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Toxin–antitoxin-stabilized reporter plasmids for biophotonic imaging of Group A streptococcus

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Abstract Bioluminescence is a rapid and cost-efficient optical imaging technology that allows the detection of bacteria in real-time during disease development. Here, we report a novel strategy to generate a wide range of bioluminescent group A streptococcus (GAS) strains by using a toxin–antitoxin-stabilized plasmid. The bacterial luciferin–luciferase operon (*lux*) or the firefly luciferase gene (*ffluc*) was introduced into GAS via a stabilized plasmid. The FFluc reporter gave significantly stronger bioluminescent signals than the Lux reporter, and was generally more stable. Plasmid-based luciferase reporters could easily be introduced into a variety of GAS strains and the signals correlated linearly with viable cell counts. Co-expression of the streptococcal ω–ε–ζ toxin–antitoxin operon provided segregational stability in the absence of antibiotics for at least 17 passages in vitro and up to 7 days in a mouse infection model. In addition, genome-integrated reporter constructs were also generated by site-specific recombination, but were found to be technically more challenging. The quick and efficient generation of various M-type GAS strains expressing plasmid-based luciferase reporters with comparable and quantifiable bioluminescence signals allows for comparative analysis of different GAS strains in vitro and in vivo.

Keywords Group A streptococcus · *Streptococcus pyogenes* · Biophotonic imaging · Bioluminescence · Toxin–antitoxin system · Firefly luciferase

Introduction

Streptococcus pyogenes, or group A streptococcus (GAS), is an important human pathogen that is responsible for a wide range of diseases ranging from uncomplicated pharyngitis/tonsillitis and impetigo to severe invasive diseases, such as necrotizing fasciitis ('flesh eating disease') and streptococcal toxic shock syndrome. In addition, GAS causes the poststreptococcal sequelae acute rheumatic fever (ARF), rheumatic heart disease (RHD), and glomerulonephritis (Cunningham 2000, 2008). It was estimated that more than 500,000 people per year die of GAS-related diseases, in particular rheumatic heart disease and invasive GAS diseases (Carapetis et al. 2005). The success of GAS as a human pathogen can be mainly attributed to a plethora of virulence factors, which include host adhesins, cytolysins, spreading factors, immune evasion factors and superantigens (Cole et al. 2011; Lynskey et al. 2011; Olsen et al. 2009).

Animal models, in particular mouse infection models, are routinely used to assess the properties of selected virulence factors. In traditional models, groups of 3–5 animals are infected and sacrificed after selected time points, followed by the removal of organs and tissue to analyze bacterial burden and dissemination. A typical experiment therefore requires between 20 and 60 animals. This number can be significantly reduced by the use of biophotonic imaging (BPI), a non-invasive and non-toxic method that is based on the detection of visible light, usually by a charge coupled

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device (CCD) camera. The light arises from either bioluminescence or the excitation of a fluorescent protein, produced by the modified bacteria. Bioluminescence is usually preferred over fluorescence as autofluorescence is generally far brighter than autoluminescence and therefore generates a lower signal/noise ratio (Troy et al. 2004).

Two reporters are commonly used for the bioluminescent labeling of bacteria: the firefly luciferase gene (*ffluc*) and the bacterial luciferin–luciferase operon (*lux*). The FFluc reporter catalyzes the most efficient bioluminescence reaction known with light emission in the 550–575 nm range producing yellow-green light (Niwa et al. 2010; Zhao et al. 2005), but there are also derivatives, such as the red-shifted firefly luciferase (FFlucRT) with a light emission peak at 610 nm (Andreu et al. 2013). The shift to a red-emitting FFluc was achieved by the introduction of six mutations (Branchini et al. 2007). The *Lux* operon is encoded in the 5.65-kb, five-gene operon (*luxCDABE*) and, in contrast to the firefly luciferase does not require administration of exogenous luciferin substrate. It emits a blue-green light with a maximum intensity at 490 nm (Meighen 1994).

Until today, more than 20 different bacterial species have been labeled for BPI, mainly by the introduction of *luxCDABE* (Andreu et al. 2011). For expression in Gram-positive bacteria, the *luxCDABE* has been modified by reorganization of the genes (*luxABCDE*) and the insertion of Gram-positive ribosome-binding sites (Francis et al. 2000). Francis and colleagues constructed the Gram-positive *Lux* transposon plasmid pAUL-A *Tn4001 luxABCDE* (Km^r) (later commercialized by Xenogen as Xen5) and successfully integrated *luxABCDE* into the *Streptococcus pneumonia* genome (Francis et al. 2001). The same construct was later used to label a *S. pyogenes* serotype M49 strain, where the *luxABCDE* operon integrated into the non-essential sugar phosphate isomerase *spy0535* gene (Park et al. 2003). To our knowledge, this is the only GAS strain for which bioluminescent labeling has been reported.

