranskii*, *Streptococcus intermedius*, *Fusobacterium periodonticum* and *Eikenella corrodens*. One microliter of each was used as a template for multiple displacement amplification. Each amplification was performed in triplicate and the yield of DNA was measured using the Picogreen™ assay (Molecular Probes, Carlsbad, CA).

Single colonies (approximately 10^8 cells) from each of *Streptococcus anginosus*, *Streptococcus intermedius*, *Streptococcus mitis*, *Fusobacterium nucleatum* subsp. *nucleatum*, *Fusobacterium nucleatum* subsp. *vincentii* and *F. periodonticum* were placed in 50 μl TE buffer in separate microcentrifuge tubes. After vortexing, 25 μl was added to 25 μl alkaline lysis

Table 1. Reference strains for preparation of DNA probes and standards

Actinobacillus actinomycetemcomitans	*	*Leptotrichia buccalis*	14201
Actinomyces gerencseriae	23860	*Neisseria mucosa*	19696
Actinomyces israelii	12102	*Peptostreptococcus micros*	33270
Actinomyces naeslundii genospecies 1	12104	*Porphyromonas gingivalis*	33277
Actinomyces naeslundii genospecies 2	43146	*Prevotella intermedia*	25611
Actinomyces odontolyticus	17929	*Prevotella melaninogenica*	25845
Campylobacter gracilis	33236	*Prevotella nigrescens*	33563
Campylobacter rectus	33238	*Propionibacterium acnes*	**
Campylobacter showae	51146	*Selenomonas noxia*	43541
Capnocytophaga gingivalis	33624	*Streptococcus anginosus*	33397
Capnocytophaga ochracea	33596	*Streptococcus constellatus*	27823
Capnocytophaga sputigena	33612	*Streptococcus gordonii*	10558
Eikenella corrodens	23834	*Streptococcus intermedius*	27335
Eubacterium nodatum	33099	*Streptococcus mitis*	49456
Eubacterium saburreum	33271	*Streptococcus oralis*	35037
Fusobacterium nucleatum subsp. *nucleatum*	25586	*Streptococcus sanguinis*	10556
Fusobacterium nucleatum subsp. *polymorphum*	10593	*Tannerella forsythia*	43037
Fusobacterium nucleatum subsp. *vincentii*	49256	*Treponema denticola*	B1
Fusobacterium periodonticum	33693	*Treponema socranskii*	S1
Gemella morbillorum	27824	*Veillonella parvula*	10790

All strains were obtained from the American Type Culture Collection (ATCC) except *Treponema denticola* B1 and *Treponema socranskii* S1, which were obtained from The Forsyth Institute.
*ATCC strains 43718 and 29523; **ATCC strains 11827 and 11828.

buffer (400 mM KOH, 100 mM dithiothreitol, 10 mM EDTA). After 10 min of incubation on ice, 25 μl neutralization buffer (400 mM HCl, 600 mM Tris–HCl, pH 0.6) was added. One microliter of the final solution was used as a template for amplification for each species. The resulting amplified DNA was measured using the Picogreen™ assay.

Checkerboard DNA–DNA hybridization

Checkerboard DNA–DNA hybridization was performed as previously described (6, 19, 20). In brief, following amplification and quantification, amplified and unamplified DNA preparations were boiled in a waterbath for 10 min. The DNAs were pipetted into the extended slots of a Minislot 30 (Immunetics, Cambridge, MA) and then concentrated onto a nylon membrane (Boehringer Mannheim) by vacuum and fixed onto the membrane by cross-linking using ultraviolet light (Stratalinker 1800, La Jolla, CA), followed by baking at 120°C for 20 min. The Minislot device permitted the deposition of 28 different samples in individual lanes on a single membrane, as well as two control lanes containing 10^5 and 10^6 cells of each test species.

The membrane with fixed DNA was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90° to the channels of the device. A 30 × 45 'checkerboard' pattern was produced. Each channel was used as a hybridization chamber for separate DNA probes.

Bound probes were detected by anti-digoxigenin antibody conjugated with alkaline phosphatase and a chemifluorescent substrate. Signals were detected using a Storm FluorImager (Molecular Dynamics, Sunnyvale, CA).

