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Escherichia coli K-12 (pEGFPluxABCDEamp): a tool for analysis of bacterial killing by antibacterial agents and human complement activities on a real-time basis

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ABSTRACT: *Photorhabdus luminescens luxCDABE* genes were integrated into *E. coli* K-12 using a high copy number plasmid containing modified *luxABCDE* genes under the control of the powerful Lac promoter. This strain emitted 10 times higher bioluminescence (BL) than *P. luminescens*. BL production under different growth conditions was studied. In both bacterial strains, the increase in BL signal correlated with the increase in optical density (OD) in a rich growth medium. However, at the logarithmic growth phase, the BL signal was roughly constant. By contrast, in minimal growth media, there was no substantial growth and the BL/cell was approximately five times higher than in the rich medium. The dynamic measurement range of BL was 10^2–10^7 colony-forming units (CFU) in *E. coli* and 10^3–10^7 CFU in *P. luminescens*. Because the decrease in the BL signal correlated with the decrease in CFU and OD, i.e. the number of bacterial cells killed, it proved to be very suitable for assessing the antibacterial effects of different antimicrobial agents. Unlike with plate counting, the kinetics of killing can be monitored on a real-time basis using BL measurements. Complement activities in different samples can be estimated using only one serum dilution. The transformed *E. coli* strain appeared to be superior to *P. luminescens* in these applications because *E. coli* was complement sensitive, the detection limit of *E. coli* was one order lower and the BL-producing system of *P. luminescens* appeared to be quite unstable. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: bioluminescence; bacterial luciferase; Escherichia coli; Photorhabdus luminescens; complement system

Introduction

Viability is traditionally defined as the ability of bacterial cells to form colonies on solid agar plates under suitable conditions and/or to proliferate in solutions with sufficient nutrients. In order to reliably monitor changes in bacterial cell number, real-time data for the whole incubation period are necessary. Optical density (OD) measurement provides such a real-time assay, but the high cell number required for turbidity measurements and an inability to distinguish between live and dead bacteria, restricts the application of this method. The colony-counting assay traditionally estimates bacterial viability and killing. Kinetic measurement of killing by colony counting is troublesome and the results are not obtained on a real-time basis because the plates require a long incubation period. Another method for measuring the number of bacterial cells is the use of bacteria with reporter genes, encoding products with detectable functionality linked to the viability of the cells, such as insect luciferase (*lucFF*) from the firefly *Photinus pyralis* or bacterial luciferase (*luxAB*) from *Vibrio harveyi*. Expression of these genes results in bacterial cells emitting bioluminescence (BL) (1,2,4–8). Green fluorescent protein (GFP) is another commonly used reporter. GFP is highly fluorescent and extremely stabile, and accumulates within the bacterial cell during growth (2–4).

Using these reporters, bacterial viability and the effects of different antimicrobial agents such as antiseptics, toxins, antibiotics and the function of immune mechanisms such as the serum complement system can be studied (2–14). However, both luciferase systems require the addition of an external substrate (4–6). Moreover, the reaction is dependent on substrate transport into the cells and thus is strongly affected by external factors such as pH. GFP works without any additional substrates or cofactors and the fluorescent signal is linked to the number of bacterial cells. However, GFP cannot be used in a killing assay because it is stable and remains fluorescent in dead cells (2,7).

Bacterial luciferase catalyses the oxidation of a long-chain fatty aldehyde and reduced flavin mononucleotide (FMN/$FMNH_2$), simultaneously emitting BL with an emission maximum at 490 nm (15–19). Bacterial luciferase is a chimeric protein with two nonidentical subunits encoded by separate genes (*luxA* and *luxB*). In the *lux* operon, these two genes are flanked by three additional genes (*luxC*, *luxD* and *luxE*) that are involved in the synthesis of its fatty aldehyde substrate. Expression of the whole operon (*luxCDABE)*, under the control of Lac promoter, produces the luciferase holoenzyme complex resulting in bacterial cells emitting BL without addition of any substrate, providing a convenient indicator of metabolic activity in the cell.

In this study, we describe an approach in which *Escherichia coli* K-12 was transformed with plasmid including a modified

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bacterial luciferase gene (*luxABCDE*) originating from the chromosomal *luxCDABE* gene of the Gram-negative soil bacterium *Photorhabdus luminescens* (16,20–24).

The aim of the study was to characterize the differences in light emission between *P. luminescens* and *E. coli* in batch culture and under conditions of biocide or serum-complement-mediated killing. Whether BL was proportional to the number of viable cells and could be used in quantitative bacterial killing assays were also studied.

Experimental

Materials

Agar, Trypton and yeast extract were obtained from Difco Laboratories (Detroit, MI, USA). Polymyxin B was purchased from Sigma (St. Louis, MO, USA) and Etax Aa (99.5% ethanol) was from Altia Oyj (Rajamäki Finland). DNA-modifying enzymes were obtained either from MBI Fermentas (Vilnius, Lithuania) or Promega (Madison, WI, USA). Ampicillin sodium salt was obtained from Sigma. All reagents were of at least analytical grade.

Construction of plasmid *pEGFPluxABCDEAmp*

Modified bacterial luciferase genes (*luxABCDE*) were ligated into *pEGFP* (Clontech, Saint-Germain-en-Laye, France), resulting in the plasmid named *pEGFPluxABCDEAmp*. The *luxABCDE* operon was excised from plasmid *pSB2025* by means of *Nco*I and *Pst*I restriction enzymes. Plasmid *pEGFPluxABCDEAmp* was transformed to *E. coli* K-12 strain M72 (Sm^R*lac*Z(Am) ΔbiouvrBΔtrpE42[λn7(Am)N53(Am)ca857ΔH1]) (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) (25) by electroporation using the Bio-Rad Gene Pulser system (Bio-Rad Laboratories, Richmond, CA, USA), and the engineered *E. coli* K-12 (*pEGFPluxABCDEAmp*) was named *E. coli*–lux. Positive transformants were identified by ampicillin resistance and BL and fluorescence emission.

Preparation of bacterial suspensions

Both *E. coli*–lux and *P. luminescens* subsp. luminescens CCM 7077^T (Czech Collection of Microorganisms, Brno, Czech Republic) were cultivated in 5 mL Luria–Bertani broth (LB) (10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.4) and incubated in a shaker (250 rpm) at 37 °C overnight. Bacterial suspensions were then transferred in 100 mL LB. The suspension was incubated in a shaker (250 rpm) at 37 °C until $OD_{620} = 0.25$, as measured with a UV-1601 photometer (Shimadzu Corporation, Japan). At this OD, the cells were in logarithmic growth and the cultures contained ~ 3.5×10^7 bacterial cells/mL. The cells were harvested by centrifugation at 2500 *g* and resuspended in LB containing 25% glycerol prior to storage at −70 °C. All *E. coli*–lux media contained 100 μg/mL ampicillin to maintain the selection pressure.

