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RESEARCH ARTICLE
Therapeutics and Prevention

Multiple Compounds Secreted by *Pseudomonas aeruginosa* Increase the Tolerance of *Staphylococcus aureus* to the Antimicrobial Metals Copper and Silver

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ABSTRACT Metal-based antimicrobials have been used for thousands of years to treat and prevent bacterial infections. Currently, both silver and copper are used in health care and industry to prevent and treat the spread of harmful bacteria. However, like most antimicrobial agents, their efficacy against polymicrobial infections has not been fully elucidated. Coinfection with *Pseudomonas aeruginosa* and *Staphylococcus aureus* and the resulting interactions have been implicated in higher virulence, antibiotic resistance, and increased chronic infections. Here, the influence of secreted compounds from *P. aeruginosa* on metal antimicrobial tolerance in *S. aureus* was examined. This study determined that multiple compounds from *P. aeruginosa* increase the tolerance of *S. aureus* to copper and/or silver when cultured in simulated wound fluid. The presence of these secreted compounds from *P. aeruginosa* during exposure of *S. aureus* to copper or silver increased the MIC from 500 μM to 2,000 μM for copper and 16 to 63 μM for silver. The contribution of specific compounds to *S. aureus* tolerance was determined using gene deletion and disruption mutants, and metabolite analysis. Compounds identified as potential contributors were then individually added to *S. aureus* during metal exposure. Copper tolerance in *S. aureus* was found to be increased by amino acids and dihydroaeruginoate (Dha) secreted by *P. aeruginosa*. The silver tolerance provided to *S. aureus* was influenced only by two amino acids, serine and threonine, as well as the *Pseudomonas* quinolone signal (PQS) molecules from *P. aeruginosa*.

IMPORTANCE Alternative antimicrobials, such as metals, are one of the methods currently used to help mitigate antibiotic resistance. Metal-based antimicrobials such as copper and silver are used currently both to prevent and to treat infections. Although the efficacy of these antimicrobials has been determined in single-species culture, bacteria rarely exist in a single-species group in the environment. Both *Pseudomonas aeruginosa* and *Staphylococcus aureus* are often found associated with each other in severe chronic infections displaying increased virulence and antibiotic tolerance. In this study, we determined that multiple compounds secreted by *P. aeruginosa* are able to increase the tolerance of *S. aureus* to both copper and silver. This work demonstrates the expansive chemical communication occurring in polymicrobial infections between bacteria.

KEYWORDS *Pseudomonas aeruginosa*, *Staphylococcus aureus*, antimicrobial, bacterial interactions, copper, metal resistance, metal tolerance, polymicrobial, silver

The presence of multidrug-resistant bacteria in health care and industry has seen a drastic increase over the past 20 years (1–4). Due to the ever-evolving state of microorganisms, research has been focused on overcoming antibiotic resistance through the development and use of alternative antimicrobial compounds, including

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metals (5–8). The use of metals as antimicrobials can be dated back to ancient civilizations including the Egyptians and Phoenicians (9, 10). These civilizations would drop coins into their water vessels or coat them to keep the water "fresh" during transport (9, 10). Currently, metals such as copper and silver are used in hospitals for indwelling medical devices, copper-coated surfaces, silver eyedrops, and bandages (11–13). Water treatment facilities also use copper-silver ionization systems to prevent bacterial contamination (14).

Two pathogens of increasing concern to health organizations around the world are *Pseudomonas aeruginosa* and *Staphylococcus aureus* (15, 16). These organisms are associated with each other in severe chronic infections such as cystic fibrosis (17–19). Silver and copper are often used to assist wound healing and for the prevention and treatment of chronic infections (20–24). The study of polymicrobial infections and interactions has begun to develop only over the past 10 years (25–29). During this period, several reviews and articles have been published examining both antagonistic and synergistic interactions between *P. aeruginosa* and *S. aureus* (19, 30–34). Some of these studies observed differences in antibiotic tolerance of either *P. aeruginosa* or *S. aureus* with secreted products from the other organism (32–36). Compounds from *P. aeruginosa* including siderophores, 2-heptyl-4-hydroxyquinoline *n*-oxide (HQNO), pyocyanin, and acylated homoserine lactones (AHLs) were all implicated in changes to antibiotic tolerance of *S. aureus* (32, 33). The influence of these compounds was also dependent on the strain, antibiotic, and growth conditions used (32–35).

