Localization of *Tropheryma whippelii* rRNA in Tissues from Patients with Whipple’s Disease

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Whipple’s disease is caused by a cultivation-resistant bacterium, *Tropheryma whippelii*. Ultrastructural studies of intestinal biopsy specimens from patients with Whipple’s disease have shown that intracellular and extracellular bacteria are present, but the preferred site of growth is unknown. Tissue sections from 8 patients with Whipple’s disease and from 19 healthy control subjects were analyzed by use of fluorescence in situ hybridization and laser scanning confocal microscopy, to determine the location of rRNA that would indicate the presence of metabolically active bacteria. *T. whippelii* rRNA was most prevalent near the tips of intestinal villi, in the lamina propria, just basal to epithelial cells. Most of the bacterial rRNA signal appeared to be located between cells and did not colocalize with the human intracellular protein vimentin. The location of bacterial rRNA in tissues from patients with Whipple’s disease provides evidence that bacteria are growing outside cells and suggests that *T. whippelii* is not an obligate intracellular pathogen.
bacterial rRNA levels tend to correlate with growth rate, and probe hybridization to rRNA has been used to assess metabolic activity in bacteria [8]. Vimentin is an intermediate filament protein that contributes to the cytoskeleton of cells derived from mesenchyme. By staining vimentin, we sought to define the boundaries of the intracellular space and to determine the relationship of the T. whippelii rRNA signal to this space.

Materials and Methods

Preparation of tissue sections and cells. Sections (5 μm) were cut from blocks of formalin-fixed, paraffin-embedded tissue and were placed on glass slides. Paraffin was removed from the tissue sections by dipping the slides in 99% octane (Sigma) for 15 s at room temperature. The tissue then was bleached by immersing the slides in a fresh solution of 100 mM Tris hydrochloride (pH 8.0) and 50 mM sodium borohydride (Sigma) for 1 h. After being rinsed in distilled water, the slides were air dried. Tissues studied included 6 duodenal biopsy specimens and 2 lymph nodes from 8 patients with Whipple’s disease. Five of the 8 cases have been described elsewhere [7, 9]. The diagnosis of Whipple’s disease was confirmed by histologic tests (PAS staining) and PCR assay for T. whippelii [7]. Control tissues consisted of formalin-fixed and paraffin-embedded intestinal biopsy specimens (17) and lymph nodes (2) from 19 patients without Whipple’s disease.

Bacteria used for whole cell hybridizations were grown on blood agar plates, and then colonies were suspended in water and were spotted on poly-lysine-coated glass slides (Poly-prep slides; Sigma). After being air dried, the cells were fixed by immersion in 10% formalin (Baxter Diagnostics) for 1 h and then were rinsed in distilled water. Bacteria studied included Escherichia coli, Cellulomonas cellulans, Cellulomonas flavigena, and Corynebacterium aquaticum (American Type Culture Collection). Like T. whippelii, the strains C. cellulans, C. flavigena, and C. aquaticum are gram-positive Actinobacteria with high guanine and cytosine content.

Probe design. The oligonucleotide probe Eub16S-338 [8] is complementary to a conserved region of bacterial 16S rRNA: 5′-GCTGCTCCCTCCGTAGGAGTG-3′. Either Cy-5 or fluorescein isothiocyanate (FITC) fluorophore was attached to the 5′ end of this oligonucleotide (all probes were synthesized by Synthentic Genetics).

A T. whippelii-specific probe was designed by selecting a unique region of 16S rRNA as a target for hybridization. Selected candidate probes were checked against the ARB database of 16S rRNA sequences (Technical University, Munich) and the Ribosomal Database Project database (RDP; Michigan State University, East Lansing; www.cme.msu.edu/RDP/html/index.html), to ensure that the probe was specific for T. whippelii rRNA. The probe Tw16S-652 (5′-TTCCGCTTCCCCCTATGCACCT-3′) was labeled with a Cy-5 fluorophore at the 5′ end. There are 2 nucleotide mismatches between this probe and the most similar 16S rRNA sequence in RDP (Microbacterium halophilum, GenBank accession no. AB-004714, a soil bacterium), whereas every other 16S rRNA sequence in GenBank, ARB, and RDP has ≥3 mismatches.

