Killing of *Staphylococcus aureus* via Magnetic Hyperthermia Mediated by Magnetotactic Bacteria

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*Staphylococcus aureus* is a common hospital and household pathogen. Given the emergence of antibiotic-resistant derivatives of this pathogen resulting from the use of antibiotics as general treatment, development of alternative therapeutic strategies is urgently needed. Here, we assess the feasibility of killing *S. aureus* cells *in vitro* and *in vivo* through magnetic hyperthermia mediated by magnetotactic bacteria that possess magnetic nanocrystals and demonstrate magnetically steered swimming. The *S. aureus* suspension was added to magnetotactic MO-1 bacteria either directly or after coating with anti-MO-1 polyclonal antibodies. The suspensions were then subjected to an alternating magnetic field (AMF) for 1 h. *S. aureus* viability was subsequently assessed through conventional plate counting and flow cytometry. We found that approximately 30% of the *S. aureus* cells mixed with uncoated MO-1 cells were killed after AMF treatment. Moreover, attachment between the magnetotactic bacteria and *S. aureus* increased the killing efficiency of hyperthermia to more than 50%. Using mouse models, we demonstrated that magnetic hyperthermia mediated by antibody-coated magnetotactic MO-1 bacteria significantly improved wound healing. These results collectively demonstrated the effective eradication of *S. aureus* both *in vitro* and *in vivo*, indicating the potential of magnetotactic bacterium-mediated magnetic hyperthermia as a treatment for *S. aureus*-induced skin or wound infections.

*S. aureus* is a major pathogen that produces toxins and superantigens causing skin and soft tissue infections in hospitals or communities (1–3). Antibiotic therapy is not highly efficient because the routine and intensive use of antibiotics has caused the emergence of both hospital- and community-associated methicillin-resistant *S. aureus*. Moreover, *S. aureus* tends to form biofilms that reduce the susceptibility of the pathogen to the immune system and topical antimicrobials, rendering treatment of infections using antibiotics less effective (4). The need to develop alternative therapeutic treatments against *S. aureus* to address this major public health problem is urgent.

Nanomaterials provide attractive options to resolve this problem. Various technologies using nanosized and microsized carriers have been developed to decrease the low rate of penetration of active agents through the skin (5, 6). Hyperthermia is a therapeutic procedure that increases tissue temperatures by using physical methods, such as microwave, radiofrequency, laser, and ultrasound. However, a bottleneck for the clinical application of hyperthermia is the difficulty of controlling the temperature temporally and spatially, particularly in deep body regions. Magnetic hyperthermia offers an attractive solution to this problem. Upon exposure to an alternating magnetic field, magnetic nanoparticles (MNPs) produce heat mainly on the basis of the mechanisms of hysteresis losses (7), relaxation losses (Néel or Brown relaxation) (8–10), and eddy current effect (11). Magnetic hyperthermia can be applied as an adjunct to radiotherapy and chemotherapy in cancer treatments and has evidently shown a beneficial effect (12). MNPs absorb energy from an alternating magnetic field and efficiently transmit energy in the form of highly localized heat to inactivate *S. aureus* within a cutaneous abscess in a mouse model of wound healing in a previous study (13). Considering the limitation in increasing the intensity and frequency of magnetic field imposed by technical, medical, and economic factors, the properties of MNPs are determining factors in magnetic hyperthermia.

However, MNPs tend to aggregate, thereby reducing heating efficiency (14). Nevertheless, these recent findings inspired us to develop a new therapeutic option to treat *S. aureus*-induced skin infections by using magnetotactic bacteria; this approach combines magnetically guided penetration with efficient magnetic hyperthermia.