Similarly to investigations into antibiotic efficacy, testing of different metal antimicrobial formulations is normally performed on a single species. However, our group has observed that similarly to antibiotics, metal susceptibility differs between single species and cocultured bacteria (37, 38). Particularly, when *P. aeruginosa* and *S. aureus* were cultured together, a higher MIC was observed for $AgNO_3$ than when either was cultured individually (37, 38). This indicates these organisms were more tolerant to silver when grown together.

The purpose of this work was to explore the influence of secreted molecules from one strain on individual metal susceptibility of the other strain. Here, we focus on compounds secreted by *P. aeruginosa* which influence the tolerance of *S. aureus* toward silver and copper metal-based antimicrobials. Extracts, and fractions thereof, from spent medium of *P. aeruginosa* grown in simulated wound fluid (SWF) were evaluated, and compounds that contributed to increased metal tolerance were identified. The findings presented here show a system of multiple biomolecules involved that demonstrates that this resistance effect is multifactorial, where different compounds differentially influence the tolerance of different metals (Ag versus Cu).

RESULTS

***Pseudomonas aeruginosa* PAO1 enhances tolerance of *S. aureus* ATCC 25923 to copper and silver.** The spent medium, containing secreted compounds from each organism during growth in simulated wound fluid (SWF) medium, was collected and added to the opposing organism exposed to $AgNO_3$ (see Fig. S1 in the supplemental material). This experiment revealed that a compound(s) contained within *P. aeruginosa* spent medium (PaS) was responsible for enhancing silver tolerance. The enhanced tolerance was also specific to SWF, as the use of other media to prepare and test the spent medium resulted in either no change or reduced $AgNO_3$ tolerance (Fig. S2). The ability of PaS to provide tolerance to *S. aureus* was determined for multiple metals, metalloids, antibiotics, and antiseptics (Table S1). Although PaS either reduced or had no impact on *S. aureus* tolerance for many of these antimicrobials, both copper and silver tolerance was enhanced. The addition of PaS to *S. aureus* was able to provide a 4-fold increase to the MIC of copper and silver (Fig. S3). Initial inhibition of *S. aureus* alone exposed to $AgNO_3$ and $CuSO_4$ was 16 and 500 μM, respectively (Fig. S3). For *S. aureus* in the presence of PaS, the MIC increased to 63 μM for $AgNO_3$ and 2,000 μM for $CuSO_4$, denoted by the inflection point that occurs at these concentrations (Fig. S3). It should be noted that although tolerance of *S. aureus* alone to $CuSO_4$ appears to