A control oligonucleotide probe, Tw16S-Cnt, is the complement of the Tw16S-652 probe: 5′-AAGGCAGAGGGGATAGCGTGAGA-3′. There is no known rRNA target for this probe, but it has a GC:AT base ratio identical to that of the Tw16S-652 probe, and hence a similar melting temperature.

In situ hybridization. Nonspecific binding to tissue sections and cells on slides was blocked by incubating with hybridization buffer (without probe) at 55°C for 1 h. Hybridization buffer consisted of 5× SET (0.75 M NaCl, 5 mM EDTA, and 0.1 M Tris [pH 7.8]), 10% dextran, 0.2% bovine serum albumin (BSA), 0.1 mg/mL polyadenosine, and 20 μg/mL salmon testes DNA (Sigma). Both blocking and hybridization occurred in Hybri-Well plastic hybridization chambers that attach to slides (Sigma). After blocking, the hybridization chamber was removed, and excess buffer was allowed to run off the slide. A second hybridization chamber was placed, and 50 μL of hybridization buffer with 50 ng of labeled probe was added to the new chamber. Chambers were sealed, and the slides were placed in humid plastic containers and were incubated for 18 h at 55°C in a water bath.

After the hybridization chambers were removed, the slides were washed twice, each by immersion in 0.2× SET at room temperature for 10 min. The slides then were washed twice, each by immersion in 0.2× SET at 55°C for 15 min, and then were rinsed in distilled water and were air dried in the dark.

RNase treatment. One control used RNase treatment of tissue, to determine whether digesting the rRNA target prevented probe binding. RNase A (Boehringer Mannheim) was dissolved in 10 mM Tris buffer with 1 mM magnesium chloride at 100 μg/mL. Before hybridization, 50 μL of this solution was applied to a tissue section of Whipple’s disease intestine on a slide. After a coverslip was placed, the slide was incubated at 37°C for 1 h and then was rinsed in distilled water. Hybridization was done as described above.

Vimentin staining. A mouse monoclonal antibody (IgG) targeting the cytoskeletal protein vimentin (Sigma) was incubated with tissue sections after in situ hybridization (see above). Antibody was diluted 1:50 with 2.5% BSA (Sigma) in 1× PBS, and 50 μL of this antibody dilution was applied to the tissue section for 1 h at room temperature. The slide then was washed in 1× PBS for 10 min, with 3 changes of buffer, for a total wash of 30 min. Goat anti–mouse IgG with a Texas Red label (Jackson ImmunoResearch) was diluted 1:50 with 2.5% BSA (Sigma) in 1× PBS for 1 h at room temperature. The slide then was washed in PBS for 10 min, with 3 changes of buffer, for a total wash of 30 min.

Nuclear staining. YO-PRO 1 fluorescent nucleic acid stain (Molecular Probes) was diluted to 1.0 μM in 1× SET buffer, and 50 μL of the stain was applied to the tissue section for 20 min at room temperature. The slide then was washed for 10 min, 3 times, in 0.2× SET buffer. The slide was rinsed in water before microscopy. For tissue sections subjected to antibody and oligonucleotide labeling, nuclear staining was the final staining method. Slides then were air dried and mounted with Fluoro Guard mountant (Bio-Rad Laboratories) and coverslips.

Confocal microscopy. Tissue sections and cells were visualized by use of a Bio-Rad MRC-1024 Laser Scanning Confocal Imaging System. Vimentin staining was revealed by scanning in the Texas Red channel (605 nm, red). rRNA probe hybridization was revealed by scanning in the Cy-5 channel (680 nm, blue pseudocolor) or, when appropriate, in the FITC channel (522 nm, green). YO-PRO 1 staining of nucleic acid was detected by scanning in the FITC channel (522 nm, green). FITC-labeled probe and YO-PRO 1 were not used on the same slides. Each image is a single optical z-section.
through the tissue. Images were collected in each fluorescence channel and then were superimposed, using computer software (Adobe Photoshop; Adobe Systems) or the imaging software of the microscope. There was no other manipulation of images using Photoshop software. More than 200 hybridizations were done using sections of tissues from patients with Whipple’s disease and normal biopsy specimens, and each section was viewed at several magnifications, ranging from ×100 to ×600. A zoom function was used for higher magnifications, such as ×3 zoom at ×600, producing an effective magnification of ×1800.

