[14] Determinations of Restriction Fragment Length Polymorphism in Bacteria Using Ribosomal RNA Genes

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Introduction

Analysis of restriction fragment length polymorphism of ribosomal RNA (rRNA) genes is a powerful and reliable tool for, but not limited to, two purposes: (1) as a taxonomic technique and (2) as a pathogen diagnostic technique. For taxonomists, the degree of conservation and variation in restriction patterns provides information about the genetics and evolution of related and distant bacteria. For diagnostic purposes as an epidemiological tool, one can determine with confidence whether clinically isolated species of a given genus represent the pathogenic form. The Southern transfer technique[1] is used in conjunction with rDNA–DNA hybridization to determine the distribution of homologous restriction fragments of the genome under comparison. If cloned DNA is not available, rRNA or transfer RNA (tRNA) can be used instead.

The rRNA and tRNA genes remain the most useful of the chronometers to study restriction site polymorphisms since they are ubiquitous and highly conserved, and different positions in the sequences change at different rates, allowing most phylogenetic relationships (including the most distant) to be measured.[2] In addition, many species have multiple identical *rrn* copies surrounded by variable sequences, allowing for the characterization of numerous forms of nonribosomal polymorphisms not possible with single-copy genes. A restriction pattern of multiple *rrn* bands of varying sizes can serve as a "hallmark" for a given bacterial species. Furthermore, owing to the high degree of conservation, a cloned rDNA from one species, namely, *Escherichia coli*, has been successfully used as the hybridization probe to explore the degree of homology among a group of many closely or distantly related organisms.[3] Thus, the most compelling reason for using rDNA as chronometers is that they can determine the hallmarks of many organisms at various taxonomic levels up to species differences. However, below the species level, the method is limited by the smaller probability of finding polymorphisms adjacent to RNA genes among strains of the same species. In this case it may be

[1] E. Southern, *J. Mol. Biol.* **98,** 503 (1975).
[2] C. L. Woese, *Microbiol. Rev.* **51,** 221 (1987).
[3] F. Grimont and P. A. D. Grimont, *Ann. Inst. Pasteur* **137B,** 165 (1986).

useful to examine other DNA probes from common single-copy genes like *trp, tna, thy,* and *lacZ* used in early studies with the Enterobacteriaceae[4–6] or *leu, thr,* and *trp* used in studies with members of the genus *Bacillus*[7,8] which provided useful information on the mechanisms that led to polymorphism such as small deletions, insertions, and other chromosomal rearrangements.

We have successfully used rRNA and tRNA as probes to determine the multiplicity, arrangement, and location of *rrn* and *trn* loci as well as differences between strains of the same species, namely, *Bacillus subtilis,* and between members of the same genus (*Bacillus*).[7–12] Ribosomal RNA restriction pattern analysis has been applied to the taxonomy of staphylococci,[4] members of *Enterobacteriaceae,*[5,6,13] *Candida* spp,[14] and *Bacillaceae,*[10,11] to cite just a few examples. The essential tools have been restriction enzymes that cut only once or not at all in the *rrn* operons and RNA probes that hybridize to chromosomal segments bounded at one end by non-*rrn* sequences. In the case of the enterobacteria (*E. coli* or *Salmonella typhimurium*),[15] Southern analysis of double-digested genomic DNA with *Bam*HI–*Pst*I revealed seven highly resolved bands. Neither restriction endonuclease cleaved within the seven operons. In the *Bacillaceae* (i.e., *Bacillus subtilis*) we used the enzyme *Bcl*I, which cuts only once in all ten operons, 79 base pairs (bp) before the 5′ end of the 23 S rDNA.[16] Southern hybridizations of *Bcl*I digests of genomic DNA from *B. subtilis* strains NCTC3610 or 168T yielded 10 distinct fragments when probed with any rDNA fragment 5′ to the internal *Bcl*I site.[9,11] Using an enzyme which cuts once in the operon is preferable because it allows the identification of clustered *rrn* operons as was found in *B. subtilis,* where 5 out of

[4] F. M. Thomson-Carter, P. E. Carter, and T. H. Pennington, *J. Gen. Microbiol.* **135,** 2093 (1989).
[5] A. Anilonis and M. Riley, *J. Bacteriol.* **143,** 355 (1980).
[6] M. Riley and A. Anilonis, *J. Bacteriol.* **143,** 366 (1980).
[7] P. Gottlieb and R. Rudner, *J. Syst. Bacteriol.* **35,** 244 (1985).
[8] P. Gottlieb, Ph.D. Dissertation, City Univ. of New York, New York (1984).
[9] G. LaFauci, R. L. Widom, R. L. Eisner, E. D. Jarvis, and R. Rudner, *J. Bacteriol.* **165,** 204 (1986).
[10] R. L. Widom, E. D. Jarvis, G. LaFauci, and R. Rudner, *J. Bacteriol.* **170,** 605 (1988).
[11] E. D. Jarvis, R. L. Widom, G. LaFauci, Y. Setauchi, I. R. Richter, and R. Rudner, *Genetics* **120,** 204 (1988).
[12] R. Rudner, A. Chevrestt, S. R. Buchhotz, B. Studamire, A.-M. White, and E. D. Jarvis, *J. Bacteriol.* **175,** 503 (1993).
[13] L. Harshman and M. Riley, *J. Bacteriol.* **144,** 560 (1980).
[14] B. B. MaGee, T. M. D'Souza, and P. T. MaGee, *J. Bacteriol.* **169,** 1639 (1987).
[15] A. F. Lehner, S. Harvey, and C. W. Hill, *J. Bacteriol.* **160,** 682 (1984).
[16] C. J. Green, G. C. Stewart, M. A. Hollis, B. S. Vold, and K. F. Bott, *Gene* **37,** 261 (1984).

