A Tick Antivirulence Protein Potentiates Antibiotics against *Staphylococcus aureus*

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ABSTRACT New strategies are needed to combat antibiotic resistance, especially against pathogens such as methicillin-resistant *Staphylococcus aureus*. A tick anti-freeze glycoprotein, IAFGP, possesses potent antibiofilm properties against a variety of clinical pathogens, including *S. aureus*. Synergy between IAFGP, or a peptide (P1) representative of a repeat region of the protein, with different antibiotics was assessed *in vitro*. Antibiotics that synergized with either IAFGP or P1 were further evaluated *in vivo* using vertebrate and invertebrate infection models. IAFGP readily enhanced the efficacy of antibiotics against *S. aureus*. Synergy with daptomycin, an antibiotic used to treat methicillin-resistant *S. aureus*, was observed *in vitro* and *in vivo* using *iafgp*-transgenic mice and flies. Furthermore, synergy with ciprofloxacin or gentamicin, antibiotics not generally used to treat *S. aureus*, was also perceived. The combined effect of the antibiotic and IAFGP was associated with improved permeation of the antibiotic into the cell. Our results highlight that synergy of IAFGP with antibiotics traditionally used to treat this pathogen, and enhancement of the potency of antibiotics not commonly used against this microbe, can provide novel alternative therapeutic strategies to combat bacterial infections.

KEYWORDS antibiotics, antifreeze protein, biofilms, *Staphylococcus aureus*

The widespread use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant microbes. *Staphylococcus aureus* and its antibiotic resistant counterpart, methicillin-resistant *S. aureus* (MRSA), within hospital and community settings, is associated with approximately 90,000 infections annually, with about 18,000 deaths in the United States (1). Antibiotic resistance among *S. aureus* isolates is compounded, in certain diseases such as endocarditis and osteomyelitis, by the capacity of this pathogen to form tenacious biofilms, endowing their resident microbes with enhanced resistance to mechanical manipulation, protection from the host immune system, and diminished efficacy of anti-infective agents such as antibiotics (2, 3). With the burgeoning rate of MRSA infections, combined with an upturn in the use of antibiotics, including vancomycin, linezolid, and daptomycin, isolates with intermediate or full resistance to each of these antibiotics have emerged (4–15). Multidrug treatment is an alternative approach to control infection, especially for patients with extensive disease that is difficult to treat due to bacterial resistance or the location of the pathogen (16, 17). The limited number of available therapeutics against MRSA, coupled with the ongoing emergence of antimicrobial resistance, necessitates the development of new approaches.

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We have previously elucidated the antivirulence function of an antifreeze protein isolated from *Ixodes scapularis* ticks (18). The *I. scapularis* antifreeze glycoprotein (IAFGP) contains highly conserved tripeptide repeats, Ala-Ala-Thr (AAT) and Pro-Ala-Thr (PAT), interspersed between a 6-amino-acid spacer sequence, Pro-Ala-Arg-Lys-Ala-Arg (PARKAR), approximately every 21 amino acids. Having demonstrated antibiofilm properties with the IAFGP protein, we proceeded to synthesize a variety of different peptides based on the original protein sequence, making variations in length, amino acid composition, and organization. In doing so, we identified peptide P1, a 24-amino-acid peptide composed of a PARKAR spacer followed by 6 AAT triplets, which phenocopied IAFGP in inhibiting Gram-positive bacterial biofilms *in vitro* and *in vivo*, including those by *S. aureus* and MRSA (18). More recently, we identified the mechanism by which IAFGP and P1 inhibited biofilms among Gram-positive pathogens, including *S. aureus*: binding and targeted manipulation of the bacterial peptidoglycan (19).

Bacterial peptidoglycan (PGN) is an essential component of almost all bacteria, and it helps maintain cell integrity and shape. Inhibition of its biosynthesis through mutations or antibiotics such as vancomycin or β -lactams can result in cell death (20–22). PGNs, among Gram-positive or-negative bacteria, are nearly identical, in that they are composed of linear glycan strands made up of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) sugar residues, cross-linked into a mesh-like framework through peptide stems attached to the NAM disaccharide repeat. The peptide stem is composed of 5 amino acid residues, L-Ala-D-isoGlu-X-D-Ala-D-Ala, where X represents the amino acid specific to Gram-positive (L-Lys) or -negative bacteria (mDap) (21). The cross-linking between glycan strands is mediated by the transpeptidase enzymes, which links the 3rd residue of one (donor) stem peptide with the 4th residue of the neighboring (acceptor) stem peptide strand with a concomitant loss of the terminal (5th) D-Ala residue from the donor stem peptide. Transpeptidase enzymes are inactivated by β -lactam antibiotics through their direct binding with the enzyme, while vancomycin binds the terminal D-Ala-D-Ala residues and inhibits the cross-linking between glycan strands (23). We have shown that IAFGP and P1 can directly bind the terminal (5th) D-alanine residue of the nascent *S. aureus* peptidoglycan and in doing so alters peptidoglycan in a nonlethal fashion, and this binding is directly correlated with an inhibition of bacterial biofilms (19). We propose the antivirulence properties of IAFGP in altering biofilms, through targeted binding and manipulation of peptidoglycan, can function synergistically with select antibiotics to improve treatment against bacterial infection.

RESULTS

IAFGP synergizes with antibiotics *in vitro*. IAFGP and the 24-amino-acid peptide derived from IAFGP, peptide P1 (PARKARAATAATAATAATAATAAT), reduce *S. aureus* biofilms, including those by methicillin-resistant *S. aureus* (MRSA) (18, 19). Having determined 0.1 mg/ml of IAFGP or P1 yields the strongest effects against *S. aureus* biofilms without any adverse effects on growth (see Fig. S1A in the supplemental material) (18, 19), using a microplate assay we evaluated whether 0.1 mg/ml IAFGP or P1 enhances traditional antibiotic therapy against *S. aureus*. We measured the MIC of each antibiotic against two *S. aureus* isolates, a laboratory strain (SA113) and a common methicillin-resistant strain (USA300 JE2), in liquid culture and in the presence of recombinant glutathione S-transferase (GST)-tagged IAFGP, GST alone, P1 peptide, or its scramble control peptide, sP1 (AATAATATAAARRAAAAPTAKTT). IAFGP or P1 synergized with 3 antibiotics, gentamicin, ciprofloxacin, and daptomycin, reducing the MIC in each case compared to antibiotic treatment alone or supplementation with either GST protein or sP1 peptide controls. The MIC of gentamicin, an aminoglycoside, was reduced 4-fold with the addition of IAFGP or P1 (Table 1). Treatment with 0.125 μ g/ml gentamicin supplemented with IAFGP or P1 reduced bacterial counts between 30- and 100-fold, respectively, compared to gentamicin alone or antibiotic-complemented controls (Fig. S1B). Ciprofloxacin, a fluoroquinolone, combined with IAFGP or P1, demonstrated markedly improved activity against SA113 (Table 1). Antibiotics like

TABLE 1 Antibiotic MIC in the presence of IAFGP or P1

Antibiotic tested	MIC ($\mu\text{g/ml}$)		MIC ($\mu\text{g/ml}$) + 0.1 mg/ml IAFGP ^c		MIC ($\mu\text{g/ml}$) + 0.1 mg/ml P1 ^c	
	SA113 ^a	USA300 ^b	SA113	USA300	SA113	USA300
Gentamicin	0.63–1.25	1.25–2.5	0.16–0.31	0.31–0.63	0.08–0.16	0.31–0.63
Ciprofloxacin	1.25–2.5	R	0.31–0.63	R	0.16–0.31	R
Daptomycin	1.25–2.5	2.5–5	0.08–0.16	0.08–0.16	0.08–0.16	0.08–0.16

^aSA113 *S. aureus* strain (ATCC 35556) is derived from laboratory strain NCTC 8325.

