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Identification of *Francisella* Species and Discrimination of Type A and Type B Strains of *F. tularensis* by 16S rRNA Analysis†

MATS FORSMAN,* GUNNAR SANDSTRÖM, AND BENGTÅKE JAURIN

Department of Cell- and Microbiology, National Defence Research Establishment, S-901 82 Umeå, Sweden

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Tularemia is a zoonotic disease, occurring throughout the Northern Hemisphere. The causative agent, the bacterium *Francisella tularensis*, is represented by two main types. Type A is found in North America, whereas type B is mainly found in Asia and Europe and to a minor extent in North America. No routine technique for rapid diagnosis of tularemia has been generally applied. We have partially sequenced 16S rRNAs of two *F. tularensis* strains, as well as the closely related *Francisella novicida*. Of 550 nucleotides analyzed, only one difference in 16S rRNA primary sequence was found. This 16S rRNA analysis enabled the construction of oligonucleotides to be used as genus- and type-specific probes. Such probes were utilized for the establishment of a method for rapid and selective detection of the organism. This method allowed identification of *Francisella* spp. at the level of genus and also discrimination of type A and type B strains of *F. tularensis*. The analysis also permitted the detection of *F. tularensis* in spleen tissue from mice infected with the bacterium. The results presented will enable studies on the epizootiology and epidemiology of *Francisella* spp.

The causative agent of tularemia, *Francisella tularensis*, can be divided in two main types (6, 24). *F. tularensis* subsp. *tularensis* (type A) has so far been found only in North America, whereas *F. tularensis* subsp. *palaearctica* (type B) occurs in Asia and Europe and to a minor extent in North America (15). Type A is associated with ticks and rabbits and is highly virulent in humans, whereas type B is found in mosquitoes and rodents and is less virulent to humans.

No antigenic difference between the types has been demonstrated (4), and accordingly, no identification method based on antigens is available. Instead, strain identification within the species is based on biochemical characterization and virulence tests (20). *F. tularensis* subsp. *tularensis* can be differentiated from *F. tularensis* subsp. *palaearctica* by its ability to ferment glycerol (23; R. B. Hornick and H. T. Eigelsbach, Letter, N. Engl. J. Med. **281**:1310, 1969) and its possession of the enzyme citrulline ureidase (20). Moreover, since the 50% lethal doses for rabbits are $<10^1$ organisms of type A and $>10^7$ organisms of type B, virulence tests on rabbits can be used as strain-specific criteria (20). However, these methods are both time and labor intensive.

Diagnosis of tularemia in humans is usually based on demonstration of homologous serum antibodies. However, demonstration of such antibodies is rather slow, since specific antibodies do not appear before the end of week 2 of illness. Trials have, however, been undertaken to evaluate rapid methods for diagnosis of tularemia. Such techniques are direct microscopy by using immunofluorescent antibodies (16) and identification of bacterial antigen in body fluids (30) and water samples (28) by an enzyme-linked immunosorbent assay. However, none of these methods seem to be generally applied for diagnosis of tularemia.

In recent years, 16S rRNA sequence analysis has been particularly useful for the determination of evolutionary relationships among numerous and diverse cellular life forms (32). As a consequence, a new approach to diagnosis based on hybridization against 16S rRNA with specific oligonucleotides has started to emerge (9, 11, 26, 27). To illustrate the application of specific hybridization probes, we have identified and synthesized oligonucleotides that are diagnostic for *Francisella* spp. The technique enabled identification at the level of genus and even permitted discrimination within species.

MATERIALS AND METHODS

Chemicals and materials. All chemicals were of the highest grade commercially available. Polynucleotide kinase, nucleotides, and NAP-5 columns were from Pharmacia, Uppsala, Sweden. Reverse transcriptase was purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany. All isotopes were purchased from Amersham International, Amersham, England. Oligonucleotides were synthesized by Symbicom, Umeå, Sweden. Nitrocellulose filters were obtained from Schleicher & Schuell, Inc., Keene, N.H.

Bacteria, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. All bacterial strains except *Francisella* strains were grown at 37°C in LB medium (1), and viable counts were performed on LB agar plates. The *Francisella* strains were grown for 3 days on modified Thayer-Martin agar plates containing Gc medium base (36 g/liter; Difco Laboratories, Detroit, Mich.), hemoglobin (10 g/liter; Difco), and IsoVitaleX (10 mg/liter; BBL Microbiology Systems, Cockeysville, Md.) at 37°C with 5% CO_2 in air. Viable counts of *Francisella* strains were performed on modified Thayer-Martin agar plates. Virulent *Francisella* strains were grown for 3 days and were thereafter heat inactivated at 65°C for 60 min.

Isolation of RNA templates. A total of 10 modified Thayer-Martin agar plates were inoculated for each *Francisella* strain used. After 3 days of growth, the cells were harvested from the surfaces of agar plates in saline, washed once, and prior to French pressure cell treatment, suspended in 15 ml of 50 mM Tris (pH 7.3)–5 mM $MgCl_2$–0.5 M NH_4Cl–20 μg of DNase per ml. A total of 200 ml of LB medium with exponentially growing *Escherichia coli* DH1 cells was harvested as described above. Total cellular RNA was purified by phenol extraction of French pressure cell lysates as described previously (25), except that ribosomes were not

* Corresponding author.

