

An Innate Bactericidal Oleic Acid Effective Against Skin Infection of Methicillin-Resistant *Staphylococcus aureus*: A Therapy Concordant with Evolutionary Medicine

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Free fatty acids (FFAs) are known to have bacteriocidal activity and are important components of the innate immune system. Many FFAs are naturally present in human and animal skin, breast milk, and in the bloodstream. Here, the therapeutic potential of FFAs against methicillin-resistant *Staphylococcus aureus* (MRSA) is demonstrated in cultures and in mice. Among a series of FFAs, only oleic acid (OA) (C18:1, cis-9) can effectively eliminate *Staphylococcus aureus* (*S. aureus*) through cell wall disruption. Lauric acid (LA, C12:0) and palmitic acid (PA, C16:0) do not have this ability. OA can inhibit growth of a number of Gram-positive bacteria, including hospital and community-associated MRSA at a dose that did not show any toxicity to human sebocytes. The bacteriocidal activities of FFAs were also demonstrated *in vivo* through injection of OA into mouse skin lesions previously infected with a strain of MRSA. In conclusion, our results suggest a promising therapeutic approach against MRSA through boosting the bacteriocidal activities of native FFAs, which may have been co-evolved during the interactions between microbes and their hosts.

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Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium and one of the major causes of fatal nosocomial infections [23]. It is estimated that annually in the United States (US) alone, *S. aureus* accounts for 12 million outpatient visits and 292,000 hospitalizations of which 126,000 are due to methicillin-resistant *Staphylococcus aureus* (MRSA) [19]. Recent studies estimated that more people die from the MRSA bacterium than from human immunodeficiency virus (HIV) in the US [31, 42], and the Center for Disease Control and Prevention estimated that more than 90,000 people die from hospital-acquired bacterial infections in the US each year [42]. Historically, MRSA infections have occurred in nosocomial settings. However, recently MRSA infections have increasingly been found among individuals in the community without healthcare exposures [55]. Although *S. aureus* including MRSA and community-associated (CA)-MRSA can cause life-threatening and systemic infections (bacteremia), skin and soft tissues are the most common sites of *S. aureus* infection and comprise more than 75% of MRSA disease [9]. Unfortunately, it remains as an unmet challenge to develop effective therapeutic approaches for MRSA treatment because of its formidable resistance against multiple traditional antibiotics such as methicillin, cloxacillin, and flucloxacillin [49, 50]. Decades of selective pressure with β -lactam antibiotics and close proximity of susceptible hosts have resulted in a high prevalence of MRSA in hospitals worldwide. Although

these factors logically explain the high incidence of hospital-associated MRSA infections, the molecular basis for the increased incidence remains incompletely defined. Recent studies indicate that strains (*e.g.*, USA300 and 400) that are the leading causes of CA-MRSA disease in the US [1, 13, 53] have enhanced virulence compared with strains that are the leading causes of hospital infections (*e.g.*, USA200).

The need for new therapy, which can effectively cripple bacterial infection and lower the risk of creating *S. aureus* resistance, is indisputable. Although vaccination is one of the modalities against *S. aureus* infection, it generally takes a long time to develop an effective vaccine. Furthermore, people may hesitate to be immunized with a preventive vaccine because they are unsure whether *S. aureus* bacteria will infect them. Even if a therapeutic vaccine is administered, protective antibodies may not be produced quickly enough to suppress bacterial spread through the body. Although systemic antibiotic therapy is currently used for treatment of *S. aureus* infections, it nonspecifically kills the majority of microbes and disrupts the homeostasis of body-resident microflora [51]. In addition, antibiotic use has a potential risk of selecting for antibiotic-resistant bacteria.

Free fatty acids (FFAs) are known to possess anti-*S. aureus* activity [8, 45, 46] and are important components of the innate immune system [10]. Many FFAs including oleic acid (OA) (a C18:1 FFA, *cis*-9) naturally exist in humans (*e.g.*, skin, breast milk, and bloodstream) and mice [8, 21, 48]. These FFAs thus can function as “innate bactericides.” It has also been known that the FFAs can anchor in the bacterial membranes to damage the cell wall structure [30]. Drugs that anchor in the bacterial membrane (*e.g.*, ceragenins and lipopeptides) or that target the bacterial membrane and proteic (lipoglycopeptides) or lipidic (glycopeptides) cell wall precursors seem to have the most potential. They show a fast and extensive bactericidal effect and are probably less prone to select for resistance owing to the difficulty that bacteria would have in modifying these targets in a way that is compatible with survival [52]. It has been known that endogenous bactericides are largely nonspecific and hold great promise to avert the development of bacterial resistance [17, 44, 46]. It has been proposed that innate bactericides and innate bactericides-resistance mechanisms have co-evolved, leading to a transient host-pathogen balance that has shaped the existing repertoire of innate bactericides [44]. More intriguingly, it has been found that two FFAs (linoleic acid and dehydrocrepenynic acid) inhibit the bacterial drug resistance by decreasing the transfer frequency of the conjugal DNA [14, 46].