^bMRSA isolate USA300 JE2 is a plasmid-cured derivative of strain LAC. MRSA clone USA300 JE2 was tested and found resistant to ciprofloxacin (>5 to $10 \mu\text{g/ml}$) (55) in the presence or absence of either IAFGP, P1, or control treatments. R, resistant.

^cGST protein or sP1 scramble peptide was used as the control and yielded MIC values identical to those from treatment with antibiotic alone.

vancomycin, linezolid, and daptomycin are potent antibiotic options against MRSA infections. While neither vancomycin nor linezolid synergized with either IAFGP or P1 (Table S1), daptomycin, a lipopeptide bactericidal agent, showed enhanced activity with IAFGP or P1 against *S. aureus*, including MRSA (Table 1). Overall, these results demonstrate synergism of three antibiotics, gentamicin, ciprofloxacin, and daptomycin, belonging to varied classes, when combined with IAFGP or P1.

Synergy of antibiotics in *iafgp*-expressing *Drosophila melanogaster* and a murine model of MRSA infection. *iafgp*-expressing *D. melanogaster* has been used to study IAFGP in invertebrates (18, 24), and these transgenic flies, compared to their control (*mCherry*) counterparts, showed enhanced survival after infection with *S. aureus* due to reduced biofilm formation and lower bacterial counts (18). The *mCherry*-expressing transgenic flies serve as a control for flies expressing an exogenous protein under the control of the same promoter (24). To investigate synergy of antibiotics within this model system, gentamicin was microinjected 1 day after bacterial infection of flies. Synergy was observed in *iafgp*-transgenic flies treated with gentamicin, with significantly enhanced survival compared with untreated *iafgp*-transgenic flies (Fig. 1A). In contrast, *mCherry* control flies, untreated or treated with gentamicin, displayed similar survival curves (Fig. 1A). These survival data were corroborated with significantly lower bacterial counts measured at 3, 4, and 5 days after gentamicin treatment in *iafgp*-transgenic flies (Fig. 1B). Similar synergistic observations were also recorded with daptomycin treatment of *iafgp*-transgenic versus control flies (Fig. S1C). These results demonstrate that antibiotics can successfully boost the ability of *iafgp*-transgenic flies to fend off biofilm-associated bacterial infections.

We previously demonstrated protection of *iafgp*-transgenic mice against MRSA (18). Since IAFGP enhanced daptomycin activity *in vitro* (Table 1), we investigated whether this antibiotic would improve protection against an intranasal lethal challenge with MRSA in the *iafgp*-transgenic mice. Daptomycin, administered 4 h postinfection, significantly improved survival of the *iafgp*-expressing mice compared to transgenic mice administered saline or wild-type mice treated with daptomycin (Fig. 2A). Experiments performed with a sublethal (10-fold-reduced) dose of MRSA showed lower bacterial counts in the blood and lungs at 24 h (Fig. 2B and C), with improved body temperature as early as 12 h after infection (Fig. 2D), among daptomycin-treated *iafgp*-transgenic mice compared to controls. Collectively, these results confirm that IAFGP synergizes with antibiotics, allowing for better protection and survival against MRSA infection.

Antibiotic synergy with P1 combats bacterial infection in *Drosophila melanogaster* and reduces bacterial adhesion to antibiotic-coated catheters. Having explored the synergistic properties of IAFGP with antibiotics using transgenic flies and mice (Fig. 1 and 2), we next sought to investigate the role of P1, an IAFGP derivative (18, 19), in synergizing with antibiotics *in vivo* using two different infection models. Similar to our experiments with the transgenic flies, we assessed synergy of antibiotics with P1 using the control *mCherry*-expressing transgenic *D. melanogaster* infected with *S. aureus*. Flies microinjected with peptide P1, prior to *S. aureus* infection, had increased

FIG 1 *Staphylococcus aureus* challenge of *iaFGP*-expressing *Drosophila melanogaster* yields improved survival by synergizing with gentamicin. *iaFGP*-transgenic and mCherry control flies were challenged with *S. aureus* SA113 (1,000 CFU/fly). Twenty-four hours postinfection, flies were treated with 2.5 μ g/ml of gentamicin (+ gent) or an equivalent volume of water control. (A) Survival data from gentamicin-treated flies (mCherry, $n = 186$; IAFGP, $n = 175$) or water controls (mCherry, $n = 192$; IAFGP, $n = 172$) were pooled from 3 independent experiments. Statistical significance was calculated using the log-rank (Mantel-Cox) test (***, $P = 0.0003$). (B) Bacterial colonization of individual flies was determined by plating serial dilutions on tryptic soy agar plates. CFU data were pooled from 3 independent experiments, with each data point representing one fly. Statistical significance was calculated using two-way ANOVA with Sidak's multiple-comparisons posttest (**, $P < 0.01$; *, $P < 0.05$).

survival compared to flies injected with control peptide sP1 (Fig. 3A, dashed line). To ensure the peptides themselves did not have any deleterious effects on fly survival, uninfected mCherry-expressing flies were assessed for survival upon P1 or sP1 injection (Fig. S2). In the absence of any bacterial infection, there was no statistically significant difference among fly survival with either peptide treatment group (Fig. S2). Treatment with 2.5 μ g/ml of gentamicin 1 day post-*S. aureus* infection significantly bolstered survival rates of the P1 group compared to those of the antibiotic-free cohort (Fig. 3A, dotted line); no significant difference was found between the sP1 groups that did or did not receive gentamicin. Synergy between P1 and gentamicin was corroborated with bacterial viability counts showing a significant decrease at days 3 and 5 (Fig. 3B). These data demonstrate that P1 functions synergistically with antibiotics to aid in clearance of the infection.

Catheter-related bloodstream infections (CRBSIs) are a substantial clinical and economic problem (25, 26). *S. aureus* accounts for at least 20% of these infections and is associated with high morbidity and mortality (27). We previously demonstrated that adsorption of IAFGP or P1 onto catheters reduced *S. aureus* attachment. However, results were more profound with P1, *in vitro* and *in vivo* (18). Rifampin and minocycline (Rif/Mino)-impregnated catheters are among the safest and most effective in diminishing the rate of catheter colonization and CRBSI (28–30). Furthermore, Rif/Mino catheters have superior antiadherence activity and prolonged antimicrobial durability against vancomycin-resistant *S. aureus* and multidrug-resistant Gram-negative organisms compared to second-generation catheters coated with chlorhexidine and silver sulfadiazine (31, 32). We therefore tested whether P1 could influence the ability of *S. aureus* to attach to these antibiotic-impregnated catheters. Having found synergy between P1 and rifampin or minocycline in a plate assay (Table S2), we investigated whether P1 coating onto Rif/Mino-impregnated catheters could prevent bacterial attachment *in vitro* and *in vivo*. *In vitro*, antibiotic-impregnated or control naive catheters were coated with P1 or controls for 24 h and then transferred into *S. aureus*

