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Redeploying β-Lactam Antibiotics as a Novel Antivirulence Strategy for the Treatment of Methicillin-Resistant *Staphylococcus aureus* Infections

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Innovative approaches to the use of existing antibiotics is an important strategy in efforts to address the escalating antimicrobial resistance crisis. We report a new approach to the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections by demonstrating that oxacillin can be used to significantly attenuate the virulence of MRSA despite the pathogen being resistant to this drug. Using mechanistic in vitro assays and in vivo models of invasive pneumonia and sepsis, we show that oxacillin-treated MRSA strains are significantly attenuated in virulence. This effect is based primarily on the oxacillin-dependent repression of the accessory gene regulator quorum-sensing system and altered cell wall architecture, which in turn lead to increased susceptibility to host killing of MRSA. Our data indicate that β-lactam antibiotics should be included in the treatment regimen as an adjunct antivirulence therapy for patients with MRSA infections. This would represent an important change to current clinical practice for treatment of MRSA infection, with the potential to significantly improve patient outcomes in a safe, cost-effective manner.

Keywords. MRSA; antibiotic; beta-lactam; virulence; attenuation.

New drug discovery, innovative deployment of existing antimicrobials, and exploration of antivirulence drugs are strategically important in efforts to combat resistance to antimicrobial drugs. Healthcare-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) remains a predominant nosocomial pathogen in which resistance to all licensed antistaphylococcal drugs has been reported. Resistance to methicillin and related β-lactam antibiotics is achieved via the acquisition of a mobile resistance cassette chromosome (SCC*mec*) carrying *mecA*, which encodes an alternative penicillin-binding protein, PBP2a, with a lower affinity for β-lactam antibiotics than the native *S. aureus* PBPs. β-Lactam resistance is highly regulated, and *mecA* expression is controlled by the *mecR1-mecI* inducer-repressor system [1]. Beyond SCC*mec*, exposure to oxacillin has pleiotropic effects because activation of *mecA* and PBP2a expression leads to repression of the accessory gene regulator (Agr) operon [2–4]. Agr downregulation by β-lactam antibiotics promotes expression of numerous surface proteins and repression of extracellular toxins and enzymes [3]. We previously reported that expression of high-level methicillin resistance, which is generally characteristic of HA-MRSA isolates, is accompanied by reduced toxin production and virulence [2–4]. In contrast, community-associated MRSA (CA-MRSA) strains are generally resistant to lower concentrations of β-lactams (CA-MRSA lineage USA300 is susceptible to >32 μg/mL oxacillin, compared with >256 μg/mL for most HA-MRSA isolates) and have emerged as an important cause of skin and soft-tissue infections, pneumonia, and sepsis in healthy individuals [5].

Because methicillin resistance is a regulated phenotype, we hypothesized that upregulation of methicillin resistance induced by exposure to oxacillin would downregulate the Agr system and virulence. Here we demonstrate that oxacillin significantly attenuates the pathogenesis of MRSA infections in mouse models of sepsis and pneumonia. RNA sequencing of the MRSA transcriptome following exposure to subinhibitory oxacillin revealed the basis of oxacillin-mediated attenuation via repression of toxin production and altered cell wall architecture, which led to enhanced complement deposition and opsonophagocytic killing with a decreased bacterial burden in the organs of infected mice. These data indicate that using β-lactam antibiotics as an adjunct treatment to control MRSA infections should significantly improve patient outcomes.

METHODS

Strains

The strains used in this study are all derivatives of USA300. A USA300 ΔlukS-PV mutant was a generous gift from Michael Otto. Mutants harboring transposon insertions in toxin genes, which were constructed in the USA300 derivative JE2, were obtained from the Nebraska Transposon Mutant library [6].

RNA Isolation

Fifty-microliter bacterial cultures were seeded at a ratio of 1:1000 from overnight cultures and grown for 20 hours in 250-mL conical flasks, followed by total RNA isolation with the Qiagen RNeasy Mini Kit, with the addition of 0.2 μg/μL of lysostaphin (Ambi Products, New York) to the lysis step. RNA quality and concentration were determined using the Life Technologies Qubit fluorometer and visualization on an agarose gel.

RNA Sequencing and Read Mapping

To compare global gene expression profiles, total RNA was extracted from 2 biological replicates of USA300 LAC grown for 20 hours in brain-heart infusion (BHI) medium and 3 biological replicates of USA300 LAC grown for 20 hours in BHI supplemented with 2.0 μg/mL oxacillin. RNA sequencing was performed using an Illumina MiSeq platform by the Biomedicum Functional Genomics Unit (Finland). We screened 301-bp paired-end RNA reads for quality, using FastQC (available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) trimmed to the first high-quality 150 bp, and the potential adaptor sequence was removed using Trimmomatic v0.32 [7]. The high-quality RNA reads had a mean length of 102 bp, with an insert size of 133 bp: 93% of these mapped to the USA300 reference chromosome (USA300_FPR3757; accession number NC_007793.1) or plasmids (accession numbers NC007790-93) using Bowtie2 [8]. The lowest and highest mean coverage levels were 83-fold and 174-fold, respectively.