Integration of the reporter genes into the genome is favored over plasmid-based systems, due to the rapid loss of plasmids in the absence of antibiotic selection markers. However, integration of genes into the genome is often far more technically challenging. Here, we describe the construction of a stabilized, bioluminescent plasmid system that was easily and efficiently expressed by a wide range of GAS strains. The system utilizes the streptococcal ω–ε–ζ toxin–antitoxin (TA) cassette, originally found on the streptococcal plasmid pSM19035 (Lioy et al. 2010) to achieve segregational plasmid stability in non-selective conditions. TA cassettes kill or inhibit the growth of plasmid-free daughter cells by post-segregational killing with the use of at least two plasmid-encoded genes. One gene encodes for a toxin whereas the second gene encodes for either an antisense RNA or a repressor protein that binds and neutralizes the toxin (Jensen and Gerdes 1995). In the streptococcal ω–ε–ζ TA cassette, the zeta-gene encodes a novel kinase that affects bacteria by inhibition of peptidoglycan synthesis (Mutschler et al. 2011) and is neutralized by the labile epsilon-antitoxin protein.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli DH5α (Invitrogen) were grown in Luria–Bertani broth (Difco, BD) at 37 °C with constant agitation. Antibiotics were added when required (100 μg/ml spectinomycin, 50 μg/ml kanamycin). GAS strains SF370 (ATCC 700294; serotype M1), MGAS315 (ATCC BAA-595; serotype M3), MGAS10750 (ATCC BAA-1066; serotype M4), MGAS10394 (ATCC BAA-946; serotype M6), MGAS8232 (ATCC BAA-572; serotype M18) and MGAS6180 (ATCC BAA-1064; serotype M28) were purchased from ATCC. GAS strain 94/229 (serotype M49) is a clinical isolate from New Zealand (Proft et al. 2000). All GAS strains were maintained in Brain Heart Infusion medium (Difco, BD) at 37 °C without agitation. When required, 100 μg/ml spectinomycin or 200 μg/ml kanamycin were added.

Construction of bioluminescent GAS

Construction of plasmids used for chromosomal integration of bioluminescent reporter genes is described in Fig. S1. Construction of TA-stabilized plasmids is described in Figs. S2 and S3. Primers used for construction of plasmids are described in Table 1. Plasmids were first transformed into chemically competent *E. coli* DH5α by the heat shock method. Plasmids were purified from *E. coli* cultures using a Plasmid Midi kit (Qiagen).

To generate electrocompetent GAS, the bacteria were grown in THY medium from single colonies to an $OD_{600\ nm}$ of approximately 0.25. After harvesting by centrifugation, the bacteria were washed twice in ice-cold 0.5 M sucrose and resuspended in 1/300 volume of 0.5 M sucrose. Next, 2 to 4 μl plasmid DNA was added to 50-μl aliquots of competent GAS and electroporated at 2,100 V, 25 μF, 200 Ω in a 2-mm cuvette using a Gene Pulser Xcell™ (Bio-Rad). One milliliter of THY medium was immediately added to the electroporated GAS, followed by incubation at 37 °C for 2 h. The bacteria were then plated onto THY agar containing appropriate antibiotics. Bioluminescent GAS were detected using the IVIS® Kinetic optical imaging system with Living Image® 3.2 software (Caliper Life Sciences). All GAS reporter strains constructed and used in this study are defined by strain codes as listed in Table 2.

Table 1 PCR primers

pFW11E.fw	5′-c**ggaattc** aaaaattgaaaaaatggtgg-3′
pFW11E.rev	5′-c**ggaattc** ttataatttttttaatctgttattta-3′
Spy0535 FR1.fw	5′-gc**ggatcc** gggaag ttacttaggt g-3′
Spy0535 FR1.rev	5′-ccc**aagctt** cttttatcttcccttctag-3′
Spy0535 FR2.fw	5′-aa**ctgcag** gagt atattaatgg agg-3′
Spy0535 FR2.rev	5′-ccc**cccggg** gcgtaaggtggtgatttg-3′
FFluc.fw	5′-**agatct** aaggagg aataaaaa atggaagacgccaaaaacat-3′
FFluc.rev	5′-**ggatcc** ttacaatttggactttccgc-3′
FFRT.fw	5′-cg**gaattc** ggagg atatat atggaagatgctaag-3′
FFRT.rev	5′-cg**gaattc** ttaagctcgggcccc-3′
Lux.fw	5′-cg**gaattc** aaggagg aataaaaaatg-3′
Lux.rev	5′-cg**gaattc** ctcaactatcaaacgcttc-3′

Restriction sites are indicated in bold. Ribosome-binding sites are underlined

Biophotonic imaging of GAS

Overnight cultures of GAS grown in THY medium were diluted 1:20 in fresh broth and grown to mid-log phase (OD_{600} of 0.5 to 0.6), before a serial 1:2 dilution was carried out in Falcon^R 96-well microtiter plates (Becton Dickinson). Fifty microliters from each well was transferred into Falcon^R white opaque 96-well microtiter plates (Becton Dickinson) and 50 μl of THY containing 100 μg/ml of luciferin (Pure Science Ltd., New Zealand) was added. For GAS expressing the *Lux* reporter, THY medium without luciferin was added. The plate was analyzed in an EnSpire™ 2300 Multilabel Reader (Perkin Elmer). The bacteria were then transferred onto Falcon^R black 96-well microtiter plates (Becton Dickinson) and analyzed on the IVIS Kinetic (Caliper Life Science). To analyze the CFUs, bacteria from selected wells were plated for enumeration. All experiments were carried out in duplicates. To determine the detection limits, the complete content of wells that showed bioluminescence just above background were plated without further dilution.

Analysis of plasmid stability

GAS M49 containing either pLZ12Km2-P23R:*ffluc* or pLZ12Km2-P23R:TA:*ffluc* (M49-FFluc (P) or M49-FFluc (P_{TA})) were cultured overnight under static, anaerobic conditions at 37 °C in BHI media supplemented with 200 μg/ml kanamycin. Cultures were passaged daily by 1/100 dilution in BHI media supplemented with or without kanamycin. Each passage was titrated and drop-plated in triplicate on both, BHI or BHI/Kan200 agar daily. The percentage of plasmid carrying cells at each passage was calculated by dividing the number of colonies counted on plates with kanamycin by colonies on plates without kanamycin.

