Published in final edited form as:

Mol Imaging Biol. 2015 December ; 17(6): 793–801. doi:10.1007/s11307-015-0856-2.

In Vivo Tracking of Streptococcal Infections of Subcutaneous Origin in a Murine Model

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Abstract

Purpose—Generation of plasmin *in vivo* by *Streptococcus pyogenes* is thought to localize the active protease complexes to the pathogen surface to aid in tissue dissemination. Here, we chose to follow cutaneous streptococcal infections by the use of non-invasive bioluminescence imaging to determine if this pathogen can be followed by this approach and the extent of bacterial spread in the absence of canonical plasminogen activation by streptokinase.

Procedures—Mice were injected subcutaneously with either bioluminescent strains of streptococci, namely Xen20 and Xen10 or *S. pyogenes* ALAB49. Bioluminescence imaging was performed daily and results were correlated with microbiological and histological analyses.

Results—Comparative analysis of chronologic non-invasive datasets indicated that Xen20 did not disseminate from the initial infection site. Contrary to this, microbiological and histological analyses of Xen20 mice for total bacterial burden indicated sepsis and widespread pathogen involvement.

Conclusions—The use of bioluminescence in microbe-based studies requires genomic and pathologic characterization to correlate imaging results with underlying pathology.

Keywords

Bioluminescence; Non-invasive imaging; *Streptococcus pyogenes*; Plasminogen; Streptokinase

Introduction

Streptococcus pyogenes, a member of the group A streptococci (GAS), is an aggressive human-specific pathogen that causes infections that range in severity from skin infections, such as pyoderma, impetigo, and cellulitis to toxic shock syndrome, glomerular nephritis, and necrotizing fasciitis [1]. The global impact of GAS infections is estimated to affect 18 million people, resulting in 517,000 deaths per year [2]. *S. pyogenes* expresses proteins that

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Conflict of Interest. The authors declare no conflicts of interest.

are covalently tethered to the cell wall for the purpose of binding host factors, including the fibrinolytic zymogen plasminogen (Pg) and the major blood clotting protein fibrin(ogen). In normal physiology, Pg is activated by proteolytic cleavage to plasmin (Pm) to degrade fibrin clots. In the context of *S. pyogenes* infection, the pathogen secretes a bacterial Pg activator called streptokinase (SK). GAS pathogenicity is now thought to be dependent on SK binding to Pg and inducing formation of an active SK•Pg* catalytic complex by the molecular sexuality mechanism [3], which then recognizes a second Pg molecule as a substrate and converts it proteolytically into Pm in the vicinity of pathogen [4, 5]. Different *S. pyogenes* strains express convergent types of receptors to bind and employ the catalytic SK•Pg*/Pm complexes, essentially coating the bacterial surface, promoting dissemination through protective fibrin barriers produced by the initial host response to infection, and indirectly activation of matrix metalloproteinases [6]. Of note, SK secreted by *S. pyogenes* lacks the ability to activate mouse Pg and streptococcal infections have increased mortality in transgenic mice that express 14–16 % plasma levels of the human Pg [7] and in mice given bolus intraperitoneal injections of human Pg [6]. Here, our goal was to follow streptococcal infection *in vivo*, using side-by-side comparison of multiple strains to support future therapeutic testing and imaging agent design. In addition, we test the dogma that mice are resistant to streptococcal infection due to a lack of pathogen-mediated Pg activation by SK.