Gene Expression Quantification

Reads mapping to genes were counted using htseq-count from HTSeq [9]. One surrogate variable was identified by surrogate variable analysis, using svaseq v3.12.0 [10]; this variable was included as an adjustment factor in the differential expression model. Differentially expressed genes was compared between samples with 0 μg/mL oxacillin versus those with 0.5 μg/mL oxacillin and between samples with 0 μg/mL oxacillin versus those with 2 μg/mL oxacillin. This was conducted using the Wald test in DeSeq2 v1.6.1 [11] where there was (1) a $\log_2$ fold change ($\log_2$ FC) of >0.5 (as specified in the Wald test) and (2) a false-discovery rate (FDR) Benjamini-Hochberg–adjusted P value of < .1. The threshold of $\log_2$ FC >0.5 was more stringent than the default value (>0). Eight genes (lacA-G and nuc) were excluded owing to conflicting direction of expression changes across comparisons.

Gene Expression in Pathways

Pathways and gene ontologies (GOs) with statistically elevated levels of differentially expressed protein-coding genes were detected using GOseq v1.18 [12], based on their QuickGO database (available at: http://www.ebi.ac.uk/QuickGO) GO terms and RefSeq identifiers. Genes present KEGG pathways were retrieved using the kegg.gsets function in GAGE v2.16.0 [13] and were corrected for length. Overrepresented GO terms and KEGG pathways had Benjamini-Hochberg–adjusted P values of < .1, using the wallenius method in GOseq.

Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Complementary DNA (cDNA) was generated from messenger RNA by using the Roche Transcriptor First Stand Synthesis kit in accordance with the manufacturer's instructions, using random hexamers. The following primers were used to amplify the cDNA: gyrB forward, CCAGGTAAATTAGCCGATTGC; gyrB reverse, AAATCGCCTGCGTTCTAGAG; mecA forward, TGCTCAATATAAAATTAAAACAAACTACGGTAAC; mecA reverse, GAATAATGACGCTATGATCCCAA; tarS forward, CTGTGGAGGAAAGTTACCGTCA; tarS reverse, TGGCTT AGCGATATCAATGCCT; dltA forward, TGGTCCAACT GAAGCTACGG; dltA reverse, ATCTTGCGCCTGGTCTTTC A; argH forward, GCGTGCACAGCCAATTTCAT; argH reverse, TGGTACCACTTAAGGCTGCTG; rot forward, TTGGGATTGTTGGGATGTTT; rot reverse, ATTCGCTTTC AATCTCGCTG; mraZ forward, GGTACACTCTAGACGAA TGGCA; mraZ reverse, GCACCAGAGAAGAACATACGC; purM forward, TGCAGTCGCAATGTGTGTTA; purM reverse, CCCATTTCAGCAGTCTCTCC; purH forward, CHAAA TAAACCGCAGCATTT; purH reverse, AGCTGCAGCACG TAACATTG; vraR forward, AAGCCCATGAGTTGAAGCCA; and vraR reverse, GCATCGGCGATATCTTTTGCA.

Standard curves were produced for each primer set by using serial dilutions of cDNA to determine primer efficiency. RT-qPCR analyses were performed using SYBR reagent (Roche) according to the manufacturer's instructions. For each RT-PCR experiment, target gene mRNA levels were compared to those for gyrB, and at least 3 biological replicates were used (ie, RNA was extracted from at least 3 independent bacterial cultures). In addition, duplicate RT-qPCRs for gyrB and each target gene were performed on RNA extracted from each biological replicate. Cycle threshold (Ct) values were subsequently determined, and for each reaction, the ratio of the target gene (x) and gyrB transcript numbers was calculated as 2 $[\text{Ct} \times gyrB^- \times \text{Ct}^x]$.

Phage Adsorption Assays

Phage ΦWS2 was initially propagated on S. aureus strain 8325-4. Fifty-milliliter bacterial cultures were seeded at a ratio of

1:1000 from overnight cultures and grown for 20 hours in 250-mL conical flasks. Where indicated, antibiotics were added 4 hours after inoculation. A 200-µL cell suspension normalized to an A_{600} of 0.5 was mixed with 100 µL of phage (at a titer of approximately 100–200 plaques/plate) and incubated at 37°C for 15 minutes to allow phage adsorption. The cells were then placed on ice for 5 minutes, and bacteria plus adsorbed phage was removed via centrifugation. Fifty microliters of supernatant containing any unbound phage was added to a 50-µL suspension of 8325-4 cells from an overnight culture, mixed with 5 mL of semisoft tryptone soy agar (TSA), and poured onto TSA plates supplemented with 5 mM $CaCl_2$ before being incubated at 37°C for 20 hours. Plaques were subsequently counted, and adsorption efficiency was calculated as the percentage of adsorbed phage relative to the number of phage used to infect the bacterial cells.