10 are arranged in two sets of tandem repeats (Fig. 1d).[10] The genomic locations of *rrn* fragments can be determined through the integrative mapping technique using transductional crosses as was done in *B. subtilis*[9,11] or through genomic restriction mapping as was done in *H. influenzae*.[17]

Among strains of *E. coli, S. typhimurium,* or *B. subtilis,* restriction site polymorphisms 5′ and 3′ to ribosomal operons were easily ascertained on either *Bam*HI–*Pst*I[15] or *Bcl*I[10] Southern hybridizations, respectively. In *B. subtilis* we found strains with naturally occurring *rrn* deletions which arose by homologous recombination between adjacent *rrn* gene sets and exhibit losses of unique fragments.[10] We also found strains with heterologous intrachromosomal rearrangements adjacent to *rrn* operons.[18] Restriction enzymes that cut more than once within the *rrn* offer Southern hybridization patterns that are useful for the identification of intragenic restriction polymorphism. They allow for the evaluation of heterogeneities in the arrangement and sequence of the individual genes, namely, 16 S, 23 S, and 5 S, and tRNAs within the *rrn* loci among strains of related species.

For example, the basic physical map of all the *rrn* operons of *B. subtilis* consists of three internal *Eco*RI and *Sma*I sites, two *Pst*I sites, and single *Bcl*I, *Bam*HI, and *Hin*dIII sites[19] (Fig. 1c). Two of the ten operons (*rrnO, rrnA*) contain tRNA genes in the spacer regions between the segments that code for 16 S and 23 S.[20] In that spacer region, one *Eco*RI site is variable in the genus *Bacillus*. It is found in the 5′ end of the 23 S gene, is present in strains of *B. subtilis* and *B. licheniformis,* and is absent in strains of *B. globigii, B. pumilus,* and *B. amyloliquefaciens*.[7] This heterogeneity allowed us to construct a dendrogram analysis of the relationships among five species of *Bacillus* and their strains[7] (Fig. 2).

In this chapter, we describe procedures which deal with the most optimal conditions required to obtain high resolution of *rrn* and *trn* fragments on Southern blots of genomic DNA from members of the genus *Bacillus*. Included are methods for the preparation of genomic DNA, restriction digests, isolation and labeling of rDNA and tDNA, gel electrophoresis including pulse-field gel electrophoresis (PFGE), Southern blotting, and hybridization analyses. The experimental approach requires the following considerations.

Choice of Restriction Endonuclease. (1) Enzymes that do not cut in *rrn* operons will generate fragments that are larger than the average size of an operon, namely, 4.8 kilobases (kb). These enzymes (i.e., *Bgl*I, *Sal*I)

[17] J. J. Lee, H. O. Smith, and R. J. Redfield, *J. Bacteriol.* **171,** 3016 (1989).
[18] E. D. Jarvis, S. Cheng, and R. Rudner, *Genetics* **126,** 784 (1990).
[19] G. Stewart, F. Wilson, and K. Bott, *Gene* **19,** 153 (1982).
[20] K. Loughney, E. Lund, and J. E. Dahlberg, *Nucleic Acids Res.* **10,** 1607 (1982).

