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EXPERIMENTAL THERAPEUTICS

Intracellular *Staphylococcus aureus* Control by Virulent Bacteriophages within MAC-T Bovine Mammary Epithelial Cells

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ABSTRACT Bacteriophages (phages) are known to effectively kill extracellular multiplying bacteria. The present study demonstrated that phages penetrated bovine mammary epithelial cells and cleared intracellular *Staphylococcus aureus* in a time-dependent manner. In particular, phage vB_SauM_JS25 reached the nucleus within 3 h postincubation. The phages had an endocytotic efficiency of 12%. This ability to kill intracellular host bacteria suggests the utility of phage-based therapies and may protect patients from recurrent infection and treatment failure.

KEYWORDS MAC-T cells, *Staphylococcus aureus*, bacteriophages

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Although traditionally considered an extracellular pathogen, *Staphylococcus aureus* is able to internalize into host cells, including professional (i.e., macrophages and neutrophils) and nonprofessional (i.e., bovine mammary epithelial cells) phagocytic cells, which is associated with chronic and recurrent infections (1, 2). Phage therapy appears to be a potent and safe alternative tool for treating such bacterial infections. Recent studies have investigated the effect of experimental phage therapy on intracellular killing of bacteria in patients' peripheral blood monocytes, polymorphonuclear neutrophils, and murine macrophages (3–6). We set out to study the intracellular killing potential of such lytic phages in nonprofessional phagocytic cells (7, 8). We investigated this question using phage vB_SauM_JS25, a broad-spectrum virulent phage belonging to the family *Myoviridae* (9).

Mammary epithelial cells play an essential role in the surveillance of mammary tissue during infection by assisting immune cell recruitment and bacterial recognition (10). Can phages penetrate within nonprofessional phagocytic cells and eliminate intracellular *S. aureus*? To answer this, bovine mammary epithelial (MAC-T) cells were cultured in 24-well plates (10^5 cells/well), incubated overnight (37°C, 5% CO_2) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma, Milan, Italy) and then infected with *S. aureus* JYG2 (10^6 bacteria/well). One hour after infection at 37°C in 5% CO_2, cells were washed three times with phosphate-buffered saline (PBS), and extracellular bacteria were killed with lysostaphin (20 μg/ml). Two hours later, cells were washed and treated with phage vB_SauM_JS25 (10^8 PFU/well) and incubated for another 3 or 12 h. Then cells were washed three times with PBS and treated with citric acid buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 2 min to inactivate any phage particles that remained on the surface (11). The cells were washed two times with medium to remove the acid buffer, followed by digestion with 0.25% trypsogen and lysis with Triton X-100 (final concentration, 0.1%)

