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

Elaine M. Waters,1,a Justine K. Rudkin,3,a Simone Coughlan,4 Geremy C. Clair,7 Joshua N. Adkins,7 Suzanna Gore,1 Guoqing Xia,2 Nikki S. Black,3 Tim Downing,5 Timo O’Neill,6 Aras Kadioglu,1,b and James P. O’Gara1,b

1Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, and 2Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health, University of Manchester, United Kingdom; 3Department of Microbiology, School of Natural Sciences, 4School of Mathematics, Statistics, and Applied Mathematics, National University of Ireland, Galway, 5School of Biotechnology, Dublin City University, and 6Department of Clinical Microbiology, Royal College of Surgeons in Ireland, Connolly Hospital, Dublin, and 7Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington

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 staphylococcal drugs has been reported. Resistance to methicillin and related β-lactam antibiotics is achieved via the acquisition of a mobile resistance cassette chromosome (SCmec) 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 SCmec, 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 ΔluxS-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 Biomedical 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 NCO007990-93) 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 were 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 log₂ fold change (log₂ 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 log₂ 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, CCAGGTAAATTAGCCGATTCG, gyrB reverse, AAATCGCCTGGGTATGAG; mecA forward, TGCTACTTATATAAATTAAACACTGTTGTAAC; mecA reverse, GAAATATGAGCCATATGACCCCA; tarS forward, CTGTGAGAGAAATCCCGTCGA; tarS reverse, TGGCTTAAGGATGATGCT; dltA forward, TTGGGATGTGGTGGT; dltA reverse, ATCTCTCAGGGTTCTCC; A; argH forward, GGCGCAGAGGACATTCACT; argH reverse, GGTGACCTTAAGGCTGCTG; rot forward, TTGGGATGTGGTGGT; rot reverse, ATTCGCTTTC AATCTCGGT; mraZ forward, GTGATGCTGACGGAA; TGGCA; mraZ reverse, GACCCAGAGAAGACATACCG; purM forward, TGGATCCATAGGTGCCTG; purM reverse, CCAATTCAGCTCTTC; purH forward, CHAA TAAACCCGAGGATT; purH reverse, AGCTGACAGCAC TACATG; vraR forward, AAGCCATGAGTTGAAGC; and vraR reverse, GATCGGCCATCTATTCGTCA.

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 \[\text{Ct} \times \text{gyrB}^{-x} \times \text{Ct}^x\].

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 × g for 20 minutes. The overlying plasma and PBMC layers were aspirated, and the neutrophil/red blood cell pellets were suspended in 1 × 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 cultured 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$^7$ HL-60 human neutrophil cells were incubated with 1 × 10$^5$ 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 × 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 (fluoxacillin 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$^8$ 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 $5 \times 10^6$ 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 $2 \times 10^8$–$3 \times 10^8$ 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 cytolitic 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 Δpvl 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 1A). 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 1B). 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 1C). 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 1D).

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 2A and 2B). Oxacillin-supplemented BHI or RPMI culture supernatants were less toxic to neutrophils, reaching significance in BHI medium (Figure 2A), whereas oxacillin-supplemented CCY supernatants were significantly more toxic (Figure 2A). Agr mutant supernatants were significantly less toxic (Figure 2B). 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 2B). Thus, potential concern about upregulation of PVL toxin by oxacillin is 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 3A) and confirmed by qPCR (Figure 3B). 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 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 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 Δpvl 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.

Using β-Lactam Antibiotics to Attenuate MRSA • JID • 5

Downloaded from http://jid.oxfordjournals.org/ at University of Manchester on November 21, 2016
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 WTA, 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-acetylgalactosamine (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-acetylgalactosamine 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 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 fluclaxacin, 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
Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copylefted and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes
Acknowledgments. We thank Michael Otto for the generous gift of USA300 ΔluxS-PV.

Disclaimer. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Financial support. This work was supported by the Irish Health Research Board (HRB_POR/2012/51) (to J. P. O.), the University of Liverpool (pump priming award to E. M. W.), and the United Kingdom Medical Research Council (to A. K.). Contributions by J. N. A. and J. C. C. were supported by the NIH National Institute of General Medical Sciences (GM103493 and GM094623).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References