In vivo bioluminescence imaging for the study of intestinal colonization by *Escherichia coli* in mice

M-L Foucault, L. Thomas, S. Goussard, B. R. Branchini, and C. Grillot-Courvalin.

Institut Pasteur, Unité des Agents Antibactériens, 75724 Paris, Cedex 15, France, and Department of Chemistry, Connecticut College, New London, CT 06320, USA.

* Corresponding author. Mailing address:

Unité des Agents Antibactériens

Institut Pasteur

25, rue du Docteur Roux

75724 Paris Cedex 15, France

Phone: (33) (1) 45 68 81 74.

Fax: (33) (1) 45 68 83 19.

E.mail: ccourval@pasteur.fr.

† Present address: Inserm, U574, Hôpital Necker-Enfants Malades, Paris, France
Bioluminescence imaging (BLI) is emerging as a powerful tool for real-time monitoring of infections in living animals. However, since luciferases are oxygenases, it has been suggested that the requirement for oxygen may limit the use of BLI in anaerobic environments such as the lumen of the gut. Strains of *Escherichia coli* harboring the genes for either the bacterial luciferase from *Photorhabdus luminescens* or the PpyRE-TS and PpyGR-TS firefly luciferase mutants of *Photinus pyralis* have been engineered and used to monitor intestinal colonization in the streptomycin-treated mouse model. There was excellent correlation between the bioluminescence signal measured in the faeces ($R^2 = 0.98$) or transcutaneously in the abdominal region of whole animals ($R^2 = 0.99$) and the CFU counts in the faeces of bacteria harboring the *luxABCDE* operon. Stability in vivo of the bioluminescence signal was achieved by constructing plasmid pAT881(pGB2ΩPami*luxABCDE*) which allowed long-term monitoring of intestinal colonization without the need of antibiotic selection for plasmid maintenance. Intestinal colonization by various *E. coli* could be compared directly by simple recording of the bioluminescence signal in living animals. The difference in spectra of light emission of the PpyR-TS and PpyGR-TS firefly luciferase mutants and dual bioluminescence detection allowed direct in vitro and in vivo quantification of two bacterial populations by measurement of red and green emitted signals, and thus to monitor the two populations simultaneously. This system offers a simple and direct method to study in vitro and in vivo competition between mutants and the parental strain. BLI is a useful tool to study intestinal colonization.
Among the wide variety of bacteria that colonize the gastrointestinal tract of mammals, *Escherichia coli* is the most abundant facultative anaerobe of the human intestinal microflora. Aside from being part of the normal flora, *E. coli* is also a versatile organism capable of causing a variety of intestinal and extra-intestinal diseases (18). The mechanisms that allow commensal *E. coli* to colonize the intestine and survive successfully in this niche remain poorly characterized. Conventional mice display natural colonization resistance by commensal *E. coli* but oral administration of streptomycin, that alters the intestinal microflora, allows colonization of the mouse large intestine by this species (25). The streptomycin-treated mouse model has been used extensively to study the factors of gram-negative bacteria implicated in the intestinal colonization process. However, this model is limited to the viable plate counts of bacteria in the faeces and misses some critical information such as the kinetics of colonization, the fate of the bacterial cells across the digestive tract, and the site of colonization. A better understanding of colonization would be facilitated by direct in vivo follow up of this process.

Bioluminescence imaging (BLI) technology is emerging as a powerful tool for the study of a wide range of biological processes in live animals, including real-time monitoring of infections (16). Bioluminescence systems emit visible light due to the luciferase-mediated oxidation of a luciferin substrate. A variety of luciferin-luciferase systems with different peak emissions have been identified in nature from numerous species (14). The luciferase of the soil bacterium *Photorhabdus luminescens* has been expressed successfully in gram-negative and gram-positive bacteria. This system emits blue-green light with an emission maximum of approximately 490 nm and does not require the addition of exogenous substrate since the luciferase operon contains the genes required for synthesis of the substrate. Therefore, this luciferase has been extensively used to monitor bacterial infections in living-mouse. One of the first investigations with *Salmonella typhimurium* transformed with the *lux* operon of *P. luminescens* evaluated the tissue distribution and the virulence of various *S. typhimurium* strains (9). Subsequent modification of the *lux* operon led to the generation of highly bioluminescent *Staphylococcus aureus* and allowed the monitoring of infections in living mice due to this species (11). The modified *lux* operon was engineered into a *lux-kan* transposon cassette for chromosomal integration in gram-positive bacteria such as *S. aureus*, *Streptococcus pneumoniae, group A Streptococcus*, and *Listeria monocytogenes* (16). Replication of *L. monocytogenes* in the lumen of the gall bladder was demonstrated for the first time by BLI (13).
Bioluminescent *E. coli* was used in the neutropenic mouse thigh model of infection to evaluate the in vivo activity of antimicrobial agents: bioluminescence was as indicative of therapeutic efficacy as CFU counts but, in addition, allowed real time monitoring of the infection and of treatment efficacy in the same animal (29); however, only short term monitoring (12 h) could be performed.

Because luciferases are oxygenases, it has been suggested that the requirement for oxygen may limit the use of BLI in anaerobic environments such as the lumen of the gut. After oral administration of bioluminescent *Salmonella* to susceptible mice, the bioluminescent signal recorded in the abdominal region was greatly enhanced after air exposure (9). It was therefore assumed that direct bioluminescence imaging of intestinal colonizing microorganisms would not be optimal unless oxygen was provided exogenously or as the result of the close interaction between cells and the bacteria (9). However, the bacterial luciferase was used to trace in real time the colonization dynamics by *Citrobacter rodentium* of the gastrointestinal tract of living animals, demonstrating that the gut represents a semi-anaerobic environment that allows the study of bacterial colonization by BLI (33).

