LETTER

Inactivation of multidrug-resistant bacteria and bacterial spores and generation of high-potency bacterial vaccines using ultrashort pulsed lasers

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Abstract
Multidrug-resistant organisms (MDROs) represent a continuing healthcare crisis with no definitive solution to date. An alternative to antibiotics is the development of therapies and vaccines using biocompatible physical methods such as ultrashort pulsed (USP) lasers, which have previously been shown to inactivate pathogens while minimizing collateral damage to human cells, blood proteins, and vaccine antigens. Here we demonstrate that visible USP laser treatment results in bactericidal effect (≥3-log load reduction) against clinically significant MDROs, including methicillin-resistant Staphylococcus aureus and extended spectrum beta-lactamase-producing Escherichia coli. Bacillus cereus endospores, which are highly resistant to conventional chemical and physical treatments, were also shown to be effectively inactivated by USP laser treatment, resulting in sporicidal (≥3-log load reduction) activity. Furthermore, we demonstrate that administration of USP laser-inactivated E. coli whole-cell vaccines at dosages as low as 10^5 cfu equivalents without adjuvant was able to protect 100% of mice against subsequent lethal challenge. Our findings open the possibility for application of USP lasers in disinfection of hospital environments, therapy of drug-resistant bacterial infections in skin or bloodstream via pheresis modalities, and in the production of potent bacterial vaccines.

KEYWORDS
bacterial spores, multidrug-resistant organisms, pathogen inactivation, ultrashort pulsed laser, vaccines
1 | INTRODUCTION

The emergence of multidrug-resistant organisms (MDROs) is among the gravest challenges in healthcare and a prominent cause of mortality in hospitalized patients worldwide. Bacterial evolution has outpaced the development of new antibiotics, with some bacterial pathogens now resistant to all available antibiotics [1–3]. In addition to MDROs, spore-forming bacteria such as Bacillus and Clostridium species represent another important source of highly transmissible nosocomial and community-acquired infectious agents which are resistant to common chemical and physical treatments and can persist in the environment for long periods of time [4–7]. Thus, there is an urgent need for new, orthogonal strategies to control these resistant pathogens.

An alternative approach to antibiotic development is the use of physical methods to inactivate pathogens in the context of therapy and vaccine production. Such a physical technique can ostensibly be applied to treat superficial infections or be coupled with a pheresis-like system to treat bloodstream infections [8, 9], while inactivated pathogens can serve as whole-cell vaccines [10]. Unfortunately, the existing physical methods including ultraviolet (UV) radiation, gamma-rays, X-rays, and heating cause extensive collateral damage to human proteins and nucleic acids, making them unsuitable for use in vivo [11–18]. The ideal physical method for clinical pathogen inactivation would be a simple one-step treatment process that inactivates a broad spectrum of pathogens, without the need to introduce chemical or biological agents. Furthermore, the method should not involve ionizing radiation or thermal heating which damage biomolecular structures. In these regards, ultrashort pulsed (USP) lasers represent a promising new approach for pathogen inactivation in the clinical setting [8]. Visible USP laser light has been shown to inactivate viruses, bacteria, mycoplasma, and fungi with minimal collateral damage to human cells and blood proteins [8, 10, 19–27]. The selective effect of USP lasers against microorganisms results from a unique inactivation mechanism, impulsive stimulated Raman scattering (ISRS), which kills microorganisms through forced mechanical vibration [8, 21, 23]. These properties make USP laser irradiation an attractive technology for clinical translation. In this report, we demonstrate the efficacy of USP laser treatment against clinically-important MDROs and against bacterial spores, for which few current therapies are effective. Furthermore, we demonstrate the potency and efficacy of a chemical-free USP laser-inactivated bacterial vaccine in mice.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture

