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Abstract
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NOTES

Monitoring of *Yersinia enterocolitica* in Murine and Bovine Feces on the Basis of the Chromosomally Integrated *luxAB* Marker Gene

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We previously integrated the *luxAB* gene into the *Yersinia enterocolitica* chromosome. In this article, we assessed, by luminometry, the survival of the engineered strain KNG1024 in the digestive tracts of mice and cows. In situ detection and a count of the released strain were performed on feces from orally inoculated BALB/c mice for 24 days. This method is a rapid and reliable system for long-term monitoring of genetically engineered bacteria. In cow feces, the count of *Y. enterocolitica* ranged from 210 to 6,000 CFU/g of feces. This very low count was not detectable by direct luminometry.

Attenuated oral pathogenic bacteria such as *Salmonella* or *Yersinia* spp. have been proposed as live, oral carriers of foreign antigens (2, 3, 12, 13). To achieve this function, they must persist in the host just long enough to induce the immune response and their subsequent spreading into the environment must be limited as much as possible. The survival, persistence, and growth of engineered bacteria in animal guts as well as in feces and stable litter should thus be thoroughly investigated. To realize this, simple, quantitative, and reliable detection methods need to be developed. A detection marker may be located on a plasmid which is introduced into the released host bacteria. However, extra-chromosomal marker systems are inherently unstable because of the tendency of many plasmids to segregate at cell division during growth after release (7, 10). In addition, it may not be good practice to use plasmid systems because of their greater potential than chromosomal systems to disseminate recombinant DNA to microbial populations. We previously designed a vector that facilitates the introduction of genetic markers into the chromosome of gram-negative bacteria by homologous recombination. As an example of the potentialities of the system, we replaced the *blaA* gene of *Yersinia enterocolitica* encoding β-lactamase A with the *luxAB* gene encoding the bacterial luciferase (for reviews, see references 4 and 6). The latter gene was expressed constitutively from the *Escherichia coli lac* promoter (8). The luciferase system is a potentially powerful tool for use in studying the fate of genetically engineered *Y. enterocolitica* strains in the environment. A plasmid-encoded *luxAB* gene has been successfully used as an in situ detection marker in *E. coli* (11). In this article, we report on a survival study in murine and bovine intestines of *Y. enterocolitica* KNG1024 serotype O:9, using bioluminescence mediated by the *luxAB* marker gene. Our data indicate that *luxAB* integrated into the chromosome could be used as a stable system to monitor strain survival in murine and bovine digestive tracts.

**Bioluminescence measurement of bacterial populations of *Y. enterocolitica***. Measurements of light emission by using *lux* gene fusions can be carried out in vivo in any growth medium without a noticeable effect on the viability of the bacteria (5). The bioluminescence assay was carried out as follows. The samples were diluted in tryptic soy broth supplemented with 0.3% yeast extract (TSB) (Difco, Detroit, Mich.). The substrate was a suspension of 0.1% *n*-decanal (Sigma Chemical Co., St. Louis, Mo.) in water resulting from by a 20-s ultrasonic treatment using a sonifier (model B-12; Branson Sonic Power Co., Danbury, Conn.). A 250-μl volume of diluted sample was mixed quickly with 50 μl of substrate suspension in a 1-ml glass vial, and the vial was immediately introduced into the counting chamber of a Pico-Lite luminometer (Packard Instrument Co., Downers Grove, Ill.). The emitted light (in counts per minute) was scored for 10 s before and for 10 s after the addition of *n*-decanal. The background luminescence value was subtracted from the values obtained with the substrate. Bioluminescence values were normalized to 1 ml of bacterial suspension. To establish the correlation between the cell density of *Y. enterocolitica* KNG1024 and the bioluminescence activity, we diluted a mid-exponential-phase culture as described above. We measured bioluminescence and counted the number of viable cells on tryptic soy agar supplemented with 0.3% yeast extract (TSA) (Difco). A plot of counts per minute per milliliter against different densities of KNG1024 gave the straight line shown in Fig. 1. As shown, the bioluminescence activity in the liquid medium was directly proportional to the number of viable cells, from 2.5 × 10⁴ to 2.5 × 10⁶ CFU ml⁻¹. According to the graph, 1 cpm was equivalent to 16 bacterial cells. No linearity was observed between the bioluminescence activity and the cell density above 2.5 × 10⁶ CFU ml⁻¹ (data not shown). The lower limit of detection was about 1.2 × 10⁴ CFU ml⁻¹.

**Monitoring *Y. enterocolitica* KNG1024 in mouse feces**. Four
BALB/c mice (bred at the University of Louvain, Woluwe, Belgium) were orally inoculated by intragastric intubation of about $10^{10}$ *Y. enterocolitica* KNG1024 cells as described by Sory et al. (13). Every 2 days, about 100 mg of feces was recovered and resuspended in 4 ml of TSB. Samples were mixed for 20 s with an Ultra-Turrax TP18/10 mixer (Janke and Kunkel, Stauffen, Germany) and allowed to decant in ice. A 250-μl volume of the upper phase of the sample was removed and analyzed in the luminometer as described above. The results, presented graphically in Fig. 2, are given in number of cells per milligram of feces. These data indicate that the method allowed the in situ enumeration of *Y. enterocolitica* KNG1024 cells in mouse digestive tracts for at least 24 days after intragastric inoculation. Similar results were obtained with the other three mice (data not shown).

