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How to cite

MANOIL, Daniel, LANGE, Norbert, BOUILLAGUET, Serge. Enzyme-mediated photoinactivation of Enterococcus faecalis using Rose Bengal-acetate. In: Journal of Photochemistry and Photobiology. B, Biology, 2018, vol. 179, p. 84–90. doi: 10.1016/j.jphotobiol.2018.01.001

This publication URL: https://archive-ouverte.unige.ch/unige:111233
Publication DOI: 10.1016/j.jphotobiol.2018.01.001

Journal of Photochemistry & Photobiology, B: Biology 179 (2018) 84–90

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Enzyme-mediated photoinactivation of *Enterococcus faecalis* using Rose Bengal-acetate

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ARTICLE INFO

Keywords:
Antimicrobial photodynamic therapy
Rose Bengal-acetate
Enterococcus faecalis
Flow cytometry
Membrane integrity

ABSTRACT

Rose Bengal-acetate (RB-Ac) is a pro-photosensitizer claimed to diffuse into target cells, where the acetate groups are hydrolyzed and the photosensitizing properties of Rose Bengal (RB) are restored. Despite promising results on tumor cells, the interaction of RB-Ac with bacteria has never been investigated. This study aimed to assess the interaction of RB-Ac with *Enterococcus faecalis* and to evaluate its potential use in antimicrobial photodynamic therapy (aPDT).

Spectrofluorometry was used to assess the ability of *E. faecalis* to hydrolyze the RB-Ac compound. Fluorescence microscopy was employed to observe the distribution and to evaluate the cellular uptake of the RB produced. The antibacterial efficiency of RB-Ac-mediated aPDT was assessed by flow cytometry in combination with the LIVE/DEAD® staining.

Results showed that RB-Ac was successfully hydrolyzed in the presence of *E. faecalis* cells. The RB produced appeared to incorporate the membrane of bacteria. Higher concentrations of RB-Ac resulted in higher incorporation of RB. The blue-light irradiation of RB-Ac-treated samples significantly reduced bacterial viability. Less than 0.01% of *E. faecalis* survived after incubation with 200 µM RB-Ac during 900 min and blue-light activation.

The current report indicates that *E. faecalis* cells can hydrolyze the RB-Ac compound to produce active RB. The use of RB-Ac did not appear to allow cytoplasmic internalization of the RB produced, which rather incorporated the membrane bilayers of *E. faecalis*. The use of RB-Ac did not provide additional advantages over RB in terms of PS localization. Nonetheless, sufficient RB was produced and incorporated into the membranes of bacteria to elicit effective aPDT.

1. Introduction

Rose Bengal (RB) is a xanthene photosensitizer (PS) which can react with visible light (450–600 nm) to produce singlet oxygen ($^1O_2$) [1]. The high oxidative properties of $^1O_2$ are employed to kill bacteria during antimicrobial photodynamic therapy (aPDT) [2]. RB-mediated aPDT using blue-light (400–500 nm) was shown to efficiently inactivate several bacterial strains, such as *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans* and various Enterobacteriaceae species [3, 4, 5]. In these reports, RB was directly incubated with bacteria and maintained in solution during light irradiation. Because this procedure yields to the production of $^1O_2$ into the bacterial suspension, the precise interaction between RB and bacteria cannot be delineated.

To more precisely investigate the interaction of RB with bacteria, Dahl et al. [6] incubated suspensions of *Salmonella typhimurium* with the PS and measured the RB retained after removing the culture medium and washing the cells. They showed that RB was capable of association with bacterial cells, likely through incorporation into the membrane bilayers [6]. More recently, the ability of RB to incorporate into the bacterial membranes of *Enterococcus faecalis* and *Fusobacterium nucleatum* was confirmed by fluorescence imaging; sufficient RB was accumulated to kill bacteria upon blue-light irradiation [7]. The ability of a PS to associate with, or penetrate into bacteria is claimed to play an important role in the efficiency of aPDT [8, 9]. Studies that used endogenous porphyrins as a PS report that the intracellular production of $^1O_2$ induces multiple cytoplasmic and DNA damages and also suggest that PS's located into the cytoplasm of cells may induce more lethal effects than PS's reacting in the vicinity of cells [10, 11]. Unfortunately, the anionic charges of the RB molecule may limit the ability of the PS to diffuse across the negatively charged glycolipids of bacterial membranes into the cytoplasm [12].

To decrease the anionic repulsion effect and to promote intracellular

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https://doi.org/10.1016/j.jphotobiol.2018.01.001
Received 12 November 2017; Received in revised form 15 December 2017; Accepted 8 January 2018
Available online 11 January 2018
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diffusion, Bottiroli et al. [13] added acetate groups to the xanthene ring of the RB molecule. In this acetylated form, the pro-photosensitizer Rose Bengal-acetate (RB-Ac) is supposed to act as a fluorogenic substrate; i.e. inactive until intracellular esterases hydrolyze the acetate groups and restore RB properties [13]. This acetylation also hinders the Π electron system of the photosensitizer so that both fluorescence and $^1O_2$ production are quenched.

