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Detection of Oxytetracycline Production by *Streptomyces rimosus* in Soil Microcosms by Combining Whole-Cell Biosensors and Flow Cytometry

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Combining the high specificity of bacterial biosensors and the resolution power of fluorescence-activated cell sorting (FACS) provided qualitative detection of oxytetracycline production by *Streptomyces rimosus* in soil microcosms. A plasmid containing a transcriptional fusion between the *tetR*-regulated P_{tet} promoter from Tn*10* and a FACS-optimized *gfp* gene was constructed. When harbored by *Escherichia coli*, this plasmid produces large amounts of green fluorescent protein (GFP) in the presence of tetracycline. This tetracycline biosensor was used to detect the production of oxytetracycline by *S. rimosus* introduced into sterile soil. The tetracycline-induced GFP-producing biosensors were detected by FACS analysis, enabling the detection of oxytetracycline encounters by single biosensor cells. This approach can be used to study interactions between antibiotic producers and their target organisms in soil.

The use of bacterial biosensors, i.e., bacteria giving an easily measurable response upon exposure to a specific compound or environmental condition, is a promising new approach in environmental biology. Their use has, however, until now been limited to measurements in more homogeneous samples such as bulk water (14, 17, 18) and soil extracts (10). Soil is a complex matrix of microhabitats conferring highly variable growth conditions for the microbiota (15). The ability to gain an understanding of soil microbial ecology and microbial processes has been severely hampered by the inability to characterize these microhabitats at a scale or resolution relevant to microbial cells. Due to their high specificity, sensitivity, and appropriate scale, whole-cell biosensors offer an approach that could deal with these issues of spatial resolution.

A major advantage of using biosensor bacteria to detect or quantify specific compounds in natural samples is that only the fraction available to the bacteria, the bioavailable fraction, is detected. Hence, the measurements made are relevant to the effects of compounds on the microbial community.

An interesting application of bacterial biosensors is the detection of bioavailable antibiotics in different environments (7). Detection of antibiotics resulting from anthropogenic or natural production is crucial for our understanding of the evolution of antibiotic resistance.

Whether antibiotics are produced in soil by indigenous soil organisms has been a scientific dispute for several decades (5, 19). Most antibiotics are excreted as secondary metabolites when the producers are grown in rich media. It is not evident that conditions in natural soil would allow the type of growth required for producing and excreting the antibiotic. The growth of potential antibiotic producers such as streptomycetes in soil is thought to be localized in discrete areas, rather than evenly distributed throughout the soil (19). Therefore, the production and presence of antibiotics are likely to be limited to a few microhabitats where conditions are favorable. This makes the detection of indigenous antibiotic production by conventional methods difficult.

Streptomyces rimosus is a known industrial producer of oxytetracycline and was originally isolated from soil (4). Indeed, most known microbial producers of the different tetracyclines are bacteria native to soil. Actinomycetes are usually present in large numbers in soil, and they constitute about 10% of the culturable microbial population, exceeding 1 million CFU/g of soil (5). Furthermore, large numbers of tetracycline resistance determinants are often found in soil samples (2, 13, 16). This has led to speculations that the tetracycline resistance genes are present in soil because tetracyclines are produced there. However, the production of tetracyclines in soil has previously never been shown by direct detection, due to the lack of detection methods with the necessary specificity and resolution power.

Most studies, aiming to examine antibiotic production in soil, have employed extraction of the antibiotic from soil prior to analysis (19). However, this method does not take the spatial distribution as well as the bioavailability of the compounds into account. If antibiotics are produced only in small amounts in a few local microhabitas, this amount will be highly diluted during extraction. This dilution could completely mask the presence of the compound, resulting in false-negative results.

We present here the construction of a biosensor bacterium which is induced to express green fluorescent protein (GFP) upon exposure to tetracycline. These experiments used soil microcosms where the oxytetracycline producer *S. rimosus* was inoculated with the biosensor. The aim of this research is to use biosensors that are distributed in the soil at high density to detect highly localized production of oxytetracycline. To detect the potentially small subpopulation of biosensor cells express-

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TABLE 1. Strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli DH5α	Auxotroph; *thi-1 argF*	6
E. coli MC4100	Prototroph	12
S. rimosus	Oxytetracycline producer	Liz Wellington (a gift)
Plasmids		
pJBA27	Ap^r $P_{A1\text{-}0403}$-*gfp*mut3	9
pUT-*tetgfp*	Km^r Ap^r P_{tet}-*atpE-gfp*	7
pTGFP1	Ap^r P_{tet}-*gfp*mut3	This study
pTGFP2	Ap^r P_{tet}-*atpE-gfp*mut3	This study

ing GFP, the bacteria were extracted from the soil and analyzed using flow cytometry. Flow cytometry enables the detection of low numbers of fluorescing cells against a high background of nonfluorescent cells. To confirm the flow cytometry results, induced biosensor bacteria were isolated using fluorescence-activated cell sorting (FACS) and examined by epifluorescence microscopy. In this study, we combine the high specificity of bacterial biosensors and the resolution power of FACS analysis. We demonstrate this method's potential for addressing environmental problems that were hitherto difficult or impossible to analyze.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are shown in Table 1.

