# Chapter 7

# Application of a *Bacillus subtilis* Whole-Cell Biosensor (*PliaI-lux*) for the Identification of Cell Wall Active Antibacterial Compounds

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## Abstract

Whole-cell biosensors, based on the visualization of a reporter strain's response to a particular stimulus, are a robust and cost-effective means to monitor defined environmental conditions or the presence of chemical compounds. One specific field in which such biosensors are frequently applied is drug discovery, i.e., the screening of large numbers of bacterial or fungal strains for the production of antimicrobial compounds. We here describe the application of a luminescence-based *Bacillus subtilis* biosensor for the discovery of cell wall active substances. The system is based on the well-characterized promoter P*liaI*, which is induced in response to a wide range of conditions that cause cell envelope stress, particularly antibiotics that interfere with the membrane-anchored steps of cell wall biosynthesis. A simple "spot-on-lawn" assay, where colonies of potential producer strains are grown directly on a lawn of the reporter strain, allows for quantitative and time-resolved detection of antimicrobial compounds. Due to the very low technical demands of this procedure, we expect it to be easily applicable to a large variety of candidate producer strains and growth conditions.

**Key words** Bio-assay, Reporter gene, Cell envelope stress, Cell wall, Antibiotic, Antimicrobial peptide, Stress response, Luminescence, Lipid II cycle

## 1 Introduction

Biosensors are "devices that use specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles, or whole cells to detect chemical compounds, usually by electrical, thermal, or optical signals," according to the IUPAC definition [1]. In recent years, whole-cell biosensors in particular have gained increasing attention in different fields of application, such as drug discovery or on-site monitoring of environmental samples, e.g., for pollutants such as heavy metal ions or xenobiotics. Compared to enzymes or the other biosensor platforms mentioned above, they offer the advantage of low costs, high stability, and ease of use [2]. Normally, whole-cell biosensors are genetically modified microorganisms that use the stimulus specificity of

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signal-transducing regulatory systems to connect an input (compound or condition to be detected) with a measurable output. The latter is usually provided by a reporter gene under control of a promoter that is regulated by the signalling system.

Three different types of microbial reporter systems are most commonly employed. The β-galactosidase (encoded by *lacZ*) is *the* classical reporter gene and was already established in the early 1970s as a quantitative and highly reproducible measure for differential promoter activity, using the chromogenic substrate ONPG (*o*-nitrophenyl-*β*-D-galactopyranoside) [3]. Despite the disadvantage of having to collect cells and perform a biochemical assay for a quantitative read-out, it is still widespread, since it only requires a standard photometer for colorimetric detection of the enzymatic reaction product at a wavelength of 420 nm. Moreover, *lacZ*-based whole-cell biosensors offer the convenience of a low-cost, simple, and fast semiquantitative readout, if X-Gal (5-bromo-4-chloro-3--indoyl-*β*-D-galactopyranoside) is used as a chromogenic substrate in plate-based whole-cell biosensor assays, which are suitable even for field work in the absence of any technical equipment [4].

More recently, two additional reporter systems have found widespread use in biosensors, namely fluorescent proteins, such as GFP, and bioluminescent reporters, derived from either bacterial or firefly luciferase. While both require more advanced documentation systems for quantitative measurements, their advantage over the β-galactosidase reporter is the possibility for a quantitative online monitoring of promoter activity in viable cells (e.g., growing liquid cultures in microtiter plates) without the need of harvesting cells and performing an assay. While GFP and its many derivatives have found widespread use in numerous biological applications, including whole-cell biosensors [5], the autofluorescence of the cells or media components often limits the dynamic range and hence sensitivity of fluorescence-based biosensors. In contrast, bioluminescence offers a virtually background-free reporter system, thereby enabling biosensors with a high dynamic range and hence sensitivity. Firefly luciferase requires the addition of luciferin as a substrate, which in the presence of ATP and oxygen leads to visible light emission. Bacterial luciferase, encoded by the *luxCDABE* operon of *Photorhabdus luminescence*, catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$) and fatty aldehydes to FMN and fatty acids, respectively, in the presence of molecular oxygen, resulting in blue-green light emission [2]. The substrates of this reaction are regenerated by the normal cellular metabolism and no addition of an external substrate is required, making bacterial luciferase the more convenient and cost-efficient alternative.