Bioluminescence as a proxy for bacterial cell burden has been reported for *Staphylococcus aureus* in models of endocarditis [8–10], osteomyelitis [11], and cutaneous wound involvement [12]. In general, light production in microbes is driven by over-expression of either the *Photobacterium luminescens lux* operon (*luxABCDE*) or the firefly luciferase (*ffluc*) plasmid. The use of bioluminescence to track *S. pyogenes* is more limited possibly due to the inherent difficulty in genetically modifying *S. pyogenes* and difficulties that stem from inserting the large *lux* operon-carrying pXen5 plasmid (~18 kb) into the cell itself [13]. For those streptococcal strains where genetic modification has been achieved, light production can also be hampered by the relatively high GC contents of the *lux* operon, the strength of the ribosomal binding sites that precedes the *luxABCDE* or *ffluc* cassettes, and whether or not stable incorporation is maintained [13]. Lastly, the detection limit of these engineered bacteria *in vivo* is also limited by the availability of cofactors necessary for the oxidation reaction, such as the flavin mononucleotide (FMNH₂), ATP, and molecular oxygen. Some examples of bioluminescent *S. pyogenes* imaging have involved colonization of the nasopharyngeal tract [14] and began with the detection of a mucosal serotype M49 strain, termed strain 591, in surrounding nasal-associated lymphoid tissue [15]. Taken together, there are many factors that mitigate the successful production of light *in vivo* and development of this technology is not trivial.

Here, we utilized wild-type mice (i.e., C57BL/6) that should be devoid of the enhanced *S. pyogenes* lethality seen in the humanized Pg transgenic mice. We tracked *in vivo* dissemination through non-invasive longitudinal imaging studies by use of pathogens containing the aforementioned *lux* operon. For this study, we compared the disease progression after infection with popular streptococcal strains using imaging and histological analysis. *Streptococcus pneumoniae* Xen10, derived from the parent strain A66.1 serotype M3, has previously been studied in pneumonia, sepsis, pouch, and meningitis models [16–

18]. For the bioluminescent *S. pyogenes*, we used the aforementioned Xen20 that was derived from the parent 591 serotype M49 strain in nasopharyngeal or impetigo murine models [15, 19–21]. We also utilized the classical *S. pyogenes* ALAB49 serotype M53 strain (*emm* pattern D or a “skin specialist” strain) [22], as a non-luminescent reference. Of note, both ALAB49 and the Xen20 (*emm* pattern E or designated a “generalist” strain that denotes equal parts “skin specialist” and “throat specialist” strains) were isolated from serious human infections, namely impetigo (Alabama, 1986) and acute post-streptococcal glomerulonephritis, respectively [23, 24]. Our side-by-side comparison demonstrated that despite the lack of SK-associated Pm production by the pathogen, *S. pyogenes* disseminates from an initial subcutaneous infection, leading to systemic disease and multiple organ involvement for both ALAB49 and Xen20, as determined by post-mortem assessment of the organs for colony forming units (CFU) determination and Gram staining. Interestingly, bioluminescence imaging did not recapitulate these findings, as the Xen20 showed no signal outside the initial site of infection despite exhibiting higher mortality than the *S. pneumoniae*-infected mice. Moreover, our studies demonstrate incorporation of the *lux* operon was done under a glucose-sensitive promoter, which may explain our divergent results as the pathogen seeds different organs. Taken together, our results indicate a Pg-independent pathway for *S. pyogenes* dissemination. Further, we conclude that *in vivo* characterization of *lux* operon insertion into *lux* operon-driven bioluminescent pathogens is necessary prior to their use as reliable proxy for bacterial burden assessment.

Materials and Methods

Animals

For this study, 36 C57BL/6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were housed at the Auburn University College of Veterinary Medicine with *ad libitum* access to alfalfa-free chow and water. All procedures were designed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Auburn University.