† This paper is dedicated to the memory of Bengtåke Jaurin, who died on 18 January 1990.

TABLE 1. Bacterial strains used in this study

Strain	Source
Escherichia coli DH1	Reference 10
Salmonella typhimurium LT2	ATCC[a] 19585
Yersinia enterocolitica 8081-C	T. F. Wetzler, University of Washington, Seattle
Bacillus subtilis	ATCC 6633
Staphylococcus aureus	ATCC 6538
Francisella tularensis live vaccine strain LVS subsp. *palaearctica*, type B (attenuated)	U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Fredrick, Md.
Francisella tularensis 38 subsp. *tularensis*, type A (avirulent)	ATCC 6223
Francisella tularensis (SBL R45) subsp. *palaearctica*, type B (moderately virulent)	National Bacteriological Laboratory, Stockholm, Sweden
Francisella tularensis subsp. *tularensis*, type A (highly virulent)	S. J. Stewart, Rocky Mountain Laboratory, Hamilton, Mont.
Francisella novicida	ATCC 15482

[a] ATCC, American Type Culture Collection.

pelleted before extraction. High-molecular-weight RNA was then prepared by precipitation with 2 M NaCl (25).

The amount of RNA was estimated by measurement of A_{260} and by agarose gel electrophoresis. Samples (10 μg) were treated with formamide and formaldehyde as described previously (14) and subjected to agarose (1.2%) gel electrophoresis in the presence of 1× MOPS buffer (20 mM 4-morpholinepropanesulfonic acid [pH 7.0], 5 mM sodium acetate, 1 mM EDTA) and 6.5% formaldehyde (7). RNA was stored at 2 mg/ml in 10 mM Tris (pH 7.4) at −70°C.

RNA sequencing. Sequencing of total RNA template was performed by using reverse transcriptase as described previously (17). Primer concentration was modified to 5 μg/ml, and the sequencing reaction was performed at 42°C for 10 min. Chase mix, composed as described previously (17), was added, and the chase reaction was prolonged to 60 min at 42°C. The terminated sequence products were separated on 0.2- to 0.6-mm-thick 6% polyacrylamide wedge-shaped sequencing gels (29).

For sequencing the 16S rRNA molecule of *Francisella* spp., universal primers A, B, and C (17) were used initially. On the basis of the *E. coli* 16S rRNA sequence (3), 10 additional primers were synthesized and used in 16S rRNA sequencing of the *Francisella* strains. The complementarity of these primers was scattered around the *E. coli* 16S rRNA molecule, with a spacing of approximately 150 nucleotides.

RNA hybridization. The procedure for RNA hybridization was essentially as described previously (12). Bacteria in a volume of 100 μl were filtered on a prewetted nitrocellulose filter in a dot blot or a slot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.). The filter with bacteria was transferred consecutively to two boxes containing filter paper (3MM; Whatman, Inc., Clifton, N.J.) presoaked with 10% sodium dodecyl sulfate (3 min at room temperature) and 3× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 [pH 7.4], and 1 mM EDTA) (15 min at 65°C).

The filter was briefly air dried and baked at 80°C in a vacuum oven for 15 min and thereafter used directly for hybridization. Synthetic oligonucleotides were end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP as detailed previously (8) and purified on NAP-5 columns prior to use. The radioactive probe was dissolved in hybridization solution (6×10^6 to 1×10^7 cpm/ml) containing 6× NET (1× NET is 0.15 M NaCl, 15 mM Tris [pH 7.0], and 1 mM EDTA), 0.5% Nonidet P-40, 1 mM Na_2HPO_4, 5× Denhardt solution (19), and 250 μg of calf thymus DNA per ml. Hybridization was carried out in sealed plastic bags submerged in a water bath, at the appropriate temperature, for 2 h. The filter was washed twice, 30 min per wash, at room temperature in 6× SSPE–0.1% Nonidet P-40. The filter was wrapped in Saran Wrap and exposed to X-ray film at −70°C using an intensifying screen. The radioactive signal was detected by autoradiography or quantified by cutting out each individual spot from the filter and placing it in a scintillation vial. The filter disks were then dried for 30 min at 65°C, 10 ml of scintillation liquid (Ready Protein$^+$; Beckman Instruments, Inc., Fullerton, Calif.) was added, and the ^{32}P activity was determined in a Beckman 5000 CE liquid scintillation counter. The time needed for a typical RNA hybridization experiment was 4 h. For each oligonucleotide used as a probe, the optimal hybridization temperature was determined. On the basis of the calculated T_m values of DNA-DNA hybrids, hybridizations were performed at different temperatures. For each probe used, the temperature giving the highest specificity was used in the experiments. The optimal hybridization temperature for the probes used was 4 to 6°C lower than the estimated T_m value.

