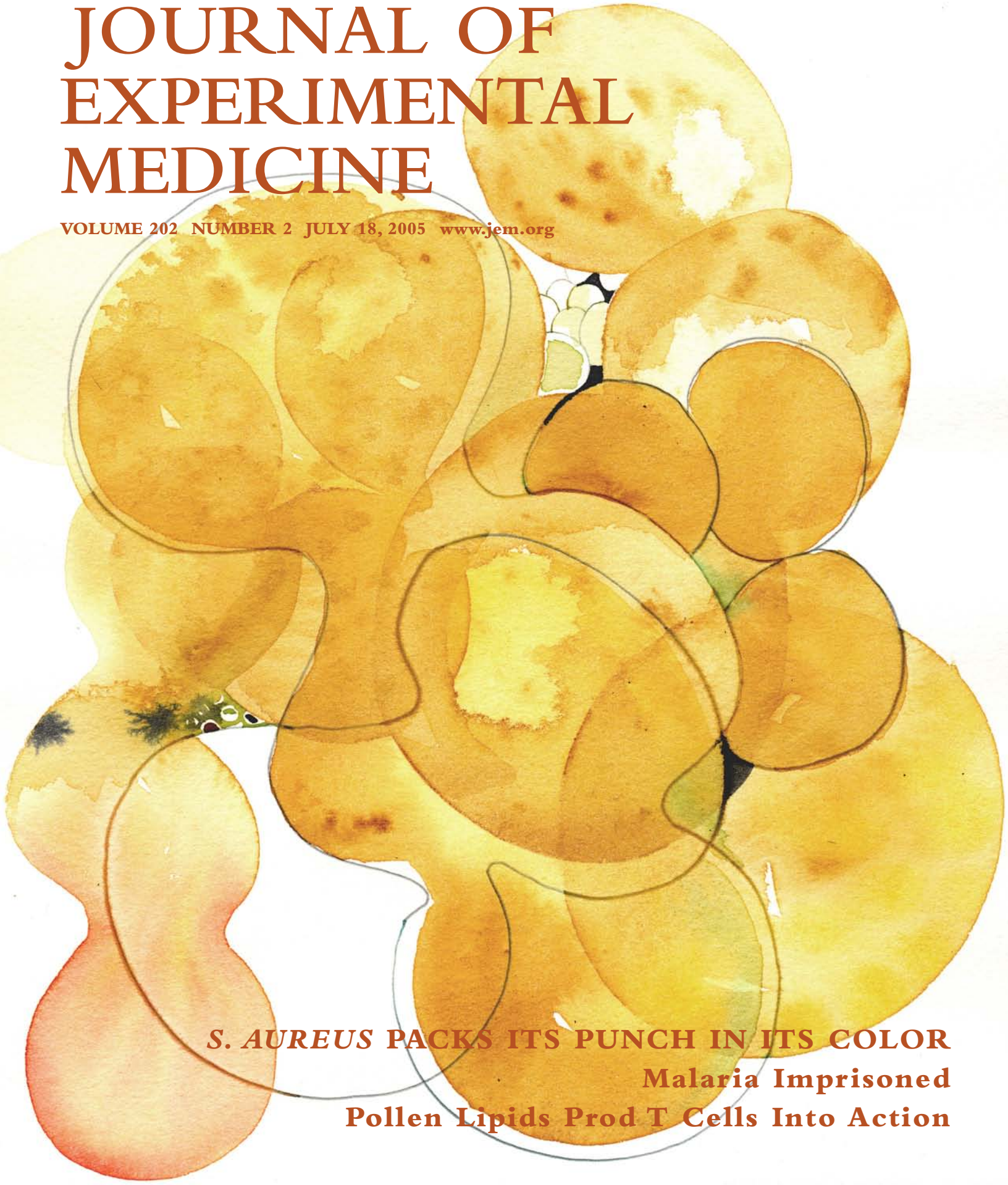
THE JOURNAL OF EXPERIMENTAL MEDICINE

VOLUME 202 NUMBER 2 JULY 18, 2005 www.jem.org



S. AUREUS PACKS ITS PUNCH IN ITS COLOR

Malaria Imprisoned

Pollen Lipids Prod T Cells Into Action

Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity

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Golden color imparted by carotenoid pigments is the eponymous feature of the human pathogen *Staphylococcus aureus*. Here we demonstrate a role of this hallmark phenotype in virulence. Compared with the wild-type (WT) bacterium, a *S. aureus* mutant with disrupted carotenoid biosynthesis is more susceptible to oxidant killing, has impaired neutrophil survival, and is less pathogenic in a mouse subcutaneous abscess model. The survival advantage of WT *S. aureus* over the carotenoid-deficient mutant is lost upon inhibition of neutrophil oxidative burst or in human or murine nicotinamide adenine dinucleotide phosphate oxidase–deficient hosts. Conversely, heterologous expression of the *S. aureus* carotenoid in the nonpigmented *Streptococcus pyogenes* confers enhanced oxidant and neutrophil resistance and increased animal virulence. Blocking *S. aureus* carotenogenesis increases oxidant sensitivity and decreases whole-blood survival, suggesting a novel target for antibiotic therapy.

