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Applications of Ribosomal *in situ* Hybridization for the Study of Bacterial Cells in the Mouse Intestine

Abstract

Localization of *E. coli* and *S. typhimurium* in the large and small intestine of streptomycin-treated mice was visualized by *in situ* hybridization with specific rRNA target probes and epi-fluorescence microscopy. Growth rates of *E. coli* BJ4 colonizing the large intestine of streptomycin treated mice were estimated by quantitative hybridization. The ribosomal contents were measured in bacteria isolated from cecal mucus, cecal contents and feces and correlated with the ribosomal contents of bacteria growing in vitro with defined rates. The data suggest that *E. coli* BJ4 grows with an apparent doubling time of 40–80 minutes in the intestinal mucosa.

Introduction

The large intestine is the most heavily colonized part of the gastrointestinal tract of mammals. At least 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 gram of feces. In this complex ecosystem the microorganisms co-exist in a fine balance. They must grow slowly enough not to overgrow the host, but fast enough not to be flushed out by the host's intestinal activities, e.g. peristaltic movements, fluid flow etc. (Borriello, 1986; Finegold *et al.*, 1983).

Most work performed on the bacterial flora of the gut has concentrated on the analysis of fecal specimens. Much less information is found about the flora of the cecum or that associated with the intestinal mucosa. *In situ* investigations of the growth physiology of intestinal bacteria are therefore of great interest. In the past, calculations of the rates of bacterial proliferation in the intestine have been based on average estimates at the level of populations. For example, the growth rate of *E. coli* in the mouse intestine has been estimated *in vivo* using radioisotope techniques, dilution by growth of a non-replicating genetic marker and simply by counting the number of viable cells (Meynell, 1959, Hiram *et al.*, 1971, Eudy and Burros, 1973, Gibbons and Kapsimalis, 1967, Freter *et al.*, 1983). Using these techniques, generation times ranging from 30 minutes to 40 hours of *E. coli* have been estimated. These systems, however, do not reflect the

physiological conditions in the gut, where entrapment of the bacteria in the mucus gel plays an important role. Furthermore, in the intestine, the bacterial cell morphology, protein profiles and growth physiology have been described to differ from what is observed during growth in laboratory media (Krogfelt *et al.*, 1993; Panigrahi *et al.*, 1992).

Bacterial growth rates can be estimated from the cellular RNA concentrations, since RNA content is dependent on the growth rate (Schaechter *et al.*, 1958; Neidhart and Magasanik, 1960; Kjelgaard and Kurland, 1963). Recently developed methods based on hybridization to whole cells with fluorophore-labelled oligonucleotide probe targeting the ribosomal RNA (rRNA) and epi-fluorescence microscopy coupled to digital image analysis allow estimations of concentration of rRNA in single cells. The ribosomal contents of the bacteria isolated from the environment can then be compared to the ribosomal contents of bacteria growing with defined rates. (DeLong *et al.*, 1989; Poulsen *et al.*, 1993). In this study, the total indigenous flora, as well as introduced streptomycin resistant strains of *E. coli* and *S. typhimurium*, were visualized and the apparent growth of *E. coli* BJ4 in the large intestine of streptomycin treated mice was estimated (Poulsen *et al.*, 1995).

In situ hybridization: The method

Sectioning and fixation

Tissue specimens from the mouse large and small intestine were prepared either by embedding in tissue glue and freezing in liquid nitrogen for cryostat sections, or by embedding in paraffin for microtome sections. Cryostate sections were fixed in 3% paraformaldehyde immediately after cryostat cutting. Tissue for microtome sections was fixed in formalin prior to embedding.

The sections on slides were air dried and stored at 4°C until they were either stained with Alcian blue PAS and Meyers Hematoxylin, or used for *in situ* rRNA-hybridization.

Bacterial cells growing in laboratory cultures and bacterial cell smears from the ceca of colonized mice were fixed in 3% paraformaldehyde. Fixed cells were stored in storage buffer (Poulsen *et al.*, 1994). Hybridizations were carried out as described by Poulsen 1994. Briefly, the speciments on slides were hybridized at 37°C in a hybridization solution containing various concentrations of formamide, depending on the melting point of the probe and on the desired specificity. The slides were then washed twice in washing solutions with decreasing specificity, rinsed quickly in distilled water and air dried.

