

Pseudomonas aeruginosa induces pigment production and enhances virulence in a white phenotypic variant of *Staphylococcus aureus*

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Abstract: Staphyloxanthin is a virulence factor which protects *Staphylococcus aureus* in stress conditions. We isolated two pigment variants of *S. aureus* and one strain of *Pseudomonas aeruginosa* from a single wound infection. *S. aureus* variants displayed white and yellow colony phenotypes. The sequence of the operons for staphyloxanthin synthesis indicated that coding and promoter regions were identical between the two pigment variants. Quorum sensing controls pigment synthesis in some bacteria. It is also shown that *P. aeruginosa* quorum-sensing molecules affect *S. aureus* transcription. We explored whether the co-infecting *P. aeruginosa* can affect pigment production in the white *S. aureus* variant. In co-culture experiments between the white variants and a selected number of Gram-positive and Gram-negative bacteria, only *P. aeruginosa* induced pigment production in the white variant. Gene expression analysis of the white variant did not indicate upregulation of the *crtM* and other genes known to be involved in pigment production (*sigB*, *sarA*, farnesyl pyrophosphate synthase gene [FPP-synthase], *hfq*). In contrast, transcription of the catalase gene was significantly upregulated after co-culture. *P. aeruginosa*-induced pigment synthesis and catalase upregulation correlated with increased resistance to polymyxin B, hydrogen peroxide, and the intracellular environment of macrophages. Our data indicate the presence of silent but functional staphyloxanthin synthesis machinery in a white phenotypic variant of *S. aureus* which is activated by a co-infecting *P. aeruginosa* via inter-species communication. Another *S. aureus* virulence factor, catalase is also induced by this co-infecting bacterium. The resulting phenotypic changes are directly correlated with resistance of the white variant to stressful conditions.

Keywords: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, staphyloxanthin, catalase, interspecies interaction

Introduction

More than 90% of all *Staphylococcus aureus* isolates from human infections are golden pigmented.¹ In 1884, Rosenbach² classified different isolates of *Staphylococci* based on pigment production as *S. aureus* (pigmented) and *S. albus* (non-pigmented). This simple classification method was later replaced by more advanced classification using other properties of the bacterium.³⁻⁵ Although most isolates of *S. aureus* produce golden colonies, it was recognized that some *Staphylococcus* isolates, which displayed all characteristics of *S. aureus*, could produce white colonies, which was partly dependent on growth media.⁶ Conclusive proof that these white colonies belonged to *S. aureus* lineage may have required modern molecular tools not available at the time. Pigment synthesis in *S. aureus* is carried out by the enzymes of the *crtOPQMN* operon, but it is not known whether the presence of an intact operon in the genome would inevitably

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lead to development of the stable golden colony phenotype. In a recent study, it was shown that a *S. aureus* isolate with a white-colony phenotype actually lacked the *crtOPQMN* operon.⁷ However, these authors proposed to reclassify the isolate and the lineage it belonged to as *S. argenteus*.

Golden pigment in *S. aureus* is the manifestation of triterpenoid staphyloxanthin biosynthesis, which is transported to and embedded in the cellular membrane.^{8,9} Staphyloxanthin is synthesized through a five-step enzymatic process by the five enzymes of the polycistronic *crtOPQMN* operon which is controlled by a single promoter.¹⁰ In this process, farnesyl diphosphate acts as the substrate for the initial step and staphyloxanthin is the product of the final step.¹¹

Staphyloxanthin has been considered one of the several virulence factors that *S. aureus* has at its disposal to withstand and survive a stressful environment. Clauditz et al have used targeted mutagenesis of the genes from the *crtOPQMN* operon to demonstrate the importance of staphyloxanthin for resistance of *S. aureus* to oxidative stress.¹² It was shown that wild type pigment-producing strains withstood exposure to higher concentrations of H₂O₂ than the isogenic, pigment knockout mutants.¹² Similarly, Liu et al reported that the golden pigment impairs neutrophil killing, promotes virulence due to its antioxidative action, and significantly contributes to the development of subcutaneous abscess in an animal model.¹³

In addition to its antioxidative activity, staphyloxanthin also affects the chemical composition and functional activity of the *S. aureus* cellular membrane by reducing membrane fluidity and thereby stabilizing the membrane structure.¹⁴ The change in membrane fluidity translates into higher resistance to host antimicrobial peptides used by the host to eradicate bacterial agents.¹⁵ The enzymes of the staphyloxanthin biosynthetic pathway are considered prime targets for drug development aimed at eradication of *S. aureus* infections. The first enzyme in this pathway, CrtM, has a similar catalytic site structure to that of human squalene synthase which is involved in cholesterol biosynthesis. Liu et al showed that inhibitors of human squalene synthase could prevent pigment production in *S. aureus*, and affect its survival.¹⁶ Two molecules, *N*-3-(3-phenoxyphenyl) propylphosphonoacetamide and phosphonosulfonate have been identified as CrtM inhibitors.¹⁷ However, specificity and in vivo applicability of these molecules need further investigation.

Wound infection is a significant problem in clinical settings, particularly in combat-related wounds where improvised explosive devices not only damage tissue but also deposit dirt and metal fragments in wounds. *S. aureus* is commonly isolated from wound infections and has become