In this study, we found that OA exerts excellent antimicrobial activity against various *S. aureus* strains, including a hospital-acquired MRSA strain (MRSA252) and a CA-MRSA strain (USA300). The use of OA as an innate bactericide is in compliance with evolutionary medicine because it is endogenously present in human skin.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

S. aureus [MRSA252, USA200, USA300, and a *S. aureus* *mprF* and *S. epidermidis* (ATCC12228; Manassas, VA, USA)] were cultured on 3% tryptic soy broth (TSB) (Sigma, St. Louis, MO, USA) agar overnight at 37°C. *B. anthracis* Sterne 34F2 spores (Colorado Serum company, Denver, CO, USA) were heated at 65°C for 30 min and then cultured in a modified nutrient broth medium [0.8% Bacto nutrient broth, 0.1% KCl, 0.012% MgSO₄, 1 mM Ca(NO₃)₂, 10 µM MnCl₂, 1 µM FeSO₄, pH 7.6], followed by incubation at 37°C with shaking. *L. monocytogenes* (EGD strain BUG) was grown overnight in Brain-heart infusion medium (Difco) at 37°C without shaking. *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) was cultured on Luria broth agar (Difco) at 26°C for 48 to 72 h. All bacteria from a single colony were cultured in their media overnight at 37°C. The overnight culture was diluted 1:100 and cultured until reaching around OD₆₀₀=1.0. Bacteria were harvested by centrifugation at 5,000 ×g for 10 min, washed with PBS, and suspended to an appropriate amount of PBS for the further experiments.

Minimal Bactericidal Concentration (MBC) Assay

To determine the MBC of OA, bacteria (1×10⁶ CFU/ml) were incubated with OA at various concentrations (0.078125–200 µg/ml) in 5% DMSO in PBS on a 96-well microplate (100 µl per well) for 10 min, 30 min, 1 h, 3 h, or 5 h. The control received only 5% (v/v) of DMSO. After incubation, the bacteria were diluted 1:10–1:10⁶ with PBS. MBC was finally examined at a 99.9% killing level and determined by spotting the dilution (5 µl) on an agar plate supplemented with bacterial media for the counting of CFUs.

Radial Diffusion Assay (RDA)

RDA with minor modifications has been described previously [36]. Briefly, bacteria in mid-log phase were centrifuged at 2,000 ×g for 10 min and washed with PBS. Bacteria (1×10⁵ CFU/ml) were dispersed in agar consisting of 1% (w/v) agarose (Sigma) and 1% (w/v) TSB in 10 mM PBS at 42°C. Subsequently, the agar was poured into Petri dishes and solidified. The wells (3 mm in diameter) with a 10-µl capacity were created by poking a pipette tip into the semi-solidified agar. Six serially diluted samples of each OA ranging in concentration from 0.625 to 200 µg/ml were prepared, and 5-µl OA aliquots solubilized in 5% (v/v) of DMSO were added to the wells. After 3 h of incubation, a 10-ml overlay gel composed of 6% TSB, 1% agarose, and 10 mM PBS (pH 7.4) was poured onto the plates, and the plates were incubated overnight to allow the surviving organisms to form microcolonies. The growth inhibition zones reflecting the antimicrobial activity of OA were observed.

Transmission Electron Microscopy

After incubation at 37°C, bacteria were harvested, washed twice with PBS, and fixed in Karnovsky's fixative (4% paraformaldehyde, 2.5% glutaraldehyde, 5 mM CaCl₂ in 0.1 M Na cacodylate buffer, pH 7.4) overnight at 4°C, followed by 1% OsO₄ in 0.1 M Na cacodylate buffer, pH 7.4, *en bloc* staining with 4% uranyl acetate in 50% ethanol, and subsequent dehydration using a graded series of ethanol solutions followed by propylene oxide and infiltration with epoxy resin (Scipoxy 812, Energy Beam Sciences, Agawam, MA, USA). After polymerization at 65°C overnight, thin sections were cut and stained with uranyl acetate (4% uranyl acetate in 50%

ethanol) followed by bismuth subnitrate. Sections were examined at an accelerating voltage of 60 kV using a Zeiss EM10C electron microscope (Carl Zeiss, Thornwood, NY, USA).