During this study, we also assessed the stability of the chromosomally integrated lacAB gene. Since strain KNG1024 was also nalidixic acid resistant, we counted strain KNG1024 cells in the feces of mice, concurrent with the luminometric assay, by plating appropriate dilutions on MacConkey medium (Difco) containing 35 μg of nalidixic acid ml⁻¹. The plates were incubated at 28°C for 2 days, and the number of colonies was scored. We found no significant discrepancy between the numbers of cells per milligram of feces as predicted by luminometric assays and the numbers of CFU per milligram of feces deduced from the viable counting method. All $2.3 \times 10^5$ colonies that were counted were luminescent in thedarkroom after being sprayed with n-decanal. Therefore, we concluded that the chromosomally integrated lacAB marker gene was stable and permitted reliable detection and quantification of released *Y. enterocolitica* cells for several days.

**Survival of *Y. enterocolitica* KNG1024 in bovine guts.** Each of four healthy cows (identified as CEB 034, CEB 052, CEB 055, and CEB 066) was inoculated orally with 165 ml of a suspension containing $1.1 \times 10^5$ cells of *Y. enterocolitica* KNG1024. The inoculation was carried out at the National Institute for Veterinary Research (Machelen, Belgium). Sampling of the fecal material was performed for 36 days. About 500 mg of feces was mixed in 10 ml of TSB medium containing 35 μg of nalidixic acid ml⁻¹ and allowed to settle for about 10 min at room temperature. A 250-μl volume of the decanted upper phase was analyzed by luminometry as described above. Contrary to the study with mice, no light output could be detected directly by luminometry. The failure to directly detect *Y. enterocolitica* KNG1024 cells by luminometry in a nonenriched sample suggested that the number of bacteria per milliliter of sample suspension was below the limit of detection of $1.2 \times 10^3$ cells ml⁻¹. This was not too surprising because, compared to the mouse system, the rumen and large intestine of the bovine system is much larger than the mere 100-fold increase used for inoculum size in the bovine studies. This dilution effect may have had an impact on the results. The sample suspensions were then enriched by overnight incubation at room temperature. After this enrichment, repetitive samples from two cows (CEB 034 and CEB 052) gave positive signals by luminometric assay.

Viable counts were carried out in parallel by plating 100 μl of feces suspension directly on MacConkey medium containing nalidixic acid. After 2 days of growth at 28°C, the colonies were sprayed with a suspension of 0.1% n-decanal and observed in a darkroom to discriminate strain KNG1024 from indigenous nalidixic acid-resistant populations. For a permanent record, colonies were photographed on Polaroid type 677 film (30-min exposure) or transferred to a sterile Hybond-N filter (Amersham) and then exposed for 10 s to an X-ray film. By using this strategy, 1 to 13 colonies of strain KNG1024 per plate could be easily identified among about 30 nalidixic acid-resistant colonies for two cows (CEB 034 and CEB 052). Hence, the cow feces contained between 200 and 2,600 CFU of *Y. enterocolitica* KNG1024 g⁻¹. The survival studies were performed for 36 days. Figure 3 summarizes the results for cow CEB 034. The graph represents the number of bioluminescent CFU per gram of feces versus the time in days after inoculation. Similar results were obtained with cow CEB 052 until day 15. The results of Fig. 3 indicate that *Y. enterocolitica* KNG1024 could survive in the guts of cows for 15 to 27 days. Beyond this period, no KNG1024 cells could be detected either by plating or by luminometry after enrichment.

**Conclusion.** Methods for direct detection and enumeration of genetically engineered microorganisms do not rely on the culturability of bacteria in the sample and consequently are more quantitative than are viable counting procedures (9). Indeed, some bacteria have been shown to become unculturable but retain their viability after exposure to the environment and have been called “non-culturable but viable” (1). Detection by using an in vivo luciferase assay as
described here has many advantages. (i) The assay is simple and quick, and the result is immediate. (ii) There is no need for an expensive chemical substrate (n-decanal). (iii) The method does not require extraction, and many samples can thus be analyzed at the same time. (iv) Provided the number of bacteria is higher than $1.2 \times 10^4$ cells per ml of resuspended sample, there is no need to culture the bacteria; therefore, the method accounts for the number of viable cells in the sample. In this study, bioluminescence has proven to be useful in the detection of recombinant Y. enterocolitica strains in mouse feces for up to 22 days. It has also allowed easy discrimination between inoculated Y. enterocolitica KNG1024 and indigenous populations in cow feces.

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FIG. 3. Survival of Y. enterocolitica KNG1024 in gut of cow CEB 034. Symbols: +, viable counts; + and −, detection and no detection, respectively, of KNG1024 cells by luminometry after enrichment.