Bottiroli et al. [13] showed that the esterases expressed by rat glioma-derived cells can hydrolyze RB-Ac and reported that acetyl-esterases exhibited a higher hydrolytic activity for the compound than esterases [13]. Later, Soldani et al. [15] used confocal microscopy to determine the cellular distribution of the RB produced after hydrolysis of RB-Ac in C6 glioma cells and human HeLa cells [14]. They showed that RB was able to penetrate the entire vacuolar system and that light-irradiation of these cells damaged multiple organelles including the endoplasmic reticulum, the Golgi apparatus and the cytoskeleton [15]. Another study by Bottone et al. [17] showed that mitochondria were also damaged during RB-Ac-mediated PDT, which was responsible for the activation of caspase factors and subsequent cell apoptosis [16, 17]. Despite promising results on tumor cells, there is no report on the interaction of RB-Ac with bacteria.

The aim of this study was to investigate the interaction of RB-Ac with *Enterococcus faecalis* and evaluate its potential use in aPDT. Specifically, fluorescence microplate reading was used to verify the hydrolysis of RB-Ac in *E. faecalis* suspensions. Epifluorescence microscopy was used to observe the distribution and evaluate the cellular uptake of the RB produced after enzymatic reaction. The antibacterial efficiency of RB-Ac-mediated aPDT has been assessed using flow cytometry (FCM).

2. Materials and Methods

2.1. Bacterial Cultures

Enterococcus faecalis (*E. faecalis* ATCC 29212) from frozen stocks was plated onto Columbia agars (Becton Dickinson AG, Allschwil, CH), transferred into liquid medium (brain heart infusion – BHI, Becton Dickinson), and incubated aerobically at 37 °C overnight. For experiments, the culture medium was removed after centrifugation (4000 *g* for 6 min) and the bacterial pellet was re-suspended into phosphate buffer saline (PBS Gibco, ThermoFisher Scientific, Reinach, CH); cell concentration was set at approximately 2×10^9 cells per mL (OD_{600nm}: 4, Biowave II, Biochrom WPA, Cambridge, GB).

2.2. Kinetics of RB-Ac Hydrolysis

To determine the ability of *E. faecalis* cells to hydrolyze RB-Ac (Santa Cruz Biotechnology Inc., Texas, USA), 200 µL of bacterial suspensions were incubated during 900 min with various concentrations of the compound (from 25 µM to 200 µM) into black 96–well plates (Greiner Bio-One GmbH, St. Gallen, CH). The fluorescence resulting from the production of RB was monitored every 10 min using a spectrofluorometer (ex/em: 544 nm/582 nm, Spectramax Paradigm, Molecular Devices, California, USA). Internal temperature of the spectrofluorometer was set at 37 °C. To compensate the loss of volume from the wells due to evaporation (900 min at 37 °C), the reading height above the plate was automatically adjusted during the experiment.

To verify whether *E. faecalis* secretes extracellular enzymes in the culture medium that may hydrolyze RB-Ac, cultures of 2×10^9 cells/mL were centrifuged to collect the supernatant. Supernatants were filtered at 0.22 µm (Millipore syringe filters, Milian SA, Vernier, CH) and incubated with RB-Ac (from 25 µM to 200 µM). Absence of bacteria in the filtrated supernatants was verified by OD_{600nm} readings and dark-field microscopy after the 900 min of incubation (data not shown). Wells containing bacteria only, supernatant only or RB-Ac only were used as controls. Experiments were performed in triplicate and repeated 3 times.

2.3. Epifluorescence Microscopy

The cellular distribution of the RB produced by enzymatic reaction was determined and quantified by epifluorescence microscopy (Axiovert 200M, Carl Zeiss, Gottingen, DE). *E. faecalis* suspensions were incubated with RB-Ac (from 25 µM to 200 µM) at 37 °C in the dark during 300 min, 600 min or 900 min. Bacteria were collected by centrifugation, and re-suspended into PBS to eliminate RB from the extracellular medium. Samples were then sonicated 20 s (Sonorex, Bandelin electronics, Berlin, DE) to disperse aggregates and aliquots of 50 µL were placed onto microscope slides (Menzel-Glasser, Braunschweig, DE). Phase contrast and fluorescence (excitation: 500/20 nm, emission: 535/30 nm) were observed using a 63× oil immersion objective, and photomicrographs were acquired with VisiView software 3.2. The mean fluorescence value of bacteria was calculated in each micrograph using ImageJ software (v1.51). For each concentration and incubation time tested, three photomicrographs were obtained, and experiments were repeated 3 times. Results express the mean of 9 photomicrographs for each condition (n = 9).