Plasminogen Activation Assay

The protocol used was described previously [25]. Briefly, the supernatants from Xen20, Xen10, and ALAB49 cultures grown to mid-log phase ($\lambda_{600\text{nm}}$ of ~0.6) were harvested, filtered, and frozen at -80°C . The aliquots were thawed, and the following components combined for each assay (all concentrations noted are final): human Glu-Pg and fibrinogen (Fbg) when added at concentrations of 20 $\mu\text{g}/\text{ml}$; 40 μl of supernatant per strain; and substrate S-2251 (D-Val-Leu-Lys-*para*-nitroanilide; Chromogenix, West Chester, OH) at a concentration of 0.17 mM, with the addition of 50 mM tris(hydroxymethyl) aminomethane pH 7.5 (Tris buffer) to a final volume of 300 μl . For the positive control, human Glu-Pg at a concentration of 20 $\mu\text{g}/\text{ml}$, purified SK at a concentration of 118 nM, and substrate S-2251 at a concentration of 0.17 mM were combined with the addition of the Tris buffer until a final volume of 300 μl was achieved. For the activity assays (Fig. 1) containing human Fbg, the human Glu-Pg and Fbg were pre-incubated before the addition of supernatant. The

samples were read by a SpectraMax® 340PC³⁸⁴ (Molecular Devices, Sunnyvale, CA) at an absorbance of 405 nm every 37 s for 1 h.

Preparation of Bacterial Injections

Strains Xen20 and ALAB49 were grown in Brain Heart infusion (BHI) broth (Research Products International (RPI), Mt. Prospect, IL) and strain Xen10 in BHI broth supplemented with 5 % sheep blood (Northeast Lab Services, Waterville, ME), both with addition of 200 µg/ml of kanamycin, for 18 h at 37 °C. Cells from stationary cultures of 1.5–2-day cultures were pelleted, washed in phosphate-buffered saline (PBS; Boston BioProducts, Ashland, MA) with 10 % glycerol twice, and resuspended in PBS with 10 % glycerol for injection with the addition of 1 mg/ml of sterile Cytodex beads (Sigma-Aldrich, St. Louis, MO).

Animal Procedures

To study the extent of bacterial dissemination throughout the mice, we induced infections in C57BL/6 mice by subcutaneous injection of between 3 and 5×10^7 colony forming units (CFU) of streptococcal bacteria 24 h prior to the first imaging session and imaged daily from days 2 to 6, as detailed in Fig. 2a. Twelve mice were used for each strain (Xen20, Xen10, and ALAB49), for the 36 aforementioned mice. For these studies, mice were anesthetized by isoflurane (Piramal Healthcare, Boston, MA) with 1–3 % medical grade oxygen using a XGI-8 Anesthesia System and the hair between the shoulder blades was removed by acute treatment with Nair (Church & Dwight Co., Inc., Ewing, NJ). The mice were imaged every 24 h for 5 min with the IVIS Lumina XRMS Imager (PerkinElmer, Waltham, MA) using a binning of four and default aperture settings. The mice were then euthanized on day 8 postinfection, and the heart, lungs, liver, kidneys, spleen, and dermis area surrounding the site of injection were collected, subjected to bioluminescent imaging as described above, and subsequently fixed in 4 % paraformaldehyde in PBS prior to histological analysis or weighed and placed in sterile PBS for homogenizing.

Histology and Ex Vivo Imaging

Tissue was rinsed with sterile PBS and dabbed dry on a gauze pad prior to positioning in partially filled disposable embedding forms. The remainder of the exposed tissue was immersed in VWR Clear Frozen Section Compound prior to submersion and freezing over an isopentane (Alfa Aesar, Ward Hill, MA) bath maintained over dry ice. The block was maintained for long periods at –40 °C and equilibrated to –20 °C prior to sectioning using a Cryostat (Thermo Scientific, Kalamazoo, MI). Slices were typically between 8 and 12 µm in thickness, depending on tissue hardness. Alternating sections were stained with either H&E or Gram stain by standard protocol and the cover slip attached by applying 2–3 drops of Cytoseal (Thermo Scientific). Histology images were captured with an Axioskop 50 (Carl Zeiss Microscopy, Jena, Germany) with a 0.76x, C-Mount adapter and using a DS-Fi1 camera head (Nikon, Melville, NY) with DS-L3 control unit. The tissue collected for homogenizing was weighed and then placed into 5 ml of either sterile PBS or 0.85 % saline solution and homogenized with a Tissue-Tearor (BioSpec Products, Inc., Bartlesville, OK). The resulting solution was serially diluted to concentrations ranging from 10^1 to 10^{-12} and plated on tryptic soy agar (TSA) supplemented with 5 % sheep blood for the strain ALAB49 or BHI agar supplemented with 5 % sheep blood and 200 µg/ml of kanamycin for the strains

Xen20 and Xen10. After 18-h incubation, the plates were imaged with a 1-s integration time with the IVIS Lumina XRMS Imager.

