

## Spatial Distribution of *Escherichia coli* in the Mouse Large Intestine Inferred from rRNA In Situ Hybridization

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**Fluorescent oligonucleotide probes targeting rRNA were used to develop an in situ hybridization technique by which the spatial distribution of *Escherichia coli* in the large intestines of streptomycin-treated mice was determined. Single *E. coli* cells were identified in thin frozen sections from the large intestines by the use of a probe specific for *E. coli* 23S rRNA. Furthermore, the total bacterial population was visualized with an rRNA probe targeting the domain *Bacteria*. By this technique, all *E. coli* cells were seen embedded in the mucosal material overlying the epithelial cells of the large intestine, and no direct attachment to the epithelium was observed.**

The large intestine is the most heavily colonized part of the gastrointestinal tract of mammals. At least 400 to 500 different bacterial species are thought to be present at any time in the healthy human intestinal tract, and up to  $10^{12}$  bacteria are found in every g of feces (2, 5). Most work performed on the bacterial flora of the gut has concentrated on the analysis of fecal specimens. Much less information about the flora of the cecum or that associated with the intestinal mucosa is found, and in situ investigations of the physiology of intestinal bacteria and information about spatial distribution are therefore of great interest.

Recent developments within molecular microbiology and molecular evolution have provided new perspectives and tools for studies in environmental microbiology in general. For example, sequence divergence among the rRNAs (5S, 16S, and 23S) from different species has been used to define a phylogenetic classification system of microorganisms (19). Data available from rRNA databases have been used to design specific hybridization probes for determinative studies in microbiology. Typically, total nucleic acids extracted from pure cultures or from the environment have been analyzed (18). The demonstration that fixed whole cells are permeable to oligonucleotide probes extended the studies to single-cell identification (1, 4, 6). By using fluorescence-labeled oligonucleotide probes targeting rRNA, the spatial distribution of *Escherichia coli* BJ4 in the colonized large intestines of streptomycin-treated mice was investigated. The total indigenous flora and indigenous *E. coli* cells in the large intestines of conventional mice were visualized.

*E. coli* BJ4, isolated from a healthy Wistar rat (9), was used for colonizing the large intestines of streptomycin-treated mice (11). A streptomycin-resistant derivative of *E. coli* BJ4 was found to colonize the gut at levels of  $10^8$  to  $10^9$  CFU/g of feces. In cecal contents, the same level of  $10^9$  CFU/g of contents was observed, while in cecal mucus the number of organisms was an order of magnitude lower, i.e.,  $10^8$  CFU/ml of mucus. A

probe specific to *E. coli* 23S rRNA (EC 1531; 5'-CACCGT AGTGCCTCGTCATCA-3') was designed for in situ hybridization of bacteria in the mouse gut. The specificity of the probe was tested by use of the CHECK-PROBE program, the Ribosomal Database Project, University of Illinois (10). The probe was labeled with fluorochromes by synthesizing the oligonucleotide with a 5' aminolinker (Aminolink 2; Applied Biosystems, Foster City, Calif.) which was subsequently used as a coupling substrate for lissamine rhodamine B sulfonyl (Molecular Probes, Eugene, Oreg.), TRITC (tetramethyl rhodamine isothiocyanate) (Molecular Probes), or CY3 dye (cyanine dye CY3.29-OSu; Biological Detection Systems, Pittsburg, Pa.). A second probe, EUB338 (17), specific to the domain *Bacteria* was used to visualize the total bacterial population of the intestine. EUB338 was labeled with fluorescein at the 5' end during the synthesis of the oligonucleotide with fluorescein-CEP (Peninsula Laboratories, Inc., Belmont, Calif.). Both labeled probes were purified by reverse-phase chromatography (9). Different fluorochromes were assessed for labeling of the *E. coli* probe in order to obtain the lowest background binding to the tissue. Using hydrophobic fluorochromes such as TRITC and, to some extent, lissamine rhodamine B resulted in a high level of unspecific binding of the probe to hydrophobic compartments of the epithelial cells. Therefore, the hydrophilic fluorochrome CY3 was tested, and the result was a significantly higher signal-to-noise ratio. In order to overcome the inherent fluorescence of epithelial cells and material trapped in the mucosal layer, we used narrow-band filters for the emission.

