Insights into Mechanisms Used by Staphylococcus aureus to Avoid Destruction by Human Neutrophils


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Polymorphonuclear leukocytes (PMNs, or neutrophils) are critical for human innate immunity and kill most invading bacteria. However, pathogens such as *Staphylococcus aureus* avoid destruction by PMNs to survive, thereby causing human infections. The molecular mechanisms used by pathogens to circumvent killing by the immune system remain largely undefined. To that end, we studied *S. aureus* pathogenesis and bacteria–PMN interactions using strains originally isolated from individuals with community-acquired (CA) and hospital-acquired infections. Compared with strains from hospital infections (COL and MRSA252), strain MW2 and a methicillin-susceptible relative, MnCop, were significantly more virulent in a mouse model of infection, comprising a global PMN challenge for Public Health, Newark, NJ 07103; and *Laboratory of Public Health Research Institute Tuberculosis Center, International Center for Public Health, Baylor University College of Medicine, Houston, TX 77030.*

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Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; CA, community acquired; HA, hospital acquired; MRSA, methicillin-resistant *S. aureus*; MnCop, Dulbecco’s PBS; LDH, lactate dehydrogenase; TEM, transmission electron microscopy; GAS group A Streptococcus.

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with no known risk factors. In contrast, MRSA252 (pulsed-field type identical to EMRSA16), is the second leading cause of healthcare-associated 
*S. aureus* infections in the United States (16). Descriptions of each strain are summarized in Table I.

*S. aureus* were grown in tryptic soy broth (BD Biosciences) containing 0.5% glucose. Bacterial cultures were inoculated from overnight cultures with a dilution of 1/200 for strains MW2, McCop, and LAC, or 1/100 for strains COL and MRSA252, and incubated at 37°C with shaking (250 rpm). For in vitro microarray analyses, *S. aureus* strains were cultured to early exponential (EE, OD$_{600}$ = 0.40), mid exponential (E1, OD$_{600}$ = 0.75; E2, OD$_{600}$ = 1.0) or early stationary (ES, OD$_{600}$ = 2.0) phases of growth and harvested immediately for RNA isolation. For all other assays, *S. aureus* were harvested at E$_1$, washed in Dulbecco’s PBS (DPBS, Sigma-Aldrich), and resuspended in RPMI 1640 medium (Invitrogen Life Technologies) buffered with 10 mM HEPES (RPMI/H, pH 7.2) to 10$^8$/ml.

**Mouse model of *S. aureus* disease**

All animal studies conformed to National Institutes of Health guidelines and were approved by the Animal Use Committee at Rocky Mountain Laboratories. Female CD1 Swiss mice were purchased from Charles River. Animals were between 10 and 12 wk of age, housed in microisolator cages, and received food and water ad libitum. *S. aureus* strains were grown to E$_1$, washed twice with sterile DPBS, and resuspended to 10$^8$/100 μl in DPBS. Mice were inoculated with 10$^7$ *S. aureus* or with sterile DPBS via the tail vein. The experiment was performed once with 10 mice per *S. aureus* strain and 5 mice for DPBS control. Criteria for determining morbidity/sickness in mice included hunched posture, decreased activity, ruffled fur, and labored breathing. Animals were euthanized if unable to eat or drink, or if they became immobile. All mice were euthanized by 48 h. Survival statistics were performed by a log-rank test (GraphPad Prism version 4.0 for Windows; GraphPad Software). To determine whether *S. aureus* had disseminated to large organs during bacteremia, liver and lung tissues were harvested at 24 h after infection, homogenized, and aliquots of each homogenate were plated on tryptic soy agar. Colonies were enumerated the following day. Alternatively, parts of the liver, lung, kidney, heart, and brain were collected in parallel for histopathology. Tissues were fixed in 10% buffered formaldehyde for 10–20 h. After fixation, tissues were processed for histology.