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Ogston coined the genus *Staphylococcus* to describe grapelike clusters of bacteria (*staphylo* means *grape* in Greek) recovered in pus from surgical abscesses (1). Shortly thereafter, Rosenbach isolated the major human pathogen in pure culture and proposed the species name *S. aureus* (golden, in Latin) for its characteristic surface pigmentation in comparison with less virulent staphylococci that normally colonize the skin surface (2). Subsequent studies of the *S. aureus* pigment have unraveled an elaborate biosynthetic pathway that produces a series of carotenoids (3). Similar carotenoids produced in dietary fruits and vegetables are well recognized as potent antioxidants by virtue of their free-radical scavenging properties and exceptional ability to quench singlet oxygen (4, 5). We hypothesized that *S. aureus* could utilize its golden carotenoid pigment to resist oxidant-based clearance mechanisms of the host innate immune system. Here we apply a molecular genetic approach of targeted mutagenesis and heterologous expression, coupled with in vitro and in vivo models of bacterial pathogenesis, to demonstrate that the *S. aureus* pigment is a virulence factor and potential novel target for antimicrobial therapy.

The online version of this article contains supplemental material.

RESULTS AND DISCUSSION

Mutagenesis, complementation, and heterologous expression of *S. aureus* pigment

The biosynthetic pathway for *S. aureus* carotenoids (6) includes the essential functions of genes *crt*M and *crt*N, encoding dehydrosqualene synthase and dehydrosqualene desaturase, respectively (Fig. 1 A). To probe the biological activities of the *S. aureus* pigment, we generated an isogenic mutant of a golden-colored human clinical isolate by allelic replacement of *crt*M (Fig. 1 A). Consistent with previous reports, pigmentation of the WT strain became apparent in the early stationary phase of growth and continued to intensify before reaching a plateau at 36–48 h (Fig. 1 B). The ΔCrtM mutant was nonpigmented and lacked the characteristic triple-peak spectral profile of WT carotenoid at 440-, 462-, and 491-nM wavelengths (Fig. 1 B). No differences in growth rate, stationary

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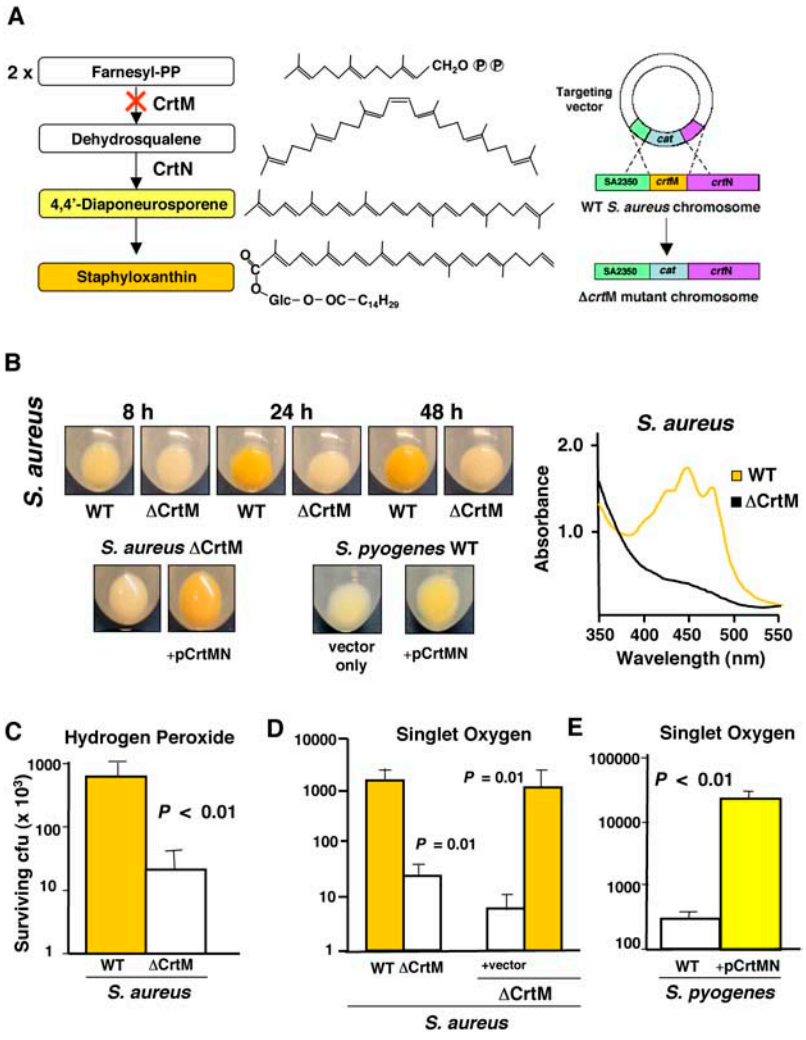