Glucose Dependence Assay

For assessment of the relative effect that D-glucose had on bioluminescence production, 1 ml of Xen20 cells was grown to stationary phase in BHI broth containing 200 µg/ml kanamycin, confirmed by an optical density measurement (OD_{600 nm}) 1, which did not fluctuate. Cell supernatants were removed after centrifugation, and the cell pellet resuspended in M9 minimal medium without D-glucose. To achieve the range of D-glucose concentrations (0–6 mM), cells were combined with D-glucose to final concentrations between 0 and 6 mM. Samples were placed at an inclined position within the IVIS Lumina XRMS on the 37 °C stage and imaged with a kinetic protocol. The imager was set to automatically adjust exposure times at 20-min time points, for a total of eight time points spanning 140 min. The flux (photons/s) of each sample was obtained from a given region of interests (ROI) for each sample using Living Image software (PerkinElmer). Statistical analysis was performed using repeated measures two-way analysis of variance (ANOVA) on R-statistical software version 3.1.2 (<http://www.r-statistics.com/on/r/>).

Results

Activation of Human Plasminogen by *S. pyogenes* Xen20 and ALAB49 Supernatants

Activation of human Pg by streptococci is a virulence determinant and involved in dissemination of the bacteria through host tissue [7]. While activation of human Pg has been well studied, we here characterized the Pm generation potential and underlying mechanisms of human Pg activation for given strains while comparing to positive and negative controls. Figure 1 demonstrates the relative activation of human Pg by SK from *S. pyogenes* ALAB49 and Xen20 in the presence of human Fbg. Xen10, which is a SK-deficient strain, and purified SK (118 nM) were used as negative and positive controls, respectively (Fig. 1a). The maximum absorbance reached was observed as an asymptote at 1.0, reached at time point 30 min, for both Xen20 and ALAB49 strains, as shown in Fig. 1b, c, respectively. SK-containing supernatants from ALAB49 and Xen20 show minor differences in their ability to activate Pg in the absence of Fbg (i.e., ~7 % for Xen20) but similar enhanced activation of human Pg in the presence of Fbg. As expected, the identical experiments using mouse Pg instead of its human counterpart showed no activation of murine Pg regardless of Fbg supplementation (data not shown).

Genomic Analysis of *S. pyogenes* Plasminogen Activation System

The dissemination of *S. pyogenes* in humans and human Pg-expressing mice is dependent on the activation of Pg by SK. Since there is some overlap in the phenotype of *emm* pattern D and E streptococcal strains, we first characterized the Pg activation system in both ALAB49 and Xen20 prior to the *in vivo* mouse challenge that would abrogate these differences. To our knowledge no other study has characterized the exact SK allele expressed by Xen20. Phylogenetic analyses of SK genes demonstrated three clusters: 1, 2a, and 2b [26]. SK of cluster 1 (SK1) demonstrates the ability to activate soluble Pg without the obligatory need for Fbg supplementation to form the SK1•Pg*•Fbg•M1-protein complex on the pathogen