Neutrophil Lysis Assays

Human neutrophils were isolated from the peripheral blood of healthy volunteers following informed consent, as approved by the University of Liverpool Ethics Committee. Heparinized blood was incubated with 6% dextran in phosphate-buffered saline (PBS; Sigma-Aldrich) for 20 minutes at room temperature. The top, clear layer containing leukocytes was transferred to a fresh tube, and the cells were underlaid with 7 mL of Histopaque (Sigma) and centrifuged at $700 \times g$ for 20 minutes. The overlying plasma and PBMC layers were aspirated, and the neutrophil/red blood cell pellets were suspended in $1 \times$ red blood cell lysis buffer (eBioscience). Cells were then washed in sterile PBS and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) buffered with 10 mmol/L HEPES (pH 7.2) to a density of 2.0×10^6 cells/mL.

For the neutrophil lysis assay, the bacteria were subcultured in brain-heart infusion (BHI) broth, RPMI medium, or CCY broth with or without oxacillin for 20 hours. Bacterial cell densities were adjusted to an A_{600} of 1, and supernatants were collected. A total of 30 µL of the neutrophil suspension was mixed with 10 µL of culture supernatant and incubated at 37°C for 30 minutes. Assays were performed in duplicate, and cell integrity was assessed using trypan blue live/dead staining microscopic analysis. Where indicated by asterisks in Figure 2, differences between groups were deemed significant as assessed using the Wilcoxon rank sum test and 2-tailed Student t tests.

C3b Deposition and Opsonophagocytic Assays

C3b deposition assays were performed as described previously [14]. Briefly, 10^7 colony-forming units (CFU) of USA300 LAC, JE2, and JE2 tarS::Tn were incubated in 20 µL of S. aureus–treated human serum (20%) for 25 minutes. Bound C3b was detected using mouse anti-human C3 monoclonal antibody (Abcam) and anti-mouse IgG2a APC (EBioscience) and subsequently measured using an Accuri C6 flow cytometer. Thiazole orange (BD) was added to each sample to isolate bacteria. The

opsonophagocytic assay was performed as described previously [15, 16]. A total of 4×10^5 HL-60 human neutrophil cells were incubated with 1×10^3 opsonized USA300 LAC, JE2, and JE2 tarS::Tn and complement. The number of CFU was counted by plating onto blood agar plates following incubation for a further 45 minutes (for HL-60 cells) or 60 minutes (for J774 cells). Intravenous immunoglobulin at a final dilution of 1:16 was used as the source for pathogen-specific antibody for opsonization. Nonopsonized staphylococci were used as a control. Statistical significance was assessed using a 2-tailed Student t test.

Mouse Infection Experiments

Age-matched, 6–8-week-old, outbred CD1 female mice (Charles River, United Kingdom) were used in a nonlethal model of sepsis and a lethal model of pneumonia. USA300 cultures were grown to an A_{600} of 1 in BHI broth for the sepsis model and overnight for the pneumonia model, washed in PBS, and adjusted to appropriate cell densities. For bacteremia, 5×10^6 CFU USA300 (10 mice/group) were injected into the tail vein and left untreated (PBS control) or treated with 7.5 mg or 75 mg oxacillin/kg/12 hours (the first antibiotic dose was administered 9 hours after infection), before being euthanized after 28 hours and 7 days. For pneumonia, mice were anesthetized with O_2 and isoflurane and infected intranasally with $2–3 \times 10^8$ CFU. Survival was followed over 7 days in untreated and oxacillin-treated mice (7.5 or 75 mg oxacillin/kg in 1 dose after 3.5 hours; 10 mice/group). Blood specimens were obtained from tails 15 hours after infection to assess bacteremia. Blood specimens collected from animals with pneumonia or homogenates of organs obtained from mice with sepsis were plated on blood agar. Statistical significance was assessed using the 2-tailed Student t test. Mouse experiments were approved by the United Kingdom Home Office (Home Office Project License Number 40/3602) and the University of Liverpool Animal Welfare and Ethics Committee.

