Chemiluminescent Universal Probe for Bacterial Ribotyping

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We describe a novel procedure for direct covalent coupling of horseradish peroxidase to rRNA and ribotyping by using enhanced chemiluminescence. Compared with their ³²P-end-labeled counterparts, chemiluminescent rRNA probes are stable and easy to synthesize and provide results as good as or superior to those obtained with isotopic labeling. Direct chemiluminescent labeling of *Escherichia coli* rRNA produces a sensitive, universal probe suitable for clinical laboratory use in the investigation of nosocomial outbreaks.

Since its first description in 1986 (6), ribotyping has emerged as one of the most powerful tools of molecular epidemiology (1-3, 5-10, 12-14, 16). This method employs labeled nucleic acid probes derived from rRNA or cloned rDNA to detect rRNA operon (*rnm*) segments which are highly conserved throughout the bacterial kingdom. *rnm* redundancy is a feature of virtually all bacterial species studied; discrimination between species, subspecies, and biotypes is possible because of genomic sequence heterogeneity, which is identified by cleavage with a variety of restriction enzymes at one or more sites within and around *rnm*. On the basis of high conservation of these genes throughout the bacterial kingdom, it has been determined that *Escherichia coli* rRNA can be used as a broad-spectrum probe (16).

Probes used for bacterial ribotyping can be generated by several methods. The most commonly used probe is homologous or heterologous rRNA that has been isotopically end labeled with ³²P. One advantage of isotopic probes is that relatively few steps are required for hybridization and washing. In addition, the strand polarity of the probe is such that hybridization with contaminating rRNA is minimized. However, the limitations of radioactive labeling of such probes for clinical laboratories become apparent when one considers the need for specially trained personnel, exposure hazards, frequent resynthesis, radioactive waste storage and removal, and cost.

Recently, several nonisotopic labeling methods, some of which have been applied to bacterial ribotyping, have been described (5, 10, 11). One such system detects digoxigenin-uridine triphosphate-labeled RNA and DNA probes with an antidigoxigenin antibody-alkaline phosphatase conjugate (Genius kit; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). An alternate system detects biotin-labeled probes by using a streptavidin-alkaline phosphatase conjugate (PhotoGene kit; GIBCO BRL, Grand Island, N.Y.). Both systems provide sensitivity that approaches that of ³²P labeling and circumvent many of the problems associated with radioisotope handling (4). However, because a second detector complex, consisting of an antibody- or avidin-coupled enzymatic reporter, is used to image the hybridized probe DNA, an extra set of incubations and washes is required, which greatly increases the labor involved. In addition, increased levels of background signal may result from the combined

effects of nonspecific hybridization and nonspecific detection (4).

We describe a novel procedure for direct covalent coupling of a horseradish peroxidase-polyethyleneimine complex to rRNA followed by enhanced chemiluminescence. Because the enzymatic reporter (horseradish peroxidase-polyethyleneimine complex) is chemically cross-linked to the RNA, the probe is functionally similar to ³²P-labeled probes; hybridization and washing are performed only once. The probe is stable for several months and simple to synthesize and may be used as a universal nonisotopic probe for bacterial ribotyping.

Random methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were obtained from the Mayo Clinic microbiology laboratory; each isolate was characterized by a positive slide agglutination test (Staphaurex; Wellcome, Temple Hill Dartford, England) and an oxacillin MIC greater than 2 µg/ml by the agar dilution method. DNA was prepared as follows. Colonies were inoculated into 4 ml of brain heart infusion broth and grown overnight at 37°C with shaking. One milliliter of each sample was centrifuged for 5 min at 14,000 × g. The pellet was washed once with TES (0.1 M NaCl, 0.01 M Tris (pH 8), 0.001 M EDTA), centrifuged for 5 min at 14,000 × g, and then resuspended in 200 µl of TES. A 10-µl volume of lysostaphin (500 U/ml; Sigma, St. Louis, Mo.) was added to the cell suspension and incubated at 37°C for 30 min. A 20-µl volume of 10% sodium dodecyl sulfate (SDS) and 1.2 µl of proteinase K (20 mg/ml; Boehringer Mannheim Biochemicals) were added, and incubation was continued at 60°C for 1 to 2 h. The DNA was then extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and then once with chloroform-isoamyl alcohol. A 1:25 volume of 5 M NaCl was added, and DNA was precipitated with 2.5 volumes of 100% ethanol for 30 min at -20°C. After centrifugation at 14,000 × g for 10 min, the pellet was washed once with 70% ethanol, Speed-Vac dried at 60°C for 15 min, and then suspended in water. DNA concentrations and A_{260/280} ratios were determined on a Perkin-Elmer lambda3B spectrophotometer. Four micrograms of DNA was digested overnight with *Eco*RI in 1× restriction buffer at 37°C, and duplicate samples were electrophoresed through a 1% SeaKem GTG agarose gel (FMC, Rockland, Maine) for 4 h at 100 V. Nonisotopic detection required DNA transfer to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) by Southern blotting (15) with subsequent baking at 80°C under vacuum for 2 h. For isotopic detection, identical samples were run in parallel and transferred to a nylon membrane (Schleicher & Schuell) and then cross-

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FIG. 1. DNAs were extracted from four isolates of MRSA, digested with *Eco*RI, electrophoresed, and transferred by Southern blotting to nitrocellulose (A) or nylon (B) membranes. (A) Chemiluminescent ribotyping, 100-min exposure. (B) Radioisotopic ribotyping, 2-day exposure.

linked in a UV transilluminator oven for a total energy exposure of 1.2×10^5 μ J.