an alarming problem due to the emergence of several methicillin-resistant strains (eg, methicillin resistant staphylococcus aureus [MRSA]).¹⁸ Wound infections are polymicrobial and *S. aureus* is commonly isolated from these infections along with a consortium of other bacteria.^{19–22} At present, it is almost impossible to define the role of each participating bacterium of these communities in the outcome of infection.²³ On the other hand, to better understand the mechanisms of persistence and in vivo survival of *S. aureus* during infections, it is necessary to understand the interaction between *S. aureus* and its co-infecting bacteria. One of the co-infecting bacterial species which is routinely isolated from clinical samples along with *S. aureus* is *Pseudomonas aeruginosa*. Samples from wound infections and lungs of patients with cystic fibrosis often contains both *P. aeruginosa* and *S. aureus*, underscoring the clinical importance of co-infection by these two bacteria.^{24,25} It has been shown that interaction of *P. aeruginosa* and Gram-positive bacteria including *S. aureus* can lead to increased virulence in *P. aeruginosa*.²⁶ There is little information available about the interaction between these two bacterial pathogens and the importance of this interaction with regards to the severity and outcome of infection. Some studies that have examined the interaction of *P. aeruginosa* and *S. aureus* suggest induction of adaptive changes in *S. aureus* which could promote its virulence. For example, *P. aeruginosa* could induce *S. aureus* to mount an adaptive response by forming small-colony variants.^{25,27,28} It has also been demonstrated that *P. aeruginosa*-produced quinolone 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) could suppress growth of *S. aureus* and a wide spectrum of other Gram-positive bacteria.^{29,30} The same molecule can also induce resistance to streptomycin or tobramycin.^{25,31} In wounds with complex infections, pathogenicity of *S. aureus* has been shown to increase in the presence of low concentrations of *P. aeruginosa*.³²

We isolated two pigment variants of *S. aureus* and one strain of *P. aeruginosa* from the same clinical sample obtained from a patient with a soft tissue wound. *S. aureus* pigment variants produced white and yellow colonies. In this study we report that the white colony variant harbors an intact *crtOPQMN* operon in spite of lack of pigmentation. Furthermore, we demonstrate that the *crtOPQMN* operon of the white variant is functional and can be triggered to produce golden pigment by the *P. aeruginosa* isolate. We also show that catalase, another *S. aureus* virulence factor, is also upregulated as a consequence of its interaction with the *P. aeruginosa*. The phenotypic changes induced by the co-infecting *P. aeruginosa* correlates to increased resistance of the white *S. aureus* variant to stressful conditions.

Materials and methods

Identification of bacteria

The two phenotypic variants of *S. aureus* and the *P. aeruginosa* strain were initially identified by an automated system for identification of bacteria (Phoenix Automated Microbiology System, Sparks, MD, USA) according to the manufacturer instructions. These results were confirmed by 16 s ribosomal DNA sequencing. Briefly, after isolation of genomic materials from both phenotypic variants, eubacterial universal 16S rDNA primers, 27 A and 1392R³³ (Table 1), were used to amplify the entire 16S rDNA gene.

Table 1 Sequencing and qRT-PCR primers

Overlapping PCR primers	Sequence	Reference
C1 F	GGTCCATGTTTCGATTTTACG	This study
C1 R	TAAAACAGGTAGCGGAATCG	
C2 F	TTGGCGTAAAGATTGTTTCTG	This study
C2 R	AGAACGTATCGAAACCAAG	
C3 F	TCAATTAGCTGATCTGCATGATT	This study
C3 R	GGATATTGTTATGGTGTGCTGG	
C4 F	AAATCTTCACCGACATCTCTT	This study
C4 R	TGCCCGTAACAGTTTATGAAGG	
C5 F	ATAGAACCGAACAACCACAACAC	This study
C5 R	GCAGACAAAATAAACGCGC	
C6 F	AATGCACCACACGCCATAG	This study
C6 R	ACTTGCGACAAATGTTAGGC	
C7 F	AAGGCATTGGCTAAATGATGG	This study
C7 R	ATAGAGACAAAGAGGGCAGAGT	
C8 F	AACTCGTTAATCTCGGTTCG	This study
C8 R	TGTTGGTGGGAAAATTGGTTC	
Universal primers		
27 F	AGAGTTTGATCMTGGCTCAG	Marchesi et al ³³
1392 R	ACGGGCGGTGTGTRC	
qRT-PCR primers		
<i>crtM</i> F	CTGCTAATTCTATGATTGGTTGTGC	This study
<i>crtM</i> R	TGGGAATATTATGCAGCTATMGAC	
<i>sigB</i> F	GAAATWGGRCWAGAATMAA	This study
<i>sigB</i> R	TTTGTCCCATTTCATYGCT	
<i>sarA</i> F	GTTTTCGTGATGTATGTCA	This study
<i>sarA</i> R	TCAATGGTCACTTATGCTGACA	
<i>FPP synth</i> F	GCGGTCAAATGTTAGATATGCAA	This study
	AGCG	
<i>FPP synth</i> R	ATCTGCTGCACTCATAACCGCAA	
<i>hfq</i> F	CGAAAACATCCAAGACAAAGC	This study
<i>hfq</i> R	AAGTGCTGATCGCATGTTTG	
<i>katA</i> F	TGCATGCCAAAGTTTCTGGTGC	This study
<i>katA</i> R	ACGGGCAAACATTTCTGGTTGCTT	
<i>agrA</i> F	TGCGAAGACGATCCAAAACAAA	This study
	GAGA	
<i>agrA</i> R	GCTTGCTCAAGCACCTCATAAG	
	GATT	

Abbreviations: PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction; *crtM*, carotenoid biosynthetic gene M; *sigB*, sigma B transcription factor gene; *sarA*, staphylococcal accessory regulator A gene; *FPP synth*, farnesyl pyrophosphate synthase gene; *hfq*, host factor gene for Q beta; *katA*, catalase gene A; *agrA*, accessory gene regulator A; F, forward; R, reverse.

The PCR amplicons were cloned into a TA-cloning vector, clonally expanded by transformation into *E. coli*, and sequenced by the dideoxy Sanger method. The three isolates were designated as *S. aureus* white and *S. aureus* yellow variants, and *P. aeruginosa* 6611.

Sequencing of *crtOPQMN* operon

Known sequences of *crtOPQMN* operon deposited in the GenBank were used in multiple sequence alignment to identify conserved regions for the design of PCR primers. These primers (Table 1) were then used for the amplification of overlapping segments covering the entire operon, including the promoter region. The amplicons were either directly sequenced or cloned into a TA-vector before sequencing. The sequencing data from the amplicons were used to assemble the entire operon including its promoter region. All sequence manipulation was carried out using commercially available software (v5.4.5; Geneious Pro, Biomatters Ltd, Newark, NJ, USA).

