In Vivo Detection and Quantification of Tetracycline by Use of a Whole-Cell Biosensor in the Rat Intestine

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An Escherichia coli biosensor strain, harboring the plasmid pTGFP2, was introduced into the gastrointestinal tract of gnotobiotic rats that continuously received drinking water containing tetracycline. Plasmid pTGFP2 contains a transcriptional fusion between a green fluorescent protein (GFP) gene and a tetracycline-regulated promoter and was shown to produce a proportional GFP signal in response to exposure to various tetracycline concentrations when harbored by an E. coli strain. The plasmid was highly unstable in the host bacteria colonizing the intestinal system of the animals, and rapid plasmid loss was observed. Reintroduction of the E. coli MC4100/pTGFP2 strain into animals already colonized by the plasmid-free E. coli strain the day before euthanasia made it possible to extract and analyze the biosensors from intestinal samples. The induction of GFP in the biosensor cells extracted from the animals was estimated on a single-cell basis by use of flow cytometry, and the mean induction of GFP in the samples was compared to a standard curve prepared from known tetracycline concentrations. The results showed that the bioavailable tetracycline concentration within the bacterial growth habitat of the intestine was proportional to the concentration of tetracycline in drinking water but represented only approximately 0.4% of the intake concentration. This is a significant finding which will help to clarify antimicrobial therapy in the intestinal environment.

Since their discovery in the 1940s, tetracyclines have been very useful antibiotics for the treatment of a whole range of bacterial infections in humans and animals and have also seen widespread use as growth promoters in animal husbandry (5). However, the emergence and characterization of some 36 different genes (17) conveying tetracycline or oxytetracycline resistance to the host bacteria by a number of different mechanisms have raised some concerns regarding the future usefulness of the tetracyclines. It is believed that subinhibitory concentrations of tetracycline have a positive selective effect on the dissemination and accumulation of tetracycline resistance genes in bacteria colonizing the gastrointestinal tract of mammals. This belief is based partly on molecular evidence, which indicates the tetracycline-induced transfer of mobile elements containing tetracycline resistance determinants (4), and partly on the observed increase in prevalence of various resistance determinants after therapeutic or prophylactic treatment with the drug (3, 9). To investigate this effect further it is important to be able to determine the actual in situ bioavailable concentration of the antibiotic in the microhabitats hosting bacterial activity. The traditional methods for measuring in situ antibiotic concentrations are based on an extraction of the compound from its natural matrix, followed by analysis by either chromatographic methods, such as high-pressure liquid chromatography (19), microbiological methods, such as bacterial inhibition assays (15), or in vitro biosensor assays (10, 12). However, the results of these methods are to some extent biased due to the extraction and homogenization steps involved and do not necessarily correspond to the actual localized bioavailable concentration to which the bacteria are exposed in situ. The recent advances in the use of bacterial biosensors in combination with flow cytometry (FCM) have made single-cell analysis possible and have thereby strengthened the spatial-resolution properties of the biosensor approach (11).

In the present study a bacterial biosensor strain, incorporating a transcriptional fusion between a tetracycline-inducible promoter and the green fluorescent protein (GFP) gene (11), was introduced into the gastrointestinal tract of tetracycline-treated rats, and samples were subsequently analyzed for GFP expression on the single-cell level by FCM. The results indicate a significant difference between the intake concentration of tetracycline and the bioavailable drug concentration in the intestinal microhabitats hosting bacterial growth.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial biosensor strain used in this study was Escherichia coli MC4100 harboring the plasmid pTGFP2, which contains a transcriptional fusion between an FCM-optimized gfp gene (mut3b) (7), encoding a stable variant of the GFP (1), and the tetracycline-regulated promoter Pr tet, pTGFP2 also carries the bla gene conveying ampicillin resistance (11). For growth in liquid culture, brain heart infusion broth (Oxoid) or Luria-Bertani (LB) broth (Merck) was used (18). Selective plating of fecal samples and intestinal contents was done on either brain heart infusion agar (Oxoid) supplemented with 100 μg of ampicillin/ml, which was selective for MC4100/pTGFP2, or MacConkey agar no. 3 (Oxoid), which was selective for all E. coli MC4100 cells.

Grouping of animals and dosing with tetracycline. Twelve female, germfree Sprague-Dawley rats, approximately 2 months old and bred at the Institute of Food Safety and Nutrition, were originally obtained from IFFA Credo, Domaine des Oncins, L’Arbresle, France. The housing, feed, temperature, and light conditions were as previously described (13). The germfree state of the animals was verified by testing fecal samples for aerobic and anaerobic growth of bacteria and yeast cells. The 12 rats were caged individually and placed in four groups with...
three animals in each group. Starting from day 6, each group received drinking water containing tetracycline (tetracycline hydrochloride; Sigma product no. T3383) in the following various concentrations: group A, 0.0 µg/ml; group B, 5 µg/ml; group C, 10 µg/ml; and group D, 50 µg/ml. Fresh drinking water was prepared every 2 or 3 days and kept in the dark at all times. The tetracycline concentration in the drinking water was tested and confirmed on three separate days during the experiment (days 11, 20, and 27) by using the E. coli MC4100/pTGFP2 biosensor strain (data not shown).

