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Amplification and Restriction Endonuclease Digestion of a Large Fragment of Genes Coding for rRNA as a Rapid Method for Discrimination of Closely Related Pathogenic Bacteria

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By use of primers specific to conserved regions of the rRNA gene cluster, a discrete amplicon of approximately 5 kb was amplified by PCR from all 21 bacterial genera investigated. Subsequent endonuclease digestion of the PCR product with *Hae*III distinguished between the three species of the human pathogen *Francisella* spp. on the one hand and four clinically relevant genomic groups of *Acinetobacter* spp. on the other hand. The described technique has the potential as a rapid method for discriminating between closely related species that are of clinical importance.

Recent advances in molecular biology have disclosed an enormous diversity in the microbial world, and at the same time they have pointed out the limitations of traditional identification techniques (1, 8). The shortcomings of the methods used at present for taxonomic analysis can be demonstrated with the bacterial genera *Acinetobacter* and *Francisella*. The genus *Acinetobacter* comprises 20 genomic species that have been differentiated on the basis of DNA-DNA hybridization studies (7, 19), although it has not been possible to distinguish them with regard to phenotypic or molecular traits. Four DNA groups (DNA groups 1, 2, 3, and 13) within this genus, known as the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex, are frequently encountered in nosocomial outbreaks (6, 7). Their identification has been hampered by the lack of convenient and rapid methods. The genus *Francisella*, on the other hand, contains only three approved species, *F. tularensis*, *F. novicida*, and *F. philomiragia* (4, 9). Despite this rather limited diversity in the genus, no available techniques conveniently distinguish the three species.

In prokaryotes, the rRNA gene loci contain all three rRNA species, 16S, 23S, and 5S. The 16S and 23S genes are separated by a spacer region varying in length from 278 bp in *Mycobacterium paratuberculosis* and *Mycobacterium avium* (20) to almost 2 kb in certain *Borrelia* species (17). Restriction fragment length polymorphism (RFLP) analysis of the amplified spacer region has been exploited for discrimination of related bacterial species (10). However, the approach has not been entirely successful. For example, RFLP analysis of the amplified spacer region did not distinguish between *M. paratuberculosis* and *M. avium* (20) or *Streptococcus anginosus* and *Streptococcus intermedius* (21).

To this end, we looked at an approach that is fairly simple and that can be performed rapidly. We reasoned that restriction analysis of almost the entire rRNA gene locus, with a total size of some 5 kb, may encompass variable regions of sufficient length to allow for discrimination between even closely related bacterial species. We used a set of primers specific for bacteria of a broad phylogenetic origin (11). The primers were complementary to the 5′ and 3′ ends of the 16S and 23S rRNA genes, respectively, thereby enabling amplification of virtually the complete rRNA gene cluster. The resulting amplicons were characterized with regard to their RFLPs. Two bacterial genera, *Acinetobacter* and *Francisella*, were used as models to demonstrate the potential of the described assay.

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MATERIALS AND METHODS

Bacterial strains. The following medically significant species were included in the study: *Listeria monocytogenes*, *Staphylococcus aureus*, hemolytic streptococci groups B, C, and G, *Streptomyces lividans*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Yersinia enterocolitica*, *Shigella flexneri*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Salmonella typhimurium*, *Haemophilus influenzae*, and *Coxiella burnetii*. One strain of each species was included. The strains were cultivated by routine techniques described elsewhere (12). DNA groups 1, 2, 3, and 13 of the genus *Acinetobacter* were each represented by four strains: DNA group 1, strains ATCC 23055, ATCC 17902, M59, and M42; DNA group 2, strains ATCC 19606, ATCC 9955, ATCC 17978, and M91; DNA group 3, strains ATCC 17922, ATCC 19004, M62, and M55; and DNA group 13, strains ATCC 17903, M89, M100, and M165. The 16 strains were characterized to the DNA group level by DNA-DNA hybridization by the hydroxyapatite method (3). Detailed information on these strains is given elsewhere (6, 19).

