

## Development of a Novel Targeting System for Lethal Photosensitization of Antibiotic-Resistant Strains of *Staphylococcus aureus*

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**Light-activated antimicrobial agents (photosensitizers) are promising alternatives to antibiotics for the treatment of topical infections. To improve efficacy and avoid possible damage to host tissues, targeting of the photosensitizer to the infecting organism is desirable, and this has previously been achieved using antibodies and chemical modification of the agent. In this study we investigated the possibility of using a bacteriophage to deliver the photosensitizer tin(IV) chlorin e6 (SnCe6) to *Staphylococcus aureus*. SnCe6 was covalently linked to *S. aureus* bacteriophage 75, and the ability of the conjugate to kill various strains of *S. aureus* when exposed to red light was determined. Substantial kills of methicillin- and vancomycin-intermediate strains of *S. aureus* were achieved using low concentrations of the conjugate (containing 1.5 µg/ml SnCe6) and low light doses (21 J/cm<sup>2</sup>). Under these conditions, the viability of human epithelial cells (in the absence of bacteria) was largely unaffected. On a molar equivalent basis, the conjugate was a more effective bactericide than the unconjugated SnCe6, and killing was not growth phase dependent. The conjugate was effective against vancomycin-intermediate strains of *S. aureus* even after growth in vancomycin. The results of this study have demonstrated that a bacteriophage can be used to deliver a photosensitizer to a target organism, resulting in enhanced and selective killing of the organism. Such attributes are desirable in an agent to be used in the photodynamic therapy of infectious diseases.**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections, and in the United States the rates of MRSA infection in intensive care units have reached 50% (15, 33). There is also an increasing incidence of community-acquired MRSA, and concern over community-acquired MRSA has led to much current research (28). Some strains of MRSA (fortunately still a minority) can also be resistant to teicoplanin and vancomycin, and the rate at which *S. aureus* can develop or acquire resistance to new antibiotics seems to be higher than the rate at which new antibiotics are discovered and developed (9, 19). Thus, it is essential that new approaches to preventing, controlling, and treating infections due to MRSA be developed.

One possible antimicrobial approach is lethal photosensitization. This involves the use of a light-activatable compound, a photosensitizer, which generates singlet oxygen and free radicals when exposed to light of a suitable wavelength (11, 16, 37). Singlet oxygen is highly reactive and cytotoxic, damaging and destroying cell walls and membranes and thus resulting in cell death (2, 3). The use of light-activated antimicrobials has been shown to be effective against a variety of gram-positive and gram-negative bacteria (16, 36), including MRSA (14), oral bacteria (40), and cutaneous microbes (42). In earlier studies,

we have demonstrated that lethal photosensitization can be effective against epidemic strains of MRSA and that the efficiency of the treatment can be increased by targeting the photosensitizer to the bacterial cell (7). This system used immunoglobulin G (IgG) as a targeting molecule to direct the photosensitizer tin(IV) chlorin e6 (SnCe6) to protein A in the cell wall of *S. aureus*, exploiting the IgG binding properties of protein A. Although this system could kill some of the MRSA strains tested, not all of the strains were efficiently killed, probably due to differences in protein A expression. We have also demonstrated that an antibody against the capsule of *S. aureus* can be used to deliver SnCe6 to the organism (8). Another possible means of delivering a photosensitizer to *S. aureus* is to use a bacteriophage. The use of a bacteriophage to deliver a photosensitizer to a target organism could have two important benefits: (i) more effective killing, since the singlet oxygen produced on irradiation would be concentrated at the bacterial cell wall, and (ii) selective killing, since binding of the phage-photosensitizer conjugate to the target organism would help to reduce collateral damage to host cells and members of the indigenous microbiota at the site.

The purposes of this study were (i) to determine whether phage could be used to deliver a photosensitizer, SnCe6, to *S. aureus* and thereby enhance killing compared to the photosensitizer itself; (ii) to determine if cell wall thickening in vancomycin-intermediate *S. aureus* (VISA) strains resulted in insensitivity to the treatment; and (iii) to investigate the effects of a phage-SnCe6 conjugate and red light on human epithelial cells.

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## MATERIALS AND METHODS

**Bacteria and bacteriophage.** The strains used in this investigation were EMRSA-1 (NCTC 11939), EMRSA-3 (NCTC 13130), EMRSA-15 (NCTC 13142), EMRSA-16 (NCTC 13143), EMRSA-17, Mu3 (a hetero-vancomycin-intermediate *S. aureus* (hetero-VISA) strain, Mu50 (a VISA strain putatively derived from strain Mu3), MW2 (a community-acquired hypervirulent strain), and the methicillin-susceptible strain NCTC 6571. It should be noted that the EMRSA strains represent different clonal genotypes on the basis of multilocus sequence typing, staphylococcal chromosomal cassette *mec*, pulsed-field gel electrophoresis, and phage typing (10, 25). Strains were maintained by subculture on Columbia agar (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 5% (vol/vol) horse blood. For experimental purposes, a few colonies were inoculated into nutrient broth no. 2 (NB2) (Oxoid Ltd.) supplemented with 10 mM CaCl<sub>2</sub> and grown aerobically for 16 h at 37°C. To obtain bacteria in different growth phases, 100 ml of NB2 containing 10 mM CaCl<sub>2</sub> was inoculated with 0.25 ml of an overnight culture and growth at 37°C was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>). Cells were harvested by centrifugation at the lag (OD<sub>600</sub>, 0.05), exponential (OD<sub>600</sub>, 0.55), and stationary (OD<sub>600</sub>, 2.3) phases as required and were resuspended in sterile 0.85% (wt/vol) saline with 10 mM CaCl<sub>2</sub> added (unless otherwise stated) to an OD of 0.05.