Co-culture experiment for induction of pigment production

Bacteria used in these experiments were grown in Luria-Bertani (LB) broth at 37°C, 220 rpm for 18–24 hours. *P. aeruginosa* 6611 was grown in stationary conditions in LB broth as a biofilm at 37°C. For co-culture experiments, an overnight culture of *S. aureus* white variant was used. *P. aeruginosa* 6611 for co-culture experiments was prepared from a 72-hours-old biofilm. Biofilm was disrupted by pipetting and vigorous vortexing. Cells were pelleted by spinning at 13,000 g for 5 minutes, supernatant was decanted, and the pellet was re-suspended in fresh LB broth and used in co-culture experiments.

Co-culture experiments were carried out by inoculating 2 µL of *P. aeruginosa* 6611 near 5 µL of *S. aureus* white variant on semi-solid LB agar plates such that the distance between the points of inoculations were a constant 1 cm. Agar plates were incubated upside down at 37°C and monitored for pigment development for 48 hours. As the experimental control, the white variant was also similarly co-cultured with *Escherichia coli* Mg1655, *Acinetobacter baumani*, co 41-00-sc1-c1, *Enterobacter* W001, and the white and yellow variants of *S. aureus*.

We also examined the supernatants from the biofilm and shaking cultures of *P. aeruginosa* for their ability to induce pigment production in the white *S. aureus* variant. The supernatants were sterile filtered through a 0.22 µm filter and spotted on an LB agar plate near the white variant. However, we did not observe any pigment induction using

this method, even though the supernatant was inoculated multiple times at the same spot during the experiment.

Quantitative real-time polymerase chain reaction (qRT-PCR) of genes involved in staphyloxanthin synthesis and control of its expression

To prepare RNA for qRT-PCR, co-culture experiments were carried out by incubation of 5 μ L of *S. aureus* white variant overnight culture on LB agar plates (six spots per plate) for 18–24 hours. *P. aeruginosa* 6611 was then spotted at 1 cm distance (see above) from the center of the *S. aureus* colonies and returned to the incubator. A plate containing only white colonies was also prepared as a control. At different time points of 1, 7, 24, and 48 hours from the start of the co-cultures, *S. aureus* colonies were collected and re-suspended in RNAProtect Bacteria Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer instructions and preserved at -80°C until RNA extraction.

RNA was extracted using the Ribo-Pure RNA extraction kit (Ambion[®], Grand Island, NY, USA). The quantity and quality of RNA was evaluated using a NanoDrop1000 spectrophotometer and Agilent Bioanalyzer 2100, respectively. The genes examined in this study were *crtM*, sigma B transcription factor (*sigB*), global transcriptional regulator (*sarA*), FPP synthase gene, post-transcriptional regulatory protein Hfq gene (*hfq*), accessory gene regulator (*agrA*), and another important virulence factor for H_2O_2 survival, catalase (*kata*). The sequence of *crtOPQMN* from the white variant was used to design qRT-PCR primers for *crtM* gene. We used the 16S rRNA gene as the internal control for the normalization of gene expression. Primers for other selected genes were designed based on the consensus sequence constructed from their known sequences deposited in the GenBank. Quantitative RT-PCR was performed using the Applied Biosystems 7500 and Power SYBR Green RNA to Ct 1-step kit (Applied Biosystems, Carlsbad, CA, USA) according to manufacturer instructions using 20 ng of total RNA per reaction. The values of Ct were calculated by 21 CFR Part 11 SDS software (v1.4.1; Applied Biosystems, Carlsbad, CA, USA) with the baseline set from 3–6 cycles, and a threshold value of 0.2. Data were analyzed using the $\Delta\Delta\text{Ct}$ method.³⁴ The results represent the average fold change of two independent experiments comparing the white variant with or without co-culturing with *P. aeruginosa* 6611.

Staphyloxanthin analysis

For spectroscopic analysis, staphyloxanthin was extracted as previously described.³⁵ Briefly, the *S. aureus* white variant was grown in the presence (co-culture) and absence of *P. aeruginosa* 6611 for 48 hours. The pigmented (yellow) *S. aureus* variant was also cultured on plates in the absence of *P. aeruginosa* 6611, and used as a positive control. After incubation, bacteria were collected from the LB agar plates and re-suspended in sterile Dulbecco's phosphate-buffered saline (DPBS). Optical density (OD_{600}) of the suspensions was adjusted to 0.50 ± 0.05 , and 5 mL of the OD-adjusted suspensions was used for the pigment extraction. The cells were collected by centrifugation for 5 minutes at 13,000 g, re-suspended in 200 μ L of 200 proof methanol (Sigma-Aldrich, St Louis, MO, USA), and heated for 5 minutes at 55°C . The cells were pelleted by centrifugation for 1 minute at 13,000 g and the supernatants were collected. The extraction step was repeated once more. Methanol extracts from both rounds of extractions were pulled, their volumes were adjusted to 1 mL, and the absorbance of the crude extract was measured at $\lambda = 465$ nm using a Genesys 20 spectrophotometer (Thermo Electron Corporation, East Greenbush, NY, USA).

For high performance thin layer liquid chromatography (HPTLC), methanol extracts were protected from light and vacuum evaporated at 45°C . After drying, samples were dissolved in 10 μ L of ethyl acetate (Sigma-Aldrich) and spotted on the HPTLC silica plates with a pore size of 60 \AA (Sigma-Aldrich). The plates were placed in a TLC tank containing 65 mL chloroform (Sigma-Aldrich), 24 mL methanol (Sigma-Aldrich), and 4 mL deionized water.³⁶ The spots were allowed to migrate upward for 15 minutes, and the plate was then air-dried and photographed.

