Ethanol extract of *Sanguisorba officinalis* L. inhibits biofilm formation of methicillin-resistant *Staphylococcus aureus* in an *ica*-dependent manner

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important nosocomial pathogen that shows resistance to many antibiotics and is usually associated with serious infections. Having the ability for biofilm formation increases resistance to antibiotics. *Sanguisorba officinalis* L. is a perennial plant that is distributed in the northern districts of China and has been used as a traditional Chinese medicine. In this study, the effect of *S. officinalis* on MRSA strain SA3 isolated from a dairy cow with mastitis was evaluated by testing the growth and biofilm formation ability of MRSA cultured with or without ethanol extracts of *S. officinalis*. The results showed that the ethanol extract of *S. officinalis* strongly inhibited the biofilm formation of MRSA. With a confocal laser scanning microscope system, we observed that the biofilm structure of the test group with the addition of *S. officinalis* appeared looser and had less biomass compared with the control group without *S. officinalis*. Furthermore, we found that the transcript levels of the *icaADBC* operon remarkably decreased upon addition of the ethanol extract of *S. officinalis*, indicating that *S. officinalis* inhibits biofilm formation of MRSA in an *ica*-dependent manner.

Key words: *Sanguisorba officinalis* L., methicillin-resistant *Staphylococcus aureus*, biofilm

INTRODUCTION

Staphylococcus aureus is an important pathogen that is a common cause of community- and hospital-acquired infections. *Staphylococcus aureus* can be well adapted to the human host by adhering to specific host substrates, evading host defenses, and resisting antibiotic therapy (Greenberg et al., 1989; Patti et al., 1994; Park et al., 1996; Joh et al., 1999; Hussain et al., 2002; Fedtke et al., 2004; Shanks et al., 2005). A variety of virulence factors

and specific mechanisms contribute to their ability to develop chronic infections in the host. One important way in which these bacteria adapted is through biofilm formation (Stewart, 2002; Gowrishankar et al., 2012). Biofilms are a population of bacteria growing on biotic and abiotic surfaces that embed themselves in a self-produced extracellular matrix of exopolysaccharide, proteins, and some micromolecules, such as DNA (Gowrishankar et al., 2012). The structure of biofilm constitutes a protected environment for the growth of bacteria by restricting antibiotic access and shielding the bacterial pathogen from host immune defenses (Joh et al., 1999; Nguyen et al., 2008; Atshan et al., 2012).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium that has developed resistance, through the process of natural selection, to β -lactam antibiotics, which include penicillins (e.g., methicillin, dicloxacillin, nafcillin, oxacillin) and cephalosporins (Barrett et al., 1968; Ubukata et al., 1985; Matsushashi et al., 1986). Serious infections are associated with MRSA, and it poses a serious threat to public health worldwide. The ability of biofilm formation in MRSA can lead to resistance to most currently used antibiotics (Ando et al., 2004). Therefore, biofilm-forming MRSA infections are life-threatening. In recent years, with the extensive use of implanted medical devices for diagnostic and therapeutic purposes, which can be easily colonized by biofilm-forming staphylococci (O'Gara, 2007), the early identification and prevention of the most serious nosocomial infections caused by biofilm-forming MRSA become more urgent and important for medical care.

Sanguisorba officinalis L. is a perennial plant that is distributed in the northern districts of China. The dried roots of *S. officinalis* display hemostatic, antiallergic, analgesic, and astringent properties, and have been used in traditional Chinese medicine for the treatment of burns, scalds, and internal hemorrhage (Liu et al., 2005; Cai et al., 2012). Several modern pharmacological studies have shown that the root contains various saponin components, which have been proven to be the main anticancer agents in some natural plants. Recently, some authors have demonstrated that aqueous extracts

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of *S. officinalis* had obvious anticancer effects by inducing cancer cell apoptosis and inhibiting angiogenesis, which has the possibility of being developed as a breast cancer therapeutic agent (Bastow et al., 1993; Liu et al., 2005). In addition, several other investigations revealed that terpenoids and triterpenoid glycosides from this herb have significant antioxidant and neuroprotective activities (Yokozawa et al., 2000, 2002; Liu et al., 2005; Nguyen et al., 2008; Zhang et al., 2012).