Phage 75 is a serogroup F staphylococcal phage used for typing staphylococci at the Laboratory of Hospital-Associated Infection, Central Public Health Laboratory, Health Protection Agency, United Kingdom. The phage was propagated on EMRSA-16 by using the phage overlay method (see below), purified by precipitation with 20% polyethylene glycol 8000 containing 1 M NaCl, resuspended in 0.85% NaCl, and filtered through a 0.45- $\mu$ m-pore-size filter (Nalgene). For propagation, 300  $\mu$ l of mid-exponential-phase EMRSA-16 was added to 15-ml Falcon tubes. Approximately 10<sup>5</sup> PFU of phage 75 was then added to the tubes and allowed to incubate at room temperature for 30 min to allow the phage to infect the bacteria. Cooled molten top NB2 agar (0.35% agar) (9 ml), containing 10 mM CaCl<sub>2</sub>, was added to the tubes, and the mixture was poured onto undried NB2 base agar (1% agar) plates. The plates were incubated at 37°C overnight. The top agar was removed by adding 1 ml of NB2 (containing 10 mM CaCl<sub>2</sub>) to each plate to aid the scraping of the top agar into a small centrifuge tube. The collected agar was then centrifuged and the supernatant collected and passed through a 0.45- $\mu$ m filter to remove any bacterial cells. The resulting phage suspension was stored at 4°C.

The titer of the bacteriophage was determined by infecting EMRSA-16 by using the phage overlay method as follows. Tenfold serial dilutions of the phage suspension were made in sterile NB2 medium with 10 mM CaCl<sub>2</sub>. A 30- $\mu$ l aliquot of each dilution was added to 300  $\mu$ l EMRSA-16 (OD<sub>600</sub>, approximately 1.0), and the mixtures were then added to 15-ml Falcon tubes containing 3 ml of molten NB2 agar containing 10 mM CaCl<sub>2</sub>. Each of these was poured onto dried NB2 agar plates, which were then incubated at 37°C overnight. The numbers of plaques on plates that contained between 30 to 300 plaques were then counted.

**Ability of bacteriophage 75 to infect strains.** Phage 75 is known to infect EMRSA-16, EMRSA-3, and EMRSA-15 (4a). The susceptibilities to infection by phage 75 of the other bacterial strains used in the study were determined by infecting mid-exponential-phase cultures of the above-described staphylococcal strains with 10<sup>-1</sup> to 10<sup>-5</sup> 10-fold serial dilutions of a high-titer phage 75 stock, according to the method described above.

**Laser and photosensitizer.** The laser used in this study was a model 127 Stabilite helium-neon laser (SpectraPhysics, Mountain View, CA), with a measured output of 35 mW, which emits light with a wavelength of 632.8 nm. The photosensitizer was SnCe6, which was custom synthesized by, and purchased from, FrontierScientific (Lancashire, United Kingdom).

**Preparation of phage-SnCe6 conjugate.** Phage 75 was conjugated to the SnCe6 by using a zero-length cross-linker in an adaptation of the method described by Embleton et al. (7). Basically, 4 mg SnCe6 was dissolved in 2.4 ml conjugation buffer [0.1 M 2-(*N*-morpholino)ethanesulfonic acid pH 6.0, 0.5 M NaCl] along with 2 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (Sigma, Poole, United Kingdom) and 5 mM sulfo-*N*-hydroxysuccinimide (Pierce, Cheshire, United Kingdom). After a 4-hour incubation with constant mixing at room temperature,  $\beta$ -mercaptoethanol was added to a concentration of 20 mM to quench the EDAC, and the reaction mix was adjusted to pH 7.6. Phage 75 in 0.85% NaCl (3 ml) was then added and the reaction mixture incubated overnight (16 h) with mixing at room temperature before quenching with ethanolamine at a concentration of 20 mM. The phage-SnCe6 conjugate was precipitated with 20% polyethylene glycol 8000 containing 1.0 M NaCl twice before being resuspended in sterile 0.85% NaCl and dialyzed against at least four changes of 0.85% NaCl so that free SnCe6 was eliminated from the preparation. The amount of SnCe6 present in the conjugate was measured by absorbance at

633 nm. To estimate the titer of the phage, an infection assay was performed. The concentration of the phage-SnCe6 conjugate was expressed in terms of the concentration of SnCe6 present in the conjugate.

**Lethal photosensitization.** The phage-SnCe6 conjugate (50  $\mu$ l) was added in duplicate to 50- $\mu$ l aliquots of bacterial suspension (containing  $1 \times 10^7$  CFU/ml) in a sterile 96-well plate. These were incubated with stirring for 30 min prior to exposure to a measured dose of red light from the HeNe laser (controls were not exposed to laser light). The typical dose was 21 J/cm<sup>2</sup>, which corresponds to a 5-min exposure using the aforementioned equipment (L+S+). The following controls were also performed: addition of saline and no exposure to laser light (L-S-), addition of unconjugated phage (at the same titer as the conjugate) in the absence of laser light, and addition of unconjugated SnCe6 and exposure to laser light. Following treatment, the survivors were enumerated by viable counting. Serial 1-in-10 dilutions in sterile phosphate-buffered saline were prepared from the contents of each well, and 25  $\mu$ l from each of these was plated in duplicate on blood agar. The number of colonies on each plate was counted following overnight incubation at 37°C. The limit of detection in all experiments was 400 CFU/ml. All experiments were carried out in duplicate on each occasion and on at least two separate occasions.

To compare killing of EMRSA-16 with epithelial cells (Hep2 cells) (see below), the experiments described above were repeated, using SnCe6 and phage-SnCe6 at final concentrations of 5  $\mu$ g/ml. However, the samples were mixed in the 96-well plates but were not stirred continuously. A lens was used to adjust the diameter of the beam to that of a well in a 96-well plate.

**Killing of Mu3 and Mu50 grown in the presence of vancomycin.** Mu3 and Mu50 were grown aerobically in NB2 broth (Oxoid) and were passaged through media containing increasing concentrations of vancomycin (0 to 16 mg/liter). The cells were harvested and exposed to phage-SnCe6 (5  $\mu$ g/ml SnCe6,  $6.3 \times 10^8$  PFU/ml) and laser light as described above, and the survivors were enumerated by viable counting.