surface. In contrast, SK of cluster 2 (SK2a or SK2b) can only activate Pg pre-bound to Fbg through the streptococcal M1 coat protein, allowing formation of the SK2b•PAM-protein complex and providing Pg to the SK2b as a neighboring (Pg/Pm)•Fbg•M1-protein complex formation. ALAB49 belongs to SK2b and the results of our kinetics mirror those reports (Fig. 1). The equivalent response of both ALAB49 and Xen20 supernatants to Fbg supplementation suggests that Xen20 is the SK2a allele evident by some activation of human Pg (~7 %) in the absence of Fbg. Others have noted that there is a strong inheritance pattern between PAM and SK2b in strains specific for skin infections such as ALAB49 [27]. Therefore, we investigated whether the parent strain of Xen20 (denoted as strain 591, serotype M49 strain) has a gene homologous to PAM through Basic Local Alignment Search Tool (BLAST) alignment with the PAM protein sequence from ALAB49 (Acc. No. G4R2S6). Although no homology between the sequences was identified, the existence of another cell wall Pg binding protein was identified. The extracellular protein factor (Epf) (Acc. No. B5XKN1), originally identified in serotype M49 and confirmed in strain 591, has been shown to bind immobilized Pg [28]. The presence of Epf has been shown to increase the ability of strain 591 cells to adhere to and internalize into human keratinocytes and binds Pg with a determined K_d value of 0.28 μ M, which is physiologically relevant, as Pg circulates at a concentration of 2 μ M [29]. Our results indicate that Xen20 and ALAB49 express cluster 2-type SK, yet Xen20 is a SK2a and ALAB49 is a SK2b. Therefore, *in vivo* comparison of their relative infectivity may discriminate the importance of these cluster types.

Diminished Bioluminescence at the Site of Infection for Xen10 and Xen20 Strains

To study dissemination of these *Streptococcus* species in wild-type mice, we monitored bioluminescence production to determine whether the absence of productive Pg binding and activation would limit spread of the pathogen to distal organs. To accomplish this, mice were injected subcutaneously with bacteria and imaged daily, as illustrated in Fig. 2a. A representative imaging signal time course is shown in Fig. 2b. Despite the decrease in bioluminescence observed for *S. pyogenes* Xen20, these infections resulted in 33 % mortality (4 of 12 mice). In contrast, all mice survived *S. pneumoniae* Xen10 infections (0 deaths in 12 infected mice) and ALAB49 infections had the highest mortality rate of 50 % (6 of 12 mice) in the span of 8 days. By bioluminescence imaging, the *S. pneumoniae* infections in the different biological replicates appeared resolved at day 5 (Fig. 2b). The bioluminescence associated with Xen20 infection sites diminished over time but did not show dissemination of the signal or other organ involvement. The continued presence of the bioluminescent signal from these pathogens over days led us to question the state of other organ involvement via histology of organs recovered from necropsy.

Gram Staining and CFU Determination for Pathogens in Distal Organs and the Skin

To address the distal organ involvement in these *Streptococcus*-infected mice, we performed histology and analyzed Gram-stained slides from each cohort. In all mice, we detected signs that an infection had occurred (Fig. 3). For Xen20- and ALAB49-infected mice (Fig. 3a, b, respectively), there was apparent dissemination from the initial skin infection to several organs, including the kidneys, heart, liver, and lungs. ALAB49 had, on average, higher relative content of the pathogen in the kidney and heart than its bioluminescent counterpart

Xen20 by gross inspection (Fig. 3b). Interestingly but not entirely unexpected, Xen10 showed the greatest bacterial burden in the lung compared to the other streptococcal strains. Examination of the Gram-stained slides for Xen10 under 400 times magnification showed Gram-positive cocci were observed as clusters or debris within splenocytes as opposed to the *S. pyogenes* infections (Fig. 3c). Therefore, Xen20 and ALAB49 infections have the capacity to overwhelm the host immune defenses, even in the absence of productive Pg activation.