FIG 2 Methicillin-resistant *Staphylococcus aureus* challenge of *iafgp*-expressing mice shows increased survival with daptomycin treatment. *iafgp*-transgenic mice (IAFGP) and C57BL/6 wild-type (WT) animals were challenged intranasally with 2.5×10^8 CFU (A) or 1×10^7 CFU (B, C, and D) of MRSA strain USA300 JE2. (A) Murine survival of transgenic or wild-type mice treated with saline (WT, $n = 20$; IAFGP, $n = 20$) or 25 mg/kg daptomycin (+dapto) (WT, $n = 20$; IAFGP, $n = 19$) was recorded. Four hours postinfection, daptomycin (dotted line) was administered. Data were pooled from 2 independent experiments, and statistical significance was calculated using the log-rank (Mantel-Cox) test (*, $P < 0.05$). (B and C) Bacterial density from blood (B) and lungs (C) 24 h postinfection for transgenic or wild-type mice treated with saline (WT, $n = 10$; IAFGP, $n = 9$) or 25 mg/kg daptomycin (-d) (WT, $n = 10$; IAFGP, $n = 10$) was recorded. Data were pooled from 2 experiments, and statistical significance was calculated using two-way ANOVA with Sidak's multiple-comparisons posttest (*, $P < 0.05$). (D) Body temperature was recorded for transgenic or wild-type mice treated with saline (WT, $n = 10$; IAFGP, $n = 9$) or 25 mg/kg daptomycin (+dapto) (WT, $n = 10$; IAFGP, $n = 10$). Four hours postinfection, daptomycin (dotted line) was administered. Data were pooled from 2 experiments, and statistical significance was calculated using two-way ANOVA with Sidak's multiple-comparisons posttest (*, $P < 0.05$).

suspensions. P1 coating on the Rif/Mino catheters significantly reduced bacterial attachment compared to sP1 or phosphate-buffered saline (PBS) controls (Fig. 3C and D). *In vivo*, antibiotic-coated or uncoated catheters were similarly treated with P1, sP1, or PBS controls, inserted subcutaneously into C57BL/6 wild-type mice, and inoculated with *S. aureus*. Three days postimplantation and infection, catheters were explanted and examined for bacterial attachment and biofilm formation. Similar to our *in vitro* results, P1 coating on the Rif/Mino catheters significantly reduced bacterial attachment to the catheters compared with controls (Fig. 3E and F). These data provide evidence that P1 synergizes effectively with Rif/Mino-impregnated catheters *in vitro* and *in vivo*, further reducing bacterial attachment.

FIG 3 Antibiotic synergy with P1 enhances survival and reduces bacterial colonization among wild-type invertebrate and vertebrate hosts. (A and B) *Staphylococcus aureus* challenge of P1-injected mCherry control *Drosophila melanogaster* yields improved survival by synergizing with gentamicin. mCherry control flies microinjected with 1 mg/ml (42 μ M) either P1 or control sP1 were challenged with *S. aureus* SA113 (1,000 CFU/fly). Twenty-four hours postinfection, flies were treated with 2.5 μ g/ml of gentamicin (+gent) or an equivalent volume of water control. (A) Survival data from gentamicin-treated flies (P1, $n = 142$; sP1, $n = 135$) (dotted lines) or water controls (P1, $n = 133$; sP1, $n = 141$) (dashed lines) were pooled from 3 independent experiments. Statistical significance was calculated using the log-rank (Mantel-Cox) test (***, $P < 0.001$; *, $P < 0.05$). (B) Bacterial colonization of individual flies was determined by plating serial dilutions on tryptic soy agar plates. CFU data were pooled from 3 independent experiments, with each data point representing one fly. Statistical significance was calculated using two-way ANOVA with Sidak's multiple-comparison posttest (***, $P < 0.001$; ****, $P < 0.0001$). (C to F) Uncoated or rifampin and minocycline (Rif/Mino) antibiotic-impregnated central venous catheter tubing was preincubated in PBS or PBS supplemented with P1 or sP1 (42 μ M) before being transferred into *S. aureus* SA113 tube suspensions (C and D) or implanted subcutaneously into the dorsal flanks of C57BL/6 wild-type mice (uncoated, $n = 6$ mice per peptide or control group; Rif/Mino, $n = 8$ mice per peptide or control group) and infected intraluminally with 5×10^5 CFU SA113 (E and F). Each dot represents one catheter, with two catheters per mouse being used. (C and E) Average CFU value $\pm$ standard errors of the means (SEMs) pooled from four independent experiments (*in vitro*) or two independent experiments (*in vivo*) are shown. (D and F) Since bacterial aggregation on the Rif/Mino catheters was reduced across all groups, the data are shown as the percent bacterial attachment on Rif/Mino-impregnated catheters relative to the uncoated naive catheters between each peptide or control group. Statistical significance was calculated using one-way ANOVA with Tukey's posttest (C and E) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$) and two-way ANOVA with Dunnett's multiple-comparison posttest (D and F) (*, $P < 0.05$; ***, $P < 0.001$).

FIG 4 IAFGP binding of β -lactams inhibits their activity. Fluorometric ELISA 96-well black plates were coated with 0.1 mg/ml either IAFGP-GST, GST alone, bovine serum albumin (BSA), or 0.01 mg/ml recombinant *S. aureus* penicillin binding protein 2a (PBP2a). Wells were incubated with various concentrations of Bocillin FL penicillin, washed, and read for their mean fluorescence intensity. Water and BSA were used as negative controls, and PBP2a served as the positive control. Values were pooled from five independent experiments (each with four technical replicates) $\pm$ SEM. Statistical significance was calculated using two-way ANOVA with Dunnett's multiple-comparison posttest (****, $P < 0.001$).

IAFGP binding of β -lactam antibiotics mitigates their bactericidal activity. We have previously demonstrated that IAFGP and P1 bind Gram-positive bacterial PGN, including PGN from *S. aureus* (18, 19). This binding to *S. aureus* PGN was specific for the terminal D-alanine residue of the stem peptide (19). Interestingly, β -lactam antibiotics themselves are analogs of D-alanyl-D-alanine (33), the same terminal amino acid residues present on the nascent PGN layer. In light of these two lines of evidence, we hypothesized that β -lactam antibiotics bind IAFGP. To explore an interaction between IAFGP and β -lactams, we coated enzyme-linked immunosorbent assay (ELISA) plates with recombinant IAFGP protein, or controls, and quantified binding using a fluorescent penicillin antibiotic conjugate. IAFGP bound the fluorescent penicillin conjugate, similar to recombinant *S. aureus* penicillin binding protein 2a (PBP2a), in a concentration-dependent manner, as represented by a decrease in mean fluorescence intensity with reducing concentrations of the fluorescent penicillin conjugate (Fig. 4). Since these data demonstrate that IAFGP directly interacts with β -lactam antibiotics, we assessed the interactions between β -lactams when coinoculated with IAFGP against *S. aureus in vitro* using our plate assays. β -Lactams, including ampicillin, penicillin G, and oxacillin, tested against *S. aureus* SA113, had severely reduced antimicrobial properties when combined with IAFGP (Table S3). In the presence of IAFGP, ampicillin bactericidal activity was reduced 2,000-fold and was not observed until a concentration of 1,024 μ g/ml was used (Table S3). Inhibition of β -lactam activity was similarly observed with penicillin G (1,024 μ g/ml) and oxacillin (2,048 μ g/ml) in the presence of IAFGP (Table S3). These data not only support the notion that IAFGP binds β -lactam antibiotics (ampicillin, penicillin G, and oxacillin), effectively preventing them from eliciting their antimicrobial activity, but also corroborates our prior findings that IAFGP binds to the terminal D-alanine residue of the stem peptide of the nascent *S. aureus* PGN (19).

In contrast to β -lactam antibiotics that were antagonistic when given in combination with IAFGP, vancomycin, an antibiotic commonly prescribed against MRSA, was observed to have neither synergistic nor antagonistic properties with either IAFGP or its peptide counterpart, P1. This glycopeptide antibiotic acts as a steric inhibitor of PGN maturation, binding the terminal D-alanyl-D-alanine residue of the growing PG chain of Gram-positive bacteria, effectively resulting in cell lysis (34). Demonstrating IAFGP and P1 binds the terminal D-alanine residue of PGN (19), we hypothesize that in the presence of both vancomycin and IAFGP or P1, there would be competition for the same site, resulting in an absence of synergy between the two molecules.