**Lethal photosensitization of epithelial cells.** For these experiments, Hep-2 cells (a human epithelial cell line) were used. The Hep-2 cells were maintained in Dulbecco's minimal Eagle's medium (Gibco, Paisley, United Kingdom) supplemented with 10% fetal calf serum (Sigma Ltd.) and incubated at 37°C in 5% CO<sub>2</sub>-air. For experimental purposes, Hep-2 cells (in Dulbecco's minimal Eagle's medium without antibiotics) were plated into 96-well plates at a concentration of  $2 \times 10^4$  cells/well and left overnight to adhere to the surfaces of the wells. The following day, the medium was removed and the following additions made: 100  $\mu$ l of SnCe6 or phage-SnCe6 (in 0.85% NaCl, 10 mM CaCl<sub>2</sub>) to a final concentration of 5  $\mu$ g/ml (L+S+) and 100  $\mu$ l 0.85% NaCl, 10 mM CaCl<sub>2</sub> (L-S-). L+S+ samples were exposed to 21 J/cm<sup>2</sup> laser light but were not stirred throughout the exposure. Instead, a lens was used to adjust the diameter of the beam to that of a well in a 96-well plate. L-S- samples were not exposed to laser light.

Following the exposures and/or additions, the added solutions were removed and fresh medium was added. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as follows. A stock MTT solution (20  $\mu$ g/well) was added to the wells, and the plate contents were incubated for 4 h at 37°C. The medium was removed, acid-isopropanol (100  $\mu$ l of 0.04 M HCl in isopropanol) was added to elute the red formazan, and the suspension was mixed thoroughly. The concentration of formazan in the solution in each well was determined on a plate Titertek Multiskan PLUS plate reader, using a test wavelength of 570 nm and a reference wavelength of 620 nm.

## RESULTS

Conjugation of bacteriophage 75 to SnCe6 resulted in suspensions containing between 10 and 15  $\mu$ g/ml of SnCe6 and  $2.5$  to  $5 \times 10^9$  PFU/ml phage 75.

**Ability of phage 75 to infect strains.** Phage 75 is a bacteriophage that is used for typing MRSA strains at the Laboratory of Hospital-Associated Infection, Central Public Health Laboratory, Health Protection Agency. The phage was found to be capable of infecting and lysing EMRSA-16 and, to a lesser extent, EMRSA-3, EMRSA-15, and NCTC 6571. It was not able to lyse any of the other strains used in the study.

**Ability of phage-SnCe6 to kill various strains of *S. aureus*.** Previous studies using SnCe6 and antibody-SnCe6 conjugates have demonstrated that the effectiveness of killing of MRSA with a targeted photosensitizer can depend on the particular

TABLE 1. Effectiveness of lethal photosensitization with the phage 75-SnCe6 conjugate for various strains of *S. aureus*<sup>a</sup>

Strain	% Kill with <sup>b</sup> :		
	Phage	SnCe6	Phage-SnCe6
MRSA-1		44	99.98
MRSA-3		65	99.99
MRSA-15		97	>99.99
MRSA-16	20	64	>99.99
MRSA-17		62	>99.99
Mu3		9	99.7
Mu50		47	99.98
MW2		72	99.14
NCTC 6571		98	100

<sup>a</sup> To six MRSA strains, one hetero-VISA strain, one VISA strain, and one MSSA strain, suspended in 0.85% NaCl-10 mM CaCl<sub>2</sub>, either phage, SnCe6 (final concentration = 1.5 μg/ml), or phage-SnCe6 (final concentration = 1.5 μg/ml [ $3.8 \times 10^8$  PFU/ml]) was added as appropriate, and aliquots were exposed to 21 J/cm<sup>2</sup> HeNe laser light.

<sup>b</sup> The percent kill of each suspension is the mean of at least four repeats relative to a control, unirradiated suspension to which neither phage nor photosensitizer was added.

strain used (7, 8). Therefore, the effectiveness of the phage-SnCe6 conjugate against different strains, including the VISA strain Mu50, the hetero-VISA strain Mu3, and the community-acquired MRSA strain MW2, was examined. As can be seen in Table 1, the phage-SnCe6 conjugate was able to achieve substantial reductions in the viable counts of a variety of strains of *S. aureus*, including MRSA, hetero-VISA, and VISA strains. Reductions of greater than 3 log<sub>10</sub> units in the viable count of EMRSA-1 and EMRSA-16 were achieved when the bacteria were irradiated with 21 J/cm<sup>2</sup> of red light in the presence of the phage-SnCe6 conjugate, whereas the phage itself, the phage-SnCe6 conjugate in the absence of laser light, and the SnCe6 (in the presence of light) did not have an appreciable effect on the viability of either of the two strains.

From Table 1 it can be seen that the susceptibility of *S. aureus* to lethal photosensitization using the unconjugated SnCe6 was markedly strain dependent; EMRSA-15, Mu50, and NCTC 6571 were particularly susceptible (>1-log<sub>10</sub>-unit loss in the viable counts). All of the strains tested were susceptible to lethal photosensitization using the phage-SnCe6 conjugate, with kills of 4 log<sub>10</sub> units achieved in most cases. With phage 75 alone, the viable count of only EMRSA-16 was reduced. For all strains, the levels of killing achieved with the conjugate were much greater than those with unconjugated SnCe6.