**PMN isolation**

PMNs were isolated from heparinized venous blood of healthy individuals in accordance with a protocol approved by the Institutional Review Board for Human Subjects, National Institute of Allergy and Infectious Diseases. Briefly, blood was drawn into BD Vacutainer® tubes containing 0.109 mol/l EDTA (BD Biosciences) and centrifuged at 1500 g at 4°C for 20 min. PMNs were isolated from packed buffy coats and washed twice in DPBS with 2.5% glutaraldehyde and washed twice in cacodylate buffer. The leukocyte-containing supernatant was centrifuged at 670 × g for 10 min and resuspended in 35 ml of 0.9% sodium chloride. The cell suspension was underlaid with 10 ml of Ficoll-PaquePLUS (1.077 g/L; Amersham Pharmacia) and centrifuged at 1000 g for 20 min at 4°C. PMNs were harvested immediately for RNA isolation. For all other assays, PMNs were harvested on ice in DPBS. PMNs (3 × 10$^7$) were added to serum-coated glass cover slips in 24-well tissue culture plates and allowed to adhere at room temperature for 15 min. Cells were chilled on ice for 10 min and AlexaFluor 488-labeled bacteria were added at a ratio of 10 bacteria:PMN. Plates were then centrifuged at 380 × g for 8 min at 4°C to synchronize phagocytosis (18). Samples were incubated at 37°C in a CO$_2$ incubator for the indicated times (t = 0 was processed immediately after centrifugation), medium was removed from the wells by aspiration, and cells were fixed on ice for 30 min with 4% paraformaldehyde. Fixative was removed by aspiration, and unengested bacteria were counterstained with AlexaFluor594 conjugated-Ab specific for AlexaFluor 488 for 15 min at room temperature. Samples were visualized with a Zeiss Axiostar 2 Plus fluorescence microscope (Carl Zeiss) and imaged with a Zeiss AxioCam digital camera. The total number of *S. aureus* bound and/or ingested was evaluated in 50 neutrophils per assay from at least five separate fields of view. Percent phagocytosis was calculated with the equation: (number of ingested bacteria per cell/total number of PMN-associated bacteria per cell, bound or ingested) × 100. Statistics were performed with a repeated-measures ANOVA and Tukey’s posttest for multiple comparisons (GraphPad Prism version 4.0 for Windows; GraphPad Software). Images were adjusted for brightness and contrast with Photoshop CS (Adobe Systems).

**PMN ROS production**

PMN ROS production was measured using a previously described method (18) with minor modifications. PMNs were incubated with 25 μM 2',7'-dihydorodichlorofluorescein diacetate (DCF, Molecular Probes) for 20–30 min at room temperature in RPMI/H. DCF-containing PMNs (10$^6$) and opsonized *S. aureus* were combined in wells of a 96-well microtiter plate at 4°C (ratio of 10 bacteria:PMN), centrifuged for 8 min at 380 × g, and transferred to a microplate fluorometer (Spectramax Gemini; Molecular Devices). ROS production was measured continuously at 1-min intervals for up to 180 min at 37°C using excitation and emission wavelengths of 485 and 538 nm, respectively. The rate of PMN ROS production over time (second-order kinetics) were determined from the $V_{max}$ (max increase in fluorescence) within each 10-min time period.

**PMN bactericidal activity**

Killing of *S. aureus* by human PMNs was determined as described (17) with some modification. Briefly, PMNs (10$^6$) were combined with ~10$^7$ opsonized *S. aureus* in 24-well tissue culture plates, centrifuged at 380 × g for 8 min and incubated at 37°C for up to 6 h. At indicated times, PMNs were lysed with 0.1% saponin (20 min on ice) and *S. aureus* were plated on tryptic soy agar. Colonies were enumerated the following day, and percent *S. aureus* survival was calculated with the equation (CFU$_{PMN}$/CFU$_{control}$) × 100. The assay measures total number of viable ingested and unengested bacteria. Statistics were performed with a repeated-measures ANOVA and Tukey’s posttest for multiple comparisons (GraphPad Prism).

**S. aureus-induced PMN lysis**

Following phagocytosis of *S. aureus*, PMN lysis was determined with a standard assay for release of lactate dehydrogenase (LDH) as described by the manufacturer (Cytotoxicity Detection Kit; Roche Applied Sciences). Alternatively, PMN lysis was determined by measuring intact intracellular hemoglobin. Statistics were performed with a repeated-measures ANOVA (LDH release) or one-way ANOVA (hemacytometer counts) and Tukey’s posttest for multiple comparisons (GraphPad Prism).

**Electron microscopy**

For transmission electron microscopy (TEM), phagocytosis assays were performed as described above, but with the following modifications. PMNs (3 × 10$^6$) were combined with 3 × 10$^7$ serum-opsonized *S. aureus* in wells of a 24-well tissue culture plate containing serum-coated Thermawan coverslips (Nalge Nunc International). Cells were incubated at 37°C with 5% CO$_2$ for up to 180 min. At the indicated times, cells were fixed in Karnovsky’s fixative containing 4% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2 (Electron Microscopy Sciences). Samples were washed twice in 0.1 M phosphate buffer, postfixed in 1% osmium/0.8% potassium ferrocyanide in 0.1 M phosphate. Following two additional washes in water, samples were stained in-block with 1% uranyl acetate in water, washed again, and then dehydrated in ethanol, and embedded in Spurr resin (Ted Pella), as described (19). Thin sections were examined on a model H7500 TEM (Hitachi High Technologies) at 80 kV. Images were captured with an Advantage HR digital camera system (Advanced Microscopy Techniques) and adjusted for brightness and contrast with Photoshop CS (Adobe Systems). For scanning transmission electron microscopy, phagocytosis assays were performed as described above, but with the following modifications. Cells were fixed in 0.15 M cacodylate buffer, pH 7.2, containing 2.5% glutaraldehyde and washed twice in cacodylate buffer.

