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Staphyloxanthin Plays a Role in the Fitness of *Staphylococcus aureus* and Its Ability To Cope with Oxidative Stress

Alexandra Clauditz,[1] Alexandra Resch,[1] Karsten-Peter Wieland,[1] Andreas Peschel,[2] and Friedrich Götz[1]*

Mikrobielle Genetik, Universität Tübingen, Tübingen, Germany,[1] and Medizinische Mikrobiologie, Universität Tübingen, Tübingen, Germany[2]

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Staphyloxanthin is a membrane-bound carotenoid of *Staphylococcus aureus*. Here we studied the interaction of staphyloxanthin with reactive oxygen substances (ROS) and showed by comparative analysis of the wild type (WT) and an isogenic *crtM* mutant that the WT is more resistant to hydrogen peroxide, superoxide radical, hydroxyl radical, hypochloride, and neutrophil killing.

Staphyloxanthin is an orange-red triterpenoid carotenoid whose biosynthesis and structure have been recently elucidated (14, 18). It is well known that carotenoids function as antioxidants, and it has been suggested that staphyloxanthin can protect *Staphylococcus aureus* against oxidative stress (12, 13). To further study the protective function of staphyloxanthin, we compared the viability of staphyloxanthin-producing *S. aureus* Newman (3) with that of its isogenic *crtM* mutant (19) (Fig. 1), which does not produce staphyloxanthin (but can be complemented by the expression plasmid pTX*crtM*) (14), to various radical and nonradical substances generated in vitro. We also tested the ability of staphyloxanthin to act as a radical scavenger.

First we investigated the oxidation of staphyloxanthin by hydroxyl radicals ($OH^{\cdot}$) and peroxynitrite ($ONOO^{-}$). We generated hydroxyl radicals by the Fenton reaction in an assay containing iron(II) chloride and hydrogen peroxide in equimolar concentrations ranging from 0.01 to 0.5 mM. Although the system is nonspecific, $OH^{\cdot}$ is likely to be among the oxidants produced, including $OOH^{\cdot}$ and $CH3^{\cdot}$ (20). Purified staphyloxanthin (14) in the reaction mixture was rapidly oxidized, as indicated by the decrease in absorbance at 478 nm (Fig. 2A). The absorbance of controls lacking iron(II) chloride, H_2O_2, or both did not decrease. The time course of staphyloxanthin oxidation in the test samples shows that after 2 min of incubation with 0.05, 0.1, and 0.5 mM equimolar amounts of iron chloride and hydrogen peroxide, 40, 50, and 60% of the staphyloxanthin was oxidized, respectively (Fig. 2C). The oxidation of staphyloxanthin by peroxynitrite was monitored for 3 h by monitoring the decrease in absorbance at the absorption maximum of staphyloxanthin in ethanol (470 nm). In this experiment, prolonged incubations were necessary because the concentration of peroxynitrite increased only gradually after addition of SIN-1. Staphyloxanthin oxidation by peroxynitrite anions was indicated by the decrease in absorption and at the same time the shift of the absorption maximum to shorter wavelengths, which indicates the reduction of double bonds (Fig. 2B). The absorbance of the controls lacking SIN-1 did not decrease.

Next we tested the viability of *S. aureus* after incubation with H_2O_2. The numbers of viable *S. aureus* Newman wild-type (WT) and *crtM* mutant cells decreased with increasing concentrations of H_2O_2 (50, 75, 100, and 150 mM) but to different extents (Fig. 3A). The number of CFU of the controls lacking H_2O_2 did not change. The survival of the bacteria in the test samples correlated with the H_2O_2 concentration. Both strains were susceptible to high concentrations of H_2O_2, but the *crtM* mutant was more susceptible than the WT at all concentrations. The viability of *S. aureus* was also investigated after incubation with superoxide radicals generated by both hypoxanthine and xanthine oxidase (XO) and phenazine methosulfate (PMS) and NADH. The generation of the radicals was monitored by measuring the reduction of cytochrome *c*. WT and the *crtM* mutant *S. aureus* cells were both sensitive to killing by hypoxanthine-XO (Fig. 3B), but the WT always revealed a better survival frequency independent of the presence of catalase, which increases XO activity, which is inhibited by H_2O_2 (Fig. 3B). The number of CFU of the control did not