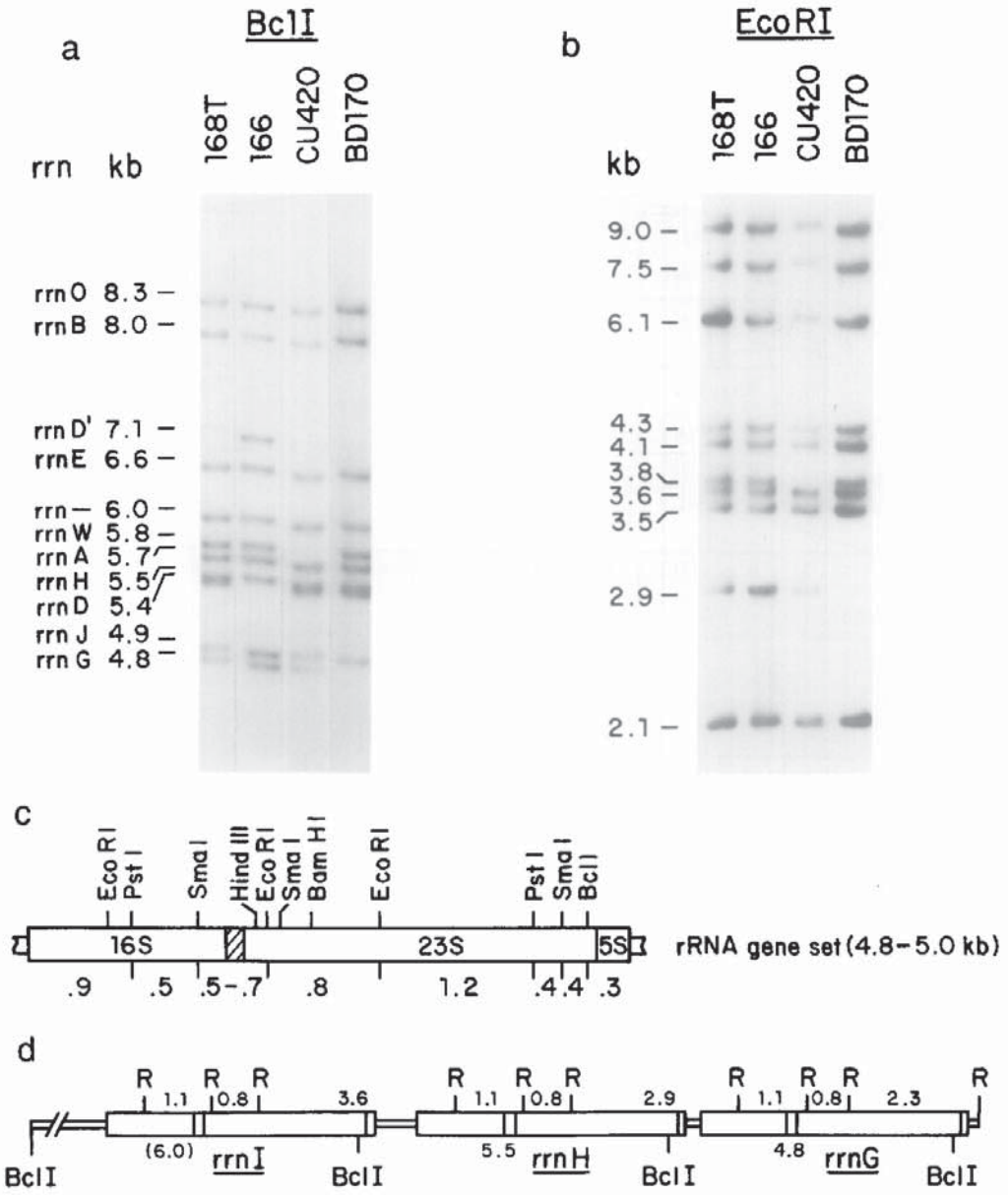

FIG. 1. Southern hybridization of total chromosomal DNAs from *B. subtilis* strains 168T, 166, CU420, and BD170 containing either a chromosomal rearrangement upstream of *rrnD* (166) or naturally occurring deletions of *rrnW* (CU420) or *rrnG* (BD170). (a) *Bcl*I digests were electrophoresed on 0.75% agarose for 5–7 days at 15–20 mA and probed with a labeled *Eco*RI–*Pst*I 23 S fragment; the assignment of the individual *rrn* operons is according to E. D. Jarvis, R. L. Widom, G. LaFauci, Y. Setauchi, I. R. Richter, and R. Rudner, *Genetics* **120,** 204 (1988). (b) *Eco*RI digests were electrophoresed on 0.75% agarose for 48 hr at 15–20 mA and probed with a labeled *Eco*RI–*Hin*dIII 23 S, 5 S fragment. (c) Generalized restriction map of a *B. subtilis* rRNA gene set as proposed by G. Stewart, F. Wilson, and K. Bott, *Gene* **153,** (1982). The hatched area represents the abutment region between 16 S and 23 S rRNA with or without tRNA genes [K. Loughney, E. Lund, and J. E. Dahlberg, *Nucleic Acids Res.* **10,** 1607 (1982)]. (d) Structure of a cluster of *rrn* operons in *B. subtilis*. The triplet *rrnI–rrnH–rrnG* is located at 14° on the map [E. D. Jarvis, R. L. Widom, G. LaFauci, Y. Setauchi, I. R. Richter, and R. Rudner, *Genetics* **120,** 204 (1988)]. The *Bcl*I and *Eco*RI (R) fragment sizes shown are those identified by restriction analysis.