Precultivation

Before growth and killing experiments, both bacteria were precultivated by adding 50 μL of freezer stock to 5 mL LB and then incubated (~ 2 h) in a shaker (250 rpm) at 37 °C until $OD_{620} = 0.25$. After incubation, bacterial cells were harvested by centrifugation at 2500 *g*, washed twice with LB, Hanks balanced salt solution (HBSS) or M9 medium (2 mM $MgSO_4$, 0.4% glucose, 0.1 mM $CaCl_2$; M9 salts: 5 mM $Na_2HPO_4.7H_2O$, 16 mM KH_2PO_4, 8.6 mM NaCl, 19 mM NH_4Cl) and resuspended in 5 mL of one of these media. All media used for *E. coli* contained 100 μg/mL ampicillin to sustain the selection pressure.

Batch cultures of *E. coli*–lux and *P. luminescens*

Fifty microliters of washed and resuspended bacterial cell suspensions in LB, M9 or HBSS were pipetted into the wells of a white clear-bottom 96-well microtiter plate (Greiner Bio-One, Düsseldorf, Germany) and 150 μL of fresh LB, M9 or HBSS were added. Plates were incubated at 37 °C in a multimode microplate reader and relative luminescence units (RLU) and OD were recorded continuously over 400 min.

Killing assays

Killing assays were performed with precultured bacteria suspended in 150 μL HBSS containing 5×10^5 to 2×10^6 bacterial cells/well, depending on the experiment. Killing was performed by adding 50 μL of ethanol, polymyxin B or human serum solutions in HBSS. Plates were incubated at 37 °C in the plate reader and both RLU and OD were recorded continuously for 200 min.

Multimode reader

A Mithras LB 940 microplate reader, Berthold Technologies (Bad Wildbad, Germany), controlled by MicroWin 2000 (Mikrotek) was programmed to measure luminescence and OD_{620} simultaneously and continuously at 60-s intervals, 0.5 s/well. The raw data were transferred and analyzed in Excel v. 2007 (Microsoft) and graphs were prepared usingt Origin v. 8 (Microcal).

Serum handling and complement measurement

Human serum was obtained from six healthy volunteers by collecting peripheral blood samples in Venosafe 4-mL serum tubes (Terumo Corporation, Leuven, Belgium). Samples were allowed to clot at room temperature for 45 min and then separated by centrifugation (1500 ***g*** for 20 min), pooled and stored at −70 °C.

The classical complement pathway was measured by adding 50 μL of various dilutions of human serum in HBSS to microplate wells containing 150 μL bacterial suspensions. For the alternative assay pathway, 0.2 M $MgCl_2$ and 10 mM ethylene glycol tetraacetic acid (EGTA) were added to various serum dilutions and incubated for 30 min at room temperature prior to measurement.

Colony-forming units (CFU) measurements

A duplicate microplate, similar to that placed into the multilabel reader, was placed in a laminar hood and incubated in a heating block at 37 °C. At intervals, 100 μL of bacterial suspension from one well at a time was diluted logarithmically 10^1–10^7-fold, and 100 and 20 μL from every dilution were pipetted into Petri dishes containing LB–agar supplemented with 100 μg/mL ampicillinin *E. coli*–lux. Colonies were counted after overnight incubation at 37 °C. The results are presented as CFU/well.

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Results

Growth and the luminescence signal

The background signal was measured from a well containing only the medium and this reading with three standard deviations added was subtracted from the readings obtained from the experimental wells. The RLU background signal was 122 (± 6.2, $n = 150$) and the OD background signal was 0.030 (± 0.001, $n = 150$). Figure 1 shows a typical batch culture experiment in LB performed in the multilabel reader at 37 °C containing bioluminescent *E. coli*–lux (Fig. 1A) and *P. luminescens* (Fig. 1B). In Fig. 1, each point represents the average of four parallel wells. The initial cell number in both cultures was ~ 1.7×10^6 cells/well. During the 120-min incubation, the RLU signal of *E. coli*–lux increased 4.2-fold (from 113,970 to 478,802), whereas the OD increased 4.3-fold (from 0.036 to 0.157). *Photorhabdus luminescens* was incubated for 400 min because of its lower growth rate. During this incubation, the increase in RLU and OD signal was 3.4-fold (from 9987 to 34,370 RLU and from 0.036 to 0.120 OD units, respectively). It must be noted that the OD of plate reader in a volume of 200 µL is 6/10 that of the normal photometer with a light path of 1 cm.

The increase in the BL signal correlated with the increase in OD. It is noteworthy, however, that at the logarithmic growth phase (shown in Fig. 1 by bars), the BL signal was roughly constant. Because the growth rate depends markedly on the composition of the growth media, we compared the generation of BL and OD in two different minimal growth media, M9 and HBSS, in *E. coli*–lux and *P. luminescens* (Fig. 2A,B) with that of LB (Fig. 1).

In both *E. coli*–lux and *P. luminescens*, the nutritionally rich LB supported the most intensive growth. In the minimal growth media, there was no growth during the 200-min incubation period. At the same time, BL/cell (RLU/OD) was approximately five times higher in minimal growth media than in LB during the whole incubation period in both bacterial species.

In order to detect the dynamics of the RLU measurement, we prepared a series dilution and measured BL, OD and CFU in LB at 37 °C. Figure 3 shows RLU and CFU as a function of OD. CFU was measured from five dilution points in *E. coli*–lux (Fig. 3A) and from 11 points in *P. luminescens* (Fig. 3B). The background OD (the lowest detection limit) was 0.030, which for both bacterial species corresponded to ~ 10^6 cells/well. RLU and CFU below this limit are shown as a function of dilution. Figure 3(A) shows that the dynamic measurement range of RLU for *E. coli*–lux is five orders of magnitude, from 10^1 to 10^6 corresponding to 10^2–10^7 CFU. Figure 3(B) shows that the dynamic range of RLU for *P. luminescens* is four orders of magnitude from 10^1 to 10^5 corresponding to 10^3–10^7 CFU.