*S. aureus* EVADE KILLING BY HUMAN NEUTROPHILS
Samples were then postfixed with 1.0% osmium tetroxide in cacodylate buffer, washed twice with water, dehydrated with ethanol, and critical-point dried through carbon dioxide. After mounting on stubs, the samples were lightly coated with iridium using an ion beam sputterer (South Bay Technology), and examined with a Hitachi S4500 field emission scanning electron microscope (Hitachi High Technologies America). Digital images were collected with an Orion system (Focused Resolutions), and adjusted for brightness and contrast with Photoshop CS (Adobe Systems).

Analysis of S. aureus gene expression

To compare transcript levels in vitro, S. aureus strains were cultured to the desired phase of growth and ~2.5 \times 10^9 cells were lysed with 700 \mu l of RLT buffer (Qiagen). Lysates were homogenized with an FP120 FastPrep system (QBiogene). Total RNA was isolated with RNeasy kits (Qiagen) and contaminating chromosomal DNA was removed by DNase treatment (Qiagen). Purified bacterial RNA was used to prepare fragmented and biotin-dUTP-labeled cDNA according to standard Affymetrix prokaryotic target preparation ([www.affymetrix.com/support/downloads/manuals/ expression_s3_manual.pdf](http://www.affymetrix.com/support/downloads/manuals/expression_s3_manual.pdf)). Briefly, cDNA was synthesized from ~10 \mu g of total RNA in 1x first-strand buffer containing 25 ng/\mu l random primers (Invitrogen Life Technologies), 10 mM DTT, 0.5 mM dNTPs (Amersham), 0.5 U/\mu l SUPERase In (Ambion), and 25 U/\mu l SuperScript II (Invitrogen Life Technologies). Remaining RNA was removed by hydrolysis with 1 N NaOH and the reaction was neutralized with 1 N HCL. cDNA (~3–6 \mu g/sample) was fragmented using ~0.6 U of DNase I (Amersham Pharmacia Biotech) per \mu g of cDNA in One-Phor-All Buffer (Amersham). The 3' termini of the fragmented cDNA was labeled using an Enzo BioArray Terminal Labeling kit with Biotin-dUTP (Enzo Diagnostics). The reaction was completed as suggested by Affymetrix using the Midi format. Biotinylated S. aureus cDNA was hybridized to custom Affymetrix GeneChips (RMLChips) containing 3961 probe sets from eight different S. aureus strains (COL, EMRSA16, MSSA476, RF122, TSS.8325, Mu50, and N315) and scanned according to standard GeneChip protocols (Affymetrix). Specific details for Affymetrix hybridization and scanning protocols can be found at the internet address provided above. Each experiment was repeated in triplicate. Data were analyzed with GeneChip Operating Software version 1.1 (Affymetrix) and GeneSpring version 6.0 (Silicon Genetics).

Data was normalized using the mean signal from the S. aureus probe sets multiplied by a scale factor to obtain the target signal. Default detection thresholds of \( p = 0.04 \) and \( p = 0.06 \) were used to determine genes that were present and absent calls were assigned to values of \( p \geq 0.06 \). The Pearson correlation (GeneSpring) was used to evaluate the replicate correlation. Any nongrouping replicates were deleted from further evaluation. Quality filters based upon Present-Absent calls and signal intensities were used to create final gene lists (Microsoft Excel 2003). Microarray data are posted on the Gene Expression Omnibus (GEO, [www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/), accession number GSE2728).

To measure S. aureus gene expression during S. aureus–PMN interaction, bacteria were grown to \( E_{6} \), washed once in DPBS, and opsonized in 50% normal human serum at 37°C for 30 min. PMNs (10^6) were combined with ~10^4 opsonized S. aureus in wells of a 12-well tissue culture plate on ice and centrifuged at 380 \times g for 10 min at 4°C to synchronize phagocytosis (17, 18). Plates were transferred to a 37°C incubator with 5% CO_2 for the desired times and samples were lysed as described above. For the microarray data derived from S. aureus–PMN interaction, RNA was isolated from the combination of three populations of bacteria: 1) S. aureus bound to PMNs; 2) S. aureus ingested by PMNs; and 3) free (uningested) S. aureus. However, we determined in separate experiments using

