Inhibition of staphyloxanthin biosynthesis in *Staphylococcus aureus* by rhodomyrtone, a novel antibiotic candidate

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Staphyloxanthin is the eponymous feature of the human pathogen *Staphylococcus aureus*, and the pigment promotes resistance to reactive oxygen species and host neutrophil-based killing. To probe the possible use of rhodomyrtone isolated from *Rhodomyrtus tomentosa* (Aiton) Hassk. leaves to inhibit pigment production in *S. aureus*, experiments were carried out to compare pigment production and the susceptibility of rhodomyrtone-treated *S. aureus* and untreated cells to oxidants *in vitro*. In addition, we observed the innate immune clearance of *S. aureus* after incubation with rhodomyrtone using an *ex vivo* assay system — human whole-blood survival. The results indicated that rhodomyrtone-treated *S. aureus* exhibited reduced pigmentation, and that rhodomyrtone treatment led to a dose-dependent increase in the susceptibility of the pathogen to *H*₂*O*₂ and singlet oxygen killing. Consequently, the survival ability of the treated organisms decreased in freshly isolated human whole blood due to less carotenoid pigment to act as an antioxidant scavenger. Rhodomyrtone may be acting via effects on DnaK and/or σ^B^, resulting in many additional effects on bacterial virulence.

INTRODUCTION

*Staphylococcus aureus* causes a wide spectrum of clinically significant hospital- and community-acquired infections in human, including skin and soft-tissue infections and life-threatening systemic infection (Daum, 2008; Pacheco et al., 2011; Karamatsu et al., 2012). The frequency of meticillin-resistant *S. aureus* (MRSA) infections has now been declared a public-health imperative (Okesola, 2011; Dhand & Sakoulas, 2012; Stefani et al., 2012). Of additional concern is the fact that these organisms are resistant to most available antibiotics, and therapeutic options for treatment and control of MRSA infections are very limited (Nuno et al., 2012).

An alternative strategy that is now gaining interest is inhibiting the synthesis of bacterial virulence factors that are essential for bacterial growth and/or survival in infected hosts (Wang et al., 2007; Escaich, 2008; Oh et al., 2010; Artini et al., 2011; Mitchell et al., 2012). Staphyloxanthin, a yellowish-orange carotenoid pigment, is one of the important virulence factors of *S. aureus* (Liu & Nizet, 2009). Lennette et al. (1985) reported that >90% of *S. aureus* isolates from human infections are pigmented. The carotenoid pigment of the pathogen provides integrity to its cell membrane (Mishra et al., 2011). Staphyloxanthin has been associated with enhancing bacterial survival in harsh environments and during infections (Giachino et al., 2001; Kahlon et al., 2010). The membrane pigment promotes resistance to reactive oxygen species (ROS) such as *O*₂⁻, *H*₂*O*₂ and *HOCl* generated by host neutrophils (Lang et al., 2000; Clauditz et al., 2006; Song et al., 2009b).

The postulated biosynthetic pathway for staphyloxanthin starts with a head-to-head condensation of two molecules of farnesyl diphasophate to form presqualene diphasophate and then dehydroxqualene (4,4'-diapophytoene), catalysed by the *S. aureus* dehydroxqualene synthase (CrtM) enzyme. The CrtN enzyme, 4,4'-diapophytoene desaturase, dehydrogenates dehydroxqualene to form the intermediate yellow pigment 4,4'-diaponeurosporene (Wieland et al., 1994; Raisig & Sandmann, 1999). Oxidation of the terminal methyl group of 4,4'-diaponeurosporene to form 4,4'-diaponeurosporenic acid is catalysed by the CrtP enzyme. CrtQ esterifies glucose at the C1' position with the carboxyl group of 4,4'-diaponeurosporenic acid to yield glycosyl-4,4'-diaponeurosporene. In the final step, the acyltransferase CrtO enzyme esterifies glucose at the C6' position with the carboxyl group of 12-methyltetradecanoic acid to give the acyl compound staphyloxanthin (Fig. 1) (Pelz et al., 2005).