Magnetotactic bacteria possess unique prokaryotic organelles called magnetosomes, which are single-domain magnetite (Fe$_3$O$_4$) or greigite (Fe$_3$S$_4$) nanocrystals that are enveloped by biomembranes (15). Biosynthesis of these crystals is genetically controlled and is enzyme catalyzed, resulting in magnetosomes that are highly homogeneous in terms of chemical composition and crystal properties. In addition, magnetosomes arrange in a chain or chains, conferring to bacteria a magnetic dipolar moment that allows them to align and swim along geomagnetic field lines, a behavior referred to as magnetotaxis. Under normal conditions, magnetosomes in bacteria do not aggregate and instead align in a chain along actin filaments. Therefore, magnetosomes are good candidates for biomedical applications in hyperthermia or thermal ablation (16, 17). Martinez-Boubeta et al. have recently demonstrated that single-domain cubic iron oxide particles, which resemble MO-1...
magnetosome crystals, demonstrate remarkable magnetic heating efficiency compared with other forms of magnetic particles (18). We have isolated an axenic culture of magnetotactic bacteria from the Mediterranean Sea (19). This magnetotactic ovoid MO-1 strain is a chemolithoautotrophic; i.e., it uses bicarbonate as a carbon source, reduces sulfur compounds as electron donors, and involves oxygen as an electron acceptor to produce energy (19). We found that each cell synthesizes approximately 11.1 magnetosomes consisting of elongated cubo-octahedral crystals approximately 64 nm by 57 nm in size. They are organized into a single chain to generate a magnetic moment of approximately $(2.6 \pm 0.8) \times 10^{-13}$ A·m$^2$ (20). MO-1 cells constantly swim forward at a velocity of up to 300 μm s$^{-1}$ along magnetic field lines (north seeking). Upon encountering obstacles, the MO-1 cells can squeeze between them or swim backward for a short distance and subsequently swim forward again to swim around them (20). We previously used the MO-1 strain to construct magnetism-guided, autopropelled microrobots that can collect S. aureus cells from suspensions and carry them to defined compartments in chips (21). The auspicious properties of the MO-1 strain, such as magnetism-guided swimming and conspicuous magnetic nanocrystals, enhance the attractiveness of the development of magnetic hyperthermia for the treatment of S. aureus-induced skin infection. To achieve this ultimate purpose, we demonstrated the feasibility of using the magnetotactic MO-1 bacteria to effectively kill the pathogen in vitro and in vivo through magnetic hyperthermia (Fig. 1).

**MATERIALS AND METHODS**

**Bacterial culture.** The ovoid magnetotactic MO-1 strain was cultured in an EMS2 medium at 23 to 26°C as previously described (19). We also cultured the MO-1 cells in an EMS2 medium without ferric quinate, which resulted in cells (named MO-1n) without or with affected small magnetosomes. The MO-1 and MO-1n cells were collected by centrifugation and resuspension in EMS2 medium without agarose, and their concentration was determined using a bacterium counter.

*S. aureus* (ATCC 25923) was grown on blood LB agar plates (Land Bridge Technology Co. Ltd., Beijing, China) at 37°C for 20 h. After incubation, colonies were streaked onto new plates. The bacterial cells were harvested from the streaked plate using an inoculating loop and then suspended in normal saline buffer (containing 0.85% NaCl). The bacterial concentration was then determined using a conventional plate counting method.

**Preparation of rabbit anti-MO-1 polyclonal antibody-conjugated MO-1 bacteria.** The MO-1 bacterial suspension (approximately $1 \times 10^{10}$ cells) harvested from the MO-1 culture medium through centrifugation was incubated with 40 μl of rabbit anti-MO-1 polyclonal antibodies in 200 μl of phosphate-buffered saline (PBS; pH 7.4) at 4°C for 30 min to form antibody-coated MO-1 cells. The antibody coating facilitates the attachment of the MO-1 cells to the S. aureus cells. To confirm the conjugation, we washed the MO-1 cells with PBS thrice and performed reactions with Cy3-conjugated goat anti-rabbit IgG antibodies (Boster Biological Technology, Ltd., Wuhan, China) for 30 min. The final product was analyzed using flow cytometry after washing.

**Attachment of antibody-coated MO-1 cells to S. aureus cells.** The suspension (250 μl) containing S. aureus and antibody-coated MO-1 cells at different ratios (1:1, 1:5, and 1:10) was incubated at room temperature for 40 min to allow cell attachment (Fig. 1B). The MO-1 cells without antibody conjugation were also mixed with S. aureus cells under identical conditions. To measure their attachment, we immersed ultrathin copper grids in the attachment suspensions and then dried the grids in a clean bench after retrieval for observation using a transmission electron microscope (TEM) (Hitachi H-7650B; Hitachi, Japan).