To confirm the Gram staining, we quantified bacterial numbers or content within each organ. This classic CFU determination per gram of isolated tissue allowed us to verify metabolically active and living cells (Fig. 4). We produced homogenates of the organs, plated dilutions, and counted the number of viable *S. pyogenes* cells (Fig. 4a, b). Only trace non-emitting colonies were detectable at the maximum Xen10 content (data not shown). Fig. 4a, b showed significant pathogen CFU amounts, yet whole organ bioluminescence imaging did not recapitulate these results, despite the presence of 10^5 – 10^7 CFU per organ, even at longer exposure times of up to 10 min (Fig. 4c). To eliminate destruction of the *lux* operon or contaminating bacteria as a cause of these results, we imaged bioluminescence in selected plates. Our results showed individual colonies reached levels of calibrated luminescence identical to the product literature (Fig. 4d). Therefore, we concluded that instability of the *lux* operon during the course of infection was not the cause for the loss of *in vivo* bioluminescent signal.

Presence of Exogenous Glucose Modulates Xen20 Bioluminescence in a Concentration-Dependent Manner

To determine if the *lux* operon was inserted into a region regulated by a particular host factor, we analyzed the genes disrupted by the transposon insertion of the bioluminescent cassette by BLAST search analysis. The plasmid pXen05, formerly termed pAULA-Tn4001 *luxABCDE Km^R*, was previously shown to have integrated at a single site in the Xen20 parent, strain 591, in the second open reading frame for a hypothetical protein (Acc. No. NP_268809) [15]. BLAST search of this hypothetical protein is 100 % homologous to a protein identified in the M1 strain of GAS as a sugar phosphate isomerase or epimerase (*iolE*) involved in carbohydrate transport and metabolism. Fluorescence microscopy studies of green fluorescence protein (GFP) driven under the *iolE* promoter in *Salmonella enterica* subspecies 1 serovar Typhimurium strain 14028 revealed strict repression of GFP-expression when grown on D-glucose-rich medium [30]. Therefore, we assayed the bioluminescence production of Xen20 cells, washed, and resuspended in M9 minimal medium with a range of D-glucose concentrations from 0 to 6 mM (Fig. 5a). Use of M9 medium limits the secondary effects of carbon sources, thereby allowing for a more reliable glucose response. Quantification of this luminescence expression revealed the addition of low concentrations of D-glucose increased the luminescence observed in the tubes up to a concentration of 4.0 mM and decreased up to 6 mM (Fig. 5b). Replicates of measurements shown in Fig. 5b were combined in Fig. 5c. Repeated measure two-way ANOVA was performed using the statistical software R 3.1.2 and revealed that both time and D-glucose concentration, as well as the interaction of these two variables, had a significant effect on light production in Xen20 ($p > 0.05$). Luminescence is recoverable by plating these separate

reactions onto BHI plates and incubating for 16 h, excluding loss of the *luxABCDE* cassette and no cell death during the 160-min incubation with D-glucose (data not shown).

Discussion

Our goals in this study were to determine whether there is an *in vivo* requirement for host Pg activation for the spread of *S. pyogenes* to distal organs. Further, we explored bioluminescence imaging of *S. pyogenes* Xen20 to non-invasively follow the spread of a cutaneous infection to other organs. We determined (i) that the SK from Xen20 and ALAB49 are cluster 2a and 2b alleles, respectively; (ii) that there was a distinct Pg-independent pathway that allows *S. pyogenes* to spread *in vivo* for both Xen20 and ALAB49; (iii) that there was an increased mortality in cohorts infected with the *S. pyogenes* Xen20 and ALAB49 compared to the *S. pneumoniae* Xen10; (iv) that the insertion site of the bioluminescent *lux* cassette into the Xen20 genome is potentially under control of a sugar phosphate isomerase/epimerase (*iolE*) promoter; and (v) that the light generation by the *S. pyogenes* Xen20 is sensitive to the local D-glucose concentrations leading to a masking of pathogen content in distal organs. In the discussion below, we relate each result to *in vivo* determinants of streptococcal infections to aid future non-invasive imaging studies.