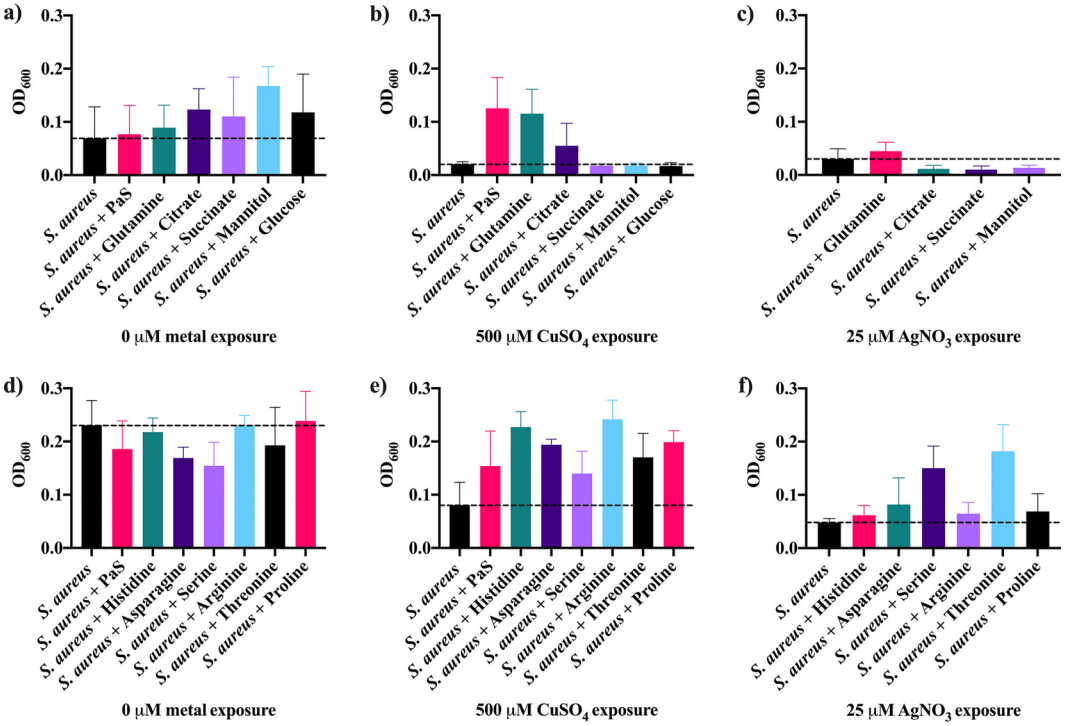

FIG 1 Optical density at 600 nm (OD_{600}) of *S. aureus* alone (a and d) or after exposure to either 500 μM $CuSO_4$ (b and e) or 25 μM $AgNO_3$ (c and f) with and without additional metabolites for 24 h at 37°C with 150-rpm shaking. Either 10 mM glutamine, citrate, succinate, mannitol, or glucose (a, b, and c) or histidine, asparagine, serine, arginine, threonine, or proline (d, e, and f) or 16% PaS was added to *S. aureus*. The average and standard deviation for three biological trials with three technical replicates each were plotted. The dashed line represents the tolerance of *S. aureus* alone with no additives.

increase slightly after 1,000 μM, this is due to slight absorbance of copper in the medium at these high concentrations.

Multiple compounds from *P. aeruginosa* are able to enhance copper and/or silver tolerance in *S. aureus*. Further experiments were performed to characterize and identify the compound(s) able to enhance tolerance of *S. aureus* to copper and silver. Preliminary characterization experiments were used to determine the heat tolerance, hydrophobicity, and approximate size of the tolerance-providing compound (Fig. S4). The spent medium was either treated with heat at 95°C for 30 min or separated with a 2:1 chloroform-methanol extraction. The spent medium was also filtered through 50-, 30-, 10-, or 3-kDa-molecular-weight-cutoff filters either prior to or after these treatments. Based on these experiments and subsequent separation on a μ reverse-phase chromatography (μRPC) C_2-C_{18} column, we observed multiple fractions that differed in their ability to provide copper and/or silver tolerance (Fig. S4 and S5).

The compounds which provided both copper and silver tolerance that were hydrophilic, were below 3 kDa, and eluted as a passthrough peak (fraction 3) using a μRPC-C_2-C_{18} column were sent for metabolic analysis. A list of compounds present in this fraction was obtained (data not shown), and the most abundant of these ($>5 \times 10^7$ relative abundance) were individually added to *S. aureus* exposed to copper or silver (Fig. 1). Glucose and citrate were also included as they may affect either *S. aureus* fitness or metal binding. The fitness of *S. aureus* unexposed to any metal was enhanced only with mannitol and citrate (Fig. 1a) though copper and silver tolerance was not impacted by these metabolites (Fig. 1b and c). All of the amino acids tested were able to enhance copper tolerance of *S. aureus* (Fig. 1e). However, none of the amino acids used increased the fitness of *S. aureus* without metal exposure (Fig. 1d). Silver tolerance of *S. aureus* was increased only with the addition of serine or threonine (Fig. 1f).

Siderophores and quorum sensing molecules from *P. aeruginosa* influence *S. aureus* copper and silver tolerance. While not identified during metabolic analysis (a

ramification of liquid chromatography-tandem mass spectrometry [LC-MS/MS] conditions), other compounds which could impact *S. aureus* tolerance include siderophores, pili and flagellar proteins, pyocyanin, hydrogen cyanide, or quorum sensing systems. Using purified pyocyanin, we found that it is not involved in our system (Fig. S6). While cyanide was able to increase *S. aureus* silver and copper tolerance, this was observed only at a concentration above what would be found in the spent medium under our conditions (39) (Fig. S7).

