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Generation and Comparison of Bioluminescent and Fluorescent *Bacillus licheniformis*

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Abstract The environmental bacterium *Bacillus licheniformis* was transformed with two different shuttle-vectors (pCSS810 and pGFPratiometric) containing insect luciferase and green fluorescent protein genes, respectively. The cells were treated with various antimicrobial agents and the emitted bioluminescence and fluorescence were measured. Plasmid harboring the green fluorescent protein gene was totally segregated without selective pressure, and fluorescent *B. licheniformis* showed a slower growth rate than the wild-type strain; those cells were bright green as visualized by epifluorescent microscopy. However, fluorescence was not correlated to the growth state of cells or affected by the antibiotic treatments. To the contrary, luminescent transformant was found to be stable without antibiotic selection and showed analogous growth behavior compared to non-plasmid-bearing cells. The luminescent strain functioned as a biosensor for the antibiotics employed. Bioluminescence measurements allowed one to determine the viability of the recombinant cells and the kinetics of the antibacterial action could be followed. Thus, the light emission was found to be a reliable, sensitive, and real-time indicator of the "well-being" of cells, whereas fluorescence allowed one to visualize both metabolically active and inactive cells.

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Introduction

Bacillus licheniformis is a Gram-positive, endospore-forming bacterium that has been isolated from soils and plant material worldwide [22]. The species has been used for decades in the large-scale industrial production of exo-enzymes [21]. Specific strains are also used to produce lipopeptide surfactants, peptide antibiotics. and other speciality chemicals [2, 8]. Additionally, there are several agricultural uses of *B. licheniformis* as a plant-growth-promoting rhizobacterium (PGPR) [16, 19, 27] and a bio-control agent [14]. An interesting endeavour is to develop *B. licheniformis* as an expression system for marker genes because the resulting recombinant strains could be useful for several goals, such as a quick and efficient selection of a growth condition suitable in large-scale industrial processes or the in situ detection of strains in the environment. Although a close relative *B. subtilis* was transformed with bioluminescence [7, 9] and fluorescent [28] phenotypes over more than a decade ago, to our knowledge there has not been any study on *B. licheniformis* genetically engineered with spectroscopically active reporter molecules. Furthermore, the recent whole genome sequence data have revealed that the transformation of *B. licheniformis* is much more difficult than the closely related *B. subtilis* because it lacks the competence stage [18].

In this article, we report the first construction of luminescent and fluorescent *B. licheniformis* recombinant strains and we focus on showing their characteristics and basic performances between the two reporter systems.

Materials and Methods

Bacterial Strains and Plasmids

Bacillus licheniformis genomovar I strain 53.3 [11] and *B. licheniformis* genomovar III strain 51.5 [11] were used in this study. The plasmid pGFPratiometric (*E. coli–L. lactis* shuttle vector with chloramphenicol resistance and carrying the *gfp* gene under the control of P32 promoter) was used to obtain the fluorescent phenotype [15]. The plasmid pCSS810 (*E. coli–B. subtilis* shuttle vector with kanamycin and chloramphenicol resistances and carrying the T5 promoter–*lac* operator upstream of the *lucFF* gene) was used to obtain the luminescent phenotype [9]. Wild-type and recombinant strains were cultivated in Luria–Bertani medium (LB) at 37°C and 250 rpm agitation, supplemented with the appropriate antibiotics when necessary.

Transformation by Electroporation

Bacillus licheniformis strains were transformed as described by Xue and collaborators [29]. To prepare electrocompetent cells, an overnight culture was diluted 20-fold in growth medium (LB containing 0.5 M sorbitol) and was grown at 37°C and 250 rpm. The only modification was that the electrocompetent cells were grown to an optical density of 1–1.1 (OD at 600 nm). The cells were harvested by centrifugation at 4°C and 5000 *g* for 5 min, washed twice with ice-cold electroporation solution (0.5 M sorbitol, 0.5 M mannitol, 10% glycerol) and resuspended in 1/40 of the same solution. The electroporation was performed in Bio-Rad (Richmond, CA) electroporation cuvettes (1 mm gap) with 50 ng of plasmid and 60 µL of ice-cold electrocompetent cells. A single electric pulse with setting of 2.1 kV was given, and the cells were then rapidly suspended in 940 µL of outgrowth medium (LB containing 0.5 M sorbitol and 0.38 M mannitol). After 3 h of incubation at 37°C and 150 rpm in a 15-mL tube, the cells were plated onto LB agar plates supplemented with the appropriate antibiotic and incubated for 3 days at 35°C.

