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 

$\text{H}_2\text{O}_2$ 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 $\sigma^B$, 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 & Sandmann, 1999). Oxidation of the terminal methyl 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 diphenylamine 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 S. aureus (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 acylphloroglucinol family (Fig. 2). The compound was isolated from Rhodomyrtus tomentosa (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; Visutthi et al., 2011). Therefore, the objective of the present study was to investigate inhibition of pigment synthesis in S. aureus 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.** S. aureus 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.

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**Qualitative assay.** S. aureus 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 microorganisms 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′-diaponeurosporene; 435 nm for 4,4′-diaponeurosporene-lactone; 455 nm for 4,4′-diaponeurosporene-succinic acid; and 462 nm for β-D-glucopyranosyl 1-O-(4,4′-diaponeurosporene-4-oate)-6-O-(12-methyltetradecanoyl) (staphyloxanthin). Ethyl acetate was used as a blank.

**Oxidant susceptibility assays.** Rhodomyrtone-treated S. aureus 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 S. aureus (10⁶ c.f.u. ml⁻¹) were prepared and incubated in 96-well plate cultures 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 S. aureus 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 S. aureus cells**

The golden colour imparted by carotenoid pigments is a virulence factor of the aggressive human pathogen S.
*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 $2 \times$ MIC (1 $\mu$g ml$^{-1}$) and $4 \times$ MIC (2 $\mu$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 $0.25 \times$ MIC and other concentrations ($0.5 \times$, $1 \times$, $2 \times$ and $4 \times$ 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 $0.5 \times$ MIC ($0.25 \mu$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. Reduction of *S. aureus* pigmentation after treatment with rhodomyrtone at $4 \times$, $2 \times$, $1 \times$, $0.5 \times$ and $0.25 \times$MIC for 18 h in broth culture. Vancomycin, penicillin G, rifampicin, ciprofloxacin, tetracycline and 1% DMSO were used as controls.](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 $0.25 \times$MIC. DMSO (1%, ◊) was used as a control. Results are shown as the mean ± SEM of triplicate experiments.](image-url)
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 survive 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 microorganisms 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...
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’-diaponeurosporenic acid, which are the precursors for staphyloxanthin production. In contrast, dehydroquazole 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 Crtn enzyme, which oxidizes dehydroquazole to 4,4’-diaponeurosporene. In contrast, the compound probably induced the activity of the Crtn enzyme, which condenses two molecules of farnesyl diphosphate to form dehydroquazole. A team of researchers discovered recently that inhibition of *S. aureus* Crtn 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 dehydroquazole, catalysed by Crtn (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* Crtn 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 dehydroquazole 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 Refaï & 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_1 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_1 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_2O_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; Haeßl & 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_2O_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_2O_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_1, resulting in many additional effects on bacterial virulence.

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