Results

Intestinal biopsy specimens from 17 patients without Whipple’s disease did not bind the T. whippelii probe and showed normal villous architecture (figure 1A). The 16S rRNA probe targeting T. whippelii bound to all tested duodenal tissues from 6 patients with Whipple’s disease. Hybridized probe was found primarily at the tips of blunted intestinal villi, especially in the most superficial area of the lamina propria (figure 1B and 1D). The distribution of bound probe (bacterial rRNA) is different than the distribution of PAS staining (polysaccharide) found in tissues from patients with Whipple’s disease (figure 1C). There appears to be more PAS-reactive material in the deeper mucosa than in the lamina propria near the villous tips. The intestinal epithelial cells themselves do not contain large amounts of T. whippelii rRNA.

Figure 2 demonstrates the relationship between bound rRNA probe targeting T. whippelii (blue pseudocolor) and human intestinal cells, as visualized by staining of the cytoskeletal protein vimentin (red fluorescence; figure 2A) and by staining of nuclei (green fluorescence; figure 2B). Vimentin antibody stained cells in the lamina propria (e.g., macrophages), as expected. Intestinal tissue from patients with Whipple’s disease bound the T. whippelii rRNA probe in a pattern that was remarkable for the number of circular punched-out areas containing no probe signal (figure 2C). Superposition of these images shows that areas without bound T. whippelii probe correspond to regions of vimentin staining (figure 2D). Most of the rRNA probe appeared to surround visible cells and to occur in spaces between cells. When the rRNA probe (blue) and vimentin antibody (red) overlap, the image is pink. Most of the images from Whipple’s disease intestine tended to be either red or blue.

T. whippelii rRNA probe was localized immediately subjacent to the basal lamina of the duodenum and was demonstrated, with decreasing signal, in deeper tissues (figure 3A). At higher magnification, the rRNA probe signal appeared to interdigitate between cells in the lamina propria (figure 3B). We could not resolve individual bacteria, even using a ×100 objective.

Fluorescence in situ hybridization (FISH) also was done on 2 lymph nodes from 2 patients with Whipple’s disease. These tissues bound probe Tw-652, revealing long linear bands of bacterial rRNA within the nodes (figure 3C and 3D).

When a broad-range bacterial probe (Eub16S-338 with Cy-5 label) was used for FISH, it bound to tissues from patients with Whipple’s disease in the same distribution as the T. whippelii–specific probe but did not hybridize to control tissues (data not shown). In some experiments, an FITC-labeled bacterial probe (Eub16S-338, green; figure 4A) and a Cy-5–labeled Whipple probe (Tw16S-652, blue; figure 4B) were used together for FISH. YO-PRO nucleic acid stain was not used in these experiments. This approach was limited by autofluorescence of fixed tissues in the FITC channel. Nevertheless, colocalization of probes to the subepithelial spaces of the lamina propria in Whipple’s disease intestine could be appreciated (figure 4C).

Several controls were done to assess the specificity of the FISH results with the T. whippelii rRNA probe. Control tissues consisting of 17 intestinal biopsy specimens and 2 lymph nodes from 19 patients without Whipple’s disease did not bind the T. whippelii probe. In addition, a control probe consisting of the complement of probe Tw16S-652 did not hybridize to tissues from patients with Whipple’s disease (data not shown). Finally, when an intestinal biopsy specimen from a patient with Whipple’s disease was treated with RNase, the T. whippelii probe no longer bound to the tissue, which suggests that the targets of the probe were destroyed (figure 4D). A similar section of Whipple’s disease intestine processed concurrently, but without RNase treatment, did bind the Whipple bacillus rRNA probe, as expected. These observations support the contention that the T. whippelii probe was binding to an RNA target in a specific fashion.

Probe specificity also was checked by whole cell hybridization, using several bacteria. Probe Tw16S-652 did not hybridize to E. coli, C. cellulans, or C. flavigena. C. aquaticum cells were faintly fluorescent when hybridized with probe Tw16S-652. All bacteria tested bound the probe Eub16S-338, as expected.