Survival in H_2O_2

After co-culturing of the *S. aureus* white variant with *P. aeruginosa* 6611 for 48 hours, pigment-induced colonies were collected from the LB agar and re-suspended in sterile DPBS. The white variant incubated in the absence of *P. aeruginosa* 6611 was used as the control in these experiments. The optical densities of the bacterial suspensions were adjusted to $\text{OD}_{600} = 0.15$ in DPBS. The number of bacteria was then adjusted to 20,000 colony forming units (CFU) per mL by serial dilutions in DPBS. Five hundred microliters of bacterial suspension containing 10,000 CFU was mixed with a final concentration of 1% H_2O_2 in 14 mL polystyrene tubes. Cells were incubated for 15 minutes at 37°C with shaking at 220 rpm. After 15 minutes of incubation with H_2O_2 , catalase at a final concentration of 1000 U/mL was added, and

incubated for 15 minutes to stop the reaction. Finally, cells were plated on LB agar with appropriate dilutions and CFUs were determined after 18–24 hours of incubation at 37°C. Survival rates were expressed as a percentage of the initial number of cells. Results are presented as the mean value of two independent experiments.

Survival in 1 mM polymyxin B

The *S. aureus* white variant with or without co-culturing with *P. aeruginosa* 6611 was collected from LB agar plates and the number of cells adjusted to 10⁴ CFU/mL in sterile DPBS. Polymyxin B (Sigma-Aldrich) at a final concentration of 1 mM was added to each sample and incubated for 30 minutes at 37°C in 3 mL polystyrene tubes. After polymyxin B treatment, bacteria were appropriately diluted and plated on LB agar, and the number of CFUs was determined after 18–24 hours. The survival rates were expressed as the percentage of the initial number of cells, and presented as an average of three independent experiments.

Intracellular survival of *S. aureus* in mouse macrophage cell line

Mouse macrophage cell line (J7741.A, ATCC, Manassas, VA, USA) was cultured according to the manufacturer instructions with ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 500 U/mL penicillin/streptomycin solution. Cells were maintained and subcultured at 37°C in an incubator in the presence of 5% CO₂. For in vitro infection experiments, cells were seeded in 6-well plates and grown until a monolayer was formed (~1.7 × 10⁶ cells/monolayer). Approximately 24 hours before the addition of bacteria, growth media containing penicillin/streptomycin was replaced with invasion media (DMEM and 10% FBS without antibiotics). For the infection assay, 1.7 × 10⁷ bacteria was added to each well (infection ratio of 1:10) and incubated at 37°C. After 30 minutes of incubation, extracellular bacteria were removed and macrophage monolayer was rinsed with 1 mL of DPBS. The infected monolayer was then incubated with 2 mL of fresh media containing

100 µg/mL of gentamicin. At different time intervals of 15 minutes (T0) and 120 minutes (T1) after the addition of the bacteria, the gentamicin-containing medium was removed, cells were washed once with DPBS, and lysed with 0.02% Triton X-100 in DPBS. Appropriate dilutions were then plated on LB agar and the number of CFUs determined after 18–24 hours of incubation at 37°C. Intracellular survival was expressed as a percentage of CFUs comparing the number of cells at T0 and T1. The survival rates were presented as the mean of three independent experiments.

Statistical analysis

Results are presented as mean ± SD. Repeats of three independent experiments were used for statistical analysis which was performed by JMP10[®] software (SAS Institute Inc, Duke University, Cary, NC, USA). Student's *t*-test was used for the evaluation of the statistical significance between the groups. *P*-values < 0.05 were considered to be significant.

Results

Sequencing of *crtOPQMN* operon and its promoter region

We initially postulated that the difference between the two pigment variants of *S. aureus* was due to the differences in sequences of the coding or promoter regions of the *crtOPQMN*. Eight primer pairs were designed and used to amplify overlapping segments of the *crtOPQMN* operons generating amplicons ranging in size from 865 to 1017 bps (Figure 1). The amplicons were sequenced and assembled to construct the full length of the operon from both the white and yellow variants of *S. aureus*. Comparison of these two operons, including the promoter regions, indicated that they were identical. Both operons contained the same gene arrangement as the canonical *crtOPQMN* operon: the promoter followed by genes coding for *glycosyl-4,4'-diaponeurosporenoate acyltransferase* (*crtO*), *phytoene dehydrogenase* (*crtI*), *4,4'-diaponeurosporenoate glycosyltransferase* (*crtQ*), *dehydrosqualene synthase* (*crtM*), and *dehydrosqualene desaturase* (*crtN*). This indicated that some mechanism other

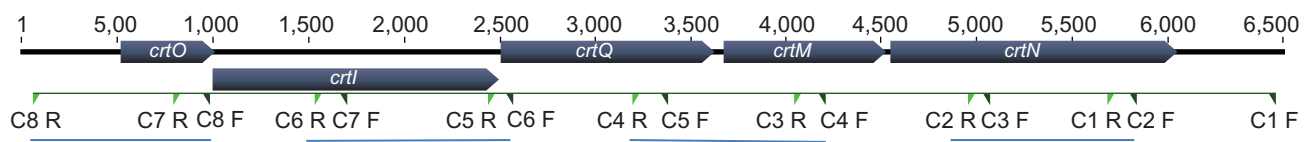


Figure 1 Positions of overlapping primers on the *crtOPQMN* operon.

Note: The green triangles indicate the position of overlapping primers used for the amplification and sequencing of the *crtOPQMN* operon.

Abbreviations: *crtOPQMN*, carotenoid biosynthetic operon; F, forward; R, reverse; *crtO*, *glycosyl-4,4'-diaponeurosporenoate acyltransferase*; *crtI*, *phytoene dehydrogenase*; *crtQ*, *4,4'-diaponeurosporenoate glycosyltransferase*; *crtM*, *dehydrosqualene synthase*; *crtN*, *dehydrosqualene desaturase*.

than mutation in the *crtOPQMN* genes or the promoter was responsible for the lack of pigment production in the white variant.