### Table I. S. aureus strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>MLST</th>
<th>spa Type</th>
<th>SCCmec Type</th>
<th>agr Type</th>
<th>PVL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW2: (pulsed-field type USA400) prototype S. aureus strain (5, 7, 36, 41, 53)</td>
<td>ST1: 1-1-1-1-1-1-1</td>
<td>spa type 131; UIJFKBPE</td>
<td>IV</td>
<td>III</td>
<td>+</td>
</tr>
<tr>
<td>CA-MRSA strain (5, 7, 36, 41, 53)</td>
<td>ST1: 1-1-1-1-1-1</td>
<td>spa type 35; UKJFKBPE</td>
<td>–</td>
<td>III</td>
<td>+</td>
</tr>
<tr>
<td>MnCop: CA-MSSA, mmTSS strain, otherwise similar to MW2 (8)</td>
<td>ST8: 3-3-1-1-4-4-3</td>
<td>spa type 1; YHGFMBQBLO</td>
<td>IV</td>
<td>I</td>
<td>+</td>
</tr>
<tr>
<td>LAC: (pulsed-field type USA300) CA-MRSA, frequently isolated from skin infections (9, 10, 12, 16, 36)</td>
<td>ST36: 2-2-2-2-3-3-2</td>
<td>spa type 16; WKGKAKAOQQ</td>
<td>II</td>
<td>III</td>
<td>–</td>
</tr>
<tr>
<td>MRSA252: HA-MRSA, predominant in healthcare settings in U.S. and U.K. (13, 16)</td>
<td>ST250: 3-3-1-1-4-16-16</td>
<td>spa type 1; YHGFMBQBLO</td>
<td>I</td>
<td>I</td>
<td>–</td>
</tr>
<tr>
<td>COL: HA-MRSA, isolate from Colindale, U.K. (14)</td>
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<td></td>
</tr>
</tbody>
</table>

* CA, community acquired; HA, hospital acquired; MLST, multilocus sequence typing; PVL, Panton-Valentine Leukocidin; mmTSS, nonmenstrual, toxic-shock syndrome.

* PVL was verified by DNA sequence analysis.

### FIGURE 1. S. aureus pathogenesis. A, Mouse survival. Mice were infected with S. aureus strains by i.v. inoculation (tail vein), and health was monitored for 48 h. Results are from 10 mice in each infection group and five mice in a PBS control group. *, \( p < 0.015 \); curves are different. B and C, Dissemination of S. aureus. Homogenates from the liver and lung of animals infected as described in A were plated for CFUs. *, \( p < 0.05 \) vs PBS control mice. D–F, Vital organ pathology in infected mice. Heart (D), kidney (E), and brain tissue (F) from several animals was examined for disease. Yellow arrows indicate acute neutrophilic inflammation, bacterial colonies (solid purple matter), and/or regions of necrosis. Black squares, regions magnified \( \times 20 \).
fluorescence microscopy, and transmission and scanning electron microscopy, that there were few, if any, free bacteria after 30 min of S. aureus-PMN interaction (see Fig. 2, and data not shown). Further, after 60 min of S. aureus-PMN interaction, typically all neutrophil-associated bacteria were internalized (data not shown). At desired times, PMNs and bacteria in each well were lysed with 700 μl of RLT buffer (Qiagen). Isolation of S. aureus RNA and microarray analyses were performed as described above, except that fold-changes for each gene were determined by comparing RMLChips hybridized with cDNA from S. aureus alone to those with cDNA from S. aureus during PMN phagocytosis (time-matched). To be included in the final gene lists (e.g., see Figs. 5–9, and supplemental Table V4), genes must have met the quality filters described above, and changes in gene expression must have been at least 2-fold in one of the strains. For those genes (>2-fold change in one strain), changes of >1.5-fold were also indicated for the other strains. PMN-S. aureus microarray experiments were performed three separate times with PMNs from three blood donors. Although the RMLChip was designed to avoid cross-hybridization with human RNA or DNA, we determined empirically (using neutrophils from three individuals) that PMN RNA failed to hybridize with RMLChips. In our experiments, 3957 of 3961 probe sets had no significant cross-hybridization with PMN RNA. The four probe sets with noted binding to neutrophil RNA were eliminated from the analysis.

TagMan real-time RT-PCR analysis

Phagocytosis experiments and RNA preparation for TaqMan analysis were performed with procedures and conditions identical to those used for the PMN-S. aureus microarray experiments. TagMan real-time RT-PCR analysis of two separate phagocytosis experiments (each assayed in triplicate) using PMNs from two human blood donors was performed with an ABI 7500 thermocycler (Applied Biosystems). Relative quantification of S. aureus genes was determined by the change in expression of target transcripts relative to gyrB (housekeeping or calibration gene) according to the manufacturer’s protocol (Applied Biosystems Relative Quantification Manual at [http://docs.appliedbiosystems.com/genindex.taf], doc. index no. 4347824).