Skin USA300 Infection Treated with OA

USA300 bacteria (1×10^7 CFU in PBS) mixed with the microcarrier (Cytodex) beads [1:1 (v/v); Sigma] were subcutaneously injected into the dorsal skins of 8- to 12-week-old female ICR mice (Harlan, Indianapolis, IN, USA). Then 100 μ l of OA (100 μ g/ml in 5% DMSO in PBS) or 5% DMSO in PBS was injected into the same location right after bacterial injection. For histological observation, the dorsal skins were cross-sectioned, stained with H&E (Sigma), and viewed on a microscope (Carl Zeiss). The lesion sizes were measured using a microcaliper (Mitutoyo, Kanagawa, Japan) 24 and 48 h after injection. The lesion sizes were examined and quantified by using Image software [National Institutes of Health (NIH), Bethesda, MD, USA]. To determine the bacterial number in lesional skin, the dorsal skin was punched with an 8-mm biopsy 24 h after bacterial injection. The punch biopsy was homogenized in 1 ml of sterile PBS with a

hand tissue grinder. Bacterial CFUs in the skin were enumerated by plating serial dilutions ($1:10^1$ – $1:10^6$) of the homogenate on a TSB agar plate. The plate was incubated for 24 h at 37°C to count colonies. All experiments using mice were conducted in a Biosafety Level 2 (BSL-2) facility and with accordance to institutional guidelines for animal experiments.

Statistical Analysis

To determine significances between groups, comparisons were made using the two-tailed t-test. For all statistical tests, a P-value of <0.05 was accepted for statistical significance.

RESULTS

OA is an Effective FFA to Inhibit *S. aureus* Growth

We have investigated a series of FFAs including OA (C18:1, *cis*-9), lauric acid (LA, C12:0), and palmitic acid (PA, C16:0) for anti-*S. aureus* activity and discovered that only OA can effectively kill *S. aureus* at reasonable doses. To determine the minimal bactericidal concentration (MBC) of FFAs, *S. aureus* multiple peptide resistance factor mutant ($\Delta mprF$) that lacks the ability to modify anionic membrane lipids with L-lysine [40] was incubated in phosphate buffer saline (PBS) with several concentrations of LA, PA, or OA for 5 h at 37°C. After incubation, the bacteria were diluted with PBS and spotted on an agar plate to count colony-forming units (CFU). Since all FFAs were dissolved in 5% (v/v) dimethylsulfoxide (DMSO), bacteria incubated with DMSO served as a control group. As shown in Fig. 1, no killing was detected when *S. aureus* was incubated with 5% (v/v) DMSO (Vehicle). The incubation of PA (1.25 to 100 μ g/ml) did not influence the growth of *S. aureus mprF* (data not shown). In contrast, we found that when the concentrations of OA and LA are higher than 3.125 and 70 μ g/ml, respectively, they started killing *S. aureus mprF* (Fig. 1A, 1B). Furthermore, MBC assays indicated that LA (0.1–200 μ g/ml) did not kill a MRSA252 strain (data not shown). To determine the cytotoxicity of FFAs, a human skin sebocyte SZ95 cell line [56] was incubated overnight with 100 μ g/ml of LA, OA, or PA. Although LA has a slight toxic effect on sebocytes, this was only seen at a concentration far exceeding that needed to kill *S. aureus*. PA and OA were not cytotoxic for SZ95 cells (Supplementary Fig. S1).

OA Kills *S. aureus* Bacteria by Breaking Down the Cell Walls

We next examine if OA can effectively inhibit the MRSA bacteria. A hospital-acquired MRSA bacterial strain (MRSA252) was incubated with OA at various concentrations in PBS for 5 h at 37°C in MBC assays. Our recent publication demonstrated that killing of MRSA252 occurred at an OA concentration greater than 5 μ g/ml, and complete killing of MRSA252 occurred at concentrations greater than 10 μ g/ml