2.4. RB-Ac Mediated Antibacterial Photodynamic Therapy – aPDT

For aPDT experiments, 200 µL of bacterial suspensions were incubated under the same experimental conditions as described for fluorescence measurements (0–200 µM/300, 600 or 900 min). Samples were washed to remove the RB from the extracellular medium and transferred into black 96–well plates (Greiner Bio-One) for irradiation. Each well was irradiated 120 s with a dental QTH lamp emitting blue-light (400 nm–500 nm), having an irradiance of 500 mW/cm^2 (Optilux 501, KerrHawe SA, Bioggio, CH). A light guide of 8 mm in diameter, delivering blue light over the whole surface of the well, was used to individually irradiate each sample. Control wells received blue-light alone or RB-Ac alone. Experiments were performed in triplicate and repeated 3 times (n = 9).

2.5. Membrane Integrity Assessment

To assess the membrane integrity of bacteria after blue-light irradiation, cultures were stained with the LIVE/DEAD BacLight Bacterial viability kit (Life Technologies, CH) as previously described [7]. This kit contains two DNA stains (SYTO 9 and propidium iodide, PI), which allow the discrimination between membrane intact and damaged bacteria through the selective entry of PI into damaged cells. After staining, samples were incubated in the dark for 15 min at room temperature and analyzed with the Accuri C6 flow cytometer (BD Accuri Cytometers, Ann Arbor, USA). SYTO 9 (ex/em; 485/500) and PI (ex/em; 535/617) fluorescence signals were collected in the FL1 (BP 533/30) and FL3 channels (LP > 670) respectively. Bacterial density (~4×10^6 cells/mL) and flow rate (14 µL/min) were set to keep events rate below 1200/s during acquisition. Bacteria were discriminated from debris and gated on FL1-H/Forward Scatter-H (FSC-H). For each sample, 20,000 events were collected in the bacterial gate. Microbial populations were identified on the basis of fluorescence detected on FL1-A/FL3-A. Untreated bacteria (SYTO 9 positive controls) and heat-treated bacteria (60 °C for 60 min – PI positive controls) were used to manually set the gates applied for population discrimination. Using such calibration, three populations are generally observed i.e.; live (SYTO 9 positive), dead (PI positive) and injured (SYTO 9/PI double positive). This so called “injured state”, which results from an incomplete uptake of PI by damaged bacteria, is associated with growth rates below 0.02% for *E. faecalis* [7, 18, 19, 20, 21, 22, 23, 24].

2.6. Statistics

Results were statistically analyzed using one-way analysis of variance (ANOVA) and Tukey multiple comparison intervals ($\alpha = 0.05$).

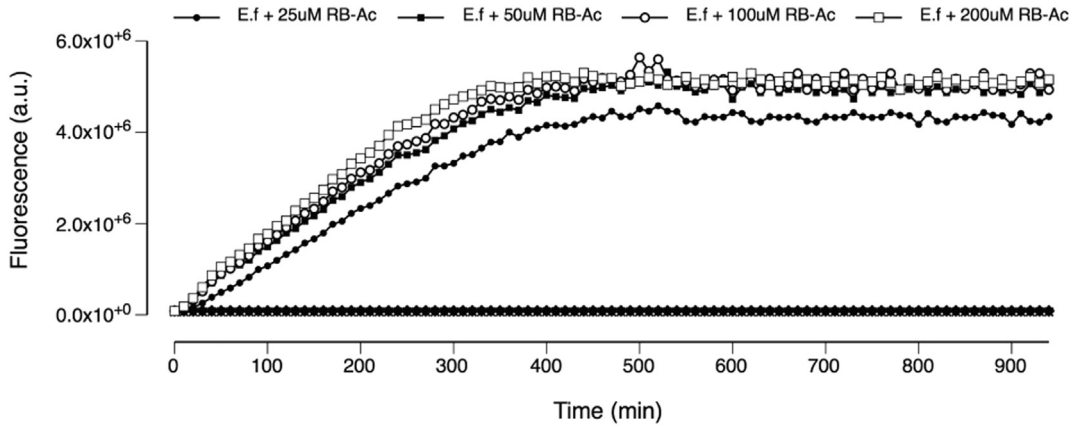

Fig. 1. Kinetic curves of RB production measured as fluorescence intensities after incubation of RB-Ac with *E. faecalis* suspensions. The concentrations of RB-Ac tested were 25 μM (●), 50 μM (■), 100 μM (○) and 200 μM (□). Controls; *E. faecalis* in PBS (◆), supernatant alone (▲), RB-Ac in PBS (X) and supernatant with RB-Ac (△) exhibited similar values and appear superposed. Wavelengths of RB excitation/emission were 544/582 nm. Experiments were done in triplicates and repeated three times (n = 9), standard deviations ≤10%.