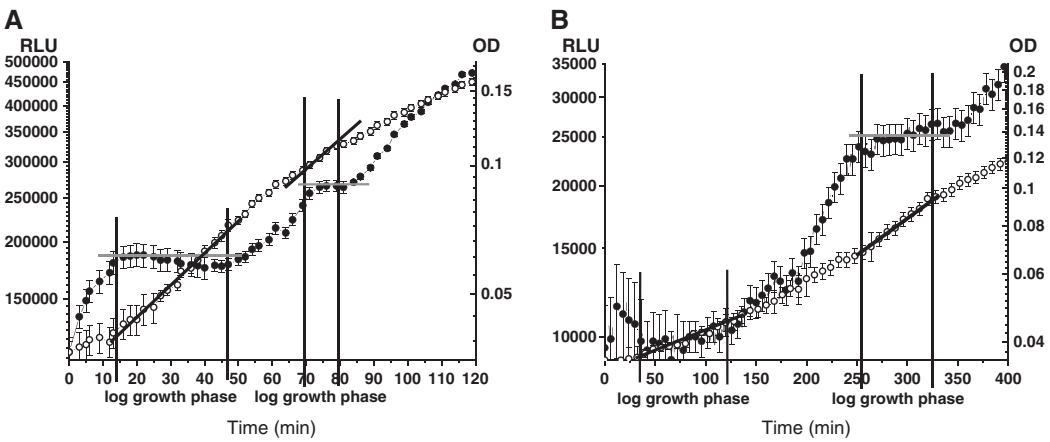

Figure 1. Batch culture (1.7×10^6 cells) of (A) *E. coli*–lux and (B) *P. luminescens* in LB at 37 °C. Bioluminescence (RLU) and optical density (OD_{620}) signals were measured simultaneously. (●) RLU, (○) OD_{620}. Results are shown as the mean ± SD of measurements from four parallel wells. Logarithmic growth phases are shown as bars.

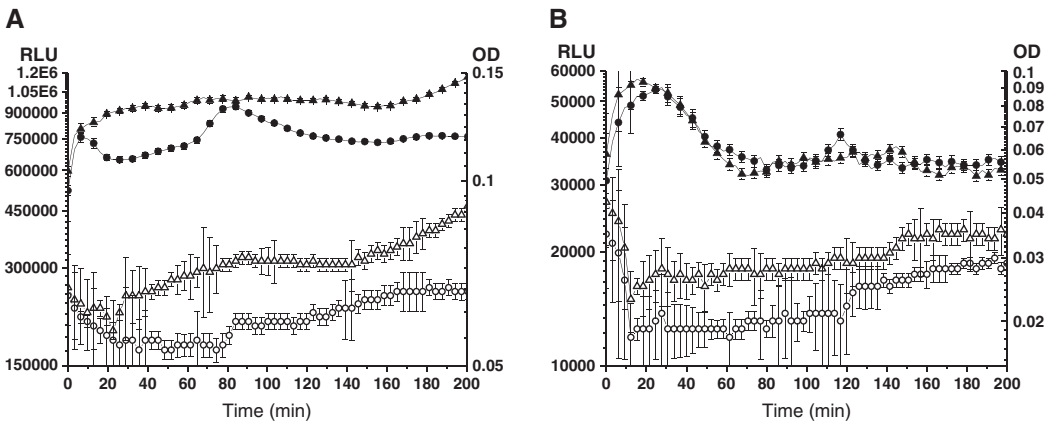

Figure 2. Bioluminescence (RLU) and optical density (OD_{620}) of (A) *E. coli*–lux (1.7×10^6 cells) and (B) *P. luminescens* (1.7×10^6 cells) incubated at 37 °C in two different minimal media (M9 and HBSS buffer). M9: (●) RLU, (○) OD_{620}; HBSS: (▲) RLU, (Δ) OD_{620}. Results are shown as the mean ± SD of measurements from three parallel wells.

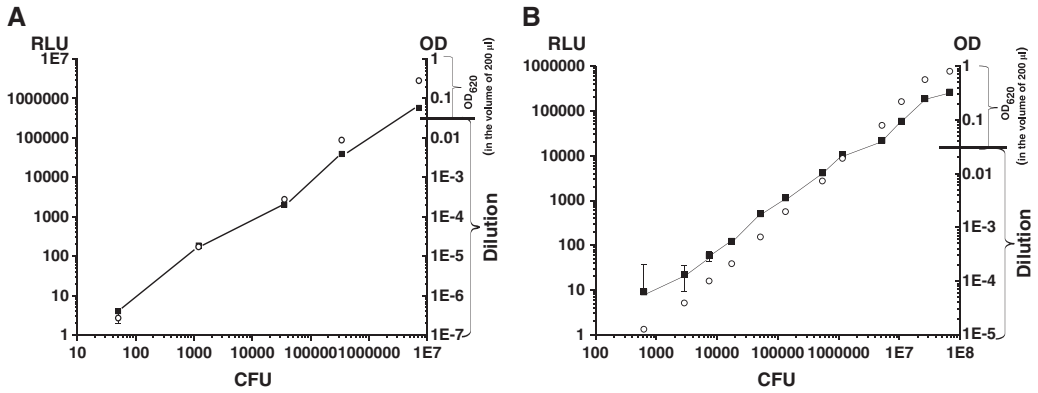

Figure 3. Bioluminescence (RLU) and optical density (OD_{620}) or dilution series as a function of colony-forming units (CFU) of (A) *E. coli*–lux and (B) *P. luminescens* in LB at 37 °C. The lower limit of OD detection (0.03) in the plate reader photometry is marked by a horizontal bar. (■) RLU; (○) OD. RLU results are shown as the mean ± SD of measurements from three parallel wells.

One *E. coli*–lux cell emitted ~ 0.1 RLU, whereas one *P. luminescens* cell emitted ~ 0.01 RLU. This difference may be explained by the fact that lux genes in *P. luminescens* are chromosomal, whereas in *E. coli*–lux they are encoded on a high copy number plasmid. Moreover, the expression of lux genes in *E. coli*–lux was controlled by the strong lac-promoter, whereas there no information regarding the strength of the natural promoter controlling lux gene expression in the *P. luminescens* genome.

Dilution series were prepared from stationary phase cells. To assess whether the proportionality was valid for the whole batch culture, we prepared a series of different cell numbers of *E. coli*–lux and *P. luminescens* (Fig. 4A,B) in LB and incubated the plate at 37 °C.

In summary, these results suggest that under normal culture conditions, RLU values are strict measures of the number of viable cells at the beginning of the exponential growth phase.

The killing assay: antimicrobial agents

We next tested the effects of various antimicrobial agents to assess whether RLU values could be used to measure viability. First, we chose two rapid antimicrobial agents, ethanol and polymyxin B, and then a serum complement system with a slower killing kinetics.