RESULTS

Oxacillin Treatment Significantly Improves Survival of Mice Infected by CA-MRSA

The therapeutic potential of oxacillin in the treatment of MRSA infections was assessed in mice. Based on clinical protocols in humans (flucloxacillin is administered intravenously at 100–200 mg/kg/day), oxacillin was intravenously administered to mice twice daily at a therapeutic concentration of 75 mg/kg/12 hours (high dose) or at one tenth the therapeutic dose (7.5 mg/kg/12 hours; low dose). To establish sepsis, groups of 5 mice were infected via the tail vein with 5×10^6 CFU of USA300, and the infection was allowed to establish for 9 hours before oxacillin or PBS (control) were administered. Thereafter, oxacillin was administered every 12 hours, and the infection was allowed to proceed for 28 hours or 7 days (Figure 1). Oxacillin reduced the bacterial burden in the kidneys and spleens of infected animals after 28 hours or 7 days,
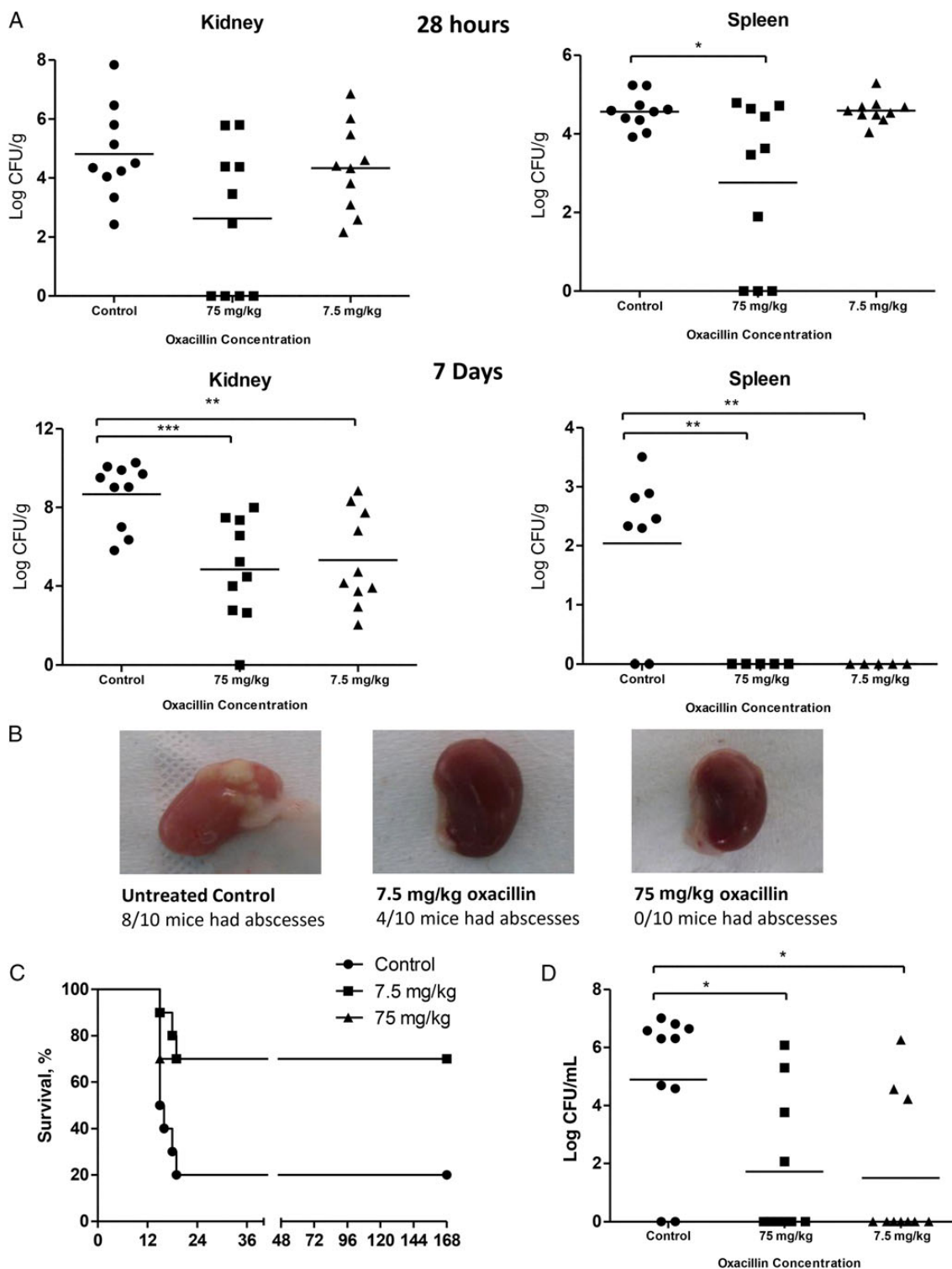
Figure 1. Attenuation of community-associated methicillin-resistant *Staphylococcus aureus* virulence by oxacillin in mouse sepsis and pneumonia infection models. *A*, The number of colony-forming units (CFU)/g of kidney and spleen recovered from mice infected by tail vein injection with 5×10^6 USA300 LAC (10 mice/group) and left untreated (control) or treated with 7.5 mg or 75 mg of oxacillin/kg per 12 hours (the first antibiotic dose was given 9 hours after infection) before being euthanized after 28 hours and 7 days. *B*, Photos of representative kidneys recovered on day 7 from untreated mice infected with USA300 LAC or mice treated with 7.5 mg or 75 mg of oxacillin/kg per 12 hours. *C*, Survival of mice over 7 days after intranasal infection of 2×10^8–3×10^8 USA300 LAC bacteria to establish pneumonia. Mice were untreated (control) or treated with 7.5 mg or 75 mg of oxacillin/kg in 1 dose after 3.5 hours, and survival was plotted as a percentage of the total number of animals (10 mice/group). *D*, The number of CFU/mL blood recovered 15 hours after infection from untreated mice with pneumonia (control) or mice treated with 7.5 mg or 75 mg of oxacillin/kg in 1 dose after 3.5 hours (10 mice/group). Statistically significant differences are indicated. ***P < .0001, **P < .001, and *P < .05, by the 2-tailed Student *t* test.
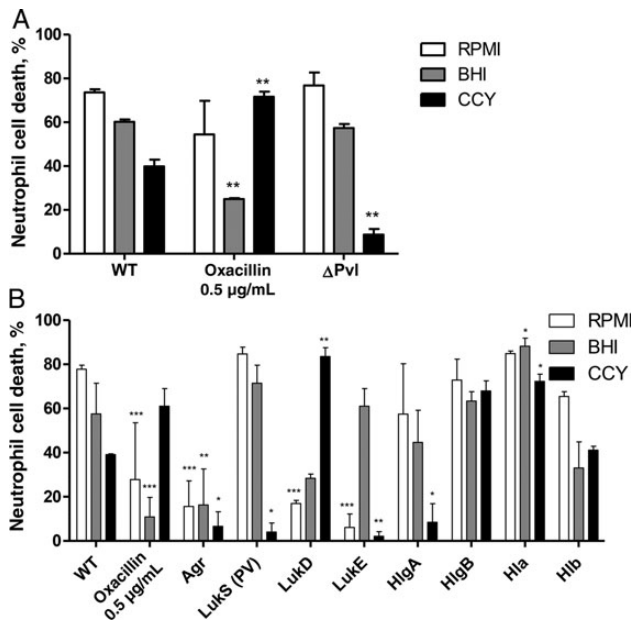
Figure 2. Oxacillin exposure represses production of cytolytic toxins. *A*, Susceptibility of neutrophils isolated from fresh human blood to lysis by supernatants from USA300 cultures grown in Roswell Park Memorial Institute (RPMI), brain-heart infusion (BHI), or CCY media in the absence of oxacillin (wild type [WT]) or in medium supplemented with oxacillin (0.5 µg/mL). USA300 LAC Δ*pvl* was included as a control. Data presented are the means of 3 independent experiments ± standard error of the mean. Statistically significant differences are indicated. *B*, Susceptibility of neutrophils isolated from fresh human blood to lysis by supernatants from *Staphylococcus aureus* JE2 *agr*, *lukS*, *lukD*, *lukE*, *hlgA*, *hlgB*, *hla*, and *hlb* transposon mutants grown for 20 hours in BHI medium. The integrity of cells stained with trypan blue was assessed using microscopy, and the data presented are the means of at least 2 independent counts ± standard error of the mean. ***P < .0001, **P < .001, and *P < .05, by the 2-tailed Student *t* test.