Colony hybridization. A mixture of *Francisella* spp. type A and type B was spread on a modified Thayer-Martin agar plate. After 3 days of growth, three consecutive lifts from the same plate were performed with Colony/Plaque Screen (Dupont, NEN Research Products, Boston, Mass.) filters. Each individual filter was placed on a new modified Thayer-Martin agar plate. On the next day, the filters with overnight colonies were treated as detailed above. Probes FT1, FT2, and FT3 were each hybridized to one filter for 2 h at 42°C (FT3) and 30°C (FT1 and FT2). Further treatments of the filters were as described above.

In vivo identification of *F. tularensis*. NRMI mice were injected intraperitoneally with 10^6 *F. tularensis* LVS organisms in 0.1 ml of saline. Mice were killed 1 and 2 days after injection by dislocation of spine. Each spleen was homogenized by squeezing the spleen through a steel net (pore size, 1 mm). Homogenate was collected in 1 ml of saline and left for sedimentation of cell debris for 10 min at room temperature. Then, the supernatant was taken and diluted 10^{-1} to 10^{-8}. Viable counts were performed from each dilution. Samples (100 μl of 10^{-1} to 10^{-4} dilutions) were filtered on a nitrocellulose filter in a slot blot apparatus as described above. For controls, mice injected with 0.1 ml of saline were used.

RESULTS

Optimization of hybridization to 16S rRNA of *E. coli* cells. Conditions for lysis of bacterial cells under which single-stranded nucleic acid (RNA) but not double-stranded nucleic acid (DNA) binds to nitrocellulose filters have been established (12). These workers also demonstrated the use of radioactively labeled oligonucleotides in hybridization to mRNA of *E. coli* cells that had been immobilized on nitrocellulose filters (12). We investigated whether this method, hereafter referred to as RNA hybridization, also could be employed for hybridization to 16S rRNA of *E. coli*. There-

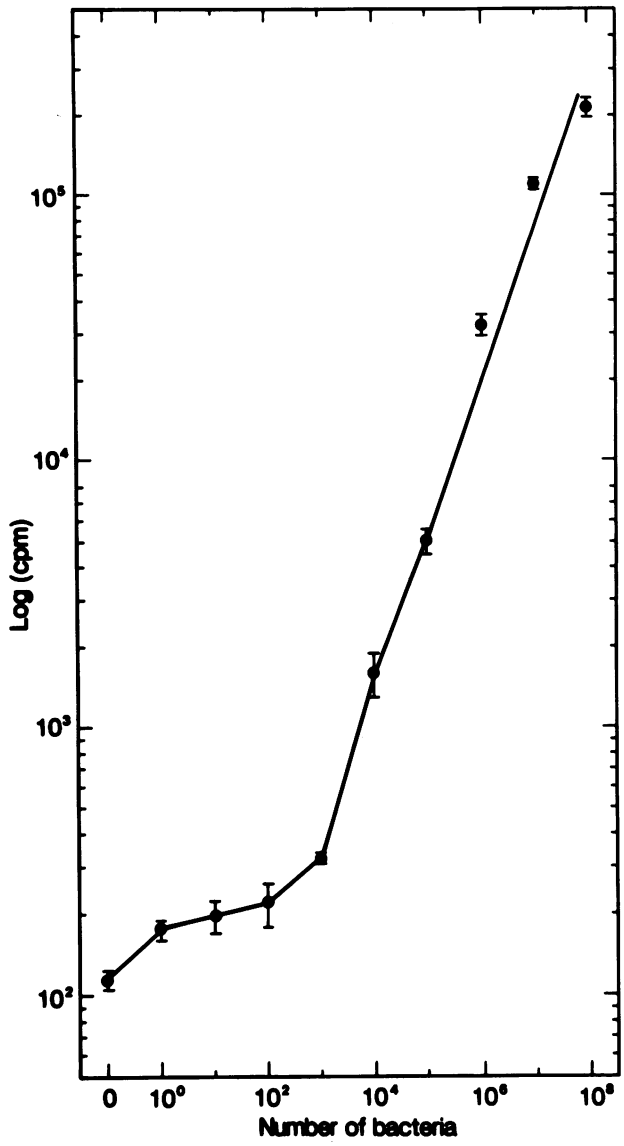

FIG. 1. Detection limit. Radioactively labeled probe EC1 (see Fig. 2) was hybridized for 2 hours at 42°C to various numbers of exponentially growing *E. coli* cells. For details, see Materials and Methods. The signals were quantified by scintillation counting. Means ± standard deviations of triplicate samples are indicated. The detection limit was estimated to be ~10^3 exponentially growing *E. coli* cells, as determined from the point where the curve forms a straight line.

fore, a number of oligonucleotides, complementary to various regions of *E. coli* 16S rRNA, were used in several hybridization experiments in order to optimize the method with respect to speed, handling, and sensitivity. By using the method as described in Materials and Methods, it was shown that this analysis permitted the detection of approximately 10^3 cells of *E. coli* in an analysis time of 4 h (Fig. 1). When the oligonucleotide used, designated EC1 (Fig. 2), and three other oligonucleotides were employed in hybridization to DNA of *E. coli* cells, it was demonstrated that hybridization to rRNA was approximately 1,000 times more sensitive per cell than hybridization to DNA (data not shown).

