Fluorometric Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization in Microdilution Wells as an Alternative to Membrane Filter Hybridization in which Radioisotopes Are Used To Determine Genetic Relatedness among Bacterial Strains

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Fluorometric hybridization in microdilution wells was developed to determine genetic relatedness among microorganisms. Total chromosomal deoxyribonucleic acid (DNA) for hybridization reactions was labeled with photoreactive biotin (photobiotin). The biotinylated DNA was hybridized with single-stranded unlabeled DNAs which had been immobilized on the surfaces of microdilution wells. After hybridization, biotinylated DNA was quantitatively detected with bet-a-D-galactosidase and a fluorogenic substrate, 4-methylumbelliferyl-beta-D-galactopyranoside. Homology values obtained with this fluorometric direct binding method were compared with values obtained with two membrane filter methods, one in which photobiotin labeling was used and one in which radioisotope labeling was used. The results showed that the fluorometric direct binding method was effective in determining genetic relatedness among microdilution wells used could be an alternative to radiolabel and membrane filter hybridization methods.

Quantitative measurement of deoxyribonucleic acid (DNA)-DNA hybridization from renaturation rates has contributed to determinations of genetic relatedness among bacterial strains (2, 4, 5, 13, 19). The methods usually used in this technique are either a free solution method in which S1 nuclease, (4), spectrophotometry, (5, 19), or hydroxyapatite is used (2) or a method in which single-stranded DNA is fixed on a solid support, such as nitrocellulose filters (1, 12). However, to carry out most of these hybridization experiments, DNA must be labeled with radioactive substances by nick translation (18) or random primed labeling (9).

Recent developments have made it possible to label DNA with nonradioactive materials without using enzymes (10, 11, 17). Biotinylation of DNA with photoreactive biotin (photobiotin) (10) is one of these recent developments. In this technique DNA is labeled by mixing it with photobiotin and irradiating the mixture with sunlight for 15 min. Biotinylated DNA was used immediately for hybridization experiments. DNA must be labeled with radioactive substances by nick translation (18) or random primed labeling (9).

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MATERIALS AND METHODS

DNAs of bacterial strains (Table 1) were prepared by the procedures of Marmur, with minor modifications (8). Under optimal conditions, 100-µl portions of a heat-denatured, purified reference DNA solution (20 µg of DNA per ml) in phosphate-buffered saline (PBS; 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2, 137 mM NaCl, 2.7 mM KCl) containing 0.1 M MgCl2 were incubated for 1 h at 37°C in microdilution plates (MicroFluor type B plates; Dynatech Laboratories, Inc., Alexandria, Va.). Each solution was then aspirated with an automatic microplate washer (model AMW2 Auto-Mini-Washer; Bio-Tec Corp., Ltd., Tokyo, Japan). The microdilution plates were washed once with PBS containing 0.1 M MgCl2 and then dried at 60°C overnight.

Photobiotinylation of DNA was performed as described previously (17). Briefly, 5 µl of photobiotin (Vector Laboratories, Inc., Burlingame, Calif.) and an equal volume of a denatured DNA solution (5 µg of DNA in distilled water) were mixed in an Eppendorf tube and then irradiated with a sunlamp (500 W) for 15 min. After irradiation, free photobiotin was removed by 2-butanol extraction. The biotinylated DNA was used immediately for hybridization experiments.

For quantitative detection of photobiotinylated DNA in microdilution wells, 200-µl portions of a prehybridization solution (2X SSC [1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5X Denhardt solution, 50% formamide) containing 200 µg of denatured salmon sperm DNA per ml were added to microdilution wells which had been coated with reference DNAs or which contained small filters coated with reference DNAs. The microdilution plates were then incubated at 37°C for 1 h. The prehybridization solution was then removed from the wells with the Auto-Mini-Washer and replaced with 100-µl portions of hybridization mixture (2X SSC, 5X Denhardt solution, 3% dextran sulfate, 50% formamide, 50 µg of denatured salmon DNA per ml) containing 50 ng of biotinylated DNA. The microplates were then covered with vinyl tape and hybridization experiments were carried out at a low temperature to minimize the evaporation of the hybridization mixture. Although 25°C lower than the thermal denaturation temperature (Tw) in 2X SSC buffer is generally accepted as an optimal temperature for hybridization, we selected 55°C lower than the Tw for quantitative hybridization because our hybridization mixture contained 50% formamide and the hybridization temperature could be brought down to a temperature 55°C lower than the Tw (15).

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was measured with a MicroFluor reader (Dynatech Laboratories, Inc., Alexandria, Va.) at a wavelength of 360 nm for excitation and 450 nm for emission.