Mouse infection

Female CD1 mice, 7–8 weeks old, were purchased from the Vernon Jansen Unit, University of Auckland, New Zealand. GAS M18 carrying pLZ12Km2-P23R:TA:*ffluc* (M18-FFluc (P_{TA})) was grown to an $OD_{600\ nm}$ of 0.5 in BHI media

Table 2 List of all GAS reporter strains used in this study

Strain code	Original GAS strain	M-type	Construct	Bioluminescent reporter
M1-wt	SF370	M1	–	–
M1-FFluc (I)	SF370	M1	Integrated	FFluc[a]
M1-Lux (I)	SF370	M1	Integrated	Lux[b]
M28-FFluc (I)	MGAS6180	M28	Integrated	FFluc
M49-FFluc (I)	94/229[c]	M49	Integrated	FFluc
M1-FFluc (P_{TA})	SF370	M1	TA plasmid	FFluc
M1-FFlucRT (P_{TA})	SF370	M1	TA plasmid	FFlucRT[d]
M1-Lux (P_{TA})	SF370	M1	TA plasmid	Lux
M3-FFluc (P_{TA})	MGAS315	M3	TA plasmid	FFluc
M4-FFluc (P_{TA})	MGAS10750	M4	TA plasmid	FFluc
M4-Lux (P_{TA})	MGAS10750	M4	TA plasmid	Lux
M6-FFluc (P_{TA})	MGAS10394	M6	TA plasmid	FFluc
M18-FFluc (P_{TA})	MGAS8232	M18	TA plasmid	FFluc
M28-FFluc (P_{TA})	MGAS6180	M28	TA plasmid	FFluc
M28-Lux (P_{TA})	MGAS6180	M28	TA plasmid	Lux
M49-FFluc (P_{TA})	94/229	M49	TA plasmid	FFluc
M49-FFluc (P)	94/229	M49	plasmid	FFluc
M49-Lux (P_{TA})	94/229	M49	TA plasmid	Lux

[a] FFluc denotes firefly luciferase

[b] Lux denotes Gram-positive modified bacterial luciferin–luciferase operon (*LuxABCDE*) (Francis et al. 2000)

[c] Proft et al. (2000)

[d] FFlucRT denotes red-shifted firefly luciferase (Andreu et al. 2013)

containing 200 μg/ml Kanamycin. Bacteria were washed once and then re-suspended in PBS. Mice were inoculated intranasally under anesthesia by depositing 50 μl of 1×10^7–1×10^8 CFU on the exterior portion of the nares. Mice were imaged daily under anesthesia, 5 min after intranasal administration of 25 μl luciferin (15 mg/ml), using the IVIS^R Kinetic optical imaging system with Living Image® 3.2 software. On day 7, all mice were euthanized and nasal regions excised and homogenized in 0.5 ml PBS using the Omni Bead Ruptor Homogenizer (Omni International™). Homogenate was serially diluted and plated for bacterial enumeration. All animal experiments were approved by the animal ethics committee, University of Auckland.

Results

Genome integrated reporter constructs

The only reported bioluminescent GAS, a serotype M49 strain, carries the *luxABCDE* operon, which was randomly integrated into the *spy0535* gene by transposon mutagenesis. Spy0535 is a hypothetical sugar phosphate isomerase and it was shown that deletion of this gene had no impact on growth kinetics and virulence (Park et al. 2003). We therefore decided to directly target this gene for the stable integration of reporter genes using an allelic replacement strategy. Flanking DNA regions of the *spy0535* gene were cloned into the multiple cloning site of vector pFW11 (Podbielski et al. 1996), which are located on both sites of the spectinomycin resistance gene *aad9*. An *Eco*RI site was introduced immediately downstream of the *aad9* stop codon to allow the insertion of reporter genes that were then co-transcribed from a strong synthetic *aad9* promoter. Two different reporter systems were used; the 5.65-kb *luxABCDE* operon and the 1.65-kb fire fly luciferase gene (*ffluc*). Integration of *aad9-luxABCDE* into the bacterial genome proved to be very difficult and could only be achieved after several attempts with the M1-serotype (M1-Lux (I)), probably due to the large size of the *lux* operon. Integration of *aad9-ffluc* was more successful and could be achieved with three out of five M-serotypes (M1-FFluc (I), M28-FFluc (I), M49-FFluc (I)).

To assess if the bioluminescent signal correlated with viable cell counts, mid-logarithmic phase cultures were serially diluted and analyzed in a multiplex plate reader. As shown in Fig. 1a, a linear correlation between bioluminescence and colony forming units (CFUs) was found (R^2>0.98), indicating that the reporter system can be used to quantitatively predict viable bacteria in real-time BPI. There was a clear difference between the two reporters, with FFluc showing a stronger bioluminescent signal compared to Lux. Only 153 CFUs of M1-FFluc (I) were required to reach 85 bioluminescence units (BLUs) and 277 CFUs gave 167 BLUs. The background signal was established by measuring unlabeled GAS at a range between 10^2 and 10^4 CFU resulting in 68±6 BLU. Therefore, the detection limit for M1-FFluc (I) was <150 CFU. In contrast, M1-Lux (I) failed to show any bioluminescence above background at <1,000 CFU (Fig. 1b). Attenuated growth of bacteria carrying the larger *Lux* operon is unlikely, as no difference in growth kinetics was observed between the two reporter strains and M1-wt (Fig. S4).