Media and culture conditions. Both *Escherichia coli* strains used were grown in a modified Luria-Bertani broth (11) containing only 4 g of NaCl per liter (LB4). LB4 with 15 g of agar per liter was used for plate counts. *S. rimosus* was continuously maintained on Trypticase Soy broth (TSB) (DSMZ medium 545) plates. *S. rimosus* spores were pregerminated in pregermination buffer (8). Antibiotics used were ampicillin and tetracycline, added to culture media to final concentrations of 100 and 10 μg/ml, respectively. Oxytetracycline at various concentrations was added to media when induction of P_{tet} was examined. All recombinant DNA manipulations were carried out by standard methods (11), except where otherwise stated. All enzymes used were purchased from Boehringer, Mannheim, Germany, and used according to the manufacturer's guidelines. Antibiotics were purchased from Sigma.

Cloning of tetracycline biosensors. Plasmids pTGFP1 and pTGFP2, which both contain transcriptional fusions between the tetracycline promoter P_{tet} and a FACS-optimized *gfp* gene from pJBA27 (9), were constructed using PCR. The tetracycline repressor *tetR* (a negative regulator of P_{tet} [1]) and P_{tet} were amplified from pUT*tetgfp* (7) using three different primers. Primer set 1 (used to construct pTGFP1) consisted of the primers 5′-AAAAGAATTCGCTGCTTTAAGACCCAC-3′ and 5′-CTATGCATGCCACTTTTCTCTATCACTG-3′, giving a 732-bp fragment containing *tetR* and P_{tet}, as well as an *Eco*RI site upstream and an *Sph*I site downstream of this regulatory region. Following digestion with *Eco*RI and *Sph*I, the PCR product was directionally ligated to *Eco*RI- and *Sph*I-digested pJBA27 using T4 DNA ligase. The ligation mix was then transformed into competent DH5α cells (11), and transformants were screened for tetracycline-inducible GFP production in an epifluorescence microscope (model Axioskop 2; Carl Zeiss, Sydney, Australia). We thereby replaced the already existing promoter region in pJBA27 ($P_{A1\text{-}04/03}$) with the *tetR*-regulated promoter P_{tet}. In this plasmid, the ATG codon that would normally (in Tn*10*) be the start codon in the *tetA* gene (encoding a tetracycline resistance efflux pump) was now the start codon of the FACS-optimized *gfp* gene.

Primer set 2 (used to construct pTGFP2) consisted of the same forward primer as in primer set 1 and 5′-CCTTTACGCATGCTGAGTCTCCAG-3′. Amplification from pUT*tetgfp* with these primers gave a 928-bp fragment that included not only *tetR* and P_{tet} but also the highly efficient *atpE* transcriptional initiation region from plasmid pUT*tetgfp*. This PCR fragment was also digested with *Eco*RI and *Sph*I, ligated into pJBA27, transformed into DH5α, and screened for tetracycline-inducible GFP production. Plasmid pTGFP2 is shown in Fig. 1. After verification by electrophoresis, the plasmids were transformed into *E. coli* strain MC4100. The recombinant strains were then examined for their GFP levels in response to oxytetracycline. Aliquots of 50 μl from exponentially growing cultures of *E. coli* MC4100 harboring either pTGFP1 or pTGFP2 were inoculated into test tubes containing 5 ml of LB4 with different concentrations of oxytetracycline. Three parallel samples were incubated for each concentration. Cultures were incubated at 30°C with shaking for 16 h. After incubation, 0.5 ml of culture from each concentration was centrifuged (6,000 × *g*, 2 min), washed once and resuspended in 3 ml of 0.9% NaCl, and transferred to cuvettes. Fluorescence (from GFP) was measured in a Perkin-Elmer LS50 luminescence spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, England). The excitation wavelength used was 488 nm, and the emission wavelength was 511 nm, both with a slit width of 2.5 nm. Optical densities of cultures were measured at 600 nm (OD_{600}) in an Ultrospec 2000 Spectrophotometer (Pharmacia Biotech, Cambridge, United Kingdom). Relative fluorescence units per OD_{600} was then plotted against the oxytetracycline concentration (Fig. 2). The performance of *E. coli* strains containing pUT*tetgfp*, pTGFP1, and pTGFP2 was tested for applicability in flow cytometry after induction of the strains with 50 ng of oxytetracycline per ml (see below).