The three *Francisella* species, *F. tularensis*, *F. novicida*, and *F. philomiragia*, were represented by 30, 3, and 7 strains, respectively. Of the 30 *F. tularensis* strains, 5 belonged to *F. tularensis* biovar *tularensis*. All *Francisella* strains were characterized to the species level through biochemical tests (utilization of glucose and glycerol, presence of citrulline ureidase, and production of H_2S and catalase) and the agglutination by a commercial *F. tularensis*-specific antiserum (Difco, Detroit, Mich.), as reported elsewhere (4, 5, 16). The virulence for rabbits has previously been characterized for several strains (16). All strains were part of our *Francisella* strain collection, and most of them have been described previously (5, 16). The following *F. tularensis* strains were included (the designation refers to their number in our strain collection): 017, 018, 020, 022, 024, 043 (type A), 090, 091, 106, 108, 138, 146, and 157 (all 13 preceding strains are human isolates); 044, 049, 050, 056, and 151 (all 5 preceding strains are environmental isolates); 033 (type A), 036, 041 (type A), 042 (type A), 045, 046 (type A), 054, 147, 148, 149, and 152 (all 11 preceding strains are animal isolates); and 155, the live vaccine strain. Moreover, *F. novicida* 040 (environmental isolate) and 156 and 159 (both human isolates) and *F. philomiragia* 037, 038, 039, 145, and 153 (human isolates) and 144 and 154 (both environmental isolates) were included.

DNA preparation and PCR. DNA was extracted by standard techniques (15). Briefly, bacterial cells were suspended in 1 ml of Tris-EDTA (TE) buffer, pelleted, and resuspended in 100 μl of TE buffer containing lysozyme (50 mg/ml), and the mixture was incubated at 37°C for 30 min. Proteinase K (20 mg/ml) and sodium dodecyl sulfate (10% [wt/vol]) were added, and the suspension was incubated for an additional 30 min. The cell lysate was purified by using the Gene Clean Kit (Bio 101, La Jolla, Calif.) and spin columns (Bio 101) according to the manufacturer's specifications. The DNA was eluted in TE buffer containing RNase (20 μg/ml), and the mixture was incubated at 37°C for 15 min to digest the

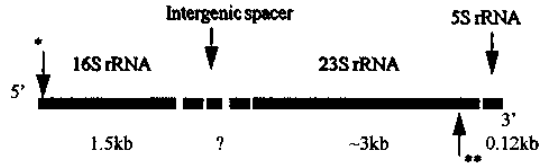

FIG. 1. Schematic representation of the bacterial genomic RNA genes. The interrupted line refers to the variable size of the 16S-23S intergenic spacer among various bacterial species. The single and double asterisks indicate the positions of the 5′ and 3′ amplification primers, respectively.

single-stranded RNA. One microliter of the eluate (approximately 10 ng) was added to a PCR master mixture containing 200 μM (each) deoxynucleoside triphosphate, 10 pmol of each primer, and 1× *Taq* extender buffer (prepared in our laboratory) containing 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 1% Triton X-100, 1.5 mM $MgSO_4$, and 1 mg of bovine serum albumin per ml in a final volume of 50 μl. The primers are complementary to positions 9 to 27 (5′-GAGTTTGATCCTGGCTCA-3′) and 2669 to 2654 (5′-CCGGTCCTCTCG TACT-3′) (11) of the 16S and 23S rRNA gene, respectively (Fig. 1). The reagents were overlaid with 50 μl of mineral oil, and the tubes were heated to 80°C before 1 U of *Taq* extender (Stratagene, La Jolla, Calif.) and 1 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.) were added to the master mixture. Immediately after adding the enzymes, the tubes were subjected to 30 cycles on a Perkin-Elmer 480 thermal cycler by the following protocol: denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 2.5 min at 72°C. The amplification process was terminated by a 10-min extension at 72°C, and the tubes were rapidly cooled to 4°C. One-tenth of each sample was subjected to electrophoresis on a 1% ethidium bromide-prestained agarose gel at 100 V for 90 min to visualize the PCR product.

For restriction endonuclease analysis, approximately 3 to 5 μl of each sample, depending on the density of the amplicon, was digested with 1 U of *Hae*III in 1× restriction buffer in a final volume of 10 μl. After incubation for 1 h at 37°C, the fragments were electrophoresed on a 2% ethidium bromide-prestained agarose gel at 100 V until complete separation was achieved.