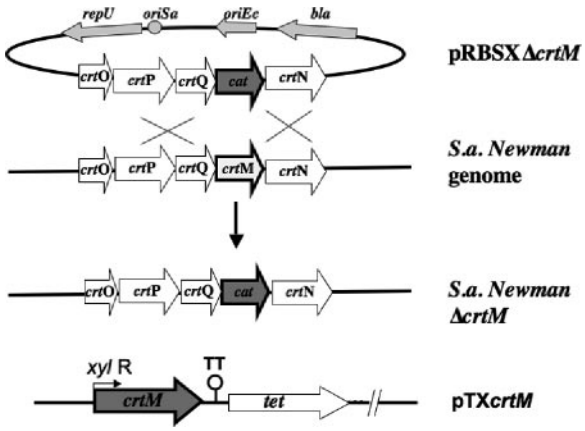

FIG. 1. Illustration of the construction of knockout plasmid pRBSXΔ*crtM* and xylose-inducible *crtM* expression plasmid pTX*crtM*, which is able to complement the *crtM* mutant in the presence of xylose as an inducer. TT, transcription terminator.

* Corresponding author. Mailing address: Mikrobielle Genetik, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany. Phone: (49) 7071 29746-36 or -35. Fax: (49) 7071 295039. E-mail: friedrich.goetz@uni-tuebingen.de.