Microcosm experiment. Samples of 2.8 g of dried soil from Sturt National Park, far northwestern New South Wales, Australia (a gift from Andrew J. Holmes, Macquarie University, Sydney, Australia), including two barley leaves (1.5 by 0.5 cm), were distributed into 15-ml polypropylene conical Falcon tubes (Becton Dickinson, North Ryde, New South Wales, Australia). This soil was chosen due to its high content of 16S ribosomal DNA sequences related to actinomycetes (data not shown). The barley leaves were intended for microscopic examination following the incubation. The microcosms were then sterilized by exposing the tubes to a dose of 8,000 Gy from a gamma source (Macquarie University cobalt-60 gamma source). From an overnight culture of biosensor *E. coli* MC4100/pTGFP2, 0.5 ml was reinoculated into 50 ml of LB4 medium. Cells were grown until OD_{600} reached 0.8. Thereafter, the biosensor cells were washed twice in phosphate-buffered saline (PBS) and resuspended in 20 ml of PBS. An aliquot (350 μl) of this suspension was then added to each tube (1.7×10^7 CFU as tested on LB4 plates containing ampicillin). Spores of *S. rimosus* were harvested from 6-day-old TSB plates, by adding 10 ml of sterile double-distilled H_2O and scraping the spore layer of the colonies with a sterile inoculation loop. The spore suspension was collected and filtered twice through a syringe filled with sterile, nonabsorbent cotton wool (8). After filtration, the spores were centrifuged and resuspended in 10 ml of sterile double-distilled H_2O. Following incubation of the spores at 50°C for 10 min, an equal volume of 2× pregermination buffer (8) was added and the spores were incubated at 37°C with shaking for 3 h. The spores were then washed twice in PBS and resuspended in 10 ml of PBS (to a final concentration of 3.2×10^5 CFU/550 μl as tested on TSB plates containing tetracycline). Nine soil microcosms were inoculated with 550 μl of undiluted

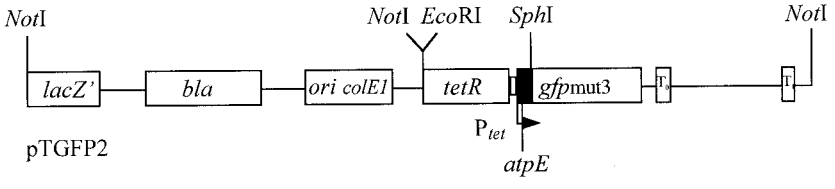

FIG. 1. Composition of pTGFP2. A928-bp *Eco*RI-*Sph*I fragment encompassing *tetR*, P_{tet}, and the *E. coli atpE* translation initiation region (in black), all obtained by PCR, was inserted into pJBA27. The *bla* gene encodes β-lactamase conferring resistance to ampicillin. *ori colE1*, origin of replication originating from pUC18-*Not*I; *lacZ′*, partially deleted *lacZ* gene; *gfp*mut3, FACS-optimized *gfp* gene encoding GFP. The two T's are transcriptional terminator sequences from pJBA27. P_{tet} is the *tet* promoter and *tetR* encodes the *tet* repressor protein, both originating from Tn*10*.