Methicillin-resistant Staphylococcus aureus (MRSA) USA400 was received from Dr Juliane Bubeck Wardenburg (Washington University School of Medicine, St Louis, MO). Extended spectrum beta-lactamase (ESBL)-producing Escherichia coli ATCC 51446 was obtained from Sonora Quest Laboratories (Tempe, Arizona) and confirmed resistant to 11 antibiotics [28]. E. coli strain 25922 was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). MRSA and ESBL E. coli were cultured in trypticase soy broth and Luria-Bertani (LB) broth, respectively, to mid-logarithmic growth phase at 37°C. Glycerol stocks were generated by addition of 50% glycerol to bacterial samples after overnight culture and frozen at −80°C until use. Bacillus cereus ATCC 14579 cultures were grown with gentle agitation in nutrient broth for 24 h at 30°C. Saturated cultures were inoculated onto nutrient agar and incubated for 48 h at 37°C to induce sporulation [29, 30]. The agar plates were flooded with 0.9% NaCl (saline), and spores were scraped from the agar surface, collected into microcentrifuge tubes, and stored at 4°C. Differential staining with malachite green and safranin and microscopic visualization were used to confirm sporulation. For experiments with E. coli 25922, bacteria were inoculated from frozen glycerol stocks into tubes containing LB media and grown at 37°C with shaking until an optical density (OD) of 0.5 was reached. Enumeration was performed via standard colony forming assays. Briefly, bacterial samples or aliquots thereof were plated on LB agar, inverted and incubated at 37°C for 18 h. Colony counts were obtained manually. A standard curve was constructed to correlate OD with bacterial counts as determined by plating.

For USP laser treatment studies, MRSA and ESBL E. coli were pelleted, washed, and resuspended in sterile saline to cell densities of approximately 10⁸ colony forming units (cfu)/mL. B. cereus was prepared in sterile saline with approximate concentrations of 10⁸ spores/mL. Prior to USP laser treatment, bacterial and spore samples (100 μL volumes) were stored on ice.

2.2 | Animals

Six-week-old male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed in designated animal facilities, fed ad libitum and inspected regularly. All animal studies were approved by the Washington University School of Medicine Animal Studies Committee (protocol number 19-0814) and...
performed in strict accordance with humane care and use of research animals.

2.3 | USP laser treatment

*Inactivation of MDR bacteria and bacterial spores:* The excitation source used in this work was described previously [9]. A diode-pumped continuous wave (CW) mode-locked Ti-sapphire laser produced a continuous train of 60 fs pulses at a repetition rate of 80 MHz. The output of the second harmonic generation system of the Ti-sapphire laser was used to treat each sample. The excitation laser operated at a wavelength of $\lambda = 420 \pm 5$ nm and with an average power of approximately 150 mW. It has a pulse width of full-width at half maximum $= 100$ fs. An achromatic lens was used to focus the laser beam into a spot about 100 μm in diameter within the sample volume. Each bacterial or spore sample with a volume of about 0.1 mL, which was placed inside a glass vial, was subjected to gentle magnetic stirring and USP laser irradiation at room temperature (22°C), followed by incubation on ice. Control samples not subjected to USP laser treatment were similarly incubated at room temperature. Cell viability was determined by plating duplicate 10-fold serial dilutions for each sample on trypticase soy agar or LB agar for MRSA and ESBL *E. coli*, respectively, and enumerating cfu after incubating plates overnight at 37°C. For *B. cereus*, samples were subjected to duplicate 10-fold serial dilutions, inoculated onto nutrient agar, and incubated overnight at 30°C.

*Generation of inactivated *E. coli* vaccines:* In order to expedite the production of large volumes of bacteria at high titers needed for vaccine experiments in mice, we employed a more powerful USP laser system which produces a train of ultrashort laser pulses with the pulse width, wavelength and repetition rate comparable with the one described above but with a much larger average power of 1.65 W. In addition, the experimental configuration was switched from the glass vial with a magnetic stirrer to a continuous-flow configuration, where the sample was passed through tubes via peristaltic pump to enable treatment by the laser beam. Samples of *E. coli* 25922 at $5 \times 10^8$ cfu/mL in PBS were inactivated by USP laser irradiation and stored at 4°C prior to immunization studies. Cell viability was determined by plating duplicate 10-fold serial dilutions for each sample on LB agar and enumerating cfu after incubating plates overnight at 37°C.

2.4 | Immunization experiments

Three groups of mice ($n = 3$ per group) were immunized twice at 3-week intervals via i.p. injection of USP laser-inactivated *E. coli* 25922 bacteria at $10^7$, $10^8$, or $10^9$ cfu equivalents suspended in 100 μL PBS, respectively, while a control group ($n = 5$) was injected with placebo (100 μL PBS). To establish the peritoneal infection model, 3 weeks after the last immunization the mice were infected via i.p. injection of $1 \times 10^7$ cfu/mouse live *E. coli* suspended in 100 μL PBS. During the first 24 h postinfection, mice were monitored approximately every 4 h for signs of morbidity including behavioral changes associated with sepsis (agitation/lethargy, shivering, piloerection), ruffling of fur, and reduced responsiveness to stimuli. After the initial 24 h, mice were inspected twice daily. Mice deemed to be terminally moribund according to these observed signs were considered having reached the mortality endpoint and were sacrificed by cervical dislocation under isoflurane anesthesia. Survival of mice over time was recorded and plotted via Kaplan-Meier analysis (GraphPad Prism).

