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# Eugenol Reduces the Expression of Virulence-Related Exoproteins in *Staphylococcus aureus*[▽]

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**Eugenol, an essential oil component in plants, has been demonstrated to possess activity against both Gram-positive and Gram-negative bacteria. This study examined the influence that subinhibitory concentrations of eugenol may have on the expression of the major exotoxins produced by *Staphylococcus aureus*. The results from a tumor necrosis factor (TNF) release assay and a hemolysin assay indicated that *S. aureus* cultured with graded subinhibitory concentrations of eugenol (16 to 128 μg/ml) dose dependently decreased the TNF-inducing and hemolytic activities of culture supernatants. Western blot analysis showed that eugenol significantly reduced the production of staphylococcal enterotoxin A (SEA), SEB, and toxic shock syndrome toxin 1 (the key exotoxins to induce TNF release), as well as the expression of α-hemolysin (the major hemolysin to cause hemolysis). In addition, this suppression was also evaluated at the transcriptional level via real-time reverse transcription (RT)-PCR analysis. The transcriptional analysis indicated that 128 μg/ml of eugenol remarkably repressed the transcription of the *S. aureus sea*, *seb*, *tst*, and *hla* genes. According to these results, eugenol has the potential to be rationally applied on food products as a novel food antimicrobial agent both to inhibit the growth of bacteria and to suppress the production of exotoxins by *S. aureus*.**

*Staphylococcus aureus* is a major nosocomial pathogen. This species is capable of causing a wide range of diseases and is often associated with high morbidity and mortality rates. The disease spectrum includes food-borne illness, cutaneous infections, endocarditis, pneumonia, septic arthritis, and osteomyelitis (24, 36). The diversity is, in part, dependent on the secretion of a broad spectrum of soluble extracellular proteins. These proteins include enterotoxins, hemolysins, toxic shock syndrome toxin 1 (TSST-1), and others (20). *S. aureus* infections are difficult to control due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. Therefore, there is a continuing need to discover new and improved antimicrobial agents to treat *S. aureus* illnesses, with potential benefits for both the food and pharmaceutical industries (35).

Enterotoxins, TSST-1, and α-hemolysin are among the major exotoxins secreted by *S. aureus*. Staphylococcal enterotoxins (SEs) are a group of major serological heat-stable enterotoxins (SEA to SEE and SEG to SEJ) (3). These toxins cause staphylococcal gastroenteritis, food poisoning, toxic shock-like syndromes, and several allergic and autoimmune diseases (3, 22, 35). TSST-1 is the key causative toxin of toxic shock syndrome (TSS), which is an acute-onset and potentially life-threatening staphylococcal syndrome (23). Furthermore, SEs and TSST-1 are also known to be pyrogenic toxin superantigens (PTSAgs) that stimulate the proliferation of T lymphocytes and the release of T-cell-derived cytokines (12). α-Hemolysin is a 33-kDa pore-forming soluble protein that has hemolytic, cytolytic, and dermonecrotic activities. Several types of human cells are affected by α-hemolysin, including erythrocytes, lymphocytes, monocytes, macrophages, and epithelial cells. Similar to the majority of proteins secreted by *S. aureus*, SEs, TSST-1, and α-hemolysin are produced primarily during the postexponential phase of growth and their expression levels are regulated by a network of interacting regulators. These include the SarA protein family (30) and many two-component regulatory systems, such as the *agr* (31) and *sae* (15) systems. The *agr* two-component system is one of the best-studied regulatory loci. Expression of *agr* is activated when bacterial cultures enter the postexponential growth phase in a cell density-dependent manner (34). Induction of *agr* expression results in RNAIII production, which leads to a reduced production of several surface proteins and facilitates the production of numerous extracellular proteins (29).

Eugenol (4-allyl-2-methoxyphenol) is a major component of clove oil and used primarily as a flavoring agent in food and cosmetic products. Studies have shown that eugenol possesses various biological abilities, including antimicrobial, antioxidant, anti-inflammatory, anticarminative, and antispasmodic activities (14, 16, 25, 27). It has long been known that certain antibiotics can influence the expression of staphylococcal exotoxins. Therefore, the goal of this study was to assess the impact of subinhibitory eugenol concentrations on the production of TSST-1, the major enterotoxins, i.e., SEA and SEB, and alpha-hemolysin by methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA).

