Research Article

First Report on Photodynamic Inactivation of Archaea Including a Novel Method for High-Throughput Reduction Measurement

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ABSTRACT

Archaea are considered third, independent domain of living organisms besides eukaryotic and bacterial cells. To date, no report is available of photodynamic inactivation (PDI) of any archaeal cells. Two commercially available photosensitizers (SAPYR and TMPyP) were used to investigate photodynamic inactivation of Halobacterium salinarum. In addition, a novel high-throughput method was tested to evaluate microbial reduction in vitro. Due to the high salt content of the culture medium, the physical and chemical properties of photosensitizers were analyzed via spectroscopy and fluorescence-based DPF assays. Attachment or uptake of photosensitizers to or in archaeal cells was investigated. The photodynamic inactivation of Halobacterium salinarum was evaluated via growth curve method allowing a high throughput of samples. The presented results indicate that the photodynamic mechanisms are working even in high salt environments. Either photosensitizer inactivated the archaeal cells with a reduction of 99.9% at least. The growth curves provided a fast and precise measurement of cell viability. The results show for the first time that PDI can kill not only bacterial cells but also robust archaea. The novel method for generating high-throughput growth curves provides benefits for future research regarding antimicrobial substances in general.

INTRODUCTION

At the very beginning of the 20th century, Oskar Raab and Hermann von Tappeiner discovered that Paramecium cells can be inactivated by simultaneous application of light and acridine dyes by a process called photodynamic mechanism (1–3). Now, it is well known that light is absorbed by such a photosensitizer molecule, which thereby generates reactive oxygen species (ROS). These ROS can destroy cells via oxidation of various cellular structures. Meanwhile, the photodynamic mechanism found its way into different medical fields termed photodynamic therapy of tumors (PDT) (4).

Another promising application of the photodynamic mechanism is PDI of microorganisms that has been proven efficient against viruses, bacteria and fungi. In PDI, a cationic photosensitizer is usually attached to the surface or taken up by the cell and ROS kill the cells or viruses via oxidative mechanisms (5). A variety of molecules were successfully tested as photosensitizers to be used in PDI like porphyrins, phthalocyanines, phenalenones, phenothiazines or flavins (5).

Photodynamic inactivation (PDI) is efficient in killing bacteria regardless their types or resistances to antibiotic substances (6,7). The photodynamic inactivation of antibiotic-resistant bacteria like methicillin-resistant Staphylococcus aureus (MRSA) was successfully shown on human skin ex vivo (8). When immobilizing photosensitizers as antimicrobial coating, a clinical study provided evidence that generated singlet oxygen can kill bacteria on near-patient surfaces (9). PDI was also successfully applied to inactivate different viruses (10–15). Also, fungi (16–20) can be killed with ease with different photosensitizers. Furthermore, photodynamic inactivation is capable of efficiently treating certain protozoa (21).

Thus, PDI can be applied to various pathogenic and non-pathogenic organisms spanning nearly all parts of known living matter. However, the scientific literature remains without any prove that this principle applies to archaea. This might be explained by the fact that there are no discrete pathogens within the archaea so far. However, scientists start to understand the importance of a healthy human microbiome that also contains archaea (22–24) although archaea are typically found in various extreme environments such as black smokers or hydrothermal vents. These organisms are called hyperthermophiles with optimal growth temperatures above 80°C. Some archaea have also been reported to be extremely tolerant to ionizing radiation such as the anaerobic Euryarchaeon Thermococcus gammatolerans (25). Picrophilus oshimae on the other hand is an organism that was isolated from a solfatara field in Hokkaido, Japan, that naturally has a pH of 2.2 and is therefore considered an acidophilic organism (26). Recently, archaeal signatures have been discovered on board the international space station that is the latest prove for archaea in artificial, human-inhabited environments (27).

Since PDI requires oxygen to generate ROS, such research in archaea is hampered since the majority of these organisms is considered anaerobic. Aerobic archaea usually require pH values at which photosensitizers are chemically destroyed (28).