The inhibition of pigment production may offer a potential novel target for treatment for complicated *S. aureus* infections. Inhibition of the CrtN enzyme by diphencylamine has been known for many years to result in colourless bacteria (Hammond & White, 1970a, b). Liu et al. (2008) reported recently that three phosphonosulfonates, including BPH-652, BPH-698 and BPH-700, were CrtM inhibitors. In addition,
Fig. 1. Biosynthetic pathways of staphyloxanthin (in *S. aureus*), cholesterol (in humans) and ergosterol (in yeasts and some parasitic protozoa). Each biosynthetic pathway involves initial formation of presqualene diphosphate, catalysed by CrtM (*S. aureus*) or by squalene synthase (SQS). In *S. aureus*, the NADPH reduction step is absent, resulting in the production of dehydrosqualene, rather than squalene.
some simple structural molecules of natural compounds have been studied that inhibit staphyloxanthin biosynthesis in the organism. Sesquiterpene farnesol, a natural plant metabolite, was reported to completely suppress staphyloxanthin production in <i>S. aureus</i> (Kuroda et al., 2007).

Rhodomyrtone [(6,8-dihydroxy-2,2,4,4-tetramethyl-7-(3-methyl-1-oxobutyl)-9-(2-methylpropyl)-4,9-dihydro-1H-xanthene-1,3(2H)-di-one] is a member of the acylphloroglucoins (Fig. 2). The compound was isolated from <i>Rhodomyrtus tomentosa</i> (Aiton) Hassk. leaves and exhibits pronounced antibacterial activity against a wide range of Gram-positive bacteria (Limsuwan & Voravuthikunchai, 2008; Saising et al., 2008; Limsuwan et al., 2009a; Voravuthikunchai et al., 2010; Saising et al., 2011). The MIC and minimum bactericidal concentration values of rhodomyrtone against MRSA range from 0.39 to 0.78 µg ml⁻¹, which is very close to those of vancomycin (Limsuwan et al., 2009b). Recently, protein profiling of rhodomyrtone-treated MRSA demonstrated alterations in the expression of several major functional classes of bacterial protein (Sianglum et al., 2011; Visuththi et al., 2011). Therefore, the objective of the present study was to investigate inhibition of pigment synthesis in <i>S. aureus</i> after treatment with rhodomyrtone.

**METHODS**

**Antimicrobial agents and chemicals.** The following commercially available compounds were purchased from the indicated manufacturers: Mueller–Hinton agar (MHA) and Mueller–Hinton broth (MHB) from Difco; tryptic soy broth from Bacto; DMSO, glycerol, H₂O₂ solution and methylene blue solution from Sigma-Aldrich; and ethanol, ethyl acetate, sodium chloride and anhydrous sodium sulfate from Fisher Scientific. All standard chemicals were of analytical grade. For rhodomyrtone purification, the active compound was isolated as described elsewhere by our research group (Hiranrat & Mahabusarakam, 2008; Limsuwan et al., 2009b). The purity of the compound was confirmed by reference to NMR and MS (Dachriyanus et al., 2002; Mohamed & Ibrahim, 2007).

**Tested bacterial strains and growth conditions.** <i>S. aureus</i> ATCC 29213 was used in this study. The pathogen was maintained in tryptic soy broth containing 20% glycerol at −80 °C until required. The organism was pre-cultured on MHA at 37 °C for 18 h. An inoculum of 100 µl culture was inoculated into 3 ml MHB and incubated at 37 °C until the mid-exponential growth phase was obtained.

![Fig. 2. Structure of rhodomyrtone.](http://jmm.sgmjournals.org)

**Effects of rhodomyrtone on staphyloxanthin synthesis**

**Qualitative assay.** <i>S. aureus</i> was inoculated into MHB and incubated at 37 °C for 3–5 h with shaking and the turbidity adjusted to McFarland standard number 4. An aliquot of the culture (18 ml) was added to 2 ml rhodomyrtone dissolved in 10% DMSO to give final concentrations ranging from 4 to 0.25 µg ml⁻¹. The treated micro-organisms were grown at 37 °C for 18 h. The cultures were centrifuged at 5000 r.p.m. for 10 min (Hettich Mikro 120 rotor) and the pellets were washed twice with normal saline solution. DMSO (1%) was used as a control. Experiments were carried out in triplicate.

**Quantitative assay.** Carotenoid extraction was performed as described elsewhere (Pelz et al., 2005). In brief, the washed bacterial cells were extracted by resuspending in ethanol and placed at 40 °C for 20 min. The resulting mixture was centrifuged at 5000 r.p.m. for 10 min (Hettich Mikro 120 rotor). The pellet was repeatedly extracted with the reagent until no visible pigment remained. The ethanolic extract was concentrated under reduced pressure. The crude pigment was shaken with ethyl acetate/1.7 M aqueous sodium chloride (1:1, v/v). The ethyl acetate layer was removed and the aqueous layer was rendered colourless by repeated extraction with ethyl acetate. The organic phase was washed with distilled water, dried over anhydrous sodium sulfate and evaporated to dryness. All procedures were carried out at room temperature and in the dark.