3. Results

3.1. Kinetics of RB-Ac Hydrolysis

Figure 1 shows the kinetics of RB-Ac hydrolysis measured as fluorescence intensities in *E. faecalis* suspensions. Controls (bacteria only) exhibited a low fluorescent signal (~8×10^4 a.u.) that did not vary throughout the 900 min of the experiment. The mean fluorescence values of culture supernatants (medium without bacteria) that received RB-Ac were similar to the RB-Ac in PBS (~6×10^4 a.u.).

When RB-Ac was incubated with *E. faecalis* cells, fluorescence increased linearly with time and reached a plateau after approximately 400 min (Fig. 1). Higher fluorescence intensities were observed at higher concentrations of RB-Ac; up to 5.3×10^6 a.u. at 200 μM. The mean rate of fluorescence production in the linear phase ($dF/dt_{@\ 300\ min}$); $1.4 \times 10^4\ min^{-1}$, was comparable between RB-Ac concentrations.

3.2. Epifluorescence Microscopy

Control suspensions of *E. faecalis* (no RB-Ac) displayed a natural fluorescence slightly above background noise. Enterococci incubated with RB-Ac acquired a significantly brighter fluorescent signal (Fig. 2). After 300 and 600 min of incubation, the fluorescence intensities of bacteria significantly increased ($p < .05$) with RB-Ac concentrations. After 900 min of incubation, fluorescence intensities varied between 1200 a.u. and 1700 a.u. independently of the RB-Ac concentration tested.

Qualitatively, fluorescence was localized at the bacterial cellular poles and less frequently at the septum region (Fig. 3D). This pattern was reproducibly observed at all concentrations and incubation times tested.

3.3. RB-Ac Mediated aPDT

Flow cytometry analysis showed that 95% of the bacteria in control cultures (no light/no RB-Ac) remained live throughout the entire experiment (up to 900 min). Neither blue-light irradiation of the culture wells nor RB-Ac incubation without exposure to light did affect viability (Fig. 4).

When bacteria were incubated with RB-Ac during 300 min before being irradiated, the percentage of live cells decreased in a concentration dependent manner. At 25 μM RB-Ac, blue-light irradiation significantly reduced ($p < .05$) the percentage of live cells to 4.2% (± 2.3%) whereas the populations of injured and dead bacteria respectively increased to 55% (± 6%) and 40% (± 20%). Incubation with 50 μM RB-Ac further decreased ($p < .05$) the population of live bacteria below 1% (± 0.3%) and increased the population of dead to 84.6% (± 3%). At 200 μM RB-Ac, 96.6% (± 1.4%) of the bacterial suspensions were dead. Figure 5 shows representative flow cytometry dot plots of the three bacterial populations observed.

Longer incubation times (600 min and 900 min) had similar effects on bacterial viability, with higher percentages of dead bacteria observed at 25 and 50 μM of RB-Ac. At 100 μM and 200 μM RB-Ac, dead cells represented up to 99.9% (± 0.13%).

4. Discussion

To the best of our knowledge, this is the first report that examines the interaction of RB-Ac with bacteria. Because acetylation of the RB converts the molecule into a pro-photosensitizer, the success of RB-Ac-mediated aPDT only depends on the ability of bacteria to hydrolyze the acetate groups. The present study confirmed that *E. faecalis* cells can successfully hydrolyze the RB-Ac compound, and that conversion of the pro-photosensitizer into RB did not occur in absence of bacteria.

The kinetic curves observed during RB-Ac incubation with bacteria were characterized by a linear increase of fluorescence followed by a plateau (Fig. 1). Previous studies that incubated RB-Ac with tumor cells described hydrolysis curves with similar shape but different kinetics [13, 25]. Whereas approximately 400 min were necessary to reach the highest RB fluorescence intensities in bacteria, only 120 min were required in tumor cells. Reasons possibly explaining this difference may include a slower diffusion of the compound across the peptidoglycan layer of *E. faecalis*, a lower enzymatic affinity or a lower enzyme concentration in bacteria. Although the kinetic curves show that RB production was stable after 500 min, incubation was prolonged to 900 min because uptake values and killing efficiencies were still increasing between 300 and 600 min.

The fluorescence photomicrographs, used to observe the distribution of the restored RB, show that the PS accumulated in spots mostly at the cellular poles of *E. faecalis* (Fig. 3). We have previously observed an identical accumulation of RB at the cellular poles of *E. faecalis*, and suggested that the fluorescent spots observed reflect an affinity of the PS for cardiolipin, a phospholipid known to cluster into membrane domains at the poles of bacteria [7, 26]. Such affinity was previously described by Tran et al. [27] who used 10-nonyl acridine orange to stain the cardiolipin-rich domains of the *E. faecalis* membrane, and reported accumulation of the dye at the same spots as RB [27].