reaching significance after 7 days at both low and high doses, reducing the number of CFUs in kidneys by 4 logs and clearing CFUs from spleen (Figure 1*A*). No visible abscesses were present on kidneys recovered from infected mice treated with high-dose oxacillin, while kidneys recovered from only 40% of infected mice treated with low-dose oxacillin had visible kidney abscesses, compared with 80% of untreated mice (Figure 1*B*). Oxacillin treatment was also therapeutically effective in a murine pneumonia model, with survival increasing from 20% in the untreated group to 60% in mice treated after 3.5 hours with high or low doses of oxacillin (Figure 1*C*). Furthermore, the number of bacteria recovered from the blood of mice with pneumonia was significantly reduced in the low- and high-dose oxacillin treatment groups—a key stage in the development of sepsis secondary to pneumonia (Figure 1*D*).

Oxacillin Represses Cytolytic Toxin Production by CA-MRSA via Downregulation of Agr

The previously reported strain-dependent effects of oxacillin on the production of a number of *S. aureus* toxins [4] prompted further investigation of oxacillin-modulated USA300 cytolytic

activity. The susceptibility of neutrophils isolated from fresh human blood (which are susceptible to lysis by *S. aureus* toxins, including Panton-Valentine leukocidin [PVL]) was tested against supernatants from cultures of USA300 or the USA300-derivative strain JE2, the parent strain of the Nebraska transposon mutant library [6], grown in BHI, RPMI, or CCY media. Previous experiments showing that upregulation and increased cytolytic activity of PVL by *S. aureus* grown in a sub–minimum inhibitory concentration (MIC) of oxacillin were performed only in CCY medium [4, 17], which appears to artificially induce higher PVL levels than measured in human abscesses or serum [18]. Western blots showed that PVL was expressed in BHI and CCY but not RPMI medium (data not shown). Mutation of *lukS*-PV in USA300 or JE2 significantly reduced neutrophil lysis but only in CCY medium (Figure 2*A* and 2*B*). Oxacillin-supplemented BHI or RPMI culture supernatants were less toxic to neutrophils, reaching significance in BHI medium (Figure 2*A*), whereas oxacillin-supplemented CCY supernatants were significantly more toxic (Figure 2*A*). Agr mutant supernatants were significantly less toxic (Figure 2*B*). Mutations in *hlb*, *hlgA*, and *hlgB* toxin genes did not attenuate toxicity (Figure 2*B*). Medium-dependent effects were associated with mutations in *lukD*, *lukE*, and *hlgA*, which attenuated toxicity in RPMI medium (*lukD*), RPMI and CCY media (*lukE*), and CCY medium (*hlgA*), respectively (Figure 2*B*). Thus, potential concern about upregulation of PVL toxin by oxacillin are ameliorated by these data showing that this was only evident in CCY medium and not in RPMI or BHI media. Collectively these data reveal a dominant role for the Agr system under all growth conditions (with a relatively minor role for other toxins) in oxacillin-induced attenuation of cytotoxicity.