The primer-probe sequences used for confirmation of microarray data were as follows: gyrB forward primer, 5′-CAATGATCACAGCATTTCGACACG-3′, gyrB probe 5′-ATCGCGTAGGCACTCTGAAACTATCCCGGAAAGAAG-3′, gyrB reverse primer 5′-CGGCCATCATCAATGACGATG-3′; cap8C forward primer, 5′-TTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′, cap8C reverse primer 5′-CTTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′, cap8C probe, 5′-TTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′; mraY forward primer, 5′-ATCGGATTTAC-3′, mraY probe, 5′-ATCGGATTTCACATGCATGCAAT-3′; cap8C forward primer 5′-TTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′, cap8C reverse primer 5′-CTTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′, cap8C probe, 5′-TTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′; vraR forward primer, 5′-TTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′, vraR reverse primer, 5′-TTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′; agrA forward primer, 5′-TTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′, agrA reverse primer, 5′-TTCTAGATCCAGAGTAGTatagaaCCATGGATC-3′.
Results

S. aureus virulence in a mouse infection model

To gain new insight into S. aureus pathogenesis, we tested virulence of strains originally isolated from individuals with CA (MW2, Mn-Cop, and LAC) and hospital-acquired (HA; COL and MRSA252) infections in a mouse model of bacteremia (Table I, and Fig. 1). All mice infected with MW2, MnCop, or LAC were moribund or noticeably sick (10 of 10 animals infected with each strain, see Materials and Methods for details). In contrast, those infected with COL or MRSA252, a strain reported as highly virulent in humans (13), appeared relatively healthy (0 of 10 animals infected with COL and 1 of 10 of those infected with MRSA252 were sick). Correspondingly, mortality was significantly increased in those infected with MW2 or MnCop (p = 0.015, log-rank test) (Fig. 1A). At the time of death, major vital organs were evaluated for signs of infection (Fig. 1, B–E). On average, more bacteria were recovered from the livers of animals infected with MW2, MnCop, or LAC compared with the other strains (Fig. 1B). There were also a greater number of S. aureus recovered from lungs of mice infected with MW2 or MnCop compared with the other strains (Fig. 1C). These observations are consistent with the known capacity of MW2 and similar strains to cause necrotizing pneumonia in healthy individuals (20).
or LAC had multifocal areas of necrosis and acute (neutrophilic) inflammation with bacterial colonies (Fig. 1D). Infection also extended to kidneys and brain in mice infected with MW2 or MnCop, providing further evidence for enhanced virulence in these strains (3 of 7 animals tested had diseased brain or spinal cord tissue) (Fig. 1, E and F). In contrast, organs from animals infected with COL or MRSA252 typically showed little or no overt signs of disease (Fig. 1, D and E, and data not shown). These results indicate that strains MW2, MnCop, or LAC are generally more virulent than either MRSA252 or COL. Inasmuch as PMNs are essential effectors of innate host defense against bacterial pathogens, the enhanced virulence of MW2, MnCop, and LAC in the mouse model may reflect increased resistance to neutrophil killing. This hypothesis is tested below.

Phagocytosis and killing of *S. aureus* by human PMNs

To determine whether differences in virulence are due in part to varied capacity of the strains to interact with the innate immune system, we evaluated phagocytosis and killing of each by human neutrophils. Although there were differences in early uptake of the strains (e.g., ingestion of COL and MW2 at 5 min was 65.1 ± 7.9% and 46.3 ± 12.9%, respectively, \( p < 0.05 \), ANOVA with Tukey’s posttest), phagocytosis of each strain occurred rapidly and was essentially complete by 15 min (Fig. 2, A and B). Notably, uptake of each strain by PMNs was virtually identical by 60 min of *S. aureus*-PMN interaction and there were few or no free bacteria (Fig. 2, and data not shown). We next determined that ingested *S. aureus* were exposed to microbicidal PMN ROS and granule contents (Fig. 2, C–E). Despite the exposure to neutrophil antibacterial components, there was significant *S. aureus* survival after phagocytosis (Fig. 2F), findings consistent with studies by Gresham et al. (21). Further, survival was significantly better for strains isolated from patients with CA infections (e.g., survival at 30 min was 58.7 ± 7.6% for MW2 vs 32.0 ± 3.0% for MRSA252, \( p < 0.001 \), ANOVA with Tukey’s posttest) (Fig. 2F). These observations provide strong support to the notion that *S. aureus* infections are caused at least in part by evasion of neutrophil-mediated killing.