Further, accumulation of the produced RB into the membrane may reveal the actual site of RB-Ac hydrolysis, owing to the fact that *E. faecalis* expresses several membrane-bound esterases, such as acetyl-O-esterase and arylesterase, all susceptible to cleave the compound [28]. This is in agreement with Pellicciari et al. [29] who demonstrated that

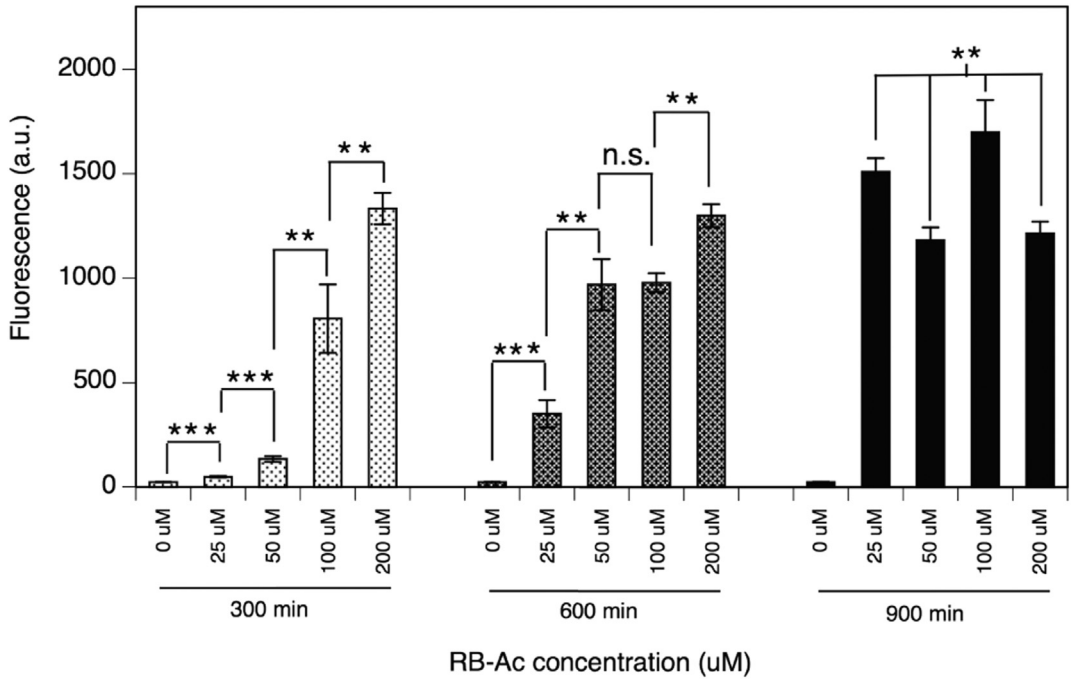

Fig. 2. Fluorescence quantifications of photomicrographs of *E. faecalis* incubated during 300 min, 600 min or 900 min with various concentrations of RB-Ac (from 0 μM to 200 μM). Stars ∗ indicate levels of statistical significance between the different RB-Ac concentrations tested (ANOVA); ∗ $p \leq .05$, ∗∗ $p \leq .01$, ∗∗∗ $p \leq .001$. Error bars indicate standard deviations, $n = 9$.

RB-Ac hydrolysis by membrane-bound esterases is the predominant mechanism of RB production in tumor cells, and that the PS localized in the membrane is subsequently internalized through endocytosis [29].

Although these data suggest that the RB produced by *E. faecalis* did not enter the cytoplasm but incorporated into the membrane, it must be noted that the membrane is an appropriate localization for antibacterial activity usually attributed to cationic PS's, which are commonly used for aPDT [30]. Caminos et al. [31] studied the mechanism of photodynamic inactivation of two cationic porphyrins, A_3B^{3+} and $TMAP^{4+}$ on *Escherichia coli*, and demonstrated that membrane damage was responsible for bacterial killing [31]. Alves et al. [32] compared the light-mediated antibacterial activity against *E. faecalis* of seven cationic porphyrins exhibiting different meso-substituent groups. Their findings demonstrated that Tri-Py+-Me-PF, the most amphipathic molecule