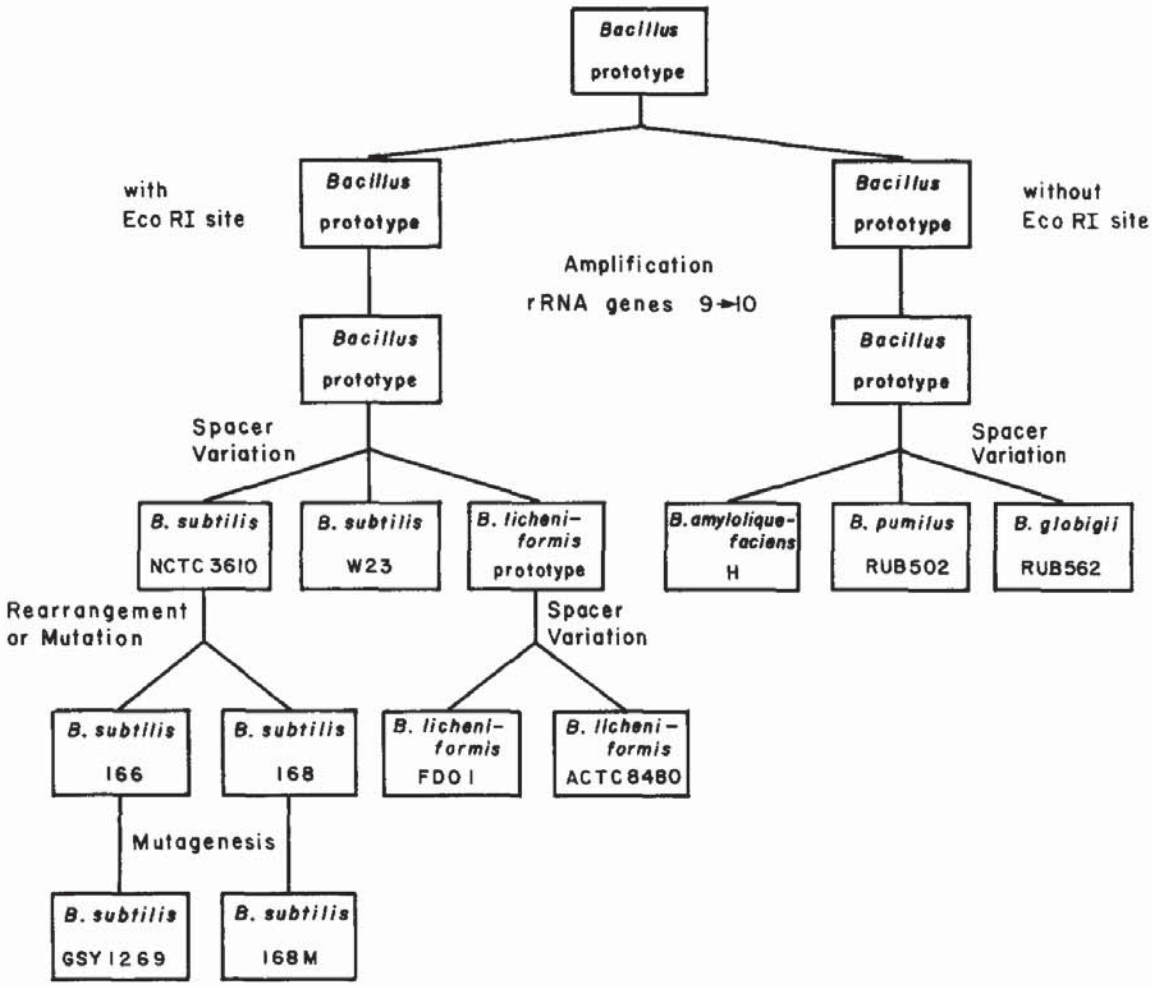

FIG. 2. Dendrogram analysis of the restriction relationships among five species of *Bacillus* and their strains. The bacteria examined were divided into two lines of descent based on the structures of the rRNA gene sets. Type I *Bacillus* species have rRNA genes possessing the 5′ 23 S sequence *Eco*RI site (see Fig. 1c), and type II species have rRNA gene sets without the *Eco*RI site. [By permission from P. Gottlieb and R. Rudner, *J. Syst. Bacteriol.* **35,** 244 (1985).]

are suitable for determining the location of *rrn* operons on a genomic restriction map, but they are not suitable for determining multiplicities, especially when clustering is involved. These enzymes might not cut between adjacent operons in a cluster. (2) Enzymes that cut once in *rrn* operons will ensure the determination of the total number of gene sets and the extent of clustering, whereby a single cutter will release operons from one another (e.g., the *Bcl*I site in the clusters shown in Fig. 1d). These enzymes (i.e., *Bcl*I, *Hin*dIII) are the preferred ones for taxonomic and diagnostic studies since differences between adjacent *rrn* operons are

easily deciphered. (3) Enzymes that cut more than once in *rrn* operons are useful for intragenic restriction polymorphism (i.e., *Eco*RI, *Pst*I).[7]

Choice of Probe. (1) All three rRNA genes (16 S, 23 S, 5 S) are suitable as probes when one restricts genomic DNA with an enzyme that does not cut inside the *rrn* operon. (2) When the genomic DNA is restricted with an enzyme that cuts only once it is desirable but not essential to probe either the 5′ side to the restriction site in the operon or the 3′ side of the restriction site. (3) When genomic DNA is cut more than once, a probe which hybridizes to internal *rrn* sequences overlapping or between the restriction sites is suitable for evaluating the existence of intragenic variations as discussed above.[7,8] (4) In the absence of cloned rDNA and restriction site analysis of a given species, isolated 16 S, 23 S, and 5 S can be synthesized as cDNA by a reverse transcriptase reaction.[21] These cDNAs can then be cloned or tested for restriction sites. In all cases we recommend probing with either 16 S or 23 S alone; otherwise, the patterns are more complex, with excessive numbers of bands.

Bacterial Strains and Isolation of Genomic DNA

Bacillus strains are best maintained on slants of tryptose blood agar base (TBAB; Difco Laboratories, Detroit, MI). Asporogenous strains are stored in vials in 20% (v/v) glycerol at −70°. To culture for the purpose of DNA isolation, the following three media are recommended: (1) VY, 2.5% veal infusion (Difco)–0.5% yeast extract (Difco); (2) LB (Luria–Bertani) broth, containing (per liter) Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g, 1 *M* NaOH 1.0 ml; or (3) BHIB, 3.7% Bacto-brain–heart infusion broth (Difco).