## MATERIALS AND METHODS

**Bacterial strains and reagents.** MSSA strain ATCC 29213 was obtained from the American Type Culture Collection (ATCC). In total, 25 *S. aureus* isolates (8 MSSA and 17 MRSA) were acquired from clinical samples at the First Hospital of Jilin University in Changchun, China. Among these, clinical MRSA strains 2985 and 3701, which have the ability to produce TSST-1, α-hemolysin, SEA, and

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SEB, were chosen for further experimentation. Mueller-Hinton broth (MHB) was purchased from BD Biosciences, Inc. (Sparks, MD), and eugenol was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

**MIC determination.** MICs of eugenol for *S. aureus* were assessed in triplicate using the microdilution method, as recommended by the Clinical and Laboratory Standards Institute (11). MICs were defined as the lowest drug concentrations that inhibited growth.

**Growth curves.** Bacteria were cultured at 37°C to an optical density at 600 nm ($OD_{600}$) of 0.3 in MHB, and 100-ml volumes of the respective cultures were aliquoted into six 250-ml Erlenmeyer flasks. Five of the cultures were supplemented with eugenol (dissolved in DMSO) at concentrations of 1/16× MIC (16 μg/ml), 1/8× MIC (32 μg/ml), 1/4× MIC (64 μg/ml), 1/2× MIC (128 μg/ml), and 1× MIC (256 μg/ml). The final DMSO concentration for all cultures was 1% (vol/vol). The control culture contained only 1% DMSO. Following the addition of eugenol (or DMSO), bacteria were grown at 37°C with constant shaking under aerobic conditions. Cell growth was monitored by measuring the $OD_{600}$ at 30-min intervals. Bacterial cultures with various concentrations of eugenol were grown to an $OD_{600}$ of 2.5, and serial 10-fold dilutions of the samples were spread onto drug-free Mueller-Hinton agar plates. The number of colonies was determined after an incubation period of 24 h at 37°C.

**TNF release assay.** The tumor necrosis factor (TNF) release assay was performed according to a slightly modified method described previously by Bernardo et al. (5). Overnight cultures of MSSA ATCC 29213, MRSA 2985, and MRSA 3701 that were grown in RPMI 1640 (Invitrogen, CA) were diluted 30-fold into 500 ml of prewarmed RPMI 1640. Following incubation at 37°C for 30 min with aeration, cultures were divided into 100-ml aliquots. Increasing concentrations of eugenol (1/16×, 1/8×, 1/4×, and 1/2× MIC) were added to the bacterial suspensions. Following the addition of eugenol, cultures were further incubated for 4 h. The final DMSO concentration for all cultures was 1% (vol/vol). The eugenol-free culture was treated with 1% DMSO alone. *S. aureus* supernatants without eugenol treatment served as control samples. All supernatants collected were filtered via a 0.2-μm filter and immediately analyzed as described below.

Animal experiments were conducted in accordance with the experimental practices and standards approved by the Jilin University Animal Welfare and Research Ethics Committee. Specific-pathogen-free BALB/c mice (male, 6 to 8 weeks old, weighing 18 to 22 g) were supplied by the Jilin University Experimental Animal Center (Changchun, China). Mice were euthanized by cervical dislocation. Single-spleen-cell suspensions were prepared in RPMI 1640 and washed and resuspended in complete RPMI 1640 (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, 15 mM HEPES, and 50 μM 2-mercaptoethanol). A total of $10^6$ (150 μl) cells were seeded into 96-well tissue culture plates. These wells were then supplemented with 50 μl of *S. aureus* culture supernatants. After incubation for 16 h, the supernatants were collected and centrifuged (1,000 × *g* for 5 min). The TNF levels from the supernatants were analyzed using the mouse TNF-α ELISA Max set standard (Biolegend, Inc., San Diego, CA), in accordance with the manufacturer's instructions.