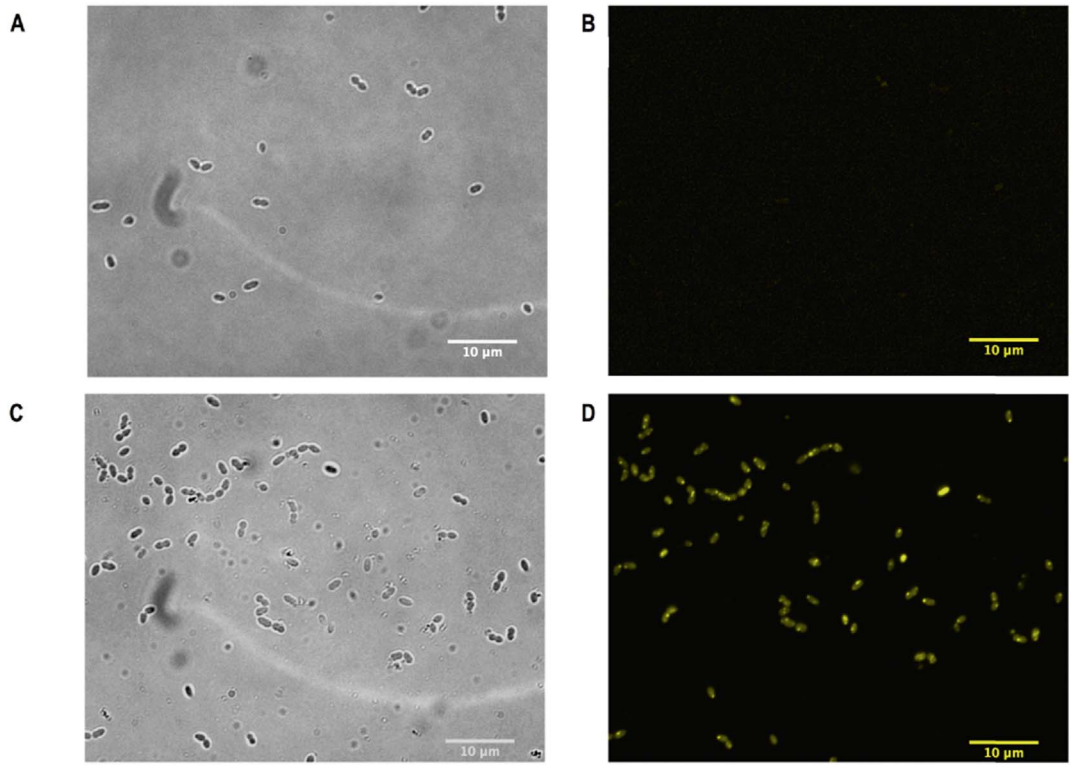

Fig. 3. Representative phase contrast and fluorescence photomicrographs of *E. faecalis* incubated 600 min with PBS (A–B) and with 200 μM RB-Ac (C–D). Yellow fluorescence: excitation 500/20 and emission 535/30. Scale bar represents 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