Comparison of DNA probes prepared from amplified and unamplified DNA

One microgram of amplified DNA from each of 14 reference strains was labeled with digoxigenin using random primer labeling (5). The probes were compared with probes prepared from unamplified DNA using checkerboard DNA–DNA hybridization (19, 20). Amplified and unamplified DNA preparations from the same strains were used as targets.

Determination of amplification bias

To test amplification bias, DNA was isolated from 20 species and a mixture containing 100 ng of each species was prepared in TE buffer. Unamplified DNA was denatured and deposited in lanes on a nylon membrane at concentrations of 10 ng and 1 ng per species (200 and 20 ng total DNA per lane) using a Minislot as described above. From the original mixture, a sample containing 10 ng DNA from each species was amplified using multiple displacement amplification as described above. The resulting amplified DNA was measured using Picogreen and 200 ng and 20 ng of the total amplified mixture was denatured and placed in lanes on the same membrane. The membrane was then analysed using checkerboard DNA–DNA hybridization using whole genomic probes to the 20 test species. Signal intensities were determined using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA). The ratio of amplified : unamplified signals was determined for each species. The amplification bias was the ratio of the largest to the smallest measured ratios (2).

Comparison of multiple displacement amplification and unamplified DNA from clinical samples

Samples of supragingival plaque, subgingival plaque, soft tissues and root canals were examined to compare the composition of the microbiota using amplified and unamplified DNA. Supragingival and subgingival plaque samples were taken from the mesiobuccal aspect of each tooth with a sterile Gracey curette and placed in separate Eppendorf tubes containing 100 μl alkaline lysis buffer. After 10 min of incubation on ice, 100 μl neutralization buffer was added and 1 μl of the final solution was used as a template for amplification.

Soft tissue samples were collected during periodontal surgery and processed similarly. Buccal mucosa samples were collected from buccal mucosa with plastic swabs.

Root canal samples were obtained by scraping the canal walls with an endodontic file. The files were aseptically cut off from their handles and transferred to 200-μl microcentrifuge tubes (Stratagene, La Jolla, CA) containing 20 μl alkaline lysis buffer. After 10 min of incubation on ice, 20 μl neutralization buffer was added and 1 μl was used as template for multiple displacement amplification. The amplified and unamplified clinical samples were examined using checkerboard DNA–DNA hybridization.

Table 2. DNA yield from purified DNA, single colonies and clinical samples after multiple displacement amplification

Type of sample	Input DNA (ng)	Mean ± SD (μg)
Reference strains		
A. naeslundii 1	10	5.02 ± 2.18
A. naeslundii 2	10	7.95 ± 0.89
A. israelii	10	11.24 ± 9.42
A. gerensceriae	10	5.63 ± 0.19
E. corrodens	10	7.68 ± 0.82
F. periodonticum	10	5.65 ± 1.00
T. socranskii	10	7.57 ± 1.54
T. denticola	10	6.33 ± 0.84
A. odontolyticus	10	6.09 ± 0.28
S. intermedius	10	6.51 ± 0.75
E. nodatum	1	6.11 ± 1.51
S. constellatus	1	6.65 ± 1.06
A. gerensceriae	1	7.82 ± 1.53
T. socranskii	1	10.57 ± 0.91
F. periodonticum	1	7.97 ± 1.90
Bacterial mixture (standard) (20 bacterial species)	10 (each species)	9.85 ± 5.78
Single colonies		
S. anginosus	6.62	7.71 ± 3.57
S. intermedius	4.74	6.92 ± 1.25
S. mitis	6.41	8.64 ± 3.04
F. nucleatum subsp. *nucleatum*	4.48	5.96 ± 1.86
F. nucleatum subsp. *vincentii*	4.77	4.19 ± 4.23
F. periodonticum	5.62	6.66 ± 2.03
Clinical samples		
Supragingival plaque	70	4.67 ± 1.19
Subgingival plaque	4.40	3.99 ± 0.53
Buccal swab	92	6.87 ± 1.47
Root canal	31	7.37 ± 0.38
Soft tissue	11	8.49 ± 1.47
Control λDNA	10	6.42 ± 0.18

The assays were performed in triplicates.