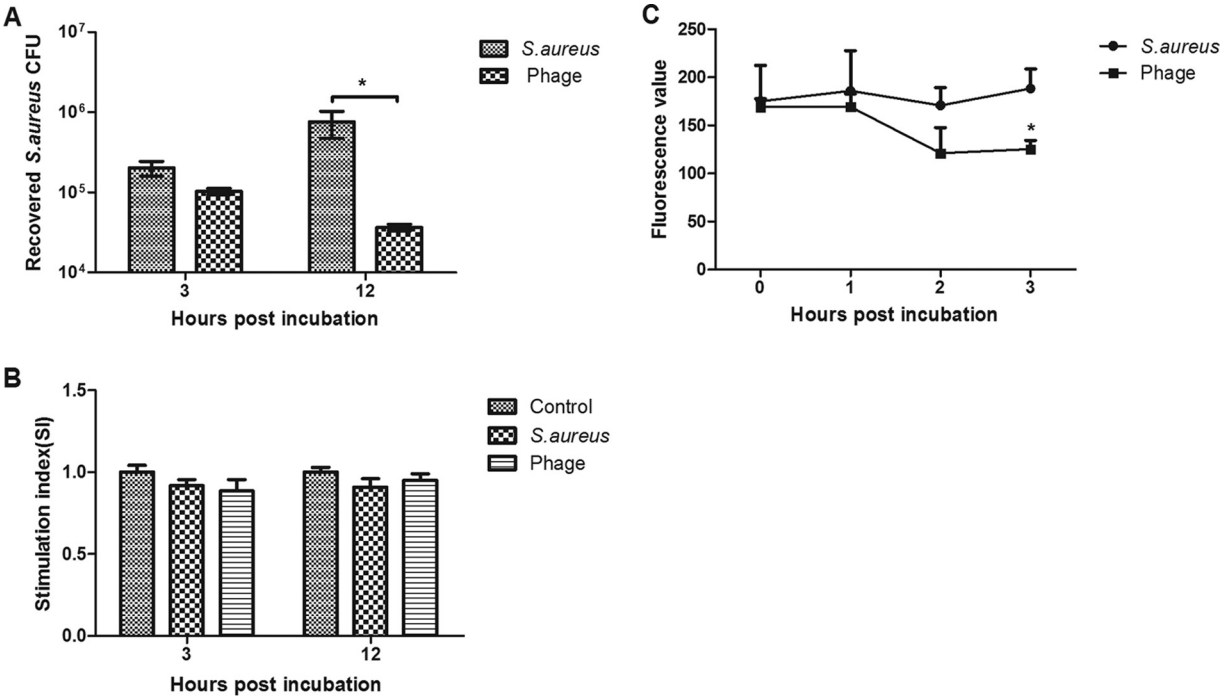

FIG 1 Bacteriophage vB_SauM_JS25 eliminates intracellular *Staphylococcus aureus* JYG2 in a time-dependent manner. (A) MAC-T cells were distributed in 24-well plates (10^5 cells/well), incubated overnight, and then infected with *S. aureus* JYG2 (10^6 bacteria/well). After incubation for 1 h at 37°C in 5% CO_2, extracellular bacteria were killed with lysostaphin (20 μg/ml). Cells were washed with DMEM. Two hours later, cells were treated with phage vB_SauM_JS25 (10^8 PFU/well) and incubated for another 3 or 12 h. Then, cells were washed with PBS and treated with citric acid buffer for 2 min to inactivate any phage particles that remained on the surface, followed by lysis and dilution. (B) Cytotoxic damage caused by *S. aureus* JYG2 and phage vB_SauM_JS25 on MAC-T cells was determined by MTT assay. Stimulation index (SI) was calculated as SI = optical density at 570 nm (OD_{570}) of treatment cells/OD_{570} of control cells. (C) MAC-T cells were infected with *S. aureus* JYG2 labeled with WGA 488 conjugate. Cells were washed with DMEM. After 2 h of treatment, cells were incubated with phage vB_SauM_JS25 (10^6 PFU/well). At the indicated time, the fluorescence in each well was measured at 495 nm for excitation and 519 nm for emission with a fluorescence plate reader. *, statistical analysis using one-way or two-way analysis of variance at $P < 0.05$.

to recover intracellular bacteria. Each lysate was then serially diluted in saline and plated on Baird-Parker agar (12). After 3 h of incubation, we observed a modest reduction in intracellular bacteria in phage-treated cells (Fig. 1A), although this was not statistically significant ($P > 0.05$). However, after 12 h of incubation, phage treatment resulted in a significant reduction in intracellular bacteria ($P < 0.05$). The longer the cells were inoculated with phage vB_SauM_JS25, the higher the observed inhibitory effect.