One of the goals of this study was to generate GAS strains that can be used for BPI in live animals. Therefore, the recombinant GAS strains were also tested in an IVIS real-time bioimager. As expected, the signals for the FFluc expressing strains was higher than for the Lux strain with a detection limit of <10^5 CFUs at 5 min exposure (Fig. 1c) compared to approximately 10^6 CFUs with the M1-Lux (I) strain. Although lower detection limits could be achieved with longer exposure times (data not shown), this is not practical for the use in animals.

Plasmid-based reporter constructs

Plasmid-based reporter constructs are expected to give stronger signals due to gene dose effects. However, selection markers are required to maintain the plasmids. Therefore, bacterial strains carrying plasmid-based reporters are considered not useful in live animals, as it is impossible to distinguish between loss of plasmid in the absence of antibiotics or eradication of the bacteria by the immune response. To circumvent this problem, we decided to introduce a streptococcal TA system into reporter plasmids. TA systems have the ability to stabilize plasmids by post-segregational killing of bacteria that lost the plasmid (Jensen and Gerdes 1995). The omega–epsilon–zeta TA operon, was excised from plasmid pBT286 (Zielenkiewicz and Ceglowski 2005) and cloned into the shuttle vector pLZ12Km2 (Okada et al. 1998) to generate pLZ12Km2:TA. In addition, we introduced the strong lactococcal promoter P23 (Que et al. 2000) to generate pLZ12Km2-P23R:TA. The *ffluc* gene was then introduced into both vectors and FFluc expression was compared in a GAS serotype M1 strain. Interestingly, FFluc expression driven from the lactococcal P23 promoter resulted in a 10- to 15-fold stronger bioluminescent signal compared to expression from the original pLZ12 promoter (Fig. S5). Therefore, all following reporter constructs were generated in pLZ12Km2-P23R:TA. Although all plasmids could easily be electroporated into any M-serotype strain, we noticed the loss of the bioluminescent signal within 1–4 days in most of the strains with the *Lux* reporter. However, this was not due to loss of plasmid, but a reduction in plasmid size probably due to recombination events. Only two strains, M4-Lux (P_{TA}) and M49-Lux (P_{TA}), retained the signal for longer than 4 days.

Similar to the chromosomally integrated reporter genes, the plasmid-based FFluc reporter gave a significantly stronger

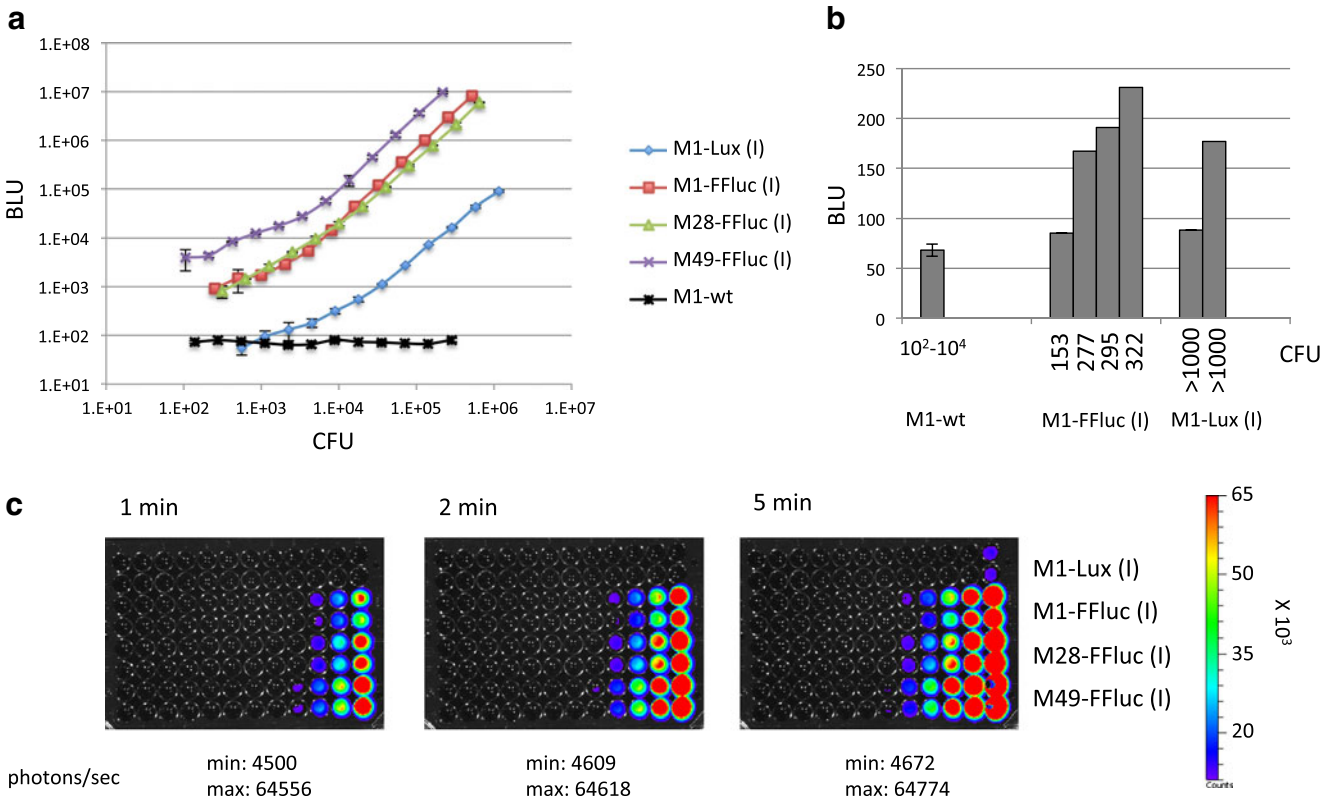

Fig. 1 Biophotonic imaging (*BPI*) of GAS strains with genome-integrated bioluminescence reporter constructs. **a** Bacteria were grown to mid-logarithmic phase and serially diluted in a 96-well microtiter plates. The signal is shown in bioluminescence units (*BLUs*) and plotted against viable cell counts showing a linear correlation. The signal was measured in an Enspire multiplex plate reader. **b** To show BPI detection limits, selected dilutions that show BLUs just above background were directly enumerated by plate counting without further dilution. **c** BPI of the serially diluted bacteria in an IVIS imaging system