**Phage-SnCe6 conjugate is effective in killing EMRSA-16 in the presence and absence of CaCl<sub>2</sub>.** The adsorption of phages onto bacteria is thought to require the presence of a divalent metal cation such as Ca<sup>2+</sup>, so it was reasoned that a divalent metal cation may be necessary for phage 75 to target SnCe6 to EMRSA-16 effectively. Therefore, experiments (using the conjugate at 1.5 μg/ml) were performed with 0.85% NaCl containing either 10 mM CaCl<sub>2</sub> or 10 mM EDTA (Fig. 1). Following exposure to laser light, the phage-SnCe6 conjugate was able to reduce the viable counts of EMRSA-16 in either case, although the killing in the presence of CaCl<sub>2</sub> was much greater than that in the absence of divalent metal ions, with 99.9% and 75% reductions in viability, respectively. The presence or absence of divalent metal ions did not affect the efficiency of killing by

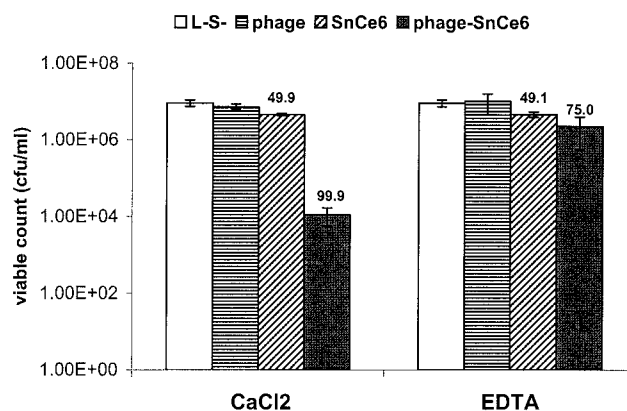


FIG. 1. Lethal photosensitization of MRSA-16 with phage-SnCe6 conjugate in the presence and absence of divalent metal ions. To suspensions of MRSA-16 in 0.85% saline with either 10 mM CaCl<sub>2</sub> or 10 mM EDTA, an equal volume of either saline (L-S-), phage suspension ( $3.8 \times 10^8$  PFU), SnCe6, or phage-SnCe6 (1.5 μg/ml) was added. The SnCe6 and phage-SnCe6 samples were exposed to 21 J/cm<sup>2</sup> HeNe laser light. Numbers above bars represent percent kills relative to the L-S- sample, to which only saline was added. Error bars indicate standard deviations.

unconjugated SnCe6, which killed approximately half of the cells in each case. A control of phage 75 alone, estimated to be at the same titer as phage in the phage-SnCe6 conjugate, was also used to determine the level of killing due to phage alone and the requirement of this for divalent metal ions. In the presence of CaCl<sub>2</sub>, the viable count was reduced by approximately 20%, while in the absence of divalent metal ion, no kills due to phage 75 alone were observed.

**Comparison of phage-SnCe6 conjugate with SnCe6.** To further investigate whether targeting the SnCe6 to MRSA by using phage 75 increased the effectiveness of lethal photosensitization, EMRSA-16 was irradiated in the presence of increasing concentrations of either unconjugated SnCe6 or phage-SnCe6 (Fig. 2). Samples irradiated in the presence of the phage-SnCe6 conjugate had much lower viable counts than those irradiated in the presence of SnCe6; at a low concentration of 1 μg/ml, the proportions of EMRSA-16 surviving irradiation with laser light in the presence of phage-SnCe6 and SnCe6 were 0.15% and 21%, respectively. As the concentration of the photosensitizer was increased, the extent of killing also increased, and the phage-SnCe6 performed consistently better than the free SnCe6.

**Effect of growth phase.** The susceptibility to lethal photosensitization of EMRSA-16 in different growth phases is shown in Fig. 3. There was little difference in the kills obtained in the different growth phases under the conditions employed, i.e., with the phage-SnCe6 conjugate containing 1.5 μg/ml of SnCe6, phage at  $1.46 \times 10^7$  PFU/ml, and a light dose of 21 J/cm<sup>2</sup>.

**Comparison of the effects of phage-SnCe6 on human epithelial cells and EMRSA-16.** The effects of the conjugate and of free SnCe6 on a human epithelial cell line, Hep2, were tested by seeding a 96-well plate with Hep2 cells and then subjecting them to treatment with SnCe6 or phage-SnCe6 at a final concentration of 5 μg/ml and with laser light (Fig. 4). Control experiments with the addition of saline only were also



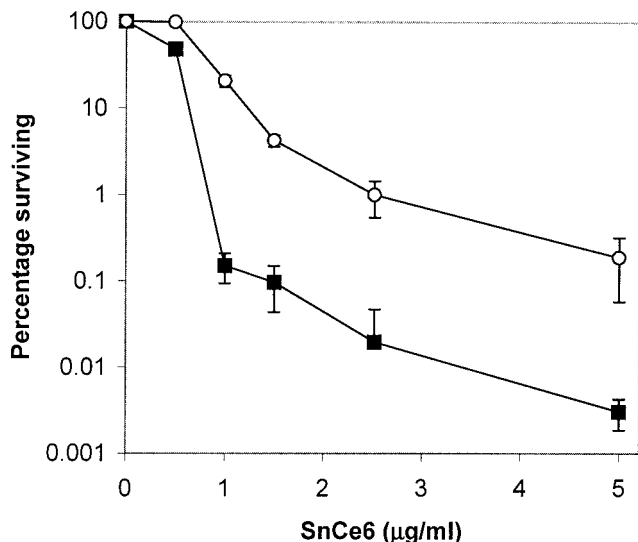


FIG. 2. Comparison of the effectiveness of SnCe6 and phage-SnCe6 conjugate for the lethal photosensitization of MRSA. SnCe6 (circles) or phage-SnCe6 (squares) was added to suspensions of MRSA-16 in 0.85% NaCl-10 mM CaCl<sub>2</sub> at the concentrations indicated and exposed to 21 J/cm<sup>2</sup> HeNe laser light. Error bars indicate standard deviations.

performed. The degree of killing was measured using the cell viability assay described above. In addition, a tandem experiment demonstrating the killing of EMRSA-16 under comparable conditions was also performed, using a colony-counting method to ascertain the level of killing. Under the conditions employed, the viability of the epithelial cells was unaffected by irradiation in the presence of either the SnCe6 or the phage-SnCe6 conjugate. In contrast, reductions of 2 and 4 log<sub>10</sub> units in the viable count of EMRSA-16 were obtained using the SnCe6 and phage-SnCe6 conjugate, respectively.