Factors essential for colonization are best studied in co-colonization experiments (7, 17). There are several luciferases with distinct emission spectra (34) that could be used in competition experiments to trace simultaneously two bacterial populations in the same living-animal. However, in order not to impose additional and different metabolic burden to the bacteria under study, the exogeneous luciferases have, ideally, to be similar to allow comparison between strains. The thermostable luciferase variants Ppy RE-TS and Ppy GR-TS, derived from wild-type luciferase from the North American firefly *Photinus pyralis*, emit red (612 nm) and green (552 nm) light, respectively, at 37°C and are encoded by single genes of 1650 bp, differing by only 9 bp (4). Bioluminescence color is determined by the Ser<sub>284</sub>Thr and Val<sub>241</sub>Ile, Gly<sub>246</sub>Ala, and Phe<sub>250</sub>Ser (Ppy GR-TS) amino acid changes (5, 34). Using optical filters, the emission spectra are readily distinguishable (4, 5). Five additional mutations provide enhanced thermostability at 37°C (4) improving the compatibility of the enzymes with bacterial culture conditions and BLI in animal models.

While the luciferase mutants and all firefly luciferases use as substrate firefly luciferin and ATP to produce light, in vivo imaging is commonly performed with endogenous ATP and requires only exogeneous administration of the luciferase substrate.

The aim of this study was to develop a dynamic mouse model using in vivo bioluminescent imaging systems to monitor bacterial colonization in situ and in real time in whole living animals. Various *E. coli* harboring either the genes for the bacterial luciferase
from *P. luminescens* or the firefly luciferase mutants (PpyRE, PpyGR) from *P. pyralis* have been engineered and used to follow bacterial intestinal colonization in mice. BLI was found to be well adapted to compare the intestinal colonizing capacity of various *E. coli* strains and to monitor co-colonisation in vivo using dual bioluminescence emission.
MATERIALS AND METHODS

Bacterial strains and plasmids. The properties of the strains and plasmids used are listed in Table 1. Spontaneous streptomycin-resistant (Str') mutants of human commensal *E. coli* K12 MG1655 (24), diarrhea-associated entero-aggregative (EAEC) *E. coli* 55989 producing aggregative adhesion fimbriae type III (2), and of *E. coli* BM2711, a RecA1 derivative of strain MM294 (12) were used. The strains were made bioluminescent by transformation with DNA of plasmids carrying the *luxABCDE* operon encoding the *Photorhabdus luminescens* bacterial luciferase or the *lucG* or *lucR* genes coding for *Photinus pyralis* firefly luciferase green and red mutants, respectively (4).

The *luxABCDE* operon was carried by three vectors. Plasmid pSB2025 (27) carries the operon cloned into the superlinker plasmid pSL1190 (Amersham Pharmacia Biotech, Uppsala, Sweden). Plasmid pAL2 is a derivative of shuttle vector pVA838 in which the *luxABCDE* operon from pSB2025 has been cloned under the control of the *P*~ami~ constitutive promoter of *Streptococcus pneumoniae* aminopterin resistance operon (1). In the third construct, the *luxABCDE* operon fused to the *P*~ami~ promoter was cloned in plasmid pGB2 (8) in three steps. First, the *luxABCD* BamHI fragment was cloned in the vector digested with the same enzyme leading to pGB2ΩluxABCD. The XbaI-PstI fragment of pGB2ΩluxABCD containing the 3' portion of *luxD* was then replaced by the XbaI-PstI fragment from pAL2 containing the 3' portion of *luxDE*, generating pGB2ΩluxABCDE. Finally, an EcoRI fragment containing the *P*~ami~ promoter from pAL2 was created by hybridization of two complementary primers and cloned in pGB2ΩluxABCDE digested with EcoRI to generate pAT881(pGB2ΩP~ami~luxABCDE).

Genes *lucR* and *lucG* for the red (Ppy RE-TS) and green (Ppy GR-TS) thermostable *P. pyralis* luciferase mutants (4) were cloned under the control of the strong *P*~tet~ promoter (21) in pCR-blunt© (Invitrogen, Cergy-Pontoise, France). The *P*~tet~lucG and *P*~tet~lucR inserts were excised with *PstI* and BamHI and ligated to pAT113 (32) digested similarly resulting in pAT882(pAT113ΩP~tet~lucR) and pAT883(pAT113ΩP~tet~lucG), respectively. Plasmid pAT113 is an integrative vector in Gram-positive bacteria and a replicative vector in gram-negative bacteria. To obtain plasmids pAT884(pTVCΩP~tet~lucG) and pAT885(pTVCΩP~tet~lucR), *P*~tet~lucG and *P*~tet~lucR were, respectively, excised from pAT883(pAT113ΩP~tet~lucG) and pAT882(pAT113ΩP~tet~lucR) by digestion with *PstI* and BamHI and ligated to the pTVC erm shuttle vector digested similarly. Plasmid DNA isolation, digestion with restriction
endonucleases, amplification by PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA), ligation with T4 DNA ligase (New England Biolabs, Beverly, MA), nucleotide sequencing, and transformation of E. coli were performed by standard methods (30). Strains were grown at 37°C in Luria-Bertani broth (LB) medium (Difco laboratories, Detroit, MI) containing streptomycin (100 µg/ml) and either erythromycin (150 µg/ml), kanamycin (50 µg/ml), spectinomycin (50 µg/ml), or ampicillin (100 µg/ml).

**In vitro plasmid stability.** Stability of the various bioluminescent plasmids in E. coli was assessed by transferring cultures (1000-fold diluted) to fresh LB broth every 24 h over 7 days. At every transfer, cells were plated on non-selective medium and the proportion of bioluminescent bacteria was determined by replica plating on the appropriate antibiotic followed by screening for bioluminescent colonies. When necessary, one ml of D-luciferin substrate (Caliper) at 150 µg/ml in PBS was added directly onto the colonies just before bioluminescence measurement.