This chapter describes the use of a luminescence-based biosensor for the detection of antimicrobial substances that interfere with the membrane-anchored steps of cell wall biosynthesis. It is derived from the *liaI* promoter (P*liaI*) of the Gram-positive model

organism *Bacillus subtilis*, which is strictly controlled by the cell-envelope stress-responsive LiaFSR three-component system [6, 7]. It was first reported to strongly respond to the cell wall antibiotics vancomycin and bacitracin [8, 9]. Subsequently, P*liaI* was thoroughly characterized and developed into a β-galactosidase-based biosensor (P*liaI-lacZ*) for the identification and characterization of *l*ipid-II *i*nterfering *a*ntibiotics (hence the name "*lia*") [4, 10, 11]. P*liaI* possesses a very low basal promoter activity and a highly dynamic response (over 100-fold induction) to its specific inducers, making it ideally suited for screening purposes. More recently, a new P*liaI*-based whole-cell biosensor has been established by employing the bacterial luciferase system from *P. luminescence* [12]. The *luxABCDE* operon used in the resulting P*liaI-lux* biosensor has been optimized for expression in *B. subtilis* [13]. In addition to its convenience and high sensitivity, its short half-life of only 5–10 min allows an almost direct monitoring of not only the induction but also the shut-off of promoter activities, thereby providing another advantage over alternative reporter systems [12].

This chapter provides a detailed protocol of how to apply the P*liaI-lux* whole-cell biosensor for a quantitative bioluminescence detection of antimicrobial compounds interfering with cell wall biosynthesis. This "spot-on-lawn" assay is based on growing a lawn of the reporter strain on solid media and applying spots of potential antibiotic producer strains to this lawn. Production of cell wall active compounds will then induce the activity of P*liaI*, resulting in a ring-shaped luminescence signal around the producer colony that can be visualized and quantified using basic chemiluminescence detection equipment. For details on applying this biosensor strain for quantitative antibiotic induction experiments in liquid cultures, the readers are referred to the previously published procedure [12]. An alternative qualitative assay based on a P*liaI-lacZ* reporter is described in Subheading 4 (*see* **Note 1**).

## 2 Materials

Prepare all media and solutions using deionized waster ($dH_2O$). All reagents can be prepared and stored at room temperature, except for agar plates (4 °C) or where stated otherwise.

All waste containing bacterial cultures should be disposed of according to local regulations. The reporter strain is a class I genetically modified organism, and all handling and disposal should follow good microbiological practice procedures.

This chapter describes standard growth conditions and media for *Bacillus subtilis*. Depending on special growth conditions that may be required by bacterial strains to be tested for antibiotic production, different media and conditions can be used, provided preliminary tests show that the *B. subtilis* reporter strain is able to grow under such conditions.

### 2.1 Media and Reagents

1. Lysogeny Broth (LB): 10 g tryptone, 5 g yeast extract, 10 g sodium chloride. Add $dH_2O$ to a volume of 1 l and autoclave. Adjustment to pH 7 is not normally necessary, but can be achieved by addition of 1 M NaOH (if the pH is too low) or 1 M HCl (if the pH is too high); if required, adjust pH before autoclaving.
2. LB agar: add 15 g/L of agar (1.5% (w/v)) to LB medium prior to autoclaving. Cool down agar to ~50 °C before adding antibiotics. Pour ca. 25 ml of agar per plate into 90 mm sterile petri dishes and let solidify.
3. LB soft agar: add 7.5 g/L of agar (0.75%) to LB medium prior to autoclaving. For immediate use, split into aliquots after autoclaving. For this, transfer 4 ml of molten soft agar into sterile tubes and keep at 50 °C until further use. For later use, let it solidify and store at room temperature until needed.
4. Chloramphenicol stock solution: dissolve 5 mg/ml in 70% ethanol. Store at −20 °C.