16S and 23S rRNAs from *E. coli* MRE600 (Boehringer Mannheim catalog no. 206911) were labeled directly by using the ECL kit (Amersham [Arlington Heights, Ill.] catalog no. RPN.3000) as follows. A 1- μ l volume of rRNA (4 μ g/ μ l) was diluted with 399 μ l of water in a 1.5-ml microcentrifuge tube to a final concentration of 10 ng/ μ l, boiled for 2 min, and then snap-cooled in an ice-water bath for 5 min. A 400- μ l volume of DNA labeling reagent was added, followed by 400 μ l of glutaraldehyde solution. The entire mixture was then incubated at 37°C for 10 min. No purification of the probe was necessary. Thirty units of RNasin RNase inhibitor (Promega Corporation, Madison, Wis.) was added. Labeled probe was stored in 50% glycerol at -20°C for up to 3 months. Southern blots were prehybridized at 40°C in a 0.125-ml/cm² concentration of a solution made with diethylpyrocarbonate-treated water containing 25 mM KPO₄ (pH 7.4), 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 $\times$ Denhardt solution, 50 μ g of salmon sperm DNA per ml, and 6 M urea. After 3 h, the prehybridization buffer was discarded and replaced with 0.125 ml of hybridization buffer (prehybridization buffer with 2.5% chondroitin sulfate A; Calbiochem, LaJolla, Calif.) per cm². The blot was then hybridized for 5 h at 40°C by using 20 ng of probe per ml of hybridization buffer. (Note: the hybridization buffer currently supplied by the manufacturer should not be used. Although the hybridization buffer from kits numbered 2000 to 2999 performed well, kits bearing numbers greater than 3000 did not produce good results. We thus formulated the prehybridization-hybridization buffers described above to provide comparable performance.) The blot was washed twice in a 6 M urea-1.1% SDS-0.5 $\times$ SSC solution prepared with diethylpyrocarbonate-treated water for 20 min at 40°C and twice in 2 $\times$ SSC for 5 min at room temperature. All 40°C hybridizations and washes were performed in a Hybaid Mini Hybridization oven (National Labnet Company, Woodbridge, N.J.). In a darkroom, the blot was incubated with equal amounts of the two detection reagents supplied (total volume, 0.1 ml/cm²) for 1 min. After excess reagents were drained off, the blot was covered with cellophane and autoradiographs (X-Omat AR; Kodak, Rochester, N.Y.) were obtained after 1 min of exposure. Subsequent exposure times were then estimated

(usually less than 60 min). Results of chemiluminescent ribotyping of four MRSA isolates are shown in Fig. 1A.

Radioisotopically labeled probe was made with the same lot of rRNA in accordance with previously published protocols (6, 16). A 50-pmol sample was end labeled with [γ -³²P]ATP (6,000 Ci/mmol; NEN Research Products, Boston, Mass.) for 2 h at 37°C, boiled in a heating block for 10 min, and then snap-cooled. After purification through a G-50 Sephadex column (Boehringer Mannheim Biochemicals), an aliquot was removed for determination of specific activity (1.1×10^6 dpm/ μ g) and the remainder of the sample was used for hybridization. Blots were prehybridized in a solution containing 5 $\times$ SSPE, 5 $\times$ Denhardt solution, 0.5% SDS, and 100 μ g of denatured salmon sperm DNA per ml at 50°C for 1 h and then hybridized in the above-described solution at 50°C for 4 h. After hybridization, blots were washed twice with 2 $\times$ SSC-0.1% SDS for 5 min at room temperature and then once with 1 $\times$ SSC-0.1% SDS for 30 min at 55°C. Autoradiographs were stored at -70°C overnight and for 2 days. Results of ³²P-labeled ribotyping of four MRSA isolates are shown in Fig. 1B.

Direct chemiluminescent labeling of rRNA was compared with isotopic labeling for ribotyping of MRSA. With the chemiluminescent probe, we obtained excellent resolution with distinctive hybridization patterns observed after 100 min of exposure. Several experiments performed on the same strain collection showed that the results obtained by using chemiluminescent probes were extremely reproducible (data not shown). In contrast, the image produced by the ³²P-labeled probe showed poorer resolution and required 2 days of exposure. Because multiple enzymatic reporters are cross-linked to each probe molecule in this procedure (in contrast to ³²P end labeling, in which the molar radioisotope-to-probe ratio is 1:1), the theoretical sensitivity of chemiluminescent probes is excellent (11). However, our experience indicates that the ³²P-labeled probe showed slightly better sensitivity albeit poor resolution after prolonged (4-day) exposure of underloaded lanes (data not shown). In either case, sensitivity is usually not a significant limitation when performing a ribotyping procedure because the amount of DNA examined in each analysis can be readily controlled.

This novel nonisotopic labeling procedure allows production of a sensitive universal probe for detection of restriction fragment length polymorphisms within and around rDNA sequences. The ease of probe synthesis and the speed with which the results are available may make investigation of nosocomial outbreaks more feasible for clinical laboratories. Autoradiograph results are available within minutes, instead of hours to days, which considerably shortens an already lengthy procedure. Although the stability of our RNA probe was not examined formally, the probe is potentially stable for many months if RNase-free solutions and supplies and RNase inhibitors are used; chemiluminescent RNA probes have been used in our laboratory up to 3 months after synthesis (6a). In summary, our results indicate that chemiluminescent rRNA probes are simple to synthesize, provide rapid results that are comparable to those obtained with isotopic labeling, and are potentially more stable than their ³²P-labeled counterparts. Experiments are currently in progress to determine the usefulness of these probes for investigation of nosocomial outbreaks.

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