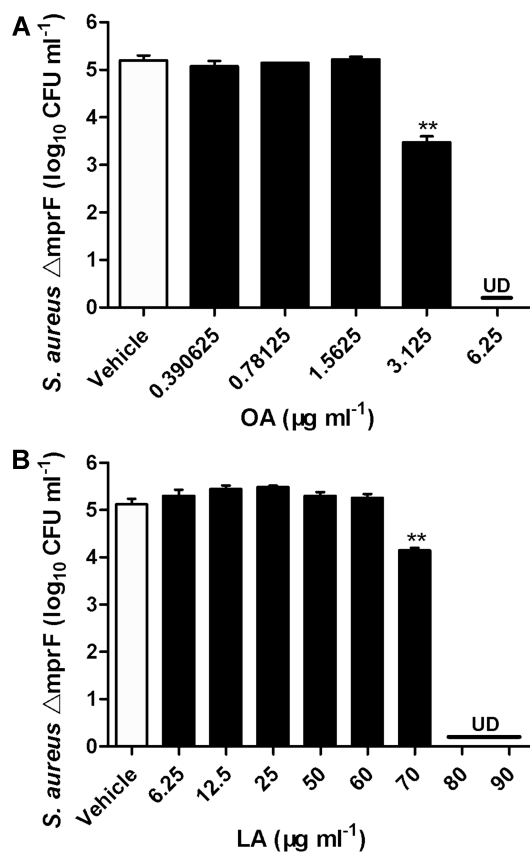


Fig. 1. OA exerts bactericidal activity against *S. aureus*. *S. aureus mprF* was cultured on Tryptic soy broth (TSB) (Sigma). For MBC assays, the bacteria (1×10^7 CFU/ml) was incubated with OA (0.375–100 μ g/ml) (A), LA (6.25–100 μ g/ml) (B), and PA (6.25–100 μ g/ml) (data not shown) in 5% DMSO in PBS on a 96-well microplate (100 μ l/well) for 5 h. Bacteria incubated with 5% (v/v) DMSO served as a control (vehicle). After incubation, the bacterial suspension was diluted 1:10–1:10⁶ with PBS, and 5 μ l of the dilutions were spotted on a TSB agar plate for CFU counts. **P<0.01. P-values were evaluated using two-tailed t-tests. Data are the mean \pm SD of three individual experiments. UD, undetectable.

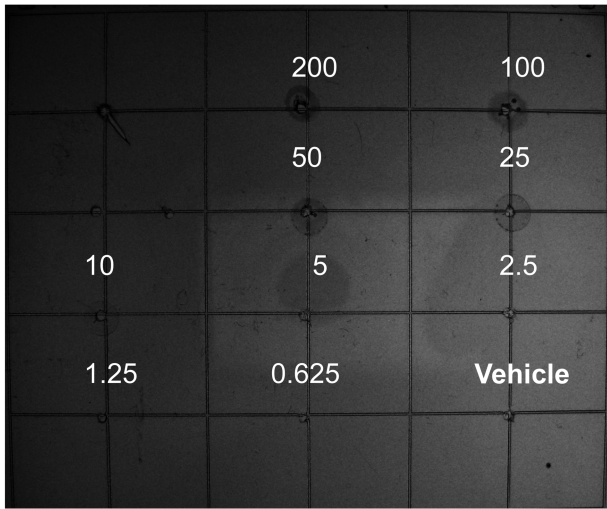


Fig. 2. Bactericidal effects of OA on MRSA252. Radial diffusion assay. Bacteria at 1×10^5 CFU per ml were dispersed in agar consisting of 1% (w/v) agarose (Sigma-Aldrich, St. Louis, MO, USA) and 1% (w/v) TSB in 10 mM sodium phosphate buffer and then poured into Petri dishes to solidify. Wells of 3 mm in diameter were made in this agar. Then 5- μ l OA (0.625 to 200 μ g/ml) aliquots solubilized in 0.01% (v/v) acetic acid were added to the wells. After 3 h of incubation, a 10-ml overlay gel composed of 6% Trypticase soy broth powder, 1% agarose, and 10 mM sodium phosphate buffer (pH 7.4) was poured onto the plates, and the plates were incubated overnight for growth inhibition zones diameters.

[24]. A sensitive radial diffusion assay (RDA) [36] as described in Materials and Methods was used to further verify the antimicrobial activity of OA against MRSA252 (Fig. 2). In consistence with MBC assays [24], the growth inhibition zones were clearly observed when bacteria were incubated with OA at a minimum effective concentration of 10 μ g/ml.

Because it has been reported that OA kills bacteria by disrupting the cell wall [30], we investigated OA-induced cell wall damage to *S. aureus* by transmission electron microscopy (Fig. 3). Vehicle control [5% (v/v) DMSO] treated bacteria showed uniform density in cytoplasmic compartments and cell separation by prominent high contrast septa (Fig. 3a). After OA treatment, disruption of the cell wall architecture was observed (Fig. 3b). The cell walls in many bacteria were completely separated from cytoplasmic compartments. This finding suggests that OA breaks down the cell wall of *S. aureus* bacteria.

OA Inhibits the Growth of USA300, a Community-Associated MRSA Bacterium

To test if OA exerts antimicrobial activity against CA-MRSA, we determined the MBC of OA for USA300, a predominant CA-MRSA that was first observed in marginalized populations and, in general, under conditions of poor hygiene and close physical contact due to overcrowding [18]. Currently, CA-MRSA is reported as the most common cause of purulent skin and soft tissue infections in the