2.4.1 | Statistical analyses

Differences between mean cfu of control vs. laser-treated bacteria were analyzed by unpaired Student's $t$-test. A $P$ value of <.05 was used as a threshold for statistical significance. Survival of vaccinated vs. control mice subsequent to live bacterial challenge was compared by standard Kaplan-Meier analyses (GraphPad Prism).

3 | RESULTS

3.1 | Prototypical MDROs are efficiently inactivated by USP laser treatment

MRSA and ESBL-producing *E. coli* represent clinically significant and prevalent MDROs in healthcare settings today. We exposed these bacteria to visible USP laser treatment and assessed the effects of the laser treatment on bacterial viability. As shown in Figure 1A,B, USP laser treatment resulted in significant $\geq 3.5$ log ($P < .0001$) and $\geq 3$ log ($P = .0442$) reductions in viable MRSA and beta-lactamase-producing *E. coli*, respectively, revealing bac- tericidal (≥99.9% reduction) activity.

3.2 | Bacterial spores are efficiently inactivated by USP laser treatment

*B. cereus* is a saprophytic, Gram-positive, spore-forming bacterium associated with toxin-mediated foodborne illnesses. To assess sporicidal inactivation, we exposed *B. cereus* endospores to visible USP laser treatment. Figure 2 shows that USP laser treatment resulted in an
average 3.8 log reduction \((P = .0001)\) in \(B. \ ceres\) endospores, revealing sporicidal (≥99.9% reduction) activity.

### 3.3 USP laser-inactivated \(E. \ coli\) vaccines at low dosages protect mice against lethal challenge

Samples of \(E. \ coli\) strain 25922 were inactivated by USP laser treatment and complete inactivation was confirmed via plating. The inactivated bacteria were administered intraperitoneally (i.p.) to C57BL/6 mice twice at a 3-week interval at dosages of \(10^7\), \(10^6\), or \(10^5\) cfu equivalents per mouse, while controls received placebo (PBS). Three weeks after the second vaccine dose, the mice were challenged i.p. with a lethal dose of live \(E. \ coli\) 25922. As shown in Figure 3, all mice in the control (placebo) group exhibited terminal morbidity or mortality within 28 h of challenge. In contrast, mice that received USP laser-inactivated vaccine showed complete protection against lethal challenge, even with vaccine dosages as low as \(10^5\) cfu equivalents/dose. Our additional experimental data indicate that, in order to completely protect the mouse against lethal challenge, two doses of vaccination with \(1 \times 10^5\) cfu/dose is required. \(1 \times 10^5\) cfu/dose is the lower limit for complete protection. Lower dosages such as \(1 \times 10^6\) cfu/dose and \(1 \times 10^5\) cfu/dose are inefficient. As a reference, bacterial vaccines have typically required dosages on the order of \(10^8\)–\(10^9\) cfu equivalents for protection using traditional inactivation methods [31–33].

### 4 DISCUSSION

The inactivation mechanism for viral pathogens via USP laser irradiation through ISRS-driven protein aggregation has been established previously [8, 21, 23]. ISRS process in a single USP laser configuration can excite Raman-active vibrational modes and break bonds in a molecule [34]. The electric field of the electromagnetic wave from the laser induces a polarization in the molecule (ie, a protein or the capsid of a virus). The induced polarization can store energy which is like charging of a capacitor. It is the variation of this energy with configuration coordinate that produces an impulsive force that drives the damped harmonic oscillators in the molecule.