**Mouse colonization.** Experiments were carried out in the streptomycin-treated mouse model that has been used extensively to study colonization of the large intestine by E. coli (25). Briefly, four 6-week-old BALB/c female mice (Janvier, Le Genest-Saint-Isle, France) were given drinking water containing 5 g/l of streptomycin sulphate for 24 h and then starved for food overnight. The bioluminescent E. coli were grown in LB medium at 37°C with shaking to the exponential phase, washed twice, and resuspended at 10⁸ CFU per ml in phosphate-buffered saline (PBS). 100 µl of the bacterial suspension mixed with 100 µl of 20% sucrose plus 6% NaHCO₃ were administered orogastrically with feeding needles, and food and streptomycin-water were returned to the mice. Fresh fecal samples collected at 24 h, 48 h, and 72 h and on every other day thereafter for 14 days were weighed, homogenized in PBS (0.1g/ml), diluted, and plated on LB agar containing streptomycin and erythromycin, or kanamycin, or spectinomycin to select, respectively, E. coli MG1655-Str², 55989-Str², or BM2711-Str² harboring bioluminescent plasmids pAL2(pVA838ΩP_amluxABCDE), pAT882(pAT113ΩP_teducR), or pAT881(pGB2ΩP_amluxABCDE). At 72 h post-feeding, some mice colonized by E. coli MG1655-Str²/pAT881(pGB2ΩP_amluxABCDE) were sacrificed and the small and large intestine (caecum plus colon) were removed. The bioluminescence signal was monitored on intact or longitudinally opened organs. The small intestine and the large intestine were then homogenized mechanically in 3 ml and 5 ml of sterile PBS, respectively, and the number of viable bacteria per organ homogenate was determined by plating onto LB...
agar containing the appropriate antibiotics. The care and the use of experimental animals complied with local animal welfare laws and guidelines.

**Bioluminescence quantification.** Bioluminescent imaging was performed using an IVIS 100 imaging system (100 series, Caliper corp., Alameda, CA) that consists of a cooled charge-coupled device camera mounted on a light-tight specimen chamber (dark box), a camera controller, a cryogenic refrigeration unit, and a Windows computer system. The bioluminescence of the bacterial luciferase was measured without filter (open). To separate spectral emissions of the green- and red-emitting firefly luciferase mutants, the imaging system was equipped with 2 band-pass filters that are 20 nm wide with central wavelengths of 540 nm and 620 nm (4, 5).

The firefly luciferase mutants require exogenous addition of D-luciferin substrate to produce light. D-luciferin (chromaGlo reagent, Promega, Madison, WI) was used for in vitro experiments and D-luciferin potassium salt (Caliper) for in vivo experiments in which optimal signals were obtained 3 h after intragastric inoculation with feeding needles of 200 µl/mouse of D-luciferin at 15 mg/ml.

For faeces analysis, 30 µl of the fecal samples homogenized in PBS (0.1g/ml) were added in a 96-well black microplate. For living animal imaging, mice were anesthetized in an oxygen-rich induction chamber with 2% isoflurane gas and anesthesia was maintained during imaging by using a nose cone isoflurane-oxygen delivery device in the specimen chamber. The microplates or the mice in ventral position were placed in the warmed specimen chamber (37°C) and photon emission was measured with exposure times ranging from 30 s to 1 min, depending on the bioluminescence intensity signal.

For quantification of red and green bioluminescent signals in mixed cultures of red and green bioluminescent *E. coli* in the faeces or in whole animals inoculated with various proportions of the two bioluminescent strains, images were acquired using the 540-nm filter to record the signal emitted by the bacterial population containing the green firefly luciferase mutant and the 620-nm filter for those producing the red firefly luciferase mutant. To determine the spectral overlaps of the luciferase mutants, the green and the red signal were also measured separately in similar mixtures of cells containing only one bioluminescent strain. The green overlap corresponds to the red emission measured with the green filter (540 nm) and the red overlap to the green emission measured with the red filter (620 nm). These independent measurements allowed subtraction of the red- and green-emission spectra overlaps with the 620-nm and 540-nm filters, respectively, leading to individual quantification of the red and green emissions when they were in mixtures.
The data are presented as pseudo-color images indicating light intensity (red being the most intense and blue the least intense) which are superimposed over the grayscale reference photographs. The region of interest was manually selected and the signal expressed as the total number of photons emitted per second [photons/s] by using IgorPro image analysis package.
RESULTS AND DISCUSSION

In vitro stability of the bioluminescent plasmids. Bioluminescence imaging has been used previously to follow in vivo infectious processes or to measure drug efficacy, but mostly in short term studies (less than 48 h). For long term studies, stable maintenance of a bioluminescence plasmid usually requires concomitant antibiotic selection which can interfere with the infectious process under study, particularly in the case of bacterial intestinal colonization. An alternative is to integrate the lux operon in the bacterial chromosome but this requires engineering of every strain used.

Stable bacterial maintenance of plasmids bearing the luciferase system in the absence of a selective agent is a prerequisite. The stability of three bioluminescent plasmids in three *E. coli* strains, MG1655-Str<sup>r</sup>, 55989-Str<sup>r</sup>, and BM2711-Str<sup>r</sup> (Table 1) was thus tested in vitro by sub-culturing in LB broth over 7 days with replica plating every 24 h of an aliquot on medium containing the appropriate antibiotic; the bioluminescent signal of the colonies on the selective plates was also monitored in parallel (Fig. 1).