**Hemolysin assay.** Bacteria were cultured in MHB at 37°C with graded subinhibitory concentrations of eugenol until the postexponential growth phase was reached ($OD_{600}$ = 2.5, equivalent to 1.0 × $10^9$ CFU/ml). Hemolytic activities of bacterial culture supernatants were assessed according to the method of Rowe and Welch (33). Bacterial cultures were centrifuged (5,500 × *g*, 4°C, 1 min), the supernatant was collected, and the residual cells were removed using a 0.2-μm filter. Prior to the addition of 25 μl of defibrinated rabbit blood, a 0.1-ml volume of culture supernatant was brought up to a volume of 1 ml through the addition of phosphate-buffered saline (PBS) buffer. After incubation for 15 min at 37°C, the unlysed blood cells were pelleted by centrifugation (5,500 × *g*, room temperature, 1 min). The hemolytic activity of the supernatant was detected by measuring the optical density at 543 nm. The control culture supernatant served as the 100% hemolysis control, and the percent hemolysis was calculated by comparison with the control culture.

**Western blot analysis.** *S. aureus* strains were cultured in a method similar to that described above, which allowed the supernatant samples to be collected in the same manner as that used for the hemolysin assay. A 25-μl volume of culture supernatant was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel after boiling in Laemmli sample buffer (21). The Western blot protocol was performed as described by Qiu et al. (32) and in the product guide for Amersham ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, United Kingdom). Antibodies to SEA, SEB, and α-hemolysin were purchased from Sigma-Aldrich and diluted to 1:10,000, 1:5,000 and 1:8,000, respectively; then, a horseradish peroxidase-conjugated anti-rabbit antiserum (Sigma-Aldrich) diluted to 1:4,000 was used as the secondary antibody. The antibody to TSST-1 (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted to 1:200 according to the manufacturer's recommendations, and then a horseradish peroxidase-conjugated anti-mouse antiserum (Sigma-Aldrich) diluted to 1:5,000 was used as the secondary antibody.

TABLE 1. Primers used for real-time RT-PCR

| Primer | Sequence | Location within gene (nucleotide positions) |
|---|---|---|
| 16S rRNA-fw | 5′-GCTGCCCTTTGTATTGTC-3′ | 287–305 |
| 16S rRNA-rv | 5′-AGATGTTGGGTTAAGTCCC-3′ | 446–465 |
| *tst*-fw | 5′-ACCCCTGTTCCCTTATCATC-3′ | 73–93 |
| *tst*-rv | 5′-AAAAGCGTCAGACCCACTAC-3′ | 159–180 |
| *sea*-fw | 5′-ATGGTGCTTATTATGGTTATC-3′ | 335–356 |
| *sea*-rv | 5′-CGTTTCCAAAGGTACTGTATT-3′ | 477–498 |
| *seb*-fw | 5′-TGTTCGGGTATTTGAAGATGG-3′ | 480–501 |
| *seb*-rv | 5′-CGTTTCATAAGGCGAGTTGTT-3′ | 612–633 |
| *hla* -fw | 5′-TTGGTGCAAATGTTTC-3′ | 485–501 |
| *hla*-rv | 5′-TCACTTTCCAGCCTACT-3′ | 569–586 |
| *agrA*-fw | 5′-TGATAATCCTTATGAGGTGCTT-3′ | 111–133 |
| *agrA*-rv | 5′-CACTGTGACTCGTAACGAAAA-3′ | 253–274 |

**Real-time RT-PCR.** Strain ATCC 29213 was grown to the postexponential growth phase ($t$ = 240 min) in MHB at 37°C either without eugenol or with 128 μg/ml of eugenol. RNA was prepared as described by Qiu et al. (32). Briefly, cells were harvested by centrifugation (5,000 × *g* for 5 min at 4°C) and resuspended into TES buffer (10 mM Tris-Cl, 1 mM EDTA, 0.5% SDS) containing 100 μg/ml of lysostaphin (Sigma-Aldrich). Following incubation at 37°C for 10 min, a Qiagen RNeasy Maxi column was used to isolate total bacterial RNA, which was in accordance with the manufacturer's instructions. The contaminating DNA was removed using the optional on-column RNase-free DNase I step (Qiagen, Hilden, Germany). RNA concentrations were detected from the $OD_{260}$, and the RNA was loaded onto an RNase-free 2% agarose gel to test for generalized degradation. The primer pairs used in real-time reverse transcription (RT)-PCR are listed in Table 1. RNA was reverse transcribed into cDNA using the Takara RNA PCR kit (AMV) version 3.0 (Takara, Kyoto, Japan), according to the manufacturer's protocol. The resulting cDNA was stored at −20°C until it was required. The PCRs were carried out in a 25-μl volume and contained SYBR Premix Ex Taq (Takara), as recommended by the manufacturer. The reactions were performed using the model 7000 sequence detection system (Applied Biosystems, Courtaboeuf, France). Cycling conditions were as follows: 95°C for 30 s; 40 cycles at 95°C for 5 s, 55°C for 30 s, and 72°C for 40 s; and one dissociation step of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. All samples were analyzed in triplicate, and the 16S rRNA housekeeping gene served as an internal control to normalize the expressional levels between samples. The relative expression levels were analyzed by the $\Delta\Delta CT$ method that is described in the Applied Biosystems user bulletin no. 2 (1).

