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

George Y. Liu, Anthony Essex, John T. Buchanan, Vivekanand Datta, Hal M. Hoffman, John F. Bastian, Joshua Fierer, and Victor Nizet

1Department of Pediatrics, 2Department of Cellular and Molecular Medicine, and 3Department of Medicine, University of California San Diego, La Jolla, CA 92093
4Department of Pediatrics, Children's Hospital and Health Center, San Diego, CA 92123
5VA San Diego Healthcare System, San Diego, CA 92161

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.

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.

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 crtM and crtN, encoding dehydroquinate synthase and dehydroquinate 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 crtM (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 ∆CrtM 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...
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 crtM and crtN together are sufficient for production of 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.

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,
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<sup>−/−</sup> is an inherited defect in phagocyte oxidative burst function commonly found in patients who have chronic granulomatous disease (CGD), and the gp91<sup>−/−</sup> 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<sup>−/−</sup> patient or gp91<sup>−/−</sup> 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.

**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<sup>6</sup> CFU), mice developed size-
able abscess lesions reaching a cumulative size of 80 mm² 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 (10⁶ CFU to 10⁷ 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 gp91Phox−/− 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).

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 gp91Phox−/− mice. (C) Streptococcus pyogenes ± expression of staphylococcal 4,4'-diaponeurosporene.
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<sup>Phox</sup>-/- mice were bred at the Veteran’s Administration Medical Center, San Diego, CA, and maintained on trimethoprim/sulfa-fanethoxazole 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 gp91<sup>Phox</sup> deficiency (homozygous ΔGT deletion in exon 2). At the start of the study, she was in good health, and her only medication was IFNγ (50 μg/m<sup>2</sup>/wk) administered three times per wk by subcutaneous injection.

**Generation of the carotenoid-deficient *S. aureus* mutant, ΔCrtM.** Precise, in-frame allelic replacement of the *crtMN* 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 *crtM* with primers *crtMupF* 5′-TTAGGAAGTCCATATCTTCAC-3′ and *crtMstartR* 5′-GGTGGTATATCCAGTGATTTTTTTCTCCATAC-3′, with ~500 bp of sequence immediately downstream of *crtM* with primers *crtMelMdownF* 5′-TCCTTCCATATATTTGGAAGTGCATATACTTCAC-3′ and *crtMendR* 5′-GGCCACCTTATACGATCATTGTCTCCTC-3′. The *crtM* start and *crtMelMdownF* 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 *crtMupF* and *crtMelMdownR*. The resultant PCR amplicon, containing an in-frame substitution of *crtM* 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 electrotransformation. 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′-CACGTCTAGAAATGGCCATTCTCATTTAAGAC-3′ and CrtR 5′-ATCGGATATCTTACATCTTACCTTCTTACGAC-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.
Spectral profile of the *S. aureus* carotenoid. Stationary phase (48-h) cultures of WT *S. aureus* PIG1 and its isogenic ΔCrtM mutant were subected 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 (H2O2) was added to 1.5% final concentration, 2 × 106 bacteria were incubated at 37°C for 1 h, and then 1,000 U/ml of catalase (Sigma-Aldrich) was added to quench residual H2O2. Dilutions were plated on Todd-Hewitt agar (THA) for enumeration of surviving CFU. For the singlet oxygen assay, 108 S. aureus or 4 × 108 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 106 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 × 108 CFU in 100 µl RPMI 1640 + 10% FCS and mixed with 3 × 103 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% CO2 incubator. Gentamycin (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 samples 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 gp91phox–/– 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 infection. In all cases, animals were killed, and skin lesions were 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 ΔCrtM 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