Results

Quantification of amplified DNA from test samples

DNA from single colonies, bacterial mixtures, pure cultures and clinical samples was successfully amplified using multiple displacement amplification (Table 2). The DNA yield ranged from 3.9 to 11.2 μg from 1 ng to 10 ng of template DNA.

Checkerboard DNA–DNA hybridization using amplified and unamplified DNA preparations

The 10 ng of amplified and unamplified DNA from reference strains were laid on a nylon membrane as targets and hybridized using DNA probes made from amplified and unamplified DNA from the same strains. MDA-amplified DNA used either as targets or probes provided signals comparable to unamplified DNA (Fig. 1).

Figure 2 presents a checkerboard DNA–DNA hybridization membrane using DNA amplified directly from single colonies. As demonstrated, the amplified DNA provided effective targets using probes prepared from amplified or unamplified DNA from the same strains.

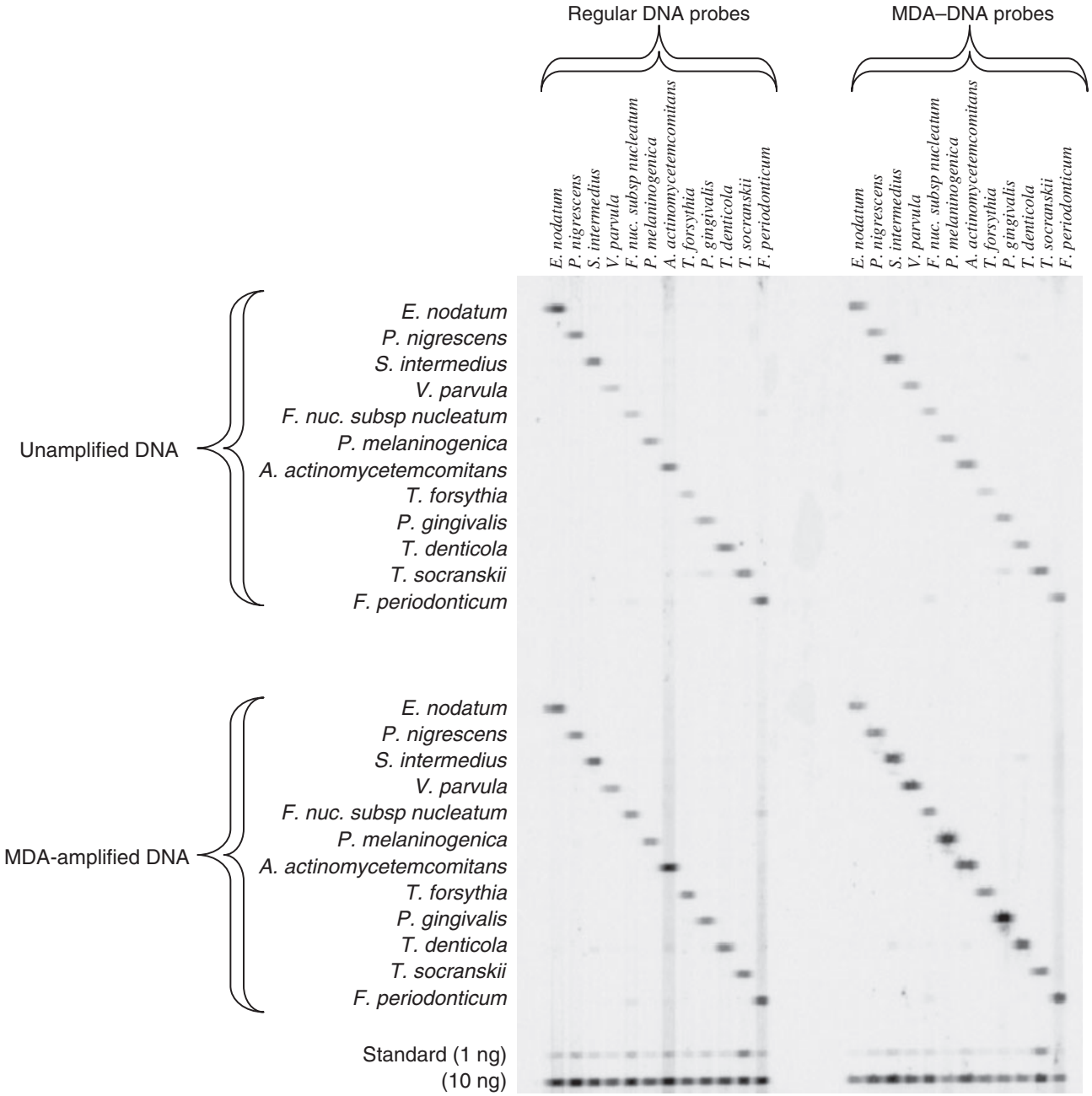

Fig. 1. Amplified and unamplified DNA from 12 species used as targets for DNA hybridization as well as for the preparation of DNA probes. Checkerboard DNA–DNA hybridization was performed using probes prepared from unamplified DNA of the test species as well as multiple displacement amplification-amplified DNA from the same organisms; 10 ng DNA from each preparation was used as the target for each test species.

Determination of amplification bias when amplifying mixed bacterial species

A membrane with the amplified and unamplified DNAs of a mixture of 20 species run in duplicate is shown in Fig. 3. In addition, the ratio of the integrated signals for the amplified and unamplified DNA is presented for each species. The maximum amplification bias was calculated to be 3.28.

The use of multiple displacement amplification to amplify clinical samples from the oral cavity is shown in Fig. 4. Amplified DNA from supragingival and subgingival plaque, soft tissues and root canals provided signals comparable to those observed using unamplified samples.

Discussion

The development of multiple displacement amplification has provided a unique tool for the study of mixed microbial populations. The ability of this technique to provide abundant, uniformly amplified DNA from small samples simplifies the production of the DNAs that are used as 'reagents' in techniques such as checkerboard DNA–DNA hybridization and facilitates the amplification of DNA from small and/or rare samples for immediate use or archival purposes.

The multiple displacement amplification technique is based on strand displacement and enables uniform whole genomic amplification of DNA targets (2). The template is replicated again and again by a 'hyperbranching' mechanism of strand