**In vitro inactivation of S. aureus through magnetic hyperthermia.** The mixed suspensions (250 μl), in which the S. aureus cells were mixed with the MO-1 cells either in free form or attached to the antibody-coated MO-1 cells in a 1.5-ml Eppendorf tube, were processed for 1 h in a laboratory-produced heating system with a frequency of 80 kHz and a magnetic field intensity of 6.9 kA/m. After the treatment, the viability of the S. aureus cells was assessed. The control samples were also maintained under

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**FIG 1** Schematic representation of magnetotactic-bacterium-mediated magnetic hyperthermia. An alternating magnetic field was applied to S. aureus suspensions with either free-form magnetic MO-1 cells (A) or antibody-coated MO-1 cells that were attached to S. aureus via affinity binding between S. aureus surface protein A and the Fc fragment of polyclonal antibodies (B). The nanocrystals of the magnetosomes (black bar inside the cells) absorb electromagnetic radiation and efficiently transmit energy in the form of highly localized heat that may kill the S. aureus cells. Flagellar filaments of MO-1 cells are shown in helices.
conditions without magnetic hyperthermia. In addition, a similar concentration of *S. aureus* cells was placed in an identical AMF to estimate the influence of the magnetic field. The MO-1n cells either in free form or after coating with polyclonal antibodies mixed with *S. aureus* were then exposed to the same AMF to probe the role of the magnetosomes chains of the MO-1 cells in the temperature increase. During magnetic hyperthermia, the real-time changes in temperature in all groups were monitored using a fiber optic sensor (FoolxTek Co., Ltd., Shenzhen, China).

**Viability assessment of *S. aureus* cells.** After the magnetic–hyperthermia procedure, each suspension was divided into two parts. One part was analyzed using flow cytometry to quantify the live *S. aureus* ratio (i.e., the proportion of live *S. aureus* cells out of the total number of cells [percent]) using LIVE/DEAD BacLight bacterial viability kits (Life Technologies, Carlsbad, CA, USA). The cells were stained according to the established procedure recommended by the supplier. Briefly, the samples (10 μl) were centrifuged at 6,000 × g for 6 min at 4°C and then resuspended in 2 ml of normal saline buffer before analysis. The cell suspensions were then incubated with a 6-μl component mixture containing SYTO 9 dye (1.67 mM) and propidium iodide (PI; 1.67 mM) for 20 min in the dark at room temperature. The stained cells were immediately analyzed in a flow cytometer (LSRFortessa; BD, USA). Data analysis, gating, and compensation were performed using BD FACSDiva 8.0 software. The samples were independently performed six times. For compensation, SYTO 9 dye or PI single-stained cells were prepared before the analyses. The mixture of *S. aureus* cells and MO-1 cells was primarily treated with 70% (vol/vol) isopropanol at room temperature for 1 h to mimic the dead cells. The cell mixture and the isopropanol-treated mixture were then stained with SYTO 9 dye and PI, respectively.

Another analysis was performed using the conventional plate counting method. The number of CFU was used to quantify the viability of the *S. aureus* cells. All analyses were independently performed six times.