In the current study, we investigated the effect of *S. officinalis* on MRSA. The growth and biofilm formation of MRSA cultured with or without ethanol extracts of *S. officinalis* was tested by performing colony-forming unit assays and microplate assays, as well as using a confocal laser scanning microscope (CLSM) system. Results showed that the ethanol extract of *S. officinalis* has a significant inhibitory effect on biofilm formation in MRSA. In addition, the mechanism of how *S. officinalis* inhibits biofilm formation was investigated by using real-time reverse transcription (RT) PCR. The results indicated that *S. officinalis* inhibits the biofilm formation of MRSA in an *ica*-dependent manner.

MATERIALS AND METHODS

Bacterial Strain and Growth Condition

Staphylococcus aureus MRSA strain SA3 used in our study were isolated from a dairy cow with mastitis. Strain SA3 is a *mecA*-positive multidrug-resistant strain. The antibiotic sensitivity assays showed that strain SA3 is sensitive to chloramphenicol and vancomycin, and resistant to ampicillin, erythromycin, and oxacillin. Strains were grown at 37°C in tryptic soy broth (TSB) containing 0.25% glucose medium (Oxoid, Basingstoke, UK).

Ethanol Extraction of *S. officinalis*

The ethanol extract of *S. officinalis* was prepared followed with the method described previously (Yu et al., 2011). *Sanguisorbae radix*, the root of *S. officinalis*, was dried at 50°C and crushed into a powder (0.3–0.5 cm × 0.1–0.3 cm) with a blender, and the crude powder was extracted with ethanol at 70°C for 3 h. Subsequently, the ethanol extracts were evaporated at 60°C under pressure, the powder was dissolved in 95% ethanol to make a stock solution (50 mg/mL), and the solution was filtered by a syringe filter with 0.22-μm pores.

Antimicrobial Activity Assay

Colonies of MRSA strain SA3 were placed into 2 mL of TSB medium and cultivated at 37°C with shaking

at 200 rpm for 16 h. Then, the overnight cultures were inoculated into fresh TSB medium and diluted to a final concentration of 0.05 by optical density at 600 nm before being dispensed into 96-well plates (Costar, Corning, Steuben, NY) containing serial dilutions of ethanol extracts of *S. officinalis*. Plates were incubated at 37°C for 12 h and then 10-fold serial dilutions of cultures were performed by successive transfer (0.1 mL) through 7 microfuge tubes containing 0.9 mL of TSB. Next, 100-μL dilutions were dropped on Luria-Bertani agar plates and viable colonies were counted via their colony-forming units on TSB agar plates after incubated at 37°C for 24 h. The survival rate of the control group without exposure to *S. officinalis* was designated as 100%. Colony-forming units of the test groups were all compared with that of the control group. Experiments were repeated 3 times with 4 parallels.

Biofilm Assays

The method for biofilm quantification was performed as described previously and modified as described herein (Mack et al., 2000; Sadovskaya et al., 2005; Yu et al., 2012). We grew MRSA strain SA3 in TSB for 16 h and diluted it 1:100 into fresh TSB. The diluted cultures were transferred into sterile 96-well flat-bottomed tissue culture plates and incubated at 37°C for 24 h. Ethanol extracts of *S. officinalis* were added to the TSB medium with diluted cultures at different concentrations. The adherent bacteria were stained with crystal violet, and the excess stain was washed off gently with slowly running water. The biomass of the biofilm was determined using a MicroELISA auto-reader (Bio-Rad, Hercules, CA) at a wavelength of 560 nm under single-wavelength mode (Ziebuhr et al., 1997; Pozzi et al., 2012). Laser scanning confocal microscopy was performed on a Zeiss LSM710 system (Carl Zeiss GmbH, Jena, Germany) with a 20 × 0.8 numerical aperture apochromatic objective. The strains were stained with acridine orange for fluorescence detection. The laser wavelength used for the CLSM was 488 nm; Z-stacks were collected at 1-μm intervals. Confocal parameters for wild-type biofilm detection were taken as standard settings. Each confocal experiment was repeated 4 times. The 3-dimensional biofilm images were rendered with Imaris 7.0 (Bitplane, Zurich, Switzerland).