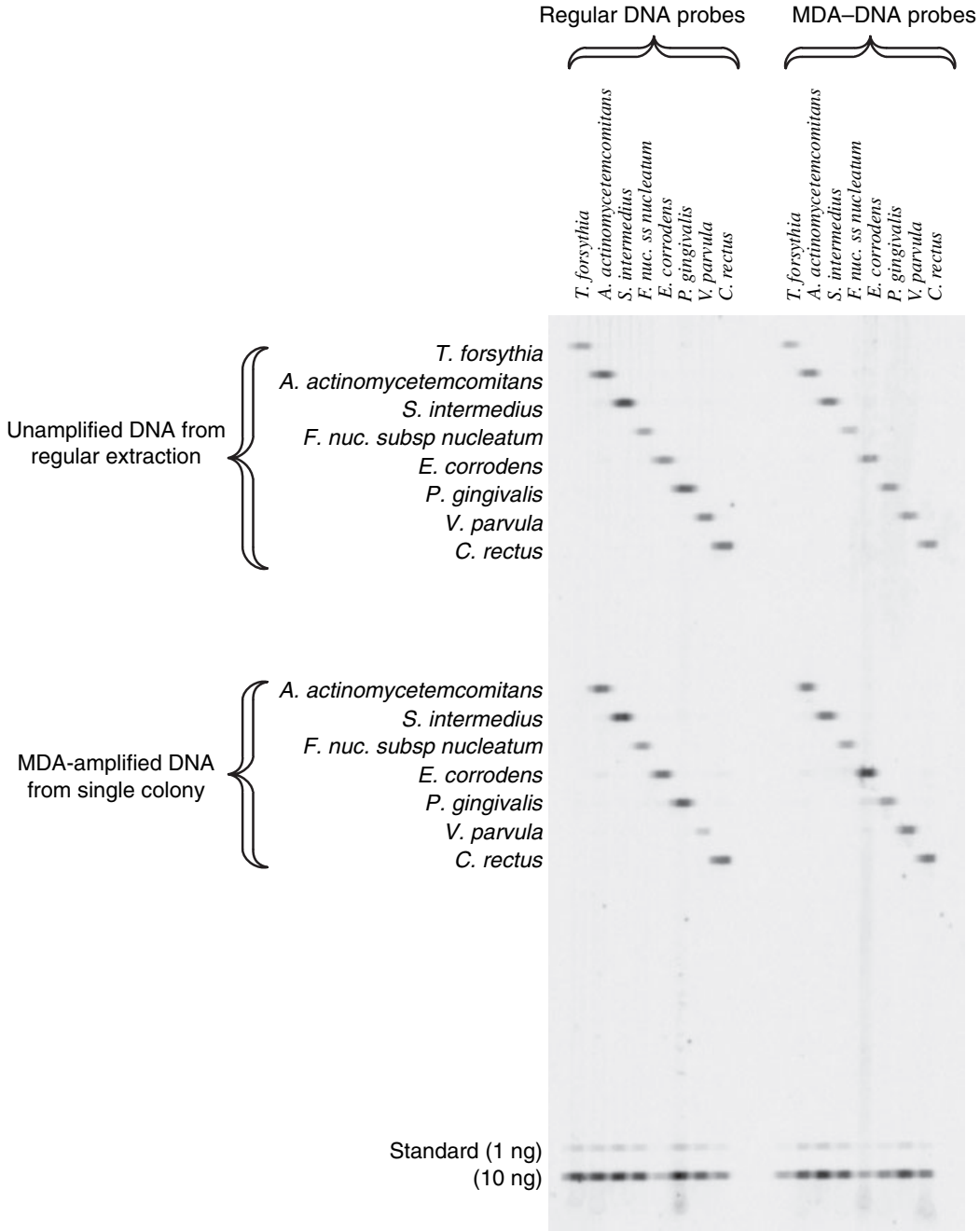

Fig. 2. Checkerboard DNA–DNA hybridization of DNA from individual species prepared using regular DNA extraction methods or multiple displacement amplification -amplified DNA from single colonies.