We performed further experiments utilizing full gene deletion mutants and transposon disruption mutants for genes involved in siderophore synthesis (*pchE*, *pchF*, and *pvdD*), quorum sensing (*lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, *pqsH*, *pqsL*, and *mvfR*), and pili (*pilA*) and flagellar (*fliC*) proteins from *P. aeruginosa* PAO1. The spent medium for each of these mutants was then collected in the same way as for wild-type *P. aeruginosa* and added to *S. aureus* to examine changes to either copper or silver tolerance. If the product of the deleted or disrupted gene was involved in enhancing either copper or silver tolerance, the spent medium from that mutant would no longer provide tolerance to *S. aureus*. Opposing this, if the product of the gene had no influence on copper or silver tolerance the provided enhancement would be the same for the mutant as wild-type spent medium.

The silver tolerance provided by *P. aeruginosa* spent medium was unaffected by the absence of *fliC*, *pchF*, or *mvfR* (Fig. S8). The absence of either *pilA* or *pvdD* in *P. aeruginosa* prevented its spent medium from increasing tolerance of *S. aureus* to silver (Fig. S8). Spent medium from the *pqsA* transposon (Tn) mutant provided only a partial tolerance phenotype to *S. aureus* (Fig. S8). Finally, the disruption of *pchE* in *P. aeruginosa* spent medium also had no influence on the conferred silver tolerance (Fig. 2).

Both the *las* and *rhl* quorum sensing systems appeared to be involved in the spent media's ability to confer silver tolerance. Spent media prepared from gene deletion mutants for *lasR*, *lasI*, *rhlR*, and *rhlI* were all unable to provide a significant enhancement to silver tolerance in *S. aureus* (Fig. S8). To examine the influence of one of these systems more closely, the signal molecule 3-*o*-C_{12}-HSL (homoserine lactone) used in the *las* system was added to *S. aureus* alone and during the preparation of both mutant and wild-type spent media (Fig. 2a). The exogenous addition of 3-*o*-C_{12}-HSL to *S. aureus* during silver exposure did not increase silver tolerance (Fig. 2a). The use of *lasI* spent medium prepared with addition of 3-*o*-C_{12}-HSL during the growth phase and after collection also had no enhancement to silver tolerance (Fig. 2a).

While the transcriptional regulator MvfR is not involved in the provided silver tolerance, other parts of the *pqs* system are. Disruption of *pqsH* or *pqsL* reduced the ability of *P. aeruginosa* spent medium to increase *S. aureus* silver tolerance (Fig. 2c and d). The protein products of *pqsH* and *pqsL*, 2-heptyl-3-hydroxy-4(1H)-quinolone synthase and monooxygenase, are responsible for the final step in synthesis of *Pseudomonas* quinolone signal (PQS) and 2-heptyl-4-hydroxyquinoline *n*-oxide (HQNO), respectively (40, 41). The effect of both HQNO and PQS individually on *S. aureus* silver tolerance and their ability to recover their respective mutant's ability to increase silver tolerance were also examined. When HQNO was added to the *pqsL* Tn mutant either during or after growth, the resulting spent medium was unable to increase silver tolerance (Fig. 2d). Spent medium was also prepared from the *pqsH* Tn mutant with addition of PQS during and after culturing. The addition of 50 μM PQS to *pqsH* spent medium during its growth was able to recover the ability to increase *S. aureus* silver tolerance (Fig. 2c). Although the addition of a lower concentration (50 μM) of PQS to the *pqsH* spent medium after growth was not enough to increase silver tolerance in *S. aureus*, 400 μM PQS alone was able to protect *S. aureus* from silver toxicity (Fig. 2c).

Unlike for silver tolerance, the provided copper tolerance was mostly unaffected by quorum sensing systems. The mutant spent media from deletion or disruption mutants for *lasR*, *rhlR*, *rhlI*, *mvfR*, *pqsA*, and *pqsH* were still able to provide copper tolerance to *S. aureus* (Fig. S8). The absence of *pilA*, *fliC*, *pchF*, or *pvdD* from *P. aeruginosa* also had no significant impact on the spent medium's ability to confer copper tolerance (Fig. S8). The use of spent medium from the *lasI* gene deletion mutant was unable to increase copper tolerance