The presence of carotenoids was determined quantitatively by measuring the absorbance of the solution in ethyl acetate at an appropriate wavelength using a quartz cuvette in a spectrophotometer. The absorbance wavelengths used were: 286 nm for 4,4′-diapophytoene; 435 nm for 4,4′-diaponeurosporene; 455 nm for 4,4′-diaponeurosporonic acid; and 462 nm for β-β-glucopyranosyl1- O-(4,4′- diaponeurosporene-4- oate)-6-O-(12-methyltetradecanoate) (staphyloxanthin). Ethyl acetate was used as a blank.

**Oxidant susceptibility assays.** Rhodomyrtone-treated <i>S. aureus</i> was prepared as described above. The treated organisms were washed twice with PBS and the turbidity adjusted to a McFarland standard number 2. H₂O₂ in PBS was added to the culture to give final concentrations ranging from 1 to 0.25 mM. The cells were incubated at 37 °C for 2 h with shaking and viable bacterial cells were enumerated. For the singlet oxygen assay, a modified method was performed as described elsewhere (Liu et al., 2005). In brief, rhodomyrtone-treated <i>S. aureus</i> (10⁸ c.f.u. ml⁻¹) were prepared and incubated in 96-well culture plates in the presence of 10 µg methylene blue ml⁻¹. The plates were placed exactly 20 cm from a 100 W light source. The samples were collected at 0, 30 and 60 min and cultured on MHA, and viable micro-organisms were enumerated. A control culture with 1% DMSO was incubated under the same conditions. Experiments were carried out in triplicate.

**Whole-blood killing assay.** The assay was carried out following a modified method of Liu et al. (2005). Briefly, rhodomyrtone-treated <i>S. aureus</i> were adjusted to a McFarland standard number 2. An aliquot of 500 ml bacterial culture was mixed with 1.5 ml freshly drawn human blood in heparinized tubes. The tubes were placed at 37 °C for 4 h with agitation and enumerated for viable bacterial colonies.

**RESULTS**

**Rhodomyrtone alters the pigmentation of <i>S. aureus</i> cells**

The golden colour imparted by carotenoid pigments is a virulence factor of the aggressive human pathogen <i>S.
aureus. Given that a protective effect is provided to the bacteria by the golden-yellow pigments, we examined whether rhodomyrtone could inhibit carotenogenesis and render S. aureus more susceptible to immune clearance. The results indicated that the treated cells possessed less pigment compared with untreated organisms and with organisms treated with antimicrobial inhibitors of pathways of macromolecular synthesis in broth culture (Fig. 3). In a quantitative assay, S. aureus treated with concentrations of the bioactive compound at $2 \times \text{MIC} (1 \, \mu\text{g ml}^{-1})$ and $4 \times \text{MIC} (2 \, \mu\text{g ml}^{-1})$ were demonstrated to produce a higher yield of dehydrosqualene than the untreated cells (Fig. 4a). In contrast, other pigments – 4,4′-diaponeurosporene, 4,4′-diaponeurosporenic acid and staphyloxanthin – were demonstrated to be present at less than twofold and less than sixfold when treated with $0.25 \times \text{MIC}$ and other concentrations ($0.5 \times$, $1 \times$, $2 \times$ and $4 \times \text{MIC}$) of rhodomyrtone, respectively (Fig. 4b–d).

**S. aureus pigment functions as an antioxidant**

We next sought to determine whether the observed antioxidant activity of rhodomyrtone-treated S. aureus translated into decreased resistance to H$_2$O$_2$. S. aureus was exposed to various concentrations of rhodomyrtone for 18 h. The treated cells were collected, followed by incubation with H$_2$O$_2$. The susceptibility of rhodomyrtone-treated cells compared with untreated cells to oxidants in vitro is shown in Fig. 5. The results indicated that the normal untreated cells of the pathogen survived better than those of the treated organisms at various concentrations of H$_2$O$_2$. After incubation with rhodomyrtone at $0.5 \times \text{MIC} (0.25 \, \mu\text{g ml}^{-1})$, the resulting S. aureus with reduced pigmentation were more susceptible to killing at all concentrations of H$_2$O$_2$ tested and the bacterial population decreased by at least 1 log. Moreover, the numbers of viable S. aureus cells after treatment with

![Fig. 3](image-url)  
**Fig. 3.** Reduction of S. aureus pigmentation after treatment with rhodomyrtone at $4 \times$, $2 \times$, $1 \times$, $0.5 \times$ and $0.25 \times \text{MIC}$ for 18 h in broth culture. Vancomycin, penicillin G, rifampicin, ciprofloxacin, tetracycline and 1% DMSO were used as controls.