**Preparation of DNA bound to nitrocellulose filters for membrane filter hybridization.** A 250-μg portion of purified, single-stranded DNA was suspended in 100 ml of 2× SSC buffer and passed through a nitrocellulose filter (type HAHY; diameter, 4.7 cm; Nippon Millipore Corp., Yonezawa, Japan). The filter was dried at 42°C for 1 h and then punched out into small disks (diameter, 0.5 cm). The filters were then baked at 80°C for 2 h and kept in a desiccator at room temperature. The filters were placed in a tissue culture plate with 48 wells (Costar, Cambridge, Mass.). Prehybridization and hybridization were carried out as described above. After hybridization, the filters were washed twice with 500 μl of 2× SSC containing 0.1% sodium dodecyl sulfate (SDS) for 15 min and twice with 500 μl of 0.2× SSC containing 0.1% SDS for 15 min. When 32P-labeled DNA was used, the hybridized filters were subjected directly to scintillation counting. When photobiotinylated DNA was used, the filters were placed in new Costar wells, and 200 μl of PBS supplemented with 2% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) and 0.1% Triton X-100 was added. The Costar plates were incubated at 37°C for 30 min, after which 2 ng of streptavidin-beta-D-galactosidase was added to each well. After 30 min of incubation at room temperature, the solution was discarded, and wells with filters were washed three times with 1 ml of PBS containing 0.1% Triton X-100. The filters were then transferred to microdilution wells, and 100 μl of a 4-methylumbelliferyl-beta-D-galactoside solution was added to each well. Fluorescence intensity was determined as described above.

**RESULTS**

Immobilization of DNAs in microdilution wells. Optimal conditions to adsorb single-stranded DNA to microdilution wells were studied by changing the compositions and concentrations of buffer. Single-stranded DNA of *Escherichia coli* K-12 was well adsorbed to a microdilution well when it was dissolved in PBS supplemented with 0.1 M MgCl₂. The incubation time for optimal DNA adsorption depended on the concentration of DNA (Fig. 1). When less than 500 ng of DNA was added to a microdilution well, maximum adsorption was obtained after overnight incubation. With 1 or 2 ng of DNA, adsorption maxima were observed within 1 h. The maximum amount of DNA adsorbed to each well was calculated to be 300 ng by measuring the supernatant recovered from each well.

To study the stability of the DNA immobilized in microdilution wells, biotinylated DNA was fixed in microdilution wells, and the remaining DNA was determined after washing with three different washing solutions (Fig. 2). When a microplate before drying was washed with 2× SSC containing 0.1% SDS, about 40% of the DNA was washed out after 40 washes. However, more than 90% of the DNA still remained after 40 washes with the same washing solution when a microplate was dried at 60°C overnight before washing. DNAs on dried microplates were also not washed out after 40 washes with PBS containing 0.1% Triton X-100.

Determination of the optimal dose of streptavidin-beta-D-galactosidase. A linear relationship between the amount of beta-D-galactosidase and fluorescence intensity is shown in Fig. 3. When 10 nU of beta-D-galactosidase was added to a microdilution well, the fluorescence intensity exceeded
FIG. 1. Determination of the optimal dose and binding time for single-stranded DNA to be immobilized in microdilution wells. Biotinylated single-stranded DNA of E. coli K-12 was added to microdilution wells. Microdilution plates were incubated at 37°C. DNA solutions were discarded after different incubation times and were washed once with PBS containing 0.1 M MgCl₂. The plates were dried. The amount of immobilized photobiotin was determined by the method described in the text. The amounts of photobiotinylated DNA added to the microtiter wells were as follows: curve A, 0.1 μg; curve B, 0.2 μg; curve C, 0.5 μg; curve D, 1.0 μg; curve E, 2.0 μg.

![Graph showing fluorescence intensity over time](image)

FIG. 2. Residual DNA immobilized in microdilution wells after washing with three different washing solutions. Curves A and a, 2× SSC; curves B and b, 0.1% SDS in 2× SSC; curves C and c, 0.1% Triton X-100 in PBS. For curves a, b, and c the microplates were washed without drying. For curves a, b, and c the microplates were washed after drying at 60°C overnight.

![Graph showing washing frequency](image)

Washing frequency

**FIG. 3.** Relationship between the amount of beta-D-galactosidase and fluorescence intensity. A 10- to 0.005-nU portion of beta-D-galactosidase was added to each well. The fluorescence intensity was read at 10, 30, 60, and 120 min after 100 μl of a 4-methylumbelliferyl-beta-D-galactopyranoside solution was added.

![Graph showing enzyme reaction](image)
were measured (Fig. 5). When the fluorescence intensity after 24 h of hybridization was calculated as 100%, about 20% of the biotinylated DNA hybridized within 2 h of hybridization at each optimal temperature (L. pneumophila at 37°C and P. aeruginosa at 42°C).

Homology values for several streptococci calculated against S. anginosus NCTC 10713 were determined after 2, 8, and 24 h of hybridization at 37°C (Fig. 6). The homology values obtained from shorter hybridization times were slightly lower than those obtained from longer hybridization times, but the values were not significantly different.

Comparison with membrane filter methods. The homology values obtained by the fluorometric direct binding method were compared with the values obtained by membrane filter methods under optimal conditions (Fig. 7). The DNA of S. anginosus NCTC 10713 was labeled with 32P by nick translation and was used for the membrane filter method. The same DNA was biotinylated by photobiotin and used both for the membrane filter method and the direct binding method. The homology values for streptococcal strains calculated against the type strain of S. anginosus, strain NCTC 10713, obtained with these three methods were very close to each other.