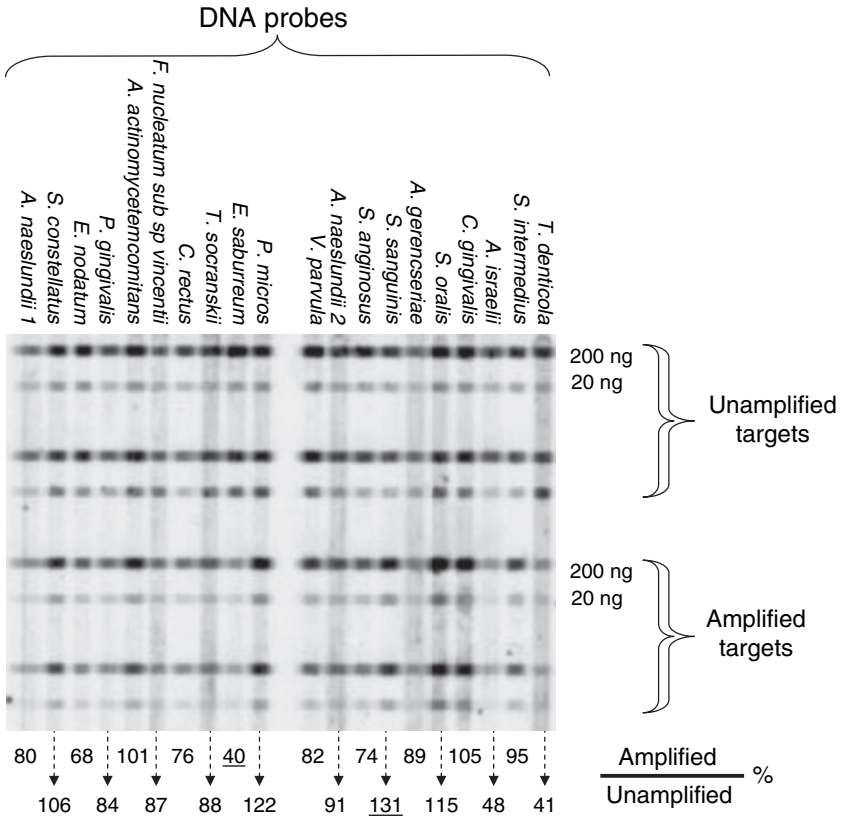

Fig. 3. Measurement of amplification bias. DNA was isolated from 20 test species and a mixture containing 1 μg of each species was prepared in TE buffer. Unamplified DNA was denatured and deposited in lanes on a nylon membrane at concentrations of 10 ng and 1 ng per species (200 and 20 ng total DNA per lane) using a Minislot device. From the original mixture, a sample containing 10 ng DNA from each species was amplified using multiple displacement amplification. The resulting amplified DNA was quantified using PicoGreen and 200 ng and 20 ng of the total amplified mixture was denatured and placed in lanes on the same membrane. The membrane was then analysed using checkerboard DNA–DNA hybridization using whole genomic probes to the 20 species. The resulting membrane with the amplified and unamplified DNAs run in duplicate is shown along with the ratio of the average integrated signals for the amplified and unamplified DNAs for each species for the 200-ng lanes. The maximum amplification bias was 131 (*Streptococcus sanguinis*)/40 (*Eubacterium saburreum*) = 3.28.

displacement synthesis (10), with the polymerase laying down a new copy as it displaces previously made copies. The DNA polymerase from bacteriophage *Φ*29, binds exceptionally tightly to DNA and continuously adds approximately 70,000 nucleotides every time it binds to the primer (1). This accounts for its ability to generate extremely long DNA products, averaging 12 kb and ranging up to 100 kb, compared with 100–1000 bp typically produced by polymerase chain reaction (PCR)-based methods (2, 9).

Samples as small as 1 ng can be amplified 1000- to 10,000-fold (11). Unlike PCR, multiple displacement amplification uniformly amplifies the entire genome (7) with minimal amplification bias (7, 12, 21). In contrast, the more widely used PCR-based amplification methods require specific primers and multiple cycles until a final product of limited size is obtained (8, 15). Multiple displacement amplification is an overnight isothermal reaction that relies on the proofreading activity of *Φ*29 DNA polymerase. It uses random primers with a total hands-on time of less than 30 min. After 16–18 h of incubation, DNA is ready to be used. The elimination of thermal cycling avoids sequence 'artifacts', such as those related to GC content that favor amplification bias, allowing equal representation of sequences because each priming event is propagated over very long distances in the genome. We demonstrated successful amplification of a wide range of bacterial species, with moles % GC content ranging from 27% to 60%. DNA from all tested species could be successfully amplified at least 1000-fold.