### 2.2 Bacterial Strains

1. Reporter strain: *B. subtilis* W168 *sacA*::P*liaI-luxABCDE* (strain TMB1858). This strain contains the promoter P*liaI* fused to the *luxABCDE* luciferase reporter operon [12]. P*liaI* is activated by lipid II cycle interfering antibiotics, such as bacitracin, nisin, ramoplanin, and vancomycin [4]. Upon promotor induction, a chemiluminescence signal is emitted (*see* **Notes 1** and **2**). Growth media should contain 5 μg/ml chloramphenicol.
2. Positive control: *B. subtilis* ATCC6633. This strain is known as a producer of the cell envelope-active antibiotic subtilin and strongly induces the promoter P*liaI* present in the reporter strain [10].
3. Negative control: *B. subtilis* W168. This strain does not produce any compounds that induce the P*liaI-luxABCDE* reporter strain.
4. Strains to be tested for production of cell envelope-active compounds.

### 2.3 Special Equipment

1. For development of this assay, a FUSION-SL™ 16 bit chemiluminescence imaging system with the analysis software FUSION-CAPT™ was used (PEQLAB). Settings described in the methods section refer to this imaging system and software and may vary for other products. Other imaging systems with comparable sensitivity can be used, but exposure times may have to be optimized.
2. Chemiluminescence is quantified using the freely available software ImageJ (http://imagej.nih.gov/ij/). Further calculations can be carried out using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA) or any other suitable software.

## 3 Methods

### 3.1 "Spot-on-lawn" Reporter Screen

1. Preparation of overnight cultures. Set up overnight cultures of the reporter strain and all bacterial strains to be tested, including the positive and negative controls. Employ sterile technique to avoid contamination. Transfer 3 ml of LB medium into a sterile 20–50 ml test tube or universal. Add antibiotics as required, e.g., chloramphenicol from stock (5 mg/ml) to a final concentration of 5 μg/ml for the reporter strain. Inoculate with a single colony of the respective strain from a fresh agar plate. Grow cultures at 37 °C overnight (~16 h) with shaking at 180–220 rpm.
2. Preparation of plates. Warm agar plates in an incubator to 20–30 °C for at least 20 min, or leave at room temperature overnight (*see* **Note 3**). Melt soft agar, transfer 4 ml of soft agar to a sterile screw-cap container, and allow it to cool down to ~50 °C to prevent killing of the cells. Add 120 μl of the reporter strain overnight culture to the soft agar and mix carefully. Pour the entire mixture onto an agar plate and swirl gently. The agar plate should be evenly covered before the soft agar solidifies (*see* Fig. 1a). Avoid air bubbles. Dry plate for ~20 min or until all condensation has disappeared in order to avoid merging of culture drops in **step 3**.
3. Carefully spot 5 μl drops of the overnight cultures of the strains to be tested, including the controls, onto the soft agar (*see* **Note 4**; Fig. 1a–c). When drops are dry, incubate at 37 °C for 1–7 days (*see* **Note 5**).

### 3.2 Luminescence Detection

1. Adjust settings of the FUSION-CAPT™ software by selecting "chemiluminescence" and choosing "full resolution" mode. Open the camera's iris to maximal aperture.
2. At each time point to be monitored during incubation, place the agar plate with the spot-on-lawn culture in the imaging system (*see* **Note 6**). Remove the lid of the plate.
3. With epi-white illumination switched on, bring the plate into focus of the camera using preview mode (*see* **Note 7**; Fig. 1d).
4. Switch off the epi-white light and start the chemiluminescence exposure (10 min). Do not open the door or switch on light during exposure.
5. Save the image (*see* Fig. 1e), place the lid on the plate, and return to the incubator until the next measurement is due. Repeat **steps 1–4**, if more than one plate is used for the assay (*see* **Note 8**).