RESULTS AND DISCUSSION

The primer set used in the PCR directed the amplification of approximately 4.5 to 5 kb of the rRNA gene cluster. In general, there were slight differences in size between amplicons of the species investigated, and these differences did not allow for the discrimination of species. However, as illustrated in Fig. 2, distinct differences in size were detected in a few cases, exemplified here by two strains of *L. pneumophila* and four strains each representing a DNA group of *Acinetobacter*. More important was the feasibility of the described amplification protocol to produce a single discrete band from all of the tested bacterial strains, which represent a wide range of phylogenetically diverse species.

Next, it was investigated whether the amplified gene clusters could be conveniently differentiated after restriction enzyme endonuclease digestion. The amplification products obtained

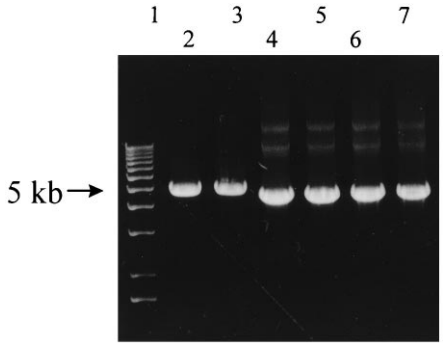

FIG. 2. Amplification of rRNA gene from *Legionella* and *Acinetobacter* species. Lane 1, 1-kb ladder (BRL); lanes 2 and 3, rRNA gene amplicon from two strains of *L. pneumophila*; lanes 4 to 7, rRNA gene amplicons from DNA groups 1, 2, 3, and 13 of the *A. calcoaceticus-A. baumannii* complex, respectively.

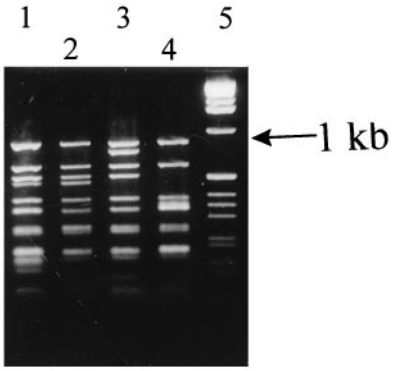

FIG. 3. Restriction fragment length polymorphism of PCR-amplified rRNA gene from *Acinetobacter* strains. Lanes 1 to 4, DNA group 1 (ATCC 23055), DNA group 2 (ATCC 19606), DNA group 3 (ATCC 17922), and DNA group 13 (ATCC 17903), respectively; lane 5, 1-kb ladder (BRL).

from the two genera *Acinetobacter* and *Francisella* were subjected to restriction by various enzymes (*Hae*III, *Sau*3A, *Alu*I, *Pst*I, *Ava*I, *Cla*I, *Eco*RV, *Mbo*I, *Msp*I, and *Rsa*I). It was found that the use of *Hae*III consistently generated fragments that could be differentiated easily after agarose gel electrophoresis.

Members of the genus *Acinetobacter* are ubiquitous in nature. They are gram-negative, strictly aerobic, diplococcoid rods that are oxidase negative and catalase positive. The taxonomy of the genus has been revised on several occasions, most recently by Bouvet and Grimont (2) and Tjernberg and Ursing (19). Although 20 different DNA genospecies have been reported in the literature, it has been possible to delineate only seven species on the basis of phenotypic characteristics. The most recent classification of members of the genus *Acinetobacter* was carried out in 1986 (2). That study proposed four new species, *A. baumannii*, *A. junii*, *A. johnsonii*, and *A. haemolyticus*, and the descriptions of the old species, *A. calcoaceticus* and *A. lwoffii*, were emended. In 1988, an additional species, *A. radioresistens*, was described (13). The other DNA-DNA hybridization groups were designated with numbers while awaiting species validation. From a clinical point of view, DNA groups 1, 2, 3, and 13 are the most important because they often are encountered in nosocomial infections. They all belong to the *A. calcoaceticus-A. baumannii* complex because of their overlapping phenotypic and genotypic traits. Thus, there is a need for rapid identification and discrimination of these groups. By using the described assay, it was possible to distinguish between strains of the four species.

Figure 3 shows the restriction analysis for one representative strain of each of the four DNA groups of *Acinetobacter* after *Hae*III digestion. Within each DNA group, identical patterns were observed. Because the amplification products were almost the same size on an agarose gel (Fig. 2), the differences are most likely due to polymorphism within the RNA genes. Considering that DNA-DNA hybridization and ribotyping are the only techniques for discriminating between these four DNA genomic species, the described assay has the potential to facilitate the identification process in clinical settings, e.g., during nosocomial infections.