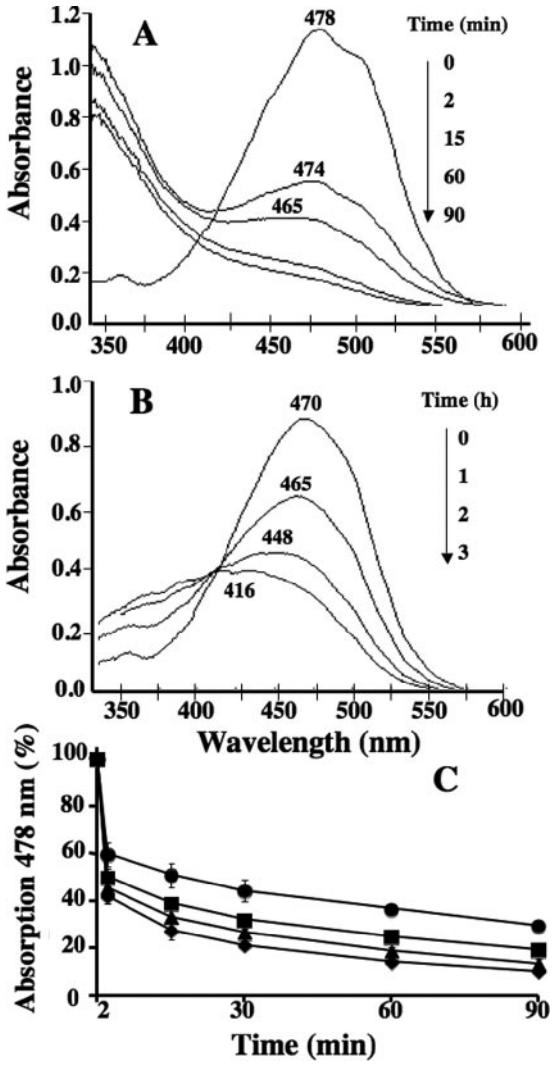

FIG. 2. Time course of staphyloxanthin oxidation by free radicals. (A) Oxidation by free radicals generated in a nonspecific Fenton reaction. The reaction mixture consisted of 12 μM staphyloxanthin in dimethyl sulfoxide-H_2O (4:1, vol/vol), 0.5 mM iron(II) chloride, and 0.5 mM H_2O_2 and was incubated under air at 25°C. Absorption spectra were recorded before the reaction with iron(II) chloride and H_2O_2 (time zero) and after 2, 15, 60, and 90 min. The absorption maxima are indicated. (B) Oxidation by peroxynitrite generated with SIN-1. The reaction mixture consisted of 12 μM staphyloxanthin and 3 mM SIN-1 in ethanol-H_2O (4:1, vol/vol) and was incubated under air at 25°C. Absorption spectra were recorded before reaction with SIN-1 (time zero) and after 1, 2, and 3 h. The absorption maxima are indicated. (C) Time course of oxidation of purified staphyloxanthin by hydroxyl radicals generated in a Fenton reaction. The reaction mixture consisted of 12.5 μM staphyloxanthin in dimethyl sulfoxide-H_2O (4:1, vol/vol) and equimolar concentrations of $FeCl_2$ and H_2O_2, i.e., 0.05 mM (●), 0.1 mM (■), 0.2 mM (▲), and 0.5 mM (◆). The mixture was incubated under air at 25°C. Oxidation of staphyloxanthin was determined by measuring the decrease in absorption at 478 nm. Data points represent the means of five independent experiments. Error bars indicate the deviation of five independent experiments.

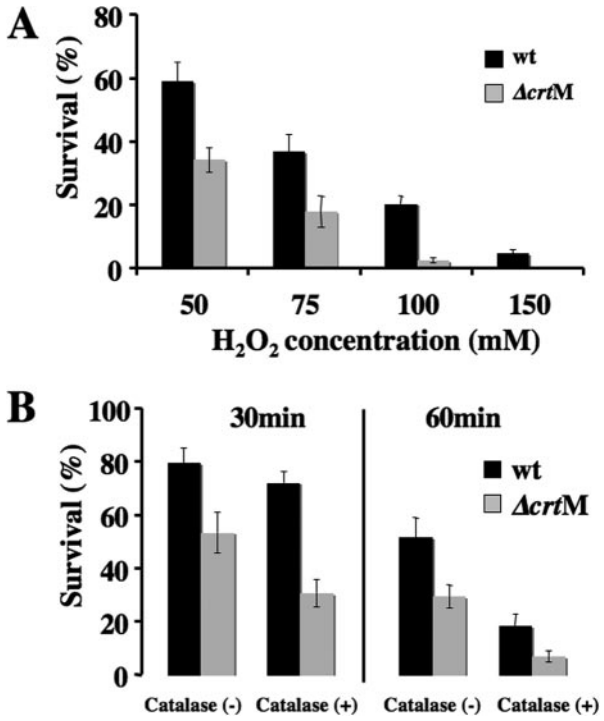

FIG. 3. Effects of H_2O_2 and superoxide radical on the survival of WT and Δ*crtM* mutant *S. aureus* Newman. (A) After 24 h of growth in basic medium, 5×10^6 CFU ml^{-1} were incubated in phosphate-buffered saline containing the indicated concentrations of H_2O_2 in the dark at 0°C. After 45 min, the reaction was stopped by destroying the remaining H_2O_2 with 2 U ml^{-1} catalase and incubation for 20 min. Diluted cells (0.1 ml) were spread on BM agar plates. Colonies were counted after 24 h of incubation at 37°C. Values are expressed as a percentage of the CFU in the control culture lacking H_2O_2. Values are the averages of five independent experiments. Error bars indicate the deviation of five independent experiments. (B) After 24 h of growth in basic medium, 5×10^6 CFU ml^{-1} were incubated in HEPES buffer containing 10 mM hypoxanthine and 0.1 U of xanthine oxidase (XO) with or without 2 U of catalase at 25°C. After incubation for 30 and 60 min, the reaction was stopped by addition of 10 μM allopurinol. Diluted cells (0.1 ml) were spread on BM agar plates. Colonies were counted after 24 h of incubation at 37°C. Values are expressed as a percentage of the number of CFU in the control culture containing only hypoxanthine (10 mM) and lacking XO. Values represent the average of five independent experiments. Error bars indicate the deviation of five independent experiments.

change. Both *S. aureus* WT and the *crtM* mutant were susceptible to 1.0 mM PMS and succinate (a source of NADH), which generate the superoxide radical O_2^-. Killing correlated with time, but the mutant was more susceptible than the WT (Fig. 4A), which showed twofold higher survival than the mutant. The number of CFU of the control did not change after incubation. Phenazine oxidizes NADH, resulting in production of O_2^-, which can react nonenzymatically with H_2O_2 to form hydroxyl radical (6) and singlet oxygen (10), leading to the peroxidation of polyunsaturated fatty acids of membrane lipids (4, 17).

