

1 **The inability of a bacteriophage to infect *Staphylococcus aureus***
2 **does not prevent it from specifically delivering a photosensitiser to**
3 **the bacterium enabling its lethal photosensitisation**

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5 **Running Title: PDT Phage**

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24 **Abstract**

25 Objectives: It has been demonstrated that the efficiency of lethal photosensitisation
26 can be improved by covalently binding photosensitising agents to bacteriophage. In
27 this study we have investigated whether a bacteriophage requires the capacity to
28 infect the bacterium to enhance lethal photosensitisation when linked to a
29 photosensitizer.

30

31 Methods: Tin (IV) chlorin e6 (SnCe6) was conjugated to bacteriophage Φ 11, a
32 transducing phage which can infect *Staphylococcus aureus* NCTC 8235-4, but not
33 EMRSA 16. The conjugate and appropriate controls, were incubated with these
34 bacteria and either exposed to laser light at 632.8 nm or kept in the dark.

35

36 Results: The SnCe6 / Φ 11 conjugate achieved a statistically significant reduction in
37 the number of viable bacteria of both 8325-4 and EMRSA 16 strains by 2.31 log₁₀
38 and 2.63 log₁₀ respectively. The conjugate could not however instigate lethal
39 photosensitisation in *E. coli*. None of the other combinations of controls; such as an
40 equivalent concentration of SnCe6 only, an equivalent titre of bacteriophage only or
41 experiments conducted without laser light; yielded significant reductions in the
42 number of viable bacteria recovered.

43

44 Conclusions: The inability of a bacteriophage to infect *S. aureus* does not prevent it
45 from specifically delivering a photosensitiser to a bacterium enabling its lethal
46 photosensitisation.

47 **Introduction**

48 Light-activated antimicrobial agents (photosensitisers) are an appealing alternative to
49 conventional antibiotics for the treatment of localised bacterial infections. Lethal
50 photosensitisation (LP) has been demonstrated to be effective at killing a range of
51 bacteria including opportunistic pathogens, commensal cutaneous species,¹
52 periodontal pathogens² and epidemic methicillin-resistant *Staphylococcus aureus*
53 (EMRSA).³ LP has two main advantages over conventional antimicrobial
54 chemotherapy. Firstly, the bactericidal effect is limited to the area that is treated with
55 both the photosensitiser and light, preventing disturbance of the wider commensal
56 microbial community.⁴ Secondly, the non-specific mode of action of liberated singlet
57 oxygen (¹O₂) against bacteria is unlikely to induce the development of protective
58 mechanisms and the subsequent proliferation of these genes through the wider
59 microbial community.

60

61 The inherent reactivity of ¹O₂ limits its ability to diffuse through an aqueous
62 environment. The lifetime of ¹O₂ in pure water is ~4 μs, which results in a theoretical
63 diffusion distance of 125 nm, if one assumes that the moiety does not interact with a
64 biological molecule.⁵ This short range action (on the scale of biological systems)
65 may possibly limit the effectiveness of LP. We have previously developed
66 methodologies to facilitate the close association of photosensitiser and bacteria
67 using targeting systems based upon the covalent conjugation of the photosensitiser
68 tin (IV) chlorin e6 (SnCe6) onto immunoglobulin G (IgG).^{3, 6} More recently, we have
69 found that covalently linking SnCe6 to *S. aureus* bacteriophage 75, commonly used
70 for typing, targets lethal photosensitisation to a range of strains of *S. aureus*
71 including MRSA.⁷ In the study reported herein, we examined whether another

72 unrelated *S. aureus* bacteriophage, phage Φ 11 a generalised transducing phage,
73 could replace phage 75 in targeting lethal photosensitisation to *S. aureus* strains.

74

75 **Materials and Methods**

76 *Bacteria and Bacteriophage*

77 The two strains of *S. aureus* used in these experiments were EMRSA-16 (NCTC
78 13143), one of the dominant nosocomial MRSA isolates in UK hospitals and 8325-4,
79 a prophage-free derivative of NCTC8325 which is methicillin-sensitive. These
80 strains were grown on Columbia agar (Oxoid Ltd., Basingstoke, United Kingdom)
81 supplemented with 5% (vol/vol) defibrinated horse blood (CBA). *E. coli* 10418 was
82 also incorporated as a Gram-negative control. In preparation for the lethal
83 photosensitisation experiments, a colony was inoculated into 20 mL of nutrient broth
84 no. 2 (NB2) containing 10 mM CaCl_2 and grown aerobically for 16 hours at 37°C in a
85 shaking incubator. The cultures were then washed by centrifugation and re-
86 suspension in PBS containing 10 mM CaCl_2 and adjusted to a final optical density of
87 0.05 at 600 nm (OD_{600}), these cell suspensions contained approximately 1×10^7
88 cfu/mL.

89

90 Bacteriophage Φ 11 is a generalised transducing phage present in *S. aureus* NCTC
91 8235 as a prophage.⁸ This bacteriophage can infect the NCTC8325 derivative 8325-
92 4, but not EMRSA16. Phage Φ 11 was propagated in *S. aureus* 8325-4 using the
93 phage overlay method and SnCe6 was covalently conjugated to the bacteriophage
94 using methods described previously.⁷ The concentration of SnCe6 bound to the
95 phage was determined by spectral analysis against a calibration curve generated
96 from known concentrations of SnCe6. In different experiments, between 7.5×10^6

97 and 4.7×10^7 cfu of bacteriophage were used in conjugation reactions and the
98 amount of SnCe6 bound varied between 3.5 and 7 $\mu\text{g}/\text{mL}$.