Oxacillin-Induced Activation of Teichoic Acid Biosynthesis Is Associated With Enhanced Complement Deposition and Opsonophagocytosis

To further investigate the basis for oxacillin-induced virulence attenuation, RNA extracted from USA300 grown for 20 hours in sub-inhibitory oxacillin (2.0 µg/mL) was sequenced. Oxacillin down-regulated 25 genes and up-regulated 174, including activation of *mecA* and concomitant repression of *agr* (Supplementary Figure S1 and Table S1). By using a 4-fold lower oxacillin concentration (0.5 µg/mL), a subset of genes exhibiting highly elevated (16) or reduced (9) expression at both antibiotic concentrations was identified (Figure 3*A*) and confirmed by qPCR (Figure 3*B*). Two major themes emerged from these data, namely that (1) 3 genes encoding major global regulators (*agr*, *rot* [repressor of toxins {Rot}], and *vraR* [vancomycin resistance regulator {VraR}]) and (2) genes encoding enzymes involved in the wall teichoic acids (WTA) biosynthesis were regulated by oxacillin. The >5-fold activation of *rot* was consistent with repression of *agr*, and VraR is known to activate
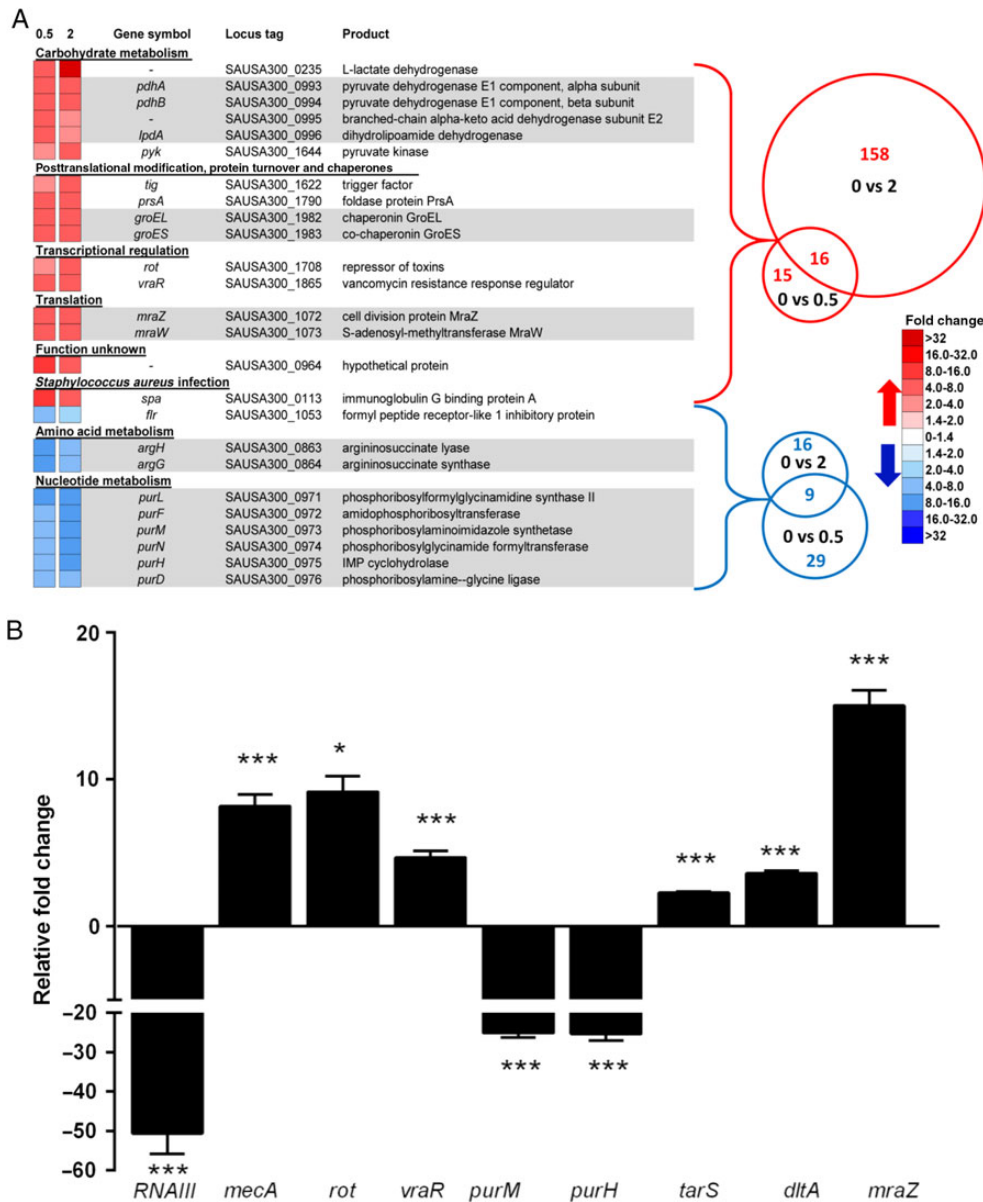
Figure 3. Oxacillin regulates genes involved in toxin production and the architecture of the cell surface. *A*, USA300 LAC genes with evidence of differential expression (DE) when grown in the presence of both 0.5 μg/mL and 2.0 μg/mL oxacillin. The genes are grouped by expression change (red for up; blue for down) and functional category. DE genes had a surrogate variable analysis–adjusted log$_2$ fold change of >0.5 and a corrected *P* value of < .1. The total number of DE genes between comparisons is shown by the circle areas. The numbers of downregulated genes between 0 μg/mL and 0.5 μg/mL (38) and 0 μg/mL and 2 μg/mL (25) were about the same as those upregulated from 0 μg/mL to 0.5 μg/mL (31), whereas the number with elevated expression between 0 μg/mL and 2 μg/mL was much higher (174). Total RNA was extracted from 2 biological replicates of USA300 LAC grown for 20 hours in brain-heart infection (BHI) medium and 3 biological replicates of USA300 LAC grown for 20 hours in BHI medium supplemented with oxacillin 0.5 μg/mL and 2.0 μg/mL. *B*, Comparison of relative gene expression by the LightCycler reverse-transcription quantitative polymerase chain reaction assay in USA300 LAC grown for 20 hours in BHI medium or BHI medium supplemented with 0.5 μg/mL oxacillin. Total RNA was extracted from cultures grown at 37°C for 20 hours to stationary phase in BHI medium. Experiments were repeated at least 3 times, and standard errors of the mean are shown. ***P < .0001 and *P < .05, by the 2-tailed Student *t* test. This figure is available in black and white in print and in color online.