To determine whether this reduction in bacteria depended on cytotoxic damage, MAC-T cell viability was measured using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO) as previously described (4). The cytotoxic damage did not differ among the three groups at any time (Fig. 1B). To confirm this result by fluorescence assay, *S. aureus* JYG2 was fluorescently labeled with Alexa Fluor 488 conjugate of wheat germ agglutinin (WGA) (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. WGA binds to *N*-acetylglucosamine residues. MAC-T cells plated in 96-well plates were infected with *S. aureus* JYG2 labeled with WGA conjugate (10^4 bacteria/well). After incubation for 1 h at 37°C in 5% CO_2, extracellular bacteria were killed with lysostaphin (20 μg/ml). Cells were washed with DMEM. After 2 h of treatment, cells were incubated with phage vB_SauM_JS25 (10^6 PFU/well). At the indicated time, fluorescence in a whole well was measured with a fluorescence plate reader (Tecan Infinite M200). Consistently, phage vB_SauM_JS25 significantly ($P < 0.05$) inhibited intracellular bacteria at 3 h postincubation (Fig. 1C).

Phages can interact with mucus-producing cells via immunoglobulin-like protein domains and reduce microbial colonization (13). To ensure that phage vB_SauM_JS25 penetrated bovine mammary epithelial cells and eliminated intracellular *S. aureus*,