Myeloperoxidase (MPO) plays a crucial role in bacterial killing by generating hypochlorous acid within neutrophil phagosomes, where it constitutes about 5% of the total neutrophil protein (1, 16). In vitro, MPO in the presence of H_2O_2 and chloride or iodide ions leads to hypochlorous acid and to the killing of many bacteria and fungi (7, 8). We analyzed the sensitivity of WT and *crtM* mutant *S. aureus* to MPO in the presence of H_2O_2 and Cl^-. Both strains were killed, but the *crtM* mutant cells were killed much faster and in greater numbers than WT cells (Fig. 4B). The bactericidal effect was de-

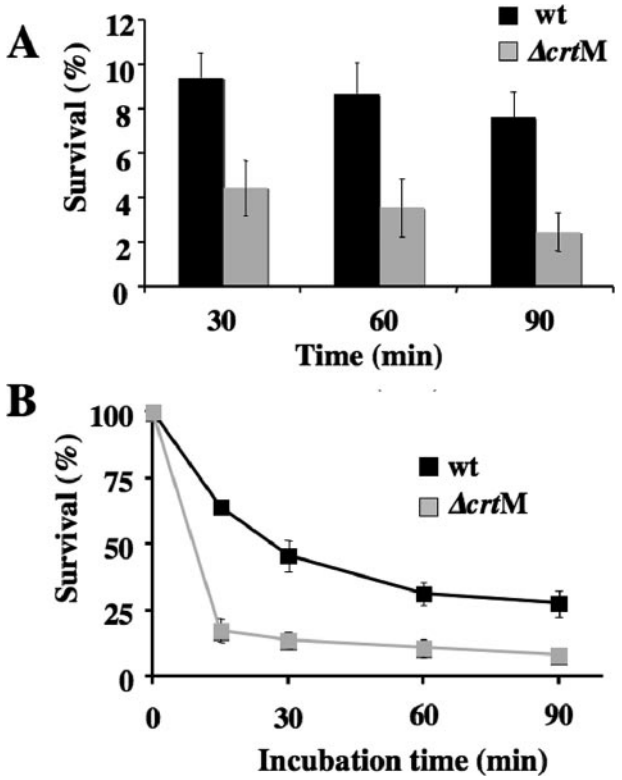

FIG. 4. Effects of PMS and MPO on the survival of WT and Δ*crtM* mutant *S. aureus* Newman. (A) After 24 h of growth in basic medium, cells were harvested and washed twice in HEPES buffer and 5×10^6 CFU ml^{-1} were incubated in 20 mM HEPES buffer containing 1 mM PMS and 2 mM succinate at 25°C. After the indicated time, 0.1 ml of diluted cells was spread on BM agar plates. Colonies were counted after 24 h of incubation at 37°C. Values are expressed as a percentage of the number of CFU in the control culture containing only succinate (2 mM) and lacking PMS. Values are the average of five independent experiments. Error bars indicate the deviation of five independent experiments. (B) After 24 h of growth in basic medium, cells were harvested and washed twice and 5×10^6 CFU ml^{-1} in phosphate-buffered saline at pH 7.4 were mixed with 0.05 U of MPO and 10 μM H_2O_2 and incubated at 25°C for 90 min. Diluted cells (0.1 ml) were spread on BM agar plates. Colonies were counted after 24 h of incubation at 37°C. The number of CFU is expressed as a percentage of the control containing only H_2O_2 (10 μM). Values are the average of five independent experiments. Error bars indicate the deviation of five independent experiments.