bioluminescence signal compared to the Lux reporter in the M49 strain and we found a linear correlation between the number of viable bacteria and the bioluminescence signal (R^2>0.98) for data points above background (Fig. 2a). The detection limit on a multiplex plate reader for M1-FFluc (P_{TA}) was <50 CFUs. Only 36 CFUs of M1-FFluc (P_{TA}) were required to reach 79 BLUs and 51 CFUs gave 105 BLUs, compared with 68±6 BLUs obtained with the unlabeled control strain. In contrast, M49-Lux (P_{TA}) could not be detected at <1,000 CFU (Fig. 2b). Similar results were obtained with M4-Lux (P_{TA}) (Fig. S6). Using the IVIS bioimager, detection limits were approximately 10^4 CFU for the FFluc reporter and 5×10^5 CFUs for the Lux reporter (Fig. 2c).

In addition to the firefly luciferase reporter, we also introduced a plasmid-based derivative with a red-shifted emission spectrum that was codon-optimised for Gram-positive bacteria (FFlucRT). Regarding the signal strength, M1-FFlucRT (P_{TA}) was indistinguishable from M1-FFluc (P_{TA}) (Fig. 2), but might be a good alternative for detection methods where a red light emission is favorable over the yellow-green light emission of the native FFluc, for example in vivo imaging.

Like the GAS strains with chromosomally integrated reporter genes, the plasmid containing strains showed no notable difference in growth kinetics compared to GAS wildtype (Fig. S7). Surprisingly, no gene dose effect was observed, as the plasmid-based constructs did not show increased bioluminescence signals compared to genome-integrated versions.

Plasmid stability

To assess the stability of the reporter plasmids carrying the TA system, GAS M49 containing the plasmid reporter with or without the TA operon (M49-FFluc (P_{TA}) or M49-FFluc (P)) was cultured overnight in the presence or absence of kanamycin. Each day, the cultures were passaged over a total period of 17 days. As shown in Fig. 3, the reporter plasmid that lacks the TA operon started to disappear after 4 passages/days and was completely gone after 9 passages/days when cultures were grown in the absence of antibiotic. In contrast, the reporter plasmid carrying the TA system was stably maintained with or without antibiotic for at least 17 passages/days.

Plasmid stability was also analyzed in vivo using a mouse colonization model. A group of five CD1 mice were infected intranasally with M18-FFluc (P_{TA}) (10^7–10^8 CFU). The mice were monitored daily in a Caliper real-time bio-imager. As shown in Fig. 4 and Fig. S8, a bioluminescence signal could