Colonization of the animals. An overnight culture of the E. coli MC4100/pTGFP2 strain was washed twice in autoclaved 0.9% (wt/vol) saline water containing peptone, and 1 ml (approximately 10^7 CFU) was given to each rat by oral gavage on day 1 and on the day before euthanasia. In addition, two different Enterococcus faecalis strains were introduced into all animals (approximately 10^9 CFU), the first (strain OG1RF) on day 0 and the second (strain OG1SS::Tn916) on day 7. Tr916 confers resistance to tetracycline in the host cell. The results obtained by using these strains were reported previously (2).

Sampling and enumeration of bacteria in samples. Fresh fecal samples were obtained directly from the rats every 2 to 3 days. After the animals were euthanized (six on day 40 and six on day 41), samples were immediately taken from the contents of the stomach, jejunum, ileum, cecum, and colon. All samples were initially diluted 10-fold (wt/vol) in saline water containing peptone, thoroughly homogenized, further diluted, and plated on appropriate selective agar plates for CFU counting.

Flow cytometry. The flow cytometer used for the analysis was a FACScalibur (Becton Dickinson ICS, San Jose, Calif.) equipped with an argon ion laser (488-nm wavelength) capable of GFP excitation, which allowed subsequent detection of the green fluorescent cells in the FL1 channel. The voltages were set at 350 V for side scatter and 600 V for detector FL1, and the E01 setting was used for forward scatter (10 times multiplication of the signal). A threshold of 170 was set on the FL1 detector in order to eliminate nonfluorescent cells. All data analysis was performed with Cellquest software (BD Biosciences). An elliptical gate was defined in a bivariate dot plot around the cells of bacterial size fluorescence as determined by side scatter and the FL1 detector (Fig. 1). The mean fluorescence of each sample was defined as the mean of the fluorescence detected per single cell within the region bounded by the cell gate. The relative induction of GFP was estimated by dividing the mean fluorescence detected in each sample by the mean fluorescence detected in a sample without tetracycline, i.e., the background fluorescence.

Standard curve. A standard curve was established in order to correlate the biosensor response to the ambient bioavailable tetracycline concentration. An overnight culture of E. coli MC4100/pTGFP2 was diluted 100-fold in 5 ml of LB broth containing various concentrations of tetracycline (three replicates) and incubated overnight at 37°C on a shaker (200 rpm). The samples were then diluted 200-fold in phosphate-buffered saline and analyzed by FCM as described above.

Bioavailable tetracycline in the gastrointestinal tract. Intestinal samples for FCM were prepared from 10-fold dilutions of intestinal contents from the colon and cecum on the day of euthanasia. The samples were vortexed and allowed to settle for approximately 30 min. The supernatant was then filtered through a sterile 30-µm-pore-size filter to remove large particles. The appropriate dilutions were analyzed by FCM as described above.

Bioavailable tetracycline in sterile feces. A setup was prepared to correlate the bioavailable tetracycline in rat feces with the actual concentration of tetracycline added to the sample. Tetracycline was added to 10-fold dilutions (wt/vol) of sterile feces to achieve eight different concentrations in three replicates (total volume in each dilution, 1 ml). Subsequently, 20 µl of a washed exponential-phase culture of MC4100/pTGFP2 (optical density at 600 nm, 0.5) was added to these samples. After overnight incubation at 37°C on a shaker (200 rpm), the samples were vortexed and allowed to settle for approximately 30 min. The supernatant was then filtered through a sterile 30-µm-pore-size filter, diluted 200-fold, and analyzed by FCM as described above, but with slightly different voltage and threshold settings.

RESULTS

Colonization. The E. coli MC4100/pTGFP2 biosensor strain readily colonized the gut of the germfree rats, and approximately 10^9 CFU/g of feces was detected in all of the animals the day after inoculation (Fig. 2 and data not shown). The total number of E. coli MC4100 cells detected in fecal samples, as counted on MacConkey agar plates, remained constant throughout the experiment; however, a gradual decline in the number of biosensor cells (MC4100 cells harboring the pTGFP2 plasmid) was observed after inoculation, and after 34 days no biosensor cells were detected (limit of detection, 500 CFU/g of feces) in fecal samples from group A (rats receiving 0 µg of tetracycline/ml). The time period in which the biosensor was detectable in fecal samples decreased with increasing tetracycline concentrations in the drinking water (data not shown). The reintroduction of E. coli MC4100/pTGFP2 the day before euthanasia resulted in an intestinal E. coli population consisting of between 1 and 25% plasmid-harboring cells in samples from five segments of the gastrointestinal tract (Fig. 3). The variation in the E. coli population in the cecum and colon segments was somewhat less, and between 2 and 8% of the total E. coli MC4100 population consisted of MC4100/pTGFP2 cells.