Ethanol. Ethanol is a known antimicrobial agent, killing organisms by denaturing their proteins and dissolving their lipids, and is effective against most bacteria and fungi (4,26). Figure 5 demonstrates the effects of different ethanol concentrations on the CFU, RLU and OD values of *E. coli*–lux (2×10^6 cells) (Fig. 5A,C) and *P. luminescence* (2×10^6 cells) (Fig. 5B,D) in HBSS buffer at 37 °C. Initial reference value with no ethanol was set as 100%. The RLU and OD signals of both bacterial species were measured simultaneously. The final alcohol concentrations in the wells were 0, 1.25, 5, 10 and 20%. CFU values were obtained by taking samples from a parallel plate at 5-min intervals. The reduction in RLU signal was proportional to the reduction in CFU (Fig. 5A,B). By contrast, the decrease in OD (Fig. 5C,D) was apparently smaller because of the much more limited dynamic measurement range.

Both bacteria were effectively killed by ethanol, but *E. coli*–lux seemed to be slightly more sensitive. It is noteworthy that in ethanol the most substantial decrease in viability occurred during the period between pipetting the compounds into the wells and the first measurements (~ 2 min).

Polymyxin B. Polymyxin B is a cationic protein, isolated from *Bacillus polymyxa*, that acts like a detergent, altering membrane permeability and killing target bacteria by destroying the bacterial cell membrane (27).

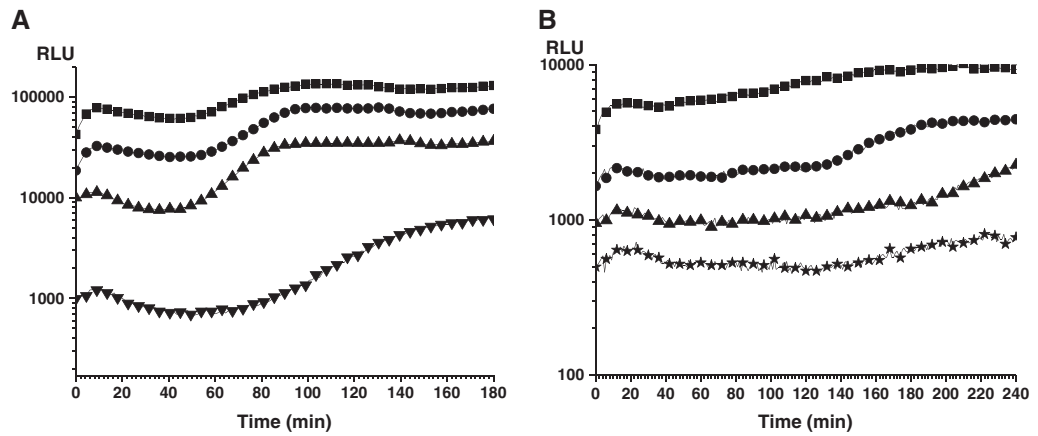

Figure 4. Batch cultures of (A) *E. coli*–lux and (B) *P. luminescens* in LB at 37 °C inoculated with various cell numbers. (■) 5×10^5, (●) 2.5×10^5, (▲) 1.25×10^5, (□) 6.25×10^4 and (▼) 1.25×10^4 bacterial cells.

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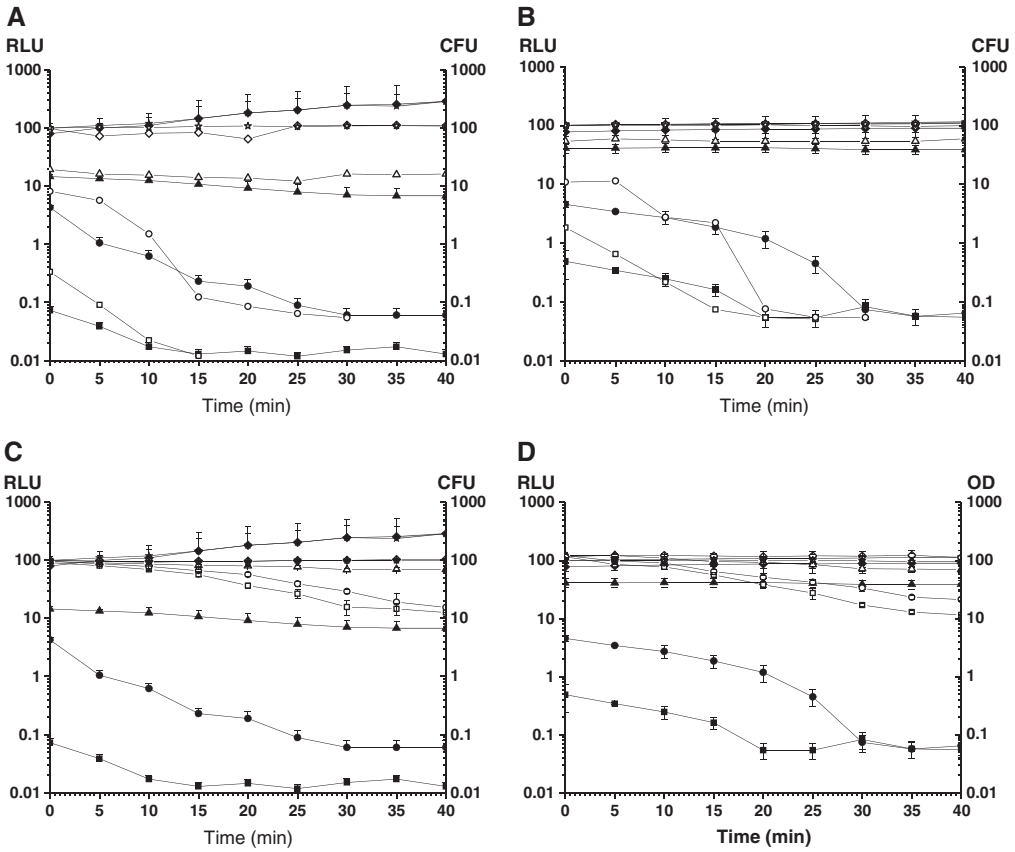

Figure 5. The viability of (A,C) *E. coli*–lux (2×10^6 cells) and (B,D) *P. luminescens* (2×10^6 cells) incubated in the presence of various ethanol concentrations in HBSS at 37 °C. The initial value with no added ethanol was set as 100%. (A–D) RLU: (■) 20%, (●) 10%, (▲) 5%, (♦) 1.25% and (□) 0% ethanol. (A, B) CFU: (□) 20%, (○) 10%, (Δ) 5%, (◊) 1% and (⋆) 0% ethanol. (C, D) OD_{620}: (□) 20%, (○) 10%, (Δ) 5%, (◊) 1.25% and (⋆) 0% ethanol. RLU and OD results are shown as the mean ± SD of measurements from three parallel wells.