For large-scale DNA preparation, a 5-ml starter culture is used to inoculate 500 ml of the above media in a 2.0-liter Erlenmeyer flask. Growth is allowed to proceed with vigorous agitation at 37° overnight. Genomic DNA is isolated and purified by a modification[22] of the procedure of Marmur.[23] The cells are collected by centrifugation at 5000 rpm, washed with a 0.15 *M* NaCl–0.1 *M* EDTA (pH 8.0) solution, and lysed at 37° in the presence of lysozyme (50 μg/ml, final concentration). The suspension of crude DNA fibers is then treated with an RNase mixture (50 μg/ml of pancreatic RNase and 50 U/ml of T1) for 30 min at 37°, followed by digestion with pronase (500 μg/ml, self-digested at 37° for 4 hr) at 37° for

[21] T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
[22] R. Rudner, H. Lin, S. Hoffman, and E. Chargaff, *Biochim. Biophys. Acta* **144,** 199 (1967).
[23] J. Marmur, *J. Mol. Biol.* **3,** 208 (1961).

at least 2 hr until the solution becomes clear. This treatment is followed by two deproteinizations with 90% phenol (pH 7.8), ethanol precipitation, and dialysis against TE buffer (10 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 7.5). High molecular weight DNA should have an $OD_{280/260}$ ratio of about 0.5 and is stored at 2 mg/ml in the cold over a drop of chloroform. All genomic DNAs used to perform "Zoo Blots" should be uniformly diluted from the stock to 100 μg/ml in TE buffer and subjected to restrictions.

Alternatively, small-scale rapid lysates for processing many bacterial species can be done when small amounts of DNA are needed. Chromosomal DNA is prepared from 5-ml overnight cultures according to the method of Jarvis *et al.*[18] The cells are centrifuged at 5000 rpm and suspended in 0.2 ml of SET buffer (20% sucrose, 50 m*M* EDTA, and 50 m*M* Tris, pH 7.6) containing 5 mg/ml lysozyme and 1 mg/ml RNase, vortexed well, transferred to a 1.5-ml Eppendorf tube, and incubated at 37° for 15 min. To the lysate 0.4 ml of 1% sodium dodecyl sulfate (SDS) is added and mixed well by inversion, followed by a 10-min incubation at 60°. An equal volume (0.6 ml) of a 1 : 1 mixture containing 80% Tris-buffered (pH 8.0) phenol : chloroform/isoamyl alcohol (24/1) is added, and the sample is vortexed and centrifuged for 5 min. The aqueous layer is removed and reextracted with chloroform/isoamyl alcohol. Nucleic acids are then precipitated with an equal volume of 2-propanol after adjustment to 0.3 *M* sodium acetate and centrifuged for 10 min. The pellet is washed twice with 70% ethanol, dried for 15 min in a Speed Vac (Savant Instruments, Inc., Hicksville, NY), and dissolved in 50–100 μl of sterile water. Typical recoveries are 25–50 μg of chromosomal DNA per sample.

Restriction Patterns of Genomic DNA and Southern Blotting

Chromosomal DNA (5 to 10 μg/ml) is digested with the desired restriction endonuclease at 3 U of enzyme per microgram of DNA for 12 hr at 37° under the conditions for digestion recommended by the supplier (New England Biolabs, Beverly, MA; Boehringer Mannheim, Indianapolis, IN; etc.). The choice of enzyme is dictated by the generalized restriction map of the *B. subtilis* rRNA gene set as proposed by Stewart *et al.*[19] (Fig. 1c). The enzymes *Bcl*I, *Bam*HI, and *Eco*RI are ideally suited because they yield a characteristic and reproducible banding pattern of the 10 *rrn* operons in *Bacillus* when hybridized to the appropriate probe (Fig. 1a). The *Hin*dIII and *Eco*RI enzymes have also been useful for locating tRNA gene clusters associated downstream of *rrn* operons.[12]

A large horizontal gel apparatus (21 × 24 cm) containing a cooling device (IBI, New Haven, CT) is used for high resolution of fragments. The comb has 20 teeth and under the conditions used forms wells able to

contain 50–60 μl of restricted DNA. Agarose type II (low electroendosmosis, EEO; Sigma, St. Louis, MO) at 0.75–0.85% (w/v) is used to cast the gel, and the running buffer is Tris–borate (TBE; 8.9 m*M* Tris–HCl, pH 8.0, 89 m*M* boric acid, 2.5 m*M* EDTA, containing 0.5 μg/ml ethidium bromide).[23] The key to high resolution is to run the gels at low voltage (5 mA and 10 V) for 2–5 days to obtain maximum separation of closely sized bands (Fig. 1a). A lane of phage λ DNA, cleaved by *Hin*dIII, is used as a fragment size marker. Gels loaded with *Bcl*I- or *Bam*HI-restricted genomic DNA are run until the fourth band of *Hin*dIII-cleaved λ (4.3 kb) reaches the bottom of the gel. The other restrictions (i.e., *Eco*RI, *Hin*dIII) need not run that long so that the internal *rrn* fragments or tRNA genes with molecular sizes 0.9–4.0 kb are retained.[7,12]

Another useful approach is the separation of large restriction fragments generated from digestions with the rare cutters *Apa*I, *Eag*I, *Nae*I, *Not*I, *Sma*I, *Sfi*I, and *Rsr*I in pulsed-field gel electrophoresis using one of the two systems: (1) OFAGF (orthogonal field alternation gel electrophoresis) and (2) CHEF (counter-clamped homogeneous field electrophoresis). Studies done in *Haemophilus influenzae* Rd with these techniques were used to generate a physical restriction map of the entire genome and to map all six *rrn*.[17] Both PFGE systems use 1% agarose gels in 0.5× TBE buffer at 0.5°–5°. The OFAGE experiments are run at 280 V for 12 hr with pulse times ranging from 1 to 36 sec, depending on the resolution range desired. The CHEF experiments are run at 195 V for 24 hr with pulse times ranging from 6 to 80 sec. The use of PFGE is particularly useful in constructing a physical map of uncharacterized genomes from clinically isolated serotypes using the *rrn* sequences as the initial probes, as was done for *Mycoplasma mobile, Caulobacter crescentus,* and *Mycoplasma pneumoniae*.