Figure 1. Genetic manipulation of *S. aureus* carotenoid pigment and its antioxidant function. (A) Biochemical pathway for *S. aureus* carotenogenesis and mutagenesis of crtM (encoding dehydrosqualene synthase) by allelic replacement. (B) Elimination of *S. aureus* pigmentation in Δ*crt*M mutant; heterologous expression of *S. aureus* 4,4′-diaponeurosporene pigment in *Streptococcus pyogenes*. Increased susceptibility of the *S. aureus* ΔCrtM mutant to killing by (C) hydrogen peroxide or (D) singlet oxygen, with restoration of WT resistance levels upon complementation with pCrtMN. (E) Decreased singlet oxygen susceptibility of *S. pyogenes* expressing 4,4′-diaponeurosporene. Results shown are representative of at least three repeated experiments. Error bars, SD of depicted variable performed in duplicate or triplicate.

phase density, surface charge, buoyancy, or hydrophobicity were observed between WT and ΔCrtM *S. aureus* (Fig. S1, A–D, available at http://www.jem.org/cgi/content/full/jem.20050846/DC1). *S. aureus crt*M and *crt*N together are sufficient for production of 4,4′-diaponeurosporene (3). To facilitate gain of function analyses, we expressed both genes in the nonpigmented *Streptococcus pyogenes*, a human pathogen associated with a disease spectrum similar to that of *S. aureus*. When transformed with the pCrtMN plasmid, *S. pyogenes* gained yellow pigmentation (Fig. 1 B) with the spectral characteristics of a carotenoid (not depicted). Complementation of the *S. aureus* ΔCrtM mutant with pCrtMN vector also fully restored pigmentation (Fig. 1 B).

S. aureus pigment functions as an antioxidant

One important mechanism by which phagocytic cells eliminate pathogens is through release of reactive oxygen species generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (7). It has been suggested that bacterial carotenoids such as those expressed by *S. aureus* could serve a protective function against these defense molecules (8–10). To prove this thesis experimentally, we first compared the susceptibility of WT and ΔCrtM *S. aureus* to oxidants in vitro. As shown in Fig. 1, C and D, the ΔCrtM mutant was killed more efficiently by hydrogen peroxide and singlet oxygen compared with the WT *S. aureus* strain. Complementation with pCrtMN restored the ability of the ΔCrtM mutant to resist singlet oxygen killing (Fig. 1 D). Similarly,

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heterologous expression of staphylococcal pigment in *S. pyogenes* led to a significant decrease in susceptibility to singlet oxygen (Fig. 1 E).

S. aureus pigment confers resistance to neutrophil and whole-blood killing

We next sought to determine whether the observed antioxidant activity of the *S. aureus* carotenoid translated to increased bacterial resistance to innate immune clearance using two ex vivo assay systems: human or mouse whole-blood survival and coculture with purified human neutrophils. WT *S. aureus* survived significantly better than the nonpigmented ΔCrtM intracellularly within human neutrophils (Fig. 2 A and Fig. S2 F, available at http://www.jem.org/cgi/content/full/jem.20050846/DC1) and in whole blood of normal mice or human donors (Fig. 2, B and E). The former effect was not explained by differences in the rate of phagocytosis, because uptake of the WT *S. aureus* and ΔCrtM mutant was comparable (Fig. S2 A). Nor were differences attributable to changes in the magnitude of neutrophil oxidative burst, because uptake of WT and mutant strains produced similar results in a nitroblue tetrazolium reduction assay (Fig. S2 B). Complementation of the *S. aureus* ΔCrtM mutant with pCrtMN restored resistance to killing by mouse whole blood (Fig. 2 B). Likewise, the pigmented *S. pyogenes* expressing staphylococcal carotenoid showed enhanced survival in human neutrophils versus the parent strain (Fig. 2 C).