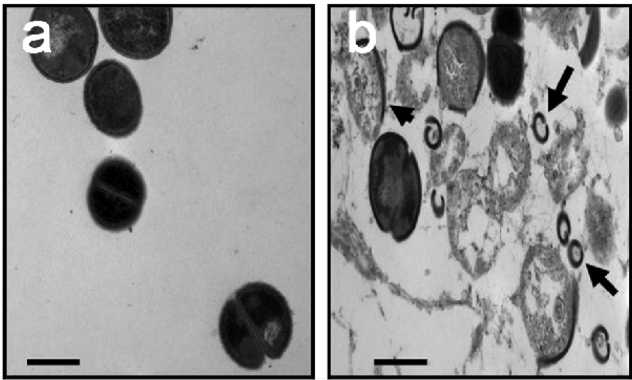


Fig. 3. OA disrupts the cell wall of *S. aureus*. *S. aureus* (MRSA252) bacteria (1×10^7 CFU per ml) were incubated with 5% DMSO in PBS (a) or with OA (100 μ g/ml dissolved in 5% DMSO) (b) for 5 h. The transmission electron microscopy of MRSA252 exposed to OA revealed cell wall (arrows) separation from cytoplasmic compartments and lysis. Bar = 0.5 μ m.

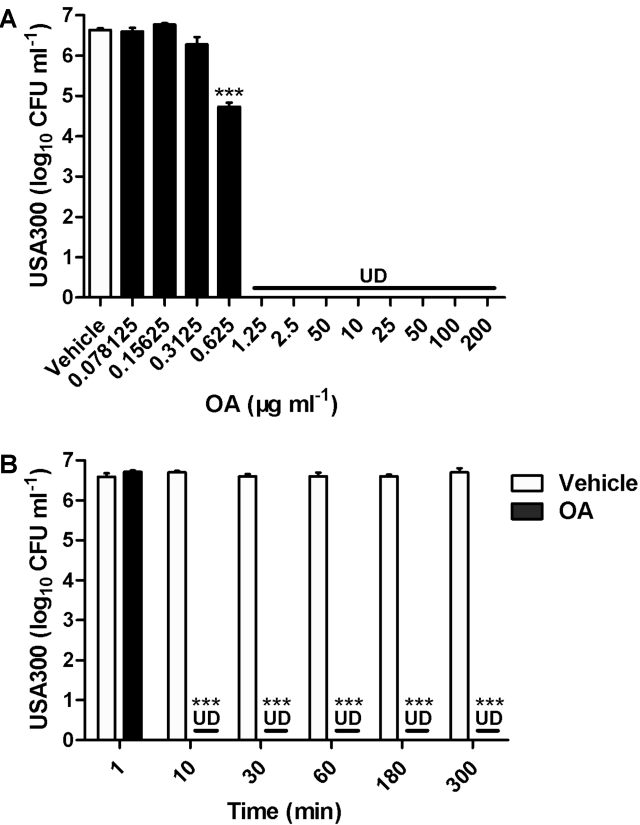


Fig. 4. Bactericidal effects of OA on USA300. USA300 (1×10^6 CFU/ml) was incubated with 0.078125–200 μ g/ml of OA in 5% DMSO in PBS for 5 h. After incubation, USA300 suspension was diluted 1:10–1:10⁶ with PBS, and 5 μ l of the dilutions was spotted on a TSB agar plate. After liquid in the OA suspension was absorbed into the agar, the plate was incubated overnight to quantify the CFU of USA300. A. MBC of OA. B. Killing time of OA to the USA300. *** $P < 0.001$. P-value was evaluated using two-tailed t-tests. Data are the mean \pm SD of three individual experiments. UD, undetectable.

Table 1. MBC values of OA on various bacteria.

Organism	Strain	MBC ($\mu\text{g/ml}$)
<i>S. aureus</i>	MRSA252	<10
<i>S. aureus</i>	USA200	<1.25
<i>S. aureus</i>	USA300	<1.25
<i>S. epidermidis</i>	ATCC12228	<2.5
<i>L. monocytogenes</i>	EGD strain BUG	<50
<i>B. anthracis</i>	Sterne 34 F2	>200
<i>E. coli</i>	BL21 (DE3)	UD