16S rRNA analysis of *Francisella* strains. rRNA was prepared from cells of *F. tularensis* subsp. *tularensis* (type A),

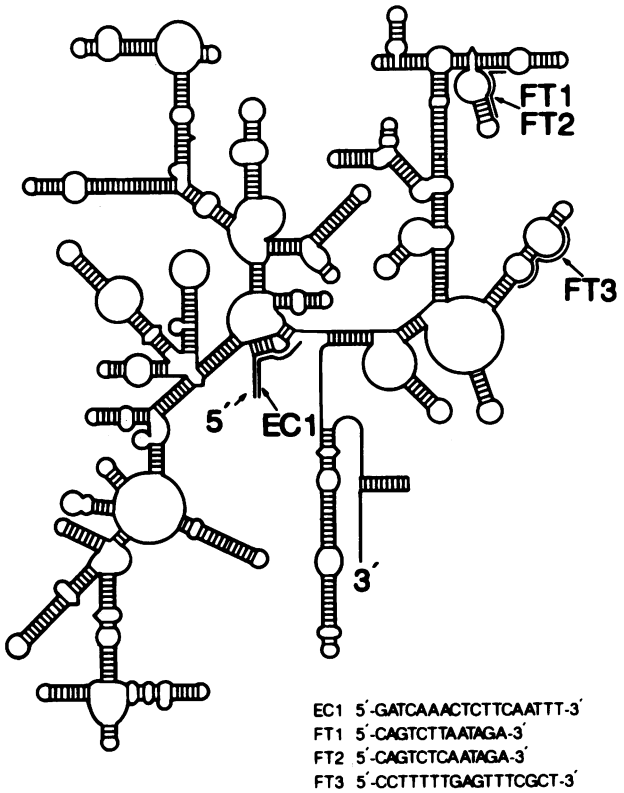

FIG. 2. 16S rRNA molecule of *E. coli*. The sequences and locations of the oligonucleotide probes used are shown. The locations indicated for FT1, FT2, and FT3 are the corresponding regions in the *E. coli* 16S rRNA molecule when compared with *Francisella* spp., based on simultaneous sequencing of both *E. coli* 16S rRNA and *Francisella* 16S rRNA.

F. tularensis subsp. *palaearctica* (type B), *F. novicida*, and *E. coli* (Table 1). A variety of oligonucleotides complementary to various parts of the *E. coli* 16S rRNA molecule (3) were synthesized and used as primers in sequencing reactions employing reverse transcriptase with each of the four RNA preparations as templates. A sequence of approximately 550 nucleotides from each 16S rRNA molecule was obtained (data not shown). Identity in 16S rRNA sequence was found between the three *Francisella* strains, except at position 1153 (numbering refers to the *E. coli* sequence [3]), where *F. tularensis* type B had an A, whereas *F. tularensis* type A and *F. novicida* had a G (Fig. 3). Several regions of the 16S rRNA sequence of the *Francisella* strains differed in primary structure from that of *E. coli*.

Identification of the genus *Francisella* by RNA hybridization. On the basis of the 16S rRNA sequences obtained, we synthesized a 17-base-long oligonucleotide (designated FT3) with an absolute complementarity to the 16S rRNA (positions 1274 to 1290) (Fig. 2) of each of the three *Francisella* strains. In RNA hybridization experiments (as described in Materials and Methods), this probe selectively detected the three *Francisella* strains, whereas cells of *E. coli*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Bacillus subtilis*, and *Staphylococcus aureus* were not recognized at all (Fig. 4). The detection limit for the *Francisella* strains was approximately 10^5 cells under the conditions used, i.e., when the *Francisella* strains had been grown for 3 days on plates. Thus, by using oligonucleotide FT3, we have presented a method for selective identification of the genus *Francisella*

