The inability of a bacteriophage to infect *Staphylococcus aureus* does not prevent it from specifically delivering a photosensitizer to the bacterium enabling its lethal photosensitization

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**Objectives:** It has been demonstrated that the efficiency of lethal photosensitization can be improved by covalently binding photosensitizing agents to bacteriophage. In this study we have investigated whether a bacteriophage requires the capacity to infect the bacterium to enhance lethal photosensitization when linked to a photosensitizer.

**Methods:** Tin (IV) chlorin e\textsubscript{6} (SnCe\textsubscript{6}) was conjugated to bacteriophage F\textsubscript{11}, a transducing phage that can infect *Staphylococcus aureus* NCTC 8325-4, but not epidemic methicillin-resistant *S. aureus* (EMRSA)-16. The conjugate and appropriate controls were incubated with these bacteria and either exposed to laser light at 632.8 nm or kept in the dark.

**Results:** The SnCe\textsubscript{6}/F\textsubscript{11} conjugate achieved a statistically significant reduction in the number of viable bacteria of both 8325-4 and EMRSA-16 strains by 2.31 log\textsubscript{10} and 2.63 log\textsubscript{10}, respectively. The conjugate could not however instigate lethal photosensitization of *Escherichia coli*. None of the other combinations of controls, such as an equivalent concentration of SnCe\textsubscript{6} only, an equivalent titre of bacteriophage only or experiments conducted without laser light, yielded significant reductions in the number of viable bacteria recovered.

**Conclusions:** The inability of a bacteriophage to infect *S. aureus* does not prevent it from specifically delivering a photosensitizer to a bacterium enabling its lethal photosensitization.

Keywords: tin (IV) chlorin e\textsubscript{6}, SnCe\textsubscript{6}, MRSA, photodynamic therapy, PDT

**Introduction**

Light-activated antimicrobial agents (photosensitizers) are an appealing alternative to conventional antibiotics for the treatment of localized bacterial infections. Lethal photosensitization (LP) has been demonstrated to be effective at killing a range of bacteria including opportunistic pathogens, commensal cutaneous species,\textsuperscript{1} periodontal pathogens\textsuperscript{2} and epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA).\textsuperscript{3} LP has two main advantages over conventional antimicrobial chemotherapy. First, the bactericidal effect is limited to the area that is treated with both the photosensitizer and light, preventing disturbance of the wider commensal microbial community.\textsuperscript{2} Second, the non-specific mode of action of liberated singlet oxygen (\(^1\text{O}_2\)) against bacteria is unlikely to induce the development of protective mechanisms and the subsequent proliferation of these genes through the wider microbial community.

The inherent reactivity of \(^1\text{O}_2\) limits its ability to diffuse through an aqueous environment. The lifetime of \(^1\text{O}_2\) in pure water is \(\sim 4 \mu s\), which results in a theoretical diffusion distance of 125 nm, if one assumes that the moiety does not interact with a biological molecule.\textsuperscript{5} This short-range action (on the scale of biological systems) may possibly limit the effectiveness of LP. We have previously developed methodologies to facilitate the close association of photosensitizer and bacteria using targeting systems based upon the covalent conjugation of the photosensitizer tin (IV) chlorin e\textsubscript{6} (SnCe\textsubscript{6}) onto immunoglobulin G (IgG).\textsuperscript{3,6} More recently, we have found that covalently linking SnCe\textsubscript{6} to *S. aureus* bacteriophage 75, commonly used for typing, targets LP to a range of strains of *S. aureus* including MRSA.\textsuperscript{7} In the study reported herein, we examined whether another unrelated *S. aureus* bacteriophage, phage F\textsubscript{11}, a generalized transducing phage, could replace phage 75 in targeting LP to *S. aureus* strains.
Materials and methods

Bacteria and bacteriophage

The two strains of *S. aureus* used in these experiments were EMRSA-16 (NCTC 13143), one of the dominant nosocomial MRSA isolates in UK hospitals, and 8325-4, a prophage-free derivative of NCTC 8325 that is methicillin-susceptible. These strains were grown on Columbia agar (Oxoid Ltd, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood (CBA). *Escherichia coli* 10418 was also incorporated as a Gram-negative control. In preparation for the LP experiments, a colony was inoculated into 20 mL of nutrient broth no. 2 (NB2) containing 10 mM CaCl₂ and grown aerobically for 16 h at 37°C in a shaking incubator. The cultures were then washed by centrifugation and re-suspension in PBS containing 10 mM CaCl₂ and adjusted to a final optical density at 600 nm (OD₆₀₀) of 0.05; these cell suspensions contained ~1×10⁷ cfu/mL.