To verify that the association of *S. aureus* carotenoid expression with enhanced phagocyte resistance was a direct consequence of its antioxidant properties, assays were repeated in the presence of the oxidative burst inhibitor diphenyleneiodonium (DPI). WT and ΔCrtM *S. aureus* survived equally well in human neutrophils (Fig. 2 D) and mouse blood (Fig. S2 C) when oxidative burst was inhibited by DPI. Gp47$^{Phox-/-}$ is an inherited defect in phagocyte oxidative burst function commonly found in patients who have chronic granulomatous disease (CGD), and the gp91$^{Phox-/-}$ mouse represents a model of human X-linked CGD (11). The survival advantage of WT over nonpigmented ΔCrtM *S. aureus* was evident only in the blood of normal humans and mice (CD1 or C57Bl/6), and not in the blood of a human gp47$^{phox-/-}$ patient or gp91$^{Phox-/-}$ mice lacking NADPH oxidase activity (Fig. 2, E and F).

It was recently reported that the apparent neutrophil killing of pathogens by reactive oxygen species could largely reflect the activation of granule proteases mediated through changes in potassium flux (12). We found no difference in the susceptibility of WT and ΔCrtM *S. aureus* to the antimicrobial action of cathepsin G, and both strains were resistant to human neutrophil elastase as previously observed for *S. aureus* (13) (Fig. S2 D). Other effector molecules of mammalian neutrophils critical to innate immune defense are the cathelicidin family of antimicrobial peptides (14). The carotenoid-deficient *S. aureus* mutant was equally susceptible to killing by the murine cathelicidin mCRAMP when compared with the WT strain (Fig. S2 E). These results support a primary role for the free-radical scavenging antioxidant properties of the *S. aureus* carotenoid in resistance to neutrophil-mediated killing.

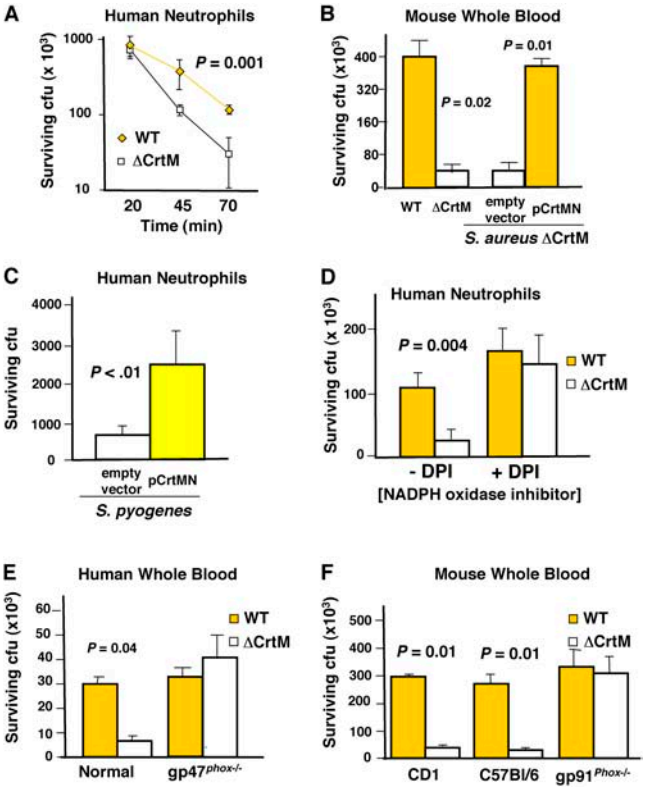

Figure 2. *S. aureus* carotenoid pigment confers resistance to oxidant killing in neutrophils and whole blood. Survival of WT and ΔCrtM *S. aureus* in (A) coculture with isolated human neutrophils and (B) murine whole blood. Also shown in B is whole-blood survival of ΔCrtM complemented with vector alone or pCrtMN. (C) Effect of plasmid expression of *crt*MN on survival of *Streptococcus pyogenes* in mouse whole blood. (D) Effect of oxidative burst inhibitor DPI on survival of WT and ΔCrtM mutant *S. aureus* human neutrophil coculture. Relative survival of WT and ΔCrtM mutant *S. aureus* is shown in (E) normal human and gp47$^{phox-/-}$ patient lacking NADPH oxidase function or (F) the blood of WT CD1 and C57Bl/6 mice and gp91$^{Phox-/-}$ mice. Results shown are representative of at least three repeated experiments, except for assay using blood from the CGD human patient, which was performed twice. Error bars, SD of depicted variable performed in duplicate or triplicate.