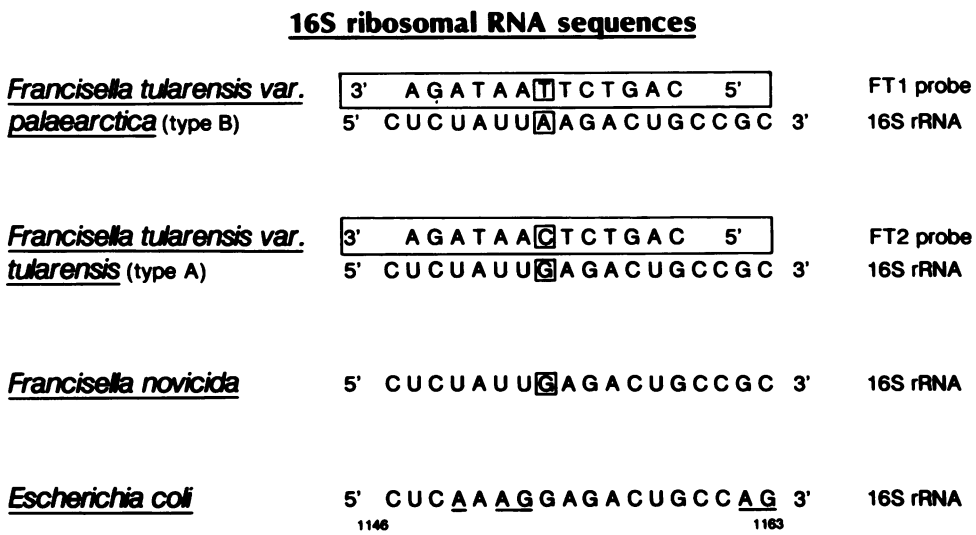

FIG. 3. 16S rRNA sequences. By using oligonucleotides complementary to various parts of the *E. coli* 16S rRNA molecule as primers in sequencing reactions employing reverse transcriptase and RNA preparations from the three strains of *Francisella* and *E. coli* as templates, a sequence of approximately 550 nucleotides from each 16S rRNA molecule was obtained. Identity in the 16S rRNA sequence was found between the three *Francisella* strains, except at position 1153 (□), where *F. tularensis* type B had an A, whereas *F. tularensis* type A and *F. novicida* had a G. On the basis of this difference, two probes were constructed: FT1 and FT2 (▭). Several positions of the 16S rRNA sequence of the *Francisella* strains differed in primary structure from that of *E. coli* (—).

by an analysis that permits screening of several samples within a time period of 4 hours.

Selective identification of *Francisella* types by RNA hybridization. Next, a method for the selective identification of type A and type B strains of *F. tularensis* was developed. Oligonucleotides complementary to the region around position 1153 of *F. tularensis* 16S rRNA (Fig. 3) were synthesized and used in RNA hybridization experiments. The FT1 probe (complementary to type B 16S rRNA) selectively detected an attenuated *F. tularensis* subsp. *palaearctica* strain (type B), whereas the FT2 probe (complementary to 16S rRNA of *F. tularensis* type A and of *F. novicida*) hybridized only to cells of an avirulent *F. tularensis* subsp. *tularensis* strain (type A) and *F. novicida* (Fig. 5). The same hybridization pattern was obtained with purified RNA (Fig. 5). Neither the FT1 nor the FT2 probe hybridized to cells of *E. coli*.

To investigate whether selective hybridization to virulent type A and type B strains of *F. tularensis* also could be obtained, strains belonging to *F. tularensis* subsp. *palaearctica* (type B) and *F. tularensis* subsp. *tularensis* (type A) were tested. Similar hybridization patterns were obtained with the virulent strains, compared with those of the avirulent and attenuated strains (Fig. 6). It should be noted that the FT1 probe also hybridized, but to a very minor extent, to cells of *F. tularensis* type A and cells of *F. novicida* (Fig. 6).

Next, to analyze in more detail the specificity of the selective identification method presented, cells of type A and type B strains of *F. tularensis* were mixed in similar ratios, grown, and treated as described in Materials and Methods. The genus-specific probe FT3 identified both strains tested, whereas the type-specific probes FT1 and FT2 selectively hybridized to strains of type B and type A, respectively (Fig. 7). Thus, this technique can be used not only to identify bacteria at the level of genus but also to discriminate different types of the same species.

In vivo detection of *F. tularensis*. Knowing that *F. tularensis* could be detected specifically, we investigated whether the method allowed the bacterium to be identified directly

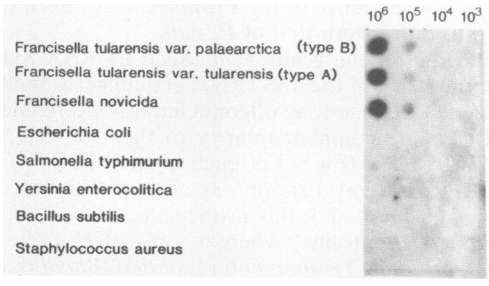

FIG. 4. Genus *Francisella* selectively identified by RNA hybridization. An oligonucleotide (FT3), complementary to 16S rRNA of each of the three *Francisella* strains indicated, was radioactively labeled and used in hybridization to bacterial cells that had been collected and lysed on nitrocellulose filters. The number of bacteria in each dot is shown at the top.

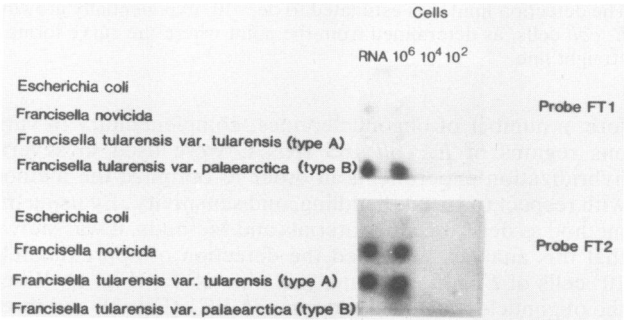

FIG. 5. *F. tularensis* type A and type B selectively detected by RNA hybridization. Two oligonucleotides, FT1 and FT2 (Fig. 3), were radioactively labeled and used separately in hybridization to purified rRNA (6 μg) or to bacterial cells that had been collected and lysed on a nitrocellulose filter. A total of 10^3 to 10^6 bacterial cells were applied to each spot.