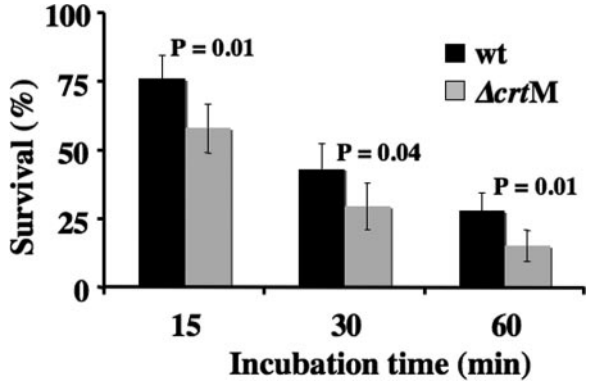

FIG. 5. Killing of WT and Δ*crtM* mutant *S. aureus* Newman by human neutrophils. After 24 h of growth in basic medium, cells were harvested and washed twice in potassium-phosphate buffer (pH 7.2) containing 0.05% human serum albumin. Bacteria (5×10^6 CFU ml^{-1}) were mixed with neutrophils (2.5×10^6/ml). Human serum was added to a final concentration of 10%, and 150 μl of prewarmed Hanks balanced salt solution was also added. Samples (500 μl) were shaken at 37°C, and the incubation was stopped after the indicated time by diluting the samples 100-fold in ice-cold distilled water. The diluted samples (0.1 ml) were spread on BM agar plates, and colonies were counted after 24 h of incubation at 37°C. The number of CFU after incubation with neutrophils is expressed as a percentage of the initial count. Values are the average of five independent experiments. Error bars indicate the deviation of five independent experiments. The significance of experimental differences was evaluated by unpaired Student test.

pendent upon the incubation time, but the overall difference between the strains remained almost constant during the 90 min of incubation; e.g., there was a fourfold difference after 15 min of incubation. The viability of the WT and the *crtM* mutant was not affected by incubation of the cells with $NO^{\cdot}$ donors (not shown). $NO^{\cdot}$ also has little effect on the viability of other bacterial species, e.g., *Escherichia coli*, *Salmonella* sp., and *Proteus vulgaris* (2, 9).

Finally, we investigated the killing of *S. aureus* by human neutrophils, which consume more O_2 after ingestion of bacteria (15). Since all of the ROS analyzed in this work are also produced during the oxidative burst, it was of interest to compare the killing of the WT and the *crtM* mutant by human neutrophils. Killing of both strains by human neutrophils increased with time, but a higher percentage of the mutant cells were killed (Fig. 5). After 15 and 60 min of incubation, the survival of the WT was 1.3- and 1.8-fold, respectively, higher than that of the *crtM* mutant. Liu et al. (13) described an approximately 10-fold higher survival frequency of the WT compared to the *crtM* mutant in human neutrophils, and they also showed that this effect is not explained by differences in the rate of phagocytosis, because the uptake of WT *S. aureus* was comparable to that of the *crtM* mutant. We saw the same tendency, although the differences between the WT and the *crtM* mutant were less pronounced. One explanation for this discrepancy could be that we used stationary-phase cells throughout our study, where staphyloxanthin production is greatest but where cells might also become more resistant to peroxides and radical species. Other groups have also described better survival of carotenoid-producing cells within human neutrophils (5, 11).

Our data indicate that staphyloxanthin scavenges free radicals with its conjugated double bonds. Since staphyloxanthin is located in the cell membrane, it probably primarily protects lipids but might also be involved in protecting proteins and DNA. Enzymes such as catalase and SOD most likely contribute to a larger extent to the survival of cells during stress and the host response, but staphyloxanthin plays an additional role in the defense against damage by ROS, thereby enhancing the virulence and fitness of the cells. Staphyloxanthin can be regarded as a biological antioxidant against hydrogen peroxide and hydroxyl radicals and might be useful as a therapeutic radical scavenger.

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