The effect of polymyxin B (0, 0.625, 1.25, 2.5, 5 and 10 μg/mL) on bacterial viability, assessed from the RLU and CFU kinetics of both *E. coli*–lux (10^6 cells) (Fig. 6A) and *P. luminescens* (10^6 cells) (Fig. 6B) was measured in HBSS buffer at 37 °C. The initial reference value with no polymyxin B was set to 100%. Polymyxin B killed ~ 10 times more *E. coli*–lux than *P. lumnescens*.

Complement system. The complement system consists of ~ 20 plasma proteins, can be activated by three different routes, and results in the assembly of the common membrane attack complex (MAC). The MAC lyses bacteria by making pores in the bacterial cell membrane (28–30).

Killing of *E. coli*–lux (2×10^6 cells) by 10% human serum in HBSS at 37 °C is illustrated in Fig. 7(A,C; classical pathway) and Fig. 7)B,D; alternative pathway, EGTA-treated serum).

As can be seen in Fig. 7, a certain period (a) is required before the values of RLU and OD start to decrease. We refer to this time as the activation phase, which we believe is the time required for formation of the MAC. The time required to achieve 90% killing by the lytic phase is designated as 'b' and for 90% killing by the

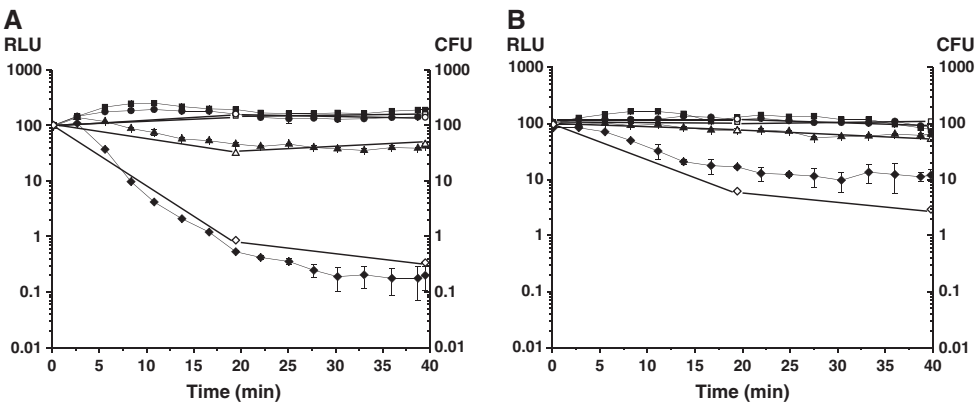

Figure 6. Viability of (A) *E. coli*–lux (5×10^5 cells) and (B) *P. luminescens* (5×10^5 cells) incubated in the presence of various polymyxin B concentrations in HBSS at 37 °C. The initial value with no added polymyxin B was set as 100%. RLU: (■) 0 μg/mL, (●) 0.25 μg/mL, (▲) 0.5 μg/mL and (♦) 1 μg/mL polymyxin B. CFU: (□) 0 μg/mL, (○) 0.25 μg/mL, (Δ) 0.5 μg/mL and (◊) 1 μg/mL polymyxin B. Results are shown as the mean ± SD of measurements from three parallel wells.

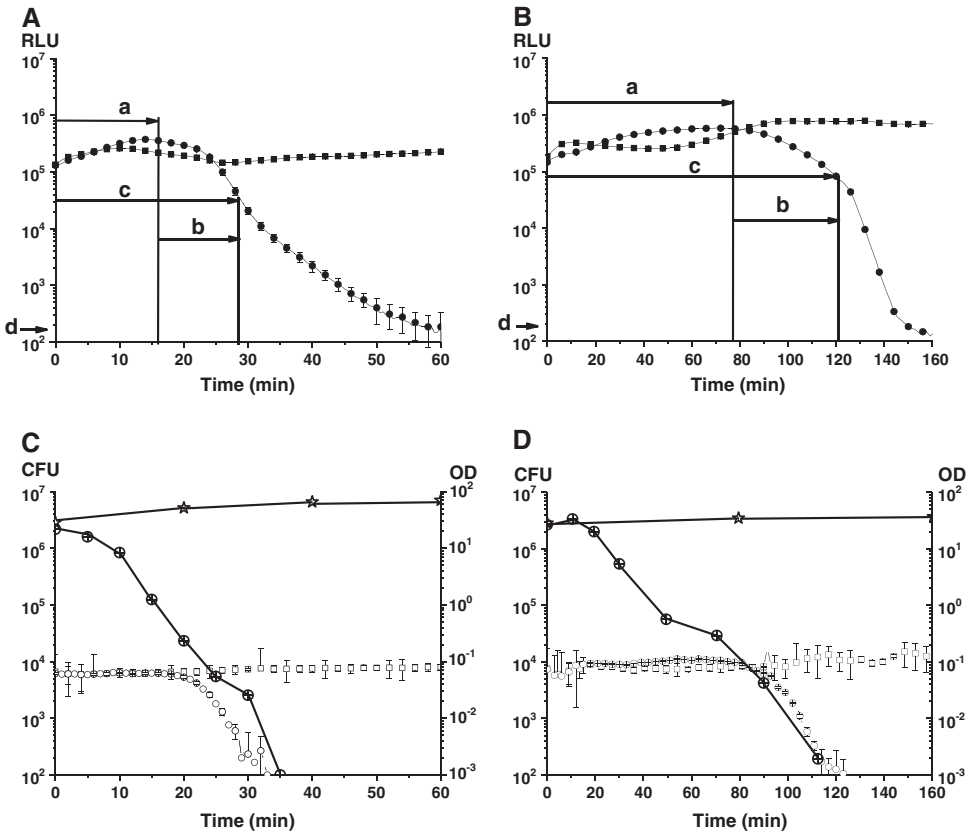

Figure 7. *E. coli*–lux (2×10^6 cells) in the presence of 10% human serum in HBSS at 37 °C. (A,C) Classical pathway of complement and (B,D) alternative pathway of complement. (■) RLU of bacteria, (●) RLU of bacteria + serum, (□) OD_{620} of bacteria, (○) OD_{620} of bacteria + serum, (⋆) CFU of bacteria, (⊕) CFU of bacteria + serum. RLU and OD results are shown as the mean ± SD of measurements from three parallel wells. a, activation phase; b, time required to reach 90% killing by the lytic phase; c, 90% killing by the entire pathway; d, residual level of BL determining the killing capacity.

entire pathway as 'c'. We chose 90% killing instead of 50% (as used in the conventional hemolytic assay) because 50% killing is poorly detectable on a logarithmic scale. The residual level of BL showing the number of residual viable bacteria is designated as 'd'. From the initial and residual number of viable cells, the killing capacity (in number of bacterial cells) of a serum dilution can be determined.