99

100 *Lethal Photosensitisation Experiments*

101 Fifty microlitres of the $\Phi 11$ -SnCe6 conjugate was added to 50 μL of bacterial
102 suspension in a sterile 96-well plate. The controls consisted of: SnCe6 alone (at the
103 same concentration as the conjugate), $\Phi 11$ alone (at the same titre as the conjugate)
104 and PBS control. All of the mixtures were incubated in the dark, with stirring, for 30
105 minutes prior to exposure to laser light. The relevant samples were then sequentially
106 exposed to laser light (632.8 nm) from a helium / neon (HeNe) gas laser with a
107 measured power output of 29.2 mW (Spectra-Physics, Darmstadt-Kranichstein,
108 Germany) for a period of 5 minutes; the mixtures were magnetically stirred
109 throughout the course of an experiment. Additional 'dark controls' were also
110 conducted for these four variables without laser light. The number of viable bacteria
111 remaining in the samples was determined immediately following exposure to the
112 laser light by serial dilution and enumeration of colony forming units on CBA. Each
113 experimental variable was repeated as a duplicate.

114

115 The duplicate experiments with both strains of *S. aureus* were conducted a total of
116 four times ($n=8$), whilst those for *E. coli* were repeated twice ($n=4$). The null
117 hypothesis was that there was no difference between the \log_{10} counts of the number
118 of colony forming units using various different experimental parameters, this was
119 analysed by student's t-test to yield p-values.

120

121

122 **Results**

123 When compared to the control, which was not exposed to laser light nor to
124 photosensitiser, the Φ 11-SnCe6 conjugate in the presence of laser light yielded a
125 2.31 log₁₀ reduction ($p < 0.05$) in the number of viable bacteria recovered from the
126 culture of *S. aureus* 8325-4 and a 2.63 log₁₀ reduction ($p < 0.05$) for the culture of
127 EMRSA16. In the presence of laser light, the Φ 11-SnCe6 conjugate did not result in
128 significant killing of *E. coli* 10418. None of the other combinations of controls (i.e.
129 SnCe6 only, phage only and 'dark controls') produced significant bacterial kills
130 (figure 1).

131

132 **Discussion**

133 We have previously shown that bacteriophage 75, a serotype F staphylococcal
134 phage, could be used to target lethal photosensitisation to a range of *S. aureus*
135 strains including strains it could not infect. The capacity of bacteriophage 75 to
136 target LP to a range of *S. aureus* strains was surprising since this phage has a
137 restricted host range. The question we asked in the current study was whether other
138 staphylophage could target LP to a range of *S. aureus* strains, once conjugated to a
139 photosensitiser, or if this was a specific trait of phage 75. We did this by
140 investigating the capacity of bacteriophage Φ 11, a prototypic group B-transducing
141 phage,⁹ to target lethal photosensitisation to *S. aureus*.

142

143 When SnCe6 was conjugated to *S. aureus* bacteriophage Φ 11, *S. aureus* strains
144 8325-4 and EMRSA16 in the presence of laser light there was an increase in the
145 killing of these bacteria by 2.39 log₁₀ and 2.35 log₁₀ respectively, when compared to
146 the equivalent concentration of SnCe6 alone (i.e. free SnCe6 that was not

147 conjugated to the bacteriophage). Since it is known that staphylophage have the
148 capacity to bind to all strains of *S. aureus*¹⁰ and bacteriophage Φ 11 is not capable of
149 infecting strain EMRSA16, the kill achieved by the Φ 11-SnCe6 conjugate suggests
150 that the photosensitiser-bacteriophage conjugate only needs to bind to the bacterial
151 cell to induce killing in the presence of laser light. The selectivity of the
152 photosensitiser-bacteriophage conjugate in targeting lethal photosensitisation to *S.*
153 *aureus* was demonstrated by the inability to cause significant killing of *E. coli* in the
154 presence of laser light.

155

156 Our results demonstrate that it is possible to use different serotypes of
157 staphylophage as vehicles to deliver photosensitiser payloads to the surface of *S.*
158 *aureus* thus enabling selective lethal photosensitization of this bacterium in the
159 presence of laser light. Such designer composites would not only possess all of the
160 advantages that photodynamic therapy has over conventional antibiotic therapy, as
161 described in the introduction, but they would also ensure there was minimal collateral
162 damage to the host and its indigenous microflora.

163

164 **Transparency Declarations**

165 None

166

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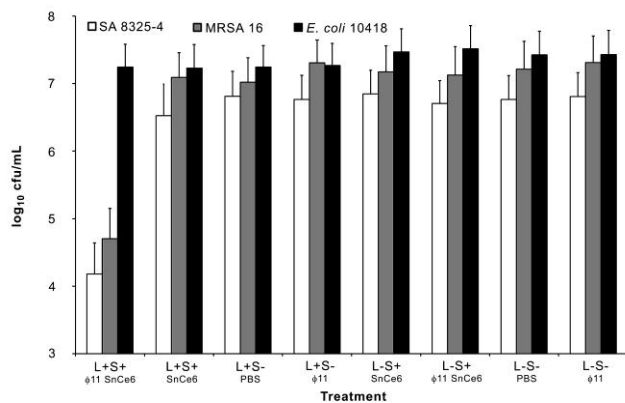
168 This project was funded internally

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172 **Figure Legend**



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174 Figure 1. The number of viable bacteria recovered following exposure of SnCe6-
175 bacteriophage 11 conjugate to laser light (leftmost columns) compared to controls.
176 The designations L+ / L- and S+ / S- refer to the presence or absence of light and /
177 or photosensitiser respectively.

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