When the photons in an ultrashort pulsed laser interact with a molecule such as a pathogen or a protein, they can excite low-frequency Raman-active vibrations on the molecule through ISRS process [34]. As the amplitude of vibration is sufficiently large, hydrogen bonds/hydrophobic contacts within the molecule will be broken. If there are no similar molecules close by, these broken hydrogen bonds/hydrophobic contacts will be reformed very rapidly to their original configuration following the passage of photons at room temperature. On the other hand, if there are similar excited molecules nearby, the broken hydrogen bonds/hydrophobic contacts in the molecules, instead of getting back to their original

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**FIGURE 1**  Inactivation of MDROs by USP laser treatment. Samples of MRSA USA400 and ESBL-producing \(E. \ coli\) ATCC 51446 were exposed to USP laser treatment for 90 min. Initial cfu (filled circles) were quantified prior to laser exposure. Control samples (filled squares) were incubated at similar temperatures in parallel with USP laser-treated samples (filled red triangles), but were not exposed to the USP laser. The dotted horizontal line represents the limit of detection. The solid horizontal bars represent the average of the respective data. The error bars represent the SEM from three experiments. *\(P = .0442\), ****\(P < .0001\); unpaired Student’s \(t\)-test

**FIGURE 2**  Inactivation of bacterial endospores by USP laser treatment. Samples of \(B. \ cereus\) ATCC 14579 spores were exposed to USP laser treatment for 90 min. Initial spores (filled black circles) were quantified prior to laser exposure. Control samples (filled blue squares) were incubated at similar temperatures in parallel with USP laser-treated samples (filled red triangles), but were not exposed to the USP laser. The dotted horizontal line represents the limit of detection. The solid horizontal bars represent the average of the respective data. The error bars represent the SEM from six experiments. ***\(P = .0001\); unpaired Student’s \(t\)-test
configurations, can become cross-linked and thus the molecules become aggregated. Therefore, the ISRS process can cause density-dependent aggregation of molecules.

For enveloped viruses, bacteria and fungi, and their spores, a lot of proteins are very tightly packed within them. When such pathogens are excited with an USP laser through the ISRS process, there is a large probability that the broken hydrogen bonds/hydrophobic contacts in these proteins, instead of getting back to their original configurations, can become cross-linked and therefore the proteins are aggregated. These aggregated proteins will lose their functionality because the functionality of a protein is intimately related to its structure. This aggregation process induced by the USP laser irradiation leads to the inactivation of nonenveloped viruses [27].

We now address the likely mechanisms of the observed inactivation of *S. aureus*, *E. coli*, and bacterial spores by the USP laser irradiation, and provide an explanation for the observed high potency of the USP laser-generated bacterial vaccine.

Lu et al previously reported the inhibition of *E. coli* respiratory enzymes by ultrashort visible femtosecond laser irradiation [35]. Based on protein gel electrophoresis studies of *E. coli* with or without USP laser treatment, they attributed the inactivation of *E. coli* to protein aggregation induced by the USP laser irradiation, a phenomenon consistent with prior viral studies [23]. Gel electrophoresis experiments on bacteria have been performed for average laser powers: 150 mW and 1.65 W, respectively. The results (which are not shown) are qualitatively similar to that reported in Reference 35, indicating that protein aggregation process prevails when bacteria are irradiated by femtosecond lasers under our experimental conditions.

As such, the most likely mechanism for the inactivation of *S. aureus* and *E. coli* observed in our work is protein aggregation within *S. aureus* and *E. coli* induced by the USP laser irradiation through the ISRS process. Within pathogens such as enveloped viruses, bacteria, and bacterial spores, there exist structures comprising proteins at high density (ie, very tightly packed within confined spaces) [8, 23, 35]. When such pathogens are excited with an USP laser through the ISRS process, there is a high probability of protein aggregation leading to loss of protein function. The inactivated pathogens remain as a whole particle due to inability of the laser to disrupt the viral envelope or bacterial cell membrane, a layer primarily composed of lipids. Furthermore, soluble proteins and many of the surface proteins of pathogens are expected to have reduced susceptibility to the abovementioned aggregation effect due to the fact that they are microscopically, relatively apart from each other.

For nonenveloped viruses, the protein aggregation effect for the enveloped viruses, bacteria, and fungi described above will be active. In addition, there is one more effect induced by the ISRS process. The capsid, which is formed by connecting subunit proteins through weak hydrogen bonds, can be disrupted by the ISRS process, leading to the disintegration of the capsid and the inactivation of nonenveloped viruses [27].

A secondary mechanism which may contribute to the overall observed inactivation may arise due to the presence of light-absorbing chromophores (such as porphyrins) in certain species of bacteria. We previously reported the inactivation of *Salmonella typhimurium* by USP laser irradiation and found that the killing efficacy for *S. typhimurium* with a deletion in a DNA-repair enzyme was slightly greater than that for *S. typhimurium*...
without the deletion [21]. This suggested that DNA damage due to excitation of porphyrins might account partially for the bacterial inactivation effect by USP laser irradiation. DNA damage was not observed previously in USP laser-inactivated viral particles, which are expected to lack such chromophores [23, 24].