Destruction of human PMNs by *S. aureus*

Between 3 and 6 h after PMN phagocytosis, net growth of each *S. aureus* strain increased significantly (Fig. 2F). Inasmuch as virtually all pathogenic *S. aureus* produce leukocidins, such as \( \gamma \)-hemolysins (22), we tested the hypothesis that late pathogen survival

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**FIGURE 4.** Global gene expression in *S. aureus* strains of diverse genetic backgrounds. A–C, Comparative microarray analysis of *S. aureus* strains cultured in vitro was measured with custom Affymetrix microarrays containing 3961 open reading frames from eight *S. aureus* genomes. Genes were included in the analysis if they were called “present” at the phase of growth indicated. A, Gene expression in MW2, COL, and MRSA252. B, Gene expression in MW2, MnCop, and LAC. C, Gene expression in LAC, COL, and MRSA252. Results are the average of three separate experiments for each strain. Phases of growth are as follows: EE, early-exponential (\( \text{OD}_{600} = 0.4 \)); E1, mid-exponential (\( \text{OD}_{600} = 0.75 \)); E2, mid-exponential (\( \text{OD}_{600} = 1.0 \)); ES, early-stationary. The individual genes from which these comparisons were made are provided in supplemental Tables II–IV.

**FIGURE 5.** Global changes in *S. aureus* gene expression during phagocytosis. *S. aureus* gene expression was measured following PMN phagocytosis as described in Materials and Methods. The number of *S. aureus* genes up (red bars) or down (blue bars) regulated at 30, 60, or 180 min after phagocytosis is indicated for assigned functional categories for each strain. The percent genes changed is based on the total number of possible coding regions in each sequenced strain (i.e., MW2, 2632 genes; MRSA252, 2673 genes; COL, 2721 genes).
and growth after phagocytosis (i.e., after 3 h) is linked to the ability of each strain to lyse human PMNs (Fig. 3). Compared with COL and MRSA252, strains MW2, MnCop, and LAC caused significantly more neutrophil destruction 6 h after phagocytosis (e.g., at 6 h release of lactate dehydrogenase was 54.3 ± 7.6% for MW2 vs 25.0 ± 4.0% for COL, p < 0.01, ANOVA with Tukey’s posttest) (Fig. 3A). These results were verified by scanning and transmission electron microscopy (Fig. 3, C and D). The observation that heat-killed MW2 failed to lyse neutrophils (Fig. 3A) is consistent with the idea that S. aureus actively produces factors to evade killing by...
PMNs. Importantly, ability of the strains to survive killing by neutrophils and cause subsequent PMN lysis correlated generally with the mouse pathogenesis data.

**Gene expression in S. aureus strains from CA and HA infections**

As a first step toward understanding the molecular basis of differences in virulence and interaction with neutrophils, we compared global gene expression in the strains grown in vitro with *S. aureus* microarrays (Table I, Fig. 4, and supplemental Tables II-IV). Collectively, expression of 210 genes differentiated MW2, MnCop, and LAC from MRSA252 and COL (supplemental Table II). Only 17 genes (not expressed in strains COL or MRSA252) were commonly expressed in the three CA strains during growth in vitro (supplemental Table II). Nine of the 17 genes encoded hypothetical proteins, and 3 others were phage-encoded (supplemental Table II). The finding that MW2, MnCop, and LAC collectively express a limited number of unique transcripts (not present in COL or MRSA252) is consistent with the genetic similarity between LAC and COL based on MLST and spa typing, and gene expression (Fig. 4C, and Table I). Although strain-specific gene expression (Fig. 4) might underlie differences in pathogenesis, it is likely that many pathogen processes used to evade PMN killing are triggered by interaction with the innate immune system.

**PMN phagocytosis induces global changes in S. aureus gene expression**

To further elucidate the molecular mechanisms used by the pathogen to evade innate host defense, we measured global changes in *S. aureus* gene expression during phagocytic interaction with human PMNs. We discovered that 21.8–39.1% of the genes in *S. aureus* were differentially regulated at any given time following phagocytic interaction (e.g., 593, 857, and 1029 genes in COL, MRSA252, and MW2, respectively, were up- or down-regulated at 180 min after phagocytosis) (Figs. 5–9, and supplemental Table V). Genes differentially expressed during PMN phagocytosis were divided into categories based on function or annotation. Genes encoding proteins with undefined function and those involved in metabolism comprised the two largest categories of *S. aureus* genes modulated by PMN phagocytosis (e.g., *n = 348 and 372* genes, respectively, for MW2 30 min after initial PMN interaction) (Figs. 5 and 8, and supplemental Table V). Notably, 26.8–38.8% of *S. aureus* genes were differentially regulated within 30 min after PMN phagocytosis, depending on the strain analyzed (e.g., 26.8% of those in COL and 38.8% of those in MW2) (Fig. 5).