Probes

Specific probes to *E. coli* 23S rRNA (Poulsen *et al.*, 1994) and *S. typhimurium* (Poulsen, unpublished) were designed in order to perform *in situ* hybridization

of bacteria in the mouse gut. The specificity of the probes was tested by use of the CHECK-PROBE program (Larsen *et al.*, 1993).

Probe EUB338 (Stahl *et al.*, 1991), specific to the Eubacterial domain was used to visualize the total bacterial population in the intestine of streptomycin treated mice. The probes were labeled with various fluorocromes, i.e. fluorescein, Lissamin Rhodamine B or CY3 as previously described (Poulsen *et al.*, 1994).

Different fluorochromes were assessed for labeling the probes in order to obtain the lowest background binding to the tissue. The use of hydrophobic fluorochromes sometimes resulted in high nonspecific binding of the probe to hydrophobic compartments of the mouse epithelial cells. When the hydrophilic fluorochromes were tested, a higher signal to noise ratio was obtained. In order to overcome the inherent fluorescence of epithelial cells and material trapped in the mucosal layer, we used narrow band by-pass filters for the emission.

Microscopy and Image analysis

The hybridizations were visualized as described in detail by Poulsen *et al.*, 1994. An Axioplan epi-fluorescence microscope was equipped with filter sets and narrow band by-pass filters depending on the exitation and emision wavelengths of the fluorocromes.

A slow scan CCD (Charged Coupled Device) camera was used for capturing digitalized images. The camera was operated at −40C. The integration time for the CCD camera varied between 500 msec and 3–6 sec depending on the intensity of the hybridized probe and on the use of narrow band by pass filters.

Image analysis was performed in order to determine the amount of fluorescence per bacterial cell volume. This ratio corresponds to the ribosomal concentration in individual bacterial cells. The applied software has previously been described (Poulsen *et al.*, 1994).

In order not to bleach the fluorocromes prior to capturing images, cells were counter stained with DAPI (4′,6′-DiAmidino-2′-PhenylIndole) which was used for focusing the camera. At least 3 different images of 200–400 cells at each growth rate were quantified. The standard deviations of these measurements were 8–25%

The streptomycin treated mouse model

Treatment of conventional mice with streptomycin-containing drinking water removes the indigenous facultative flora, and thereby allows orally introduced streptomycin resistant strains of *S. typhimurium* SL5319 (Franklin *et al.*, 1990) or *E. coli* (Krogfelt *et al.*, 1993) to colonize.

This animal model has been studied in great detail (Franklin *et al.*, 1990; Krivan *et al.*, 1992; Myhal *et al.*, 1982 and Wadolkowsky *et al.*, 1988), and was therefore chosen for the present work.

Visualization of specific bacterial strains in the intestine

As an example of the application of the hybridization method to *S. typhimurium* SL5319, in figure 1 we show a hybridized section of the ileum of a mouse colonized with this strain. Panels A and B show the section stained with Mayers Hematoxylin and Alcian blue. In Panel C one single bacterial cell caught by the