![Fig. 4](image-url)  
**Fig. 4.** Absorbance of dehydrosqualene at OD$_{286}$ (a), 4,4′-diaponeurosporene at OD$_{435}$ (b), 4,4′-diaponeurosporenic acid at OD$_{455}$ (c) and staphyloxanthin at OD$_{462}$ (d) produced by S. aureus ATCC 29213 after treatment with rhodomyrtone at $4 \times$ (●), $2 \times$ (×), $1 \times$ (▲), $0.5 \times$ (▲) and $0.25 \times \text{MIC}$ (■). DMSO (1%, ◊) was used as a control. Results are shown as the mean ± SEM of triplicate experiments.
rhodomyrtone at 0.25 × MIC also decreased by >1 log with increasing concentrations of H₂O₂ up to 1 mM and the treated bacterial cells were susceptible to 0.5 and 0.25 mM of H₂O₂. In contrast, pathogen treated with rhodomyrtone at 0.125 × MIC and DMSO-treated S. aureus were able to survival under the H₂O₂ conditions tested.

In a further experiment, we tested the viability of rhodomyrtone-treated S. aureus after incubation with singlet oxygen. Samples were collected at various time intervals and viable micro-organisms were enumerated. The bacterial population of 1 % DMSO-treated micro-organisms after exposure to 10 μg methylene blue ml⁻¹ for 1 h remained unchanged (Fig. 6). In contrast, rhodomyrtone-treated S. aureus cells were less able to survive under this extreme condition. When S. aureus treated with rhodomyrtone at the MIC was tested for its survival ability after exposure to methylene blue for 30–60 min, the bacterial population decreased by at least 2 logs. Rhodomyrtone-treated S. aureus at other concentrations of the active compound also failed to grow under singlet oxygen conditions.

S. aureus pigment confers resistance to whole-blood killing

We investigated the killing of rhodomyrtone-treated S. aureus by human whole blood. The results showed that the number of 1 % DMSO-treated culture cells did not change. In contrast, increased susceptibility of the rhodomyrtone-treated S. aureus to killing by human whole blood was observed. After incubation at all concentrations of rhodomyrtone tested (0.125 ×, 0.25 × and 0.5 × MIC), the resulting pathogen with reduced pigmentation was less able to survive in freshly isolated human whole blood, as the organisms contained less carotenoid pigment to act as an antioxidant scavenger (Fig. 7). Our in vitro and ex vivo results suggested that S. aureus pigment is both necessary and sufficient to promote oxidant resistance and whole-blood survival, suggesting a novel target for antibiotic therapy.

DISCUSSION

In the search for the next generation of antibiotics, recent efforts have targeted virulence factors rather than essential gene functions (Hung et al., 2005). Staphyloxanthin of S. aureus is a virulence factor for the organism (Song et al., 2009a, b). The golden-coloured pigment is a typical secondary metabolite that is not essential for growth and reproduction of the pathogen (Liu et al., 2008) but might aid invasiveness in vivo (Pelz et al., 2005). To investigate the biological activities of rhodomyrtone on S. aureus pigment production, carotenoids were extracted from rhodomyrtone-treated S. aureus. The treated S. aureus

![Fig. 5. log reduction in survival of rhodomyrtone-treated S. aureus ATCC 29213 after incubation with 0.25 mM (black bars), 0.5 mM (grey bars) and 1 mM (white bars) H₂O₂. PBS was used as a control. Results are shown as the mean ± SEM of triplicate experiments.](http://jmm.sgmjournals.org)

![Fig. 6. Survival of S. aureus ATCC 29213 treated with rhodomyrtone at 1× (●), 0.5× (○), 0.25× (▲) and 0.125×MIC (■) after incubation with 10 μg methylene blue ml⁻¹ for 0, 30 and 60 min. DMSO (1 %, ◦) was used as a control. Results are shown as the mean ± SEM of triplicate experiments.](http://jmm.sgmjournals.org)