Improved permeability of peptidoglycan is directly correlated with permeation of antibiotics. We have previously demonstrated that binding of IAFGP to *S. aureus* causes a severe, nonlethal alteration in the bacterium's peptidoglycan with improved permeability of small-molecular-weight fluorescent dyes (19). Having observed synergy with either IAFGP or P1, both *in vitro* and *in vivo*, we hypothesized synergy with antibiotics could be attributed to improved uptake of some antibiotics on account of

FIG 5 IAFGP or P1 improves antibiotic permeation into cells. (A) Bacterial cells cultured in TSB or medium supplemented with GST alone, GST-tagged IAFGP, and P1 and sP1 peptides were incubated with gentamicin-conjugated Texas Red molecule (iGTTR) and imaged by phase-contrast (left phase) and epifluorescence microscopy (middle). (B) A pseudo-colored merge of each micrograph is shown on the right. Population analysis is from values measured at the single-cell level (n values shown above bars). Total fluorescent signal intensities were normalized by cell area and are represented as mean fluorescence (arbitrary units [AU]). Analysis in medium supplemented with PBS served as an added control. Statistical significance was calculated using two-way ANOVA with Dunnett's multiple-comparison post-test (***, $P < 0.005$). Scale bar, 2 μm .

a more permeable peptidoglycan (35). In order to assess the improved permeability of gentamicin, we employed the gentamicin (GT) antibiotic conjugated to Texas Red (TR) via an inflexible linker (i), iGTTR (36). *S. aureus* preincubated with either IAFGP or P1, treated with a low concentration of iGTTR (12 $\times$ less than the gentamicin MIC of 0.075 $\mu\text{g}/\text{ml}$; Table 1), revealed higher fluorescent signal intensities versus their respective protein (GST) or peptide (sP1) controls (Fig. 5A). Population analysis of cells across multiple fields of view provided quantitative confirmation of the immunofluorescence data (Fig. 5B). These data demonstrate that alterations mediated by IAFGP or P1 binding of *S. aureus* peptidoglycan yields a more porous peptidoglycan, a critical link toward increased permeation and synergy with antibiotics.

DISCUSSION

IAFGP is the first antifreeze protein demonstrated to have an important secondary function, namely, the ability to alter bacterial biofilm formation (18, 19). Maintaining bacteria in a planktonic state allows for enhanced access for antibiotics, which can help to eliminate microbes. We now demonstrate that IAFGP works in conjunction with traditional antibiotics to combat infection. IAFGP and its peptide derivative, P1, synergize with representative antibiotics (gentamicin, ciprofloxacin, and daptomycin) of 3

different classes of compounds (aminoglycosides, fluoroquinolones, and lipopeptides) against *S. aureus*. The synergistic interactions between IAFGP/P1 and antibiotics may also reduce the selective pressure for the development of resistance in *S. aureus*. Gentamicin is not commonly used to treat *S. aureus*, except in some cases of combinatorial therapy associated with the treatment of endocarditis (37); ciprofloxacin and other fluoroquinolones are often used for broad-spectrum coverage; and daptomycin is a potent lipopeptide-based antibiotic prescribed against MRSA infections. IAFGP and P1 increased the activity and efficacy for each of these antibiotics against *S. aureus* and its drug-resistant counterpart. These studies highlight the diversity of IAFGP's synergistic capacity: enhancement in the activity of antibiotics such as gentamicin or ciprofloxacin, which are not usually used specifically for *S. aureus*, or the improved effect of highly specific antibiotics such as daptomycin.

Synergy between IAFGP and antibiotics was not limited to *in vitro* assays but was also validated *in vivo* within fly and mouse model systems. Daptomycin is approved for the treatment of complicated skin and skin-structure infections caused by *S. aureus*, including strains resistant to methicillin (MRSA) and for the treatment of bacteremia and right-sided endocarditis (38, 39). However, despite potent *in vitro* activity, binding to pulmonary surfactants yielded inadequate efficacy *in vitro* and in phase 3 clinical studies against *Streptococcus pneumoniae* and *S. aureus*, the most common Gram-positive pathogens in community-acquired pneumonia (40, 41). Despite these mitigating factors, daptomycin, in our own murine nasal infection models, yielded a 20 to 50% survival advantage with half the optimal dosage, 25 mg/kg of body weight versus 50 mg/kg (42), among *iafgp*-transgenic mice, demonstrating synergy in more complex biological systems at a lower dose versus traditional therapeutics strategies. We also observed synergy in a surgical catheter implant model, highlighting a potential application of P1 as a prophylactic coating for catheters and other indwelling devices, such as heart valves, pacemakers, or prosthetic implants. *S. aureus* infection in flies is associated with biofilm formation (18). Morbidity during these bacterial infections can be attributed to systemic bacterial dissemination from the biofilm architecture. Our observations using P1 alone, and in concert with antibiotics within control flies, demonstrates the prophylactic utility of P1 as a systemic antivirulence agent that can target bacterial biofilms. Moreover, the absence of *in vitro* cytotoxicity adds further weight toward either protein or peptide as a potent prophylactic (see Fig. S3 in the supplemental material). In battling infections, disabling microbial virulence attributes *in vivo* can shift the advantage to the host and render bacteria increasingly susceptible to host defenses and antibiotics.

While IAFGP synergized with some antibiotics, other antibiotics, such as vancomycin and the β -lactams, did not synergize with either IAFGP or P1. Reasons for these contrasting observations are directly related to our earlier findings, which highlight the IAFGP and P1 site of binding, the terminal D-alanine residue of the bacterial peptidoglycan (PGN) (19). PGN plays a key role in maintaining cell shape, providing an attachment site for surface-exposed virulence factors and avoiding modifications in internal osmotic pressure (21, 43). Alterations during the course of PGN biosynthesis through mutations or introduction of PGN-acting antibiotics such as β -lactams or vancomycin can result in cell death. The PGN glycan strands are cross-linked by the transpeptidase enzymes to provide a tight mesh-like layer around the bacterial cell membrane. This cross-linking between strands occurs between the 5 amino acid residues (L-Ala-D-isoGlu-X-D-Ala-D-Ala) containing stem peptides. During this process, the 3rd amino acid residue of one (donor) stem peptide, on one glycan strand, is cross-linked with the 4th residue of the neighboring (acceptor) stem peptide strand. This results in a concomitant loss of the terminal (5th) D-Ala residue from the donor stem peptide and an interpeptide bridge forming between the two glycan strands. β -Lactam antibiotics such as ampicillin, penicillin G, or oxacillin elicit their bactericidal activity by blocking this cross-linking of PGN units, inhibiting the peptide bridge formation reaction catalyzed by transpeptidase enzymes, which are members of the penicillin-binding protein (PBP) family (44).

In contrast with gentamicin, ciprofloxacin, and daptomycin, both vancomycin and β -lactams are PGN-acting antibiotics, albeit at different stages of PGN biosynthesis. The absence of synergy with vancomycin, typically used against MRSA infections, with either IAFGP or P1 is attributed to both antibiotic and protein/peptide sharing the same terminal D-alanine residue binding site on PGN (19). In contrast, the antagonism with β -lactams ampicillin, penicillin G, and oxacillin is by virtue of these antibiotics being D-alanyl-D-alanine analogs, the same terminating residues of bacterial PGN. As a result, IAFGP binds the antibiotic directly, potentially sequestering them, allowing for bacterial cultures to grow uninhibited. While IAFGP and P1 potentiate the action of some antibiotics (gentamicin, ciprofloxacin, and daptomycin), it is no surprise that IAFGP and P1, by binding to the terminal D-alanine residue of the PGN stem peptide chain, hinder or inhibit the action of PGN-acting antibiotics, adding credence to our original observations on IAFGP's and P1's site of action (19). Moreover, the importance of the terminal D-alanine residue has been previously demonstrated by Hochbaum et al. with *S. aureus* and by Leiman et al. with *Bacillus subtilis*, wherein addition of alternative D-amino acids can replace the terminal D-alanine residue directly interfering with the protein component of the biofilm matrix (45, 46).