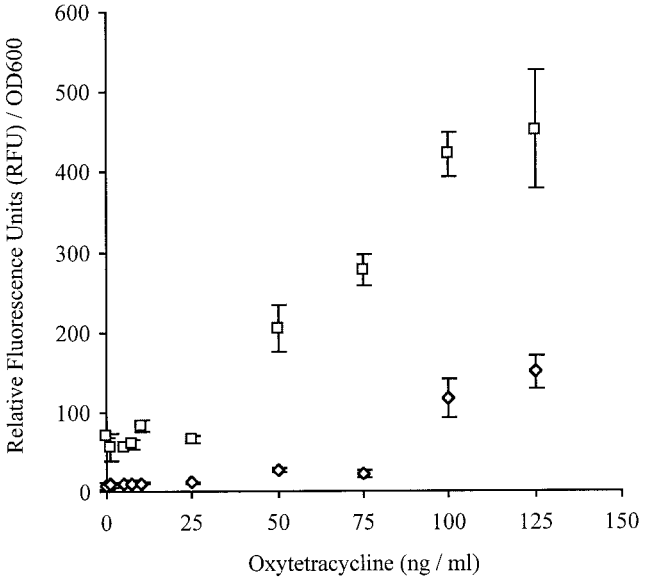

FIG. 2. GFP in cultures of MC4100 harboring pTGFP1 or pTGFP2. Strains were grown overnight in LB4 containing increasing concentrations of oxytetracycline. Diamonds represent MC4100/pTGFP1, and squares represent MC4100/pTGFP2. Vertical bars show the standard deviations ($n = 3$).

spore suspension (series A, 8.6×10^4 spores/g of wet soil), nine soil microcosms were inoculated with 550 μl of a 10^{-2} dilution of spores (series B, 8.6×10^2 spores/g of wet soil), nine soil microcosms were inoculated with 550 μl of a 10^4 dilution of spores (series C, 8.6 spores/g of wet soil), and nine control microcosms were supplemented with 550 μl of PBS (series D, no spores). Thus, all the microcosms contained 900 μl of liquid. In order to verify that the *S. rimosus* spore inoculum did not contain any oxytetracycline, exponentially growing cells of *E. coli* MC4100/pTGFP2 were inoculated with a dilution series of sonicated (5 min in a 250/450 Sonifier [Branson Ultrasonics Corp., Danbury, Conn.]) and sterile-filtered *S. rimosus* inoculum and shaken at 37°C for 16 h. A tube containing *E. coli* MC4100/pTGFP2 plus PBS and a tube containing MC4100/pTGFP2 plus 50 ng of oxytetracycline per ml were included. The cultures were washed twice in PBS, and fluorescence was determined using an LS 50B luminescence spectrometer (Perkin-Elmer). A scan of light emission from 495 to 525 nm was recorded when samples were excited at 488 nm. The FACS-optimized GFP has an emission peak at 511 nm.

Microcosms were incubated at room temperature, and triplicate samples were taken at given times. At each sampling, 10 ml of PBS was added to each microcosm sampled, the tubes were vortexed for 1 min, and the soil slurry was allowed to settle for 1 h to avoid background fluorescence from soil particles. Subsamples (2 ml) of the supernatant were filtered through a 38-μm-pore-size stainless steel mesh (using Swinnex filter holders [Millipore]; 13-mm diameter). A dilution series was made in PBS for plate counts of both *S. rimosus* (on TSB containing tetracycline) and *E. coli* MT4100/pTGFP2 (on LB4 containing ampicillin). The undiluted filtrate was analyzed using a Becton Dickinson FACScalibur flow cytometer (Becton Dickinson, North Ryde, New South Wales, Australia) to detect and enumerate biosensor bacteria which had been exposed to oxytetracycline (see below).

Flow cytometry. Flow cytometry analyses were performed using a FACScalibur flow cytometer equipped with an argon ion laser (488 nm) capable of GFP excitation. Voltages were set at 380 V for side scatter (SSC), 600 V for detector FL1, and 650 V for detectors FL2 and FL3 (fluorescence detectors). Sheath fluid consisted of undiluted Osmosol (Lab Aids Pty Ltd., Narrabeen, New South Wales, Australia). Fluorebrite beads (6 μm; Polysciences, Warrington, Pa.) were run to check instrument performance before analysis of samples. This setup was used to test pure cultures.

For analysis of soil samples, the instrument was set up for environmental analysis (AusFlow protocol) by swapping FL1 and FL2 detectors. Compensation was set at FL2 − %FL1 = 99.9% and FL2 − %FL3 = 99.9% to reduce natural autofluorescence found in environmental samples. Samples were analysed in triplicate for every condition, and the instrument was decontaminated by running 10% (wt/vol) sodium hypochlorite solution followed by distilled water between each sample. Positive control samples consisted of soil slurries (extracted as described above) with biosensor bacterium *E. coli* MC4100/ pTGFP2, which had been induced with 50 ng of oxytetracycline per ml prior to mixing with the soil. These samples were analyzed first, and the threshold was set, using the green fluorescence detector (FL2), to just above the majority of the background of fluorescent particles (Fig. 3).

A polygonal gate (R1) was defined around the population of positive control bacteria on a bivariate dot plot of log SSC versus log forward scatter (Fig. 3). A second dot plot of log SSC versus log green fluorescence was then analyzed by gating on R1, and an ellipse region (R2) was defined around the bacteria expressing GFP. Negative samples without fluorescent cells were analyzed to determine the level of background fluorescence occurring within R2.