**Effect of growth with vancomycin on the susceptibility to lethal photosensitization of VISA strains.** To examine the ef-

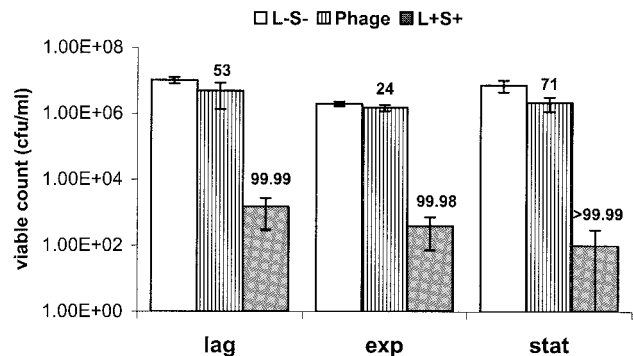


FIG. 3. Lethal photosensitization of MRSA-16 in different growth phases, using phage-SnCe6. To suspensions of MRSA-16 in 0.85% saline, an equal volume of either saline (L-S-), phage suspension (final concentration = 1.46 × 10<sup>7</sup> PFU/ml), or phage-SnCe6 (final concentration equivalent to 1.5 µg/ml of SnCe6) was added. The phage-SnCe6-containing suspensions were exposed to 21 J/cm<sup>2</sup> HeNe laser light. Numbers above bars represent percent kills relative to the L-S- suspension, to which only saline was added. exp, exponential phase; stat, stationary phase. Error bars indicate standard deviations.

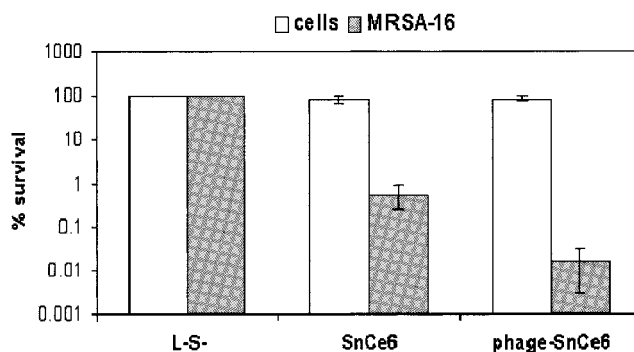


FIG. 4. Relative susceptibilities of MRSA and human epithelial cells to lethal photosensitization. To either human epithelial cells (Hep2 cells) or EMRSA-16, SnCe6 or phage-SnCe6 conjugate was added to give a final concentration of 5 µg/ml, and the cultures were then exposed to 21 J/cm<sup>2</sup> of laser light. To L-S- samples, only saline was added and the suspensions were not exposed to laser light. Error bars indicate standard deviations.

fects of growing vancomycin-intermediate strains in the presence of vancomycin on their susceptibility to lethal photosensitization by phage-SnCe6 and red light, Mu3 and Mu50 were grown in media with and without vancomycin. Both strains were passed through media containing increasing concentrations of vancomycin (0 to 16 mg/liter) and then irradiated with laser light (21J/cm<sup>2</sup>) in the presence of the phage-SnCe6 conjugate (5 µg/ml SnCe6, 6.3 × 10<sup>8</sup> PFU/ml) (Fig. 5). Both Mu3

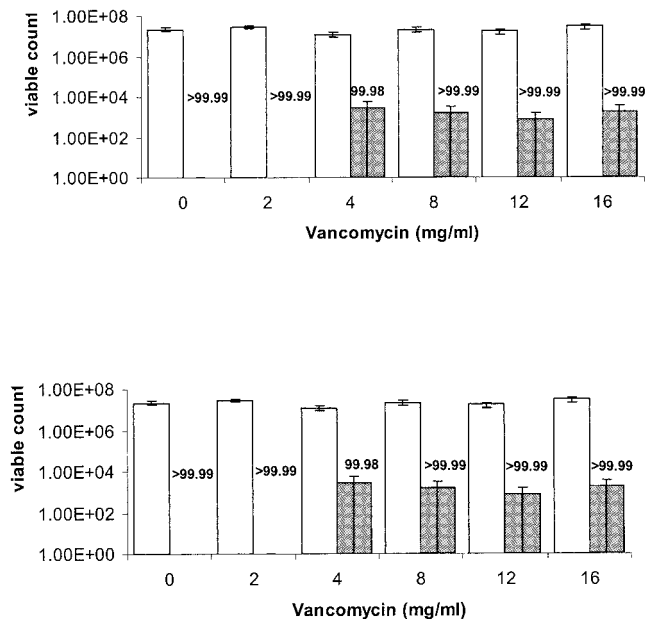


FIG. 5. Effect of growth in vancomycin on the killing of vancomycin-intermediate strains Mu3 (top) and Mu50 (bottom). The organisms were grown in medium with no added vancomycin and then passed through media containing increasing vancomycin in a stepwise fashion. To bacterial suspensions in 0.85% NaCl-10 mM CaCl<sub>2</sub>, 0.85% NaCl (L-S-) or phage-SnCe6 (L+S+) was added and exposed to laser light. Numbers above the bars show the percent kills achieved. The limit of detection was 400 CFU/ml. Error bars indicate standard deviations.

and Mu50 were very susceptible to killing in all cases, with a reduction in the viable count of over 3 log<sub>10</sub> units regardless of the vancomycin concentration.