In *E. coli* MG1655-Str<sup>r</sup>, pSB2025 appeared stable during the first two days of culture but thereafter rapid plasmid loss was observed (Fig. 1A). There was even more rapid loss of the bioluminescence signal with only 24% bioluminescent colonies among the ampicillin-resistant colonies after two transfers (Fig. 1B-left panel). Plasmid pAL2, that has been reported to be stable in vitro in *S. pneumoniae* (1), was also gradually lost from MG1655-Str<sup>r</sup> without additional loss of the *luxABCDE* operon; 100% of the erythromycin resistant cells remaining bioluminescent (Fig. 1B). The stability of both plasmids was similar in *E. coli* 55989-Str<sup>r</sup> (Fig. 1A) and a loss of the bioluminescence signal was also observed for the plasmid pSB2025 during the successive transfers (data not shown). Surprisingly, in *E. coli* BM2711-Str<sup>r</sup> both pSB2025 and pAL2 were remarkably stable with 100% and 77% of bioluminescent colonies after 7 days of sub-culture, respectively (Fig. 1A, 1B-right panel).

Low copy number plasmid pAT881(pGB2Ω<sub>amp</sub>luxABCDE) was remarkably stable in *E. coli* MG1655-Str<sup>r</sup>, 55989-Str<sup>r</sup>, and BM2711-Str<sup>r</sup> with, respectively, 98%, 94%, and 100% of bioluminescent and spectinomycin resistant colonies after growth for 7 days (Fig. 1A).

For the firefly luciferase system, plasmid pAT884(pTVCΩ<sub>tet</sub>lux<sup>R</sup>) was very unstable in *E. coli* 55989-Str<sup>r</sup> with almost 50% loss after overnight culture (Fig. 1A) and was not used. After 3 days of growth, 63% and 73% of the bacteria still harbored plasmids pAT882 and
pAT883, respectively, and 100 % of the kanamycin resistant cells remained bioluminescent indicating no luciferase gene loss by the plasmid (data not shown). These data confirm that plasmid stability depends on the plasmid, the genetic background of the host (15), and the presence of another plasmid in the strain (e.g. in 55989-Str', (2). The new plasmid pAT881(pGB2ΩP_am1uxABCDE) displayed good in vitro stability in the three E. coli studied and appears as a good candidate for long term in vivo studies.

**Bioluminescence monitoring of mouse intestinal colonization.** Oral streptomycin treatment, which selectively removes facultative anaerobes of the intestinal tract while leaving the anaerobic microflora essentially intact, allows mouse intestinal colonization by E. coli K12 (25). In this model, colonization ability is assessed by monitoring daily the number of CFU per gram of faeces after intra-gastric inoculation. However, fecal counts are tedious and imply, in case of co-colonization, that strains have to be tagged with different selection markers. Moreover, this approach does not provide any direct indication about the anatomical localisation of the colonization. The very good correlation obtained with serial dilutions of faeces from mice fed with E. coli MG1655-Str'/pAL2 between the CFU counts and the bioluminescent signal in photons/sec (R² = 0.98 versus R² = 0.99 for bioluminescent bacteria directly diluted in culture medium, Fig. 2C) indicated that photon emission levels accurately reflect bacterial numbers in the faeces. The bioluminescence system allowed the detection of bacterial quantities as low as 10³ CFU/ml in broth medium and ca. 10⁵ CFU/g of faeces in faeces samples.

*E. coli* MG1655-Str'/pAL2(pVA838ΩP_am1uxABCDE) was used to monitor intestinal colonization of streptomycin-treated mice by bioluminescence. Sets of four mice were intragastrically inoculated with 10⁷ CFU in 200 µl PBS with 10 % sucrose plus 3% NaHCO₃ and the bioluminescent signal was monitored, both transcutaneously on whole animals and in the faeces (Fig. 2). As early as 24 h post-feeding, a strong bioluminescent signal was observed transcutaneously in the abdominal region (Fig. 2A) and in the faeces (Fig. 3A). Counts on streptomycin containing plates, which represent all *E. coli* CFU, showed that MG1655-Str'/pAL2 had the classical colonization profile of *E. coli* K12 with an initiation stage (0 to 3 days post-feeding) followed by a maintenance stage (3 days post-feeding and beyond) at 10⁹ CFU/g of faeces (7) indicating that the presence of pAL2 in the strain did not interfere with its colonization ability. The signal in the faeces remained at a plateau of 10¹⁰ photons/s/g of faeces until day 3 and then slowly decreased to 10⁷ photons/s/g of faeces at day 9 (Fig. 3A);
the similar decrease, from $10^9$ to $10^6$ CFU/g of faeces of colonies on erythromycin-supplemented plates, indicated loss of plasmid pAL2 by the host. Similarly, the signal measured in whole animals decreased slowly from $10^9$ photons/s at day 3 post-inoculation to $10^5$ photons/s at day 9 of colonization (Fig. 3E). There was very good correlation ($R^2 = 0.98$) between the bioluminescent signal in the abdominal region measured transcutaneously in whole animals and the CFU counts of bioluminescent bacteria in the faeces (Fig. 2C). These results indicated that intestinal colonization can be detected directly by simple recording of the bioluminescent signal in living animals. However, because of its instability in *E. coli* MG1655-Str<sup>+</sup>, the bioluminescent reporter plasmid pAL2 allowed real-time monitoring only during the initial steps of intestinal colonization.

Long-term study of intestinal colonization was performed with *E. coli* MG1655-Str<sup>+</sup> harboring pAT881(pGB2Ω<sub>ami</sub>luxABCDE) which was remarkably stable in vitro (Fig. 1A). The level of colonization by this strain was similar to that obtained with MG1655-Str<sup>+</sup> (Fig. 3B) and the bioluminescent signals obtained in the faeces (Fig. 3B) and transcutaneously (Fig. 3E) remained stable until at least day 13 (data not shown). Long term colonization can thus reliably be assessed by bioluminescence monitoring of MG1655-Str<sup>+</sup>/pAT881.