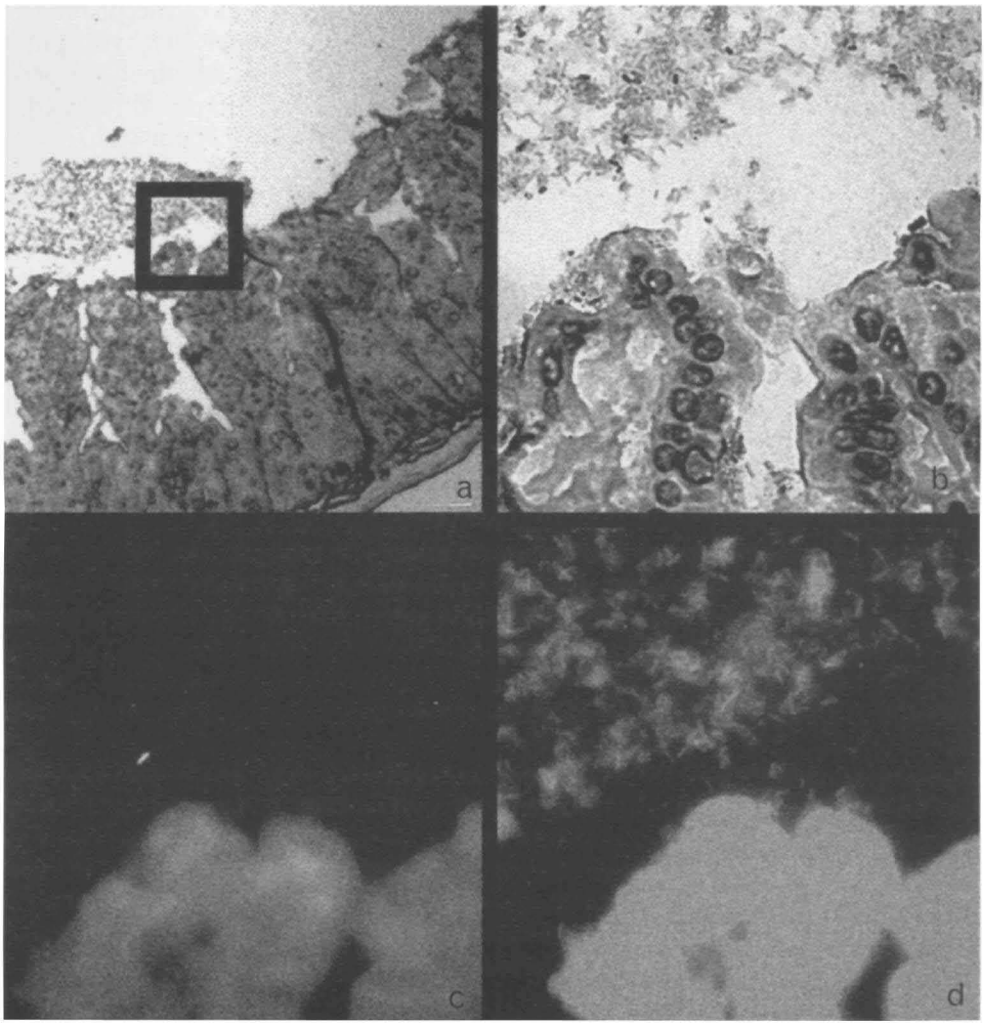

Fig. 1. 5 μm thin microtome section of the ileum of a mouse colonized with *Salmonella typhimurium* SL5319. In panels A) and B) are shown the staining of the section with Meyers heamatoxylin and Alcian blue, enlarged 100X and 630X respectively. The simultaneous probing of the same section with a probe specific for *Salmonella* (C) and a probe targeting the eubacterial domain (D) are also shown.

Salmonella specific probe is visualized, while in panel D bacteria responding to the universal probe, targeting all the eubacteria, are shown.

In this experiment, *S. typhimurium* colonized the mouse cecum at levels of 10^8 to 10^{10} CFU per gram, while the concentration of CFU found in the ileum was four orders of magnitude lower.

E. coli BJ4 was found to colonize the gut at levels of 10^8 to 10^9 CFU per gram feces. In cecal contents the same levels of 10^9 CFU per gram were observed, while in cecal mucus the concentration of CFU was an order of magnitude lower, i.e. 10^8 CFU per gram of mucus (Poulsen *et al.*, 1995).

E. coli BJ4 (Krogfelt *et al.*, 1993) was easily detected on sections from the large intestine of the colonized mice. The bacterial cells were embedded in the mucosal material and were never observed to bind to the intestinal epithelium (Poulsen *et al.*, 1994)

Unchallenged streptomycin-treated mice were used as controls. No *E. coli* or *S. typhimurium cells* were detected in sections from their intestines.

Determination of ribosomal cell content of bacteria growing *in vitro* and *in vivo*, and correlation between growth rates and ribosomal concentrations

In vitro measurements upon growth in defined laboratory media.

Pure cultures were grown in minimal media with different carbon sources, resulting in a series of different generation times. Growth rates were measured by following the optical density at 450 nm. The ribosomal contents of *E. coli* BJ4 cells growing under different conditions in various media supporting various growth rates were determined by whole cell rRNA hybridization.

A linear correlation between bacterial growth rate and ribosomal concentration was observed (Poulsen *et al.*, 1995).

In vivo measurements upon growth in the mouse intestine.

Growth rates of *E. coli* BJ4 were estimated in samples from feces, cecal contents, and cecal mucus taken from mice colonized with this strain. The samples were fixed, spread in a monolayer on a hybridization slide, and hybridized as described above. Digital images were captured for image analysis and the mean signal intensity measured. By use of a linear standard curve obtained from cultures grown in defined laboratory media, the mean signal intensities could be converted into apparent growth rates.