**Statistical analysis.** SPSS 12.0 statistical software was applied to analyze the experimental data. The data are presented as the mean values ± standard deviations (SD). An independent Student's $t$ test was used to determine statistical differences, and a $P$ value of $<0.05$ was considered to be statistically significant.

## RESULTS

**Influence of eugenol on *S. aureus* growth.** The eugenol MIC for each of the 26 *S. aureus* strains examined was assessed. The values obtained ranged from 128 to 512 μg/ml. The MIC values of eugenol against *S. aureus* ATCC 29213, MRSA 2985, and MRSA 3701 were 256 μg/ml. These data indicate that the eugenol structure could be used as a basic structure for the development of novel and more potent drugs to treat *S. aureus* infections.

To examine the effect of subinhibitory eugenol concentrations on the production of bacterial virulence factors, we ini-

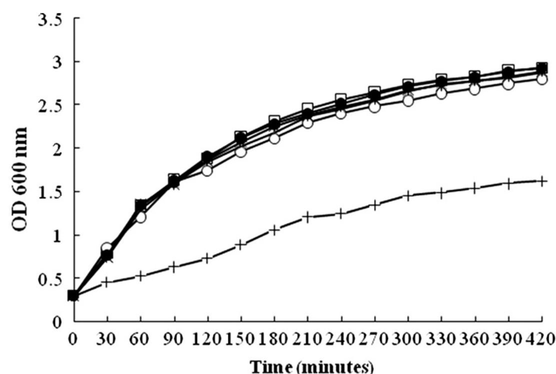


FIG. 1. Growth curves for *S. aureus* ATCC 29213 cultured in MHB with various concentrations of eugenol. Symbols: □, eugenol-free *S. aureus*; ●, *S. aureus* supplemented with 16 μg/ml eugenol; *, *S. aureus* supplemented with 32 μg/ml eugenol; △, *S. aureus* supplemented with 64 μg/ml eugenol; ○, *S. aureus* supplemented with 128 μg/ml eugenol; +, *S. aureus* supplemented with 256 μg/ml eugenol (1× MIC). These curves represent the results of one of three reproducible experiments.

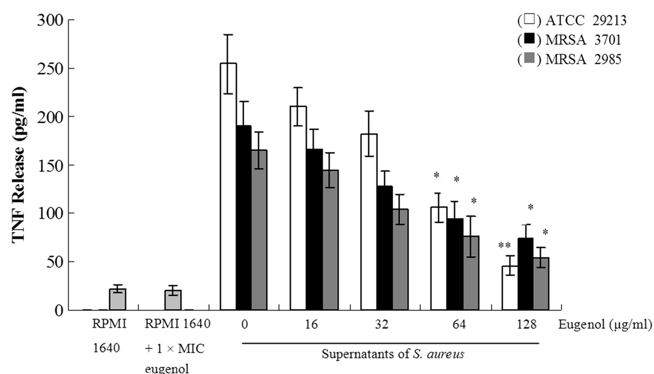


FIG. 2. Release of TNF-α from splenocytes that were stimulated with RPMI 1640-grown *S. aureus* culture supernatants in the presence of graded subinhibitory eugenol concentrations. Values are averages for three independent experiments. The error bars represent ±1 standard deviation. *, $P < 0.05$; **, $P < 0.01$.

tially investigated the influence of graded sublethal concentrations of eugenol on the growth of *S. aureus*. To do this, a growth curve assay was performed. As shown in Fig. 1, eugenol concentrations from 1/16× MIC to 1/2× MIC had no significant effects on the growth of *S. aureus*. However, when cultured with 1× MIC of eugenol, the growth rate was significantly decreased: after 30, 210, and 420 min of eugenol treatment, the $OD_{600}$ values of *S. aureus* ATCC 29213 cultured in MHB were 58.1%, 49.2%, and 55.6%, respectively, those of cultures receiving no treatment or lower concentrations of eugenol. Although the growth kinetics can vary greatly between strains, the growth of MRSA 2985 and MRSA 3701 were affected by these concentrations of eugenol in a similar manner. In other words, MRSA 2985 and MRSA 3701 supplemented with 1/16×, 1/8×, 1/4×, and 1/2× MIC of eugenol had no significant influence on growth (data not shown).