Since bioluminescence emission is optimal in an aerobic environment (14), the signal recorded transcutaneously may underestimate the actual colonisation due to limitation in oxygen availability in the intestinal cavity. The small and large intestine (caecum and colon) of mice colonized with *E. coli* MG1655-Str<sup>+</sup>/pAL2 was dissected at 72 h and the bioluminescent signal was recorded on intact or longitudinally opened organs (Fig. 2B). A ca. 10-fold higher bioluminescent signal was observed for the caecum and large intestine when these organs were opened. No significant bioluminescent signal was recorded from the intact small intestine but a slight signal was detected, albeit inconsistently, in the distal part of the small intestine when it was longitudinally opened, (Fig. 2B). The CFU counts confirmed that the large intestine was the major site of *E. coli* colonisation ($10^9$ CFU per organ) whereas only ca. $10^6$ CFU were grown from the small intestine. These results are consistent with previous investigations that identified the mucus overlying the epithelial cells of the large intestine as the principal site of colonization by various *E. coli* strains such as MG1655 (25, 26). Taken together, these data indicate that transcutaneous BLI appears therefore as a good indicator of intestinal colonization. These observations support the recently established notion that aerobic respiration, required for optimal colonization by commensal and pathogenic *E. coli*, can take place in the intestine in mice (17).
Comparison of intestinal colonization by various *E. coli* by bioluminescence. Strain 55989-Strr is a diarrhoea-associated enteroaggregative *E. coli* with particular adhesion properties (fimbriae type III) and constitutes an emerging pathotype responsible for enteric illness (2). The biochemical characteristics and virulence factors implicated in host infectivity and in intestinal colonization ability of this pathogenic strain have been recently studied (3, 22). The level of colonization by 55989-Strr/pAL2, between $10^8$ to $10^9$ CFU/g of faeces, and the bioluminescence, from $10^9$ to $10^{10}$ photons/sec/g of faeces, were similar to those obtained with MG1655-Strr/pAL2 (Fig. 3C). *E. coli* BM2711-Strr is a RecA1 derivative of K12 MM294 (Table 1). The level of intestinal colonization by *E. coli* BM2711-Strr/pAL2, from $10^7$ to $10^8$ CFU/g of faeces and $10^9$ photons/s/g of faeces (Fig. 3D), was lower than those of 55989-Strr/pAL2 and MG1655-Strr/pAL2. This difference is within the range observed when strains with colonizing defects, related for example to differences in sugar consumption, are studied (10). The difference in colonization ability between MG1655-Strr, 55989-Strr, and BM2711-Strr was easily detected by bioluminescence measure of whole animals; the transcutaneous photon emission of MG1655-Strr/pAL2 and 55989-Strr/pAL2 was ca. $10^8$ photons/s whereas BM2711-Strr/pAL2 colonization resulted in a signal of only ca. $10^6$ photons/sec (Fig. 4C). Transcutaneous BLI can therefore be used for rapid screening of the colonizing ability of mutant strains or of enteropathogenic *E. coli*.

Streptomycin treatment regimen for intestinal colonization. The standard protocol for intestinal colonization of mice has remained as originally described in 1982 by Myhal et al. (25) with mice fed orally with 5 g/l of streptomycin in the drinking water. To determine the minimal dose of streptomycin required to eliminate the resident facultative anaerobic microflora to allow colonization by *E. coli*, mice were given, prior to colonization, water containing various concentrations of streptomycin (20 mg/l, 0.5 g/l, 1 g/l, 2.5 g/l, and 5 g/l). Colonization by MG1655-Strr/pAL2 was monitored by faeces counts (Fig. 4A) and bioluminescence (Fig 4B). There was no colonization in the absence or in the presence of 20 mg/l of streptomycin. However, at a streptomycin concentration as low as 1 g/l, colonization reached levels similar to those obtained with the standard concentration of 5 g/l. When mice were treated with 0.5 g/l, colonization reached the same high level but decreased slowly from 10 to 8 Log_{10}CFU/g of faeces and from 8.5 to 5.5 Log_{10}photons/sec/g of faeces between days 1 and 3, suggesting that this concentration was too low to maintain colonization in the
digestive tract of mice. Bioluminescence is therefore a useful tool to assess directly host parameters involved in intestinal colonization such as the role of the innate immune system.

**Firefly luciferase can be used to trace intestinal colonization by *E. coli***. The 6-kb *lux* operon from *P. luminescens* encodes both the luciferase and the luciferin substrate required for the bioluminescent reaction (23). Mutants PpyRE-TS and PpyGR-TS, derived from wild-type firefly luciferase, are encoded by single genes of 1650 bp that are 99.4% identical (4). Bacteria expressing the firefly luciferases do not produce firefly luciferin and the substrate, identical for the two variants, has to be added exogenously whereas the ATP is available endogenously. D-luciferin is usually injected via intraperitoneal route and distributes rapidly throughout the mice (31). After intraperitoneal injection of 200 µl/mouse of the substrate at 15 mg/ml in PBS to mice colonized by *E. coli* 55989-Str/r/pAT882(pAT113ΩPtetlucR) or *E. coli* 55989-Str/r/pAT882(pAT113ΩPtetlucG), the bioluminescent signal peaked 15 to 20 min post-inoculation (6.6 Log10 photons/s) and decreased rapidly (data not shown). However, after administration of the same amount of substrate via the intra-gastric route, the transcutaneous red- and green-bioluminescent signals increased regularly during the first 2 h (approximately 2 Log10) to reach a plateau that lasted at least 4 h (7.9 Log10 photons/s for PpyGR and 9.2 Log10 photons/s for PpyRE) and then started to decrease slowly 6 h post inoculation (Fig. 5). The transcutaneous signals obtained with PpyRE and the bacterial luciferase were comparable (10 Log10 photons/s). In all subsequent experiments, the bioluminescent signal was therefore measured at an early stage of the plateau, i.e. 3 h after intragastric inoculation of the substrate. Of note, the red signal was always approximately one Log10 higher than the green one (Fig. 5), consistent with the notion that blue and green emission spectra are more absorbed by tissue haemoglobin than the red-emission spectrum (35).

