International Journal of Antimicrobial Agents xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Short Communication

Influence of antibiotic pressure on bacterial bioluminescence, with emphasis on *Staphylococcus aureus*

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ARTICLE INFO

Article history:
Received 22 July 2015
Accepted 15 September 2015

Keywords:
luxA gene
Vancomycin
Ciprofloxacin
Erythromycin
Chloramphenicol

ABSTRACT

Bioluminescence imaging is used for longitudinal evaluation of bacteria in live animals. Clear relations exist between bacterial numbers and their bioluminescence. However, bioluminescence images of *Staphylococcus aureus* Xen29, *S. aureus* Xen36 and *Escherichia coli* Xen14 grown on tryptone soy agar in Etests demonstrated increased bioluminescence at sub-MICs of different antibiotics. This study aimed to further evaluate the influence of antibiotic pressure on bioluminescence in *S. aureus* Xen29. Bioluminescence of *S. aureus* Xen29, grown planktonically in tryptone soy broth, was quantified in the absence and presence of different concentrations of vancomycin, ciprofloxacin, erythromycin or chloramphenicol and was related to expression of the *luxA* gene under antibiotic pressure measured using real-time PCR. In the absence of antibiotics, staphylococcal bioluminescence increased over time until a maximum after ca. 6 h of growth, and subsequently decreased to the detection threshold after 24 h of growth owing to reduced bacterial metabolic activity. Up to MICs of the antibiotics, bioluminescence increased according to a similar pattern up to 6 h of growth, but after 24 h bioluminescence was higher than in the absence of antibiotics. Contrary to expectations, bioluminescence per organism (CFU) after different growth periods in the absence and at MICs of different antibiotics decreased with increasing expression of *luxA*. Summarising, antibiotic pressure impacts the relation between CFU and bioluminescence. Under antibiotic pressure, bioluminescence is not controlled by *luxA* expression but by co-factors impacting the bacterial metabolic activity. This conclusion is of utmost importance when evaluating antibiotic efficacy in live animals using bioluminescent bacterial strains.

1. Introduction

Q2 Over the past decades, increasing use of biomaterial implants and devices has been accompanied by a concurrent increase in the incidence of biomaterial-associated infections (BAIs). BAIs have now become the main cause of prosthetic implant and device failure [1], and patients with prosthetic joint infection, for instance, find themselves at a risk of mortality exceeding that of many cancers [2]. Along with the combined search and development of new antibiotic drugs to fight bacterial resistance to antibiotics, new antimicrobial prophylactic and therapeutic measures have been developed to treat bacterial biofilms. Therewith, the need for appropriate methods to evaluate these measures in vitro and in vivo has become of paramount importance, especially in case of BAIs where clinical trials are difficult, requiring large patient groups owing to the relatively low incidence of BAI. Recently, the combination of sensitive bio-optical imaging systems and the availability of bioluminescent bacteria has enabled real-time non-invasive monitoring of the spatiotemporal persistence of bacteria in live animals, and the number of in vitro- and in vivo-based papers relying on bacterial bioluminescence as an indicator of bacterial persistence is rapidly increasing [3–8]. Clear relations have been found between bioluminescence arising from bacterially contaminated biomaterials in animals and ex vivo bacterial counts after culturing organisms from explanted materials after sacrifice in multiple papers [4,5,7–10].

Bioluminescent bacteria are genetically engineered by stably integrating the *lux* operon into their genome or on a plasmid and are equipped with a luciferase reporter system capable of emitting visible light that can be detected by highly sensitive camera systems [6]. The total bioluminescence observed depends on the number of bacteria involved and the bioluminescence per

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http://dx.doi.org/10.1016/j.ijantimicag.2015.09.007
0924-8579/

individual organism. Bioluminescence per organism is controlled by five essential genes (*luxABCDE*) [11] as well as different co-factors (Supplementary Fig. S1).

Although several studies have confirmed that bioluminescence relates well to bacterial numbers in a biofilm [3,4], it has also been reported that bioluminescence per individual organism changes during the different bacterial growth phases [7,12–14]. Furthermore, discrepancies have been pointed out between the number of viable bacteria in a biofilm and their total bioluminescence following exposure to antibiotics [14]. For an illustration of this phenomenon, we present bioluminescence images overlaid on black & white photographs of the agar plates for *Staphylococcus aureus* Xen29, *S. aureus* Xen36 and *Escherichia coli* Xen14 grown on tryptone soy agar (TSA) in Etests against vancomycin, ciprofloxacin, erythromycin and chloramphenicol pressures (Supplementary Fig. S2).

The aim of this study was to further evaluate the influence of the presence of different antibiotics on staphylococcal bioluminescence and expression of *luxA* during growth in a commercially available and often used [7,10,15] bioluminescent strain, namely *S. aureus* Xen29.

2. Materials and methods

2.1. Bacterial strain

Staphylococcus aureus Xen29, originating from meticillin-susceptible *S. aureus* ATCC 12600, was made bioluminescent by inserting a modified *Photorhabdus luminescens lux* operon (*luxABCDE*) into the bacterial genome [9], situated within the open reading frame of the hypothetical gene SA2154 [16]. The strain was obtained commercially from PerkinElmer (Waltham, MA).