In addition, we further investigated the relationship between the OD values and number of CFU. The results obtained indicate that the levels of bacteria in the control culture and the eugenol-treated (1/16×, 1/8×, 1/4×, and 1/2× MIC) cultures were approximately $1.0 \times 10^9$ CFU/ml when grown to an $OD_{600}$ value of 2.5.

**Eugenol represses TNF-inducing and hemolytic activities of *S. aureus* culture supernatants.** It is well known that secreted proteins in *S. aureus* have the ability to stimulate T lymphocytes to release proinflammatory cytokines (e.g., TNF-α), while hemolysins produced by *S. aureus* cause hemolysis of rabbit erythrocytes. Therefore, TNF release and hemolysin assays were carried out to investigate the influence of eugenol on the TNF-inducing and hemolytic activities of *S. aureus* culture supernatants.

As shown in Fig. 2, the culture supernatants of *S. aureus* grown in the presence of graded subinhibitory concentrations of eugenol elicited much lower levels of spleen cell TNF-α production. A 1× MIC of eugenol dissolved in RPMI 1640 alone did not directly induce or reduce TNF release at (data not shown). Of importance, eugenol diminished the TNF-inducing activity of *S. aureus* supernatants in a dose-dependent manner.

When *S. aureus* strains ATCC 29213, MRSA 2985, and MRSA 3701 were treated with 1/16× MIC of eugenol, the hemolysis of culture supernatants from those strains were 41.8%, 51.5%, and 60.4% of their control cultures, respectively. Remarkably, no hemolytic activities were detected when these strains were cultured with eugenol at concentrations of 1/4× MIC or 1/2× MIC (Table 2). This dose-dependent hemolysis reduction was observed in all of the investigated strains. Furthermore, treatment with eugenol at 1× MIC or 2× MIC did not directly cause or inhibit hemolysis of rabbit erythrocytes. Additionally, there was little influence on the hemolytic activity of culture supernatants when preincubated with a 2× MIC of eugenol (data not shown).

**Eugenol represses TSST-1, SEA, SEB, and α-hemolysin levels in *S. aureus* culture supernatants.** Among *S. aureus* secreted proteins, TSST-1 and enterotoxins are the most important exoproteins that could act as superantigens, stimulating release of T-cell-derived cytokines by spleen cells. Additionally, α-hemolysin is the major hemolysin that is responsible for the hemolytic activity of *S. aureus* culture supernatants. To determine whether the reduced TNF-inducing and hemolytic activities of *S. aureus* culture supernatants in the presence of various subinhibitory concentrations of eugenol were due to the diminished production of TSST-1, SEA, SEB (i.e., the major enterotoxins of *S. aureus*), and α-hemolysin, the culture supernatants were subjected to Western blot analysis. *S. aureus*

TABLE 2. Hemolytic activities of *S. aureus* culture supernatants treated with graded subinhibitory concentrations of eugenol

| Strain | % hemolysis of rabbit erythrocytes by culture supernatant with eugenol at[a]: | | | | |
|---|---|---|---|---|---|
| | 0 | 1/16× MIC (16 μg/ml) | 1/8× MIC (32 μg/ml) | 1/4× MIC (64 μg/ml) | 1/2× MIC (128 μg/ml) |
| ATCC 29213 | 100 | 41.8 ± 5.8* | No | No | No |
| MRSA 2985 | 100 | 51.5 ± 4.9* | 10.5 ± 4.2** | No | No |
| MRSA 3701 | 100 | 60.4 ± 6.3 | 25.3 ± 3.8** | No | No |

[a] The drug-free culture supernatants served as the 100% hemolysis control. No, no observed hemolytic activity. Values are the means ± standard errors of results of three independent experiments. *, $P < 0.05$; **, $P < 0.01$, compared to the results for the corresponding control.