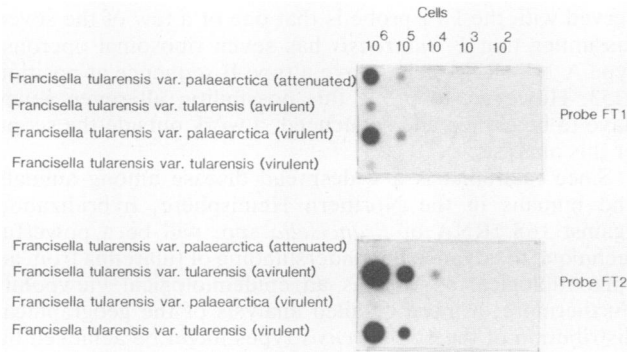

FIG. 6. rRNA hybridization patterns of the virulent strains of *F. tularensis* subsp. *tularensis* (type B) and *F. tularensis* subsp. *palaearctica* (type A) identical to those of the avirulent and attenuated strains. Two oligonucleotides, FT1 and FT2, based on the 16S rRNA sequences of *F. tularensis* (Fig. 3), were used separately in hybridization to heat-inactivated virulent strains and avirulent and attenuated strains. The number of heat-inactivated cells in each slot is indicated at the top.

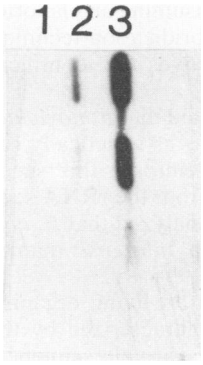

FIG. 8. In vivo identification of *F. tularensis* subsp. *palaearctica* in mice by RNA hybridization. Homogenized spleens from mice injected intraperitoneally with 10^6 *F. tularensis* LVS organisms were filtered on a nitrocellulose filter and subjected to RNA hybridization (at 30°C), using oligonucleotide FT1 as a probe. Lanes: 1, spleen homogenate from control mice injected with saline and killed 2 days after injection; 2, spleen homogenate from mice injected with 10^6 *F. tularensis* LVS organisms and killed 1 day after injection; 3, spleen homogenate from mice injected with 10^6 *F. tularensis* LVS organisms and killed 2 days after injection. Initially, after killing, the homogenized spleen suspension contained (as determined by viable counts) 10^5 *F. tularensis* LVS organisms per ml (day 1) and 10^7 *F. tularensis* LVS organisms per ml (day 2). Vertically, 10-fold dilutions, from 10^{-1} to 10^{-4}, of the homogenized spleen filtrates are shown.

from infected animals. Therefore, mice were infected with *F. tularensis* LVS (type B). Homogenized spleen samples from uninfected mice and from mice infected 1 or 2 days prior to testing were analyzed in RNA hybridization experiments using the oligonucleotide FT1 as a probe. *F. tularensis* could readily be identified in samples from infected mice, whereas uninfected animals gave no signal at all (Fig. 8). Also, the signal of hybridization was significantly increased in samples obtained from mice 2 days after infection, compared with the signal from mice infected 1 day prior to testing. This increase most probably reflects growth of the bacterium in the infected animals; this idea is also supported by an increase in plating counts of bacteria from spleen tissue (Fig. 8).

DISCUSSION

This paper describes a unique method for the detection of bacteria belonging to the genus *Francisella*. The method was also found to be novel, insofar as it could discriminate between types within the species *F. tularensis*. Hybridization against *F. tularensis* 16S rRNA was found to be highly specific, since no reactivity with unrelated bacteria was observed. In comparison with immunoassays for the detection of *F. tularensis* (16, 28, 30), the specificity is similar, because cross-reactions between *F. tularensis* and other clinically important gram-negative bacteria are rare (22).

F. tularensis exhibits a unique composition of fatty acids (13, 21), a property which can be utilized for genus identification. A drawback with this technique, as well as *F. tularensis*-specific immunoassays, is that it does not distinguish between types of *F. tularensis*. Biochemical characterization has been useful, because type A can be separated from type B by its ability to ferment glycerol (23; Hornick and Eigelsbach, Letter) and its possession of the enzyme citrulline ureidase (20). Measurement of such differences,

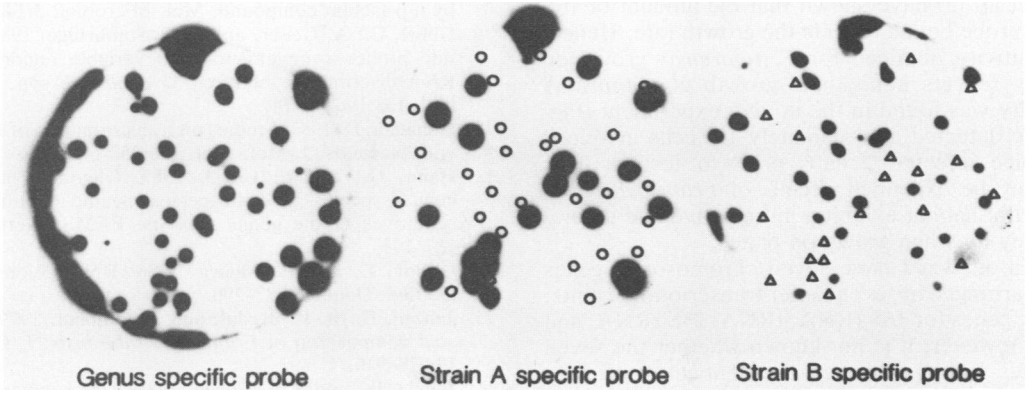

FIG. 7. Discrimination of *F. tularensis* type A and type B by colony hybridization. Three replicas were made from the same plate that contained a mixture of *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *palaeartica* (type B). The replicas were hybridized with the *Francisella* genus-specific probe FT3 (Fig. 2), the FT2 probe (type A; Fig. 3), or the FT1 probe (type B; Fig. 3). Dark spots represent signals obtained with each probe. Open circles indicate colonies not reacting with strain A-specific probe; open triangles show location of colonies not reacting with strain B-specific probe.