IAFGP binding alters peptidoglycan in a nonlethal manner, resulting in a less dense, more permeable peptidoglycan (19). Improved permeability of small molecules through the peptidoglycan layer can validate our current understanding of how some antibiotics are synergistic with IAFGP/P1. We hypothesize that IAFGP's improved permeability of peptidoglycan facilitates smaller antibiotics penetrating into the cell, supporting synergism between IAFGP and the antibiotic counterpart. While the body of this work focused on *S. aureus* and its drug-resistant counterpart as model organisms, other important Gram-positive clinical pathogens, including *Enterococcus* spp. and *Streptococcus* spp., are also significantly inhibited for biofilm formation by IAFGP and P1 on account of their similar peptidoglycan structure (19). With burgeoning rates of antibiotic resistance among these other clinically relevant Gram-positive pathogens and varied treatment regimens associated with their respective clinical sequelae (47), we would predict synergy of IAFGP and P1 with antibiotics against these clinical pathogens as well. The ability to examine the efficacy of IAFGP and P1 against a wide array of Gram-positive microbes, in a logical fashion, is now possible.

Antibiotic resistance represents a major cause of human morbidity and mortality. Bacterial biofilms compound these concerns, with resident bacteria becoming up to 1,000-fold more resistant to antibiotics and insensitive to the host immune response (48). The dissemination of antibiotic resistance genes among diverse pathogenic bacteria, such as *S. aureus*, coupled with the dearth of new antibiotics has led to a situation in which many microbial infections are multidrug resistant and extremely difficult or impossible to treat. An alternative approach is to develop antivirulence-based drugs that disarm pathogens within their host (44, 49). Targeting bacterial virulence attributes like biofilm formation, with IAFGP or a derivative peptide, in conjunction with antimicrobial therapy offers promising opportunities to counter the burgeoning rates of antibiotic resistance while altering infectivity and treating infections.

MATERIALS AND METHODS

Strains and growth conditions. *S. aureus* strains tested include strain SA113 (ATCC 35556), derived from laboratory strain NCTC 8325, and MRSA isolate USA300 JE2, a plasmid-cured derivative of strain LAC that was isolated in 2002 from a skin infection. Liquid cultures were maintained in either tryptic soy broth (TSB) (Sigma, St. Louis, MO) or their agar plates.

Cloning, expression, and purification of IAFGP. Purified IAFGP-GST was prepared as previously described (18). Briefly, *iafgp*, without its signal sequence, was PCR amplified from pGEMT*iafgp* (50) and cloned into pGEX-6p-3 (GE Healthcare, Piscataway, NJ). Protein expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and bacteria were lysed using a French pressure cell press at 20,000 lb/in². Following centrifugation, IAFGP-GST was affinity purified using GST Sepharose (GE Healthcare, Piscataway, NJ) eluted and stored in aliquots at -80°C . Precision protease treatment or dialysis resulted in IAFGP degradation. Therefore, the GST-tagged protein in elution buffer was used for experiments. Recombinant GST was purified from pGEX-6p-3 by following the same protocol and used as a control.

Antibiotic MIC and synergy assay. Gentamicin (Life Technologies, Grand Island, NY), ampicillin, penicillin G, oxacillin, ciprofloxacin, rifampin, minocycline (Sigma-Aldrich, St. Louis, MO), and daptomycin (Cubist Pharmaceuticals, Lexington, MA) were maintained according to the manufacturer's recommendations. MIC assays were generally performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines, with minor modifications. Briefly, overnight cultures of bacteria were adjusted in TSB to obtain standardized populations by measuring the turbidity with a spectrophotometer (Nanodrop 2000c; Thermo Scientific, Waltham, MA). The bacterial strains were grown to mid-log phase (1×10^6 CFU/ml) and inoculated into TSB, and 0.2 ml was dispensed, per well, into a 96-well microtiter plate. Susceptibility tests were performed by 2-fold standard broth microdilution for the above-mentioned antibiotics. After 18 h of incubation at 37°C, the minimal concentration required to prevent growth was defined as the MIC. Bacterial growth in each well was confirmed visually and by the measurement of the optical density at 600 nm (OD_{600}) using a microtiter ELISA plate reader (BioTek, Winooski VT).

Synergy between protein or peptide and the antibiotics was assessed by incubating standardized populations of bacteria into individual wells of a 96-well plate with purified glutathione S-transferase (GST), recombinant GST-tagged IAFGP (IAFGP) (1.6 μ M and 0.9 μ M, respectively), synthetic peptide P1 or sP1 (40 μ M each; >87% purity) (ThermoFisher Scientific, Waltham, MA), or their respective buffers (elution buffer or PBS) for 45 min at 37°C. Twofold standard broth microdilutions of the antibiotics listed above thereafter were supplemented into the wells. Plates were incubated for 18 h at 37°C, and bacterial growth in each well was confirmed visually or by the measurement of OD_{600} using a microtiter ELISA plate reader (BioTek, Winooski VT). The minimal concentration required to prevent growth was defined as the MIC.

Fly propagation and fly infection. *iafgp*-expressing and control *mCherry*-expressing flies were generated as described previously (24). *D. melanogaster* was maintained using standard procedures. The fly colony was maintained at 21 to 23°C. Following infection, flies were incubated at 29°C to increase upstream activation sequence (UAS)-mediated transgene expression. Five- to 9-day-old females were used for experiments. Twenty to 30 flies per vial were infected by microinjection, and survival was monitored (51). Microinjection was performed into the thorax below the wing using a Nanojet microinjector (Drummond Scientific, PA). Bacteria in stationary growth phase were diluted in PBS to an OD_{600} of 0.1 and 9.2 nl was injected, corresponding to an infectious dose of approximately 1,000 CFU/fly. Antibiotics (gentamicin and daptomycin at 2.5 μ g/ml) were administered 24 h postinfection in a similar fashion. Individual flies were homogenized in 500 μ l PBS using a bullet blender and steel beads (Nextadvance, NY). Serial dilutions were plated on brain heart infusion agar plates and incubated at 37°C overnight. Microinjection of *mCherry*-expressing wild-type flies with either peptide (P1 or sP1) was done 1 day prior to infection at a final dose of 1.0 mg/ml (42 μ M). The concentration administered was 10 times higher than that previously used to inhibit biofilms (18) to prevent repeat injections. Infections with *S. aureus* and injections with the appropriate antibiotic were given on consecutive days thereafter.

In vitro catheter binding assay. Central venous catheters (Cook Medical Inc., Bloomington, IN) that were impregnated with rifampin/minocycline or absent of any antibiotic were incubated for 18 h in peptides dissolved in PBS (46 μ M), washed with PBS, and incubated in bacterial suspensions (OD_{600} of 0.015) for 24 h. Following removal of nonadherent bacteria with repeated washing, adherent bacteria and biofilms were dissociated using sonication in PBS. Bacterial titers were quantified by plating serial dilutions on agar plates and were calculated as CFU per catheter.

Murine challenges with bacteria and surgeries. Murine catheter implant surgery was performed as described previously (52). Mice were anesthetized using isoflurane inhalation and a dorsal area was shaved. Following surface disinfection, a 3- to 4-mm incision was made on each mouse flank. Using a blunt probe to form subcutaneous pockets, a 2-cm catheter tube (Cook Medical Inc., Bloomington, IN) was inserted into each cavity. Incisions were sealed using tissue glue, and approximately 5×10^6 CFU of exponentially growing *S. aureus* SA113, washed and diluted in PBS, were injected through the skin into the lumen of the catheters. Three days postimplant the devices were removed, rinsed once, and then sonicated in PBS. The number of attached bacteria was determined by plating serial dilutions on agar plates.

