Appendix B
Ribotyping Protocol

Institute for Environmental Health

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Genomic DNA Isolation and Restriction Endonuclease Digestion

Confluent growth was scraped with a sterile flat-head toothpick and suspended in 200 microliters (μL) 50mM Tris, 50mM EDTA (pH 8.0). Six hundred μL more of 50mM Tris, 50 mM EDTA was then added and the suspension was thoroughly mixed by pipetting up and down. Then 45 μL 20% sodium dodecyl sulfate (SDS) and then 10 μL proteinase K (20 μg/mL; Pharmacia, Piscataway, N.J.) were added. This solution was then incubated at 40°C for 1 hour. After an equal volume of phenol was added to each tube, samples were vortexed, and centrifuged for 5 minutes. The top layer was extracted, and an equal volume of chloroform was added. The preparation was vortexed again, centrifuged, and extracted. Two and a half volumes of absolute ethanol were added and the DNA was precipitated out and spooled onto a glass capillary pipette. The DNA was washed with a few drops of absolute ethanol, dried, and re-suspended in 50μL dH2O.

Separate restriction endonuclease digestion reactions were set up using *Eco*R1 and *Pvu*II, 10 units/μL (Boehringer Mannheim, GmbH, Germany) as instructed by the manufacturer using 2 μL DNA. They were incubated at 37°C overnight. The samples were then centrifuged and 0.5 μl of enzyme was added. The samples were re-incubated at 37°C for a minimum of 3 hours. They were centrifuged again and 3 μL stop dye was added. Every batch of restriction enzyme reaction contained two reactions with a positive control strain that was included on two lanes on each gel.

Gel Electrophoresis and Southern Blot Hybridization

Samples were run on a 0.8% agarose gel in 1X Tris-borate-EDTA at 22 volts and 17 milliamps, for 17 hours. λ HindIII was used as a size standard along with a known *E. coli* isolate designated as 3915. Each agarose gel was assigned a number, and when more than one gel was run, the position of the first standard reference strain was changed in each gel (1st lane on the first gel, to the Nth lane on the Nth gel). Electrophoresis gels were stained in ethidium bromide. If two gels were stained in a single container, one corner of the gel with the higher number was clipped. The label for each gel was also transferred to the staining container. Each gel was then photographed and a hard copy of the print was labeled with the gel sheet (containing the isolate numbers loaded on each lane, and the enzyme used to cut the DNA, plus date, gel number, voltage, current, gel strength, buffer strength, and electrophoresis time information). After photography each gel was returned to the same staining container.

The DNA fragments were then transferred to a Nitran filter (Schleicher & Schuell, Keene, N.H.), baked at 80°C for 1 hour, and probed with ^{32}P-labeled copies of *E. coli* rRNA, which were made by extension of random hexanucleotide primers using Avian Myeloblastosis Virus reverse transcriptase (Stratagene, La Jolla, California) under conditions specified by the supplier. Each membrane filter was labeled with the gel number, restriction enzyme designation, date, and technician's initials. Hybridization was done in 5X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS,

1mM EDTA, and 50% formamide at room temperature overnight. Salmon sperm DNA and blocking reagent (Boehringer Mannheim GmbH, Germany) were used to block non-specific binding. Three washes were done with a solution of 2X SSC and 0.1% SDS, once at 25°C for 20 minutes and twice at 65°C for 20 minutes to wash off low-homology, non-specific binding. Blots were then exposed with an intensifying screen to x-ray film (Kodak, Rochester, New York) for 24 hours at -70°C. Two to three exposures were done to ensure all possible bands would show up.

All reagents and buffers were made according to formulas in the MEI standard operating procedures. Reagents and buffers were tested for sterility.

Restriction Fragment Length Polymorphism Analysis

Each ribotype was then analyzed by assigning an alphanumeric pattern based on the distance between the bands. Bands more than 3 mm apart were counted as singles while bands that were within 3 mm of each other were counted as doubles or triples. For example, two bands that were closer than 3 mm were designated "2" and a group of three bands with 3 mm or less between each band were designated "3." A "1" designated a single band more than 3 mm distant from another band. Each unique banding pattern was called a ribotype and assigned an alphanumeric pattern.

Two isolates that had the same numeric value but different banding patterns were assigned letters to differentiate the two ribotypes. For example, two isolates with an identical numerical pattern of 2122111, but with the bands shifted so the two isolates did not have identical banding patterns, were labeled 2122111A and 2122111B.

The ribotypes were then entered into a Microsoft Access® database and compared to the other ribotypes of known source in the library database. Ribotype patterns that numerically appeared to be similar were compared side-by-side visually to judge matching.

Isolates with the same *Pvu*II and *Eco*R1 ribotypes are deemed to be members of the same ribogroup. Using this approach only isolates with two identical ribotypes were grouped together.