Sample filtrates from microcosms (100 μl) were added to 100 μl of PBS in 5-ml Falcon tubes (BD Biosciences, San Jose, Calif.) and were vortexed prior to FACS

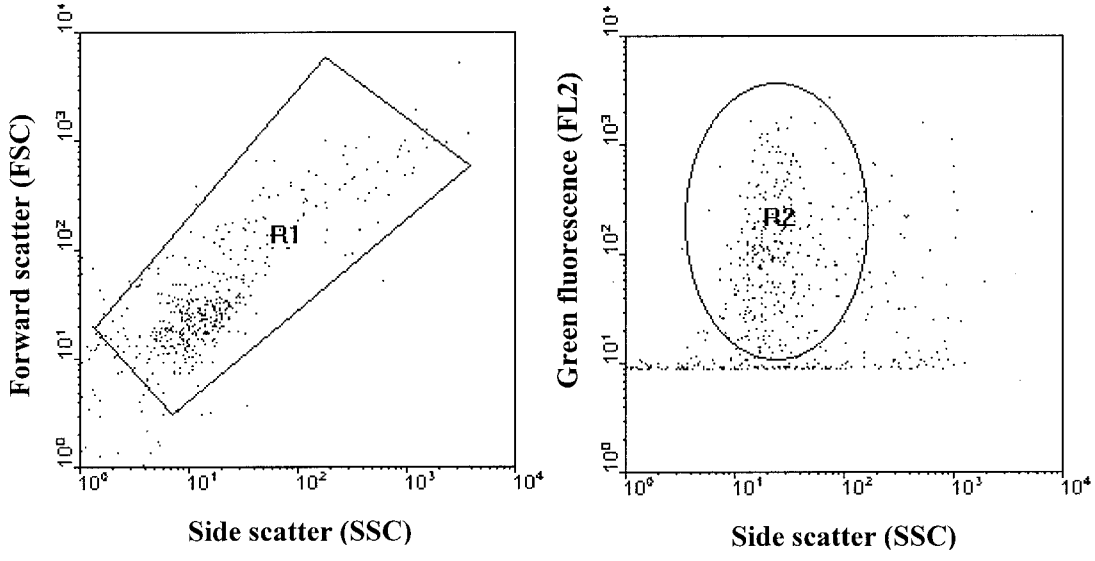

FIG. 3. Flow cytometric analysis of *E. coli* MC4100/pTGFP2 cells (induced with 50 ng of oxytetracycline per ml) within soil. Region 1 defines where bacteria lie according to size (left). The second dot plot (right) is gated on bacteria (region 1 [R1]). Region 2 (R2) defines the population of GFP-expressing bacteria.

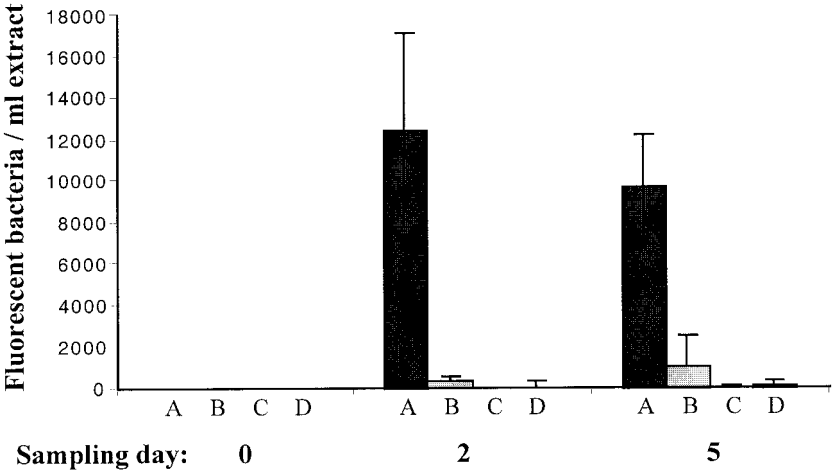

FIG. 4. Enumeration of induced biosensor bacteria (*E. coli* MC4100/pTGFP2). Soil microcosms were extracted with 10 ml of PBS, sedimented for 1 h, and filtered through a 38-μm-pore-Size mesh. Soil extracts were then analyzed on a FACScalibur flow cytometer, and only bacteria lying in the R2 region (shown and defined in Fig. 3) are counted as positive. Values and standard deviations in the negative area are not shown. Series A, B, C, and D were initially inoculated with 8.6×10^4, 8.6×10^2, 8.6, and 0 spores per g of wet soil, respectively.