An Axioplan epifluorescence microscope (Carl Zeiss) was used to visualize the hybridizations. The microscope was equipped with a 100-W mercury lamp and filter sets 1, 10, 14 (Carl Zeiss), and XF40 (Omega Optical, Brattleboro, Vt.) to visualize DAPI (4',6-diamidino-2-phenylindole), fluorescein, TRITC and CY3, and lissamine rhodamine B, respectively. In combination with filter set 14 a narrow-band bypass filter, 590/10 nm (Oriol Corporation, Stratford, Conn.), was used, while in combination with filter set 10 two narrow-band bypass filters, 530/10 nm (Oriol Corporation) and 546/12 nm (DELTA Light and Optics, Lyngby, Denmark), were used. In all cases, a 63×/1.25 Plan Neofluar Ph3 oil immersion objective (Carl

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Zeiss) was used. A slow-scan charged coupled device (CCD) camera was used for capturing digitalized images. The CCD camera was a CH250 camera (Photometrics, Tucson, Ariz.) equipped with a KAF 1400 chip (pixel size, 6.8 by 6.8  $\mu\text{m}$ ). The camera was operated at  $-40^\circ\text{C}$ . The integration times for the CCD camera were 500 ms and 4 to 6 s without and with the narrow-band filters, respectively. Image analysis was done in 12 bits with the PMIS software version 1.5 (Photometrics) or converted to 8-bit files to be analyzed by Gene Join (release 7) (13) or Adobe Photoshop version 2.5 (Adobe Systems Inc., Mountain View, Calif.). A DOS-based 486 computer was used as a controller for the CCD camera, and a Macintosh Quadra 950 was used to run the Macintosh software. Prior to visualization, the hybridized samples were mounted in emission oil except for those of hybridizations with fluorescein-labeled probes, which were mounted in Citifluor (Citifluor Ltd., London, United Kingdom).

Tissue specimens from the mouse large and small intestines (approximately 3 mm long) were cut, placed on filter paper, embedded in tissue glue (Tissue-Tek, OCT compound; Milles Inc.), and frozen immediately by being embedded in 2-methylbutan (Merck) cooled in liquid nitrogen. Frozen tissue was stored at  $-80^\circ\text{C}$  until it was sectioned on a Leitz 1720 cryostat. The sections (10  $\mu\text{m}$  thick) were placed on six-well Teflon-coated slides (Novakemi, Enskbede, Sweden) that had been gelatin treated (1). Tissue sections were then fixed in 3% paraformaldehyde for 30 min and washed three times in phosphate-buffered saline (pH 7.4). The sections on slides were air dried and stored at  $4^\circ\text{C}$  until they were stained with Alcian blue PAS and Meyers hematoxylin or used for in situ rRNA hybridization. Bacterial cell smears from the ceca of colonized mice were also fixed on Teflon-coated slides (1). Hybridizations were carried out with 10  $\mu\text{l}$  of hybridization solution (35% formamide, 100 mM Tris [pH 7.5], 0.1% sodium dodecyl sulfate [SDS], 0.9 M NaCl, 25 ng of probe), and the samples were kept in a humidified chamber for 16 h at  $37^\circ\text{C}$ . Thereafter, the slides were rinsed in water and washed in 100 ml of prewarmed ( $37^\circ\text{C}$ ) washing solution I (35% formamide, 100 mM Tris [pH 7.5], 0.1% SDS, 0.9 M NaCl) for 15 min and subsequently for 15 min at  $37^\circ\text{C}$  in 100 ml of prewarmed washing solution II (100 mM Tris [pH 7.5], 0.9 M NaCl). The slides were then rinsed quickly in distilled water and air dried.

When stained with DAPI (Sigma), the slides were rinsed in water, washed in washing solution I for 15 min at  $37^\circ\text{C}$ , and then transferred to 100 ml of 100 mM Tris (pH 7.5)–0.9 M NaCl–0.625 g of DAPI for 5 min at  $21^\circ\text{C}$ . Subsequently, the slides were transferred to 100 ml of washing solution II for 15 min at  $37^\circ\text{C}$  and finally rinsed with distilled water.

Two conventional mice (untreated mice) and two streptomycin-treated mice unchallenged with *E. coli* BJ4 were used as controls. No *E. coli* organisms were detected in thin sections from the intestines of unchallenged streptomycin-treated mice and extremely few organisms were detected in the conventional mice by in situ hybridization. The experiments were repeated twice.

Ten streptomycin-treated mice challenged with *E. coli* BJ4 were used for determination of the spatial distribution of the bacteria in the gut. Two mice were sacrificed at days 1, 3, 5, 7, and 10 after challenge. Specimens from the small and large intestines were collected, frozen in liquid  $\text{N}_2$ , and used for thin sectioning. Counts of viable *E. coli* BJ4 organisms were determined for feces, cecal contents, and cecal mucus from each mouse.