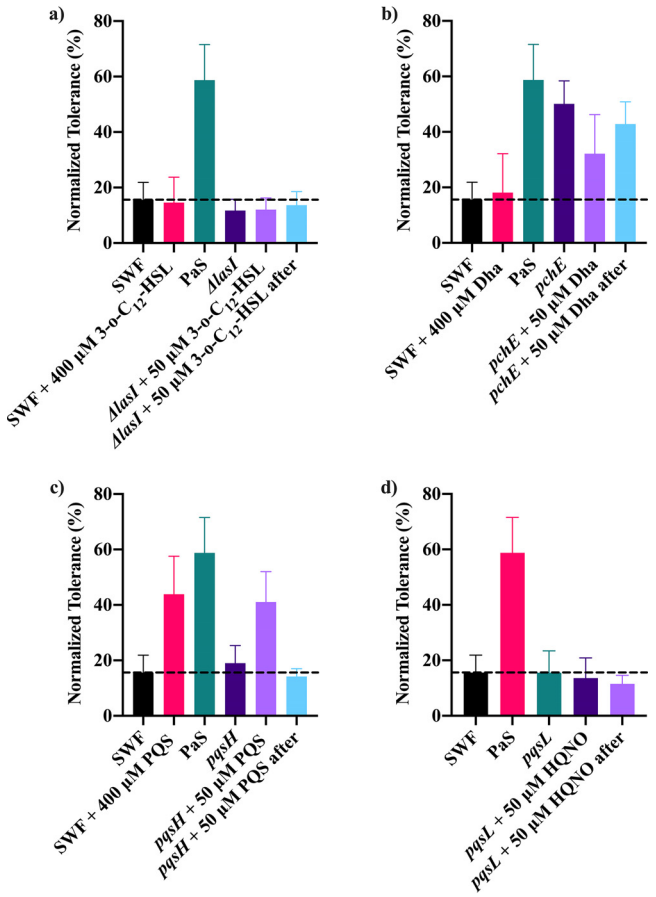

FIG 2 The normalized tolerance of *S. aureus* to 40 μM $AgNO_3$ was determined using a bioassay with and without the addition of spent medium from wild-type *P. aeruginosa* PAO1 (PaS) or gene deletion or disruption mutants for *lasI* (a), *pchE* (b), *pqsH* (c), or *pqsL* (d). Spent medium was also prepared with the addition of 50 μM 3-*o*-C_{12}-HSL to *lasI*, 50 μM Dha to *pchE*, 50 μM PQS to *pqsH*, and 50 μM HQNO to *pqsL* during and after culturing. A higher concentration (400 μM) of each of the compounds was also added to *S. aureus* exposed to silver alone during the bioassay. The average and standard deviation from three biological trials with three technical replicates each are plotted. The dashed line represents the copper tolerance of *S. aureus* alone (SWF).

compared to *S. aureus* alone, but this was not significantly lower than the tolerance provided by wild-type PaS (Fig. 3a). The addition of exogenous 3-*o*-C_{12}-HSL directly to the bioassay or during growth of Δ*lasI* spent medium also did not impact copper tolerance (Fig. 3a). Though the addition of 3-*o*-C_{12}-HSL to Δ*lasI* spent medium after its preparation was able to increase copper tolerance, this was very minor.

The only gene which appeared to have a significant influence on copper tolerance was *pqsL*, as its removal resulted in a significant reduction in the conferred copper tolerance (Fig. 3d). The role of *pqsL* in conferring copper tolerance is similar to what was observed for silver tolerance. Neither the addition of HQNO to *pqsL* Tn mutant spent medium during nor addition after growth was able to recover the spent medium's ability to increase copper tolerance (Fig. 3d).

While the primary siderophore in *P. aeruginosa*, pyoverdine, does not influence copper tolerance, pyochelin does, particularly the precursor to pyochelin, dihydroaeruginoic acid (Dha). The *pchE* Tn mutant spent medium was still able to confer copper tolerance to *S. aureus*, but exogenous Dha also increased *S. aureus* copper tolerance (Fig. 3b).