Protoplast Transformation

Protoplast preparation and transformation driven by polyethylenglycole (PEG) was performed in accordance with the method described by Quax [17]. An additional regeneration medium used by Gray and Chang [5] was also employed to improve the regeneration of protoplasts. Electroporation of protoplasts was made as reported by Romero and collaborators [20].

Bacillus licheniformis Wild-Type and Recombinants Growth Curves

Sterile LB medium (40 µg/mL chloramphenicol for pGFP-ratiometric; 20 µg/mL kanamycin and 20 µg/mL chloramphenicol for pCSS810) was inoculated with 20% (v/v) overnight culture and incubated at 250 rpm at 37°C. Samples were taken at 1-h intervals for optical density measurement (OD at 600 nm) in Gene Quant Pro (Amersham).

Plasmid Stability

Plasmid segregation was determined by colony assay as described by Cutting and Vander Horn [4].

Light Emission Measurement

Light emission from growing *B. licheniformis* 51.5/pCSS810 cells was measured by adding 100 µL of substrate solution (1 mM D-luciferin in 0.1 M sodium citrate buffer pH 5.0) to 100-µl suitable culture dilutions at room temperature in a 96-well microtiter plate luminometer (Plate Chameleon Multilabel Detection Platform; Hidex, Finland).

Fluorescence Measurement

Green fluorescent protein production of *B. licheniformis* 51.5/pGFPratiometric was measured with a Plate Chameleon Multilabel Detection Platform in a 96-well microtiter plate, suspending 200 µL of growing culture in 0.1 M phosphate buffer, pH 7.5. The 485-nm excitation filter and 535-nm emission filter were used for the fluorescence measurements. Fluorescent cells were visualized under epifluorescence microscopy (Axiostar plus; Carl Zeiss).

Testing of Antimicrobial Agents

Twofold dilution series were made from gentamycin, tetracycline, and carbenicillin starting from 10, 0.8, and 0.4 µg/mL, respectively. For luminescence monitoring, 50 µL of diluted (1:100, v/v) overnight culture of pCSS810-bearing cells were added to 100 µL of each antibiotic dilution; D-luciferin (50 µLl) was added just before light emission measurement. For fluorescence testing of pGFP-ratiometric recombinant cells, 100 µL of diluted (1:2, v/v) overnight culture were added to 100 µL of each antibiotic dilution; the wild-type strain was considered to evaluate the autofluorescence of cells. The microtiter plates were incubated at 35°C and the emitted light and fluorescence were measured after 0, 1, 2, 3, and 4 h.

Results

Transformation, Plasmid Stability, and Growth Rates of Wild Type and Recombinants

The transformation by electroporation of both *B. licheniformis* strains with the two plasmids used in this study gave positive colonies only for strain 51.5. Strain 53.3 was not transformable either by protoplast electroporation or protoplast transformation with PEG even though high protoplast regeneration values were obtained (data not shown). This strain was naturally resistant to chloramphenicol (20 μg/mL), kanamycin (5 μg/mL), and presented mucoid colonies. On the other hand, electroporation of *B. licheniformis* 51.5 with pGFPratiometric using 40 μg/mL of chloramphenicol as the selection, gave a transfer efficiency of 7.4×10^3 CFU/μg of plasmid, and brightly fluorescent cells were obtained (Fig. 1). A significant difference was observed in the transformation yields using pCSS810 selected with 20 μg/mL of kanamycin and 20 μg/mL of chloramphenicol, which gave only one luminescent colony. After 20–25 cell doublings in the absence of antibiotics, the frequency of plasmid-containing cells of *B. licheniformis* 51.5/pCSS810 was 77%, whereas for *B. licheniformis* 51.5/pGFPratiometric, no colonies were obtained without selective pressure.

Differences in growth were obtained for *B. licheniformis* wild-type strain and recombinants. Unlike the wild-type and luminescent strains, which presented the same growth curves, the green fluorescent protein (GFP) recombinant strain showed a slow growth rate and it never reached the same optical density values.

Light Emission and Fluorescence Measurements

Light emission and fluorescence measured as a function of cell growth are shown in Fig. 2. Bioluminescence increased proportionally to cell growth. After 23 h of growth, luminescence was no longer detectable (Fig. 2a), whereas fluorescence did not decrease in the end of the stationary growth state (Fig. 2b).