a cell-wall-stress regulon in response to β-lactam and glycopeptide antibiotics [19].

The highly conserved *mraZ* and *mraW* genes, located at the head of the division and cell wall *dcw* (division and cell wall)/*mra* (murein A) gene cluster, may also contribute to altered cell wall architecture in response to oxacillin. The transcriptional regulator MraZ controls expression of genes within and outside the *dcw* cluster [20] and *mraW* (*rsmH*) encodes a 16S ribosomal RNA (rRNA) methyltransferase. rRNA methyltransferases have been implicated in control of ribosome function in response to various stresses [20, 21]. Repression of the purine biosynthetic operon (apparently via activation of the *purR* repressor) was

the largest fold change (>20-fold) identified, and although the biological reason for this remains unclear, the purine nucleotide signaling molecules cyclic diadenosine monophosphate and ppGpp have been implicated in homogeneous oxacillin resistance [2, 22, 23], perhaps suggesting a physiological requirement to maintain intracellular purine homeostasis under oxacillin stress. Activation of the *spa* gene (presumably mediated by Rot [24]), which encodes protein A, an antibody-evasion protein, was not consistent with reduced virulence and may suggest that oxacillin-mediated attenuation of virulence is primarily associated with increased susceptibility to innate, rather than acquired, immune responses.