analysis. Samples were analyzed on medium flow rate for 3 min (equivalent to 40 μl of filtrate sample). All events satisfying threshold requirements were collected and saved into data files. All data analyses were carried out using Cellquest software (BD Biosciences). The software WinMDI obtained from Joseph Trotter (Salk Institute for Biological Studies, La Jolla, Calif.) was used for graphic presentation of data. Verification of the GFP-expressing population (Fig. 3) (R2) was carried out using FACS of GFP-induced cultures. A FACScalibur flow cytometer modified for environmental analysis was used to select target cells, allowing the confirmation of these by epifluorescence microscopy (Axioskop 2; Carl Zeiss. Confirmation of GFP-expressing bacteria was carried out using ×40 and ×100 (oil immersion) objectives. Excitation of GFP was through a 100-W mercury vapor arc lamp with a filter block that excited GFP between 450 and 490 nm, allowing visualization at 520 nm.

Statistics. Cell numbers found on different days were compared by the use of Student's *t* test. Probabilities of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Biosensor construction. Detection of tetracycline in soil by FACS analysis was in this study achieved by the construction of a bacterial biosensor which contained the GFP gene under the regulation of the tetracycline-responsive promoter P_{tet}.

An existing tetracycline-induced GFP-producing biosensor construct (7) did not produce sufficient fluorescence for detection in a FACS sorter (data not shown). The GFP produced from this construct has an optimal excitation wavelength of 395 nm compared to the FACS, which excites using a 488-nm laser beam. It was especially inadequate when soil samples were analyzed, since the Sturt National Park soil used in this experiment contained many weakly fluorescent particles giving forward scatter and SSC signals in the same region as the *E. coli* MC4100 cells (data not shown).

Two new biosensor cassettes were therefore cloned using a FACS-optimized *gfp* gene (*gfp*mut3) fused to the *tet* repressor gene *tetR* and the *tet* promoter P_{tet}. GFP produced from this gene has a 21-fold increase in fluorescence intensity when excited at 488 nm compared with the wild-type GFP (3). This makes it more suitable for FACS analysis. Two versions of this biosensor construct were made. Plasmid pTGFP1 contains *tetR*-$P_{tet,}$ the "natural" translation initiation region from the *tetA* gene, fused to the FACS-optimized *gfp* gene. Plasmid pTGFP2 (Fig. 1) contained, in addition to the components of pTGFP1, the highly efficient translation initiation region of the *E. coli atpE* gene.

The fluorescence produced from pTGFP1 was much lower than the fluorescence from pTGFP2 (Fig. 2). This was true both for basal-level GFP expression (at 0 μg of oxytetracycline per ml) and at higher concentrations. *E. coli* MC4100 containing either plasmid showed an increase of fluorescence in response to increased oxytetracycline concentrations. Both cultures induced with 50 ng of oxytetracycline per ml were then tested in the FACS sorter. At this oxytetracycline concentration, clear induction was seen in *E. coli* MC4100/pTGFP2 (Fig. 2). The two induced cultures were added to the soil which was to be used in the microcosm experiments, extracted with PBS, filtered, and run through the FACS sorter. Only the induced culture of *E. coli* MC4100/pTGFP2 was readily distinguishable from the other particles in the soil. The result is shown in Fig. 3.

Microcosms. Four sets of sterile soil microcosms containing high numbers of cells of the tetracycline biosensor bacterium *E. coli* MC4100/pTGFP2 were set up. To each set, a decreasing number of *S. rimosus* spores were added. Following incubation, microcosms were sacrificed and the content of induced biosensor bacteria was determined by FACS analysis.

No fluorescent bacteria (in the R2 region [Fig. 3]) were detected above the background level on day 0 (Fig. 4). On day 2, however, there was a fluorescent population in the series A samples (highest spore inoculum) of 1.24×10^4 R2 counts per ml of extract corresponding to 28% of the total biosensor population calculated from the plate counts. The number of fluorescent bacteria in the series A samples remained at approximately the same high level on day 5 (9.64×10^3 R2 counts/ml of extract). Due to the overall growth of the biosensor in these samples on day 5, the fluorescent population had dropped to constitute only 2.4% of the total number of biosensors. Figure 5 shows the distribution of fluorescence counts in a typical series A sample from day 0 and day 2. Only counts in the R2 region are summarized in Fig. 4. The increase in the