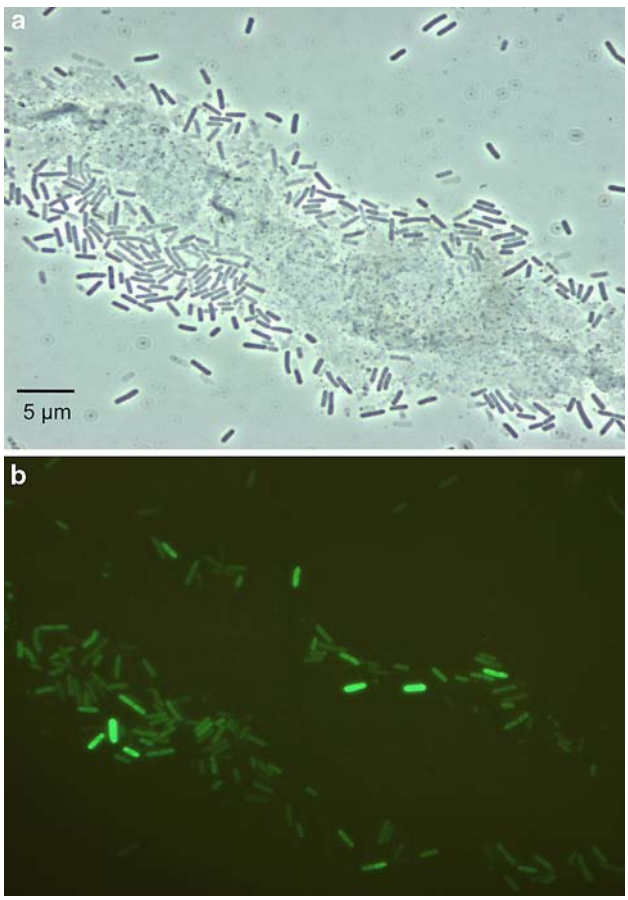

Fig. 1 Images of recombinant *B. licheniformis* 51.5/pGFPratiometric grown overnight under **(a)** a phase contrast microscope and **(b)** an epifluorescence microscope

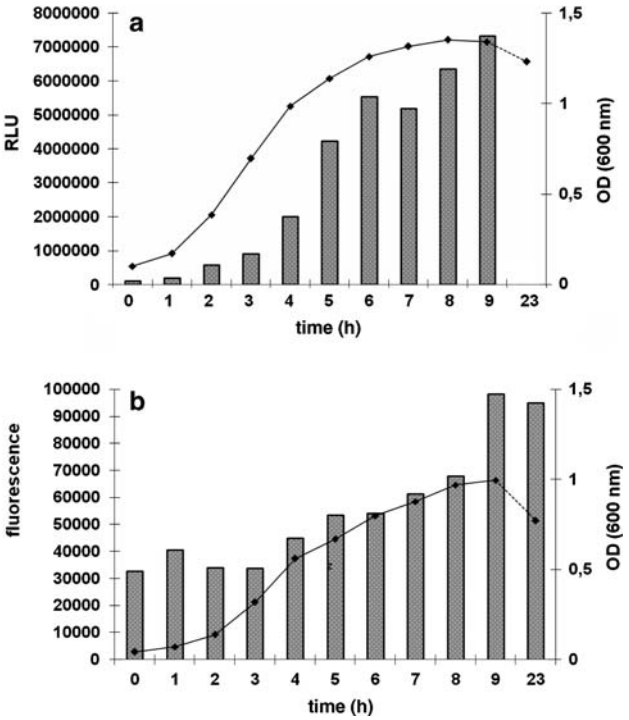

Fig. 2 (**a**) Growth curve of recombinant *B. licheniformis* 51.5/pCSS810 as a function of time and bioluminescence (bars), starting from 2.5×10^7 CFU/mL as determined by plate counting. (**b**) Growth curve of recombinant *B. licheniformis* 51.5/pGFPratiometric as a function of time and fluorescence (bars), starting from 6.5×10^7 CFU/mL as determined by plate counting. The values are means of two independent experiments and the variation between the measurements was less than 10%

Effect of Antimicrobial Agents on Light Emission and Fluorescence

The effect of selected model antibiotics with different mechanism of action was tested against *B. licheniformis* 51.5/pCSS810 (luminescent phenotype) and against *B. licheniformis* 51.5/pGFPratiometric (fluorescent phenotype). The minimum inhibitory concentrations (MICs) of gentamycin and tetracycline were 10 and 1 μg/mL, respectively. *B. licheniformis* 51.5 was not sensitive to carbenicillin. The ffects on light emission were dependent on the antibiotic tested and was seen after 4 h of incubation. Carbenicillin did not affect light emission, even at the highest concentration (Fig. 3a). Gentamycin and tetracycline showed time- and dose-dependent inhibition of light production. Moreover, gentamycin inhibited light production only at the highest concentration (10 μg/mL) (Fig. 3b), whereas tetracycline showed a linear dose response (Fig. 3c).