Standard curve. The relative induction of GFP increased with increasing tetracycline concentrations (Fig. 4a), and at tetracycline concentrations between 0.05 and 0.2 µg/ml, a lin-
ear correlation between drug concentration and relative mean induction of GFP was observed. Tetracycline concentrations below 0.02 μg/ml did not induce a detectable response of the *E. coli* MC4100/pTGFP2 biosensor strain.

**Bioavailable tetracycline in the gastrointestinal tract.** The FCM data obtained from the samples retrieved from the cecum and colon segments of the intestinal tract were very similar, and consequently a mean value for the three replicates in

![Graph](https://example.com/graph.png)

**FIG. 2.** CFU of biosensor *E. coli* MC4100/pTGFP2 (closed squares) and *E. coli* MC4100 (open squares) in fecal samples from rats not receiving tetracycline in drinking water. Each point represents a geometric average of values obtained for three animals. Error bars designate standard errors of the means.

![Graph](https://example.com/graph2.png)

**FIG. 3.** CFU of biosensor *E. coli* MC4100/pTGFP2 (closed squares) and *E. coli* MC4100 (open squares) in fecal samples from rats receiving either 0.0 (a), 5 (b), 10 (c), or 50 (d) μg of tetracycline/ml of drinking water. Samples were taken from five segments of the gastrointestinal tract immediately after euthanasia. Each point represents a geometric average of values obtained for three animals. Error bars designate standard errors of the means.
both segments was used for further analysis. The mean fluorescence levels observed for samples from groups B (rats receiving 5 \( \mu \)g of tetracycline/ml), C (10 \( \mu \)g of tetracycline/ml), and D (50 \( \mu \)g of tetracycline/ml) were significantly higher than the fluorescence for samples from group A (0 \( \mu \)g of tetracycline/ml), which was used as the background level in this experimental setup. The relative mean fluorescence values for samples from groups B, C, and D were 1.2, 1.6, and 7.6, respectively.

**DISCUSSION**

After the initial colonization of the *E. coli* biosensor strain MC4100/pTGFP2 in the intestinal systems of the rats, high plasmid instability was observed for all four groups of animals (Fig. 2 and data not shown). This observation is in line with the general belief that high-copy-number cloned plasmids, such as pTGFP2, convey a selective disadvantage to the host cell when no selection for the plasmid is present (6). In this case, tetracycline increases the selective disadvantage by inducing GFP synthesis, which requires increased energy. If all plasmids are lost from one *E. coli* cell through segregational and structural instability, or if the inoculum contains plasmid-free MC4100 cells, these cells will inevitably be in close proximity to other isogenic, plasmid-harboring cells in the dynamic gastrointestinal environment. The in situ growth rates of the competing cell lines in the same microhabitat will determine the ratio between the two cell types and ultimately lead to loss of the type with the slowest in situ growth rate, i.e., the plasmid-harboring bacteria. The observed rapid plasmid loss from the intestinal *E. coli* population indicated a highly competitive intestinal environment.

The day after the reinoculation of the biosensor strain *E. coli* MC4100/pTGFP2 into the *E. coli* MC4100-colonized animals, the CFU counts for samples obtained from five segments of the gastrointestinal tract showed an approximately constant ratio throughout the gastrointestinal system (Fig. 3). In samples from the cecum and colon seg-

![Graph](http://aac.asm.org/)  
**FIG. 4.** Relative mean fluorescence values estimated by FCM analysis of biosensor *E. coli* MC4100/pTGFP2 cells exposed to various concentrations of tetracycline in LB broth (a) and estimated bioavailable tetracycline concentrations in sterile diluted fecal samples supplemented with various tetracycline concentrations (b). Each point represents an average of three values. Error bars designate standard errors of the means. The line in panel b represents a linear regression of the obtained values \( R^2 = 0.979 \).

![Graph](http://aac.asm.org/)  
**FIG. 5.** Estimated bioavailable tetracycline concentrations, within the bacterial growth habitat of the intestine, in animals receiving various tetracycline concentrations in drinking water. Each point represents an average value for samples extracted from the cecum and colon segments of three animals (two animals for the concentration of 10 \( \mu \)g/ml). Error bars designate standard errors of the means. The line represents a linear regression of the obtained values \( R^2 = 0.999 \).
ments, between 2 and 8% of the E. coli MC4100 population carried the pTGFP2 plasmid. These findings indicate the ability of the biosensor strain to coexist with an isogenic strain in the intestine.