Discussion

We have shown, using FISH, that bacterial rRNA from T. whippelii is located in the lamina propria of small-bowel biopsy specimens from patients with Whipple’s disease, as expected. Although some uptake of the T. whippelii rRNA probe was noted within human cells, most of the bacterial rRNA signal found in the intestine appeared to be located between visible cells, not within cells. These results were confirmed by using a T. whippelii rRNA probe alone, which ruled out quenching from other fluorophores as a cause of this phenomenon. This observation suggests that metabolically active bacteria exist primarily in the extracellular spaces. Furthermore, the distribution of T. whippelii rRNA was different from the distribution of PAS reactivity in several tissues studied. PAS reactivity was most evident within cells of the deeper mucosa, whereas the most intense bacterial rRNA signal occurred in the lamina propria near villous tips.

In tissues from patients with Whipple’s disease stained with vimentin antibody (red) and the T. whippelii rRNA probe (blue),
Figure 1. Fluorescence in situ hybridization images and histologic analysis of intestinal tissues. A, Duodenal tissue from a patient without Whipple's disease (original magnification, ×200), using conditions as in B, did not bind the Whipple probe. B, Duodenal tissue from a patient with Whipple's disease (original magnification, ×100). The Whipple rRNA probe Tw16S-652 stains blue, nuclear stain YO-PRO 1 (Molecular Probes) is green, and vimentin antibody stains red. Note the abundant bacterial rRNA signal in the lamina propria near the villus tips. C, Periodic acid–Schiff (PAS)–stained section of Whipple's disease intestine (original magnification, ×200). The section of villus at left is from the same tissue specimen and region shown in D. Note the relative paucity of PAS reactivity in the lamina propria near the villus tips, compared with that in the deeper mucosa. D, Triple-stained section of Whipple's disease intestine (original magnification, ×400) showing infiltration of the lamina propria with vimentin-staining cells and *Tropheryma whippelii* rRNA.
Figure 2. Triple-stained section of Whipple's disease intestine showing individual emission channels for each fluorophore and combined image. 

A. Cytoskeletal protein vimentin stains red in Texas Red channel. Intestinal epithelial cells do not contain vimentin but show some red staining due to autofluorescence.

B. Cell nuclei stain green in fluorescein isothiocyanate channel due to uptake of YO-PRO 1 (Molecular Probes) nucleic acid stain. Note that a faint green signal also is present and corresponds to the rRNA signal in C and probably represents staining of bacterial nucleic acid.

C. Tropheryma whippelii 16S rRNA has a blue pseudocolor in the Cy-5 channel. Note the punched-out appearance of the bacterial probe signal.

D. Superimposed images A–C demonstrate the relationship between cells in the lamina propria and T. whippelii rRNA. Several punched-out areas seen in C are filled by vimentin protein, indicating intracellular compartment (original magnification, ×1200).
Figure 3.  

A. Region of Whipple’s disease intestine (original magnification, ×600). 

B. Same region with ×2 zoom, producing an effective original magnification of ×1200. Note that the most intense bacterial rRNA signal appears between cells and is not associated with the intracellular compartment. 

C. Triple-stained section of a Whipple’s disease lymph node. Note the blue signal from Tropheryma whippelii rRNA probe (original magnification, ×400). 

D. When visualized against a background of red tissue autofluorescence, the T. whippelii rRNA probe produces long bands of signal within a Whipple’s disease lymph node (original magnification, ×600).
Figure 4. Colocalization of the broad-range and specific rRNA probes and effect of RNase treatment. A–C, Colocalization of the broad-range bacterial rRNA probe Eub-338 and the *Tropheryma whippelii* rRNA probe Tw-652. A, Bacterial rRNA probe Eub-338 labeled with fluorescein isothiocyanate (FITC; green) hybridized to spaces within the lamina propria of Whipple’s disease intestine as revealed in the FITC channel. Note some autofluorescence of the tissue. B, Binding of Cy-5–labeled *T. whippelii* probe to the same section of tissue is seen, using the Cy-5 channel. C, Superposition of images A and B shows that the broad-range bacterial probe and the Whipple bacillus–specific probe colocalized to the same areas in the tissue, as demonstrated by the blue-green color. D, Section of Whipple’s disease intestine treated with RNase no longer bound probe Tw-652 (Cy-5 channel, blue). Tissue architecture is revealed by observing autofluorescence in the FITC channel (green) and superimposing images (original magnification, ×200).
the rRNA signal appeared to be dissociated from the vimentin, which represents the intracellular compartment of human cells in the lamina propria. Vimentin is an intermediate filament protein found in cells of mesenchymal origin, such as fibroblasts, lymphocytes, and macrophages.