**In vivo treatment of the mouse model with the *S. aureus*-infected wound.** The 6-week-old to 8-week-old BALB/c male mice were used to develop a *S. aureus*-infected-wound model. We created an approximately 5-mm-long wound in the tails by using a scalpel after the mice were anesthetized with pentobarbital sodium. The tail wound was inoculated with 1 μl (1 × 10^6 CFU) *S. aureus*. Thirty-six infected mice were divided randomly into the following four groups according to treatment: a no-treatment group (infection group), an AMF treatment group (infection + AMF group), an antibody-coated MO-1 cell treatment group (infection + ab-coated MO-1 group), and a group treated with both antibody-coated MO-1 cells and AMF (infection + ab-coated MO-1 + AMF group). The *S. aureus*-infected wounds in the mouse tails were treated using magnetic hyperthermia on day 2 postinfection. Prior to hyperthermia treatment, 1 × 10^10 polyvalent antibody-coated magnetotactic MO-1 cells were added into the infected wound, and a permanent-magnet block (NdFeB; 20 mm by 10 mm by 10 mm [the latter dimension of 10 mm represents the magnetization direction]) (surface field, 0.4 T) was used to induce the magnetic moment (i.e., the surface field). The length of the wound was measured before and after magnetic hyperthermia for 7 days. A statistical analysis was performed using SPSS 20.0 software (SPSS; IBM, Chicago, IL, USA). The temperature changes in each group were measured thrice and expressed as means ± standard deviations (SD). One-way analysis of variance (ANOVA) with repeated measurements was used to investigate the differences in temperature increase. Viability experiments using flow cytometry and the conventional plate counting method were conducted in duplicate for each time point and repeated six times. An average was obtained from each set of duplicate experiments. The minimum and maximum values among the six averages were removed, and the remaining four averages were used for calculations. Two-way ANOVA was applied to determine the effects of AMF and MO-1 cells on the viability of the *S. aureus* cells. The Mann-Whitney U test was used to determine the differences in the live *S. aureus* ratios. Student’s t test was used to evaluate the differences between the CFU counts in the plate counting experiments. As described for the *in vivo* experiments, we used one-way ANOVA with repeated measurements to determine the differences in the wound lengths among the groups of infected mice. A P value of <0.05 was considered significant in all statistical tests.

**RESULTS**

**Attachment of the MO-1 cells to the *S. aureus* cells.** Overall 99% of the MO-1 cells were effectively coated with rabbit anti-MO-1 polyclonal antibodies (see Fig. S1 in the supplemental material). We then verified the attachment of the magnetotactic MO-1 cells to the *S. aureus* cells under the TEM. The micrographs clearly showed the magnetic nanocrystal chain in the MO-1 cells (a representative image is shown in Fig. 2A). Without precoating of the MO-1 cells with antibodies, the MO-1 (black arrows) and *S. aureus* (red arrows) cells dispersed in the suspensions (Fig. 2B). In contrast, the precoated MO-1 cells aggregated with the *S. aureus* cells (Fig. 2C and D). Figure 2D shows that the magnetic MO-1 cells were tightly attached to the *S. aureus* cells. We also found that the mixture of 1 × 10^10 antibody-coated MO-1 cells with 1 × 10^9 *S. aureus* cells in normal saline buffer (250 μl) for 40 min at room temperature led to complete attachment between the *S. aureus* cells and the coated MO-1 cells. Our analysis of magnetic hyperthermia was based on this condition.

**Thermal energy generated by magnetosomes subjected to alternating magnetic fields.** We analyzed the heating capacity of the magnetic nanocrystals of the MO-1 cells subjected to AMF at 80 kHz frequency and 6.9 kA/m intensity. The temperature of the *S. aureus* suspensions without MO-1 cells (control) increased from 27.0°C to approximately 39.0°C when the cells were exposed to the field for 60 min (Fig. 3). This increase is possibly attributable to the eddy current effect. When magnetotactic MO-1 cells were added, the temperature of the *S. aureus* suspensions increased significantly from 27.0°C to approximately 43.0°C (Fig. 3). A similar curve of temperature increase was observed in the suspension of *S. aureus* cells attached to the magnetic MO-1 cells (Fig. 3). The presence of the magnetic MO-1 cells resulted in a faster increase in temperature and higher final temperatures in both *S. aureus* suspensions than in the suspension without the MO-1 cells. One-way ANOVA with repeated measurements using the group as a variable showed that the temperature of the mixed suspension containing either the free-form (*P < 0.01*) or the attached (*P < 0.05*) magnetic MO-1 cells was significantly different from that of the suspension of *S. aureus* alone. However, no significant temperature difference was observed in comparisons of the suspensions of the free-form and the attached MO-1 cells (*P > 0.05*). To confirm the magnetosome-mediated hyperthermia effect, we performed a control experiment with MO-1n cells. As shown in Fig. S2 in the supplemental material, incubation without addition of ferric quinate to the growth media abolished magnetosome production in 76% of the MO-1n cells and the rest of the cells contained an average of only 3.9 magnetosomes per cell, which is significantly less than that seen with the MO-1 cells, with an average of 11.1 magnetosomes per cell when incubated under normal conditions (see Fig. S2A and S2B). We found that the temperature increase in the mixed suspension of MO-1n cells (antibody coated or not) and *S. aureus* cells was similar to that observed in the suspension of *S. aureus* alone (*P > 0.05*), indicating that the higher temperature increase after addition of magnetosomes was not contributed by...
the cell body but by the magnetosomes (Fig. 3). The temperature increase was observed only when the suspensions were subjected to an alternating magnetic field (data not shown).