## DISCUSSION

The lethal photosensitization of microbes is one of several alternatives to antibiotics that are being developed for use in the treatment of infections due to antibiotic-resistant organisms, and a number of studies have demonstrated its effectiveness in animal models (12, 17, 20). The light used (usually generated by a laser) in such an approach must, obviously, not have any adverse effects (e.g., due to phototoxicity or heating) on host tissue. The helium/neon laser is a device emitting light with a wavelength of 632 nm, and, at the light energy doses and densities needed to activate photosensitizers such as tin chlorin e6, it does not result in damage to human tissue. Indeed, irradiation of wounds with light from a helium/neon laser (without the application of a photosensitizer) has met with some success as a means of stimulating wound healing in humans (29, 30). One possible problem with the use of light-activated antimicrobial agents is that the singlet oxygen produced during the process has the potential to also damage neighboring host cells. There is, therefore, great interest in developing methods of targeting the photosensitizer to the infecting organism. As well as achieving selectivity, another advantage of using a targeted photosensitizer is that the antimicrobial effectiveness of lethal photosensitization is also increased. This is because, following binding of the targeted photosensitizer to the organism, subsequent irradiation results in the generation of singlet oxygen only in the vicinity of the pathogen and not at extraneous sites. Consequently, less photosensitizer needs to be applied, and because there is less attenuation of the incident light by unbound photosensitizer, a lower light dose can be used. Those targeting systems which have shown promise in laboratory and in vivo studies include the use of antibodies (4, 7, 8) and chemical modification of the photosensitizer itself (12, 32). In the present study we investigated the possibility of another targeting strategy, one based on the use of bacteriophages.

Bacteriophages, in their own right, are exciting prospects as antibacterial agents, although problems could arise due to the induction, or the prior existence, of an immune response to the particular bacteriophage being used (22, 35). However, studies with humans have demonstrated that bacteriophages may be applied topically and systemically without inducing adverse effects (22, 34, 35). One of the problems with using bacteriophages in the treatment of infections due to *S. aureus* is their restricted host range. There are a number of polyvalent staphylophages which lyse a broad range of *S. aureus* strains, but again, certain strains remain resistant (26, 27). All staphylophages are thought to recognize the same receptor located in the peptidoglycan-teichoic acid complex on the surface of staphylococci, and thus differences in the susceptibility of strains are believed to be due to postadsorption phenomena (26). Indeed, Pantucek et al. (27) have shown that the polyvalent phage  $\phi$ 812, which was able to lyse over 80% of 782 *S. aureus* strains tested, did in fact bind to strains that were resistant to this staphylophage. It is apparent from these studies that the use of a polyvalent phage such as  $\phi$ 812 as a

treatment for staphylococcal infections would not provide full protection. However, since bacteriophages also bind to staphylococci that are not susceptible to infection, they could serve as a delivery system for photosensitizers. Bacteriophages have already been used successfully to deliver other antimicrobial agents to bacteria. For example, Westwater et al. (39) have used a nonlytic phage of *Escherichia coli* as a vehicle to deliver DNA encoding bactericidal proteins to *E. coli* in vitro and in a mouse bacteremia model and have found that target bacterial cell numbers were reduced by several orders of magnitude.

The results presented here have demonstrated that it is possible to use bacteriophage 75 to deliver the photosensitizer SnCe6 to various strains of *S. aureus* and thereby enable killing of the organisms upon irradiation with red light. Furthermore, not only was the phage-SnCe6 conjugate more effective at killing *S. aureus* than the unconjugated photosensitizer, it was effective at killing EMRSA-16 and under similar conditions (but in separate experiments) did not affect the viability of human epithelial cells.

The phage 75-SnCe6 conjugate was able to sensitize a variety of strains of *S. aureus* to killing by red light; these included strains responsible for epidemics in the United Kingdom (1, 21), community-acquired MRSA strains (6, 28), and vancomycin-intermediate strains (13, 38). Killing was not the result of phage-mediated lysis, as no significant reduction in the viable count was achieved in control cultures containing only the phage (with the exception of EMRSA-16). Furthermore, strains that were not lysed by phage 75 (e.g., EMRSA-3, Mu3, and Mu50) were also killed on irradiation in the presence of the phage-SnCe6 conjugate, implying that the bactericidal effect was due to lethal photosensitization.

Binding of bacteriophage to *S. aureus* is known to be affected by Ca<sup>2+</sup> ions and/or other divalent metal ions. The presence of the metal ion chelator EDTA resulted in a decrease in the kills obtained using either phage 75 alone or the phage-SnCe6 conjugate, although it had no effect on killing by the unconjugated photosensitizer. That the enhanced killing with phage-SnCe6 required the presence of divalent metal ions further supports the idea that the phage-SnCe6 is indeed binding to the strains and that this is sufficient for targeted lethal photosensitization.

The effect of growth phase on the susceptibility of *S. aureus* to lethal photosensitization appears to depend on the nature of the photosensitizer used. For example, killing induced by aluminum disulfonated phthalocyanine is not affected by the growth phase of the organism (41), whereas in the case of SnCe6, lag-phase cells are less susceptible to light-induced killing (7). The efficacy of some targeted photosensitizers can also be growth phase dependent. For example, a conjugate of IgG and SnCe6 is a less effective photosensitizer of lag-phase cells of MRSA than of cells from other growth phases. As the IgG targets the protein A on the organism, this reduced effectiveness may be attributable to reduced production of protein A during the lag phase of growth. In contrast, SnCe6 conjugated to an antibody against the capsule of *S. aureus* has a reduced efficacy against stationary-phase cells of MRSA (8). The phage-SnCe6 conjugate appears to be equally effective against MRSA in all growth phases.

One of the features common to vancomycin-intermediate strains of *S. aureus* is thickening of the cell wall in the presence

of the antibiotic (5, 18). Such thickening is thought to be a major contributing factor to vancomycin resistance, as it results in entrapment of molecules of the antibiotic, thereby preventing them from reaching their intracellular target. Cell wall thickening may result in reduced susceptibility of the organism to lethal photosensitization for a number of reasons. First, the major targets of singlet oxygen are the bacterial cell wall and membrane, so that any increase in the thickness of the cell wall could protect both structures against the harmful effects of singlet oxygen. Second, it may alter the number and/or distribution of phage receptors and so could affect the number of molecules of the phage-SnCe6 conjugate adsorbed to the bacterial surface. The results of this study revealed that after growth of the strains in up to 16 mg/liter of vancomycin, the Mu3 and Mu50 strains remained highly susceptible to lethal photosensitization using the phage-SnCe6 conjugate.