For monomicrobial infection, *iafgp*-expressing mice (18) were infected with the *S. aureus* MRSA isolate USA300 JE2. A total of 5×10^8 CFU logarithmic-growth-phase bacteria per mouse were applied intranasally in 50 μ l PBS. Four hours postinfection, mice were administered 25 mg/kg USP-grade daptomycin (Cubist Pharmaceuticals, Lexington, MA) or an equivalent volume/body weight of sterile 0.9% sodium chloride (Hospira Inc., Lake Forest, IL). Mice were monitored at regular intervals for up to 36 h for the following signs of moribund conditions: sustained hypothermia (body temperature of <30°C for more than 1 h as measured by the infrared thermometer), decreased mobility, dehydration, change in the color of mucous membranes, and labored breathing. We determined that mice showing more than 3 of the indicated signs were moribund and euthanized them promptly with CO₂. Transgenic mice were similarly infected with a 10-fold lower dose of bacteria, treated with daptomycin or left untreated as described above, to enumerate viable bacteria from blood or explanted lung tissue. Additionally, the body temperature of these mice was recorded every 4 h.

Ethics statement. All studies with mice were carried out by following animal protocol numbers 2014-07941 (catheter model) and 2014-11208 (MRSA inhalation model), approved by Yale University's Institutional Animal Care and Use Committee (IACUC). The IACUC is governed by applicable federal and state regulations, including those of the Animal Welfare Act (AWA), Public Health Service (PHS), NIH Office of Laboratory Animal Welfare Assurance number A3230-01, and the United States Department of

Agriculture (USDA), license and registration number 16-R-0001, and is guided by the U.S. government principles for the utilization and care of vertebrate animals used in testing, research, and training.

IAFGP- β -lactam ELISA. Recombinant GST-tagged IAFGP binding to β -lactams was performed using a slight modification to the standard sandwich ELISA protocol outlined by BioLegend (BioLegend, San Diego, CA). Briefly, GST-tagged IAFGP or GST alone and bovine serum albumin (BSA) were diluted to 0.1 mg/ml in carbonate coating buffer, pH 9.5, and coated onto 96-well black immunoplates (ThermoFisher Scientific, Waltham, MA). After overnight incubation at 4°C, the wells were blocked in PBS with 1% milk for 1 h at 37°C. Wells were washed thoroughly with PBS and 0.1% Tween 20 (PBS-T) and thereafter incubated, in the dark for 2 h at 37°C, with various concentrations of Bocillin FL penicillin (ThermoFisher Scientific, Waltham, MA) diluted in PBS-T. The wells were washed repeatedly with PBS-T, and fluorescence was read using a TECAN Infinite 200 Pro series (Tecan US Inc., Morrisville, NC). BSA and water were used as negative controls. *Escherichia coli*-purified recombinant *S. aureus* penicillin binding protein 2a (PBP2a) was used at 0.01 mg/ml and served as a positive control (53) (RayBiotech, Norcross, GA).

Fluorescence microscopy experiments. Overnight cultures of *S. aureus* SA113 bacteria were subcultured for 2 h in fresh TSB or medium supplemented with GST alone, IAFGP-GST, P1, or sP1 control peptide (each at 0.1 mg/ml) or an equivalent volume of PBS control. Cells were harvested and washed with PBS to prevent further treatment. Each sample was resuspended in PBS supplemented with 1 mM CaCl_2 and incubated with 0.075 $\mu\text{g/ml}$ of gentamicin conjugated to Texas Red (iGTTR) and incubated at room temperature for 10 min. Excess stain was removed by two washes and then resuspension in PBS. Cell suspensions were spotted on 2% agarose pads containing PBS, and phase/epifluorescence microscopy images were acquired using an Eclipse Ti-U microscope (Nikon, Japan) outfitted with an Orca-ER camera (Hamamatsu, Japan). Image processing and quantitative analysis were performed using MATLAB (MathWorks, Natick, MA). Each cell included in the analysis was detected using a modified version of MATLAB scripts described elsewhere (54). Integrated, total signal intensities were normalized on a per-cell basis, according to area, and the average signal intensity for each population was reported.

Lactate dehydrogenase activity assay. Human embryonic kidney (HEK) and human hepatocyte (HepG2) cells were obtained from the ATCC and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2 unless otherwise noted. Human lung epithelial cells (A549) were obtained from the ATCC and were maintained in Ham's F-12 medium (F12) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2 unless otherwise noted. The lactate dehydrogenase assay was performed using the lactate dehydrogenase activity assay kit as described by the manufacturer (Sigma, St. Louis, MO). Briefly, 1,200 to 2,000 actively growing cells in 200 μl were seeded into individual wells of a 96-well plate and were incubated for 24 h. DMEM or F12 supplemented with 2% FBS was diluted 1:1 with PBS containing 0.4 mg/ml, 0.2 mg/ml, 0.1 mg/ml, or 0 mg/ml of IAFGP, GST, P1, or sP1. One hundred microliters of treated medium was added to wells of the 96-well plate and allowed to incubate for 24 h. The supernatant was used in the colorimetric assay, and the 96-well plate was read at A_{450} using a microtiter ELISA plate reader (BioTek, Winooski VT). Each treatment was performed in duplicate, and two biological replicates were performed for each cell line. Two biological replicates containing two technical replicates were performed for each cell line and medium condition.

Statistical analysis. All experiments were repeated independently at least 3 times, if not noted otherwise. One representative experiment or pooled data are shown. Statistical differences between experimental and control groups were analyzed using Prism 6.0 software (GraphPad Software Inc., CA). Significance was determined using two-way analysis of variance (ANOVA) with Sidak's or Dunnett's multiple-comparison posttest. Differences in survival were calculated using the log-rank (Mantel-Cox) test. Analysis was performed using two-sided tests. A *P* value of <0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00113-17>.

SUPPLEMENTAL FILE 1, PDF file, 3.6 MB.

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N.M.A. and E.F. conceived the studies and wrote the manuscript. N.M.A. performed most of the experiments, with the help of L.L., B.L.J., K.M., A.A., T.O.Y., E.S., and M.H. L.L. helped with microinjections for the fly infections, B.L.J. helped with the fluorescence microscopy and analysis, K.M. performed *in vitro* toxicity assays, and T.O.Y. provided valuable support for the MRSA mouse infections. Data analysis was performed by

N.M.A. All authors discussed the results and commented on the manuscript. E.F. and C.J.-W. directed the overall investigation.

We have no conflicts of interest to declare related to the publication of the manuscript.