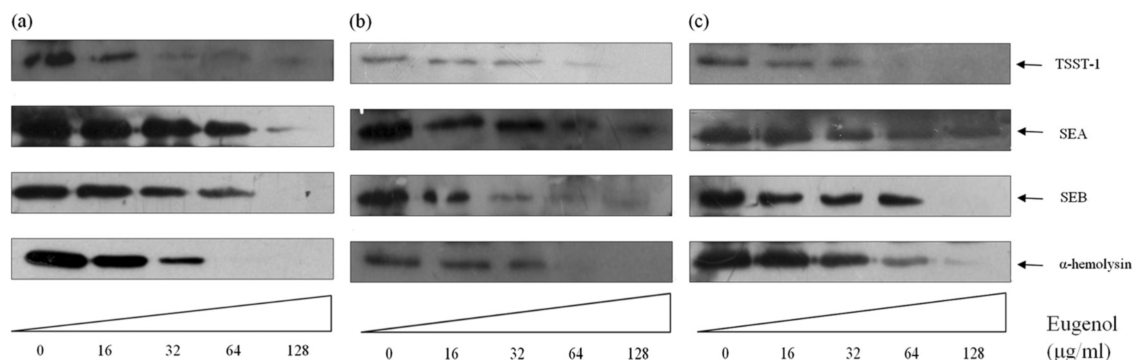


FIG. 3. Western blot analysis of TSST-1, SEA, SEB, and α-hemolysin produced by *S. aureus* ATCC 29213 (a), MRSA 2985 (b), and MRSA 3701 (c) grown with increasing concentrations of eugenol.

ATCC 29213, MRSA 2985, and MRSA 3701 were grown with graded subinhibitory concentrations of eugenol to the postexponential growth phase (an $OD_{600}$ value of 2.5). As shown in Fig. 3, treatment with eugenol resulted in a dose-dependent decrease in the production of TSST-1, SEA, SEB, and α-hemolysin.

**Eugenol represses the transcription of *S. aureus tst*, *sea*, *seb*, *hla*, and *agrA*.** As eugenol remarkably decreased the production of *S. aureus* TSST-1, SEA, SEB, and α-hemolysin, we therefore hypothesized that eugenol could affect the transcription of *tst*, *sea*, *seb*, and *hla*. Therefore, real-time RT-PCR was performed to detect the relative expression levels of *tst*, *sea*, *seb*, and *hla*. Additionally, the production of TSST-1, SEB, and α-hemolysin are positively regulated by the *agr* two-component system (30); therefore, the transcription level of *agrA* was also evaluated. As expected, the transcription levels of these genes were significantly decreased in strain ATCC 29213 upon treatment with eugenol (Table 3). When cultured with a 1/2× MIC of eugenol, the transcriptional levels of *tst*, *sea*, *seb*, *hla*, and *agrA* were decreased by 10.4-, 6.3-, 12.8, 15.6, and 5.5-fold, respectively.

## DISCUSSION

Similar to that of other Gram-positive bacteria, the pathogenicity of *S. aureus* is, to a great extent, dependent upon the secretion of numerous extracellular virulence factors. Consequently, the clinical performance of antibiotics for the treatment of *S. aureus* is determined not only by their respective bactericidal or bacteriostatic activities but also by their effects on the release of virulence factors (5). In addition, an alternative strategy for the treatment of *S. aureus* infections, one that is now gaining great interest, is targeting bacterial virulence. This alternative strategy provides promising opportunities to abate pathogenicity and its consequences without placing an immediate life-or-death pressure on the target bacterium (8).

Previous studies have shown that the use of many antibiotics at suboptimal concentrations, which have little or no influence on cell growth, could substantially affect the expression of bacterial exotoxins. This mode of suppression has been reported to include the production of proteins such as α- and γ-hemolysin, enterotoxins A and B, coagulase, and TSST-1 (5, 13, 17, 20). These antibiotics are recommended for the treatment of severe staphylococcal illnesses associated with the secretion of these toxins. In the present study, we have shown via transcriptional, expressional, and phenotypic analyses that subinhibitory concentrations of eugenol dose-dependently decrease the production of α-hemolysin, TSST-1, SEA, and SEB in both MSSA and MRSA. These data suggest that the structure of eugenol could be used as a fundamental structure for the development of antimicrobial agents aimed at the bacterial virulence factors.