Identification of organisms by rapid hybridization. Microplate hybridization was used for the identification of gram-positive cocci (6) and gram-negative bacteria (Fig. 8). Hybridization was carried out for 2 h under stringent conditions. To identify members of the genus Legionella, we selected 45°C for the hybridization temperature, although the optimal temperature for these organisms was calculated to be 37 to 40°C because the G+C contents of their DNAs ranged from 38 and 43 mol%. The G+C contents of the DNAs of members of the genus Pseudomonas ranged from 58 to 71 mol%, and 50°C was selected for the hybridization temperature for these organisms. By using 2 h of hybridization under stringent conditions, all of the legionellae and pseudomonads in Table 1 could be differentiated. Examples of hybridization data for eight species of legionellae and pseudomonads are shown in Fig. 8.

DISCUSSION
In the early stages of our experiments, we expected that DNA immobilized in microdilution wells would be washed out by posthybridization washing. Indeed, 40% of the DNA immobilized in microdilution wells was washed out when a washing solution containing 0.1% SDS was used. Therefore, we removed SDS from our washing buffer when the direct binding method was used. Originally, we irradiated the microdilution plates with ultraviolet light to promote the immobilization of DNA. However, in preliminary experiments, the absolute fluorescence intensity of microtiter wells not irradiated with ultraviolet light was higher than the intensity of wells irradiated with ultraviolet light (data not shown). Therefore, ultraviolet irradiation was not used in succeeding work.

The values obtained from both membrane filter methods corresponded well to the values obtained from the direct binding microdilution method. However, the background for the membrane filter method in which photobiotin was used was higher than the background for the fluorometric direct binding method.
FIG. 6. Homology values for six selected strains of streptococci against \( S.\ anginosus\) NCTC 10713\(^T\), calculated after 2, 8, and 24 h of hybridization. Hybridization experiments were carried out at 37°C in 2× SSC hybridization mixture containing 50% formamide. The hybridization experiments were carried out at 37°C in 2× SSC hybridization mixture containing 50% formamide. The bars at the tops of the columns indicate standard deviations. The average homology values were determined after six experiments. Unlabeled competitive DNAs were from \( S.\ anginosus\) NCTC 10713\(^T\) (strain 1), \( S.\ anginosus\) GIFU 10035 (strain 2), \( S.\ anginosus\) ATCC 27335 (strain 3), \( S.\ anginosus\) ATCC 27823 (strain 4), \( S.\ pyogenes\) ATCC 12344\(^T\) (strain 5), \( S.\ agalactiae\) NCTC 8181\(^T\) (strain 6), and \( E.\ coli\) K-12 (strain 7).

The homology values calculated after 2 h of hybridization were not significantly different from the values calculated after 24 h of hybridization, even though the extent of hybridization was only 20% of that obtained after 24 h (Fig. 6). Thus, we used 2-h hybridization times for determinations of genetic relatedness among bacterial strains and for identification of bacteria. Another reason that we selected the 2-h hybridization times is that sometimes when the 24-h times were used, the fluorescence intensity reached the maximum level within a few minutes after the 4-methylumbelliferyl-beta-D-galactopyranoside was added. The names of the organisms on the graphs indicate lateral homology values among the test strains could not be calculated. On the other hand, when 2-h hybridization times were used, it always took 15 to 60 min to reach the maximum intensity that could be read by the Microfluor fluorescence reader.

Hybridization in microdilution wells has several advantages. It is possible to automate the procedure, and the
microdilution plates on which reference DNAs are fixed can be stored in a desiccator at room temperature.

DNA labeling with photobiotin is simpler than enzymatic labeling methods, such as nick translation (18) or random primed DNA labeling (11). Membrane filter hybridization in which photobiotin is used requires extensive washing; fluorometric direct binding in microdilution wells requires less washing.

We prepare microdilution plates in which reference DNAs of medically important bacteria are fixed, and the plates are stored under dry conditions (8). When an unknown organism is isolated, its DNA is extracted from 1 to 3 ml of an overnight culture broth by using a small-scale DNA extraction method (15). The DNA is then labeled with photobiotin, and hybridization is carried out to identify the most closely related organism among the organisms whose reference DNAs are fixed in microdilution wells. This method is used successfully for identification of clinically important human bacteria. In many cases, it is not possible to estimate the exact G+C content of the DNA of a new isolate before hybridization. Thus, we prepare two hybridization temperatures, 45 and 50°C. When an isolate is thought to be a vibrio, campylobacter, bacteroides, legionella, streptococcus, staphylococcus, or anaerobic coccus, 45°C is selected for the hybridization temperature because these organisms have DNA G+C contents that are less than 50 mol%.

Hybridization at 50°C is used for identification of pseudomonads and micrococci, which have G+C contents greater than 60 mol%.

LITERATURE CITED