Intestinal colonization by *E. coli* 55989-Str/r was monitored with the firefly PpyrRE luciferase reporter gene using plasmid pAT882(pAT113ΩPtetlucR) (Table 1). In two independent experiments, *E. coli* 55989-Str/r/pAT882 gave colonization levels similar to those of *E. coli* 55989-Str/r/pAL2(pVA838ΩPamiluxABCDE) by CFU counts and bioluminescence detection (7.0 ± 0.3, 6.9 ± 0.4 Log10 CFU/g of faeces plated on medium containing erythromycin and 8.6 ± 0.2, 8.3 ± 0.4 Log10 photons/s/g of faeces, respectively) suggesting that plasmid pAT882 did not interfere with the colonization capacity of the host strain and that the two luciferases have similar levels of luminescence emission in vivo in the microaerobic environment of the mouse intestine.
One potential way to improve luc gene efficiency is to optimize codon usage for bacteria and even more so to the specific host strain. We recently found that human codon optimization of a related red emitting luciferase led to a 10-fold improvement in luciferase detectability in the HEK293 cell line (6). Stability of the bioluminescence signal could be improved by insertion of the luciferase reporter gene in the chromosome (20). Preliminary colonization studies have been undertaken with bioluminescent-engineered strains of Enterococcus faecalis that contain the red firefly luciferase gene mutant stably inserted in the chromosome at a single copy. Luminous bacteria could be easily detected transcutaneously and in the feces of gnotobiotic mice during long-term colonization without signal loss (unpublished data). Thus, bioluminescence technology can be applied to other intestinal colonizers, such as Enterococcus, for which specific factors implicated in the colonization process are being characterized (28).

Quantification in vitro and in vivo of two simultaneous bioluminescent signals. Co-colonization by two strains is frequently performed to study growth rates or nutrient requirements during intestinal colonization or to compare commensal or pathogenic E. coli (10). Bioluminescence could be used to distinguish the strains without the need for introducing distinct selectable markers that could alter differently the fitness of the host. The green and red luciferase mutants have emission spectra at 546 and 610 nm at 25°C respectively, that can be separated by narrow band pass filters to minimize spectral overlap (Fig. 6). The green overlap with the red filter (620 nm) is more important than the negligible red overlap with the green filter (540 nm). To determine the consequences of spectral overlap on the ability to measure accurately the individual red and green bioluminescence emissions, the signals of E. coli 55989-Str' suspensions composed of various proportions of bacteria harboring pAT882(pAT113ΩP_{ed}ufR) or pAT883(pAT113ΩP_{ed}ufG) were measured in vitro (Fig. 6). The results were then compared with the bioluminescent signals obtained from green or red emitting bacteria mixed in the same proportion with non-bioluminescent 55989-Str'. The green and red signals of the bacterial mixtures measured simultaneously were, after correction for overlap, proportional to the corresponding bacterial numbers in the suspension (correlation coefficient $R^2 = 0.98$ for both the red and the green signal) (Fig. 6). These results indicate that the dual bioluminescence detection system allows direct in vitro quantification of two bacterial populations by simultaneous measurement of red and green emitted signals, thus avoiding bacterial enumeration.
Various proportions (20:80, 50:50, 80:20, 100:0) of red and green emitting *E. coli* 55989-Str were inoculated oro-gastrically into streptomycin-treated mice and the bioluminescent signal was measured transcutaneously and in the faeces 3 h after inoculation of the substrate (Fig. 7). The mice were assigned to three groups: the G group was inoculated with various proportions of *E. coli* 55989-Str with or without pAT883(pAT113Ω\*P\*tet\*lucG), the R-group with mixtures of *E. coli* 55989-Str with or without pAT882(pAT113Ω\*P\*tet\*lucR), and the M-group was inoculated with various mixtures of *E. coli* 55989-Str containing pAT882 or pAT883. The groups inoculated with bacteria emitting a single bioluminescent signal allowed determination of the spectral-overlaps of the 540- and 620-nm filters. Since the red and green emitting bacteria in a mouse were indistinguishable on the basis of antibiotic resistance, the CFU counts of the two types of bacteria in the mixture were deduced from the counts of the bioluminescent bacteria in the R- and G-group. Twenty-four hours post inoculation, there was very good correlation between the bioluminescent red and green signals and the red and green emitting CFU counts in the faeces (Fig. 7B).

Similar results were observed when the bioluminescent signals were measured transcutaneously in whole animals but, as already mentioned, the red signal was stronger due to greater green absorption by animal tissues (Fig. 7A). However, the transcutaneous signal was more variable than that in the faeces (data not shown).

These results indicate that monitoring of two bacterial populations in the gut of a single animal can be achieved by quantification of bioluminescence signals, either in the faeces or transcutaneously in whole animals. This system, which allows direct monitoring of two bacterial populations simultaneously, offers a simple and direct method to study in vitro and in vivo competition between mutants and the parental strain.