The results of a number of *in vitro* studies have shown that the light dose and photosensitizer concentration required to achieve killing of bacteria have little effect on the viability of host cells in tissue culture (31, 32). Furthermore, studies with animals have reported little evidence of damage to host tissues following the successful lethal photosensitization of infecting bacteria (20, 23, 24). Nevertheless, it is important to take all possible precautions against the possibility of collateral damage to host tissues during antimicrobial photodynamic therapy, and this is one of the potential benefits of using a photosensitizer-targeting system. The results of this study have shown that a light dose and phage-SnCe6 concentration able to achieve appreciable killing of MRSA had no effect on the viability of human epithelial cells *in vitro*. These results imply that damage to host tissue during antimicrobial photodynamic therapy may be avoidable. However, the results of such studies are very preliminary and certainly do not constitute a model of the situation *in vivo*. Issues such as the effect of light on conjugate-coated bacteria attached to, or within, phagocytes and other host cells need to be addressed. It is essential, therefore, that further investigations be carried out using appropriate models so that the likely effects on the host of the combined effect of light and the photosensitizer conjugate can be assessed.

Use of appropriate bacteriophages could enable targeting of other microbes responsible for diseases of humans and other animals. Bacteriophage-photosensitizer conjugates could, therefore, provide an effective means of selectively killing infecting organisms without damaging host tissues.

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#### REFERENCES

- Aucken, H. M., M. Ganner, S. Murchan, B. D. Cookson, and A. P. Johnson. 2002. A new UK strain of epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA-17) resistant to multiple antibiotics. *J. Antimicrob. Chemother.* **50**:171–175.
- Bertoloni, G., F. M. Lauro, G. Cortella, and M. Merchat. 2000. Photosensitizing activity of hematoporphyrin on *Staphylococcus aureus* cells. *Biochim. Biophys. Acta* **1475**:169–174.
- Bhatti, M., A. MacRobert, S. Meghji, B. Henderson, and M. Wilson. 1998. A study of the uptake of toluidine blue O by *Porphyromonas gingivalis* and the mechanism of lethal photosensitisation. *Photochem. Photobiol.* **68**:370–376.
- Bhatti, M., A. MacRobert, B. Henderson, P. Shepherd, J. Cridland, and M. Wilson. 2000. Antibody-targeted lethal photosensitization of *Porphyromonas gingivalis*. *Antimicrob. Agents Chemother.* **44**:2615–2618.
- Communicable Disease Surveillance Centre. 1995. Epidemic methicillin-resistant *Staphylococcus aureus*. *Communicable Dis. Rep.* **5**:165–168.
- Cui, L., X. Ma, K. Sato, K. Okuma, F. C. Tenover, E. M. Mamizuka, C. G. Gemmell, M. N. Kim, M. C. Ploy, N. El-Solh, V. Ferraz, and K. Hiramatsu. 2003. Cell wall thickening is a common feature of vancomycin resistance in *Staphylococcus aureus*. *J. Clin. Microbiol.* **41**:5–14.
- de Sousa, M. A., and H. de Lencastre. 2004. Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. *FEMS Immunol. Med. Microbiol.* **40**:101–111.
- Embleton, M. L., S. P. Nair, B. D. Cookson, and M. Wilson. 2002. Selective lethal photosensitization of methicillin-resistant *Staphylococcus aureus* using an IgG-tin (IV) chlorin e6 conjugate. *J. Antimicrob. Chemother.* **50**:857–864.
- Embleton, M. L., S. P. Nair, B. D. Cookson, and M. Wilson. 2004. Antibody-directed photodynamic therapy of methicillin-resistant *Staphylococcus aureus*. *Microb. Drug Resist.* **10**:92–97.
- Enright, M. C. 2003. The evolution of a resistant pathogen—the case of MRSA. *Curr. Opin. Pharmacol.* **3**:474–479.
- Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt. 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci. USA* **99**:7687–7692.
- Fischer, F., G. Graschew, H. Sinn, W. Maier-Borst, W. Lorenz, and P. Schlag. 1998. A chemical dosimeter for the determination of the photodynamic activity of photosensitizers. *Clin. Chim. Acta* **274**:89–104.
- Gad, F., T. Zahra, K. P. Francis, T. Hasan, and M. R. Hamblin. 2004. Targeted photodynamic therapy of established soft-tissue infections in mice. *Photochem. Photobiol. Sci.* **3**:451–458.
- Gemmell, C. G. 2004. Glycopeptide resistance in *Staphylococcus aureus*: is it a real threat? *J. Infect. Chemother.* **10**:69–75.
- Griffiths, M. A., B. W. Wren, and M. Wilson. 1997. Killing of methicillin-resistant *Staphylococcus aureus* *in vitro* using aluminium disulphonated phthalocyanine, a light-activated antimicrobial agent. *J. Antimicrob. Chemother.* **40**:873–876.
- Haddadin, A. S., S. A. Fappiano, and P. A. Lipsett. 2002. Methicillin-resistant *Staphylococcus aureus* (MRSA) in the intensive care unit. *Postgrad. Med. J.* **78**:385–392.
- Hamblin, M. R., and T. Hasan. 2004. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem. Photobiol. Sci.* **3**:436–450.
- Hamblin, M. R., D. A. O'Donnell, N. Murthy, C. H. Contag, and T. Hasan. 2002. Rapid control of wound infections by targeted photodynamic therapy monitored by *in vivo* bioluminescence imaging. *Photochem. Photobiol.* **75**:51–57.
- Hanaki, H., K. Kuwahara-Arai, S. Boyle-Vavra, R. S. Daum, H. Labischinski, and K. Hiramatsu. 1998. Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50. *J. Antimicrob. Chemother.* **42**:199–209.
- Johnson, A. P., and N. Woodford. 2002. Glycopeptide-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **50**:621–623.
- Komerik, N., H. Nakanishi, A. J. MacRobert, B. Henderson, P. Speight, and M. Wilson. 2003. *In vivo* killing of *Porphyromonas gingivalis* by toluidine blue-mediated photosensitization in an animal model. *Antimicrob. Agents Chemother.* **47**:932–940.
- Livermore, D. M. 2000. Antibiotic resistance in staphylococci. *Int. J. Antimicrob. Agents* **16**(Suppl. 1):S3–S10.
- Merril, C. R., D. Scholl, and S. L. Adhya. 2003. The prospect for bacteriophage therapy in Western medicine. *Nat. Rev. Drug Discov.* **2**:489–497.
- Millson, C. E., M. Wilson, A. J. MacRobert, and S. G. Bown. 1996. *Ex vivo* treatment of gastric *Helicobacter* infection by photodynamic therapy. *J. Photochem. Photobiol.* **32**:59–65.
- Millson, C. E., W. Thurrell, G., Buonaccorsi, M. Wilson, A. J. MacRobert, and S. G. Bown. 1997. The effect of low-power laser light at different doses on gastric mucosa sensitised with methylene blue, haematoporphyrin derivative or toluidine blue. *Laser Med. Sci.* **12**:145–150.
- Murchan, S., P. Kaufmann, A. Deplano, R. de Ryck, M. Struelens, C. Elsborg Zinn, V. Fussing, S. Salmenlinna, J. Vuopio-Varkila, N. El Solh, C. Cuny, W. Witte, P. Tassios, N. Legakis, W. van Leeuwen, A. van Belkum, A. Vindel, I. Lacaoncha, J. Garaizar, S. Haggman, B. Olsson-Liljequist, U. Ransjo, G. Coombes, and B. Cookson. 2003. Harmonization of pulsed-field gel electrophoresis for epidemiological typing of methicillin-resistant *Staphylococcus aureus* by consensus in European laboratories and its application for tracing the spread of related strains. *J. Clin. Microbiol.* **41**:1574–1585.
- Novick, R. 1990. The staphylococcal genome, p. 1–12. *In* R. Novick (ed.), *Molecular biology of the staphylococci*. VCH Publishers Inc., New York, N.Y.
- Pantucek, R., A. Rosypalova, J. Doskar, J. Kailerova, V. Ruzickova, P. Borecka, S. Snopkova, R. Horvath, F. Gotz, and S. Rosypal. 1998. The polyvalent staphylococcal phage phi 812: its host-range mutants and related phages. *Virology* **246**:241–252.
- Said-Salim, B., B. Mathema, and B. N. Kreiswirth. 2003. Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging pathogen. *Infect. Control Hosp. Epidemiol.* **24**:451–455.
- Schindl, A., G. Heinze, M. Schindl, H. Pernerstorfer-Schon, and L. Schindl.