Therefore, in this study the extremely halophilic organism Halobacterium salinarum (29), which grows aerobically at neutral pH values was investigated. Cells of the genus Halobacterium are rods or disk-shaped cells that stain Gram-negative and...
often contain gas vacuoles (30). The present study shows PDI of an Archaeum (*Halobacterium salinarum*) for the first time. Another goal of this study was the application of growth curves in order to evaluate the logarithmic reduction of viable cells like archaea. The application of growth curves allows a much higher throughput of samples compared to other methods such as pour plate, spread plate or drop plate methods (31,32).

**MATERIALS AND METHODS**

**Photosensitizers.** The Photosensitizer TMPyP was purchased from Sigma-Aldrich with a minimum dye content of 97%, while SAPYR, an exclusively singlet oxygen producing photosensitizer, was purchased from TriOptoTec GmbH, with a minimum dye content of 97%. Photosensitizer solutions were prepared in adequate concentrations of 2, 10, 20, 50 and 100 µmol L⁻¹ in sterile sodium chloride solution (10% w/v).

**Organism and cultivation.** For inactivation, *Halobacterium salinarum* DSM 3754 was used. It was cultivated at 37°C for 48 h in a modified *Halobacterium* medium containing per liter 5 g yeast extract (Becton, Dickinson and Company, Sparks), 5 g casamino acids (Becton, Dickinson and Company), 1 g sodium glutamate (Merck KGaA, Darmstadt, Germany), 2 g potassium chloride (Carl Roth GmbH+Co. KG, Karlsruhe, Germany), 5 g sodium tetra citrate (Merck KGaA), 20 g magnesium sulfate heptahydrate (Merck KGaA) and 200 g sodium chloride (Carl Roth GmbH+Co. KG). Besides the organic compounds, all substances were provided in analytic grade. The medium was autoclaved at 121°C for 20 min in 50 mL Erlenmeyer flasks containing 20 mL medium.

**Cell preparation.** Cells were harvested via centrifugation of 10 mL medium transferred into sterile 15 mL reaction tubes. Centrifugation took place for 7 min at 4500 g. The supernatant was discarded, and the pellet was suspended in 5 mL of sterile sodium chloride solution (10% w/v). These steps were repeated thrice in order to remove remaining culture medium. After harvesting, the optical density measured at 600 nm of the cells was adjusted to 0.6.

**Photodynamic inactivation.** The assays were conducted in 96-well plates. Column 1 of the plate contained 25 µL of archaeal cells with 25 µL of sodium chloride solution (10% w/v). Next five columns contained TMPyP as a photosensitizer in ascending concentration, namely 1, 5, 10, 25 and 50 µmol L⁻¹, and 25 µL of archaeal cells. Columns 7–12 were prepared accordingly with SAPYR. Experiments were conducted at low ambient light conditions as described elsewhere (33). The assays were illuminated beneath a commercial blue light source (blue_v, Waldmann GmbH, Villingen-Schweningen, Germany) with a radiant exposure of 10.8 J cm⁻². Additionally to the illuminated sample, a dark control was treated in the same manner without irradiation for dye concentrations of 0 and 50 µmol L⁻¹. After illumination, 20 µL of the cell suspension were immediately transferred into 180 µL of prewarmed (37°C) culture medium in 96-well plates. Twelve wells of the well plate contained medium without cells and served as blank control. These so prepared well plates were placed in a spectral photometer (CLARIOStar, BMG LABTECH GmbH, Ortenberg, Germany). Internal incubator was set to 37°C, while the internal shaker was turned on every 5 min shaking at 200 rpm for 30 s. After every 5 min, the spectral photometer measured the optical density at 600 nm of all wells. In total, the measurement was carried out for 2245 min with an additional last measurement carried out at 4115 min in order to increase the limit of detection to around 3 orders of magnitude. As each experiment was performed in triplicates, mean values of the optical density values were calculated and plotted via SigmaPlot against the incubation time.