In conclusion, we have demonstrated that intestinal colonization can be monitored in whole living animals with genetically-engineered *E. coli* that produce either the bacterial luciferase from *P. luminescens* or the firefly luciferase mutants PpyRE and PpyGR from *P. pyralis*, and that two bioluminescent signals can be simultaneously monitored and quantified in the same living-mouse. BLI will therefore be a very useful tool to compare the relative colonization efficiency of commensal and pathogenic *E. coli* strains and further elucidate the mechanism of colonization resistance (19). Stability of the bioluminescence signal in vivo has been improved by constructing plasmid pAT881(pGB2Ω\*ami\*luxABCDE) which allows long-term intestinal colonization without the need for antibiotic selection plasmid maintenance. This reporter system has numerous potential applications for monitoring the evolution of
infectious diseases in animal models in the absence of antibiotic selective pressure and without killing the animals.
We thank P. S. Cohen for providing *E. coli* MG1655-Str*^r^*, C. Le Bouguénec for providing *E. coli* 55989-Str*^r^* and for helpful discussions, M.-A. Nicolas for help with bioluminescence experiments, and P. E. Reynolds for critical reading of the manuscript. This work was supported by grants from the European Commission, grant n° LSHM CT 2005 518152-EAR that included a fellowship in support of M-L F. and from the Air Force Office of Scientific Research (FA9550-07-1-0043), the National Science Foundation (MCB0842831) and the Hans & Ella McCollum 21 Vahlteich Endowment.
FIG. 1. In vitro stability of bioluminescent plasmids. (A) Stability of the plasmids in *E. coli* MG1655-Str<sup>r</sup>, 55989-Str<sup>r</sup>, and BM2711-Str<sup>r</sup> was assessed by sub-culturing each strain in LB-broth over 7 days and by replica plating on the appropriate antibiotic. Stability of a plasmid is expressed as the percentage of colonies on the selective plate relative to that on the non-selective plate. (B) The bioluminescent signal of every ampicillin- or erythromycin-resistant colony of *E. coli* MG1655-Str<sup>r</sup>/pSB2025 (left) and *E. coli* MG1655-Str<sup>r</sup>/pAL2 (right) after 2 days of culture was determined.

FIG. 2. Monitoring of intestinal colonisation by bioluminescence imaging in whole animals. (A) $10^7$ *E. coli* MG1655-Str<sup>r</sup>/pAL2(pVA838Ω<sub>pami</sub>luxABCDE) were inoculated intragastrically into four streptomycin-treated mice and the bioluminescent signal was measured transcutaneously 24 h post feeding in whole animals. The intensity of the transcutaneous photon emission is represented as a pseudo-color image. (B) The small (top) and large (bottom) intestine were dissected at 72 h and the photon/sec per organ was quantified on intact (left) or longitudinally opened (right) organs. (C) Linear correlation between bacterial counts and the bioluminescent signal by serial dilutions of bioluminescent bacteria in culture medium or in the faeces (left). Correlation between transcutaneous photon emission measured in the whole animal and the number of bioluminescent bacteria in the faeces during colonization (right).

FIG. 3. Comparison of the colonizing ability of various *E. coli* by bioluminescence. Sets of four mice were fed with $10^7$ CFU of (A) MG1655-Str<sup>r</sup>/pAL2(pVA838Ω<sub>pami</sub>luxABCDE), (B) MG1655-Str<sup>r</sup>/pAT881(pGB2Ω<sub>pami</sub>luxABCDE), (C) 55989-Str<sup>r</sup>/pAL2(pVA838Ω<sub>pami</sub>luxABCDE), or (D) BM2711-Str<sup>r</sup>/pAL2(pVA838Ω<sub>pami</sub>luxABCDE). At the indicated times, colonization was monitored by bacterial enumeration in the faeces plated on either streptomycin (100 µg/ml), erythromycin (150 µg/ml), or spectinomycin (50 µg/ml), and by measuring the bioluminescent signal in faeces or (E) transcutaneously in whole animals. At every time point, Log<sub>10</sub> means of the CFU per gram of faeces, photons/sec per gram of faeces, and photons/sec in whole animals for each set of four mice in a given experiment are plotted with standard errors.
FIG. 4. Intestinal colonization by *E. coli* MG1655-Str⁺/pAL2 of mice treated with various concentrations of streptomycin. The Log\(_{10}\) means of CFU/g of faeces (A) and photons/s in whole animal (B) for each set of two mice are presented for every time point with standard error bars.

FIG. 5. Transcutaneous bioluminescence kinetic profile after intragastric administration of D-\(\text{D-luciferin}\). D-luciferin was administered intragastrically 24 h after bacterial inoculation of mice colonized with *E. coli* 55989-Str⁺ harboring pAT882(pAT113\(\Omega\)P\(_{tet}\)lucR) or pAT883(pAT113\(\Omega\)P\(_{tet}\)lucG), and the red ( nhiên) or green ( ) bioluminescent signal was monitored for 22 h.

FIG. 6. In vitro simultaneous bioluminescent monitoring of various bacterial mixtures of green and red emitting *E. coli* 55989-Str⁺ (A) Spectral emission of the green (Val\(_{241}\)Ile/Gly\(_{246}\)Ala/Phe\(_{250}\)Ser) and red (Ser\(_{284}\)Thr) luciferase mutants. (B) ca. \(10^8\) CFU/ml suspensions of mixed green- and red-emitting bacteria at the following ratios: 0:100, 20:80, 40:60, 60:40, 80:20, and 100:0 were analysed. Similar ratios of green- or red-emitting bacteria with non-bioluminescent *E. coli* 55989-Str⁺ were analysed. The enzymatic reaction was initiated by adding Chroma-Glo reagent just before imaging. Mean photons/s red and green values are plotted for each dilution and standard errors are indicated by bars.