The aggregation of specific intracellular proteins and the preservation of whole bacterial particles after USP laser irradiation provide a plausible explanation for the high potency of USP laser-inactivated bacterial vaccines. We previously showed that USP laser-inactivated influenza virus showed complete preservation of its spike protein, hemagglutinin, which serves as the major antigen for effective vaccine-elicited immune responses [10]. This USP laser-inactivated influenza virus vaccine was shown to have at least 10-fold greater potency relative to the formaldehyde-inactivated vaccine. Viral capsid and matrix proteins in the interior of enveloped virions have been shown to be aggregated by USP laser treatment [23]. Aggregation is well-known to increase the immunogenicity of proteins, a phenomenon that may lead to augmented immune responses elicited by such USP laser-generated vaccines [36, 37].

In order to get better insight on why the bacterial vaccine prepared by the USP laser treatment elicits strong immune reactions, experiments involving more mice per group and a comprehensive analysis of humoral and cellular immune response induced by the potent bacterial vaccine are being planned.

5 | CONCLUSIONS

In this report, we demonstrate that MRSA and ESBL-producing E. coli, as well as Bacillus cereus endospores which are highly resistant to conventional chemical and physical treatments, are effectively inactivated by USP laser treatment. These results open the possibility for USP laser disinfection of hospital environments, and superficial or pheresis-coupled bloodstream therapy of drug-resistant bacterial infections. We have also shown that immunization of mice with USP laser-inactivated E. coli vaccines was able to protect 100% of the mice against lethal challenge without the use of adjuvants. This finding paves the way for the production of chemical-free and highly potent bacterial vaccines via USP laser irradiation.

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CONFLICT OF INTEREST

Shaw-Wei D. Tsen and Kong-Thon Tsen hold patents on “System and method for inactivating microorganisms with a femtosecond laser” (publication no. US20080299636 A1).

ETHICS STATEMENT

This manuscript complies with the Committee on Publication Ethics (COPE) Code of Conduct and with the originality and authorship requirements of this journal.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are included within this article.

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REFERENCES

[1] M. E. Falagas, I. A. Bliziotis, Int. J. Antimicrob. Agents 2007, 29, 630. https://doi.org/10.1016/j.ijantimicag.2006.12.012.
[2] E. Martens, A. L. Demain, J. Antibi. (Tokyo) 2017, 70, 520. https://doi.org/10.1038/ja.2017.30.
[3] E. D. Brown, G. D. Wright, Nature 2016, 529, 336. https://doi.org/10.1038/nature17042.
[4] Salton, M. R. J. & Kim, K. S. in Medical Microbiology (ed S. Baron) (University of Texas Medical Branch at Galveston, 1996).
[5] P. Setlow, J. Appl. Microbiol. 2006, 101, 514. https://doi.org/10.1111/j.1365-2672.2005.02736.x.
[6] A. D. Russell, Clin. Microbiol. Rev. 1990, 3, 99. https://doi.org/10.1128/cmrr.3.2.99.
[7] J. L. Sagripanti, A. Bonifacino, Appl. Environ. Microbiol. 1999, 65, 4255.
[8] S. W. Tsen, K. T. Tsen, Selective Photonic Disinfection: A Ray of Hope in the War Against Pathogens, Morgan & Claypool Publishers, San Rafael, CA 2016.
[9] S. W. Tsen, T. C. Wu, J. G. Kiang, K. T. Tsen, J. Biomed. Sci. 2012, 19, 62. https://doi.org/10.1118/1423-0127-19-62.
[10] S.-W. D. Tsen, N. Donthi, V. La, W. H. Hsieh, Y. D. Li, J. Knoff, A. Chen, T.-C. Wu, C.-F. Hung, S. Achilefu, K. T. Tsen, J. Biomed. Opt. 2015, 20, 051008. https://doi.org/10.1117/1.JBO.20.5.051008.
[11] T. Douki, J. Cadet, Biochemistry 2001, 40, 2495.
[12] M. H. Gaber, J. Biosci. Bioeng. 2005, 100, 203. https://doi.org/10.1263/jbb.100.203.
[13] J. H. Seo, J. H. Kim, J. W. Lee, Y. C. Yoo, M. R. Kim, K. S. Park, M. W. Byun, Int. Immunopharmacol. 2007, 7, 464. https://doi.org/10.1016/j.intimp.2006.11.012.