The initial stages of bacterial colonization were investigated by administering a low amount of bacteria to the mice. Two mice were sacrificed at 1, 3, 5, 24, 40, 96 and 120 hours after challenge and mucus samples were taken for CFU determinations and hybridization. *E. coli* BJ4 colonization of the intestinal mucus increased rapidly as shown in Figure 2A. Intensity measurements followed by hybridization showed ribosomal contents corresponding to genera-

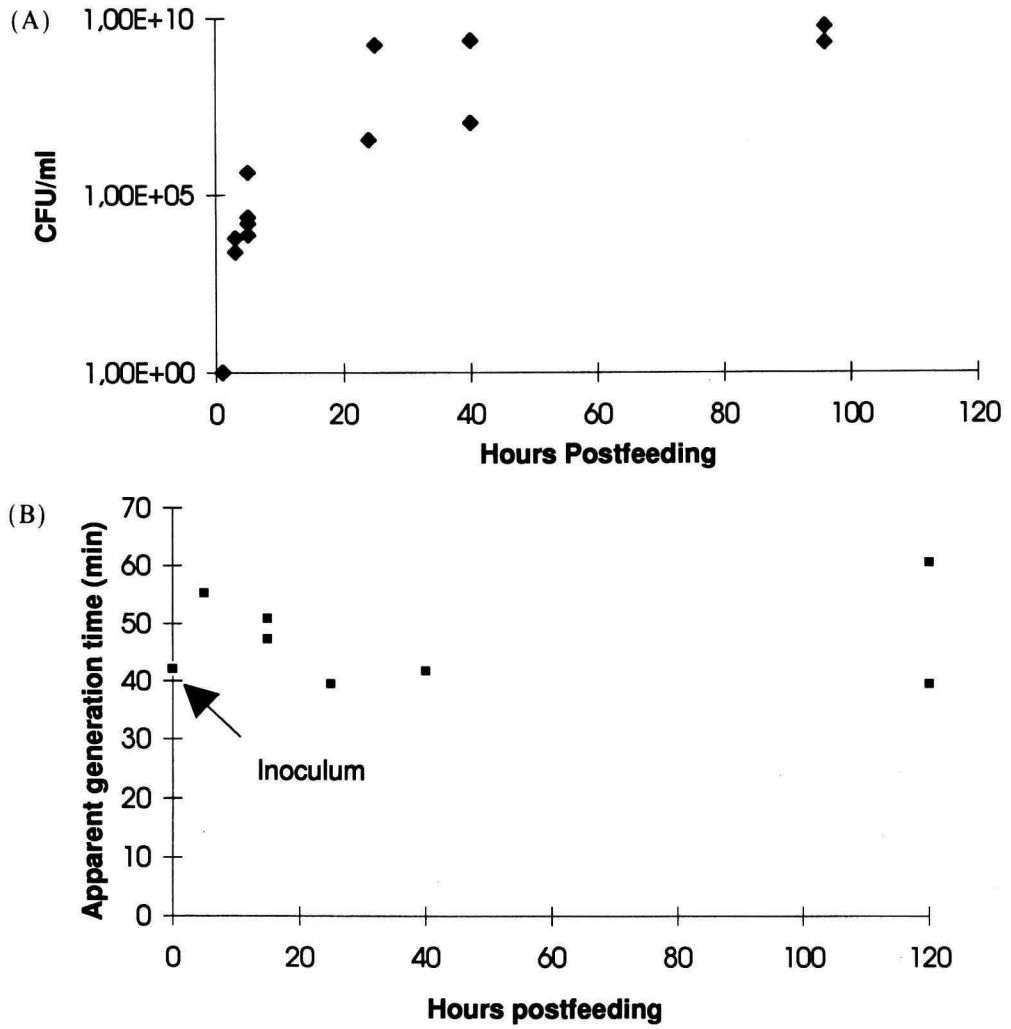

Fig. 2. Colonization of the mouse large intestine by *E. coli* BJ4 during the first 120 hours post inoculation. A) *E. coli* BJ4 CFU/ml in mucus. B) Generation times in cecal mucus inferred by ribosomal hybridization.

tion times of 40–80 minutes for all samples taken between 1 and 120 hours after challenge (Fig.–2B). The apparent generation times of 40–80 minutes remained constant throughout the 20 days investigated (not shown). Colony forming units of *E. coli* BJ4 remained constant throughout the experiment at the levels of 10^9 and 10^8 CFU/ml in cecal contents and cecal mucus respectively, and 10^9 CFU/g in the fecal samples.

