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

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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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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 ($\alpha = 0.05$).