2002. Systemic effects of low-intensity laser irradiation on skin microcirculation in patients with diabetic microangiopathy. *Microvasc. Res.* **64**:240–246.
30. **Schindl, A., M. Schindl, H. Pernerstorfer-Schon, U. Mossbacher, and L. Schindl.** 2000. Low intensity laser irradiation in the treatment of recalcitrant radiation ulcers in patients with breast cancer—long-term results of 3 cases. *Photodermatol. Photoimmunol. Photomed.* **16**:34–37.
31. **Soukos, N. S., M. Wilson, T. Burns, and P. M. Speight.** 1996. Photodynamic effects of toluidine blue on human oral keratinocytes and fibroblasts and *Streptococcus sanguis* evaluated in vitro. *Lasers Surg. Med.* **18**:253–259.
32. **Soukos, N. S., L. A. Ximenez-Fyvie, M. R. Hamblin, S. S. Socransky, and T. Hasan.** 1998. Targeted antimicrobial photochemotherapy. *Antimicrob. Agents Chemother.* **42**:2595–2601.
33. **Stefani, S., and P. E. Varaldo.** 2003. Epidemiology of methicillin-resistant staphylococci in Europe. *Clin. Microbiol. Infect.* **9**:1179–1186.
34. **Sulakvelidze, A., Z. Alavidze, and J. G. Morris.** 2001. Bacteriophage therapy. *Antimicrob. Agents Chemother.* **45**:649–659.
35. **Summers, W. C.** 2001. Bacteriophage therapy. *Annu. Rev. Microbiol.* **55**:437–451.
36. **Usacheva, M. N., M. C. Teichert, and M. A. Biel.** 2001. Comparison of the methylene blue and toluidine blue photobactericidal efficacy against gram-positive and gram-negative microorganisms. *Lasers Surg. Med.* **29**:165–173.
37. **Wainwright, M.** 1998. Photodynamic antimicrobial chemotherapy (PACT). *J. Antimicrob. Chemother.* **42**:13–28.
38. **Walsh, T. R., and R. A. Howe.** 2002. The prevalence and mechanisms of vancomycin resistance in *Staphylococcus aureus*. *Annu. Rev. Microbiol.* **56**:657–675.
39. **Westwater, C., L. M. Kasman, D. A. Schofield, P. A. Werner, J. W. Dolan, M. G. Schmidt, and J. S. Norris.** 2003. Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for treatment of bacterial infections. *Antimicrob. Agents Chemother.* **47**:1301–1307.
40. **Wilson, M.** 2004. Lethal photosensitisation of oral bacteria and its potential application in the photodynamic therapy of oral infections. *Photochem. Photobiol. Sci.* **3**:412–418.
41. **Wilson, M., and J. Pratten.** 1995. Lethal photosensitisation of *Staphylococcus aureus* in vitro: effect of growth phase, serum, and pre-irradiation time. *Lasers Surg. Med.* **16**:272–276.
42. **Zeina, B., J. Greenman, W. M. Purcell, and B. Das.** 2001. Killing of cutaneous microbial species by photodynamic therapy. *Br. J. Dermatol.* **144**:274–278.