In Fig. 7(A,C) the activation phase 'a' was 17 min, 'b' was 29 min and 'c' was 46 min for both RLU and OD. In the alternative pathway (Fig. 7B,D) the values of 'a', 'b' and 'c' were 78, 43 and 121 min, respectively.

The killing kinetics in CFU did not show any activation phase at all (Fig. 7C) or just the short one as seen in Fig. 7(D). This is possibly because the killing activity of the complement system continues overnight in samples transferred to agar plates. This can be seen in Fig. 7(C) where, at the zero incubation time, 25% of the bacteria were already killed.

Figure 8 demonstrates the effect of the human complement system against *P. luminescens* in HBSS at 37 °C. No antimicrobial effect was discovered during this incubation.

Figure 9(A) shows the effects of different dilutions of human serum, all from the same volunteer human donor, against *E. coli*–lux (5×10^5 cells) in HBSS at 37 °C. The final serum dilutions 0, 1, 2, 3, 4 and 5% formed a dose-dependent kinetics. During the 180-min incubation, 1% serum dilution demonstrated a bacteriostatic effect. By increasing the serum concentration in the reaction mixture, both activation and lytic phase were shortened. Only 5 and 4% serum dilutions reached the total

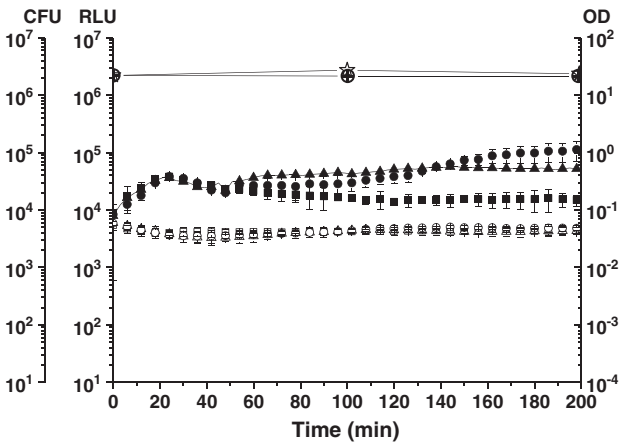

Figure 8. *Photorhabdus luminescens* (2×10^6 cells) in the presence of 10% human serum in HBSS at 37 °C. (■) RLU of bacteria, (●) RLU of bacteria + serum, (▲) RLU of bacteria + serum + EGTA, (□) OD_{620} of bacteria, (○) OD_{620} of bacteria + serum, (Δ) OD_{620} of bacteria + serum + EGTA, (⊕) CFU of bacteria + serum. RLU and OD results are shown as the mean ± SD of measurements from three parallel wells.

killing capacity during the 3 h of incubation, meaning that the RLU signal was reduced to the background level.

Figure 9(B) shows the bacteriolytic activity of the classical pathway in 5% serum from three donors in four parallel measurements. *E. coli*–lux (5×10^5 cells) and serum samples were incubated in HBSS at 37 °C. It can be seen that the reproducibility

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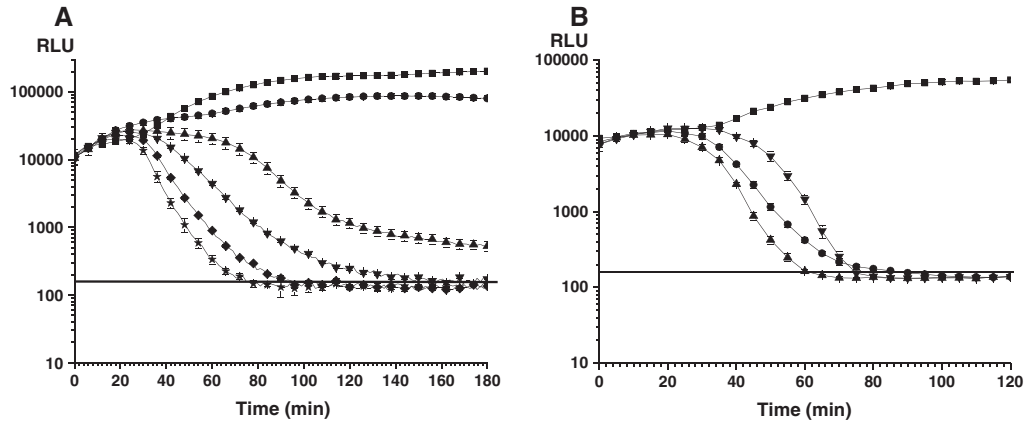

Figure 9. *E. coli*–lux (5×10^5 cells) incubated in the presence of various concentrations of (A) human serum: (■) 0%, (●) 1%, (▲) 2%, (▼) 3%, (♦) 4%, (□); or (B) 5% serum from three different healthy human donors in HBSS at 37 °C: (■) bacteria alone, (●) donor 1, (▲) donor 2 and (▼) donor 3. All results are shown as the mean ± SD of measurements from four parallel wells. The horizontal line represents the background signal + 3 × STDV: [(120 RLU + (3 × 6.3 RLU)].

of this assay is high and the differences between the donors must reflect differences in the complement compound concentration or functionality.

Discussion

Photorhabdus luminescens luxCDABE genes were integrated into *E. coli* K-12 using a high copy number plasmid containing modified *luxABCDE* genes under the control of the powerful Lac promoter. This recombinant strain *E. coli*–lux emitted 10 times higher BL than *P. luminescens*. The likely reason for this is that in *P. luminescens* the original lux genes were located in the chromosome and moreover, were under the influence of upstream regulatory genes *luxR* and *luxI* that control the expression of bacterial luciferase, e.g. by quorum sensing (31–33). The critical cell number of *P. luminescens* for quorum sensing appeared to be ~ 5×10^4 bacterial cells/well in batch culture since 1.25×10^4 cells/well did not emit above-background BL (Fig. 4B).

We have previously used *luxAB* genes from *V. harveyi* in *E. coli* (1,6,34). The problem with this construct was that during the logarithmic growth phase the BL signal elevated considerably more than indicated by OD signal and at the beginning of the stationary phase BL emission collapsed. Moreover, the optimum temperature of used *luxAB* construct was 30 °C (34). The same phenomenon was observed even by using *luxCDABE* from *V. fisheri* in *E. coli* and with *luxABCDE* from *P. luminescens* in *S. aureus* (23,35). The reason for this decreasing BL was suggested to be the decline in the supply of the intracellular reducing power for luciferase (23). Such phenomena were not observed in the present study using *E. coli* transformed with the *luxABCDE* from *P. luminescens*. On the contrary, during fast growth (logarithmic growth in LB) the RLU signal did not increase either in a plasmid derived *E. coli* or chromosome-derived *P. luminescens* systems. The same has been reported when *luxABCDE* genes from *P. luminescens* were integrated into the *E. coli* chromosome (36). The availability of fatty aldehyde has been reported to be the limiting factor in *P. luminescens* luciferase-linked BL emission (1,37). We also added a fatty aldehyde (decanal) into a growth medium for recombinant *E. coli*–lux and observed that the BL signal increased to the level indicated by the increase of the OD in the logarithmic phase (data not shown). Moreover, the bacterial growth in minimal media (M9 and HBSS) was marginal, but the BL signal was significantly higher than in rich LB medium (Figs 1 and 2). Obviously, the availability of fatty aldehyde is higher under slow growth conditions.