The electrophoretically separated DNA fragments are transferred onto either nitrocellulose filters (Schleicher & Schuell, Keene, NH; BA-S NC) or Nytran nylon membranes (Schleicher & Schuell) according to the procedure of Southern[1] following a modified approach of the established protocols[23] and recent advances. After electrophoresis, the gel is soaked for 1 hr in a denaturing solution (1.5 *M* NaCl–0.5 *M* NaOH), followed by a 1-hr soak in a neutralizing buffer (1 *M* Tris-HCl, pH 7.5, 1.5 *M* NaCl). The gel is soaked in 10× SSC (1× SSC is 150 m*M* NaCl, 15 m*M* sodium citrate) for 30 min, followed by blotting on a flat surface onto a membrane which had been presoaked in 10× SSC for 5 min. The DNA bands are fixed to the membrane by UV-mediated cross-linking using a UV 300 Transilluminator (Fotodyne, New Berlin, WI) at 254 nm for 90–120 sec. The membranes are kept moist throughout to allow for rehybridization with a second probe. The blot is prehybridized in a solution containing

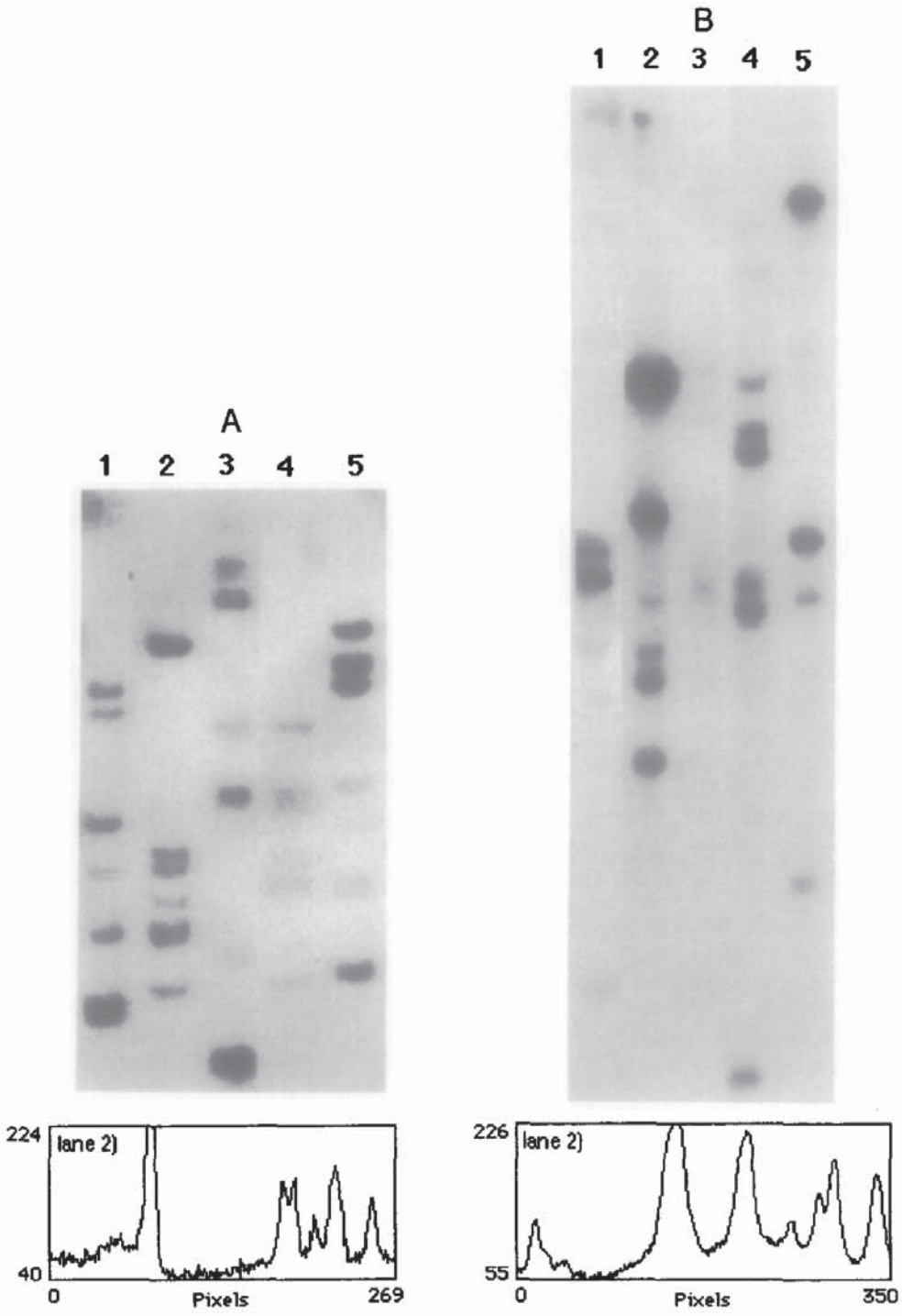

FIG. 3. Southern hybridization using *B. subtilis* rRNA and tRNA as probes of total chromosomal DNAs from the following five members of the genus *Bacillus:* (1) *B. subtilis* 168T, (2) *B. subtilis* W23, (3) *B. pumilus* RUB502, (4) *B. licheniformis* ACTC8480, and (5) *B. amyloliquefaciens* H. (A) *Bcl*I digests were probed with a labeled 2.0-kb *Hin*dIII–*Pst*I fragment of the 23 S gene (see Fig. 1c). The gel was electrophoresed as described for Fig. 1a. A density profile from a line plot of lane 2 (*B. subtilis* W23) was done with the NIH