however, is time consuming and laborious, and therefore the rapid and simple hybridization technique against 16S rRNA described here improves the identification of *F. tularensis* strains.

It has been suggested that *F. novicida*, the other species of the genus *Francisella*, is genetically related to *F. tularensis* (31); these results reinforce this suggestion. Moreover, it might be suggested from the rRNA sequences of *F. tularensis* and *F. novicida* analyzed that *F. novicida* is more related to *F. tularensis* subsp. *tularensis* than to *F. tularensis* subsp. *palaearctica*.

rRNA is found in all living organisms. Among different bacteria, rRNA is characterized by the presence of highly conserved and more variable sequences. This, in combination with the relatively large size of rRNA (1,500 to 3,000 nucleotides) and the ease with which rRNA can be isolated, makes rRNA ideal for measurement of phylogenetic relationships (32). Consequently, hybridization with short oligonucleotides targeted for unique regions in the rRNA molecules offers a new approach for direct classification and identification of bacteria. This has, by hybridization to 16S rRNA, recently been demonstrated for representatives of the genus *Proteus* at the level of genus, species, and even subspecies (11). Furthermore, by using specific 16S rRNA probes, discrimination between *Mycoplasma* species (9) and specific identification of *Vibrio anguillarum* in kidneys from fish (26) have been reported. Phylogenetic group-specific fluorescently labeled oligonucleotides against 16S rRNA could identify single microbial cells by hybridization when viewed by fluorescence microscopy (5). Also, by using quantitative microfluorimetry, it was possible to show that the amount of rRNA-specific probe bound reflects the growth rate (5).

We have shown that the RNA hybridization method used gave a detection limit of approximately 10^3 cells of *E. coli* (Fig. 1). Each cell of *E. coli* contains at exponential growth approximately 20,000 ribosomes (18). The sensitivity offered by RNA hybridization could be compared with that of approximately 10^6 cells when DNA is the hybridizing target. Thus, the increase in sensitivity correlates well with the copy number of the hybridizing target. When cells of *F. tularensis* were grown on plates and analyzed by RNA hybridization, the sensitivity was significantly lower than that with exponentially growing cells of *E. coli*. It is known that the number of ribosomes per cell of *E. coli* is highest at exponential growth and declines during stationary phase (18). DeLong et al. (5) have shown that the amount of 16S rRNA-specific probe bound reflects the growth rate. Hence, the lower sensitivity obtained for *F. tularensis* grown on plates probably reflects nonoptimal growth conditions. A higher sensitivity was found in the in vivo experiment (Fig. 8), in which we detected approximately 10^4 cells in spleen tissue from mice infected 2 days prior to testing. This discrepancy may be explained if cells of *Francisella* spp. grown in a natural habitat exhibit a more active and natural growth than they do when grown on plates.

Most bacteria analyzed have seven chromosomal genes for 16S rRNA, arranged in seven larger transcriptional units, each containing genes for 16S rRNA, tRNA, 23S rRNA, and 5S rRNA (2). However, it is not known whether the seven 16S rRNA genes within a species are identical in primary sequence. The FT1 probe hybridized efficiently to 16S rRNA of *F. tularensis* type B, whereas it hybridized weakly to cells of *F. tularensis* type A and *F. novicida* (Fig. 6). The FT2 probe did not show any cross-hybridization at all. One possible explanation for the minor cross-hybridization observed with the FT1 probe is that one or a few of the seven (assuming that *F. tularensis* has seven ribosomal operons) type A 16S rRNA genes have a type B sequence at position 1153. However, to prove this possibility, all seven genes have to be cloned and sequenced, a work outside the scope of this analysis.

Since tularemia is a widespread disease among animals and humans in the Northern Hemisphere, hybridization against 16S rRNA of *Francisella* spp. will be a powerful technique to advance the understanding of tularemia from an epizootiological as well as an epidemiological viewpoint. Furthermore, a more detailed analysis of the geographical distribution of the *F. tularensis* types might be achieved by using the present identification method.