Induction of pigment production in white phenotypic variant and pigment analysis

In some bacteria, pigment production is controlled by quorum-sensing signals.^{37–39} It has also been demonstrated that *P. aeruginosa* affects gene expression in *S. aureus* through secreted quorum-signaling molecules.²⁸ We sought to determine if *P. aeruginosa* 6611 isolated along with the white *S. aureus* variant could induce pigment production in the latter. Interestingly, after 24 hours of co-culturing with *P. aeruginosa* 6611 the white *S. aureus* variant developed golden pigment, the intensity of which maximized at 48 hours. This induction of pigment was not seen when the white variant was co-cultured with bacterial strains *Escherichia coli* Mg1655, *Acinetobacter baumani* co 41-00-sc1-c1, and *Enterobacter* W001 (Figure 2). To quantify pigment production in the white variant, we carried out a co-culture experiment between *P. aeruginosa* 6611 and the white variant. We also used the pigmented yellow variant in the co-culture experiments as a positive control. Spectrophotometric measurement of absorbance (A_{465}) of crude methanol extracts of the white variant at 48 hours showed an approximately 4-fold increase in absorbance. We did not observe any such increase of absorption in crude extracts from the yellow variant. Interestingly, the

absorbance (A_{465}) of crude extracts from *S. aureus* white and yellow variants, after co-culture with *P. aeruginosa* 6611, were comparable (data not shown). This observation suggests that co-culture with *P. aeruginosa* had minimal effect on the yellow variant's pigment production. The extracted pigments were also analyzed by HPTLC, which clearly demonstrated the induction of pigment production in the white phenotypic variant after co-culture with *P. aeruginosa* 6611 (Figure 3). Therefore, the *crtOPQMN* operon in the white variant of *S. aureus* is functional and staphyloxanthin production can be induced by environmental factors such as secreted molecules from *P. aeruginosa* 6611.

Quantitative RT-PCR of genes relevant for expression and/or control of staphyloxanthin

Since the *crtOPQMN* operon of the white variant has an intact coding region and promoter, the induction of staphyloxanthin production by *P. aeruginosa* 6611 is either controlled at a step upstream of the staphyloxanthin biosynthetic pathway, or at a post-transcriptional step. We examined the expression of the *crtM* gene as well as several other genes that could control the *crtOPQMN* operon.

The expression of *crtM* did not change significantly after 1 hour and 7 hours of co-culture with *P. aeruginosa* 6611 (Figure 4). Although there was a slight increase in expression at 7 hours (~1.7-fold), this may not represent a significant change. The expression of genes coding for

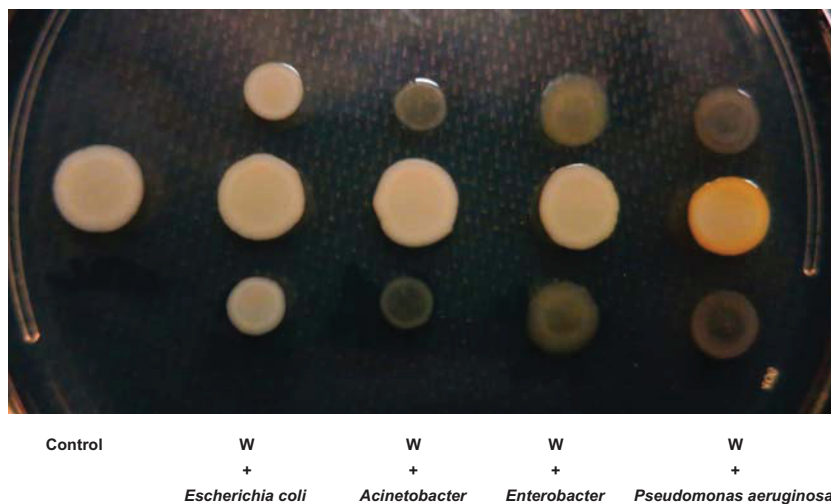


Figure 2 The effect of co-culture with different bacteria on pigment production by the *Staphylococcus aureus* white variant.

Notes: The co-culture experiment was carried out on an LB agar plate for 48 hours between the *Staphylococcus aureus* white variant (W) and *Escherichia coli* Mg1655, *Acinetobacter baumani* co 41-00-sc1-c1, *Enterobacter* W001, or *Pseudomonas aeruginosa* 6611. Only *P. aeruginosa* 6611 induced pigment production in the white variant (yellow colony).

Abbreviation: LB, Luria-Bertani.



Figure 3 HPTLC analysis of staphyloxanthin in crude methanol extract from *Staphylococcus aureus*. Crude methanol extracts of staphyloxanthin from the *S. aureus* white variant (W), the *S. aureus* white variant co-cultured for 48 hours with *Pseudomonas aeruginosa* 6611 (Wp), and the *S. aureus* yellow variant (Y) were analyzed by HPTLC on silica plates (pore size 60 Å). The results indicated a significant difference in pigment-band intensity between the white variant and the white variant co-cultured with *P. aeruginosa* 6611.

Notes: Numbers below the bands represent the absorbance of the crude methanol extracts at A_{465} (W = 0.121, Wp = 0.463, Y = 0.381). There is ~4-fold increase in absorbance in crude extracts of the white variant after co-culture with *P. aeruginosa* 6611, indicating substantial increase in pigment production.

Abbreviation: HPTLC, high performance thin layer liquid chromatography.

transcription factor sigma B (*sigB*) and global transcriptional regulator (*sarA*), both of which have been shown to positively control *crtOPQMN* operon^{10,40} also did not change significantly after the first and seventh hours. The gene for farnesyl diphosphate synthase (FPP synthase gene) is involved in the synthesis of farnesyl diphosphate, which is the substrate for the first step of the staphyloxanthin biosynthetic pathway. Expression of this gene also did not

change during the early time intervals. Post-transcriptional regulator gene (*hfq*) has recently been shown to participate in RNA-mediated inhibition of *crtOPQMN* operon transcription.⁴¹ The expression of this gene was also not affected during the first two time intervals. With the exception of *sarA*, expression of the abovementioned genes was significantly downregulated after a 24-hour co-culture with *P. aeruginosa* 6611.