Fluorescence was not reduced with any antibiotic tested (data not shown).

Discussion

Natural competence is a feature of few *B. licheniformis* strains [6]. *B. licheniformis* 53.3, isolated from soil, was not transformable by both electroporation and protoplast transformation and presented antibiotic-resistant colonies that arose simply because the acquisition of spontaneous resistance. This strain produced extremely mucoid colonies, which suggested the accumulation of glutamyl polypeptide [13] and could be implicated as a potential barrier to transformation [25]. For this reason, we focused our efforts on strain 51.5, which is genotypically different from strain 53.3 [11] and it did not present a mucous appearance growing as well-separated colonies. In accordance to our observation, strain 51.5 gave a transformation yield of 7.4×10^3 CFU/μg of plasmid by electroporation with pGFPratiometric. This result is comparable with the efficiency of the commonly transformable *B. subtilis* that usually shows a transformation yield up to 10^4 CFU/μg of DNA [30]. The fluorescent plasmid in *B. licheniformis* cells determined a lower growth rate and was found to be totally segregated after growing in nonselective medium, limiting the utilization of pGFPratiometric in systems allowing the antibiotic selection. Similar data were obtained for *E. coli*, whereas the presence of the plasmid in *Lactococcus lactis* did not reduce the growth rate [15]. The use of the *gfp* gene as the reporter system found several applications [12], because the GFP of the jellyfish *Aequorea aequorea*, unlike other biomarkers, does not require any substrate or additional cofactors in order to fluoresce [3]. Another useful

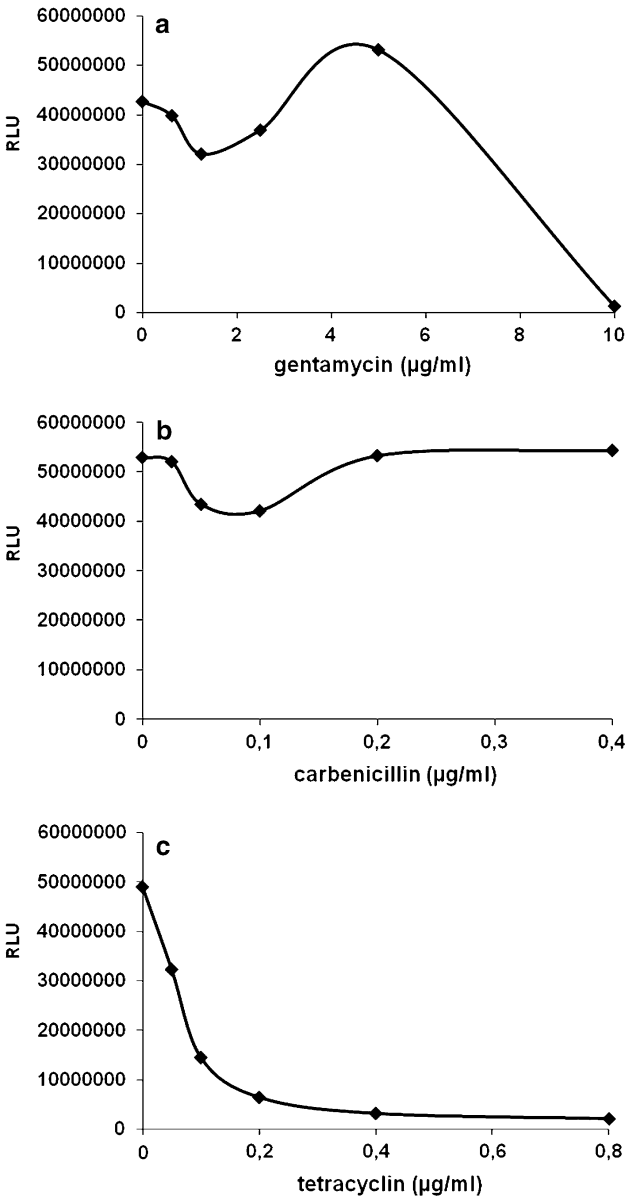

Fig. 3 Effect of carbenicillin **(a)**, gentamycin **(b)**, and tetracycline **(c)** on light emission by *B. licheniformis* 51.5/pCSS810. Luminescence was monitored after 4 h. The values are means of two independent experiments and the variation between the measurements was less than 10%