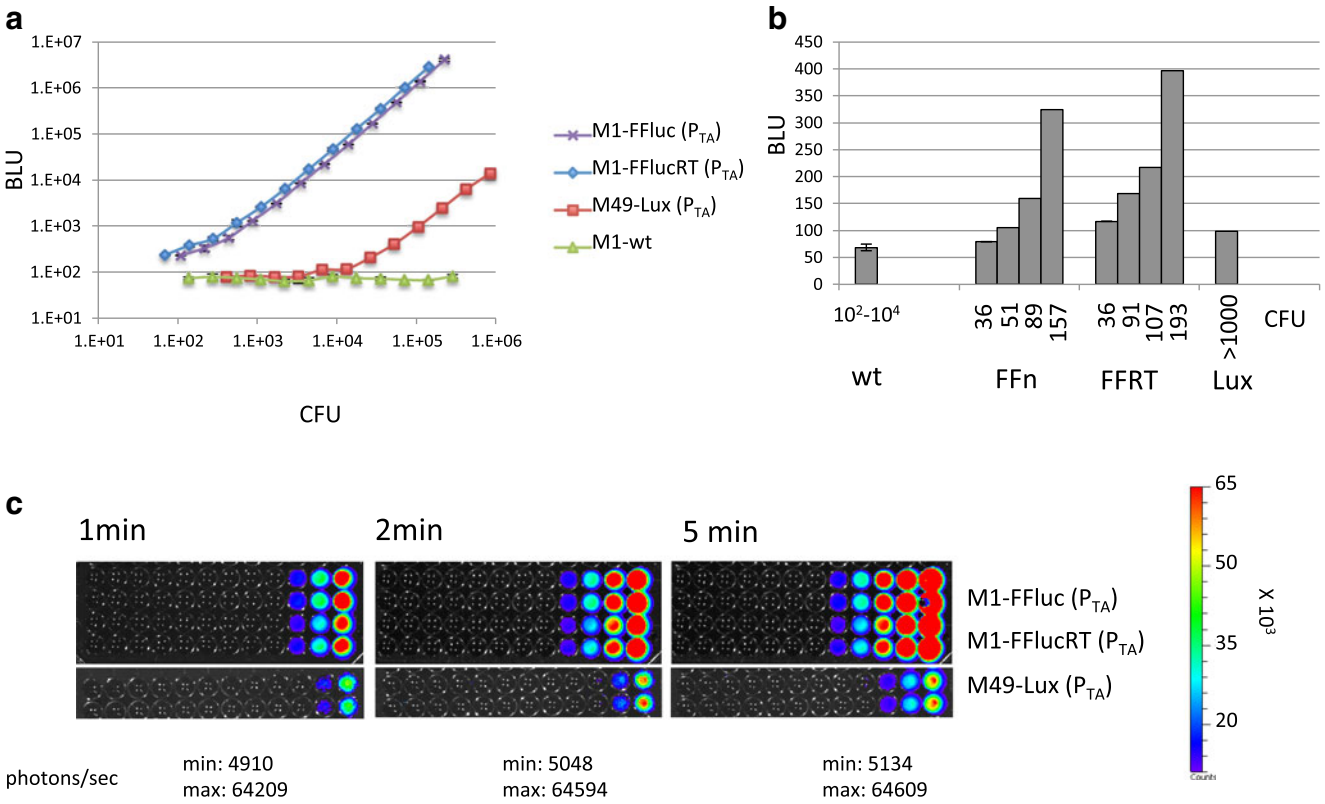

Fig. 2 Biophotonic imaging (*BPI*) of GAS strains carrying a toxin–antitoxin stabilized reporter plasmid. **a** Bacteria were grown to mid-logarithmic phase and serially diluted in 96-well microtiter plates. The signal is shown in bioluminescence units (*BLUs*) and plotted against viable cell counts showing a linear correlation. The signal was measured in an Enspire multiplex plate reader. **b** To show BPI detection limits, selected dilutions that show BLUs just above background were directly enumerated by plate counting without further dilution. **c** BPI of the serially diluted bacteria in an IVIS imaging system

be detected in 5/5 mice at day 3, in 3/5 mice at day 4 and day 5, and in 1/5 mice at day 6 and day 7. After 7 days, the experiment was terminated, the nasal regions of the infected mice were homogenized and bacteria were enumerated on plates containing kanamycin. No bacteria were recovered in mice A, C, D, and E. In contrast, 3.5×10^3 CFU were recovered from mouse B, which correlates with the bioluminescent signal observed in the bioimager. The loss of bioluminescence in the other four mice is not unexpected, as mice are not natural carriers of GAS and, at non-lethal doses, the bacteria are usually cleared by the immune response.

Discussion

We have generated bioluminescent GAS using FFluc and Lux reporter systems, both genome-integrated and by using TA-stabilized reporter plasmids. Although firefly luciferase reporters

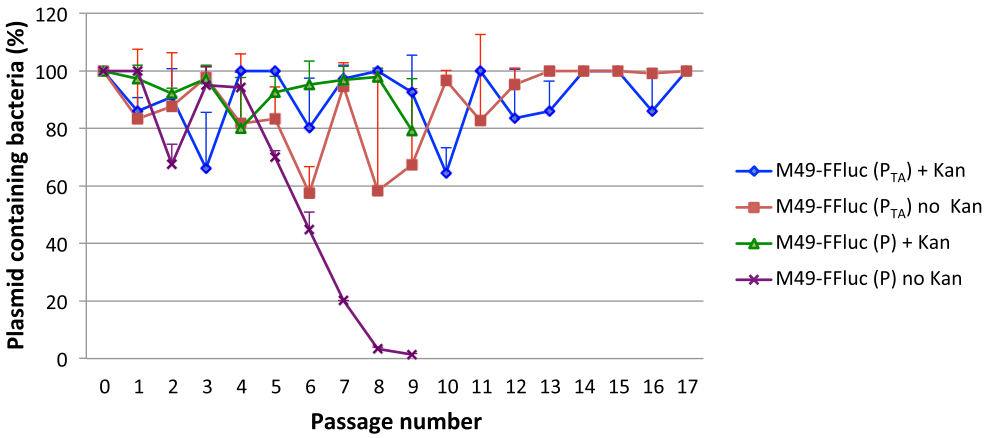

Fig. 3 Stability of the reporter plasmid carrying the toxin–antitoxin system. GAS M49 containing either pLZ12-Km2-P23R:*ffluc* (M49-FFluc (P)) or pLZ12-Km2-P23R:TA:*ffluc* (M49-FFluc (P_{TA})) were cultured overnight in the presence or absence of kanamycin. Each day, the cultures were plated for bacterial enumeration and passaged over a total period of 17 days