**Calculation of microbial reduction.** The method used in this report for calculation of the microbial reduction was performed in accordance with another report in a modified manner (34). Therefore, doubling time of the archaeal cells was calculated as Δq between an optical density of 0.1 and 0.2 in early exponential growth phase. The doubling times were calculated for the untreated control. The time difference in reaching the optical density of 600 nm of 0.1 of the control and illuminated cells was calculated and is further on called Δt. The logarithmic reduction shortly called q was subsequently calculated as described in the formula below.

\[
q = \log \frac{2^{\Delta t}}{2}
\]

\[
\rho = \log 2^{\Delta t}
\]

**Attachment of photosensitizers to cells.** In order to investigate the attachment of the photosensitizer to the cell surface, a culture grown as mentioned before was centrifuged at 4500 g for 7 min and final optical density at 600 nm was adjusted to 0.6. The supernatant was discarded and the cells were suspended in 500 µL of 10% (w/v) NaCl and 500 µL of 20 µmol L⁻¹ of photosensitizer solution. Cells were mixed thoroughly and centrifuged again. The supernatant was measured in a photometer at a wavelength of 421 nm for TMPyP and 370 nm for SAPYR, resembling the corresponding absorption maxima. As blank, a 10% (w/v) NaCl solution was used. Control consisted of 5 µmol L⁻¹ in a 10% (w/v) NaCl solution.
DPBF (1,3-Diphenylisobenzofuran) assays. For analyzing the production of singlet oxygen production under high salt concentrations, photosensitizer in final concentrations of 1, 5, 10, 25 and 50 µmol L\(^{-1}\) and sodium chloride (20% w/v) were mixed and resuspended with the DPBF reagent with MeOH as a solvent to a total volume of 200 µL. Final concentrations were 1, 5, 10, 25 and 50 µmol L\(^{-1}\) DPBF and 10% (w/v) NaCl. DPBF was excited in a spectral photometer (CLARIOStar, BMG LABTECH GmbH) at 411 nm while emission was detected at 451 nm. Relative fluorescence was calculated from a control without PS as:

\[
\text{Relative fluorescence} = \frac{\text{Fluorescence}_{\text{PS}}}{\text{Fluorescence}_{\text{Control}}}
\]

DPBF assays indicate that the production of singlet oxygen takes place with both dyes under high sodium chloride concentrations (Fig. 2). TMPyP generated more singlet oxygen when compared to SAPYR under the experimental conditions. For the highest concentration of 50 µmol L\(^{-1}\), the same fluorescence is observed, indicating that either the same amount of oxygen was produced or all the available oxygen was depleted within the reaction. After 10 s of illumination, 50 µmol L\(^{-1}\) TMPyP (Fig. 2a) yielded a relative fluorescence of 10.3% and 11.0% for 50 µmol L\(^{-1}\) SAPYR (Fig. 2b), respectively. 25 µmol L\(^{-1}\) of photosensitizer showed a relative fluorescence of 10.2% for TMPyP and 55.6% for SAPYR, respectively. At 10 µmol L\(^{-1}\), barely reduced relative fluorescence for TMPyP after 10 s was detected (10.5%) while SAPYR exhibited a tremendously higher relative fluorescence with 80.3%. 32.9% for 5 µmol L\(^{-1}\) TMPyP and 75.1% for 5 µmol L\(^{-1}\) SAPYR were the measured relative fluorescence values for mentioned concentration. 1 µmol L\(^{-1}\) of photosensitizer led to a relative fluorescence of 80.7% for TMPyP and 102.4% for SAPYR. Water controls after 10 s of illumination for SAPYR (data not shown) showed values of 22.6% for 50 µmol L\(^{-1}\), 39.9% for 25 µmol L\(^{-1}\), 64.2% for 10 µmol L\(^{-1}\), 76.9% for 5 µmol L\(^{-1}\) and 107.4% for 1 µmol L\(^{-1}\). Water controls for TMPyP showed after 10 s of illumination: 10.2% for 50 µmol L\(^{-1}\), 10.3% for 25 µmol L\(^{-1}\), 10.5% for 10 µmol L\(^{-1}\), 16.5% for 5 µmol L\(^{-1}\) and 84.5% for 1 µmol L\(^{-1}\). Fig. 2c compares the relative fluorescence for SAPYR and TMPyP after 10 s of illumination in dependence of the concentration of the photosensitizer.