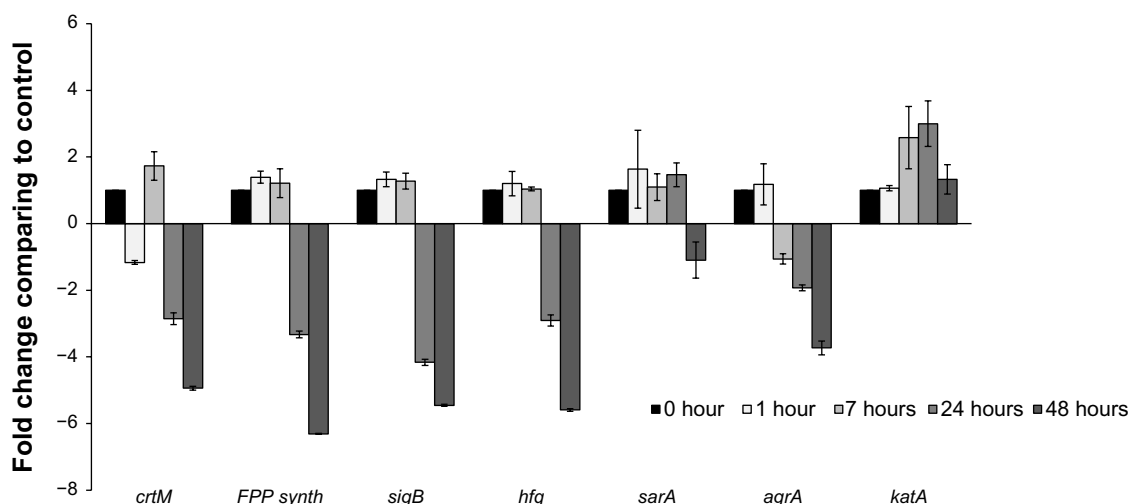


Figure 4 Transcriptional analysis of selected genes from the *Staphylococcus aureus* white variant after co-culture with *Pseudomonas aeruginosa*. Several genes involved in pigment production were analyzed by qRT-PCR after co-culture with *P. aeruginosa* 6611. These genes included *crtM*, which is directly involved in staphyloxanthin synthesis. Transcriptional factors SigB and SarA control the expression of the *crtOPQMN* operon, and product of the *hfq* gene may be involved in post-transcriptional control of the operon. FPP-synthase gene is involved in the synthesis of farnesyl-diphosphate which is the substrate for the *crtOPQMN* operon. The results indicate no significant changes in expression of these genes through the first 7 hours of co-culture. In the later time intervals of 24 hours and 48 hours, these genes (with the exception of *sarA* which was slightly downregulated) were significantly downregulated. The *agrA* gene could be involved in the downregulation of staphyloxanthin. The expression of the *katA* gene may be correlated to the production of staphyloxanthin since both confer resistance to H_2O_2 . The transcription of *agrA* is downregulated after 7 hours and into the later time intervals. In contrast, *katA* gene is significantly upregulated after 8 hours and reached its peak (more than 3-fold) at 24 hours.

Notes: The columns represent fold change in gene expression of co-cultured *S. aureus* white variant as compared to the white variant that was not co-cultured. Time points represent data gathered after 1, 7, 24, and 48 hours after the start of co-culture with *P. aeruginosa* 6611.

Abbreviations: qRT-PCR, quantitative reverse transcription polymerase chain reaction; *crtM*, carotenoid biosynthetic gene M; *sigB*, sigma B transcription factor gene; *sarA*, staphylococcal accessory regulator A gene; *FPP synth*, farnesyl pyrophosphate synthase gene; *hfq*, host factor gene for Q beta; *katA*, catalase gene A; *agrA*, accessory gene regulator A; *crtOPQMN*, carotenoid biosynthetic operon.

We also examined the expression of two other genes that code for two important virulence factors, AgrA and catalase. A component of the AgrC/AgrA two-component signal transduction system, increased expression of Staphylococcal accessory regulator gene (*agrA*) promotes dispersal of *S. aureus* biofilms,⁴² and downregulates staphyloxanthin biosynthetic genes.⁴³ Catalase is an important virulence factor which protects *S. aureus* against H₂O₂.⁴⁴ The expression of catalase could be coordinated with the expression of staphyloxanthin to provide the maximum protection against H₂O₂. Therefore, we also examined the expression of catalase in co-culture experiments. The expression of *agrA* followed a pattern similar to other genes examined and was downregulated after 7 hours. In contrast, catalase transcription was upregulated to about 2.5-fold after 7 hours and more than 3-fold after 24 hours. Both downregulation of *agrA* and upregulation of catalase can be considered to promote the survival of *S. aureus* in stressful conditions. The trend in expression of genes examined in this study could represent a global adaptive response by *S. aureus* to the presence of *P. aeruginosa* 6611, which consists of slowing of metabolism, promotion of biofilm formation, and increased resistance to oxygen free radicals.

Survival in the presence of H₂O₂

Staphyloxanthin, due to its antioxidant properties, has a role in survival in hyperoxic environments and during neutrophilic bursts.¹³ We sought to determine if pigment induction in the *S. aureus* white variant can enhance survival in the presence of H₂O₂. After co-culturing the white variant with *P. aeruginosa* 6611, the newly pigmented white variants were treated with 1% H₂O₂ and assessed for their survival as compared to the non-pigmented white variants. Our results indicated that survival of the white variant significantly improved from 8.9% ± 0.7% in absence of pigmentation to 21.8% ± 3.1% after *P. aeruginosa*-induced pigment production (Figure 5A). The role of staphyloxanthin in the protection of *S. aureus* against H₂O₂ is well established by previous independent studies,^{12,14} and our experiments served merely as confirmation of those results. Therefore, we believe our results are valid in demonstrating the importance of staphyloxanthin in the survival of bacterium in a hyperoxic environment despite only two repeats of independent experiments.