S. aureus pigment contributes to virulence in a subcutaneous abscess model

Our in vitro and ex vivo results demonstrate that *S. aureus* carotenoid is both necessary and sufficient to promote oxidant resistance and phagocyte survival. To assess the significance of these observations to disease pathogenesis, we used a murine subcutaneous challenge model. In these studies, individual animals were injected simultaneously in one flank with the WT *S. aureus* strain and in the opposite flank with the ΔCrtM mutant. At the site of WT injection (10^6 CFU), mice developed size-

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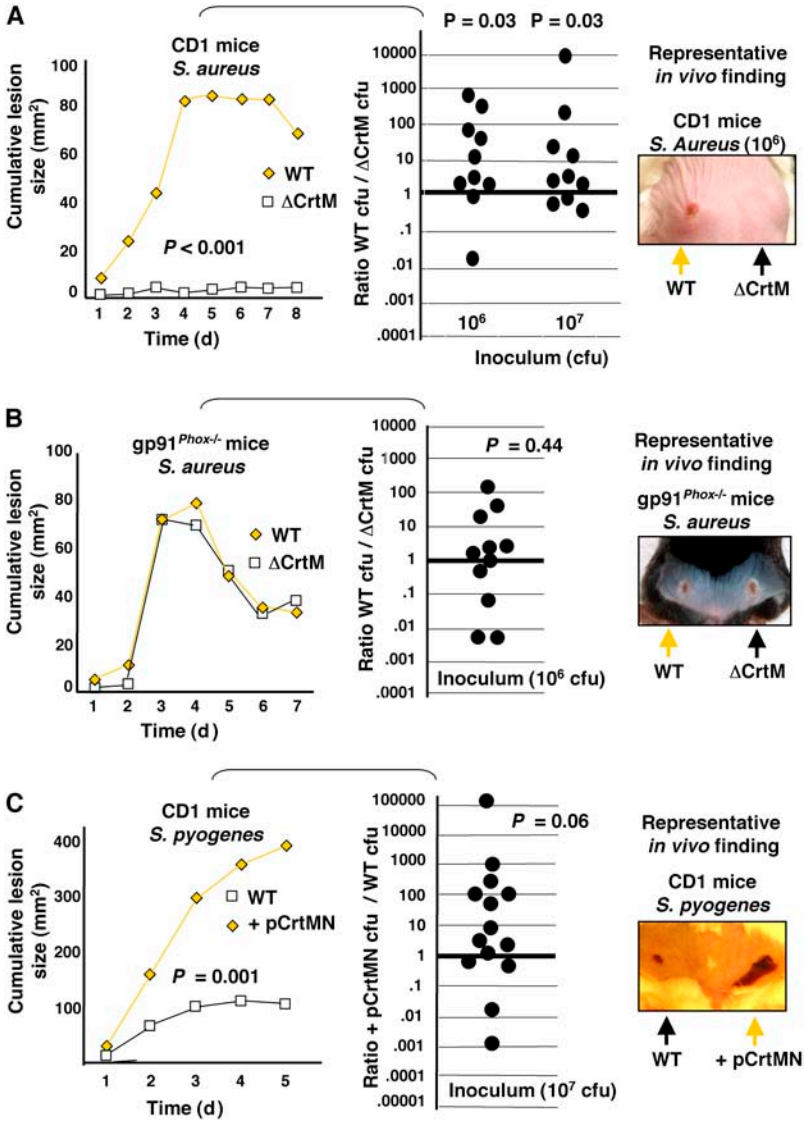

Figure 3. *S. aureus* carotenoid contributes to virulence in a subcutaneous abscess model. Mice were injected subcutaneously in opposite flanks with the two bacterial strains under comparison. Line graphs depict sum cumulative skin lesion size generated by the indicated bacterial strain. Dots on scatter graphs indicate ratio of CFU of pigmented versus nonpigmented strains recovered from skin lesions in each individual mouse. Photographic image depicts representative mouse in each treatment group. (A) WT versus ΔCrtM mutant *S. aureus* in CD1 mice. (B) WT versus ΔCrtM mutant *S. aureus* in gp91$^{Phox-/-}$ mice. (C) *Streptococcus pyogenes* ± expression of staphylococcal 4,4′-diaponeurosporene.