The genus *Francisella* comprises three species, but almost all knowledge originates from studies with *F. tularensis*, which is a highly virulent bacterium that causes the zoonotic disease tularemia (4, 18). *F. novicida* and *F. philomiragia* are also of medical significance, but they usually cause disease only in immunocompromised individuals (9). *F. tularensis* exists in two biovars (4, 14), of which *F. tularensis* biovar *tularensis*, also denoted type A, is only found in North America and *F. tula-*

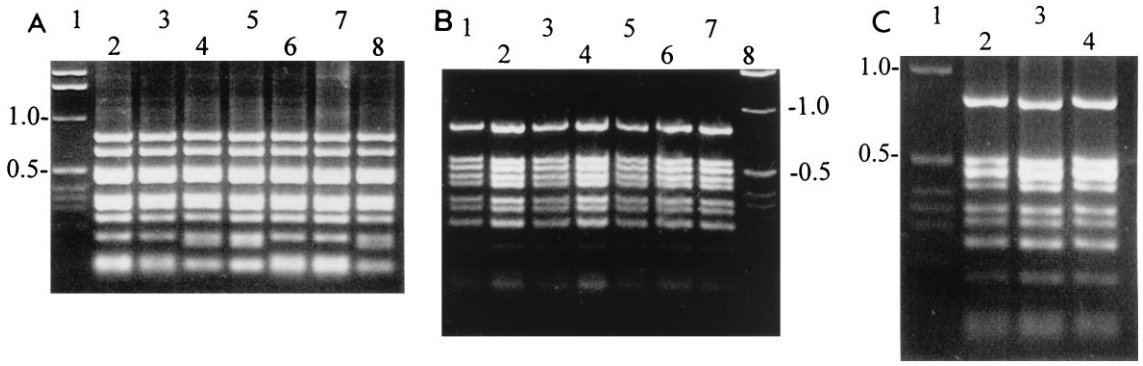

FIG. 4. RFLP of PCR-amplified rRNA genes from *Francisella* isolates. (A) *F. tularensis*. Lane 1, 1-kb ladder (BRL); lanes 2 to 8, *F. tularensis* FSC 017, FSC 024, FSC 033, FSC 041, FSC 043, FSC 149, and FSC 157, respectively. (B) *F. philomiragia*. Lanes 1 to 7, *F. philomiragia* FSC 037, FSC 038, FSC 039, FSC 144, FSC 145, FSC 154, and FSC 155, respectively; lane 8, 1-kb ladder (BRL). (C) *F. novicida*. Lane 1, 1-kb ladder (BRL); lanes 2 to 4, *F. novicida* FSC 040, FSC 156, and FSC 159, respectively.

rensis biovar *palaearctica*, also designated type B, can be isolated in Europe, Asia, and North America. The traditional criteria used to identify and distinguish members of the genus *Francisella* have been based on the metabolism of carbohydrates and an unusual fatty acid composition (4, 9).

By using the described assay to analyze 40 *Francisella* strains, four different restriction patterns were obtained, two for *F. tularensis* (Fig. 4A) and one each for *F. philomiragia* (Fig. 4B) and *F. novicida* (Fig. 4C). In Fig. 4A, one pattern is found in lanes 2, 3, 6, and 7 and the other pattern is found in lanes 4, 5, and 8. These same four patterns were also found when 23 other *Francisella* strains were analyzed by the technique.

Although the two patterns for *F. tularensis* did not show any correlation to geographical origin or the virulence of the strains, the described method is more advantageous than traditional methods, because it allowed for the rapid discrimination of the *Francisella* species. Moreover, it allowed samples to be treated in such a way that laboratory-acquired disease can be avoided.

In conclusion, the protocol presented here appears to give reproducible patterns, at least for the strains investigated, but it still allows for the resolution of closely related species, such as members of the genera *Acinetobacter* and *Francisella*. The technique has some inherent beneficial properties because no sophisticated equipment is required and it is reasonably rapid. Moreover, it can be combined with hybridization or direct sequence analysis of the amplified fragment. The combination of these techniques will result in a highly specific and potentially promising strategy for taxonomic analysis. In view of its relative simplicity, the method has a potential for use in the rapid typing of closely related bacterial isolates, for example, strains of medical importance.

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