Survival in the presence of polymyxin B

Polymyxin B is a broad spectrum antibiotic that is mostly used against Gram-negative bacteria, but has also been shown to be effective against *S. aureus*.⁴⁵ Polymyxin B binds to the lipopolysaccharide layer of the bacterial cell

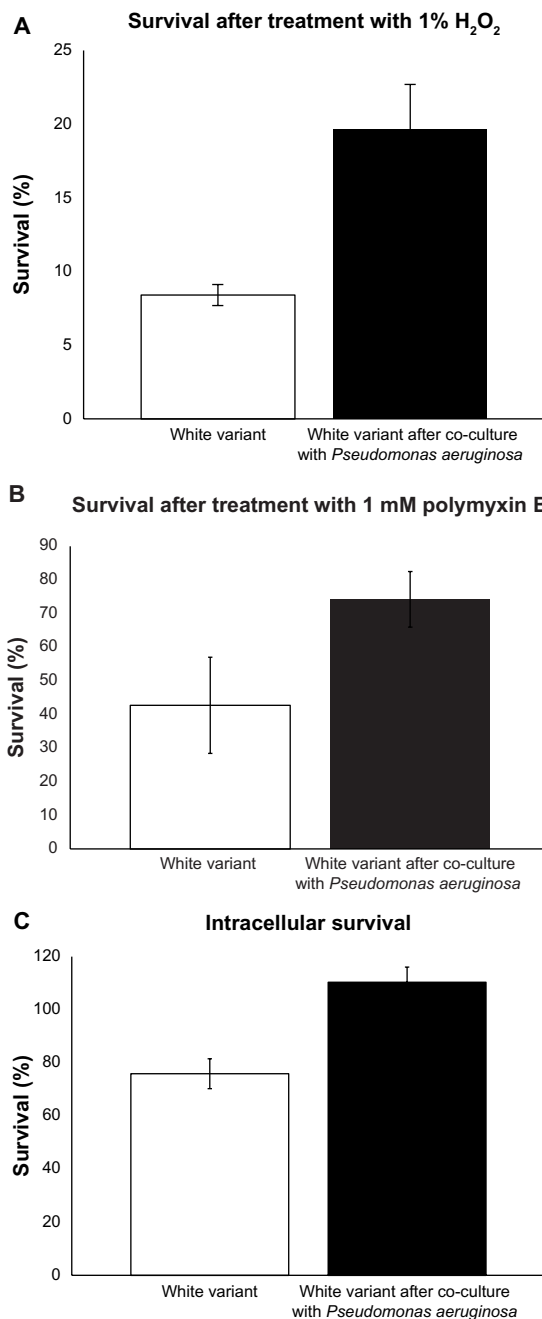


Figure 5 Induction of resistance to stress in the *Staphylococcus aureus* white variant by *Pseudomonas aeruginosa* 6611. **(A)** Co-culture with *P. aeruginosa* 6611 for 48 hours led to enhanced survival of the white variant in the presence of 1% H₂O₂ as compared to the white variant without co-culturing (21.8% ± 3.1% vs 8.9% ± 0.7%, $P < 0.05$). Induction of staphyloxanthin production and upregulation of catalase gene could account for increased resistance of the white variant to H₂O₂. **(B)** Co-culture with *P. aeruginosa* 6611 for 48 hours increased the resistance of the white variant to 1 mM polymyxin B. The difference between survival of the white variant after co-culturing and the white variant without co-culturing is almost 2-fold (75.3% ± 8.3% vs 42.7% ± 14.9%). **(C)** Co-culture with *P. aeruginosa* 6611 for 48 hours resulted in enhanced survival of the white variant within the mouse macrophage cell line J7741.A. After 2 hours of infection, the number of co-cultured white variant increased slightly (110.4% ± 4.1%). In the parallel experiment with the white variant without co-culturing, the number of survived bacteria decreased after 2 hours of infection (75.9% ± 3.9%, $P < 0.05$).

Notes: All results represent the mean from either two experiments (panel A) or three experiments (panels B and C), expressed as a percentage of the initially inoculated number of the *S. aureus* white variant (white bars) and the pigmented white variant after 48 hours of co-culture with *P. aeruginosa* 6611 (black bars). The P -value was determined using Student's t -test.

wall with its fatty acid portion inserted into the hydrophobic region of the plasma membrane. This increases membrane fluidity, allowing water to enter the cytoplasm. We hypothesized that staphyloxanthin production, due to its membrane stabilization effects, will increase bacterial survival during exposure to polymyxin B. As expected, the induction of staphyloxanthin production after co-culture with *P. aeruginosa* 6611 increased resistance of the white variant to this antibiotic. Pigmented *S. aureus* white variants had a survival rate of $75.3\% \pm 8.3\%$, as compared to $42.7\% \pm 14.9\%$ for the non-pigmented white variants (Figure 5B). Despite the almost two times higher survival rate of the pigment-induced and non-pigmented white variants, this difference failed to reach threshold for statistical difference. However, this data supports the view of staphyloxanthin stabilizing activity in the plasma membrane.

Intracellular survival

We examined the impact of *P. aeruginosa* 6611 co-culture on the white variant's survival within macrophages. The mouse macrophage cell line J7741.A was infected for 2 hours with *P. aeruginosa*-induced pigmented white variant and its survival was compared to the non-pigmented white variant. We calculated the intracellular survival rate as the percentage of the initial (T0) number of bacteria that survived after 2 hours (T1). Our results showed $110.4\% \pm 4\%$ intracellular survival of pigmented bacteria as compared to $75.9\% \pm 3.9\%$ for the non-pigmented bacteria ($P < 0.05$) (Figure 5C). The 110% represents an approximately 10% increase in the population of pigmented white variant after 2 hours within the intracellular environment of macrophages. These results suggest that *S. aureus* white variant co-cultured with *P. aeruginosa* 6611, not only survived better than the control white variant, but may have also divided within the macrophages during the 2-hour infection period. The data from this experiment indicates a clear intracellular survival advantage for *S. aureus* white variant after co-culture with *P. aeruginosa* 6611.

Discussion

Interspecies interactions play a significant role in wound infections since these infections are commonly polymicrobial with constant physical and chemical communication between the microbes. Modulation of gene expression as a result of this interaction could have great impact on the pathogenicity of microbes and consequently clinical outcomes of the infection. This study demonstrates the importance of such interspecies communication with respect to pathogenic phenotypes of coinfecting bacteria.

P. aeruginosa, which is often isolated along with *S. aureus* from wound infections,⁴⁶ induced the production of at least two virulence factors, staphyloxanthin and catalase, in a white variant of *S. aureus*. This had a direct effect on its resistance to stress and survival within host macrophages. Golden pigment staphyloxanthin and catalase enzyme are both antioxidative defense mechanisms that confer survival advantage during infection. Staphyloxanthin also provides protection against membrane-disrupting antimicrobial peptides.