Increasing concentrations of tetracycline in drinking water caused an increase in the mean fluorescence of the E. coli MC4100/pTGFP2 biosensor bacteria extracted from the cecum and colon segments of the gut. This finding showed that the concentration of tetracycline encountered by active biosensor bacteria in these segments was proportional to the concentration of tetracycline in the drinking water. Pilot experiments indicated that drinking water containing 1 μg of tetracycline/ml or less did not induce a significant biosensor response in the rats and consequently did not affect the intestinal microbial population (data not shown). This finding is in line with that of other investigations showing that the minimum selective concentration of tetracycline in drinking water is between 1 and 10 μg/ml (8, 15). However, it is still possible that tetracycline concentrations below 1 μg/ml could have a long-term effect on the microbial population.

In order to correlate the observed induction of the biosensor with the actual bioavailable concentration of tetracycline in the bacterial growth habitat, a number of issues must be addressed. It has been suggested (16) that there is a lateral movement of intestinal bacteria, produced by growth in the mucus layer, into the luminal contents followed by excretion in feces. In connection with this lateral movement, it is plausible that the biosensor bacteria encounter various tetracycline concentrations; however, this is only translated into a GFP response if the cells are active and if protein synthesis occurs. Two factors point towards low bacterial activity in the luminal contents: first, previous studies of E. coli bacteria have shown that the major bacterial growth compartment in the gut is within the mucus layer and that little growth takes place in the luminal contents (14, 16, 21). Second, in a parallel study, inhibitory bioavailable tetracycline concentrations were measured in extracts from the cecum and colon segments and in fecal samples from groups B, C, and D (2). The measured concentrations represented between 6 and 10% of the administered drug concentration in drinking water in the cecum and colon segments and between 13 and 34% in the fecal samples. These ranges are similar to those measured in other investigations of fecal samples from tetracycline-treated mice (8, 15) and cause inhibition of growth of protein synthesis in the E. coli biosensor cells, especially in group D. Furthermore, the FCM data showed a well-defined biosensor population with respect to green fluorescence, indicating that the cells did not respond to a different concentration of tetracycline when shed to the luminal contents (Fig. 1). This finding suggests a fast transition from the growth habitat in the mucus layer to the luminal contents. We propose that (i) the biosensor bacteria respond to the relatively low concentration of bioavailable tetracycline within the mucus layer, which constitutes a partially tetracycline-protective environment, and (ii) the biosensor bacteria do not respond to higher concentrations when shed into the luminal contents, due to the lack of protein synthesis. Consequently, it was possible to estimate the actual bioavailable tetracycline concentrations in the fraction of the gastrointestinal tract which hosted the growth of the biosensor bacteria by comparing the relative mean fluorescence values obtained from the animal experiment with the standard curve (Fig. 4a). The bioavailable tetracycline concentration within the bacterial growth habitat of the intestine was proportional to the concentration of tetracycline in the drinking water and represented approximately 0.4% of the intake concentration of the drug (Fig. 5). This is significantly less than the proportion of bioavailable tetracycline in sterile diluted fecal samples supplemented with tetracycline (Fig. 4b) and explains the ability of the sensitive E. coli cells to proliferate in the intestinal environment, even when tetracycline concentrations in drinking water, as well as in luminal contents, far exceeded inhibitory concentrations. The rapid plasmid loss observed in the intestinal system (Fig. 2) could reduce the plasmid copy number in some biosensor bacteria and cause a slight underestimation of the tetracycline concentration due to lower gfp expression. However, the experience gained in our laboratories with the biosensor strain shows that gfp expression is not very sensitive to the plasmid copy number. This view is supported by a recent study of the effect of plasmid copy number on protein expression levels (20).

The results of that study showed that the two isogenic E. faecalis strains were able to coexist in the intestines of rats receiving up to 50 μg of tetracycline/ml of drinking water, which is well above the inhibitory concentration for the sensitive strain and suggests the existence of tetracycline-depleted microhabitats in the intestinal environment. These findings further indicate that the in situ bioavailable tetracycline concentrations were much lower than the intake concentration.

This is, to our knowledge, the first time that a bacterial biosensor strain has been used to quantify in vivo tetracycline concentrations directly in the bacterial growth compartments of the gastrointestinal tract. Because the induction levels of the biosensor bacteria were almost identical in intestinal samples from the cecum and colon segments, and no bacterial activity was present in the luminal contents, it seems probable that analysis of tetracycline in excreted feces would reveal similar results. This would further allow real-time detection and quantification of the in situ bioavailable tetracycline contents in the intestinal environment. The results obtained from this study and in future work will contribute importantly to our understanding of antimicrobial therapy in the intestinal environment.

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