Cecal mucus and cecal contents from two streptomycin treated unchallenged mice were hybridized with the *E. coli* specific probe. No hybridization was observed, thereby confirming the specificity of the probe used.

Discussion

Studies on the growth of bacterial cells have usually been performed with pure cultures growing in well defined liquid laboratory media under well controlled conditions. In natural environments bacterial growth is influenced by a number of factors that are often unknown. The complexity of the natural ecosystems makes it difficult to mimic the conditions *in vitro*. Therefore, it is important to develop methods by which bacterial growth physiology can be studied *in situ*.

The method of *in situ* rRNA hybridization in whole fixed cells has proven very useful in several complex environmental contexts for specific identification of single bacterial species. Moreover, quantification of the hybridization signal may be achived by the aid of digitilized video cameras (CCD-cameras) and fluorescence microscopy. Through such measurements ribosomal concentrations in bacteria can be determined (Poulsen *et al.*, 1993).

In the present study, we have applied rRNA hybridization to detect the spatial distribution of bacterial cells in the small and large intestines of mice. Furthermore, by use of this method we have measured the cellular ribosomal concentration and thereby estimated the bacterial growth rates in the large intestine.

In a series of growth experiments supporting growth rates of *E. coli* BJ4 betweeen 30 and 180 min. followed by rRNA hybridizations, a linear relationship between growth rate and ribosomal contents was observed. Having this standard curve, *E. coli* BJ4 cells isolated from the cecal mucus of mice colonized for 20 days with this strain showed a rRNA content corresponding to a generation time of 40–80 minutes. Hybridizations on *E. coli* BJ4 isolated from cecal contents and feces showed approximately the same rRNA content (intensity/bacterial volume) as cells isolated from mucus, indicating the same doubling times. However, we observed that *E. coli* BJ4 did not grow in fecal pellets after excretion during a period of 24 hours at room temperature (data not shown), and it is also unlikely that *E. coli* grows in the intestinal contents (Wadolkowski *et al.*, 1988). We therefore assume that the high ribosome content observed in the *E. coli* BJ4 cells isolated from cecal contents and fecal pellets reflects that the bacteria have been growing rapidly while associated with the mucosal layer, and that the ribosomes needed to maintain this high growth rate stay intact in cecal contents and feces (Poulsen *et al.*, 1995).

That fast growth does indeed occur in the mucosal layer is supported by the increase in CFU counts observed during the initial stages of colonization (figure 2A). Even if factors such as washout, mucosal turnover and release of bacteria from the mucosal layer are not taken into consideration, the bacteria must grow with a generation time of at least 80 minutes to achieve the increase in CFU's observed in the period between 3 and 24 hours after challenge.

Later, when the number of CFU in the intestine stays at a constant high level, the low bacterial generation times in mucus estimated by *in situ* hybridization are supported further by the following considerations:

Since the total mucosal population stays constant, the mean stay of bacteria in mucus must be equal to the mean growth rate of the mucosal population. The

hybridization data suggest an average generation time of about one hour, and thus the bacteria must stay associated with the mucosal layer for a similar period. If we assume that no bacterial growth occurs neither in luminal contents, nor in feces, the minimum size of the population in mucus needed to produce the 10^9 BJ4 cells counted in faeces from 24 hours is $10^9/24 = 4*10^7$ CFU. This correlates well with the actual plate counts showing a total cecal mucosal BJ4 population of about 10^7 CFU (Poulsen *et al.*, 1995).

Thus, the generation time of 40–80 min for *E. coli* BJ4 in the mucus of the large intestine of mice estimated by *in situ* hybridization is in agreeement with the counted colony forming units, if we assume that growth mainly takes place in mucus.

In summary, it is clear that *in situ* rRNA hybridization can be applied in the complex environment of the intestine, and reveal useful information about bacterial distribution. In addition, the concentration of ribosomal RNA in a given bacterial cell as measured by *in situ* hybridization can provide further information about the physiological state of the cell.

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