The growth curves displayed in Fig. 3a clearly show that for the highest concentrations of SAPYR (25 and 50 µmol L\(^{-1}\)), no growth of *H. salinarum* was detected. Even after 4115 min of incubation, optical density values have not reached values above 0.1 (data not shown). The calculated log\(_{10}\) reductions for SAPYR are displayed in Fig. 3b. Significant log\(_{10}\) reductions were obtained for photosensitizer concentrations of 25 and 50 µmol L\(^{-1}\).
The illuminated control without photosensitizer exhibited a logarithmic reduction of 0.05.

When using the same concentrations of TMPyP and radiant exposure, the inactivation of *H. salinarum* was more efficient as shown by the growth curves in Fig. 4a compared to SAPYR (Fig. 3). The lowest photosensitizer concentration was 5 µmol L⁻¹ to achieve maximal logarithmic reduction of 3.22. At 4115 min, TMPyP concentrations from 5 to 50 µmol L⁻¹ showed that optical density did not exceed values above 0.1 resulting in a microbial reduction of at least 3 orders of magnitude. Dark control for TMPyP at 50 µmol L⁻¹ showed no relevant logarithmic reduction with a calculated value of 0.09.

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**Figure 3.** (a) Growth curves of *H. salinarum* after PDI treatment with SAPYR as photosensitizer. Different colors reflect the different SAPYR concentrations used for PDI treatment. Y-axis indicates the optical density measured at 600 nm. X-axis indicates the time in minutes. (b) Calculated log₁₀ reduction displayed as bar chart diagram.

**Figure 4.** (a) Growth curves of *H. salinarum* after PDI treatment with TMPyP as Photosensitizer. Different colors reflect the different photosensitizer concentrations used for PDI treatment. Y-axis indicates the optical density measured at 600 nm. X-axis indicates the time in minutes. (b) Calculated log₁₀ reduction displayed as bar chart diagram.
DISCUSSION

The presented results clearly show that the Archaeum *Halobacterium salinarum* can be efficiently inactivated (>99.9%) using PDI with either photosensitizer at low concentrations. The results also show that high concentrations of sodium chloride, for example, 10%, did not lead to a chemical alteration of the photosensitizers used. The loss of concentration after illumination for SAPYR is a general feature of the molecule, observed even in water without further substances present.

The structure of archaeal cells shows significant differences when compared to eukaryota or bacteria. One of the most striking differences is their outer structure. Besides an S-Layer, which is also known for some bacteria, archaea are characterized by their extraordinary membrane structure. The phospholipid bilayer membrane consists of side chains of 20 carbon atoms built from isoprene attached with an ether linkage to glycerol compared to eukarya and bacteria that form ester bonds with fatty acids (35,36). Another obvious feature of halophilic archaea is the pink-to-purple color of the cells due to retinal bound to rhodopsin-like molecules named bacteriorhodopsin (37). Bacteriorhodopsin is an integral membrane protein with seven near-parallel alpha helices, whereas between the helices a retinal molecule is integrated as ligand forming a trimer composed of 21 helices and three retinal molecules (38). This protein functions as a light-driven proton pump contributing to the formation of a proton gradient for energy conservation (39). As rhodopsin and bacteriorhodopsin are structurally and chemically very similar, the singlet oxygen-quenching rate constant for rhodopsin should be quite similar to bacteriorhodopsin, at least within the same order of magnitude. The reported value for the quenching rate is 1.1 × 10^7 L mol⁻¹ s⁻¹, therefore being similar to the ones found in literature for the azide ion (40–43). The presented results clearly show that archaeal structures compared to bacteria should have an impact on the efficiency but do not lead to a certain resistance or tolerance. Recently, van der Oost and coworkers engineered an *E. coli* cell capable of producing and incorporating archaeal lipids into the cell membranes (44). The impact of archaeal lipids concerning the susceptibility toward ROS could be studied in a sophisticated manner in future research utilizing the mentioned *E. coli* with the hybrid membrane.