Bacteriophage Φ₁₁ is a generalized transducing phage present in *S. aureus* NCTC 8325 as a prophage. This bacteriophage can infect the NCTC 8325 derivative 8325-4, but not EMRSA-16. Phage Φ₁₁ was propagated in *S. aureus* 8325-4 using the phage overlay method and SnCe6 was covalently conjugated to the bacteriophage using methods described previously. The concentration of SnCe6 bound to the phage was determined by spectral analysis against a calibration curve generated from known concentrations of SnCe6. In different experiments, between 7.5×10⁶ and 4.7×10⁷ pfu of bacteriophage was used in conjugation reactions and the amount of SnCe6 bound varied between 3.5 and 7 μg/mL.

LP experiments

Fifty microlitres of the SnCe6/Φ₁₁ conjugate was added to 50 μL of bacterial suspension in a sterile 96-well plate. The controls consisted of: SnCe6 alone (at the same concentration as the conjugate); Φ₁₁ alone (at the same titre as the conjugate); and PBS. All of the mixtures were incubated in the dark, with stirring, for 30 min prior to exposure to laser light. The relevant samples were then sequentially exposed to laser light (632.8 nm) from a helium/neon (HeNe) gas laser with a measured power output of 29.2 mW (Spectra-Physics, Darmstadt-Kranichstein, Germany) for a period of 5 min; the mixtures were magnetically stirred throughout the course of an experiment. Additional ‘dark controls’ were also conducted for these four variables without laser light. The number of viable bacteria remaining in the samples was determined immediately following exposure to the laser light by serial dilution and enumeration of cfu on CBA. Each experimental variable was repeated as a duplicate.

The duplicate experiments with both strains of *S. aureus* were conducted a total of four times (n=8), whilst those for *E. coli* were repeated twice (n=4). The null hypothesis was that there was no difference between the log₁₀ counts of the number of cfu using various different experimental parameters; this was analysed by Student’s *t*-test to yield *P* values.

Results

When compared with the control, which was not exposed to laser light or to photosensitizer, the SnCe6/Φ₁₁ conjugate in the presence of laser light yielded a 2.31 log₁₀ reduction (*P*, 0.05) in the number of viable bacteria recovered from the culture of *S. aureus* 8325-4 and a 2.63 log₁₀ reduction (*P*, 0.05) for the culture of EMRSA-16. In the presence of laser light, the SnCe6/Φ₁₁ conjugate did not result in significant killing of *E. coli* 10418. None of the other combinations of controls (i.e. SnCe6 only, phage only and ‘dark controls’) produced significant bacterial kills (Figure 1).

![Figure 1](image-url)

Figure 1. Number of viable bacteria recovered following exposure of SnCe6/bacteriophage Φ₁₁ conjugate to laser light (leftmost columns) compared with controls. The designations L+ and L− refer to the presence or absence of light, respectively, and the designations S+ and S− refer to the presence or absence of photosensitizer, respectively.
Phage-mediated delivery of a photosensitizer

Discussion

We have previously shown that bacteriophage 75, a serotype F staphylococcal phage, could be used to target LP to a range of \textit{S. aureus} strains including strains it could not infect. The capacity of bacteriophage 75 to target LP to a range of \textit{S. aureus} strains was surprising since this phage has a restricted host range. The question we asked in the current study was whether other staphylophage could target LP to a range of \textit{S. aureus} strains, once conjugated to a photosensitizer, or if this was a specific trait of phage 75. We did this by investigating the capacity of bacteriophage \textit{F}11, a prototypic group B-transducing phage,\textsuperscript{9} to target LP to \textit{S. aureus}.

When SnCe6 was conjugated to \textit{S. aureus} bacteriophage \textit{F}11, for \textit{S. aureus} strains 8325-4 and EMRSA-16 in the presence of laser light there was an increase in the killing of these bacteria by 2.39 log\textsubscript{10} and 2.35 log\textsubscript{10}, respectively, when compared with the equivalent concentration of SnCe6 alone (i.e. free SnCe6 that was not conjugated to the bacteriophage). Since it is known that staphylophage have the capacity to bind to all strains of \textit{S. aureus}\textsuperscript{10} and bacteriophage \textit{F}11 is not capable of infecting strain EMRSA-16, the kill achieved by the SnCe6/\textit{F}11 conjugate suggests that the photosensitizer/bacteriophage conjugate only needs to bind to the bacterial cell to induce killing in the presence of laser light. The selectivity of the photosensitizer/bacteriophage conjugate in targeting LP to \textit{S. aureus} was demonstrated by the inability to cause significant killing of \textit{E. coli} in the presence of laser light.

Our results demonstrate that it is possible to use different serotypes of staphylophage as vehicles to deliver photosensitizer payloads to the surface of \textit{S. aureus} thus enabling selective LP of this bacterium in the presence of laser light. Such designer composites would not only possess all of the advantages that photodynamic therapy has over conventional antibiotic therapy, as described in the introduction, but they would also ensure there was minimal collateral damage to the host and its indigenous microflora.

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Transparency declarations

None to declare.

References