An important finding in this study was that a naturally occurring white variant of *S. aureus* carries an intact and functional *crtOPQMN* operon for the synthesis of golden pigment staphyloxanthin. The fact that this operon does not produce staphyloxanthin in the absence of environmental stimuli (eg, the molecules originated from *P. aeruginosa*) indicates an intricate regulatory mechanism in the control of pigment production. More interesting may be the mechanism of this control system. We examined the transcription of several genes that are directly or indirectly involved in the synthesis of staphyloxanthin. These included the *crtM* gene that mediates the first reaction in the staphyloxanthin biosynthetic pathway, as well as transcription factors *sigB* and *sarA* genes which positively control its expression. *P. aeruginosa*-induction of pigment production in the *S. aureus* white variant did not lead to upregulation in the transcription of *crtM* or the two transcription factors. This finding is contrary to previous studies that reported increased transcription of *sigB* after exposure to signals from *P. aeruginosa*.²⁸ This discrepancy between our results and those of others may be partly due to distinct bacterial strains used in different studies. Furthermore, in choosing time points of 1, 7, 24, and 48 hours after the addition of *P. aeruginosa* 6611 in co-culture experiments, we may have missed transient upregulation of genes involved in staphyloxanthin biosynthesis.

We also explored the possibility that synthesis of the upstream substrate for staphyloxanthin synthesis could be affected by *P. aeruginosa*. The substrate of the first enzymatic reaction for *S. aureus* staphyloxanthin biosynthesis is (2E,6E)-farnesyl diphosphate, which itself is synthesized from isopentenyl diphosphate through a 3-step enzymatic process. Farnesyl-diphosphate synthase, which also has geranyltransferase activity, carries out the last two steps of this process. Examination of farnesyl-diphosphate synthase mRNA transcript indicated no upregulation after 7 hours of incubation of the *S. aureus* white variant with *P. aeruginosa* 6611. Furthermore, after 24 hours, transcription of the gene coding for this enzyme was significantly

decreased, as was the case with most other genes analyzed in this study.

Control and modulation of gene expression can also be accomplished by post-transcriptional mechanisms. In *E. coli*, the Hfq protein mediates interaction between some small RNA species and their target mRNAs, which could affect mRNA translation. In a recent study, Liu et al showed that deletion of the *hfq* gene in a strain of *S. aureus* led to increased expression of surface pigment.⁴⁷ It appears that in certain strains of *S. aureus*, Hfq may directly interact with the target mRNA, thereby controlling gene expression at the level of translation. We did not see any significant decrease in transcription of the *hfq* gene in the white *S. aureus* variant after 7 hours of incubation with *P. aeruginosa* 6611. However, at the 24- and 48-hour time intervals, there was considerable decrease in transcription of most of the genes examined, including the *hfq* gene. Since *P. aeruginosa*-induced pigmentation appears after 24 hours, we cannot rule out a role for Hfq in mediating this outcome. Therefore, the exact mechanism by which *P. aeruginosa* 6611 controls staphyloxanthin production in the white *S. aureus* variant remains to be elucidated.

We also examined the expression of AgrA, (a part of the AgrC/AgrA two-component signal transduction system) which positively regulates the dispersal of *S. aureus* biofilms⁴² and downregulates the expression of staphyloxanthin.⁴⁴ Expression of the *agrA* gene did not change during the first 7 hours of co-culture with *P. aeruginosa* 6611. The expression of *agrA* was, however, significantly downregulated after 24 hours and correlated to upregulation of pigment production. Therefore, downregulation of the *agrA* gene in presence of *P. aeruginosa* 6611, may promote pigment production in the white variant.

Catalase gene, is considered an important virulence factor which protects *S. aureus* against H₂O₂.⁴³ Since both catalase and staphyloxanthin protect the microorganisms against the same stress condition, it is plausible that their expression would be coordinated. Interestingly, the transcription of the catalase gene of the white *S. aureus* variant was significantly upregulated after 7 hours of co-culture with *P. aeruginosa* 6611 and peaked after 24 hours. Although the transcription of the catalase gene contrasts that of other genes, it is consistent with the trend in the adaptation of the white variant to a more H₂O₂-resistant phenotype. This bacterium appears to responds to *P. aeruginosa* 6611 stimulation by changing its gene expression to acquire a more stress-resistant phenotype, enabling it to withstand oxidative stress, as well as the intracellular environment of macrophages.

Although our results demonstrating the antioxidant role of staphyloxanthin is consistent with those of other authors, it is important to draw a clear distinction between these studies. In other studies, gene knockout experiments¹³ and antagonist drugs targeting *crtOPQMN* operon^{17,36,48} were used to show the importance of staphyloxanthin for the survival of *S. aureus* in hyperoxidative environments. In our study, the *S. aureus* white variant was a naturally occurring bacterium isolated from a clinical sample, rather than being artificially constructed by genetic manipulation or by the administration of antagonistic drugs. Furthermore, our study demonstrates an important example of interspecies interaction that may be occurring in polymicrobial infections.

The medical community is beginning to recognize the significance of polymicrobial infections and the role of the human-associated microbial community in promoting or averting such infections. Many therapies are now taking into account the polymicrobial etiology of diseases and the attendant implications for treatment and prevention.²³ Our results demonstrate that *P. aeruginosa* 6611 can induce transcriptional changes in *S. aureus*, which contribute to its virulence and intracellular survival. We show that a phenotypically white variant of *S. aureus* carries a functional apparatus for pigment synthesis, which can be activated by a co-infecting *P. aeruginosa* 6611, presumably via secreted signals. Additionally, another *S. aureus* virulence factor, catalase, can also be induced as a result of this interspecies interaction. To the best of our knowledge, this is the only example in the literature thus far demonstrating interaction between co-infecting *S. aureus* and *P. aeruginosa* which could impact the virulence and intracellular survival of at least one of them. Further investigation of interactions between *S. aureus* and *P. aeruginosa* will provide deeper understanding of the mechanisms that control their virulence.

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Disclosure

The authors report no conflicts of interest in this work.

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