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LITERATURE CITED

1. **Bertani, G.** 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62**:293–300.
2. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148**:107–127.
3. **Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *E. coli*. Proc. Natl. Acad. Sci. USA **75**:4801–4805.
4. **Carlisle, H. N., V. Hinchliffe, and S. Saslaw.** 1962. Immunodiffusion studies with *Pasteurella tularensis* antigen-rabbit antibody system. J. Immunol. **89**:638–644.
5. **DeLong, E. F., G. S. Wickman, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243**:1360–1363.
6. **Eigelsbach, H. T., and V. G. McGann.** 1984. Gram-negative aerobic cocci, p. 394–399. *In* N. R. Krieg and J. G. Holts (ed.), Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore.
7. **Fellous, M., U. Nir, D. Wallach, G. Merlin, M. Rubinstein, and M. Revel.** 1982. Interferon-dependent induction of mRNA for the major histocompatibility antigens in human fibroblasts and lymphoblastoid cells. Proc. Natl. Acad. Sci. USA **79**:3082–3086.
8. **Forsman, M., L. Lindgren, B. Häggström, and B. Jaurin.** 1989. Transcriptional induction of *Streptomyces cacaoi* β-lactamase by a β-lactam compound. Mol. Microbiol. **3**:1425–1432.
9. **Göbel, U., A. Geiser, and E. J. Stanbridge.** 1987. Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* spp. J. Gen. Microbiol. **133**:1969–1974.
10. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
11. **Haun, G., and U. Göbel.** 1987. Oligonucleotide probes for genus-, species-, and subspecies-specific identification of representatives of the genus *Proteus*. FEMS Microbiol. Lett. **43**: 187–193.
12. **Ivanov, I., and L. Gigova.** 1986. RNA colony hybridization method. Gene **46**:287–290.
13. **Janzen, E., B. P. Berdal, and T. Omland.** 1979. Cellular fatty acid composition of *Francisella tularensis*. J. Clin. Microbiol. **10**:928–930.
14. **Jaurin, B., and S. N. Cohen.** 1984. *Streptomyces lividans* RNA polymerase recognizes and uses *Escherichia coli* transcriptional signals. Gene **28**:83–91.
15. **Jusatz, H. J.** 1961. The geographical distribution of tularemia throughout the world, 1911–1959, p. 38. *In* World-atlas of epidemic diseases, vol. 3. Falk Verlag, Hamburg, Federal

Republic of Germany.
16. **Karlsson, K. A., and O. Söderlund.** 1973. Studies of the diagnosis of tularemia. Contrib. Microbiol. Immunol. **2**:224–230.
17. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc. Natl. Acad. Sci. USA **82**:6955–6959.
18. **Maaløe, O., and N. O. Kjeldgaard.** 1966. Control of macromolecular synthesis: a study of DNA, RNA, and protein synthesis in bacteria, p. 117–124. W. A. Benjamin, New York.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Marchette, N. J., and P. S. Nicholes.** 1961. Virulence and citrulline ureidase activity of *Pasteurella tularensis*. J. Bacteriol. **82**:26–32.
21. **Nichols, P. D., W. R. Mayberry, C. P. Antworth, and D. C. White.** 1985. Determination of monosaturated double-bond position and geometry in the cellular fatty acids of the pathogenic bacterium *Francisella tularensis*. J. Clin. Microbiol. **21**:738–740.
22. **Ohara, S., T. Sato, and M. Homma.** 1974. Serological studies on *Francisella tularensis*, *Francisella novicida*, *Yersinia philomiragia*, and *Brucella abortus*. Int. J. Syst. Bacteriol. **24**:191–196.
23. **Olsufjev, N. G., and O. S. Emelyanova.** 1962. Further studies of strains of tularemic bacteria of the old and new world. J. Hyg. Epidemiol. Microbiol. Immunol. **6**:193–205.
24. **Olsufjev, N. G., O. S. Emelyanova, and T. N. Dunayeva.** 1959. Comparative study of strains of *B. tularensis*. II. Evaluation of criteria of virulence of *Bacterium tularense* in the old and the new world and their taxonomy. J. Hyg. Epidemiol. Microbiol. Immunol. **3**:139–149.
25. **Pace, B., E. A. Matthews, K. D. Johnson, C. R. Cantor, and N. R. Pace.** 1982. Conserved 5S rRNA complement to tRNA is not required for protein synthesis. Proc. Natl. Acad. Sci. USA **79**:36–40.
26. **Rehnstam, A.-S., A. Norqvist, H. Wolf-Watz, and Å. Hagström.** 1989. Identification of *Vibrio anguillarum* in fish by using partial 16S rRNA sequences and a specific 16S rRNA oligonucleotide probe. Appl. Environ. Microbiol. **55**:1907–1910.
27. **Romaniuk, P. J., and T. J. Trust.** 1987. Identification of *Campylobacter* species by Southern hybridization of genomic DNA using an oligonucleotide probe for 16S rRNA genes. FEMS Microbiol. Lett. **43**:331–335.
28. **Sandström, G. E., H. Wolf-Watz, and A. Tärnvik.** 1986. Duct ELISA for detection of bacteria in fluid samples. J. Microbiol. Methods **5**:41–47.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
30. **Tärnvik, A., S. Löfgren, L. Öhlund, and G. Sandström.** 1987. Detection of antigen in urine of a patient with tularemia. Eur. J. Clin. Microbiol. **6**:318–319.
31. **Tyeryar, F. J., Jr., and W. D. Lawton.** 1970. Factors affecting transformation of *Pasteurella novicida* J. Bacteriol. **104**:1312–1317.
32. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**: 221–271.