FIG. 7. In vivo BLI of mice colonized with mixtures of red and green emitting *E. coli*. Various proportions (20:80, 50:50, 80:20, 100:0) of red and green emitting 55989-Str⁺ were inoculated orogastrically into streptomycin-treated mice. The bioluminescence signal was measured transcutaneously (A) and in fecal samples; (B) 24 h after inoculation to mice, using optical filters. Values are means of photons/s/g of faeces from two mice.
REFERENCES


<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655-Str&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Spontaneous Str&lt;sup&gt;r&lt;/sup&gt; mutant of MG1655 (CGSC n° 7740) F&lt;sup&gt;−&lt;/sup&gt;, λ&lt;sup&gt;+&lt;/sup&gt;, ilvG&lt;sup&gt;−&lt;/sup&gt;, gfb 50, rph-1, Str&lt;sup&gt;r&lt;/sup&gt;</td>
<td>24</td>
</tr>
<tr>
<td>55989</td>
<td>Enteroaggregative strain producing AAF-III fimbriae</td>
<td>2</td>
</tr>
<tr>
<td>55989-Str&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Spontaneous Str&lt;sup&gt;r&lt;/sup&gt; mutant of 55989</td>
<td>22</td>
</tr>
<tr>
<td>BM2711</td>
<td>thi-1, endA1, hsdR17 (r&lt;sub&gt;λ&lt;/sub&gt; m&lt;sub&gt;λ&lt;/sub&gt;), glnV44, recA1, Δ(lac)X74</td>
<td>12</td>
</tr>
<tr>
<td>BM2711-Str&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Spontaneous Str&lt;sup&gt;r&lt;/sup&gt; mutant of BM2711</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSB2025</td>
<td>Superlinker plasmid pSL1190&lt;sub&gt;Ω&lt;/sub&gt;luxABCDE, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>27</td>
</tr>
<tr>
<td>pAL2</td>
<td>pVA8380&lt;sub&gt;Ω&lt;/sub&gt;&lt;&lt;sup&gt;p&lt;/sup&gt;luxABCDE, p15A and pVA749 ori, Em&lt;sup&gt;r&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>pGB2</td>
<td>pSC101 derivative, Sp&lt;sup&gt;r&lt;/sup&gt;, Sm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td>pAT881</td>
<td>pGB2&lt;sub&gt;Ω&lt;/sub&gt;&lt;&lt;sup&gt;p&lt;/sup&gt;luxABCDE</td>
<td>This work</td>
</tr>
<tr>
<td>pGEX-6P-2&lt;sup&gt;Ω&lt;/sup&gt;lucR</td>
<td>Source of luc&lt;sub&gt;R&lt;/sub&gt; (Ser&lt;sub&gt;284&lt;/sub&gt;Thr)</td>
<td>4</td>
</tr>
<tr>
<td>pGEX-6P-2&lt;sup&gt;Ω&lt;/sup&gt;lucG</td>
<td>Source of luc&lt;sub&gt;G&lt;/sub&gt; (Val&lt;sub&gt;241&lt;/sub&gt;Ile, G1y&lt;sub&gt;246&lt;/sub&gt;Ala, Phe&lt;sub&gt;250&lt;/sub&gt;Ser)</td>
<td>4</td>
</tr>
<tr>
<td>pAT113</td>
<td>pACYC184 derivative, Mob+, attTn5545, Em&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>32</td>
</tr>
<tr>
<td>pAT882</td>
<td>pATT113&lt;sub&gt;Ω&lt;/sub&gt;&lt;&lt;sup&gt;p&lt;/sup&gt;luc&lt;sub&gt;R&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pAT883</td>
<td>pATT113&lt;sub&gt;Ω&lt;/sub&gt;&lt;&lt;sup&gt;p&lt;/sup&gt;luc&lt;sub&gt;G&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pTVC-erm</td>
<td>pACYC184 derivative, Mob+, ori pAMβ1, Em&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>P. Trieu-Cuot unpublished</td>
</tr>
<tr>
<td>pAT884</td>
<td>pTVC&lt;sub&gt;Ω&lt;/sub&gt;&lt;&lt;sup&gt;p&lt;/sup&gt;luc&lt;sub&gt;R&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pAT885</td>
<td>pTVC&lt;sub&gt;Ω&lt;/sub&gt;&lt;&lt;sup&gt;p&lt;/sup&gt;luc&lt;sub&gt;G&lt;/sub&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

<sup>a</sup> Resistance to: Ap, ampicillin; Em, erythromycin; Km, kanamycin; Sm, streptomycin plasmid; Sp, spectinomycin; Str, streptomycin chromosome.
FIG. 1

A

MG1655-Str

55989-Str

BM2711-Str

% plasmid stability

0 2 4 6 8

Days

B

pAT881(pGB2Ωamt_luxABCDE)
pAL2 (pVA838Ωamt_luxABCDE)
pSB2025(pSL1190ΩluxABCDE)
pAT882(pAT113Ωtet_lucR)
pAT883(pAT113Ωtet_lucG)
pAT884(pTVCΩtet_lucR)

FIG. 1
FIG. 2
FIG. 3

A  MG1655-Str\(^R\)/pAL2  

B  MG1655-Str\(^R\)/pAT881  

C  55989-Str\(^R\)/pAL2  

D  BM2711-Str\(^R\)/pAL2  

E  

- Total CFU/g of faeces (Str\(^r\))  
- CFU/g of faeces (Em\(^r\) or Sp\(^r\))  
- photons/s/g of faeces  

FIG. 3
FIG. 4

Streptomycin concentration:
- 0 g/L
- 20 mg/L
- 0.5 g/L
- 1 g/L
- 2.5 g/L
- 5 g/L
FIG. 5
FIG. 6
FIG. 7