**Genes encoding proteins that moderate oxidative stress, and those involved in metabolism and capsule synthesis are induced during phagocytosis**

Consistent with the finding that *S. aureus* was exposed to PMN-derived ROS (Fig. 2C), at least 25 stress response genes were up-regulated in the pathogen during phagocytic interaction (e.g., 593, 857, and 1029 genes in COL, MRSA252, and MW2, respectively, were set down-regulated at 180 min after phagocytosis) (Figs. 5–9, and supplemental Table V). Genes differentially expressed during PMN phagocytosis were divided into categories based on function or annotation. Genes encoding proteins with undefined function and those involved in metabolism comprised the two largest categories of *S. aureus* genes modulated by PMN phagocytosis (e.g., *n = 348 and 372* genes, respectively, for MW2 30 min after initial PMN interaction) (Figs. 5 and 8, and supplemental Table V). Notably, 26.8–38.8% of *S. aureus* genes were differentially regulated within 30 min after PMN phagocytosis, depending on the strain analyzed (e.g., 26.8% of those in COL and 38.8% of those in MW2) (Fig. 5).
30 or 60 min). This idea is supported by the lack of net *S. aureus* growth for up to 3 h after ingestion (Fig. 2F).

**Phagocytosis up-regulates *S. aureus* genes encoding proteins that mediate virulence**

Within 30 min of *S. aureus*-PMN interaction, genes involved in virulence were up-regulated in each of the strains (Figs. 5 and 6, and supplemental Table V). For example, at least 36 genes encoding virulence factors and/or toxins were induced in MW2 30 min after phagocytic interaction with neutrophils (Figs. 5 and 6, and supplemental Table V). These genes encode toxins, secreted exoproteins, and cell-wall associated adhesions, such as extracellular matrix and plasma binding protein (ssp), epidermin immunity/latex immunoglobulin protein (eitA), epidermin immunity/latex immunoglobulin protein (eitF), fibronectin-binding proteins (fnbA, fnbB), staphylocoagulase, and clumping factor (clfA) (Fig. 6).

Genes encoding two-component/His-dependent hemolysins (hlgA, hlgB) and hlgC, which are known to destroy leukocytes, were induced in all strains during PMN phagocytosis (Fig. 6) (22). In contrast, genes encoding several toxins, such as an exotoxin 2 homologue, set7B, set11, set14, lukD, lukE, and enterotoxin type C3 (sec3), as well as those encoding numerous hypothetical or exported/surface-associated proteins, were up-regulated only in strains causing CA infections (Figs. 6 and 7, and supplemental Table V). Further studies are needed to determine whether these genes underlie the noted variances in strain pathogenesis (Fig. 1) and enhanced ability of MW2, MnCop, and LAC to circumvent killing by neutrophils (Fig. 3).
**FIGURE 9.** Phagocytosis triggers differential regulation of *S. aureus* genes involved in cell division, replication, and biosynthetic activity. Gene expression was determined as described in the legend of Fig. 6.

*S. aureus* genes involved in transcriptional regulation are modulated by neutrophil phagocytosis

*S. aureus* adapts to varied host environments and thus inhabits skin, mucous membranes, blood, and deeper tissues (1–6). This characteristic is due partially to several gene-regulatory systems that tightly regulate expression of *S. aureus* virulence genes (25). Transcripts encoding *S. aureus* gene-regulatory systems VraSR, SaeSR, and SarA were up-regulated significantly by PMN phagocytosis (Fig. 6). The VraSR two-component gene regulatory system...
induced by cell-wall synthesis inhibitors and/or cell envelope damage (26), observations most compatible with our results. SarA is known to control expression of S. aureus virulence factors and is thus important for pathogen survival during host-pathogen interaction (21, 27) (Fig. 6). Our observation that sarA is induced, and alternative sigma factor B (sigB) and accessory gene regulator (agr) operons are repressed during PMN phagocytosis, corresponded with concomitant regulation of a number of putative and proven virulence factors, such as the gene encoding β-hemolysin (hld) and the anti-holin encoding operon lgrAB (Fig. 6, and supplemental Table V) (28–30). Taken together, these findings suggest that evasion of human innate immunity by S. aureus is regulated at the level of gene transcription.

Confirmation of microarray data by TaqMan real-time RT-PCR (TaqMan analysis)

We used TaqMan real-time RT-PCR to confirm changes in gene expression identified by microarray analysis (Fig. 10). Eight S. aureus genes identified by microarrays as differentially transcribed during PMN phagocytosis were evaluated by TaqMan analysis. Genes were selected from several functional categories and transcript levels were measured at 60 and 180 min after PMN phagocytosis in strains MRSA252 and MW2. There was a strong correlation between TaqMan and microarray results (83.3%), consistent with previous comparisons (17, 19, 31–35).