40% formamide, 4× SSC, 50 m*M* sodium phosphate, pH 6.5, 250 μg/ml sonicated heat-denatured salmon sperm DNA, 1% glycine, and 5× Denhardt's solution (1× Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) as described by Ostapchuck *et al.*[24] in a heat-sealable bag at 42°–45° for 3–5 hr. After removal of the prehybridization buffer, hybridization buffer containing 40% formamide, 4× SSC, 20 m*M* sodium phosphate, pH 7.5, 100 μg/ml sonicated denatured salmon sperm DNA, 1% Denhardt's solution, and 0.1% SDS is added (4 ml per each 100 cm^2 of membrane), followed by the addition of ^{32}P-labeled rRNA, or single-stranded DNA, or polymerase chain reaction (PCR) fragment probes. The filters are incubated overnight at 42°–45° in a water bath. Alternatively, the HB 1100D Red Roller II (Hoeffer Scientific Instruments, San Francisco, CA) designed for incubating and hybridizing membranes from Southern transfers is recommended as a suitable oven that holds up to 6 large or small hybridization tubes; the apparatus can perform hybridizations with minimal volumes of solutions, as little as 5 ml.

The hybridization solution is removed and the membrane is then washed in 2× SSC–0.5% SDS for 15 min followed by a second wash in 2× SSC–0.1% SDS at room temperature, and then in 0.1× SSC–0.5% SDS at 42° for 1 hr with gentle rocking, followed by a stringent wash in 0.1× SSC–0.5% SDS at 68°–72° for 1 hr. The same procedure is used when the probe is the end-labeled RNA. The membrane is kept moist, sealed in a heat-sealable bag, and exposed to Kodak (Rochester, NY) XAR-5 film at −70° with (or from room temperature, when sharper bands are desired) Du Pont Cronex Lightning Plus intensifying screens for several days. Fragment sizes are determined from the autoradiogram by measuring the migration distances and applying the relationship of Bearden.[25] Typical restriction patterns of *BclI* and *Eco*RI digests of genomic DNA from strains of *B. subtilis* and from five species of the genus *Bacillus* obtained by the conventional gel electrophoresis–Southern blot analysis are shown in Figs. 1 and 3.

[24] P. Ostapchuk, A. Anilionis, and M. Riley, *Mol. Gen. Genet.* **180,** 475 (1980).
[25] J. Bearden, *Gene* **6,** 221 (1979).

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image analysis software. (B) *Eco*RI digests were probed with a labeled 1.6-kb PCR fragment containing the amplified 16 tRNA gene cluster of *trnD* [R. Rudner, A. Chevrestt, S. R. Buchhotz, B. Studamire, A.-M. White, and E. D. Jarvis, *J. Bacteriol.* **175,** 503 (1993)]. The gel was electrophoresed as described for Fig. 1b. A density profile of lane 2 (*B. subtilis*) is also presented.

Hybridization Probes and Densitometry

Ribosomal RNA for use as a probe is isolated from *B. subtilis* 168T grown in VY medium and purified by the method of Margulies *et al.*[26] The 5 S, 16 S, and 23 S rRNA species are resolved in low melting point agarose (BRL, Gaithersburg, MD) or Sea-Plaque (FMC, Rockland, ME). The RNA bands are excised from the gel are 5′ end labeled with [γ-^{32}P]adenosine triphosphate and T4 polynucleotide kinase according to the method of Maizels.[27] Moreover, ^{32}P-labeled cDNAs of these rRNAs can be synthesized in agarose by a reverse transcriptase reaction according to published procedures.[21] Alternatively, cloned rDNA, tDNA, or PCR fragments,[7,9–12] all gel-purified and cleaned with GlasPac (National Scientific Supply Co., San Rafael, CA), can be used as hybridization probes. Labeling is done either by nick translation[28] or with the random primer extension kit as directed by the supplier (USB, Cleveland, OH) using [α-^{32}P]dCTP and/or [α-^{32}P]dATP. The labeled DNA is freed from low molecular weight material by passage through a 1-ml spun column of Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with TE buffer. The rRNA and the DNA are routinely labeled to a specific activity of 0.5×10^8 to 2×10^8 counts/min (cpm)/μg. For hybridization, 1×10^6 to 4×10^6 cpm of ^{32}P-labeled RNA or DNA probe is added per lane of the hybridization membrane.