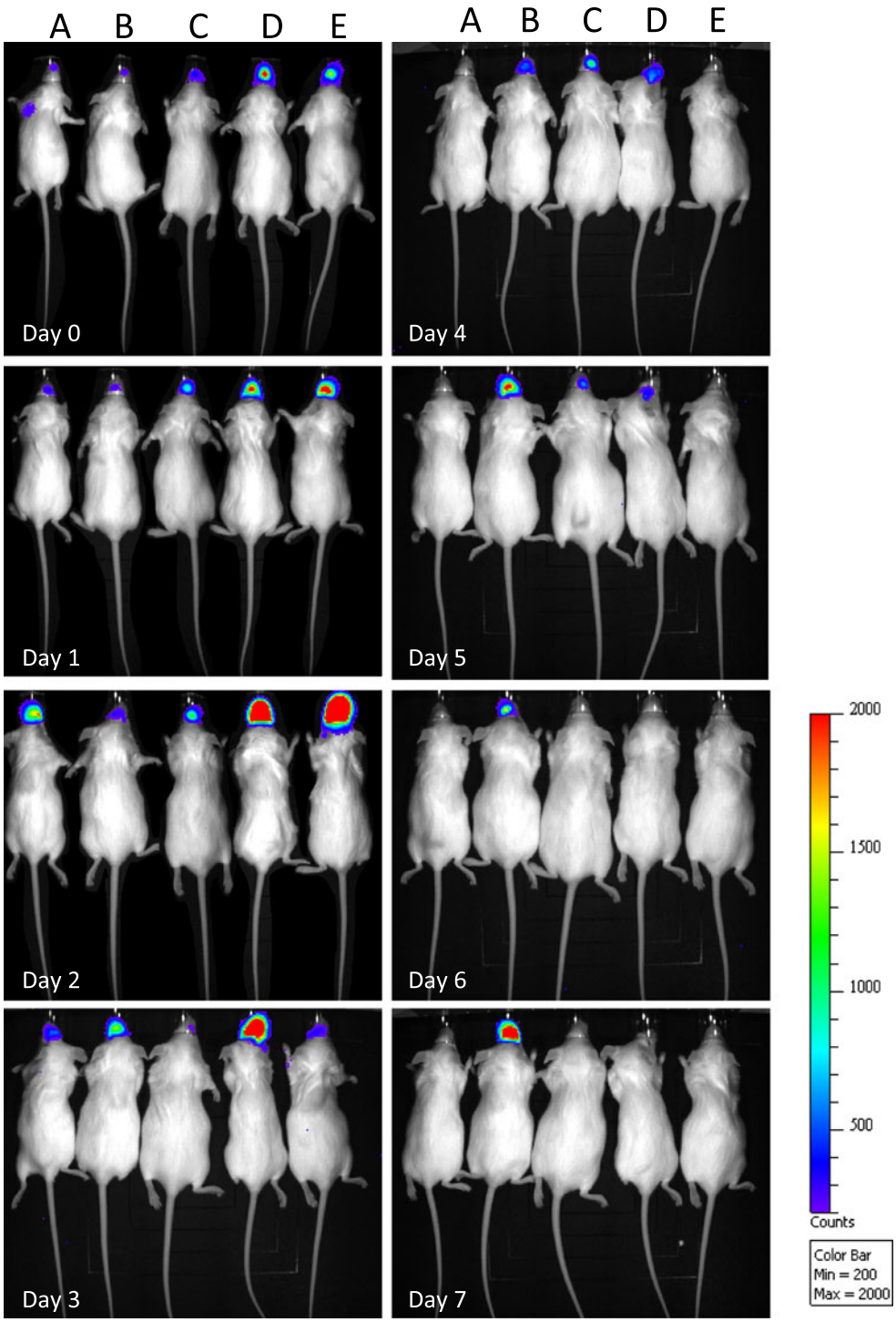

Fig. 4 Intranasal infection of mice with GAS serotype M18 carrying the pLZ12Km2-P23R:TA:*ffluc* reporter plasmid (M18-FFluc (P_{TA})). The mice were monitored over 7 days using a Caliper real-time live animal bioimager

have the disadvantage that a substrate (luciferin) is required for imaging, the bioluminescent signal was far stronger than from the *Lux* reporter. The reason for this difference is not clear, but substrate concentration may play a role. With the *Lux* reporter, the substrate is encoded in the operon and expressed together with the luciferase enzyme. The amount of substrate available may therefore be the limiting factor when comparing with FFluc in which we can provide excess exogenous substrate. In addition, we observed some instability of the *lux* operon in the pLZ12Km2-P23R:TA plasmid probably due to recombination events. This was observed in only some GAS M-types and did not involve the TA cassette. As this happened only with the Lux reporter, we assume that the size of the reporter (5.65 kb *luxABCDE* operon vs. 1.65 kb *ffluc* gene) was responsible for the recombination of the plasmid and hence loss of bioluminescent signal.

There was only a marginal difference in signal strength between the genome-integrated and the plasmid-based reporters. A possible reason could be differences in the promoter regions. While expression of the genome-integrated reporter genes is driven by the *aad9* promoter of plasmid pFW11 (Podbielski et al. 1996), the lactococcal promoter P23 (Que et al. 2000) was used for expression of the plasmid-based reporter genes. However, the plasmid-based system still has a major advantage over the genome-integrated system. Integration of reporter genes require extensive analysis of each modified strain to ensure that allelic replacement has occurred and neighboring genes are unaffected, as this might impact on the fitness of the bacteria. On the other hand, the TA-stabilized plasmids carrying the *ffluc* gene could easily be transformed into all tested M-type strains (M1, M3, M4, M6, M18, M28 and M49) and showed comparable bioluminescence signals, which were proportional to the number of viable cells (Fig. S6). In addition, we have successfully introduced the plasmids into 25 clinical isolates of GAS comprising 20 different M serotypes (data not shown). Due to the ease of cloning reporter genes into the TA-stabilized plasmid, it would be interesting to compare signal strength of other luciferases. For example the luciferase from the marine copepod *Gaussia princeps* (*Gluc*) has also been cloned and compared to the *luxAB* reporter when expressed in *Mycobacterium smegmatis* (Wiles et al. 2005). Authors showed that *Gluc* exhibited enhanced stability to low pH, hydrogen peroxide, and high temperature. In addition luminescence was also retained at stationary phase, in contrast to *luxAB*.

Our approach facilitates the simple and rapid assessment of bacterial loads from a variety of GAS strains without using time-consuming plate enumeration methods. In addition, the signal strength is sufficient for BPI in live animals, which allows analysis of spatial and temporal dynamics of the bacteria during infection. Plasmid-based reporter systems are generally regarded as unsuitable in animal models, as application of antibiotics to provide selective pressure for the plasmid might interfere with the disease model. We have shown that the TA-stabilized FFluc reporter plasmids are stable without antibiotics for at least 17 days in vitro. Plasmid stability in live animals is not easy to assess, as the bacteria are usually cleared by the host immune system within a few days. However, we were able to show plasmid stability in mice for up to 7 days in the absence of antibiotics.