Discussion

S. aureus is one of the most prominent human pathogens and is responsible for diverse infections worldwide (1). Risk of S. aureus infection is increased during or after hospitalization, and there is a serious problem with antibiotic resistance in the pathogen in healthcare settings (1, 2). In contrast, there has been a dramatic increase in the number of S. aureus infections occurring in healthy individuals with no apparent risk factors for disease (2, 7, 9, 10, 12, 15, 36–43). The molecular basis for these infections is largely unknown, although it is likely that a combination of pathogen virulence mechanisms and host susceptibility factors contribute to disease.

Neutrophils are a critical component of innate immunity and are essential for controlling bacterial infections (44–50). Defects that alter normal PMN function, such as chronic granulomatous disease and leukocyte adhesion deficiency, predispose individuals to serious S. aureus infections (reviewed in Ref. 44). Further, it has long been known that individuals with neutropenia are more susceptible to bacterial infections, including those caused by S. aureus (45–48). Recent studies in animal models of S. aureus disease support these observations unambiguously (49, 50). Inasmuch as evasion of innate immunity plays a critical role in bacterial pathogenesis (17, 19, 51), a significant part of our study investigated the interaction of human PMNs with S. aureus. Of the S. aureus strains used in our study, those originally isolated from individuals with CA infections (no established risk factors) evaded killing by human neutrophils better than those isolated from hospital infections (Table I and Fig. 2F). Differences in pathogen survival were not due to altered neutrophil function, because phagocytosis, ROS production and degranulation were ultimately comparable for each of the strains tested (Fig. 2). Rather, the data indicate that strains MW2, MnCop, and LAC are more resistant to destruction per se and/or produce factors that cause more rapid host cell lysis by comparison (Fig. 3). Notably, there was no net growth by any of the strains during phagocytic interaction with human neutrophils until sometime after 3 h of culture (Fig. 2F). The increased survival/growth by MW2, MnCop, and LAC at 6 h was reflected generally by the degree of PMN lysis caused by the strains (compare Fig. 2F with Fig. 3, A and B). Thus, our data indicate that some S. aureus survived intraphagosomally without multiplying, but produced factors that cause PMN lysis.

Results from the neutrophil-S. aureus experiments were paralleled by the mouse pathogenesis data, which indicate MW2, MnCop, and LAC (those from community infections) are more virulent than COL or MRSA252 (Fig. 1), consistent with recent studies indicating strain-to-strain variability in S. aureus virulence (52). Strains LAC (pulsed-field type USA300) and MW2 (pulsed-field type USA400) are MRSA strains closely associated with community outbreaks of S. aureus disease in the United States (5, 7, 9, 10, 12, 36, 38, 41, 53). For example, recent reports indicate USA300 is a causative agent of staphylococcal necrotizing fasciitis (9) and severe community-onset pneumonia in healthy adults (12). In contrast, MRSA252 is a leading cause of hospital infections in the United States and United Kingdom (16), but to our knowledge is not a typical source of disease in individuals without associated risk factors. We note also that methicillin resistance has little to do with virulence per se, because MnCop, an MSSA strain closely related to MW2, was similar to MW2 in the mouse model of infection and in each of our host-pathogen assay systems (Figs. 1–3,
An enhanced understanding of the molecular mechanisms used by *S. aureus* to circumvent the human innate immune system will facilitate development of new treatments for bacterial infections. Our previous work with the prominent human pathogen GAS indicated that ~16% of its genes are differentially regulated during phagocytic interaction with neutrophils (19). By comparison, we determined that up to ~39% of the genes in *S. aureus* changed following phagocytosis (Fig. 5, see MW2 at 180 min). Differences in the sensitivity of the methods used (spotted vs oligonucleotide microarrays) may account for some of the differences. However, the two pathogens are quite distinct with regard to genome size, their interaction with human neutrophils, and in the types of disease caused by each. For example, GAS produces numerous molecules that inhibit PMN phagocytosis (reviewed in Ref. 56), whereas *S. aureus* is readily ingested. Nonetheless, each pathogen uses strategies to prolong intraphagosomal survival and cause eventual host cell lysis (Figs. 2 and 3) (19, 51, 56). Thus, there likely exist some common molecular mechanisms for immune evasion between the two pathogens.

We used microarrays to generate a global view of the genes and gene regulatory networks involved in bacterial pathogenesis and therefore generated a vast repository of information from which many new studies of *S. aureus* pathogenesis will emerge. The global pathogen response revealed by our studies identified dozens of potential vaccine Ags and targets for therapeutics designed to control *S. aureus* infections.

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### Disclosures

The authors have no financial conflict of interest.

### References


#### Key points

- Uncertainty and variation in microarray methods
- Importance of microarray studies in understanding pathogenesis
- Global pathogen response revealed by microarray studies
- Identification of potential vaccine Ags and targets for therapeutics
- Critical review of the manuscript and technical assistance from Layre Parkins
- No financial conflict of interest

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