Total RNA Isolation, cDNA Generation, and Real-Time PCR Processing

Overnight cultures of MRSA strain SA3 were diluted 1:100 in TSB containing 0.5% glucose and grown to the late exponential phase in 24-well plates (Costar). Cells were collected and resuspended in Tris-EDTA

Table 1. Oligonucleotide primers used in this study

Primer name	Oligonucleotide (5'-3')
rt-icaA-f	TTTCGGGTGTCTTCACTCTAT
rt-icaA-r	CGTAGTAATACTTCGTGTCCC
rt-icaB-f	CCTATCCTTATGGCTTGATGA
rt-icaB-r	CATTGGAGTTCGGAGTGA
rt-icaC-f	TACTGACAACCTTGAATTACCA
rt-icaC-r	AATAGCCATACCATTGACCTAA
rt-icaD-f	CCAGACAGAGGGAATACC
rt-icaD-r	AAGACACAAGATATAGCGATAAG
rt-icaR-f	TTATCTAATACGCTGAGGAAT
rt-icaR-r	GGATGCTTTCAAATACCAACT

buffer (pH 8.0) containing 10 g/L of lysozyme and 40 mg/L of lysostaphin. After incubation at 37°C for 5 min, cells were prepared for total RNA extraction using the Trizol method (Invitrogen, Waltham, MA), and residual DNA was removed with DNase (RNase free; TaKaRa, Shiga, Japan). Real-time RT-PCR was performed with a PrimeScript 1st Strand cDNA synthesis kit and SYBR Premix Ex Taq (TaKaRa) using a StepOne real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). The quantity of cDNA measured by real-time PCR was normalized to the abundance of 16S cDNA (Chen et al., 2000). All of the real-time RT-PCR assays were repeated at least 3 times with similar results. The primers used in our study are listed in Table 1.

Statistical Analysis

The data were analyzed using the statistical software SPSS (ver. 19.0, IBM Corp., Armonk, NY) by a one-way ANOVA method; the test results were shown as mean $\pm$ SD. The paired *t*-test was used for statistical comparisons between groups. The level of statistical significance was set at a *P*-value of ≤ 0.01 .

RESULTS

Inhibitory Effect of Ethanol Extract of *S. officinalis* on *Staph. aureus*

To examine the antibacterial activity of the ethanol extract of *S. officinalis* against *Staph. aureus* in vitro, the antibacterial assays were performed. After exposure to the extract of *S. officinalis* at different concentrations for 12 h at 37°C, the cells of MRSA strain SA3 were inoculated into fresh TSB and then spread onto the TSB agar plates. After cultivating for 24 h at 37°C, the colony-forming units of the bacteria were counted and compared. As shown in Figure 1A, the survival rate of the control group without exposure to *S. officinalis* was designated as 100%. With the increase

Figure 1. (A) Colony-forming unit assays of methicillin-resistant *Staphylococcus aureus* (MRSA) strain SA3. Colony counts of SA3 following 12 h of incubation at 37°C with or without ethanol extract of *Sanguisorba officinalis* were compared. The survival rate of the control group without exposure to *S. officinalis* was designated 100%. The colony counts of the test group cultured with *S. officinalis* at different concentrations were all compared with that of the control group. An asterisk (*) represents means significantly different from the control group with no ethanol extract of *S. officinalis* (*P* < 0.05). (B) The growth curves of MRSA strain SA3 cultured in tryptic soy broth medium with or without ethanol extract of *S. officinalis*. The results represent a mean of 3 independent experiments. Ctrl = a negative control in which the ethanol extract of *S. officinalis* was substituted by equal volume of ethanol.

in the concentration of *S. officinalis* L., the survival rate of MRSA strain SA3 decreased accordingly. When the concentration of *S. officinalis* reached 10 mg/mL, the survival rate of cells dropped to 10%. These data indicated that *S. officinalis* has antibacterial activity against *Staph. aureus*.