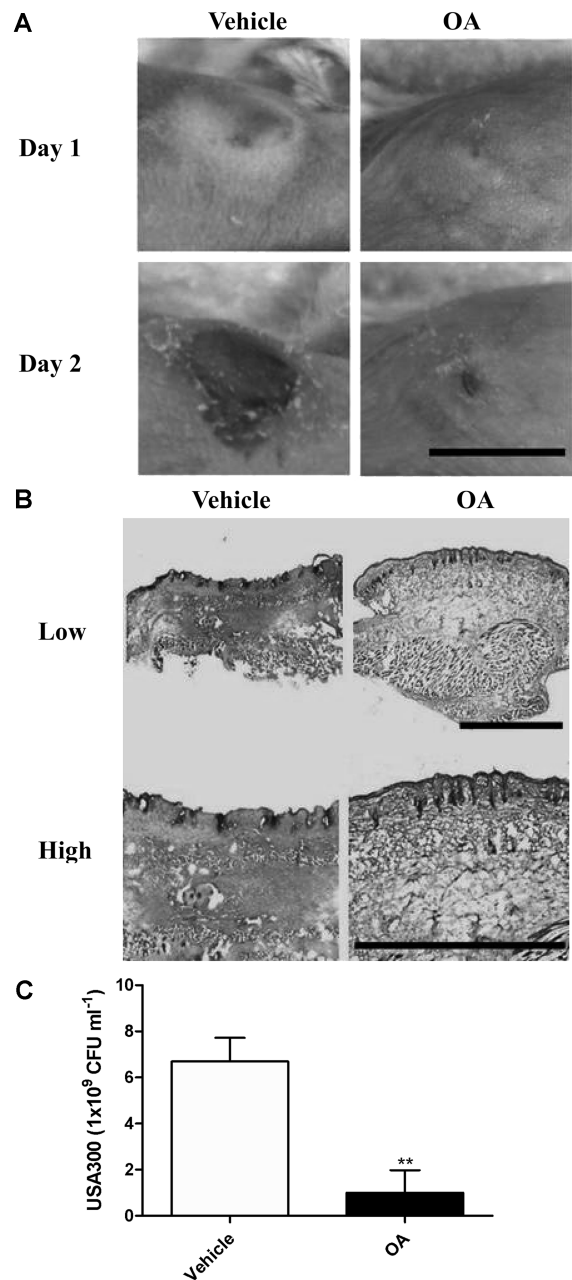
UD, undetectable bactericidal effect at any dose tested

United States [28]. To determine the MBC of OA for USA300, bacteria were incubated with OA at various concentrations in PBS for 5 h at 37°C. After incubation, the bacteria were diluted with PBS and spotted on an agar plate to count CFUs. We found that OA effectively suppressed the growth of USA300 at an OA concentration greater than 0.625 $\mu\text{g/ml}$, and completely killed the USA300 at concentrations greater than 1.25 $\mu\text{g/ml}$ (Fig. 4A). Taken together with data in Fig. 1A, 2, and 4A, the findings suggest that OA may have broad-spectrum antimicrobial activity against *S. aureus* bacteria. To reveal how fast OA can efficiently kill USA300, bacteria were incubated for 1 min, 10 min, 30 min, 1 h, 3 h, and 5 h with OA, respectively. The results illustrated that the USA300 bacteria were completely eliminated after incubation with OA (100 $\mu\text{g/ml}$) for 10 min (Fig. 4B).

To determine if OA exerts this antimicrobial activity to other bacteria, we measured the MBC values of OA for Gram-positive bacteria including *Staphylococcus epidermidis* (*S. epidermidis*; American Type Culture Collection (ATCC) 12228], *Listeria monocytogenes* (*L. monocytogenes*; EGD strain BUG), *Bacillus anthracis* (*B. anthracis*; Sterne 34F2 spores), and a Gram-negative bacterium [*Escherichia coli* (*E. coli*); BL21 (DE3)] (Table 1). The growth of all Gram-positive bacteria, but not *E. coli*, could be significantly suppressed by OA at a concentration lower than 200 $\mu\text{g/ml}$, suggesting that Gram-positive bacteria are more susceptible to OA than are Gram-negative bacteria.

OA Alleviates USA300-Induced Skin Lesions

We injected USA300 bacteria (1×10^7 CFU per 50 μl in PBS) or PBS (100 μl) subcutaneously into dorsal skins of Institute Cancer Research (ICR) mice (Fig. 5). The subcutaneous injection of USA300, but not PBS, induced a skin lesion approximately 4.61 ± 1.57 mm in diameter 2 days later. To determine if OA provided protection against USA300 infection *in vivo*, mouse dorsal skins injected with bacteria or PBS were subsequently injected with 100 μl OA (100 $\mu\text{g/ml}$ in 5% DMSO in PBS) or 5% DMSO. The bacteria-induced skin lesion was significantly attenuated in OA-treated mice (1.03 ± 0.98 mm²) compared with that in DMSO-treated mice (15.79 ± 4.18 mm²) (Fig. 5A).