*E. coli* BJ4 was not found in the small intestines of the mice by in situ hybridization. Only a few very faintly fluorescing cells could be detected 24 h postfeeding in the lumen of the small

intestine by in situ hybridization. These cells are believed to be residual cells from the original inoculum given per os to the mice. The *E. coli* BJ4 strain was easily detected on sections from the large intestines of the colonized mice. The bacterial cells were embedded in the mucosal material and were never observed to bind to the intestinal epithelium (Fig. 1A and B).

The spatial distribution of *E. coli* BJ4 relative to the total intestinal bacterial population was determined by the use of a second probe, targeting the entire bacterial domain. By labeling the species-specific probe with one fluorochrome (red emission) and the general bacterial probe with a second fluorochrome (green emission), the probes could be used simultaneously. Upon hybridization, three monochrome images were captured, one for each probe, which were subsequently pseudocolored and superimposed (red and green in Fig. 1), and a third image (blue in Fig. 1) captured by phase-contrast microscopy to visualize the epithelial cells; the epithelial cells could not be seen by epifluorescence microscopy because of the use of narrow-band filters. This image was then merged onto the previously superimposed images. The mucus layer was observed by phase-contrast microscopy but not visualized in Fig. 1. Thus, we were able to visualize simultaneously the epithelial cells of the intestine and the spatial distribution of the total intestinal bacterial population as well as the localization of *E. coli* BJ4. Figure 1 shows in situ hybridizations of *E. coli* BJ4 as well as the total bacterial population in thin sections of the large intestines of streptomycin-treated mice at days 1 (Fig. 1A) and 10 (Fig. 1B) postchallenge. The relative abundance of *E. coli* BJ4 was clearly reduced during the first 10 days of colonization.

In addition, the total microbial population was simultaneously stained with DAPI and probed for *E. coli* BJ4. By this method, *E. coli* BJ4 was estimated to constitute 16% of the total microbial population of the cecal contents at day 1 and to slowly decrease to about 4% by day 10. It is likely that a period of approximately 1 week is required before the intestinal microenvironment is stabilized and the introduced strain is established without being outcompeted by the residual flora. Colonization experiments supported these results (data not shown). Furthermore, during this time, the bacterial cells changed in shape to coccoid cells, as previously described (9).

The spatial distribution of naturally occurring *E. coli* in the large intestine was also determined. The rRNA probe used in this work specifically targets all *E. coli* strains and was therefore used for in situ hybridization of thin sections of the large intestines of conventional mice. Figure 1C shows the results of such a hybridization experiment. Naturally occurring *E. coli* cells were also found in the mucosa and not attached to the epithelial cells. Indigenous *E. coli* cells were present in very low numbers in the conventional mice, since only a couple of cells were observed on each of the prepared sections from the intestines of the conventional mice in comparison with the 50 to 80 times more *E. coli* cells observed in similar sections obtained from colonized mice. In support of this observation, the feces of conventional mice contained *E. coli* at levels of  $10^5$  to  $10^6$  CFU/g of feces (data not shown), while streptomycin-treated mice were colonized with  $10^8$  to  $10^9$  CFU of *E. coli* BJ4 per g of feces. Furthermore, in early histological studies of the gastrointestinal flora of adult mice, only infrequently were coliforms observed in the cecum (3). It is worth noting, in Fig. 1C, that in the large intestines from the conventional mice fusiform bacteria are in abundance, but they are eliminated when streptomycin is administered to the mice, as previously described (12, 16).

It has previously been reported that indigenous microorganisms adhere to the membrane of intestinal epithelial cells via