To focus our studies on *S. pyogenes* dissemination *in vivo*, we asked whether longitudinal and non-invasive imaging of Xen20 correlated with disease progression and if we could use the bioluminescent signal from Xen20 as a surrogate for overall bacterial burden. Because virulence of streptococcal pathogens is related to the expression of microbial virulence factors, including exotoxins (i.e., strep-tolysin O and S, streptococcal pyogenic exotoxin A and C), hyaluronidase, C5a peptidase, streptococcal chemokine protease, and SK, we evaluated streptococcal infections in a Pg-independent model of cutaneous infection, thereby testing whether activation of host Pg is strictly required for *S. pyogenes* virulence. One example of these Pg-independent disseminative virulence factors is the cysteine protease SpeB, which is found in all GAS strains and cleaves cytokine precursors, immunoglobulins, and immune system-associated receptors [31]. Inactivating SpeB decreases the GAS dissemination, potentially through inactivation of SK causing modulation of Pg activation [32, 33]. Streptococcal deoxyribonuclease, called strepto-dornase, and phage-acquired DNases function to clear the infection site to aid in dissemination and immune system evasion [31]. Therefore, we sought to determine if these factors, along with others, are sufficient to allow for the spread of the *S. pyogenes* infections in the absence of a productive SK•Pg system [34]. In this model, there is a potential for formation of an inactive mouse SK•Pg complex on the pathogen surface, which should mimic the human infection, yet minus the Pg activation and its resultant Pm burst.

We compared two *S. pyogenes* and one *S. pneumoniae* strains by assessing the migration of the light source as the disease developed. To better understand the *S. pyogenes* strains that we chose, initial characterization of ALAB49 and Xen20 secretomes was performed using both human and mouse proteins. Both ALAB49 and Xen20 belonged to the cluster 2 alleles of SK (Fig. 1), but genomic analysis revealed that the anchoring of the Pm to the surface of the pathogen was different in that Xen20 is PAM-negative (SK2a) and ALAB49 is PAM-

positive (SK2b). Since Fbg-enhanced human Pg activation by both pathogens was similar, it is reasonable to assert both pathogens would be impaired in a similar way *in vivo*, given the lack of mouse Pg activation. Because ALAB49 is a skin specialist strain and Xen20 is a generalist strain, our side-by-side comparison between these two *S. pyogenes* strains was done at the phenotypic imaging level, asking the question whether the same inoculum lead to abscess formation in the kidney or other organs and whether or not bioluminescence recapitulates the bacterial burden in distal organs.

Our *in vivo* results were somewhat unexpected in that the total bacterial burden exceeded our bioluminescence assessment (Fig. 3). We saw no signal originating in the vicinity of the distal organs (i.e., the liver, kidney, and spleen) for the Xen20 pathogen-infected mice, despite a significant 50 % mortality for both the *S. pyogenes* strains (Xen20-ALAB49) on day 7. Indeed, bioluminescent imaging of excised organs showed no additional signal. This lack of light production in the infected organs could be caused by several factors, including (i) reduction of cellular molecular oxygen to catalyze the redox reaction by which luminescent substrates are oxidized to produce light, (ii) diminished presence of reducing equivalents (i.e., FMNH₂ and/or ATP) in stationary phase growth seen by the microbe in tissues, or (iii) exhausted substrate pool for bacterial luciferase due to shifts in carbon source bioavailability. Taken together, our data show that even in the absence of Pg activation, both *S. pyogenes* strains ALAB49 and Xen20 cause systemic infections.

Since we now know that the *lux* operon incorporated into the Xen20 genome may be under the control of the *iolE* promoter, it is conceivable that the observed *in vivo* signal may be caused by a shift, rather than a decrease, in metabolic activity. Increasing utilization of D-glucose as the preferred carbon source may have lead to a decrease of imaging signal below the detection threshold [35, 36]. It is also conceivable that this glucose-dependent promoter decreases the expression of *lux* operon-encoded proteins which convert endogenous fatty acids to usable substrates for the luciferase enzyme, decreasing the overall pool of substrates, as previously suggested. Due to the observed effect of D-glucose on bioluminescence expression by Xen20 (Fig. 5), further study is required to determine the specific effects of carbon source availability on regulation of bioluminescence expression and maintenance in *S. pyogenes* strains under carbon-limiting conditions. The effects of D-glucose on BLI signal by Xen20 *in vitro* strongly reflects the *in vivo* condition if one considers murine blood glucose ranges from ~70 to 90 mg/dl, which is ~3.9 to ~5 mM [37] and can be as high as 100 mg/dl (~5 mM) [38]. Consequently, the pathogen would be in an environment that is similar to the lower level of light detected in the *in vitro* time course (Fig. 5b, c).