Furthermore, the growth rates of the cells were tested when they were grown in TSB medium with *S. officinalis* at different concentrations. The results showed that the growth rates of the bacteria decreased to a small extent when the external concentration of *S. officinalis* was 2.5 mg/L compared with the wild type without the addition of *S. officinalis* L.; when the concentration of *S. officinalis* was 10 mg/mL, the growth of the bacteria

was remarkably inhibited (Figure 1B). These data indicated that only at high concentrations can *S. officinalis* show considerable antibacterial activity against *Staph. aureus*.

Inhibitory Effect of the Ethanol Extract of *S. officinalis* on Biofilm Formation of *Staph. aureus*

To examine whether the ethanol extract of *S. officinalis* affects biofilm formation of *Staph. aureus*, we performed biofilm assays. The cells of MRSA strain SA3 were grown in TSB for 16 h and diluted 1:100 in fresh TSB. The ethanol extract of *S. officinalis* was added to the TSB medium to final concentrations of 0, 1, 2.5, 5, and 7.5 mg/mL. Cultures were transferred to sterile 96-well flat-bottomed tissue culture plates and incubated for 16 h at 37°C. After crystal violet staining, we observed biofilm biomass in the 96-well plates. As shown in Figure 2A, strains of the control group without ethanol extract of *S. officinalis* formed obvious biofilms, whereas with the increase of the concentration of *S. officinalis* biofilm formation of the bacteria significantly decreased, and when the concentration of *S. officinalis* reached 5 mg/mL, no biofilm was observed. In addition, the quantity of biofilm formation was further tested using a MicroELISA autoreader (Thermo Scientific, Pittsburgh, PA). We found that biofilm quantity decreased with the increase of *S. officinalis* L concentration (Figure 2A). To directly observe the effect of ethanol extract of *S. officinalis* on biofilm formation of *Staph. aureus*, we used the CLSM system and the strains were stained with acridine orange for fluorescence detection. After 12 h of incubation at 37°C in TSB containing 0.5% glucose, strains of the control group without ethanol extract of *S. officinalis* produced intact and rough biofilms, as monitored by CLSM (Figure 2B). In contrast, biofilms produced by strains of the experimental group with 2.5 mg/mL of ethanol extract of *S. officinalis* were nearly undetectable (Figure 2B). These data revealed an interesting phenomenon that although the antibacterial activity of *S. officinalis* at a low concentration is not obvious, the effect of that on biofilm formation is significant.

Ethanol Extract of *S. officinalis* Significantly Inhibited MRSA Biofilm Formation by Decreasing Transcription of the *icaADBC* Operon

To investigate the mechanism of how *S. officinalis* regulates biofilm formation in MRSA strain SA3, the transcript levels of biofilm-associated genes were tested by performing real-time RT-PCR experiments. We examined the transcript levels of the *icaADBC* operon, which encodes the polysaccharide intercellular adhesin

Figure 2. Effect of *Sanguisorba officinalis* on biofilm formation in methicillin-resistant *Staphylococcus aureus* (MRSA) strain SA3. Biofilm formation of SA3 cultured in tryptic soy broth containing 0.5% glucose in 96-well plates for 12 h at 37°C with or without ethanol extract of *S. officinalis* (A, a) Photographs of the 96-well plates after staining with crystal violet; (b) cells that adhered to the plate after staining with crystal violet were measured by optical density at 560 nm. (B) Biofilm integrity and fluorescence were monitored by CLSM: (a) control group with no ethanol extract of *S. officinalis* L., and (b) the external concentration of *S. officinalis* is 2.5 mg/mL. Biofilm integrity and green fluorescent protein (GFP) fluorescence were monitored by confocal laser scanning microscope and the 3-dimensional images were rendered with Imaris 7.0 (Bitplane, Zurich, Switzerland). The results represent a mean of 3 independent experiments. Double asterisks (**) means significantly different from the control group with no ethanol extract of *S. officinalis* ($P < 0.01$).