This study also showed that archaeal structures like S-layer, rhodopsin or ether lipids did not prevent binding of the photosensitizer. It should be noted that the present investigation cannot differentiate whether photosensitizer molecules are taken up by the archaeal cells or are attached to the cellular wall only. The amounts of photosensitizer bound to the cells differ for both photosensitizers used. This difference might be explained by the different charges of the photosensitizers. SAPYR molecule exhibits only one positive charge while TMPyP four. Thus, TMPyP should attach much better to negatively charged outer structures of cells in general. As for bacterial cells, the S-layer of *Halobacterium* is negatively charged as the charge also maintains the lattice of the S-layer (45–47).

DPBF assays in general are sufficient to detect the generation of singlet oxygen. Our data suggest that under the experimental conditions, TMPyP produced more singlet oxygen than SAPYR. However, the different extinction coefficients along with emission spectrum of the light source and the photosensitizer concentrations must be considered. The impact of the absorbed photons on photosensitizers was thoroughly investigated elsewhere (48). According to that publication, the amounts of absorbed photons per second were compared. Additionally, the different singlet oxygen quantum yields were considered which are 0.77 for TMPyP (49) and 0.99 for SAPYR (50). Under present experimental conditions, SAPYR generates 94% or 62% less singlet oxygen than TMPyP for 1 or 50 µmol L⁻¹ photosensitizer concentrations, respectively. These data should explain to some extent the difference in singlet oxygen production (DPBF assays; Fig. 2) and consequently also in PDI of *Halobacterium salinarum* for both photosensitizers (Figs. 3 and 4). Nevertheless, a reduction of viable cells is achieved for both photosensitizers with more than 3 log₁₀ at 5 µmol L⁻¹ (TMPyP) or 25 µmol L⁻¹ (SAPYR). Our study showed that photodynamic inactivation of archaea works well and efficiently even at high salt concentrations. However, when compared to other model organisms like *E. coli*, *B. atrophaeus* or *E. faecalis*, *Halobacterium salinarum* seems to be less susceptible to PDI (33,48,50).

Various studies concerning photodynamic inactivation focus primarily on pathogenic bacteria causing severe skin infection (8,51–53). Nevertheless, the contribution of archaea to the human health in general is highly debated and highlighted (54–56). Up to date, there are no representatives known within the archaea that can cause pathogenic infections. However, it is known that archaea are able to colonize the human body (23,54,57–59). The role of *Methanobrevibacter oralis* in gingivitis and brain abscesses has been described (60,61).

When looking at the second goal of the present study, the data indicate that the presented method for high-throughput measurement of the antimicrobial potential can be applied to any microorganism in liquid medium. The presented method offers several advantages compared to other methods like spread plating, pour plating or drop plating. First, the method offers huge time saving as the process of measuring and partly evaluation is automated. Compared to plating methods, a dilution series is not necessary, as well as plating is obviously not performed. Furthermore, the method is much cheaper than plating methods as no agar medium is necessary and the experiments can be performed within the microvolume range. Drop plating methods have some issues like accuracy and limitations to certain cells; however, the problems were mentioned and tried to optimize elsewhere (32).

CONCLUSIONS

The presented results are the first record for the photodynamic inactivation of archaea, therefore proving the applicability of PDI toward all domains of life. The novel method for generating high-throughput growth curves will prove useful in the future concerning research of antimicrobial substances in general, as the method is applicable for all organisms that grow in liquid media. Our future research concerning photodynamic inactivation of archaea will focus on the role of archaeal lipids concerning photodynamic inactivation.

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