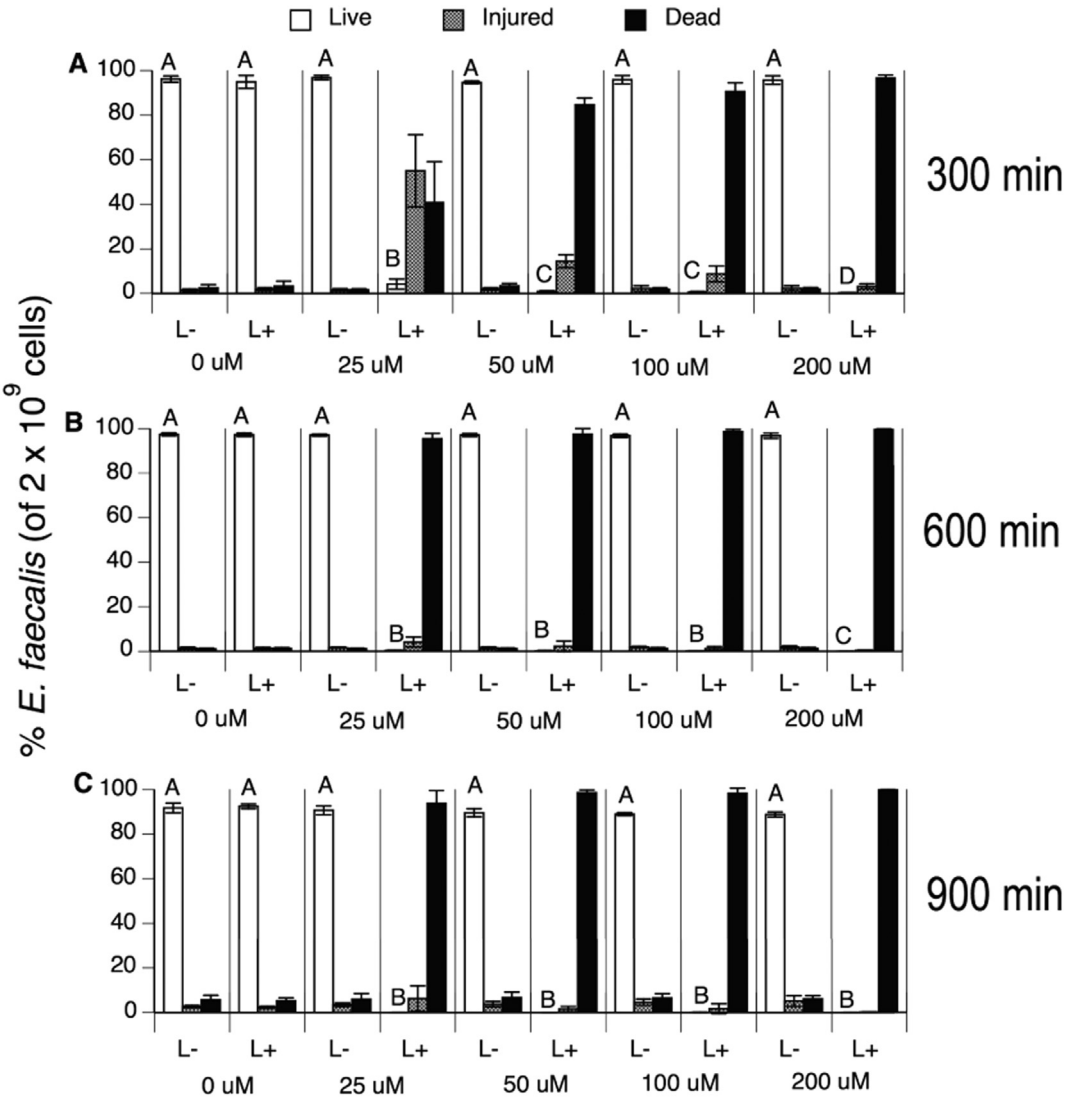

Fig. 4. Effect of various RB-Ac concentrations on *Enterococcus faecalis* viability after incubation during 300 min (A), 600 min (B) or 900 min (C) and 120 s bluelight irradiation. Histograms represent the three populations as observed after FCM analysis; live cells (white bars), injured cells (gray bars) and dead cells (black bars). Letters indicate statistical differences between live cells (ANOVA, Tukey post-hoc comparisons, $\alpha = 0.05$). Error bars indicate standard deviations, n = 9.

among the seven porphyrins tested, displayed the highest antibacterial activity likely due to an increased ability to associate with bacterial membranes [32]. Despite the anionic charges of RB, there is evidence showing that the partition coefficient of the molecule (Log K*p* water/octanol = 0.59) confers amphipathic characteristics to this PS and consequently the ability to incorporate into membrane bilayers [33]. Several reports indicate that irradiation of PS's located into bacterial membranes induce lipid hydroperoxidation, cleavage of fatty acids, cross-linking of membrane proteins all leading to physiological and structural membrane damage and eventually death [34, 35, 36].

For aPDT experiments, *E. faecalis* was incubated in PBS for periods up to 900 min and viability of bacteria remained stable as evidenced by FCM analysis (Fig. 4). This is in agreement with Figdor et al. [37] who showed that *E. faecalis* can survive 7 days in PBS and also resist prolonged periods of starvation (> 4 months) [37]. As expected, the viability of *E. faecalis* was not affected by blue-light irradiation, because this species does not produce endogenous porphyrins and therefore is almost insensitive to blue-light (Fig. 4) [10, 38]. Also, in absence of blue-light irradiation, RB-Ac up to 200 µM had no effect on *E. faecalis* viability, indicating that the compound does not exhibit a dark toxicity. In contrast, RB has been reported to display dark toxicity at concentrations ranging between 5 and 10 µM [39, 40]. Together, this confirms that the killing effect observed was due to the combination of blue-light and enzymatically produced-RB.

Upon irradiation, viability decreased with RB-Ac concentrations. As shown in figure. 5B, light-irradiation of bacteria incubated with 25 µM RB-Ac during 300 min mostly damaged the cells (~55% injured) without eliciting a complete bacterial death (Fig. 5B). However at 50 µM, more dead than injured cells were observed, indicating that bacteria exposed to increasing concentrations of RB-Ac progressively lost their membrane integrity before dying (Fig. 5C, D). Longer incubation times also resulted in increased membrane damage; ~40% of dead bacteria were observed after 300 min of incubation with 25 µM RB-Ac, and this ratio increased to ~95% after 900 min of incubation (Fig. 5A, B and C). The current results indicate that increased membrane damage was associated with higher RB uptakes and that uptake values increased with concentrations and incubation time of RB-Ac (Fig. 2). However, at 900 min of incubation, this trend was not observed anymore. It is possible that a saturation of the sites where RB was shown to accumulate occurred before the end of the incubation period.

