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 anti-staphylococcal drugs has been reported. Resistance to methicillin and related β-lactam antibiotics is achieved via the acquisition of a mobile resistance cassette chromosome (SCCmec) 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 SCCmec, 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 sequenced 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 NC_007790-93) 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 and 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 0.5 µg/mL oxacillin.

**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, CCAGTGAATATTGGCGATTTGC; gyrB reverse, AAATCCTCGGTTTCTAGAG; mecA forward, TGGCTCATATAAATTTAAAACAAACTCGTAAAC; mecA reverse, GAAATGACGCTATGATCCAA; tarS forward, CTGGTGAGGAAAGTACCCTGCTA; tarS reverse, TGGGCTTACGGATATCAGCTC; dltA forward, TGGTCCAGTACCCG; dltA reverse, ATCTTTGCGCTGTCTTCA; argH forward, GCGGGACAGCAATTTTCAT; argH reverse, TGGTACCACCTAAGGCTGTG; rot forward, TTGGGATTGGGAGATTGTT; rot reverse, ATTCGGTTCAATCCTCGT; mraZ forward, GTGACACTCATAGCAGAA; mraZ reverse, GCACCAGAGAAGACAATCAGC; purM forward, TGGTGTCGAAATGTTGTTGTT; purM reverse, CCCATTGAGCAGTCTCCTC; purH forward, CHAAGAAACCCGAGCATT; purH reverse, AGCTGCAAGCAGTACATGTG; vraR forward, AAGCCCATGATTGAAGCA; and vraR reverse, GCATGGGCAATCCTTTGCA.

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[Ct × gyrB - 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
sities were adjusted to an broth with or without oxacillin for 20 hours. Bacterial cell den-
in brain-heart infusion (BHI) broth, RPMI medium, or CCY.

C3b deposition assays were performed as described previously

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 × 10⁶ 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 A₆₀₀ 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, 10⁶ 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

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 (fluoroacinil 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 × 10⁶ 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.
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 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 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
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 dwc (division and cell wall)/mra (murein A) gene cluster, may also contribute to altered cell wall architecture in response to oxacillin. The transcripational regulator MraZ controls expression of genes within and outside the dwc 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

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 log2 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.
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 s, 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 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 flucluocillin, 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 copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

**Notes**

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