Conclusions

S. pyogenes Xen20, a genetically modified bioluminescence-expressing strain, and the related non-bioluminescent ALAB49, showed dissemination to distal organs in the absence of productive utilization of Pg by the pathogen. From an initial cutaneous infection, both *S. pyogenes* strains caused significant morbidity as opposed to the *S. pneumoniae*. *S. pyogenes* Xen20 and ALAB49 showed dissemination to the heart, lung, kidneys, and spleen, while *S. pneumoniae* Xen10 was cleared by the host and did not result in mortality. These results also

indicate a slight decrease in the CFU recovered from distal tissue of mice infected with Xen20 *versus* ALAB49. Despite the presence of bacteria in these tissues, there was no detectable bioluminescent signal, indicating a hurdle for the use of bioluminescence as a sole readout of bacterial burden for this specific strain. The decrease in bioluminescent signal was not caused by *lux* transposon instability or decreased bacterial numbers and may be related to glucose or carbon source availability in the microenvironment of the pathogen in distal organs. In addition, these results highlight the importance of characterizing each bioluminescent-expressing bacterial strain in *in vivo* model systems to prevent false-negative results and obtaining an unbiased assessment of the disease state.

Acknowledgments

This research was supported by the National Institutes of Health through grants provided by the National Heart, Lung, and Blood Institute, R01 HL056181 and R01 HL071544 (to P. E. B.), R00 HL094533 (to P. P.), and R01 HL114477 (to P. P. and M. N.), and the National Institute of Allergy and Infectious Diseases grant 2R44AI085840-02 (to P. P.).

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Fig. 1.

Human plasminogen activation by the streptococcal secretomes. **a** Activation of human plasminogen (Pg) with and without fibrinogen (Fbg) for supernatants of log phase streptokinase (SK)-deficient *S. pneumoniae* (Xen10) and a SK (118 nM)-positive control. **b**, **c** Activation of Pg with and without Fbg for supernatants of *S. pyogenes* strains ALAB49 and Xen20, respectively.

Fig. 2.

Time course of streptococcal infection monitored by bioluminescence imaging. **a**

Experimental design. **b** Daily imaging of mice after subcutaneous injection of *S. pyogenes* (Xen20) and *S. pneumoniae* (Xen10). Bioluminescence imaging for a representative of the mice for the light-producing microbes, shown at the indicated time points, $n=12$ for each group.

Fig. 3. Histological analysis of streptococcal strains reveals widespread dissemination. **a** Xen20 (*S. pyogenes*). **b** ALAB49 (*S. pyogenes*). **c** Xen10 (*S. pneumoniae*). Histological analysis of isolated organs Gram-positive *Streptococcus* species (blue) and tissue (yellow) are shown at 50 times magnification, with the area of interest (black rectangle) shown at 400 times magnification. Representative data from two injections of two mice from each group for a total $n=4$.

Fig. 4. Evidence of bioluminescent colonies retrieved from *in vivo* infection. Mean colony forming units (CFU) per organ recovered from homogenates of two separate injections of two mice each for **a** ALAB49 or **b** Xen20, with $n=4$. **c** Bioluminescence from organs (from *left to right*): heart, lungs, spleen, liver, kidneys, and infection site. Signal is only observed from the skin at the infection site, despite CFUs ranging from 1×10^5 to 2×10^7 present within the organs. **d** Bioluminescent colonies of Xen20 recovered from the spleen from panel **b**.