**Killing efficiency of magnetic hyperthermia.**

The effect of magnetic hyperthermia on the viability of the *S. aureus* cells was first analyzed using high-throughput flow cytometry. Approximately $1 \times 10^8$ *S. aureus* cells in 250-μl suspensions were subjected to AMF. Two-way ANOVA was used to analyze the effects of AMF and MO-1 cells on the pathogen. The results showed that both the AMF (*P* < 0.01) and addition of MO-1 cells in either free form or antibody-coated form (*P* < 0.01) significantly affected the viability of *S. aureus*.

As shown in Fig. S3 in the supplemental material, the magnetotactic MO-1 cells and the *S. aureus* cells could be easily separated. The application of AMF barely reduced the live-cell ratio (from 97.1% to 94.7%) in the *S. aureus* suspension without magnetotactic bacteria (Fig. 4A). In contrast, the percentage of live *S. aureus* cells significantly decreased (*P* < 0.05) by approximately 25% in the suspensions containing free magnetotactic MO-1 cells that were subjected to AMF (Fig. 4B). More importantly, our flow cytometric analysis showed that the attachment of the antibody-coated MO-1 cells reduced by approximately 50% the number of

**FIG 2** Attachment of magnetotactic MO-1 cells to *S. aureus* cells. (A) A representative magnetotactic MO-1 cell with the chain of single-domain magnetite particles (indicated by white arrow). (B) Mixed suspension of free-form MO-1 cells (black arrows) with *S. aureus* cells (red arrows). (C) Aggregates that formed between magnetotactic MO-1 cells, which were coated with rabbit anti-MO-1 polyclonal antibodies (black arrows), and *S. aureus* cells (red arrows). (D) An enlarged area of the inset shown in panel C.

**FIG 3** Curve of temperature increase during magnetic hyperthermia. The suspensions of *S. aureus* alone (−), mixed with free-form MO-1 cells (+ MO-1) or MO-1n cells (+ MO-1n), and attached to MO-1 cells (+ ab-coated MO-1) or MO-1n cells (+ ab-coated MO-1n) were treated with an alternating magnetic field with a frequency of 80 kHz and an intensity of 6.9 kA/m. The temperature (mean ± SD) of each group (*n* = 3 per group), which was recorded using a fiber optic sensor, is shown. *, *P* < 0.05; **, *P* < 0.01 (versus the suspension with *S. aureus* alone; results were analyzed using one-way ANOVA).
live *S. aureus* cells after hyperthermia treatment (*P* < 0.05) (Fig. 4). In addition, no significant differences in the live-cell ratio were observed in the *S. aureus* suspensions of three groups when no magnetic field was applied. The data obtained from the plate counting analysis (see Fig. S4) showed similar results. All of these data clearly demonstrated the efficient killing effect of magnetotactic MO-1 cell-mediated hyperthermia on *S. aureus* cells.

MO-1-mediated magnetic hyperthermia improved healing of the *S. aureus*-infected wound in vivo. *In vitro* experiments demonstrated that the antibody-coated magnetotactic MO-1 cells could induce much more death of *S. aureus* under conditions of magnetic hyperthermia. We measured the length of the *S. aureus*-infected wound in mouse tails to evaluate the effect of magnetic hyperthermia mediated by antibody-coated MO-1 cells on wound healing.