The detection limit of our recombinant *E. coli*–lux was a few tens of bacterial cells per well, whereas that of a *P. luminescens* was a few hundred cells (Fig. 3). The BL emission of recombinant *E. coli*–lux also exceeded that of *E. coli* transformed with the *V. harveyi luxAB* gene or *Photinus pyralis lucFF* genes (detection limit ~ 10^4 bacterial cells) used in our laboratory.

The luminometric approach provides a reliable estimation of bacterial viability and killing because the BL signal is directly connected to the metabolic activity of the bacterial cells and thus to the viability of the bacterial population. Because the reduction in RLU signal was correlated with the number of killed bacteria this method proved to be very suitable for assessing the antibacterial effects of different agents. Three separate antimicrobial effects were observed. First, when the signal did not decrease but remained lower than that of the control cells, this represented bacteriostasis, which was visible in OD, CFU and RLU measurements with 1.25% ethanol (Fig. 5) and in RLU measurements with 1% serum (Fig. 9A) (2,5). Second, the decrease in the signal represented a bateriocidal effect, visible in RLU and CFU measurements (10 and 20% ethanol dilution in Fig. 5; serum dilutions > 2% in Fig. 9) (5,38). Third, the decrease in OD signal represented bacteriolysis (Figs 5 and 7) (5,13,38–40).

When incubated in LB, both bacterial species grew and the generation time for *E. coli*–lux was ~ 40 min, whereas that for *P. luminescens* was ~ 230 min (Fig. 1). By contrast, there was no substantial growth in HBSS and M9 (Fig. 2). Hence, in the presence of rapid antimicrobial agents such as ethanol and polymyxin B there was no concern regarding the possible proliferation of viable bacteria in the incubation medium. In the presence of complement, however, especially in the alternative pathway, the activation period was long, allowing bacterial proliferation. Therefore, the use of HBSS, instead of LB, is essential in complement killing assays.

In the complement activity assay, the activation phase 'a' (Fig. 7) was defined as the time required for bacterial cell lysis to begin (5). Lysis of the cells was observed in the reduction in OD. Because the reduction in OD commenced simultaneously with the reduction in RLU and both processes continued with the same kinetics, we assumed that the reduction in RLU also represents bacteriolysis. However, we did not see such an activation phase in CFU readings (Fig. 7C,D). This may be

explained by the observation that complement components transferred with the sample cells into the plates continued their activity during overnight incubation of the plates. This is visible even in the zero time samples in which the CFU was 25% lower than the control value (Fig. 7C). Moreover, when we compared the CFU values in different dilutions of the sample, we noticed that the number of viable cells was always considerably lower in less diluted serum samples (data not shown).

The total hemolytic assay (classical pathway CH_{50} and alternative pathway AH_{50}) is a conventional method for complement activation assessment (12,41). The hemolytic value reflects the ability of the serum complement system to lyse 50% of sheep red blood cells in a given time (90 min). In our real-time application, we could select one serum dilution and then compare the differences in parameters 'a', 'b' and 'c' between samples (Figs 7, 9). Several samples can be analyzed simultaneously and in a fraction of the time needed for traditional CH_{50} and AH_{50}. Moreover, the real-time reaction kinetics of these samples was readable and comparable throughout the whole measurement period (42).

The transformed *E. coli*–lux strain appeared to be superior to *P. luminescens* in these applications because *E. coli*–lux was human-complement-sensitive and the detection limit of *E. coli*–lux was one order of magnitude lower. Moreover, the BL-producing system of *P. luminescens* appeared to be quite unstable because the emission properties gradually disappeared in the deep freeze storage.

The real-time application is useful for both toxicological and immunological monitoring. *Escherichia coli* is known to resist some toxins by preventing their entrance into the cell. For the analysis we can use agents, such as polymyxin B, at moderate concentrations, to increase the permeability of the plasma membrane allowing transportation of the toxic agents inside the bacterial cell (14). This application is convenient for the estimation of complement and phagocyte activity by providing a model with justified targets to these immune systems, living microbes. The advantage is also that this application allows the high throughput screening of large number of samples.