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REFERENCES

- Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK, Active Bacterial Core Surveillance (ABCs) MRSA Investigators. 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 298:1763–1771. <https://doi.org/10.1001/jama.298.15.1763>.
- Foster TJ. 2005. Immune evasion by staphylococci. *Nat Rev Microbiol* 3:948–958. <https://doi.org/10.1038/nrmicro1289>.
- Jefferson KK. 2004. What drives bacteria to produce a biofilm? *FEMS Microbiol Lett* 236:163–173. <https://doi.org/10.1111/j.1574-6968.2004.tb09643.x>.
- Cunha BA, Perez FM. 2009. Daptomycin resistance and treatment failure following vancomycin for methicillin-resistant *Staphylococcus aureus* (MRSA) mitral valve acute bacterial endocarditis (ABE). *Eur J Clin Microbiol Infect Dis* 28:831–833. <https://doi.org/10.1007/s10096-008-0692-2>.
- Endimiani A, Blackford M, Dasenbrook EC, Reed MD, Bajaksouszian S, Hujer AM, Rudin SD, Hujer KM, Perreten V, Rice LB, Jacobs MR, Konstan MW, Bonomo RA. 2011. Emergence of linezolid-resistant *Staphylococcus aureus* after prolonged treatment of cystic fibrosis patients in Cleveland, Ohio. *Antimicrob Agents Chemother* 55:1684–1692. <https://doi.org/10.1128/AAC.01308-10>.
- Gould IM. 2013. Treatment of bacteraemia: methicillin-resistant *Staphylococcus aureus* (MRSA) to vancomycin-resistant *S. aureus* (VRSA). *Int J Antimicrob Agents* 42(Suppl):S17–S21. <https://doi.org/10.1016/j.ijantimicag.2013.04.006>.
- Gu B, Kelesidis T, Tsiodras S, Hindler J, Humphries RM. 2013. The emerging problem of linezolid-resistant *Staphylococcus*. *J Antimicrob Chemother* 68:4–11. <https://doi.org/10.1093/jac/dks354>.
- Hayden MK, Rezaei K, Hayes RA, Lolans K, Quinn JP, Weinstein RA. 2005. Development of daptomycin resistance in vivo in methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* 43:5285–5287. <https://doi.org/10.1128/JCM.43.10.5285-5287.2005>.
- Ikeda-Dantsuji Y, Hanaki H, Sakai F, Tomono K, Takesue Y, Honda J, Nonomiya Y, Suwabe A, Nagura O, Yanagihara K, Mikamo H, Fukuchi K, Kaku M, Kohno S, Yanagisawa C, Nakae T, Yoshida K, Niki Y. 2011. Linezolid-resistant *Staphylococcus aureus* isolated from 2006 through 2008 at six hospitals in Japan. *J Infect Chemother* 17:45–51. <https://doi.org/10.1007/s10156-010-0085-1>.
- Jones T, Yeaman MR, Sakoulas G, Yang SJ, Proctor RA, Sahl HG, Schrenzel J, Xiong YQ, Bayer AS. 2008. Failures in clinical treatment of *Staphylococcus aureus* infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding. *Antimicrob Agents Chemother* 52:269–278. <https://doi.org/10.1128/AAC.00719-07>.
- Murthy MH, Olson ME, Wickert RW, Fey PD, Jalali Z. 2008. Daptomycin non-susceptible methicillin-resistant *Staphylococcus aureus* U S A 300 isolate. *J Med Microbiol* 57:1036–1038. <https://doi.org/10.1099/jmm.0.2008.000588-0>.
- Saravolatz LD, Pawlak J, Johnson LB. 2012. In vitro susceptibilities and molecular analysis of vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus* isolates. *Clin Infect Dis* 55:582–586. <https://doi.org/10.1093/cid/cis492>.
- Sharma M, Riederer K, Chase P, Khatib R. 2008. High rate of decreasing daptomycin susceptibility during the treatment of persistent *Staphylococcus aureus* bacteremia. *Eur J Clin Microbiol Infect Dis* 27:433–437. <https://doi.org/10.1007/s10096-007-0455-5>.
- Stevens DL, Bisno AL, Chambers HF, Dellinger EP, Goldstein EJ, Gorbach SL, Hirschmann JV, Kaplan SL, Montoya JG, Wade JC. 2014. Practice guidelines for the diagnosis and management of skin and soft tissue infections: 2014 update by the Infectious Diseases Society of America. *Clin Infect Dis* 59:e10–e52. <https://doi.org/10.1093/cid/ciu296>.
- Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* 302:1569–1571. <https://doi.org/10.1126/science.1090956>.
- Fitzgerald JB, Schoeberl B, Nielsen UB, Sorger PK. 2006. Systems biology and combination therapy in the quest for clinical efficacy. *Nat Chem Biol* 2:458–466. <https://doi.org/10.1038/nchembio817>.
- Levy SB, Marshall B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* 10:S122–S129. <https://doi.org/10.1038/nm1145>.
- Heisig M, Abraham NM, Liu L, Neelakanta G, Mattessich S, Sultana H, Shang Z, Ansari JM, Killiam C, Walker W, Cooley L, Flavell RA, Agaisse H, Fikrig E. 2014. Antivirulence properties of an antifreeze protein. *Cell Rep* 9:417–424. <https://doi.org/10.1016/j.celrep.2014.09.034>.
- Abraham NM, Liu L, Jutras BL, Yadav AK, Narasimhan S, Gopalakrishnan V, Ansari JM, Jefferson KK, Cava F, Jacobs-Wagner C, Fikrig E. 2017. Pathogen-mediated manipulation of arthropod microbiota to promote infection. *Proc Natl Acad Sci U S A* 114:E781–E790. <https://doi.org/10.1073/pnas.1613422114>.
- Mengin-Lecreux D, van Heijenoort J. 1985. Effect of growth conditions on peptidoglycan content and cytoplasmic steps of its biosynthesis in *Escherichia coli*. *J Bacteriol* 163:208–212.
- Vollmer W, Blanot D, de Pedro MA. 2008. Peptidoglycan structure and architecture. *FEMS Microbiol Rev* 32:149–167. <https://doi.org/10.1111/j.1574-6976.2007.00094.x>.
- Vollmer W, Joris B, Charlier P, Foster S. 2008. Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev* 32:259–286. <https://doi.org/10.1111/j.1574-6976.2007.00099.x>.
- Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. *Cold Spring Harb Perspect Biol* 2:a000414.
- Neelakanta G, Hudson AM, Sultana H, Cooley L, Fikrig E. 2012. Expression of *Ixodes scapularis* antifreeze glycoprotein enhances cold tolerance in *Drosophila melanogaster*. *PLoS One* 7:e33447. <https://doi.org/10.1371/journal.pone.0033447>.
- Eggimann P, Sax H, Pittet D. 2004. Catheter-related infections. *Microbes Infect* 6:1033–1042. <https://doi.org/10.1016/j.micinf.2004.05.018>.
- Pittet D, Tarara D, Wenzel RP. 1994. Nosocomial bloodstream infection in critically ill patients. Excess length of stay, extra costs, and attributable mortality. *JAMA* 271:1598–1601.
- Walz JM, Memtsoudis SG, Heard SO. 2010. Prevention of central venous catheter bloodstream infections. *J Intensive Care Med* 25:131–138. <https://doi.org/10.1177/0885066609358952>.
- Hockenhull JC, Dwan KM, Smith GW, Gamble CL, Boland A, Walley TJ, Dickson RC. 2009. The clinical effectiveness of central venous catheters treated with anti-infective agents in preventing catheter-related bloodstream infections: a systematic review. *Crit Care Med* 37:702–712. <https://doi.org/10.1097/CCM.0b013e3181958915>.
- O'Grady NP, Alexander M, Burns LA, Dellinger EP, Garland J, Heard SO, Lipsett PA, Masur H, Mermel LA, Pearson ML, Raad II, Randolph AG, Rupp ME, Saint S. 2011. Summary of recommendations: guidelines for the prevention of intravascular catheter-related infections. *Clin Infect Dis* 52:1087–1099. <https://doi.org/10.1093/cid/cir138>.
- Ramritu P, Halton K, Collignon P, Cook D, Fraenkel D, Battistutta D, Whitby M, Graves N. 2008. A systematic review comparing the relative effectiveness of antimicrobial-coated catheters in intensive care units. *Am J Infect Control* 36:104–117. <https://doi.org/10.1016/j.ajic.2007.02.012>.
- Raad I, Mohamed JA, Reitzel RA, Jiang Y, Raad S, Al Shuaibi M, Chafar AM, Hachem RY. 2012. Improved antibiotic-impregnated catheters with