![Fig. 7. log reduction in survival of rhodomyrtone-treated S. aureus ATCC 29213 after incubation with human whole blood for 4 h. DMSO (1 %) was used as a control. Results are shown as the mean ± SEM of triplicate experiments.](http://jmm.sgmjournals.org)
exhibited less pigment and lacked the measurable absorbance of staphyloxanthin at OD_{462}. Moreover, the treated organisms were unable to produce 4,4′-diaponeurosporene and 4,4′-diaponeurosporenolic acid, which are the precursors for staphyloxanthin production. In contrast, dehydrodrosqualene production in rhodomyrtone-treated S. aureus at 2× and 4× MIC levels was shown to produce a higher yield than the untreated cells. Depending on the concentration, rhodomyrtone might inhibit the Crt enzyme, which oxidizes dehydrodrosqualene to 4,4′-diaponeurosporene. In contrast, the compound probably induced the activity of the CrtM enzyme, which condenses two molecules of farnesyl diphosphate to form dehydrodrosqualene. A team of researchers discovered recently that inhibition of S. aureus CrtM and CrtN reduced bacterial survival during infections, suggesting a novel virulence-targeted approach for antibiotic therapy (Hammond & White, 1970a, b; Liu et al., 2008). The first committed step in the staphyloxanthin biosynthetic pathway proceeds through presqualene diphosphate and then dehydrodrosqualene, catalysed by CrtM (Pelz et al., 2005), and is very similar to that catalysed by SQS in plants, animals and some protozoa, as demonstrated in Fig. 1 (Song et al., 2009b). Liu et al. (2008) recently observed that the crystal structure of S. aureus CrtM is very similar to that of human SQS used in cholesterol biosynthesis in humans (Liu et al., 2008). However, a distinction occurs in the last step of catalysis. In S. aureus, the NADPH reduction step is absent, resulting in the production of dehydrodrosqualene rather than squalene (Pelz et al., 2005), whereas the eukaryotic SQS catalyses an NADPH-dependent reduction to yield squalene (Pandit et al., 2000).

Very recently, the effects of rhodomyrtone on the cellular proteins of MRSA have been elucidated. The results indicated that rhodomyrtone inhibited the synthesis of DnaK protein in the treated pathogens (Sianglum et al., 2011). DnaK protein is associated with a wide variety of cellular processes (Hu et al., 2006; Kurt et al., 2006; Singh et al., 2007; Al Refaii & Alix, 2009; René & Alix, 2011), and deletion of dnaK in S. aureus demonstrated an increase in susceptibility to oxidative stress conditions and reduced carotenoid production (Singh et al., 2007). Sigma factor σ^{B} was significantly downregulated in rhodomyrtone-treated S. aureus cells (Sianglum et al., 2012). This gene is necessary for the synthesis of staphyloxanthin in S. aureus (Kullik et al., 1998; Bischoff et al., 2004; Pelz et al., 2005). We hypothesized that inhibition of the expression of DnaK protein and the σ^{B} gene may also influence the production of staphyloxanthin and have an impact on S. aureus pathogenesis.

To probe the possible use of rhodomyrtone for inhibition of pigment production, we performed experiments to compare the susceptibility of rhodomyrtone-treated S. aureus and untreated cells to oxidants in vitro. We found that blocking S. aureus pigment formation led to an increase in the susceptibility of the pathogen to H_{2}O_{2} killing. Moreover, the treated organism was killed more efficiently by singlet oxygen compared with untreated cells. In previous work, it was demonstrated that colourless S. aureus is more susceptible to killing by ROS (Liu et al., 2005; Daum, 2008; Haebich & Von Nussbaum, 2008; Walsh & Fischbach, 2008). Similarly, carotenoids in dietary fruits and vegetables have been reported to have potent antioxidant activity by virtue of their free-radical scavenging properties (El-Agamey et al., 2004).

Staphyloxanthin is a virulence factor of the human pathogen S. aureus and the microbial pigment contributes to resistance to ROS and host neutrophil-based killing. We next sought to investigate the observed innate immune clearance of the pigment after incubation with rhodomyrtone using an ex vivo assay system—human whole-blood survival. Untreated cells of S. aureus survived better than the rhodomyrtone-treated cells in freshly isolated human whole blood. Liu et al. (2005) reported that blocking S. aureus carotenogenesis made the organism more susceptible to killing by 1.5% H_{2}O_{2} and decreased whole-blood survival. Moreover, non-pigmented S. aureus cells were more susceptible to innate immune clearance in a mouse infection model (Liu et al., 2008).

In conclusion, this finding demonstrated that rhodomyrtone-treated S. aureus exhibited reduced pigmentation and that rhodomyrtone treatment led to a dose-dependent increase in the susceptibility of the pathogen to H_{2}O_{2} and singlet oxygen killing. Consequently, the survival ability of the treated organisms decreased in freshly isolated human whole blood. Rhodomyrtone may be acting via effects on DnaK and/or σ^{B}, resulting in many additional effects on bacterial virulence.

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