At the highest aPDT dose tested (900 min incubation with 200 µM RB-Ac) only 0.01% of the bacteria survived. According to the American Society of Microbiology, "any new approach must achieve a reduction of at least 3 $\log_{10}$ colony forming units (killing efficiency of 99.9% or

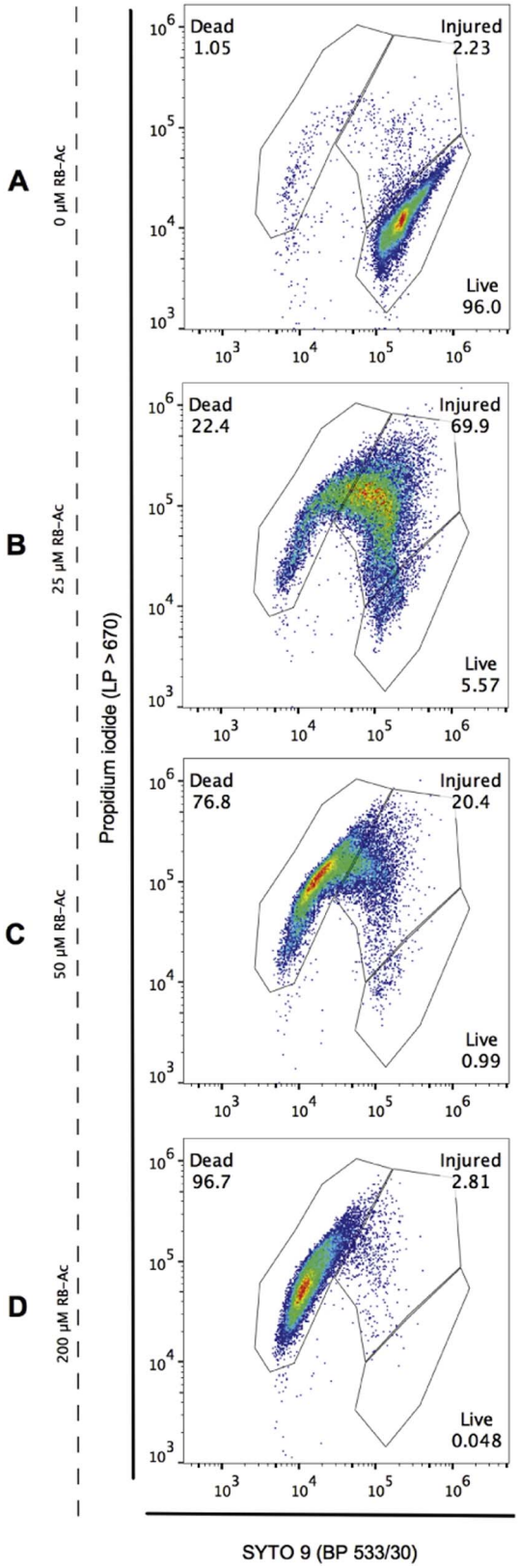

Fig. 5. Flow cytometric dot plots of *Enterococcus faecalis* stained with SYTO 9 and PI after 300 min incubation with PBS (A), with 25 µM RB-Ac (B), with 50 µM RB-Ac (C) or with 200 µM RB-Ac (C) and light irradiated for 120 s. SYTO 9 fluorescence (BP 533/30 nm) on the X-axis is plotted against PI fluorescence (LP > 670 nm) on the Y-axis. Numbers in each gate represent events frequencies.

more) to be termed antimicrobial or antibacterial" [41]. The bacterial counts provided by flow cytometry in this report, indicate that RB-Ac-mediated aPDT achieved a 4 $\log_{10}$ reduction in viability and can therefore be termed antibacterial.

Together these results indicate that RB production and antibacterial activity were highest after 900 min of incubation. Long incubation time may be used in clinical applications where the compound could be administrated, maintained in place and subsequently activated during a second visit. As an example, the treatment of infected dental roots may benefit from this approach because several visits are generally needed to disinfect a root canal [42]. During the first visit RB-Ac could be placed into the root canal, then hydrolyzed by bacteria in-between the appointments and finally light activated during a second visit. There is evidence showing that aPDT in combination with conventional root canal disinfection results in superior bacterial reduction and improved healing [43].

Because RB-Ac hydrolysis was shown to occur only in close contact with bacteria, this mechanism could offer a selective delivery of RB in areas of high bacterial density, such as biofilms. The hydrophobic nature of the RB-Ac compound may facilitate its penetration into the biofilm exopolysaccharide matrix known to restrict the diffusion of hydrophilic and positively charged molecules [44]. Further investigations on biofilms seem therefore warranted. Future studies may also investigate different chemical modifications to produce pro-photosensitizers with faster enzymatic kinetics or specificity towards bacterial strains.

Within the limitations of this study, it was shown that RB-Ac can be hydrolyzed in the presence of *E. faecalis* cells. The use of RB-Ac did not appear to allow cytoplasmic internalization of the RB produced, which rather incorporated the membrane bilayers of the bacterium. Therefore, the use of RB-Ac did not provide additional advantages over RB in terms of PS localization. Nonetheless, sufficient RB was produced and incorporated into the bacterial membranes to achieve successful bacterial killing using RB-Ac-mediated-aPDT.

Acknowledgments

Manufacturers of the light sources used in this study (KerrHawe SA, Bioggio, CH) are acknowledged for their support. No competing financial ties exist.

Funding Information

This study was supported by Grant #31003A-149962 of the Swiss National Science Foundation.

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