marker is the insect luciferase, a common used reporter gene that present a direct linkage to ATP metabolism. For this feature, the luciferase activity in vivo has been successfully used as an indicator of the intracellular state of cells and has been demonstrated to be suitable for several applications [23, 26]. The luminescent phenotype of the transformable *B. licheniformis* strain 51.5 was obtained with the plasmid *E.coli–B.subtilis* shuttle vector pCSS810, which gave only one luminescent colony on an antibiotic selective agar plate, however producing high levels of bioluminescence (Fig. 2a). Analogous results were achieved for

the closely related *B. subtilis* species by Lampinen and collaborators [9]. Nonetheless, *B. licheniformis* 51.5/pCSS810 showed analogous performances compared to non-plasmid-bearing cells and was almost stable without selective pressure, suggesting its possible employment in experiments without the antibiotic selection. Comparing the growth curves of recombinant *B. licheniformis* with light emission and fluorescence production, we observed that bioluminescence was affected by the growth phase, as obtained for *Streptococcus mutans* [10]. Differently, higher fluorescent values were found immediately after the inoculum and kept until 23 h of growth. Moreover, high values of autofluorescence were obtained and the sensitivity was lowered. In order to evaluate the behavior of *lucFF*- and *gfp*-based reporter systems in *B. licheniformis* as a function of cellular state conditions, we tested three different antimicrobial agents. With gentamycin, which irreversibly binds to the 30S ribosome and freezes the 30S initiation complex, a good correlation between bioluminescence and MIC was found, as light emission was affected only with a concentration of 10 μg/mL. Similar results were obtained with tetracycline, which did not cause immediate cell death, inhibiting the protein synthesis; a lower concentration clearly affected the light emission but not the growth on liquid medium, indicating disturbances in the intracellular state long before the actual growth was inhibited. Carbenicillin did not affect either bioluminescence or growth in liquid medium. Thus, by measuring the light emission, the viability or steady state of cells was determined. As is evident, the effects of different antibiotics are already seen in a time scale of 4 h compared to conventional overnight cultivation experiments (Fig. 3). Several publications are available describing bioluminescent bacteria as a tool for the high-throughput detection of antimicrobial compounds [1, 10, 24]. In contrast, relative fluorescence was not affected by the antibiotic treatment of cells. Our results demonstrate that only bioluminescence was a real-time indicator of the viability of the cells. *B. licheniformis* strain 51.5 showed biological control properties against fungal plant pathogens (unpublished data) and the light-emitting recombinant could be used in ongoing studies to monitor the physiological state of bacterial biocontrol cells on plant surfaces. Plants' surfaces are indeed characterized by a low nutrients' availability and harbor a wide range of autochthon organisms, which are antibiotic producers. On the other hand, green fluorescent recombinant could be used to study the localization and distribution of *B. licheniformis* 51.5 on plant tissues, as it is an ideal marker due to easy microscopic evaluation.

To summarize, *B. licheniformis* 51.5 was found to be transformable by electroporation with both shuttle vectors, pCSS810 and pGFPratiometric, resulting in a bioluminescent and fluorescent phenotype, respectively. Light-emitting and fluorescent transformants were found to give complementary information. The expression of *lucFF* gene products in *B. licheniformis* 51.5 allowed the determination of the metabolic activity and the viability of the transformed cells by detection of their bioluminescence. On the other hand, GFP expression allowed visualizing metabolic inactive, but also active, cells. Even though the sensitivity of GFP might be compromised by the prevalence of other fluorescent molecules found in many biological systems, it has advantages because it requires no exogenous substrates and is thus not influenced by the kinetics of cellular uptake of substrate or logistics of substrate consumption. For these reasons, fluorescent and luminescent recombinants could be versatile tools for studying the role of *B. licheniformis* individual cells in complex systems (i.e., soil and plants)in which it is naturally found as an autoctone organism.

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