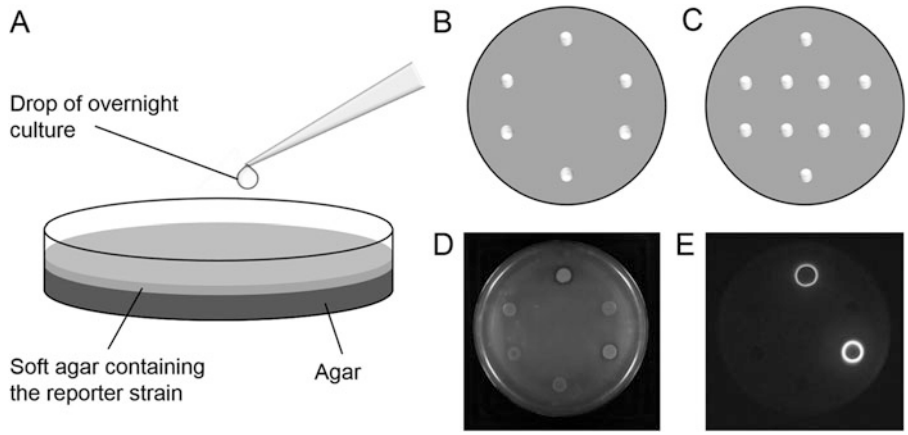


**Fig. 1** Preparation of plates. (**a**) The agar plate is covered by a layer of soft agar containing the reporter strain (*light gray*). Culture drops of potential antimicrobial peptide producers are spotted carefully onto the thoroughly dried plate. (**b**, **c**): Recommended distribution patterns of culture drops. (**d**) Epi-white light image of a plate with spots of potential producer strains growing on the reporter strain lawn. (**e**) Luminescence image of the same plate shown in panel (**d**). Production of cell wall-active compounds (spots) induces the P*lial* promotor of the reporter strain (lawn). Induction results in a ring-shaped luminescence signal of the reporter strain surrounding the producer colony

### *3.3 Luminescence Quantification*

1. After starting the ImageJ software, choose your analysis settings ("*Analyze*" → "*Set Measurements…*"). Tick the box "*Min & max gray value.*"
2. Open the image of the first time point ("*File*" → "*Open…*" or type *O* using the keyboard). Identify the precise location of each colony using the epi-white image of the same plate as a reference (*see* **Note 9**).
3. Click on the measurement tool depicting a straight line (******straight******, *see* Fig. 2). Set a line across the ring-shaped luminescence signal around a colony and select "Measure" ("*Analyze*" → "*Measure*" or type *M* using the keyboard; *see* Fig. 2, orange line). The measurement result will be automatically added to the "*Results*" window.
4. Repeat **step 3** until 20 independent lines have been measured (*see* **Note 10**). Ensure these lines are arranged randomly to measure the different sections of the luminescence circle. Right click on "*Summarize*" to receive the mean maximum value *LS* and the standard deviation *ΔLS*. Repeat measurements for all other colonies on the plate. Transfer all data to Microsoft Excel® or equivalent software for further calculations.
5. To determine the brightness of luminescence intrinsically produced by the reporter strain, follow the same procedure as in **step 4** to set 20 straight lines onto an area of the agar plate

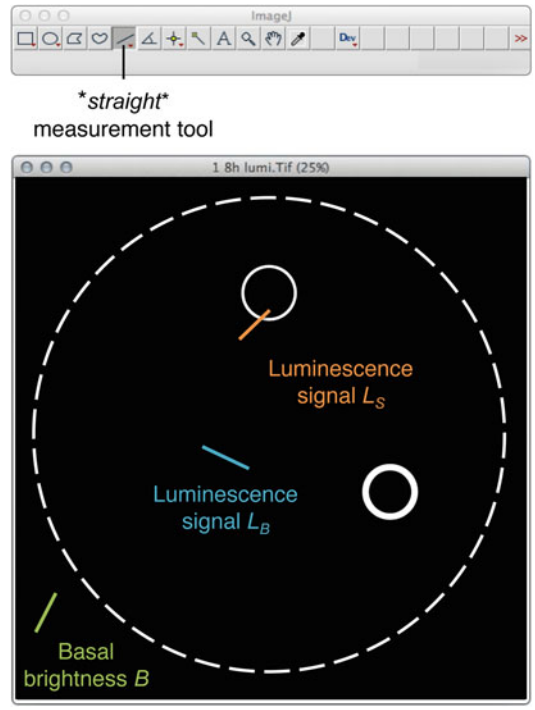


**Fig. 2** Luminescence quantification using ImageJ. For determination of the luminescence signal *LS* of the reporter strain around the colonies, linear measurements are taken across the luminescent ring (*orange line*). The background luminescence signal *LB*, caused by autoinduction of the reporter strain [14], is determined by linear measurements in an area containing no producer colony (*blue line*). For determination of the basal image brightness *B*, linear measurements are taken on areas that are in the image but not on the plate (*green line*). The edge of the agar plate is indicated by a *dashed white circle*, and luminescence around producer colonies is indicated by *solid white circles*. Measurements are automatically summarized by the software in the "*Results*" window

image where no colonies are located. These measurements will yield the mean maximum value *LB* and the standard deviation $\Delta LB$ needed to correct the signal determined for the producer colonies (*see* **Note 11**; Fig. 2, blue line).