2.2. Total bioluminescence of Staphylococcus aureus Xen29 in planktonic culture in the absence and presence of antibiotic pressure

Bacteria were cultured from cryopreservative beads onto TSA (Oxoid Ltd., Basingstoke, UK) in the presence of 200 mg/L kanamycin and were incubated for 24 h at 37 °C in ambient air. Prior to each experiment, one colony was used to inoculate 10 mL of tryptone soy broth (TSB) (Oxoid Ltd.) and was planktonically cultured at 37 °C for 24 h in ambient air. Then, 500 μL of each culture was used to inoculate 10 mL of TSB growth medium and was planktonically grown at 37 °C with continuous shaking at 150 rpm for 16 h. Staphylococci were suspended in TSB to a concentration of 5×10^6 bacteria/mL as counted in a Bürker-Türk counting chamber.

Next, 200 μL aliquots of bacterial suspension in TSB with an antibiotic concentration of 0 (no antibiotic) up to its minimum inhibitory concentration (MIC) were incubated at 37 °C in sterile 96-well plates (Falcon®; Corning Inc., Corning, NY). Total bioluminescence was measured over the area of each individual well at 2, 3, 4, 5, 6 and 24 h after inoculation using a highly sensitive, cooled charge-coupled device camera (IVIS® Lumina Imaging System; PerkinElmer) and was expressed as photons per second (p/s). Note that the total bioluminescence observed is the product of bacterial bioluminescence per individual organism and the number of CFU in the well culture.

Four antibiotics were applied that differ in their mode of antibacterial action: vancomycin is an inhibitor of bacterial cell wall synthesis; ciprofloxacin is an inhibitor of bacterial nucleic acid synthesis; and erythromycin and chloramphenicol prevent bacterial protein synthesis. MICs of *S. aureus* Xen29 against the different antibiotics were determined using Etest strips (AB BIODISK, Solna, Sweden) on TSA and were read from the photographs presented in Supplementary Fig. S2: 0.75 mg/L for vancomycin; 0.31 mg/L for ciprofloxacin; 0.25 mg/L for erythromycin; and 2.7 mg/L for chloramphenicol.

2.3. Number of viable bacteria

To determine the number of CFU responsible for the total bioluminescence observed, staphylococci were grown for various periods of time in the absence and presence of vancomycin, ciprofloxacin, erythromycin or chloramphenicol at their MIC in 200 mL of TSB. Aliquots of 1 mL were taken from the bacterial suspension at different time points and were serially diluted, after which 100 μL of the diluted bacterial suspensions were plated on TSA plates and were incubated at 37 °C. CFU were counted after 24 h of incubation and were expressed as CFU/mL. From the same 200 mL culture, 200 μL aliquots were taken and the total bioluminescence (p/s) was measured in a 96-well plate in order to calculate the bioluminescence per CFU while accounting for the different volumes (p/s/CFU).

2.4. RNA isolation and luxA expression

Expression of the *luxA* gene in *S. aureus* Xen29, harvested from the same bacterial cultures as used for CFU determination, was measured using real-time PCR as described previously [17] after incubation for different times in the absence and presence of vancomycin, ciprofloxacin, erythromycin or chloramphenicol at the different MICs of the antibiotics.

The sequence of *S. aureus* NCTC 8325-4 was used to design primer sets for *gyrB* and of *P. luminescens* for *luxA* (gyrB f3, 5′-ATATAGGATCGACTTCAGAG-3′; gyrB r4, 5′-TGAATATCAACTGGGATACC-3′; LuxA f1, 5′-GTATTTCTGAGGAGTGTGGT-3′; LuxA r2, 5′-CTGTTATTCATATCCGTGCC-3′). Then, 100 nM of each primer was used under a two-step protocol with an annealing temperature of 60 °C. Under the selected conditions, primer efficiency was between 90% and 110% as determined using serial dilutions of chromosomal DNA of *S. aureus* Xen29.

Total RNA was isolated from aliquots of the growing suspension after different time periods in the absence and presence of the antibiotics up to 24 h at their respective MICs. Bacteria were harvested by centrifugation and were frozen at −80 °C. Samples were thawed slowly on ice and RNA isolation was carried out using a RiboPure™-Bacteria Kit (Ambion, Foster City, CA). DNA was removed using the Ambion® DNA-free™ Kit (Applied Biosystems, Foster City, CA) and the absence of genomic DNA was verified by reverse transcription PCR (RT-PCR) prior to reverse transcription. For all samples, 35 cycles of PCR using the *gyrB* primer set did not result in any detectable signal, confirming the absence of genomic DNA in the RNA preparation. RNA concentrations were determined using an ultraviolet spectrophotometer (NanoDrop, Wilmington, DE), and 250 ng of total RNA was used for cDNA synthesis (iScript™; Bio-Rad, Veenendaal, The Netherlands). PCR reactions were prepared in triplicate using a CAS-1200 pipetting robot (Corbett Life Science, Sydney, Australia). Expression levels of *luxA* in staphylococci were analysed using the $2^{-\Delta\Delta C_T}$ method [18] with *gyrB* as reference and expressed per CFU.

3. Results

Total staphylococcal bioluminescence after 2, 3, 4, 5, 6 and 24 h of planktonic growth was plotted as a function of antibiotic concentration for each of the antibiotics involved (Fig. 1). In the absence of antibiotics, staphylococcal bioluminescence increased over time until a maximum occurred after ca. 6 h of growth, and subsequently decreased to the detection threshold after 24 h of growth owing to reduced metabolic activity of the bacteria. Up to the MICs of the

Please cite this article in press as: Daghighi S, et al. Influence of antibiotic pressure on bacterial bioluminescence, with emphasis on *Staphylococcus aureus*. Int J Antimicrob Agents (2015), http://dx.doi.org/10.1016/j.ijantimicag.2015.09.007