The *Φ*29 DNA polymerase has a very low error rate of 1 in 10^6 to 1 in 10^7 nucleotides in its intrinsic enzymatic activity (4) and during amplification (12), in contrast to 3×10^4 for *Taq* DNA polymerase (3). Thus, the accumulation of mutations following a 10,000-fold amplification is just 3 per 10^6 nucleotides (12). PCR-based amplification results in bias that varies from 10^2 to 10^6, while multiple displacement amplification bias for human genomic DNA has been estimated to be less than three-fold (2). Low amplification bias was confirmed in the present study, when comparing amplified and unamplified DNA from a mixture of subgingival species. The species were amplified in comparable ratios, with maximum amplification bias of 3.28.

Current knowledge of the microbiology of oral biofilms, particularly the etiology and treatment of periodontal diseases, has been facilitated by checkerboard DNA–DNA hybridization (18, 19) because this method delivers comprehensive information efficiently. However, the performance of the technique relies on the availability of high-quality DNA obtained through culture followed by phenol extraction. These tedious, time-consuming processes may yield DNA of variable quantity and quality.

The results of the present investigation suggest that multiple displacement amplification might be an important aid in the checkerboard DNA–DNA hybridization technique. The reduction in cost and time and increased feasibility would allow the use of the technique particularly in laboratories where resources are directed to other goals and the use of the technique would not otherwise be possible.

Whole genomic probes have several advantages that make them suitable for the checkerboard DNA–DNA hybridization technique. However, cross-reactivity among closely related species appears to be a weakness of those probes. We are now devising two methods to overcome this limitation. The experiments presented in this manuscript were performed using Genomiphi™ reagents that contain random primers. One of the approaches we are currently developing combines the efficacy of the *Φ*29 DNA polymerase and the specificity of oligonucleotides. Instead of employing random primers, we have been using specific 24-mers targeting the 16S rRNA gene. That way one expects to obtain probes that are more specific than their whole genomic counterparts. Another approach under way involves increasing the sensitivity of the already specific oligonucleotide probes. Until now, this would require a PCR step to amplify the target,