**Fig. 5.** OA alleviates lesions and decreases bacterial colonization in the USA300-infected skins.

A. The dorsal skins of ICR mice were injected subcutaneously with USA300 bacteria (1×10^7 CFU per 100 μl in PBS). Then 100 μl of OA (100 $\mu\text{g/ml}$ in 5% DMSO in PBS) or 5% DMSO (vehicle) was subsequently injected right after bacterial injection. Skin lesions were pictured on day 1 and day 2 after injection. Bars=5 mm. **B.** Inflamed skins (low and high powers) were observed in the H&E-stained frozen sections of skins injected with USA300 and with vehicle. By contrast, inflammation was significantly alleviated in the skins injected with USA300 plus OA. The scale bar of low power and high power was 200 and 50 μm , respectively. **C.** The skin biopsy was homogenized in 1 ml of sterile PBS with a hand tissue grinder. Two days after injection, the CFUs in the skin injected with USA300 plus vehicle or OA were enumerated by plating serial dilutions ($1:10^1$ – $1:10^6$) of the homogenate on a TBS agar plate. *** $P < 0.001$. P-values were evaluated using two-tailed t-tests. Data are the mean \pm SD of six skin lesions from three mice per group.

Histological observation in hematoxylin and eosin (H&E) stained tissue sections revealed that injection of USA300 inflamed the skin and broke down the epidermal layer (Fig. 5B). Treatment with OA, but not DMSO, alleviated the skin damage response to USA300 infection. To determine the intensity of bacterial colonization, skins treated with OA or DMSO were homogenized to estimate the CFU. The bacterial numbers in skins injected with USA300/DMSO and USA300/OA were $6.7 \times 10^9 \pm 1 \times 10^9$ and $1.0 \times 10^9 \pm 9.8 \times 10^8$ CFU, respectively, suggesting that OA considerably decreased the growth of USA300 in the skin lesions (Fig. 5C).

DISCUSSION

Evolutionary (Darwinian) medicine has proven valuable in explaining the reasons for the development of antibiotic resistance. The use of non-endogenous antibiotics for bacterial treatments may not be in compliance with evolutionary medicine since bacteria can fight back by developing the ability to neutralize these antibiotics. For example, overuse of broad-spectrum antibiotics, such as second- and third-generation cephalosporins, greatly hastens the development of methicillin resistance [33]. It has been reported that FFAs endogenously exist in human organs [27] including skin [4] and can function as innate bactericides against various pathogens by altering the hydrophobicity of the bacterial cell wall [30]. Here, we demonstrated that OA, but not LA and PA, can effectively kill *S. aureus* bacteria including both MRSA252 and USA300 *in vitro* (Fig. 2 and 4) and significantly suppress USA300-induced skin lesions and bacterial colonization in mice (Fig. 5). USA300 is one of the aggressive CA-MRSA that became notable for extreme antibiotic resistance and being responsible for rapidly progressive, fatal diseases including necrotizing pneumonia and severe sepsis [5]. As shown in Fig. 4, complete eradication of USA300 bacteria can be found as early as 10 min after incubation with OA, suggesting that OA may be able to efficiently clear bacteria at the early stage of CA-MRSA infection. OA is an unsaturated FFA, whereas LA and PA are saturated FFAs. In general, unsaturated FFAs tend to have greater potency against bacteria than saturated FFAs [11]. However, our previous studies demonstrated that, compared with OA and PA, LA showed the strongest bacteriocidal activity against *P. acnes*, a Gram-positive bacterium [56]. It has been reported that the hydrophobicity of the bacterial cell wall can affect the interactions between bacteria and FFAs [30]. Thus, we speculate that, besides the structure of FFAs, the hydrophobicity of the bacterial cell wall may influence the bacteriocidal activities of FFAs.

Our data have demonstrated that OA, PA, and LA are not detrimental to a human skin cell (sebocyte) (Fig. S1). The bacteriocidal actions of FFAs are typically broad spectrum and of potencies comparable to antimicrobial peptides

(AMPs). Although both FFAs and AMPs are endogenous antimicrobial agents, FFAs with diverse structures are relatively unstable and they also have a tendency to bind nonspecifically to proteins [12]. Treatment of sebocytes with OA or PA up-regulated the expression of human beta-defensin (hBD)-2 and -3 and induced a release of hBD-2 [38]. An *in vivo* study from our laboratory [38] demonstrated the up-regulation of mouse β -defensin 4, a mouse ortholog for hBD-2, in mouse ear skin after epicutaneous application of OA. In addition, hBD-2 synergistically killed *P. acnes* in combination with LA [38], suggesting cooperative effects between FFAs and AMPs. Recently, it has been reported that FFA induced faster wound closure in mice [7] and increased the wound healing tissue mass in rats [43]. FFAs induced vascular endothelial growth factor- α (VEGF- α) and interleukin-1 β (IL-1 β) in the inflammatory phase of wound healing in rats [43]. In addition, FFAs were able to stimulate the production of cytokine-induced neutrophil chemoattractant in inflammation 2 α/β [43], suggesting that the pro-inflammatory effect of OA may speed up the wound healing process [7]. The above results suggest that endogenous FFAs not only function as bactericides but also as native enhancers of innate immunity. Moreover, the OA-induced cytokine profiles in infected and non-infected mice may be worth establishing in future experiments.