able abscess lesions reaching a cumulative size of 80 mm^2 by d 4; injection of an equivalent inoculum of the carotenoid-deficient mutant on the contralateral flank failed to produce visible lesions (Fig. 3 A). Quantitative culture from skin lesions at two different challenge doses (10^6 CFU to 10^7 CFU) consistently demonstrated significantly higher numbers of surviving WT *S. aureus* compared with the ΔCrtM mutant in the individual mice (Fig. 3 A). To corroborate that an antioxidant effect is key to the mechanism of protection afforded by the *S. aureus* carotenoid in vivo, the subcutaneous infection experiment was repeated in gp91$^{Phox-/-}$ mice. In the absence of host NADPH oxidase function, WT and ΔCrtM mutant *S. aureus* produced lesions of similar cumulative size, and no survival advantage was detected on quantitative abscess culture (Fig. 3 B). Finally, we asked whether *S. aureus* carotenoid was sufficient to enhance bacterial virulence by comparing the course of infection produced by *S. pyogenes* expressing CrtMN with that in controls transformed with vector alone. As shown in Fig. 3 C, lesions generated by the carotenoid-expressing strain were significantly larger and contained greater numbers of surviving bacteria than those produced by the WT strain. Raw data from the in vivo experiments are provided in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20050846/DC1).

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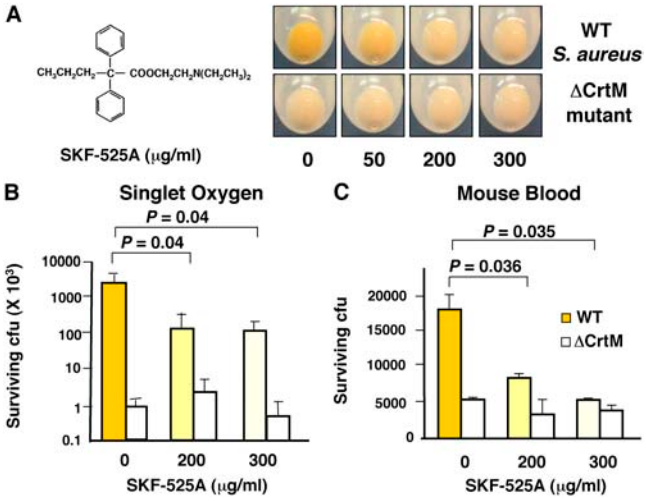

Figure 4. Inhibition of *S. aureus* pigment production increases oxidant sensitivity and phagocytic clearance. WT and ΔCrtM mutant *S. aureus* were cultured in the presence or absence of SKF 525-A at the indicated concentrations. Depicted are the observed effects on (A) pigmentation phenotype, (B) singlet oxygen susceptibility, and (C) survival in murine whole blood. Results shown are representative of at least three repeated experiments. Error bars, SD of depicted variable performed in duplicate or triplicate.

S. aureus pigment production is a potential novel target for antimicrobial therapy

Given the protective effect provided to the bacteria by the golden yellow pigments, we examined whether a pharmacologic agent that inhibited carotenogenesis might render *S. aureus* more susceptible to immune clearance. The mixed function oxidase inhibitor 2-diethylaminoethyl-2,2-diphenyl-valerate (SKF 525-A; Calbiochem) was previously shown to inhibit pigment formation in *S. aureus* (15), although a moderate residual accumulation of δ carotenoid intermediate was noted in those experiments. As shown in Fig. 4 A, we found a dose-dependent decrease in pigment production in our WT strain of *S. aureus* grown in the presence of this agent. Blocking *S. aureus* pigment formation led to a dose-dependent increase in the susceptibility of the organism to singlet oxygen killing (Fig. 4 B) and a decrease in the ability of WT S. *aureus* to survive in murine whole blood (Fig. 4 C). As a control, the ΔCrtM mutant was exposed to SKF 525-A in parallel experiments with no significant effects on oxidant susceptibility or blood survival (Fig. 4, B and C).

Golden color imparted by carotenoid pigments is the eponymous feature of the human pathogen *S. aureus*. We have used a molecular genetic analysis pairing mutagenesis and heterologous expression to show that this hallmark phenotype is in fact a virulence factor, serving to protect the bacterium from phagocytic killing through its antioxidant properties. In the present era, effective control of this important disease agent is compromised by rapid evolution of antimicrobial resistance in both community and hospital settings (16, 17). In principle, the inhibition of carotenogenesis may offer a novel therapeutic approach to the treatment of complicated *S. aureus* infections, effectively rendering the pathogen more susceptible to clearance by normal host innate immune defenses.