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References

1. Blouin K, Walker SG, Smit J, Turner R. Characterization of in vivo reporter systems for gene expression and biosensor applications based on *luxAB* luciferase genes. Appl Environ Microbiol 1996 Jun;62(6):2013–21.
2. Lehtinen J, Lilius EM. Promethazine renders *Escherichia coli* susceptible to penicillin G: real-time measurement of bacterial susceptibility by fluoro-luminometry. Int J Antimicrob Agents 2007 Jul;30(1):44–51.
3. Lehtinen J, Nuutila J, Lilius EM. Green fluorescent protein–propidium iodide (GFP–PI) based assay for flow cytometric measurement of bacterial viability. Cytometry A 2004 Aug;60(2):165–72.
4. Lehtinen J, Virta M, Lilius EM. Fluoro-luminometric real-time measurement of bacterial viability and killing. J Microbiol Method 2003 Oct;55(1):173–86.
5. Kilpi MK, Atosuo JT, Lilius EM. Bacteriolytic activity of the alternative pathway of complement differs kinetically from the classical pathway. Dev Comp Immunol 2009 Oct;33(10):1102–10.
6. Korpela M, Mantsala P, Lilius EM, Karp M. Stable-light-emitting *Escherichia coli* as a biosensor. J Biolumin Chemilumin 1989 Jul;4 (1):551–554.
7. Lehtinen J, Jarvinen S, Virta M, Lilius EM. Real-time monitoring of antimicrobial activity with the multiparameter microplate assay. J Microbiol Method 2006 Sep;66(3):381–9.
8. Nikoskelainen S, Bylund G, Lilius EM. Effect of environmental temperature on rainbow trout (*Oncorhynchus mykiss*) innate immunity. Dev Comp Immunol 2004 May 17;28(6):581–92.
9. Nikoskelainen S, Lehtinen J, Lilius EM. Bacteriolytic activity of rainbow trout (*Oncorhynchus mykiss*) complement. Dev Comp Immunol 2002 Nov;26(9):797–804.
10. Virta M, Karp M, Ronnemaa S, Lilius EM. Kinetic measurement of the membranolytic activity of serum complement using bioluminescent bacteria. J Immunol Method 1997 Feb 28;201(2):215–21.
11. Virta M, Lineri S, Kankaanpaa P, Karp M, Peltonen K, Nuutila J, *et al*. Determination of complement-mediated killing of bacteria by viability staining and bioluminescence. Appl Environ Microbiol 1998 Feb;64(2):515–19.
12. Porcel JM, Peakman M, Senaldi G, Vergani D. Methods for assessing complement activation in the clinical immunology laboratory. J Immunol Method 1993 Jan 4;157(1–2):1–9.
13. Rozenberg-Arska M, Porsius JC, Jaarsma EY, Verhoef J. Bactericidal, bacteriolytic and opsonic activity of human serum against *Escherichia coli*. J Med Microbiol 1986 Sep;22(2):143–9.
14. Virolainen NE, Pikkemaat MG, Elferink JW, Karp MT. Rapid detection of tetracyclines and their 4-epimer derivatives from poultry meat with bioluminescent biosensor bacteria. J Agric Food Chem 2008 Dec 10;56(23):11065–70.
15. Baldwin TO, Christopher JA, Raushel FM, Sinclair JS, Ziegler MM, Fisher AJ, *et al*. Structure of bacterial luciferase. Curr Opin Struct Biol 1995;5:798.
16. Close DM, Patterson SS, Ripp S, Baek SJ, Sanseverino J, Sayler GS. Autonomous bioluminescent expression of the bacterial luciferase gene cassette (lux) in a mammalian cell line. PLoS One 2010 Aug 27;5(8):e12441.
17. Francis KP, Joh D, Bellinger-Kawahara C, Hawkinson MJ, Purchio TF, Contag PR. Monitoring bioluminescent *Staphylococcus aureus* infections in living mice using a novel luxABCDE construct. Infect Immun 2000 Jun;68(6):3594–600.
18. Meighen EA. Genetics of bacterial bioluminescence. Annu Rev Genet 1994;28:117.
19. Meighen EA. Bacterial bioluminescence: organization, regulation, and aplication of lux genes. FASEB J 1993;7:1016.
20. Babic I, Fischer-Le Saux M, Giraud E, Boemare N. Occurrence of natural dixenic associations between the symbiont *Photorhabdus luminescens* and bacteria related to *Ochrobactrum* spp. in tropical entomopathogenic *Heterorhabditis* spp. (Nematoda, Rhabditida). Microbiology 2000 Mar;146(3):709–18.
21. Daborn PJ, Waterfield N, Silva CP, Au CP, Sharma S, Ffrench-Constant RH. A single *Photorhabdus* gene, *makes caterpillars floppy* (*mcf*), allows *Escherichia coli* to persist within and kill insects. Proc Natl Acad Sci USA 2002 Aug 6;99(16):10742–7.
22. Fischer-Le Saux M, Viallard V, Brunel B, Normand P, Boemare NE. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. Int J Syst Bacteriol 1999 Oct;49(4):1645–56.
23. Qazi SN, Counil E, Morrissey J, Rees CE, Cockayne A, Winzer K, *et al*. agr expression precedes escape of internalized *Staphylococcus aureus* from the host endosome. Infect Immun 2001Nov;69(11):7074–82.
24. Hyrsl P, Ciz M, Lojek A. Comparison of the bioluminescence of *Photorhabdus* species and subspecies type strains. Folia Microbiol (Praha) 2004;49(5):539–42.
25. Bernard HU, Remaut E, Hershfield MV, Das HK, Helinski DR, Yanofsky C, *et al*. Construction of plasmid cloning vehicles that promote gene expression from the bacteriophage lambda pL promoter. Gene 1979 Jan;5(1):59–76.
26. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 1999;12(1):147.
27. Katz M, Tsubery H, Kolusheva S, Shames A, Fridkin M, Jelinek R. Lipid binding and membrane penetration of polymyxin B derivatives studied in a biomimetic vesicle system. Biochem J 2003 Oct 15;375 (2):405–13.

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28. Dankert JR, Esser AF. Bacterial killing by complement. C9-mediated killing in the absence of C5b-8. Biochem J 1987 Jun 1;244(2):393–9.
29. Friec GL, Kemper C. Complement: coming full circle. Arch Immunol Ther Exp (Warsz) 2009 Nov–Dec;57(6):393–407.
30. Sodetz J, Plumb M. Complement: Terminal Pathway. els. 1999. DOI: 10.1038/npg.els.0003977
31. Waters CM, Bassler BL. The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. Genes Dev 2006 Oct 1;20(19):2754–67.
32. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 2005;21:319–46.
33. Waters CM, Lu W, Rabinowitz JD, Bassler BL. Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of vpsT. J Bacteriol 2008 Apr;190(7):2527–36.
34. Winson MK, Swift S, Hill PJ, Sims GG, Bycroft BW, Williams P, *et al*. Engineering the *luxCDABE* genes from *Photorhabdus luminescens* to provide a bioluminescent reporter for constitutive and probe plasmid and mini-Tn5 construct. FEMS Microbiol Lett 1998;163:193.
35. Koga K, Harada T, Shimizu H, Tanaka K. Bacterial luciferase activity and the intracellular redox pool in *Escherichia coli*. Mol Gen Genom 2005;274:180.
36. Sitaras C, Beyde A, Malekazari P, Herrington MB. Light producing reporter plasmids for *Escherichia coli* K-12 that can be integrated into the chromosome. Plasmid 2011x May;65(3):232–8.
37. Yagur-Kroll S, Belkin S. Upgrading bioluminescent bacterial bioreporter performance by splitting the lux operon. Anal Bioanal Chem 2011 May;400(4):1071–82.
38. Taylor PW. Bactericidal and bacteriolytic activity of serum against Gram-negative bacteria. Microbiol Rev 1983;47(March):46.
39. Ginsburg I. The role of bacteriolysi in the pathophysiology of inflammation, infection and post-infectious sequelae. APMIS 2002;110:753–70.
40. Taylor PW. Sensitivity of some smooth strains of *Escherichia coli* to the bactericidal action of normal human serum. J Clin Pathol 1974;27:626–9.
41. Mollnes TE, Jokiranta TS, Truedsson L, Nilsson B, Rodriguez de Cordoba S, Kirschfink M. Complement analysis in the 21st century. Mol Immunol 2007 Sep;44(16):3838–49.
42. Buchtíková S, Vetešniková Šimková A, Rohlenová K, Flajšhans M, Lojek A, Lilius E, *et al*. The seasonal changes in innate immunity of the common carp (*Cyprinus carpio*). Aquaculture 2011;318 (1):169–75.