Autoradiographed films are scanned for hybridization intensity using various commercial densitometers equipped with recorders such as the Zeineh soft laser scanning densitometer (Model SL-DNA; Biomed Instruments, Fullerton, CA) or Wayne Rasband's NIH image analysis software using a photometric charge-coupled diode (CCD) camera system (Biological Detection Systems). In our initial studies, densitometry measurements[7,8] were done with a linear transport drive containing a holder for X-ray film strips inserted between the spectrometer monochromatic light source (580 nm) and the spectrophotometer (Gilford Instruments). Scan profiles were made by a recorder (Model 6051, Gilford) equipped with a pyroscribe device onto heat-sensitive chart paper (Honeywell). Peak areas are determined with a planimeter (Nobis Instruments) equipped with a mobile cursor to scan the densitometric tracing. The intensities of the hybrid bands on autoradiograms of "Zoo Blots" reflect the relative amounts of probe DNA that is bound and therefore serve as a rough measure of the total amount of DNA present in a particular restriction

[26] L. Margulies, V. Remeza, and R. Rudner, *J. Bacteriol.* **107,** 610 (1971).
[27] N. Maizels, *in* "ICN–UCLA Symposium on Molecular and Cellular Biology" (G. Wulcox, J. Abelson, and C. F. Fox, eds.), Vol. 8, p. 247. Academic Press, New York, 1977.
[28] P. W. J. Rigby, M. Dieckmann, C. Rhodes, and P. Berg, *J. Mol. Biol.* **113,** 237 (1977).

fragment of the related species that is well matched with the corresponding DNA sequences in the probe preparation. The sum of the measured intensities for all hybrid bands in a bacterial chromosome then serves as an estimate of the relative homology index to the *B. subtilis* 168T probe DNA.

Analysis of Differentiation and Similarity Coefficients

To determine the extent of conservation of sequences in *rrn* regions and the extent of strain differentiation the following analysis is performed.[5] A "similarity coefficient" (S) representing a fractional estimate of the conserved fragment sizes between two chromosome digests is expressed as $S = 2a/(x + y)$, where a equals the pairs of size-conserved fragments in paired digests of chromosomal DNA and x and y are the total number of homolog fragments observed in paired autoradiogram lanes. The fraction of conserved fragments is related to the fraction of base substitutions[29] and to the number of fragments with a specific size range. For example, as seen in Fig. 3A, the *B. subtilis* 168T chromosome has 10 *Bcl*I restriction fragments ($x = 10$) and W23 has 7 ($y = 7$), with four bands being similar ($a = 4$); therefore, the calculated S value is 0.47. The same analysis between *B. subtilis* and *B. pumilus* gives an S value of 0.11. Data previously compiled on eight members of the genus *Bacillus* revealed variations between two restriction digests (*Eco*RI and *Hin*dIII) at all levels of similarity coefficients from highly conserved ($S = 0.86$) to random ($S = 0.00$–0.14).[8] We note that the *Eco*RI restriction patterns of the *Bacillus* species are considerably more conserved than those obtained with the *Hin*dIII enzyme.[8] Similarly, rRNA gene restriction pattern analysis in seven *Staphylococcal* species and their strains revealed a greater band conservation among the *Eco*RI homologs than among the *Hin*dIII fragments.[4]

Concluding Remarks

The use of *E. coli* rRNA (16 S, 23 S) as a broad-spectrum probe has allowed the application of fingerprinting of genomic DNA from a large number of isolates from diverse gram-positive and gram-negative bacteria.[3] This single RNA probe reacted with the *rrn* genes in the genomes of species as phylogenetically remote from *E. coli* as *Bacillus, Brucella, Acinetobacter, Mycobacterium,* and *Listeria*. Each species generated by *Eco*RI, *Hin*dIII, and *Bam*HI digests gave a novel band pattern of rDNA restriction fragments. When these patterns are combined they constitute the beginning of a database which could easily serve for the identification

[29] W. B. Upholt, *Nucleic Acids Res.* **4,** 1257 (1977).

of unknown isolates. Ideally, the comparison of the restriction pattern of an unknown isolate with the corresponding pattern of a known species would require screening the database, assuming that all the techniques have been standardized.

Determination of rDNA provides a novel means for detecting minor genomic differences and can be used to characterize clinical isolates, which apparently show fewer obvious variations in total DNA fingerprints compared to the differences seen between species.[3,4,14,30] As an epidemiology tool, rRNA gene restriction patterns are highly reproducible and easy to compare if one limits the number of bands attainable by the use of highly defined probes. The assignment of strains to a particular group or subgroup is arbitrary unless computer-assisted analyses are used.

[30] R. J. Owen, A. Beck, P. A. Dayal, and C. Dawson, *J. Clin. Microbiol.* **26,** 2161 (1988).

[15] DNA Fingerprinting of *Mycobacterium tuberculosis*

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Introduction

The use of genetic markers to type pathogenic mycobacterial strains is of great value for studying the epidemiology of tuberculosis. This is particularly important because multidrug resistant organisms are increasingly prevalent and because the acquired immunodeficiency syndrome (AIDS) pandemic is posing new challenges to tuberculosis control strategies. Until recently, mycobacteriophage and drug susceptibility patterns were the only markers available for *Mycobacterium tuberculosis* strain typing. This method has limited utility because it is technically difficult to perform and only a limited number of types can be distinguished.[1]

Mycobacterium tuberculosis belongs to the *M. tuberculosis* complex group of bacteria which includes *M. tuberculosis, Mycobacterium bovis, M. africanum,* and *Mycobacterium microti.* These species constitute a genetically closely related group of bacteria which are often difficult to differentiate using biochemical markers and growth characteristics. This close taxonomic relatedness is also reflected in DNA–DNA hybridization studies which have shown nearly 100% chromosomal homology between

[1] J. Crawford and J. H. Bates, *in* "The Mycobacteria: A Sourcebook," Part A, (G. P. Kubica and l. G. Wayne, eds.), pp. 123. Dekker, New York, 1984.