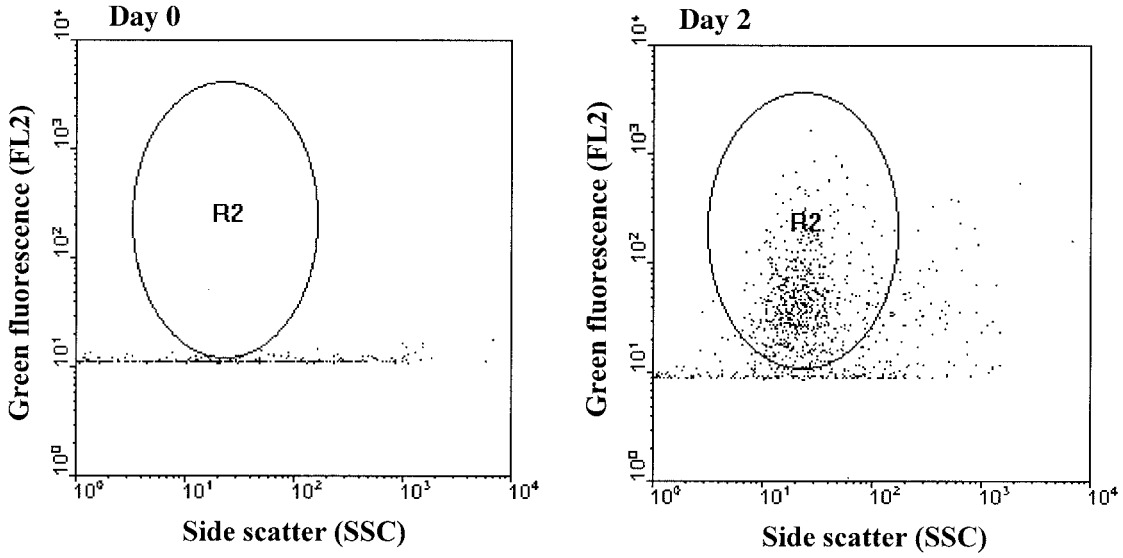

FIG. 5. Flow cytometric analysis at day 0 (left) and day 2 (right) of soil extract from microcosms inoculated with a high density of *S. rimosus* spores (series A).

numbers of fluorescent biosensors is most likely caused by oxytetracycline production in the soil microcosms. Cell sorting, followed by microscopy, confirmed that the particles counted by FACS indeed were induced biosensor cells.

In series B samples (samples with a 10^2-diluted *S. rimosus* inoculum) from day 2 and day 5, the number of induced biosensors appeared to be higher than in both the series C and the series D samples. The difference was, however, not statistically significant due to large fluctuations within replicates in series B, day 5 (see error bars in Fig. 4). In any case, it suggests that tetracycline production is occurring in this series as well. FACS signals in samples from series C and series D remained at the same background level throughout the whole period.

No detectable concentrations of GFP were found in the controls when a sonicated spore inoculum was examined as described above, indicating that no oxytetracycline was added to the microcosms together with the *S. rimosus* spore inoculum (data not shown).

Background fluorescence stemming from soil particles averaged 192 R2 counts/ml of extract (data not shown). This background level was subtracted from the counts of each microcosm harvested. Only readings above this background level are shown in Fig. 4.

Selected barley leaves were inspected in the epifluorescence microscope in order to produce photographic evidence of the interaction between *Streptomyces* hyphae and associated biosensor cells. However, the concentrations of the organisms were apparently too low, as no fluorescing cells were found.

Cell growth and inhibition in the microcosms. Bacterial numbers showed that neither growth nor decline of the biosensor bacteria was taking place during incubation in sterile soil (Fig. 6). On day 5, however, the numbers of biosensors had increased significantly (10-fold) in the samples with the highest *S. rimosus* spore inoculum. This could be due to excretion of metabolites from *S. rimosus* as it grows and degrades the soil polymer components such as chitin and hemicellulose or the death of *S. rimosus* hyphae and subsequent release of nutrients into the soil matrix. The production of oxytetracycline in the experiment indicated that *S. rimosus* was metabolically active in the soil microcosms. On day 5, the R2 numbers remained high (see above), but the percentage of biosensors which were induced had dropped from 28 to 2.4%. This is not an unexpected development. If the induced biosensors were exposed to tetracycline in concentrations that slow down their growth, it would give a growth advantage to the bacteria that are situated in areas without tetracycline. An overall growth in biosensor numbers would therefore favor the uninduced bacteria, hence the lower percentage. The induced biosensor cells used for defining the R2 region were grown in the presence of