(PIA) synthetases. The cells of MRSA strain SA3 were cultivated for 16 h in TSB with *S. officinalis* at concentrations of 0, 1, and 3 mg/mL. Subsequently, the cells were harvested and the transcript levels of the *ica* operon were tested. As shown in Figure 3A, the transcript levels of *icaA*, *icaB*, *icaC*, and *icaD* were significantly decreased upon the addition of *S. officinalis* L., and with the increase of *S. officinalis* concentration the inhibitory effect of *S. officinalis* on the *ica* operon was stronger, indicating that *S. officinalis* affects the transcription of the *ica* operon in a concentration-dependent manner. To further investigate how *S. officinalis* regulates the *ica* operon, we examined the transcript level of *icaR*, which has been identified as the repressor of the *ica* operon. Results showed that the transcript level of *icaR* increased by adding *S. officinalis* in the culture medium in *Staph. aureus*. (Figure 3B), confirming that *S. officinalis* influences the *ica* operon through the transcriptional regulator *icaR*.

DISCUSSION

Bacterial biofilms have become a major impediment to the cure of many chronic human infections, which are characterized by prolonged inflammation and respond poorly to conventional treatments (Secor et al., 2011). The resistance to antimicrobial agents among bacteria growing in biofilms is many times higher than that of their planktonic counterparts (Yu et al., 2011). The insensitivity of biofilm bacteria to antibiotics is due to the presence of large amounts of exopolysaccharides, the expression of biofilm-specific resistance genes, and suitable conditions for growing slowly (Rezaei et al., 2013). Methicillin-resistant *Staphylococcus aureus* is an important nosocomial pathogen that shows resistance to many antibiotics and has emerged as a serious threat to public health worldwide. On the other hand, biofilm formation ability makes MRSA exhibit stronger resistance to antibiotic agents, thus increasing difficulties in eradication of the pathogen from the host. In the current study, we found that the ethanol extract of *S. officinalis* inhibited biofilm formation ability of a MRSA strain. This indicates that, with the combined use of antibiotics and this medicine, the dose of antibiotic used in the treatment can be minimized. Our study provides new clues to the effective therapy for chronic infections caused by MRSA.

Sanguisorba officinalis is one kind of common Chinese medicine. Previous studies on *S. officinalis* have seldom reported its inhibitory effect on MRSA. In the present study, we found that *S. officinalis* remarkably inhibited the growth of MRSA at a high concentration (>7.5 mg/mL). Interestingly, we found that at a low concentration (<2.5 mg/mL), although *S. officinalis*

only slightly inhibited the growth of the bacteria, the inhibitory effect of *S. officinalis* on biofilm formation in the MRSA strain was obvious. For example, the growth curve of the experimental group was slightly changed with the addition of *S. officinalis* at a concentration of 2.5 mg/mL compared with the control group without the addition of *S. officinalis* L.; however, at the same concentration, the MRSA strain almost lost the ability of biofilm formation.

Furthermore, based on detailed investigation into this interesting phenomenon, we found that the addition of *S. officinalis* at a low concentration can increase

Figure 3. Comparative measurement of transcription of the biofilm-associated genes and their regulators. Relative transcript levels of *icaA*, *icaB*, *icaC*, and *icaD* and their regulatory gene *icaR* were determined by real-time reverse transcription PCR in methicillin-resistant *Staphylococcus aureus* (MRSA) strain SA3 cultured with or without ethanol extract of ethanol extract of *Sanguisorba officinalis*. Error bars indicate standard deviations. The results represent a mean of 3 independent experiments. Double asterisks (**) represent means significantly different from control group with no ethanol extract of *S. officinalis* ($P < 0.01$).

the transcript level of *icaR*, thus leading to the strong inhibitory effect on the transcription of the *icaADBC* operon, which encodes the polysaccharide intercellular adhesin synthetases. These data provide an explanation of how *S. officinalis* strongly inhibits the biofilm formation of MRSA only at low concentrations.

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