**FIG 4** Ratios of live *S. aureus* cells analyzed by flow cytometry. (A) Fluorescence-activated cell sorter (FACS) analysis of SYTO 9- and propidium-iodide-stained *S. aureus* (SA) cells in the suspension containing *S. aureus* alone, a mixed suspension, and an attached suspension after magnetic hyperthermia was induced. Live cells with intact membrane were stained only by SYTO 9 dye with green fluorescence, whereas the dead cells with damaged membrane were stained simultaneously by the PI dye with red fluorescence and by SYTO 9 dye. (B) The ratio of live *S. aureus* cells was statistically obtained from the FACS analysis. The values are means ± SD (shown with error bars) (*n* = 4). *, *P* < 0.05.
healing. In the absence of magnetosomes, the wound length in mouse tails with or without alternating magnetic field treatment was slightly reduced, probably resulting from self-healing (Fig. 5). Addition of antibody-coated MO-1 bacteria slightly improved the rate of wound healing (Fig. 5). Moreover, application of both antibody-coated MO-1 cells and AMF significantly reduced the wound length compared with that seen in the infection group (\( P < 0.01 \) (versus infection-only group)); ##, \( P < 0.01 \) (versus infection + AMF group); &\#, \( P < 0.05 \) (versus infection + ab-coated MO-1 group). Data were analyzed using one-way ANOVA with repeated measures.

DISCUSSION

This study assessed the feasibility of the use of magnetotactic bacterial nanocrystals as a thermal source in hyperthermia treatment against *S. aureus* both *in vitro* and *in vivo*. We observed that in hyperthermia, the magnetosomes effectively mediated the heating of pathogen suspension to 43°C, which can kill pathogens. More importantly, our protocol for the use of magnetotactic MO-1 bacteria-mediated magnetic hyperthermia against *S. aureus* (1×10^9 CFU) at wound sites. Then, 1×10^10 antibody-coated MO-1 cells (1 µl) were added to the wound sites on day 2 postinfection. A magnetic block (NdFeB; 20 mm by 10 mm by 10 mm [the latter dimension of 10 mm represents the magnetization direction]) (surface field, 0.4 T) was used to induce antibody-coated MO-1 cells to penetrate the infected wound. The infected wound tails were subjected to an AMF of 6.9 kA/m and 80 kHz for 1 h. The control groups contained infected mouse models without any treatment (infection group), those with only antibody-coated MO-1 cells (infection + ab-coated MO-1 group), and those with only antibody-coated MO-1 cells (infection + AMF group), and those with only antibody-coated MO-1 cells (infection + ab-coated MO-1 group). The lengths of the infected wounds were measured for 7 days. Wound length was normalized to the value measured before AMF treatment on day 2 postinfection (\( n = 9 \)). **, \( P < 0.01 \) (versus infection-only group); ##, \( P < 0.01 \) (versus infection + AMF group); &\#, \( P < 0.05 \) (versus infection + ab-coated MO-1 group). Data were analyzed using one-way ANOVA with repeated measures.
S. aureus aggregation induced by the antibodies on the surface of the MO-1 cells, thereby reducing the infectivity of the pathogen. Moreover, application of AMF strongly promoted wound healing, suggesting that magnetic hyperthermia plays an important role in the healing of S. aureus-infected wounds.

Topical drug application tends to cause systemic side effects that are less severe than those seen with systemic application. Targeting tumors or infected tissues using MNPs can significantly improve the effectiveness of magnetic hyperthermia and reduce unacceptable coincidental heating of healthy tissues. The use of appropriate means to inject and target MNPs into the infected tissues is a major concern in the application of hyperthermia. In contrast to the currently used passive targeting methods, we propose a new perspective using magnetism-guided and autopropelled microrobots that actively swim into the infected tissues and attach to S. aureus through high-affinity binding between the surface protein A of the pathogen and the Fc fragment of the coated polyclonal antibodies. The associated auspicious properties, such as efficient penetration and active binding, will be unprecedentedly effective in the use of magnetic hyperthermia against S. aureus-induced skin infections and probably against other types of infections as well as against cancers. However, physiologically low-salt environments limit the long-term survival of the magnetic MO-1 cells, which may affect the microrobots as an ultimate magnetism-hyperthermia system for skin infections. Despite this limitation, we found that the MO-1 cells can survive and remain motile in mouse blood at 37°C for more than 10 min, which might be sufficient to allow the magnetotactic MO-1 cells to cross the S. aureus biofilm at the surface of an infected wound. Furthermore, the osmolality of the physiological buffer may be slightly increased by adding salts to the MO-1 suspension to increase the survival and mobility of the MO-1 cells. In the future, the efficacy and safety of this treatment must also be verified by nonclinical studies and subsequently by clinical studies.

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