Previous studies have demonstrated that FFAs of various chain lengths and with different levels of unsaturation were primarily effective against Gram-positive bacteria, but not against a number of Gram-negative bacteria [3, 15, 22, 25, 29, 32, 40, 47]. Consistent with these studies, our results indicate that OA has antibacterial activity against a range of Gram-positive bacteria, but not a Gram-negative *E. coli* bacterium (Table 1). Cell walls in Gram-positive bacteria consist of many layers of peptidoglycan without a lipid outer membrane. Cell walls in Gram-negative bacteria, on the other hand, have only one or a few layers of peptidoglycan but possess an outer membrane consisting of various lipid complexes. It has been shown that the outer membrane layer of Gram-negative organisms may screen the cells against fatty acids to prevent their accumulation on the inner cell membrane. Therefore, Gram-negative bacteria, protected by their outer lipid membrane, are resistant to the destructive powers of OA [47]. It has been reported that use of a chelating agent to remove the outer lipid membrane will overcome the limitation of FFAs to Gram-negative organisms [26], raising the possibility of using OA for treatment of Gram-negative organism-infected skin. On the surface of the skin, triglycerides produce FFAs by catalytic reactions in the presence of bacterial hydrolases [34]. Although the FFA content in human and mouse skins is different, it has been reported that human skin surface is rich in FFAs including LA, myristic acids (8.7 nmol/cm^2), PA (15 nmol/cm^2), stearic acids

(4.8 nmol/cm²), linoleic, and linolenic acids, palmitoleate, and OA (9.5 nmol/cm²) [39, 54]. As shown in Fig. 5, subcutaneous injection of 100 µg (approximately 354 nmol) OA significantly decreased the growth of USA300 in the skin lesions. The incomplete elimination of bacteria by OA may be due to the possibility that OA cannot access endocytosed USA300 within immune cells. Recently, *flk/flk* mutant mice (an *N*-ethyl-*N*-nitrosourea-induced recessive germ line mutation of C57BL/6 mice) showed reduced sebum production and were unable to synthesize the palmitoleate and OA [16]. The mutant mice had a defect in the clearance of skin infections by *Streptococcus pyogenes* (*S. pyogenes*) and *S. aureus*. Positional cloning and sequencing revealed that *flk* is a novel allele of the stearoyl coenzyme A desaturase 1 gene (*Scd1*), which is an enzyme responsible for the biosynthesis of OA and POA [41] and can be strongly up-regulated by Toll-like receptor 2 (TLR2) [16]. Thus, the essential role of endogenous OA in combating *S. aureus*/MRSA infection can be addressed in the future by comparing the severity of *S. aureus*/MRSA-induced skin lesions in *flk/flk* mutant and wild-type mice.

It is also unknown how many endogenous FFAs in skin can actively eliminate infected *S. aureus*. The amount of endogenous FFAs in the human body was insufficient to completely eradicate the overgrowth of *S. aureus* and/or bacteria with mutations or drug resistance. The use of exogenous FFAs including OA at different doses can efficiently alleviate mild to severe stages of *S. aureus*/MRSA infection. Unfortunately, one challenge of using FFAs for medical practice stems from their poor water-solubility. DMSO was used to dissolve OA in this study. Although DMSO is an anti-inflammatory agent that has been shown clinically to relieve pain [6], it has been reported that topical application of DMSO can cause skin irritation [57]. To circumvent this problem, we have recently incorporated OA onto the surface of a liposome (LipoOA) [56]. Our published result demonstrated that LipoOA exhibited higher potency than free OA in terms of the suppression of the growth of MRSA252 [56]. Without toxicity to normal skin, LipoOA was highly effective in curing skin infections caused by MRSA252 [24]. Previous studies demonstrated that liposomes enriched in OA are less susceptible to oxidation and show less pro-inflammatory activity when exposed to oxidizing conditions [35]. In addition, it has been known that liposomal antibacterial agents are able to kill intracellular bacteria [2]. Thus, LipoOA may be able to optimize the potency of OA against *S. aureus* infection in humans.

Resistance of *S. aureus* to β -lactam antibiotics is usually caused by the production of β -lactamases. Production of β -lactamase is controlled by the *bla* regulatory apparatus, which is homologous to the *mec* system responsible for the regulation of *mecA*, the gene encoding methicillin resistance, in many strains with inducible resistance [20]. It has been

reported that FFAs in sebum have the capability of inhibiting the induction of β -lactamase in *S. aureus* [8]. Here, we emphasize that OA is an endogenous molecule that may be part of innate immunity against *S. aureus*/MRSA infection. Novel bactericides using OA and its derivatives may gain a new set of modalities for fighting antibiotic resistance.

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