6. Similarly, determine the mean maximum value and the standard deviation of the basal brightness *B* of the image by analyzing areas that are still in the image but not on the plate (*see* Fig. 2, green line).
7. To calculate the luminescence signal *S* over the basal brightness *B* of the image, subtract the mean maximum value of the basal brightness *B* of the image from the mean maximum value of the measured luminescence *LS* or the plate background *LB*.

$$S = L - B$$

8. The error propagation of several error-prone values is determined according to the following formula, simplified for two error-prone values in a subtraction.

$$\Delta S = \Delta L + \Delta B$$

*S*: Signal over basal brightness; *L*: Mean maximum value of luminescence (*LS* or *LB*); *B*: Mean of basal brightness; *Δ*: Standard deviation of each parameter; *ΔS*: Uncertainty of *S*.

9. Analysis of the same plate and colonies at multiple time points allows quantification of the signal over time and thus displays the time course of production of cell wall-active compounds (*see* Fig. 3).

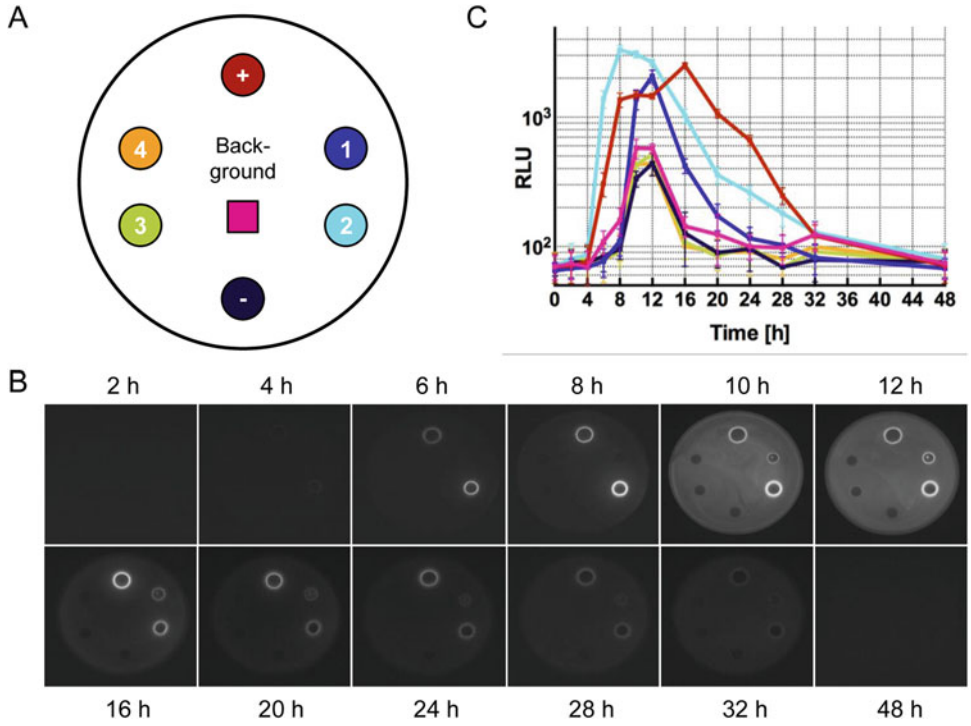


**Fig. 3** Time-resolved quantification of antibiotic production. (**a**) Legend detailing the distribution of spots and identity of tested strains. Numbers indicate candidate producer strains; +, positive control (*B. subtilis* ATCC6633); –, negative control (*B. subtilis* W168); "Background" indicates the quantification of autoluminescence of the reporter strain. Colors match those used in panel (**c**). (**b**) Time course of antimicrobial production for example candidate strains 1–4, as well as positive and negative control strains. Spots of the producer strains are grown on a *B. subtilis* P*lial-luxABCDE* reporter lawn. (**c**) Quantified luminescence of potential producer strains 1–4, the controls, and the autoluminescence of the reporter strain ("Background") caused by stationary phase induction of the reporter strain [14]. Note that tested strains 1 and 2 produce similar levels of luminescence as the positive control strain (i.e., production of a cell wall active compound), while strains 3 and 4 fail to produce a signal above the negative control or reporter strain background level of luminescence (i.e., no production of a cell wall active compound)