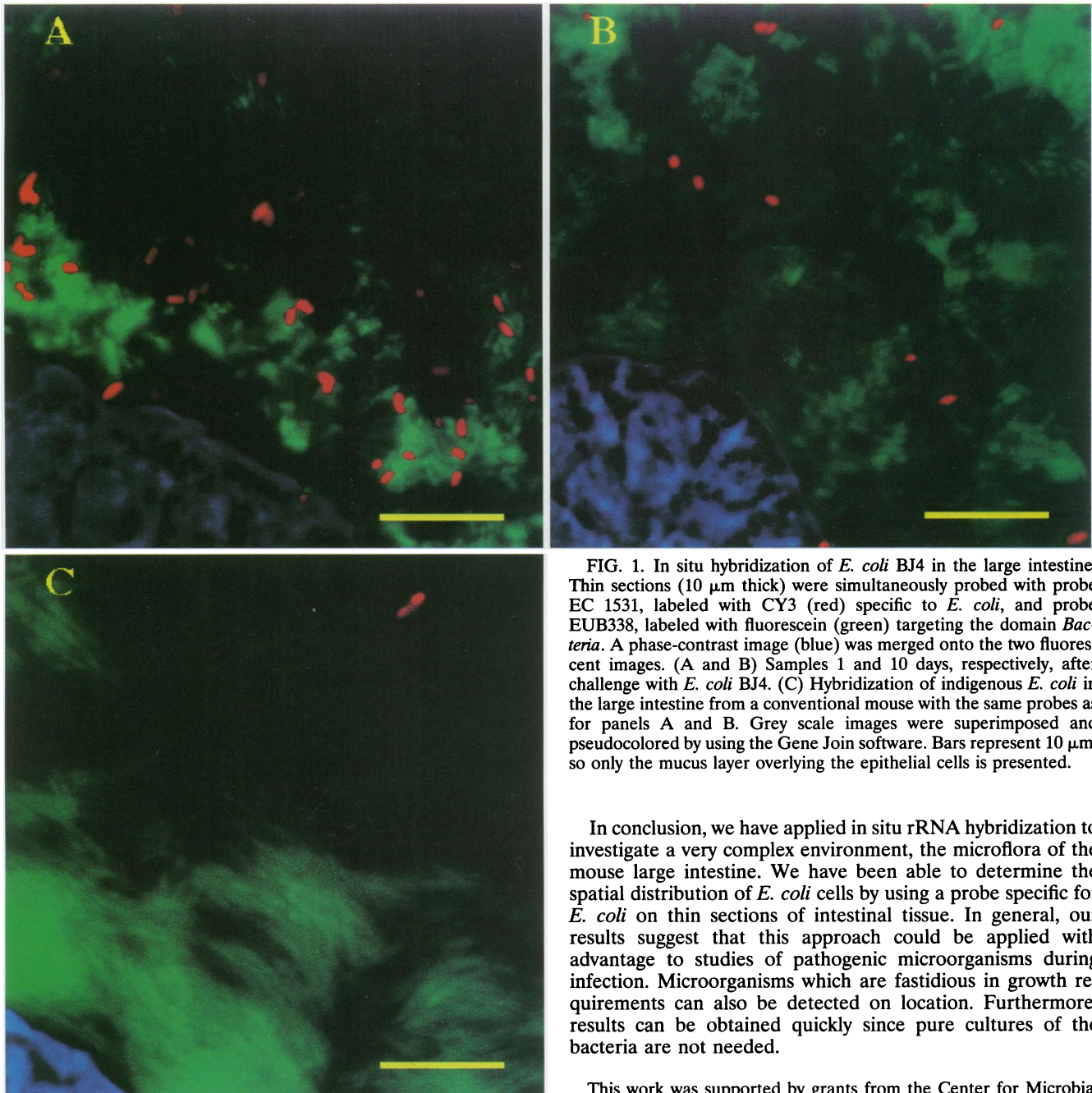


FIG. 1. In situ hybridization of *E. coli* BJ4 in the large intestine. Thin sections (10  $\mu\text{m}$  thick) were simultaneously probed with probe EC 1531, labeled with CY3 (red) specific to *E. coli*, and probe EUB338, labeled with fluorescein (green) targeting the domain *Bacteria*. A phase-contrast image (blue) was merged onto the two fluorescent images. (A and B) Samples 1 and 10 days, respectively, after challenge with *E. coli* BJ4. (C) Hybridization of indigenous *E. coli* in the large intestine from a conventional mouse with the same probes as for panels A and B. Grey scale images were superimposed and pseudocolored by using the Gene Join software. Bars represent 10  $\mu\text{m}$ , so only the mucus layer overlying the epithelial cells is presented.

long thin filaments, i.e., fimbriae, to avoid being swept away down the lumen (7, 14). Despite the fact that *E. coli* BJ4 possesses type 1 fimbriae that are produced in vivo (8, 9), *E. coli* BJ4 cells do not attach to the epithelial cell surface of the large intestine (Fig. 1A and B). The same is also true for the indigenous *E. coli* cells in the intestine of a conventional mouse (Fig. 1C).

Localization of microorganisms in the host organs is usually performed by classical histological and immunohistological techniques, which are restricted to the surface characteristics of the bacteria known only from cells grown under laboratory conditions. Even when specific antibodies are available for in situ studies, the thick mucus layer above the epithelial cells tends to block the antibodies. Moreover, extensive washing to remove the mucus layer displaces the microorganisms (15).

In conclusion, we have applied in situ rRNA hybridization to investigate a very complex environment, the microflora of the mouse large intestine. We have been able to determine the spatial distribution of *E. coli* cells by using a probe specific for *E. coli* on thin sections of intestinal tissue. In general, our results suggest that this approach could be applied with advantage to studies of pathogenic microorganisms during infection. Microorganisms which are fastidious in growth requirements can also be detected on location. Furthermore, results can be obtained quickly since pure cultures of the bacteria are not needed.

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