REFERENCES

1. Ogston, A. 1881. Report upon micro-organisms in surgical diseases. BMJ. 1:369–375.
2. Rosenbach, F.J. 1884. Mikro-Organismen bei den. In Wund-Infectionen-Krankheiten des Menschen. J.F. Bergman, editor. Wiesbaden, Germany. 1–122.
3. Marshall, J.H., and G.J. Wilmoth. 1981. Pigments of *Staphylococcus aureus*, a series of triterpenoid carotenoids. J. Bacteriol. 147:900–913.
4. Krinsky, N.I. 1993. Actions of carotenoids in biological systems. Annu. Rev. Nutr. 13:561–587.
5. El-Agamey, A., G.M. Lowe, D.J. McGarvey, A. Mortensen, D.M. Phillips, T.G. Truscott, and A.J. Young, 2004. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. Anh. Biochem. Biophys. 430:37–48.
6. Wieland, B., C. Feil, E. Gloria-Maiercker, G. Thumm, M. Lechner, J.M. Bravo, K. Poralla, and F. Gotz. 1994. Genetic and biochemical analyses of the biosynthesis of the yellow carotenoid 4,4’-diaponeurosporene of *Staphylococcus aureus*. J. Bacteriol. 176:7119–7126.
7. Fang, F.C. 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol. 2:820–832.
8. Rosen, H., and S.K. Klebanoff. 1979. Bacterialicidal activity of a superoxide anion-generating system. A model for the polymorphonuclear leucocyte. J. Exp. Med. 149:27–39.
9. Dahl, T.A., W.R. Middon, and P.E. Hartman. 1989. Comparison of killing of gram-negative and gram-positive bacteria by pure singlet oxygen. J. Bacteriol. 171:2188–2194.
10. Liu, G.Y., K.S. Doran, T. Lawrence, N. Turkson, M. Pultti, L. Tissi, and V. Nizet. 2004. Sword and shield: linked group B streptococcal Phox and V. Nizet. 2004. Sword and shield: linked group B streptococcal β-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. Proc. Natl. Acad. Sci. USA. 101:14491–14496.
11. Pollock, J.D., D.A. Williams, M.A. Gifford, L.L. Li, X. Du, J. Fisherman, S.H. Orkin, C.M. Doerschuk, and M.C. Dinauer. 1995. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. Nat. Genet. 9:202–209.
12. Reeves, E.P., H. Lu, H.L. Jacobs, C.G. Messina, S. Bohover, G. Gaella, E.O. Potma, A. Warley, J. Roes, and A.W. Segal. 2002. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. Nature. 416:291–297.
13. Belaaouaj, A., R. McCarthy, M. Baumann, Z. Gao, T.J. Ley, S.N. Abraham, and S.D. Shapiro. 1998. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. Nat. Med. 4:615–618.
14. Nizet, V., T. Ohtake, X. Lauth, J. Trowbridge, J. Rudisill, R.A. Dorschner, V. Pestonjamasp, J. Praino, K. Huttner, and R.L. Gallo. 2001. Innate antimicrobial peptide protects the skin from invasive bac-
15. Hammond, R.K., and D.C. White. 1970. Inhibition of carotenoid hydroxylation in *Staphylococcus aureus* by mixed-function oxidase inhibitors. *J. Bacteriol.* 103:607–610.

16. Weigel, L.M., D.B. Clewell, S.R. Gill, N.C. Clark, L.K. McDougal, S.E. Flannagan, J.F. Kolonay, J. Shetty, G.E. Killgore, and F.C. Tenover. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science.* 302:1569–1571.

17. Naimi, T.S., K.H. LeDell, K. Como-Sabetti, S.M. Borchardt, D.J. Boxrud, J. Etienne, S.K. Johnson, F. Vandenesch, S. Fridkin, C. O’Boyle, et al. 2003. Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. *JAMA.* 290:2976–2984.

18. Kansal, R.G., A. McGeer, D.E. Low, A. Norryby-Teglund, and M. Kotb. 2000. Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases. *Infect. Immun.* 68:6362–6369.

19. Jeng, A., V. Sakota, Z. Li, V. Datta, B. Beall, and V. Nizet. 2003. Molecular genetic analysis of a group A *Streptococcus* operon encoding serum opacity factor and a novel fibronectin-binding protein, SfbX. *J. Bacteriol.* 185:1208–1217.

20. Lewis, A.L., V. Nizet, and A. Varki. 2004. Discovery and characterization of sialic acid O-acetylation in group B *Streptococcus*. *Proc. Natl. Acad. Sci. USA.* 101:11123–11128.

21. Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, et al. 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet.* 357:1225–1240.

22. Bunce, C., L. Wheeler, G. Reed, J. Musser, and N. Barg. 1992. Murine model of cutaneous infection with gram-positive cocci. *Infect. Immun.* 60:2636–2640.