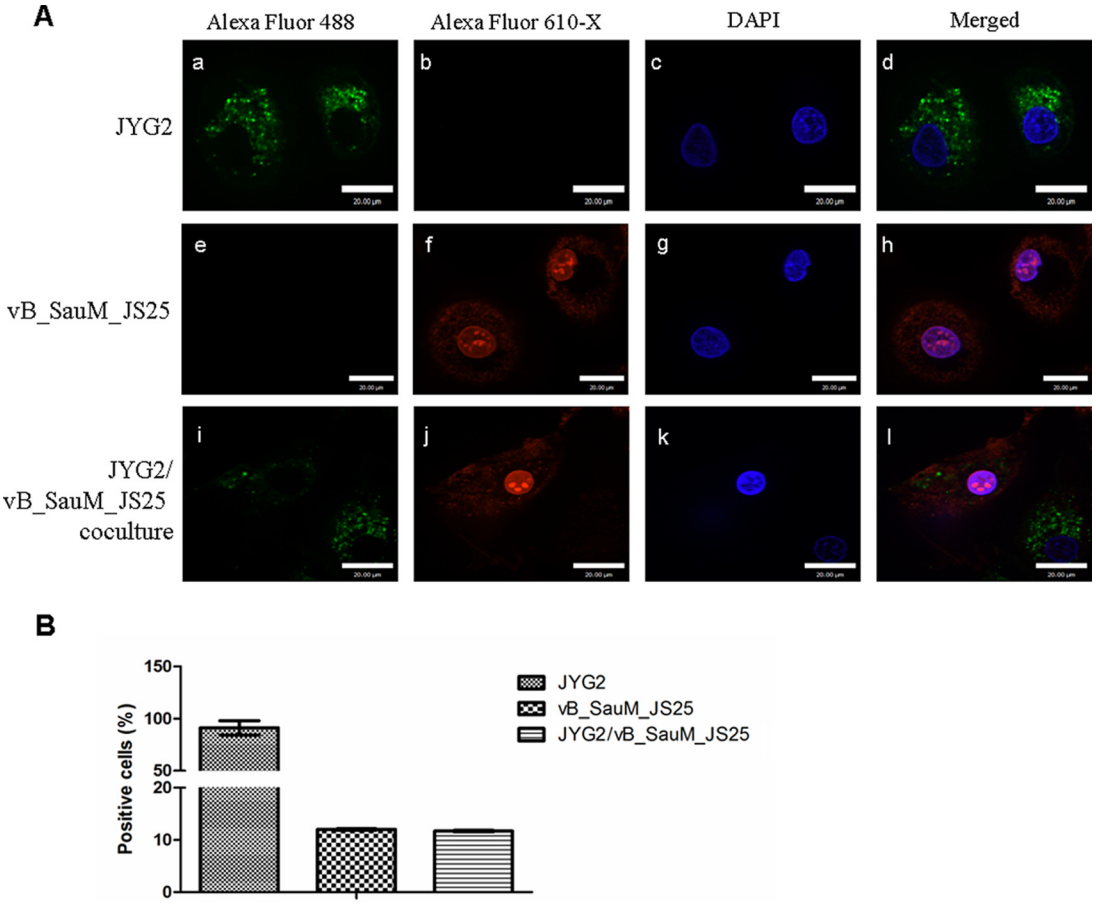

FIG 2 Confocal microscopy of internalized phage vB_SauM_JS25 and intracellular *S. aureus* JYG2. (A) Nucleus (blue, DAPI, c, g, k), JYG2 (green, Alex Fluor 488 conjugated WGA, a, e, i), and vB_SauM_JS25 (red, Alex Fluor 610-X, b, f, j) are shown in the same focal plane. vB_SauM_JS25 (red) is colocalized with JYG2 (green) in the merged image (d, h, l). The maximum intensity projection shows all Z-stacks simultaneously. Scale bar, 20 μm. (B) Percentage of MAC-T cells positively labeled after being infected with stained JYG2 and vB_SauM_JS25, individually or combined. A total of 200 cells from 10 randomly selected fields at $\times$400 magnification were analyzed by confocal microscopy.

phage vB_SauM_JS25 and *S. aureus* JYG2 were fluorescently labeled with Alexa Fluor 488 conjugate and 610-X *N*-hydroxysuccinimide (NHS) ester (Invitrogen), respectively. The NHS ester of 610-X can be used to label the primary amines (R-NH2) of proteins. The phages were ultrafiltrated through a cellulose membrane (Millipore, Billerica, MA) with a nominal molecular mass limit of 100 kDa to remove excess Alexa Fluor 610-X. *S. aureus* JYG2 was washed three times before infection. MAC-T cells were plated in a 12-mm glass-based dish (Nunc, Thermo Fisher Scientific, Waltham, MA). The cells were challenged with *S. aureus* JYG2 labeled with WGA conjugate (10^6 bacteria/well). One hour later, cells were washed with DMEM, and extracellular bacteria were killed with lysostaphin (20 μg/ml). After 2 h of treatment, cells were washed and incubated with Alexa Fluor 610-X conjugate-labeled phage vB_SauM_JS25 (10^8 PFU/well). Three hours later, cells were treated with citric acid buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0). Then washed cells were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) in PBS for 5 min and subsequently washed again for 10 min. Glass-based dishes were mounted onto microscope slides, and the samples were examined using an UltraView VoX confocal microscope (PerkinElmer) (14). The percentage of Alexa Fluor 488- or 610-X-labeled positive cells was determined by counting 200 cells from 10 randomly selected fields at $\times$400 magnification on a confocal microscope as previously described (15). Briefly, the phage positive rate was recorded as (number of 610-X-labeled positive cells/200 cells) $\times$ 100%. Results indicated that phage vB_SauM_JS25 crossed MAC-T cell membranes and cleared intracellular *S. aureus* (Fig. 2A).

In particular, phage vB_SauM_JS25 reached the nucleus within 3 h postincubation (Fig. 2Af, h, j, and l). The reason for entrance of labeled phage into the nucleus is unclear and needs further investigation. The endocytotic efficiency of phage vB_SauM_JS25 was ~12% (Fig. 2B). Previous studies showed that the phage particles transferred to the interior of macrophages and significantly reduced the damage caused by the cytotoxic effects of bacteria on phagocytes (4, 16). Moreover, Barr et al. (13) reported that interaction of phages and mucus-producing cells does occur in the elimination of bacteria, and this interaction is based on immunoglobulin-like domains. The interaction via immunoglobulin-like domains may help phages cross MAC-T cell membranes. The mechanism by which phages are internalized in MAC-T cells will be further studied.

Our *in vitro* results with MAC-T bovine mammary epithelial cells suggest that phage vB_SauM_JS25 may be a potential therapeutic candidate for treatment of *S. aureus* infection of bovine mammary epithelial cells *in vivo*.

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