Numerous studies of bacteria, using FISH, have demonstrated that the level of rRNA probe hybridization correlates with the ribosome content of the bacteria. The ribosome content of bacterial cells is, in turn, related to growth rate, and ribosome numbers may change within an organism over time, depending on metabolic activity [8, 10, 11]. Actively growing cells have a higher ribosome content and bind more rRNA probe than do metabolically quiescent cells. For instance, there is an excellent correlation between rRNA probe binding in marine bacteria and uptake of radioactively labeled amino acids, as measured by autoradiography [12].

In one possible scenario suggested by these results, macrophages migrate into the lamina propria of Whipple’s disease intestine, ingest and destroy T. whippelii (including rRNA), and then migrate out of the zone of maximal bacterial growth, retaining partially digested, PAS-reactive bacterial cell wall. To quote a previous hypothesis based on ultrastructural studies [3], “in Whipple’s disease the rate of phagocytosis appears to be insufficient to cope with bacillary growth, so that the number of bacteria increases in untreated patients; the bacilli ingested by macrophages are unable to grow and are degraded” (p. 1012).

In our FISH analysis of T. whippelii-infected tissues, using an rRNA probe, individual bacteria were not visualized even when a ×100 objective was used, with an effective magnification of ×1000. Instead, a diffuse pattern of rRNA-directed DNA probe binding was noted in tissues. The failure to detect individual bacteria probably is related to the small size of the bacterium (0.2 × 2 μm). The practical limit of resolution in fluorescence microscopy is ~0.5 μm [13]. For light sources separated by a distance smaller than the limit of resolution, the images merge into one. With the longer wavelength of light emitted by the Cy-5 fluorophore, one can expect even less resolution. In addition, the close packing of bacteria in tissue sections may make resolution of individual organisms difficult because of overlapping signals.

The predominant extracellular location of the Whipple bacillus in tissues is an important clue in understanding this bacterium. The failure to propagate this microbe on routine cell-free bacteriologic medium cannot be explained by the requirement for intracellular growth. Our understanding of bacterial growth requirements clearly lags behind our ability to detect them by using molecular methods [14]. Attempts have been made to cultivate the Whipple bacillus in cell lines, on the basis of the hypothesis that this organism is an intracellular pathogen [4, 5]. Our data contradict this hypothesis. A recent report describes propagation of T. whippelii in a human fibroblast cell line [5]. Although these investigators detected bacteria or their degradation products within cultured cells by immunofluorescence staining and by electron microscopy, they did not demonstrate that T. whippelii replicates in the intracellular compartment. Regardless of how T. whippelii replicates in the laboratory, on the basis of FISH of rRNA, the habitat for metabolically active bacteria in human intestinal tissue appears to be the extracellular spaces of the lamina propria.

To be formally named, a newly identified bacterium should ideally be cultivated, to define its phenotypic characteristics and metabolic needs, thereby fulfilling requirements set forth in the International Code of Nomenclature of Bacteria [15]. Many, if not most, bacteria in the biosphere cannot be cultivated in the laboratory [16, 17]. Recognizing this problem, a new taxonomic status, designated “Candidatus,” has been implemented for prokaryotes that resist cultivation and are identified through unique nucleic acid sequences [18, 19]. One requirement for inclusion in this provisional taxonomic status is that the bacterium should be localized in its natural habitat, using in situ hybridization and a specific probe. Here we provide evidence from FISH results that the unique rRNA sequence from T. whippelii is present in infected tissues from patients with Whipple’s disease. Accordingly, we propose the formal name of Candidatus Tropheryma whippelii for the Whipple bacillus.

Koch’s postulates traditionally have been used to prove that a microbe is the cause of a disease. For cultivation-resistant microbes, such as T. whippelii, it is difficult to fulfill Koch’s postulates. We have suggested some guidelines for proving microbial disease causation, using sequence-based evidence [20]. A critical element of causal evidence using these guidelines is the ability to link, using in situ hybridization, a putative pathogen’s nucleic acid sequence to visible tissue pathology in a disease. Our FISH results show that the 16S rRNA sequence from T. whippelii is present in tissues from patients with Whipple’s disease, thereby supporting the causal role of T. whippelii in Whipple’s disease.

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References