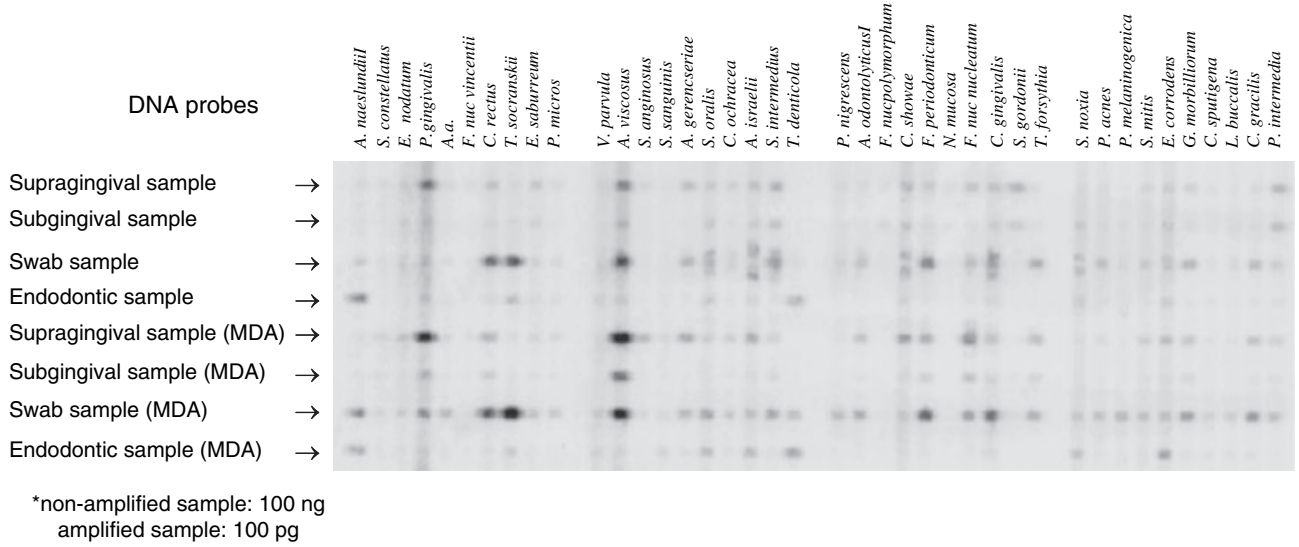

Fig. 4. Comparison of multiple displacement amplification-amplified and unamplified DNA from bacterial samples taken from various intraoral locations. The samples were split; 1/200 of each sample was amplified using multiple displacement amplification and then 3 μl of the 20-μl amplified product was run using the checkerboard DNA–DNA hybridization assay. The remaining 199 μl of the original sample was used directly in the checkerboard assay.

thereby introducing bias to the reaction. We are optimizing a technique to amplify the target using multiple displacement amplification and so improve the sensitivity of the oligonucleotide probes.

The present study also demonstrated the suitability of multiple displacement amplification for the amplification of different oral samples with minimal bias. This finding opens a new venue for microbiological analysis of clinical samples, demonstrating the potential for an extended range of analyses on a single sample. In its original format, checkerboard DNA–DNA hybridization analysed samples for the presence of a set of 40 bacterial species and used the entire sample. Multiple displacement amplification of the sample would permit virtually limitless numbers of DNA probes to be used to assay the same sample.

The technique also seems to be a promising tool in the study of 'precious' or rare biofilm samples: i.e. clinical samples that are difficult to obtain. The availability of a renewable source of sample will permit 'banking' of samples for future analysis, as well as analysis for the presence of 'uncultivable' bacteria or of ancient samples. While a very effective method, degraded DNA, poor-quality extractions or low amounts of template might produce bias (9). Thus, results from amplification of formalin-fixed and paraffin-embedded tissue samples might have to be carefully interpreted. However, archival frozen plasma has been demonstrated as a suitable source of amplifiable DNA (16). Also, there have been reports on successful amplification of minute samples, containing one single bacterium (14).

The multiple displacement amplification technique provides a simple and reliable method to amplify DNA for use in checkerboard DNA–DNA hybridization. It is also a useful tool in the amplification of clinical samples.

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