- extended-spectrum activity against resistant bacteria and fungi. *Antimicrob Agents Chemother* 56:935–941. <https://doi.org/10.1128/AAC.05836-11>.
32. Raad I, Reitzel R, Jiang Y, Chemaly RF, Dvorak T, Hachem R. 2008. Anti-adherence activity and antimicrobial durability of anti-infective-coated catheters against multidrug-resistant bacteria. *J Antimicrob Chemother* 62:746–750. <https://doi.org/10.1093/jac/dkn281>.
 33. Kelly JA, Knox JR, Zhao H, Frere JM, Ghayson JM. 1989. Crystallographic mapping of beta-lactams bound to a D-alanyl-D-alanine peptidase target enzyme. *J Mol Biol* 209:281–295. [https://doi.org/10.1016/0022-2836\(89\)90277-5](https://doi.org/10.1016/0022-2836(89)90277-5).
 34. Kohanski MA, Dwyer DJ, Collins JJ. 2010. How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol* 8:423–435. <https://doi.org/10.1038/nrmicro2333>.
 35. Sarre SG, Hahn FE. 1967. Mode of action of gentamicin. *Trans N Y Acad Sci* 29:575–578. <https://doi.org/10.1111/j.2164-0947.1967.tb02427>.
 36. Escobedo JO, Chu YH, Wang Q, Steyger PS, Strongin RM. 2012. Live cell imaging of a fluorescent gentamicin conjugate. *Nat Prod Commun* 7:317–320.
 37. Baddour LM, Wilson WR, Bayer AS, Fowler VG, Jr, Bolger AF, Levison ME, Ferrieri P, Gerber MA, Tani LY, Gewitz MH, Tong DC, Steckelberg JM, Baltimore RS, Shulman ST, Burns JC, Falace DA, Newburger JW, Pallasch TJ, Takahashi M, Taubert KA, Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease, Council on Cardiovascular Disease in the Young, Councils on Clinical Cardiology, Stroke, and Cardiovascular Surgery and Anesthesia, American Heart Association, Infectious Diseases Society of America. 2005. Infective endocarditis: diagnosis, antimicrobial therapy, and management of complications: a statement for healthcare professionals from the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease, Council on Cardiovascular Disease in the Young, and the Councils on Clinical Cardiology, Stroke, and Cardiovascular Surgery and Anesthesia, American Heart Association: endorsed by the Infectious Diseases Society of America. *Circulation* 111:e394–434. <https://doi.org/10.1161/CIRCULATIONAHA.105.165564>.
 38. Arbeit RD, Maki D, Tally FP, Campanaro E, Eisenstein BI, Daptomycin 98-01 and 99-01 Investigators. 2004. The safety and efficacy of daptomycin for the treatment of complicated skin and skin-structure infections. *Clin Infect Dis* 38:1673–1681. <https://doi.org/10.1086/420818>.
 39. Fowler VG, Jr, Boucher HW, Corey GR, Abrutyn E, Karchmer AW, Rupp ME, Levine DP, Chambers HF, Tally FP, Vigliani GA, Cabell CH, Link AS, DeMeyer I, Filler SG, Zervos M, Cook P, Parsonnet J, Bernstein JM, Price CS, Forrest GN, Fatkenheuer G, Gareca M, Rehm SJ, Brodt HR, Tice A, Cosgrove SE, S. aureus Endocarditis and Bacteremia Study Group. 2006. Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus*. *N Engl J Med* 355:653–665. <https://doi.org/10.1056/NEJMoa053783>.
 40. Pertel PE, Bernardo P, Fogarty C, Matthews P, Northland R, Benvenuto M, Thorne GM, Luperchio SA, Arbeit RD, Alder J. 2008. Effects of prior effective therapy on the efficacy of daptomycin and ceftriaxone for the treatment of community-acquired pneumonia. *Clin Infect Dis* 46: 1142–1151. <https://doi.org/10.1086/533441>.
 41. Silverman JA, Mortin LI, Vanpraagh AD, Li T, Alder J. 2005. Inhibition of daptomycin by pulmonary surfactant: in vitro modeling and clinical impact. *J Infect Dis* 191:2149–2152. <https://doi.org/10.1086/430352>.
 42. Mortin LI, Li T, Van Praagh AD, Zhang S, Zhang XX, Alder JD. 2007. Rapid bactericidal activity of daptomycin against methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* peritonitis in mice as measured with bioluminescent bacteria. *Antimicrob Agents Chemother* 51: 1787–1794. <https://doi.org/10.1128/AAC.00738-06>.
 43. Holtje JV. 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiol Mol Biol Rev* 62:181–203.
 44. Rasko DA, Sperandio V. 2010. Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov* 9:117–128. <https://doi.org/10.1038/nrd3013>.
 45. Hochbaum AL, Kolodkin-Gal I, Foulston L, Kolter R, Aizenberg J, Losick R. 2011. Inhibitory effects of D-amino acids on *Staphylococcus aureus* biofilm development. *J Bacteriol* 193:5616–5622. <https://doi.org/10.1128/JB.05534-11>.
 46. Leiman SA, May JM, Lebar MD, Kahne D, Kolter R, Losick R. 2013. D-amino acids indirectly inhibit biofilm formation in *Bacillus subtilis* by interfering with protein synthesis. *J Bacteriol* 195:5391–5395. <https://doi.org/10.1128/JB.00975-13>.
 47. Rivera AM, Boucher HW. 2011. Current concepts in antimicrobial therapy against select gram-positive organisms: methicillin-resistant *Staphylococcus aureus*, penicillin-resistant pneumococci, and vancomycin-resistant enterococci. *Mayo Clin Proc* 86:1230–1243. <https://doi.org/10.4065/mcp.2011.0514>.
 48. Musk DJ, Jr, Hergenrother PJ. 2006. Chemical countermeasures for the control of bacterial biofilms: effective compounds and promising targets. *Curr Med Chem* 13:2163–2177. <https://doi.org/10.2174/09298670677935212>.
 49. Parry DJ, Jr, Pierson E, Hung DT. 2007. Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol* 3:541–548. <https://doi.org/10.1038/nchembio.2007.24>.
 50. Neelakanta G, Sultana H, Fish D, Anderson JF, Fikrig E. 2010. *Anaplasma phagocytophilum* induces *Ixodes scapularis* ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold. *J Clin Investig* 120:3179–3190. <https://doi.org/10.1172/JCI42868>.
 51. Apidianakis Y, Rahme LG. 2009. *Drosophila melanogaster* as a model host for studying *Pseudomonas aeruginosa* infection. *Nat Protoc* 4:1285–1294. <https://doi.org/10.1038/nprot.2009.124>.
 52. Cassat JE, Lee CY, Smeltzer MS. 2007. Investigation of biofilm formation in clinical isolates of *Staphylococcus aureus*. *Methods Mol Biol* 391: 127–144. https://doi.org/10.1007/978-1-59745-468-1_10.
 53. Fuda C, Suvorov M, Vakulenko SB, Mobashery S. 2004. The basis for resistance to beta-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *J Biol Chem* 279: 40802–40806. <https://doi.org/10.1074/jbc.M403589200>.
 54. Parry BR, Surovtsev IV, Cabeen MT, O'Hern CS, Dufresne ER, Jacobs-Wagner C. 2014. The bacterial cytoplasm has glass-like properties and is fluidized by metabolic activity. *Cell* 156:183–194. <https://doi.org/10.1016/j.cell.2013.11.028>.
 55. Seidl K, Leimer N, Palheiros Marques M, Furrer A, Senn G, Holzmann-Burgel A, Matt U, Zinkernagel AS. 2014. USA300 methicillin-resistant *Staphylococcus aureus* in Zurich, Switzerland between 2001 and 2013. *Int J Med Microbiol* 304:1118–1122. <https://doi.org/10.1016/j.ijmm.2014.08.005>.