Another advantage of the plasmid-based system is its potential use in isogenic knock-out mutants for a direct comparison with wildtype bacteria, which will assist in the analysis of novel and known virulence factors.

To the best of our knowledge, this is the first report of bioluminescent GAS, apart from a previously described M49 strain expressing a genome-integrated *Lux* reporter (Park et al. 2003) and will facilitate future analysis of GAS pathomechanisms.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Andreu N, Zelmer A, Wiles S (2011) Noninvasive biophotonic imaging for studies of infectious disease. FEMS Microbiol Rev 35:360–394

Andreu N, Zelmer A, Sampson SL, Ikeh M, Bancroft GJ, Schaible UE, Wiles S, Robertson BD (2013) Rapid in vivo assessment of drug efficacy against *Mycobacterium tuberculosis* using an improved firefly luciferase. J Antimicrob Chemother 68:2118-2127

Branchini BR, Ablamsky DM, Murtiashaw MH, Uzasci L, Fraga H, Southworth TL (2007) Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications. Anal Biochem 361:253–262

Carapetis JR, Steer AC, Mulholland EK, Weber M (2005) The global burden of group A streptococcal diseases. Lancet Infect Dis 5:685–694

Cole JN, Barnett TC, Nizet V, Walker MJ (2011) Molecular insight into invasive group A streptococcal disease. Nat Rev Microbiol 9:724–736

Cunningham MW (2000) Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13:470–511

Cunningham MW (2008) Pathogenesis of group A streptococcal infections and their sequelae. Adv Exp Med Biol 609:29–42

Francis KP, Joh D, Bellinger-Kawahara C, Hawkinson MJ, Purchio TF, Contag PR (2000) Monitoring bioluminescent *Staphylococcus aureus* infections in living mice using a novel luxABCDE construct. Infect Immun 68:3594–3600

Francis KP, Yu J, Bellinger-Kawahara C, Joh D, Hawkinson MJ, Xiao G, Purchio TF, Caparon MG, Lipsitch M, Contag PR (2001) Visualizing pneumococcal infections in the lungs of live mice using bioluminescent *Streptococcus pneumoniae* transformed with a novel gram-positive lux transposon. Infect Immun 69:3350–3358

Jensen RB, Gerdes K (1995) Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol Microbiol 17:205–210

Lioy VS, Pratto F, de la Hoz AB, Ayora S, Alonso JC (2010) Plasmid pSM19035, a model to study stable maintenance in Firmicutes. Plasmid 64:1–17

Lynskey NN, Lawrenson RA, Sriskandan S (2011) New understandings in *Streptococcus pyogenes*. Curr Opin Infect Dis 24:196–202

Meighen EA (1994) Genetics of bacterial bioluminescence. Annu Rev Genet 28:117–139

Mutschler H, Gebhardt M, Shoeman RL, Meinhart A (2011) A novel mechanism of programmed cell death in bacteria by toxin–antitoxin systems corrupts peptidoglycan synthesis. PLoS Biol 9:e1001033

Niwa K, Ichino Y, Kumata S, Nakajima Y, Hiraishi Y, Kato D, Viviani VR, Ohmiya Y (2010) Quantum yields and kinetics of the firefly bioluminescence reaction of beetle luciferases. Photochem Photobiol 86:1046–1049

Okada N, Tatsuno I, Hanski E, Caparon M, Sasakawa C (1998) *Streptococcus pyogenes* protein F promotes invasion of HeLa cells. Microbiology 144(Pt 11):3079–3086

Olsen RJ, Shelburne SA, Musser JM (2009) Molecular mechanisms underlying group A streptococcal pathogenesis. Cell Microbiol 11: 1–12

Park HS, Francis KP, Yu J, Cleary PP (2003) Membranous cells in nasal-associated lymphoid tissue: a portal of entry for the respiratory mucosal pathogen group A streptococcus. J Immunol 171:2532–2537

Podbielski A, Spellerberg B, Woischnik M, Pohl B, Luetticken R (1996) Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). Gene 177:137–147

Proft T, Moffatt SL, Weller KD, Paterson A, Martin D, Fraser JD (2000) The streptococcal superantigen SMEZ exhibits wide allelic variation, mosaic structure, and significant antigenic variation. J Exp Med 191:1765–1776

Que YA, Haefliger JA, Francioli P, Moreillon P (2000) Expression of *Staphylococcus aureus* clumping factor A in *Lactococcus lactis subsp. cremoris* using a new shuttle vector. Infect Immun 68:3516–3522

Troy T, Jekic-McMullen D, Sambucetti L, Rice B (2004) Quantitative comparison of the sensitivity of detection of fluorescent and bioluminescent reporters in animal models. Mol Imaging 3:9–23

Wiles S, Ferguson K, Stefanidou M, Young DB, Robertson BD (2005) Alternative luciferase for monitoring bacterial cells under adverse conditions. Appl Environ Microbiol 71:3427–3432

Zhao H, Doyle TC, Coquoz O, Kalish F, Rice BW, Contag CH (2005) Emission spectra of bioluminescent reporters and interaction with mammalian tissue determine the sensitivity of detection in vivo. J Biomed Opt 10:41210

Zielenkiewicz U, Ceglowski P (2005) The toxin–antitoxin system of the streptococcal plasmid pSM19035. J Bacteriol 187:6094–6105