## 4 Notes

1. Should no sufficiently sensitive equipment for luminescence detection be available, the assays can be performed in a qualitative manner using a reporter strain carrying a P*liaI-lacZ* construct [4]. In this case, both the base agar and soft agar overlay should be supplemented with 100 μg/ml XGal, added after autoclaving from a stock solution of 50 mg/ml XGal dissolved in dimethylformamide. Induction of the reporter will result in the formation of a blue ring surrounding the producer colony. In theory, the intensity of the blue coloration can be quantified with an image analysis software such as ImageJ. However, in contrast to luminescence, which is a transient emission of photons and where the reporter protein has a very short half-life [12], the beta-galactosidase produced by induction of the *lacZ*-reporter is stable and the blue product of XGal cleavage will accumulate over time. It is therefore not possible to perform time-resolved analyses as described for the luciferase reporter in Fig. 3.
2. The P*liaI*-reporter will respond to cell envelope stress and is therefore a nonspecific reporter for production of substances that interfere with cell envelope integrity, including but not restricted to lipid II cycle interfering antibiotics. If a more specific screen is desired, two alternative reporters can be used. The target promoter of the BceRS-BceAB resistance system of *B. subtilis,* P*bceA*, is known to respond to bacitracin, the lantibiotics mersacidin and actagardine, and the fungal defensin plectasin [10]. The target promoter of the paralogues PsdRS-PsdAB system, P*psdA*, responds to a broad range of cell wall active antimicrobial peptides, such as nisin, subtilin, actagardine, gallidermin, and enduracidin [10]. It is assumed that these lists of inducing substances are not exhaustive and new substrates may be identified from further screens. Fusions of these promoters to the *luxABCDE* reporter may be used, following the same procedures as described here, instead of the P*liaI* reporter to provide some further information on the nature of the substance produced by the strains under investigation.
3. If the agar plate is too cold, the soft agar will solidify before it is evenly spread onto the plate.
4. Culture spots should be distributed equally over the plate (*see* Fig. 1b), as their location on the plate and their proximity to each other could affect the luminescence signal. For an initial screening of producer strains, a higher number of cultures can be tested on a single plate (*see* Fig. 1c). To avoid bacterial aerosols and splashes, gently push the drop halfway out of the pipette tip before placing the drop carefully on the soft agar.

5. Plates may dry out when using a small incubator or longer incubation times. Therefore, it is helpful to wrap the plates in foil.
6. Time points depend on the growth rate of the tested strains and the desired time lapse resolution of antibiotic production and therefore may vary from 2 h intervals to daily measurements over 1–7 days. An initial screening helps to choose the optimal time points and overall duration of experiments.
7. Saving an image of the plate with the light switched on will help to remember the orientation of the plate in case of no or only low luminescence.
8. It might be necessary to enhance the brightness of the image to be able to see the luminescence on screen.
9. Do not adjust the image brightness before or during the analysis. This is essential to allow comparison of the signal strength between the time points.
10. The lines should be distributed around the luminescent ring, as the luminescence signal can be uneven around a colony. The precise length of the measurement lines is not of importance, because only the maximum value will be used for further analysis. Information of angle and length is displayed in the status bar or can be checked in the "*Results*" table.
11. The P*liaI* promoter was found to be autoinduced during transition of *B. subtilis* to stationary phase [14], which results in a transiently increased luciferase activity of the reporter strain in this assay. It is necessary to consider the effect of autoinduction to avoid false positive signals during luminescence quantification. Determination of the background luminescence signal, *LB*, allows appropriate correction of the actual reporter signal, *LS*, obtained for producers of cell wall-active compounds.

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