In the food industry, essential oils (EOs) extracted from plants have been used primarily for flavoring and fragrance (4). It has long been known that EOs have antimicrobial activities, and recent studies have demonstrated that these properties are attributable to the presence of numerous substituted aromatic molecules. These molecules include eugenol, cinnamaldehyde, and carvacrol (18, 19, 26). Currently, there is a trend in food processing to avoid the application of chemical preservatives such as NaCl and nitrates. Thus, the use of bioactive compounds derived from EOs as alternative antimicrobial agents is garnering great interest (14, 37). Eugenol, the major phenolic compound and antimicrobial component of clove oil, has been registered by the European Commission for use as a flavoring ingredient in foodstuffs and is considered to pose no risk to consumer health (7). Eugenol has been reported to inhibit the growth of *Escherichia coli*, *Listeria monocytogenes*, and *S. aureus* (4, 6). In addition to antibacterial activities, eugenol also possesses antifungal and antiviral activities (2, 9). Because of its antimicrobial properties, eugenol has the potential to be used as a food antimicrobial agent.

Staphylococcal gastroenteritis and food poisoning do not result from the ingestion of *S. aureus* itself but rather from enterotoxins that are preformed within the food (22, 35). More

TABLE 3. Relative expression levels of *tst*, *sea*, *seb*, *hla*, and *agrA* in *S. aureus* ATCC 29213 after culture with eugenol at 1/2× MIC (128 μg/ml)

| Gene | Product | Fold change ± SD[a] |
|---|---|---|
| *tst* | Toxic shock syndrome toxin 1 | −10.4 ± 2.5 |
| *sea* | Enterotoxin A | −6.3 ± 1.5 |
| *seb* | Enterotoxin B | −12.8 ± 1.8 |
| *hla* | α-Hemolysin | −15.6 ± 2.2 |
| *agrA* | Accessory gene regulator A | −5.5 ± 0.8 |

[a] A minus sign indicates a reduction, and the SDs were calculated based on three independent experiments.

importantly, SEs are heat-stable enterotoxins that can resist high temperature without losing their biological activity (3). Therefore, apart from the inhibition of the growth of vegetative bacterial cells, food microbiologists are also interested in the inhibition of toxin expression (7). The findings in our study that eugenol could substantially inhibit the production of enterotoxins when used at sublethal concentrations without an effect on bacterial growth may increase the likelihood for eugenol to be used as a novel natural food preservative.

Indeed, many genes encoding virulence factors are coordinately regulated in response to a variety of intracellular and extracellular signals. It has been shown that subinhibitory concentrations of antibiotics may interfere with the translation of one or more regulatory gene products in *S. aureus*, which in turn affects transcription of exoprotein-encoding genes. For instance, the effect of subinhibitory concentrations of licochalcone A on α-hemolysin production may partially depend on the inhibition of the *agr* two-component system (32). Furthermore, subinhibitory concentrations of clindamycin differentially repress the transcription of *S. aureus* exoprotein genes and act partially through *sar* (17). Therefore, it is tempting to speculate that eugenol-induced inhibition of global regulators might lead to a decrease in the secretion of virulence-related exoproteins. To address this hypothesis, real-time RT-PCR was carried out to evaluate the effect of eugenol on the *S. aureus agr* locus. Our results indicate that *agrA* transcription was significantly reduced when *S. aureus* was cultured with a 1/2× MIC of eugenol to the postexponential growth phase. Nevertheless, the mechanisms by which *S. aureus* regulates virulence gene expression are extremely complicated. This regulation involves an interactive, hierarchical regulatory cascade among the *agr*, *sar*, and other regulatory gene products (10). Since the expression levels of TSST-1, SEB, and α-hemolysin are positively controlled by *agr* (1a, 28), we presume that the influence of subinhibitory concentrations of eugenol on the production of these toxins may depend, in part, on eugenol-induced inhibition of the *agr* two-component system. The *agr* locus has no effect on SEA expression (1a, 28). Therefore, it is clear that the effect of eugenol on SEA secretion cannot be mediated through interactions with *agr*. However, the action of regulatory genes other than *agr* on SEA production in *S. aureus* remains unclear. Consequently, the regulatory mechanisms involved in the eugenol-induced reduction of SEA remain to be determined.

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