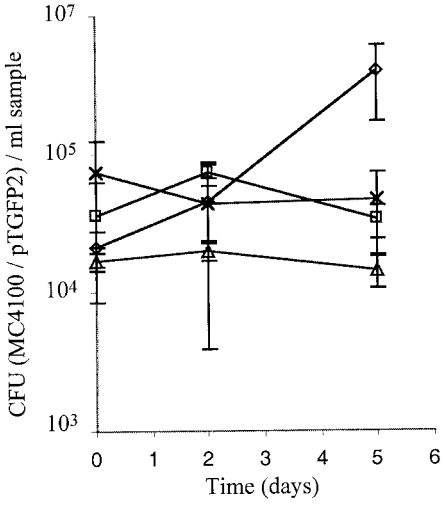

FIG. 6. Enumeration of biosensor bacteria (*E. coli* MC4100/pTGFP2) on LB4 containing ampicillin. Open diamonds, microcosms containing undiluted *S. rimosus* spore inoculum (A series); open squares, 100×-diluted spore inoculum (B series); open triangles, 10,000×-diluted spore inoculum (C series); multiplication signs, no spores added (D series).

50 ng of oxytetracycline per ml. This concentration was chosen since it gives an induction easily distinguishable from soil particles in the FACS. Concentrations of oxytetracycline above 50 ng/ml have in our hands slowed down growth of *E. coli* MC4100. It is therefore likely that tetracycline was produced by *S. rimosus* locally in soil in high enough concentrations to inhibit bacterial growth in the vicinity of the producer. This indicates that antibiotic production can give the producer a selective advantage under natural conditions. It also suggests that indigenous actinomycetes may provide selective conditions for antibiotic-resistant bacteria in natural soil. However, since growth conditions in culture cannot be compared to those in soil microcosms, the response of the biosensor is qualitative rather than quantitative in this experiment. The number of biosensor bacteria determined by plate counts accounted for only a fraction of the biosensor bacteria added. Between 1.7×10^4 and 7.3×10^4 CFU of *E. coli* MC4100/pTGFP2 per ml of extract was recovered from the microcosms on day 0 (1 h after inoculation). This is equivalent to a recovery of the biosensor of between 1 and 5% of the number of biosensors inoculated into the soil. The low recovery could be due to the long (1-h) sedimentation period. Apparently, the bacteria precipitated rapidly together with the soil particles in this type of soil. As a control, samples were taken after 10 min of sedimentation on day 2 and plated onto selective media. The CFU counts after 10 min were 5- to 10-fold higher than in the same samples after 1 h of sedimentation (data not shown). The 10-min samples were, however, not suitable for analysis in the FACS, because of a high level of particles interfering with the detection of fluorescent bacteria.

Likewise, *S. rimosus* numbers in the extract were low compared to the number of CFU added. *S. rimosus* could be detected by plating only in the series with the highest inoculum density. The counts were 3.5×10^2 on day 0, 9.8×10^1 on day 2, and 4.4×10^2 CFU/ml of sample on day 5. The recovery in the sample with the highest inoculum density was approximately 1%. Samples from the 10-min sedimentation on day 2 (see above) showed the same sedimentation for *S. rimosus* as for *E. coli* MC4100/pTGFP2. The growth or decline of *S. rimosus* CFU could not be detected via plate counts in this experiment.

Application potential. We have introduced a new approach in environmental microbiology by combining the high specificity of bacterial biosensors and the resolution power of a fluorescence-activated cell sorter. Application of these techniques provided detection of oxytetracycline production by *S. rimosus* in soil microcosms. This has not been possible with traditional methods, possibly due to the very low oxytetracycline concentrations in the bulk soil.

Analyzing the samples by FACS has the major advantage that fluorescence is detected per individual bacterial cell. This allowed the detection of a few fluorescent cells exposed to inducing concentrations of oxytetracycline among a larger number of nonfluorescing bacteria.

Moreover, the use of a whole-cell biosensor permits detection of only the bioavailable fraction of tetracycline. This gives the opportunity to study biological effects of antibiotics produced in the environment or introduced as pollutants in manure and sewage sludge.

We believe that an improved method of extracting biosensor cells from the soil can give higher recovery of biosensor cells and less variable detection of oxytetracycline production. This will provide the means for tetracycline detection at lower densities of *S. rimosus* cells. We are currently working on applying this approach in nonsterile and unamended soil using different gram-negative bacterial hosts for the biosensor construct.

A combination of this setup and confocal scanning laser microscopy could give additional information on the spatial diffusion of antibiotics and its effect on the surrounding microflora, in situ.

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