MATERIALS AND METHODS

Bacteria, mice, and chemical reagents. WT *S. aureus* strain (Pig1), isolated from the skin of a child with atopic dermatitis, was a gift from D. Leung (National Jewish Medical and Research Center, Denver, CO). *S. pyogenes* strain 5448 is a well-characterized serotype M1T1 clinical isolate (18). CD1 and C57Bl/6 mice were purchased from Charles River Laboratories. The gp91$^{Phox-/-}$ mice were bred at the Veteran's Administration Medical Center, San Diego, CA, and maintained on trimethoprim/sulfamethoxazole prophylaxis until 3 d before experiments. *S. aureus* and *S. pyogenes* were propagated in Todd-Hewitt broth (THB) or on THB agar (Difco). Unless otherwise indicated, all experiments were performed with bacteria derived from light-protected *S. aureus* 36–48-h stationary phase cultures or *S. pyogenes* 24-h stationary phase cultures, a point at which pigmentation phenotypes were readily apparent.

Human CGD patient. The human CGD patient was an 18-yr-old female with a gp47^{phox} deficiency (homozygous ΔGT deletion in exon 2). At the time of study, she was in good health, and her only medication was IFNγ (50 μg/m^2) administered three times per wk by subcutaneous injection.

Generation of the carotenoid-deficient *S. aureus* mutant, ΔCrtM. Precise, in-frame allelic replacement of the *S. aureus crt*M gene with a chloramphenicol acetyltransferase (*cat*) cassette was performed using PCR-based methods as described for *S. pyogenes* (19) or *Streptococcus agalactiae* (20), with minor modifications. Primers were designed based on the published *S. aureus* crtMN sequence (6) cross-referenced to genome *S. aureus* strain N315 (21). PCR was used to amplify ~500 bp upstream of *crt*M with primers *crt*MupF 5′-TTAGGAAGTGCATATACTTCAC-3′ and *crt*MstartR 5′-GGTGGTATATCCAGTGATTTTTTTCTCCATAC-TAGTCCTCCTATATTGAAATG-3′, along with ~500 bp of sequence immediately downstream of *crt*M with primers *crt*MendF 5′-TAC-TGCGATGAGTGGCAGGGCGGGGCGTAACAAAGTATTTAGTA-TTGAAGC-3′ and *crt*MdownR 5′-GGCACCGTTATACGATCATCGT-3′. The *crt*MstartR and *crt*MendF primers were constructed with 25-bp 5′ extensions corresponding to the 5′ and 3′ ends of the *cat* gene, respectively. The upstream and downstream PCR products were then combined with a 650-bp amplicon of the complete *cat* gene (from pACYC184) as templates in a second round of PCR using primers *crt*MupF and *crt*M-downR. The resultant PCR amplicon, containing an in-frame substitution of *crt*M with *cat*, was subcloned into temperature-sensitive vector pHY304 to create the knockout plasmid. This vector was transformed initially into permissive *S. aureus* strain RN4220 (provided by P. Sullam (Veteran's Affairs Medical Center, San Francisco, CA) and then into *S. aureus* strain Pig1 by electroporation. Transformants were grown at 30°C, shifted to the nonpermissive temperature for plasmid replication (40°C), and differential antibiotic selection and pigment phenotype were used to identify candidate mutants. Allelic replacement of the crtM allele was confirmed unambiguously by PCR reactions documenting targeted insertion of *cat* and absence of crtM in chromosomal DNA isolated from the final mutant ΔCrtM.

Complementation and heterologous expression studies. Primers CrtF 5′-CAGTCTAGAAATGGCATTTCAATATAGGAG-3′ and CrtR 5′-ATCGAGATCTCTCACATCTTTCTCTTAGAC-3′ were used to amplify the contiguous CrtM and CrtN genes from the chromosome of WT *S. aureus* strain Pig1. The fragment was directionally cloned into the shuttle expression vector pDCerm (19) and the recombinant plasmid (pCrtMN) used to transform by electroporation the *S. aureus* ΔCrtM mutant and *S. pyogenes* strain 5448. All stocks of pCrtMN-transformed *S. pyogenes* were destroyed by autoclaving at the conclusion of the project.

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Spectral profile of the *S. aureus* carotenoid. Stationary phase (48-h) cultures of WT *S. aureus* Pig1 and its isogenic ΔCrtM mutant were subjected to methanol extraction. The absorbance profile of the extracts was measured with a MBA 2000 spectrophotometer (PerkinElmer).