The *dlt* operon, *tarS*, and *tagH*, which are involved in modification and export of WTAs, were activated by oxacillin. Furthermore, activation of the Rot- and Agr-regulated *dlt* operon is consistent with oxacillin-induced activation of *rot* and repression of *agr* [24, 25]. WTA in *S. aureus* is composed of multiple ribitol phosphate units modified with D-alanine and O-N-acetylglucosamine (O-GlcNAc) in either an α or β configuration [26, 27] and is implicated in cell division, biofilm formation, and virulence. α-O-GlcNAc and β-O-GlcNAc WTA modifications are catalyzed by the TarM and TarS glycosyltransferases, respectively [26], and loss of WTA or deletion of *tarS* alone increases MRSA susceptibility to β-lactams [26]. Furthermore WTA N-acetylglucosamine residues contribute to opsonophagocytosis by activating the complement system [27]. The approximately 5-fold activation of *tarS* (Supplementary Figure S2) can be predicted to contribute to increased resistance to β-lactams, enhanced complement deposition and opsonophagocytosis, and promote interactions with phage for which glycosylated WTA acts as a receptor [27]. Binding of phage WS2 to USA300 and JE2 was *tars* dependent, and WS2 binding to wild-type cells grown in subinhibitory oxacillin (0.5 µg/mL) was significantly elevated, increasing to 100% in cells exposed to high-dose oxacillin (data not shown). TarS-dependent deposition of complement protein C3b on oxacillin-grown cells was also significantly increased, which in turn was associated with significantly higher levels of opsonophagocytosis, using HL-60 neutrophil cells (Figure 4A and 4B). Apparently, increased oxacillin-induced β-O-GlcNAcylation of WTA promotes opsonophagocytosis. Collectively, among the pleiotropic effects of oxacillin, activation of *rot*, repression of *agr*, activation of WTA biosynthesis, increased WTA β-O-GlcNAcylation, and enhanced opsonophagocytosis are predominant effectors of oxacillin-induced virulence attenuation.

DISCUSSION

The widespread and generally safe clinical use of β-lactam antibiotics means there are few barriers to their redeployment as antivirulence agents in combination with other antibiotics. Although our data are limited to USA300, which was chosen because its oxacillin MIC can be significantly increased in the
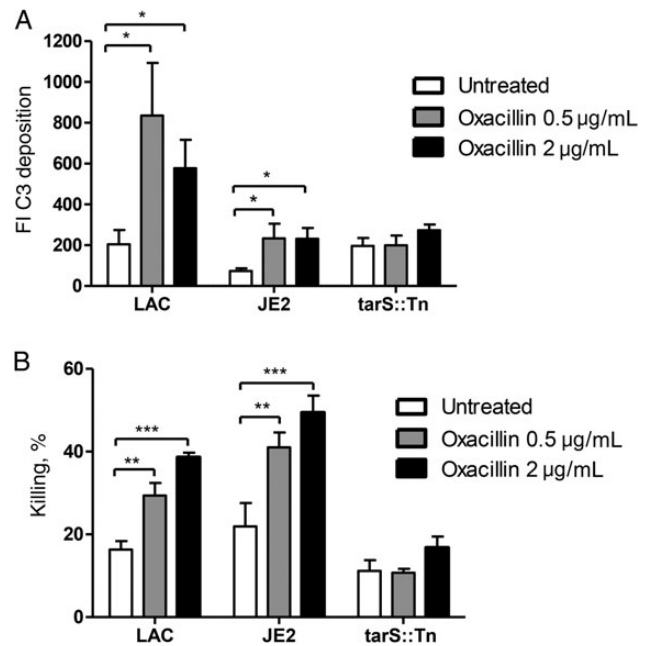

Figure 4. Oxacillin exposure promotes opsonophagocytic killing. *A*, Deposition of complement protein C3b on USA300 LAC, JE2, and JE2 *tarS*::Tn cells grown in brain-heart infusion (BHI) medium or BHI medium supplemented with oxacillin (0.5 µg/mL or 2 µg/mL). *B*, Opsonophagocytic killing of USA300, JE2, and JE2 *tarS*::Tn grown in BHI medium or BHI medium supplemented with oxacillin (0.5 µg/mL or 2 µg/mL) by HL-60 (neutrophil) cells. The data presented are the means of at least 3 independent experiments ± standard error of the mean. ***$P < .0001$, **$P < .001$, and *$P < .05$, by the 2-tailed Student *t* test.

presence of the antibiotic, the dominant phenotypes associated with MRSA exposure to oxacillin were entirely consistent with virulence attenuation. The USA300 data presented here are supported by a 1981 study showing the therapeutic potential of an ampicillin/β-lactamase inhibitor combination against nafcillin-resistant *S. aureus* in a rabbit model of endocarditis [28] and by more-recent studies showing that β-lactam antibiotics can potentiate the activity of antimicrobial peptides against methicillin-susceptible *S. aureus* and MRSA [29, 30]. Potential concern about upregulation of PVL toxin by oxacillin [4] was ameliorated by data showing that this was only evident in CCY medium and not in RPMI or BHI media. In the animal experiments described in this study, the bacteria were grown in BHI medium to ensure that PVL levels were not artificially activated and, thus, that they were more physiologically relevant [18]. A recent randomized, controlled study with 60 patients revealed that the duration of bacteremia was reduced from 3 to 1.9 days in patients treated with both vancomycin and flucloxacillin, compared with those treated with vancomycin alone [31], further indicating that potential induction of PVL by β-lactams does not exacerbate MRSA infections. In addition, our findings provide mechanistic insights that explain and support the therapeutic benefits observed in this clinical trial. The therapeutic benefit and cost-effectiveness of using β-lactams to repress toxin production,

alter cell wall architecture, and increase bacterial susceptibility to host immune cell killing is compelling.

Supplementary Data

Notes

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