Oxidant susceptibility assays. Tests for susceptibility to oxidants were performed either in PBS (*S. aureus*) or THB (*S. pyogenes*). Hydrogen peroxide (H_2O_2) was added to 1.5% final concentration, 2×0^9 bacteria were incubated at 37°C for 1 h, and then 1,000 U/ml of catalase (Sigma-Aldrich) was added to quench residual H_2O_2. Dilutions were plated on Todd-Hewitt agar (THA) for enumeration of surviving CFU. For the singlet oxygen assay, 10^8 S. *aureus* or 4×10^8 S. *pyogenes* were incubated at 37°C in individual wells of a 24-well culture plate in the presence or absence of 1–6 μg/ml methylene blue and situated exactly 10 cm from a 100-W light source. Bacterial viability was assessed after 1–3 h by plating dilutions on THA. Control plates handled identically but wrapped in foil or exposed to light in the absence of methylene blue did not show evidence of bacterial killing.

Whole-blood killing assays. Bacteria were washed twice in PBS, diluted to an inoculum of 10^4 CFU in 25 μl PBS, and mixed with 75 μl of freshly drawn human or mouse blood in heparinized tubes. The tubes were incubated at 37°C for 4 h with agitation, at which time dilutions were plated on THA for enumeration of surviving CFU.

Neutrophil intracellular survival assays. Neutrophils were purified from healthy human volunteers using a Histopaque gradient (Sigma-Aldrich) per manufacturer's directions. Intracellular survival assays were performed as follows. Bacterial cultures were washed twice in PBS, diluted to a concentration of 4.5×10^6 CFU in 100 μl RPMI 1640 + 10% FCS and mixed with 3×10^5 neutrophils in the same media (multiplicity of infection = 15:1), centrifuged at 700 *g* for 5 min, then incubated at 37°C in a 5% CO_2 incubator. Gentamicin (final concentration 400 μg/ml for *S. aureus* and 100 μg/ml for *S. pyogenes*; GIBCO BFL) was added after 10 min to kill extracellular bacteria. At specified time points, the contents of sample wells were withdrawn, centrifuged to pellet the neutrophils, and washed to remove the antibiotic medium. Neutrophils were then lysed in 0.02% Triton X, and CFU were calculated by plating on THA. Several assays were repeated with the addition of a step involving preincubation of the bacterial inoculum with 10% autologous human serum for 15 min on ice.

Murine model of subcutaneous infection. 10–16-wk-old CD-1 or $gp91^{Phox-/-}$ mice were injected subcutaneously in one flank (chosen randomly) with the bacterial test strain and simultaneously in the opposite flank with a different strain for direct comparison. Bacterial cultures were washed, diluted, and resuspended in PBS mixed 1:1 with sterile Cytodex beads (GE Healthcare) at the specified inoculum, following an established protocol for generating localized *S. aureus* and *S. pyogenes* subcutaneous infection (14, 22). Lesion size, as assessed by the maximal length × width of the developing ulcers, was recorded daily. Cumulative lesion size represents the total sum of lesion sizes from all animals in each treatment group on a given day. At day 8 (*S. aureus*) or day 5 (*S. pyogenes*), animals were killed, and skin lesions were excised, homogenized in PBS, and plated on THA for quantitative culture.

Statistics. The significance of experimental differences in oxidant sensitivity, blood killing, and neutrophil survival were evaluated by unpaired Student's *t* test. Results of the mouse in vivo challenge studies were evaluated by paired Student's *t* test.

Assurances. All animal experiments were approved by the University of California, San Diego (UCSD) Committee on the Use and Care of Animals and performed using accepted veterinary standards. Experimentations using human blood were approved by the Dual Tracked UCSD Human Research Protection Program/CHSD IRB. Prior informed consents were obtained from the human subjects. Experimental protocols were approved by the UCSD Biosafety Committee.

Online supplemental material. Fig. S1 provides basic characterization of the WT and ΔCrtM mutant *S. aureus* isolates in terms of growth rate, buoyancy, hydrophobicity, and surface charge. Fig. S2 contains data comparing WT and DCrtM mutant *S. aureus* with respect to rate of phagocytic uptake in neutrophils, induction of neutrophil oxidative burst, mouse whole-blood survival with and without NADPH oxidase inhibition, sensitivity to granule proteases and cationic antimicrobial peptides, and survival in human neutrophils after preopsonization. Table S1 contains detailed data on lesion size and bacterial counts from the in vivo mouse challenge experiments shown in Fig. 3. A brief supplemental Materials and methods section is provided for those experiments appearing only in the supplemental figures. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050846/DC1.

This work was supported by a Howard Hughes Medical Institute Fellowship and Burroughs–Wellcome Career Award (to G.Y. Liu), an Edward J. Mallinckrodt, Jr., Scholar Award (to V. Nizet), and National Institutes of Health grant no. AI048694 (to V. Nizet).

The authors have no competing financial interests.

Submitted: 28 April 2005
Accepted: 1 June 2005

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