DISCUSSION

The impact of interspecies interactions on antimicrobial tolerance presents a significant issue during treatment of chronic infections. By understanding the factors which

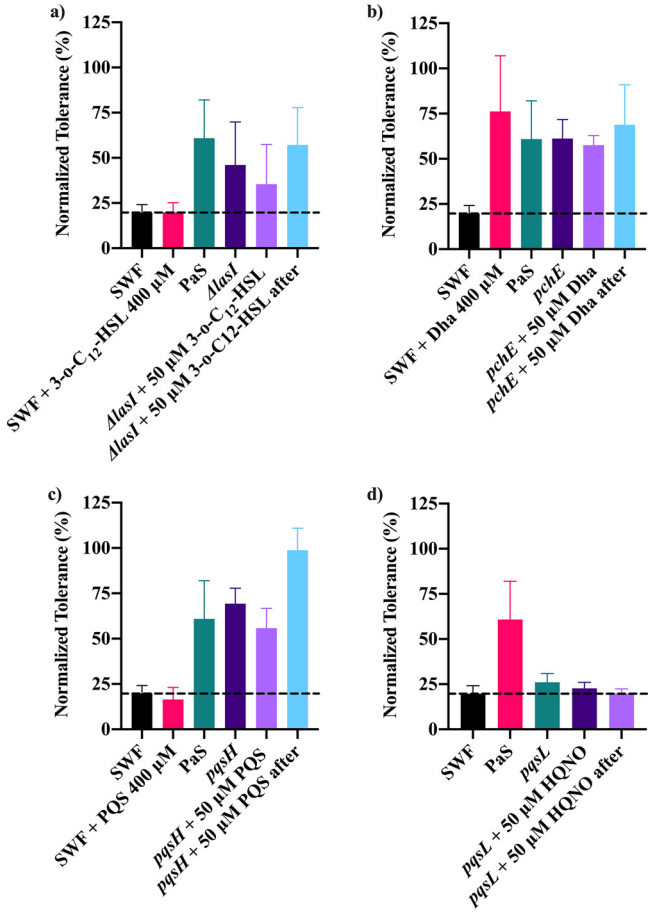

FIG 3 The normalized tolerance of *S. aureus* to 500 μM $CuSO_4$ was determined using a bioassay with and without the addition of spent medium from wild-type *P. aeruginosa* PAO1 (PaS) or gene deletion or disruption mutants for *lasI* (a), *pchE* (b), *pqsH* (c), or *pqsL* (d). Spent medium was also prepared with the addition of 50 μM 3-*o*-C_{12}-HSL to *lasI*, 50 μM Dha to *pchE*, 50 μM PQS to *pqsH*, and 50 μM HQNO to *pqsL* during and after culturing. A higher concentration (400 μM) of each of the compounds was also added to *S. aureus* exposed to copper alone during the bioassay. The average and standard deviation from three biological trials with three technical replicates each are plotted. The dashed line represents the copper tolerance of *S. aureus* alone (SWF).

increase or decrease the metal tolerance during interspecies interactions, preventative measures may be taken. Previous reports identified an increased tolerance to $AgNO_3$ during coculture of *P. aeruginosa* and *S. aureus* compared to their individual cultures (37, 38). However, a mechanism responsible for this observation was not proposed. The findings from the current study indicate that multiple compounds secreted by *P. aeruginosa* can increase *S. aureus* tolerance to $AgNO_3$ and $CuSO_4$. In contrast to this, compounds secreted by *S. aureus* have no significant impact on tolerance of *P. aeruginosa* to $AgNO_3$ (see Fig. S1 in the supplemental material).

Although previous reports identified a link between pyocyanin and silver tolerance provided by *P. aeruginosa*, this was not the case in our system using SWF as the culture medium (35, 42). Other researchers have also reported a link between hydrogen cyanide and interactions between *P. aeruginosa* and *S. aureus* (33). Though the presence of hydrogen cyanide did increase both silver and copper tolerance, the concentration needed is above what would be secreted by *P. aeruginosa* (39).

The compounds present in *P. aeruginosa* spent medium that impact $AgNO_3$ tolerance differ from those influencing $CuSO_4$ tolerance. In both cases, binding interactions which reduce the bioavailability of the metal in the medium influence the observed tolerance. However, different binding coordination chemistry and affinities are involved for each metal. The conferred silver tolerance was directly linked to serine, threonine,

and PQS. The ability of serine and threonine to increase silver tolerance was largely unexpected, as they are not normally associated with silver atom binding sites (43). The addition of serine or threonine did not increase *S. aureus* growth without metal exposure, signifying that increased fitness is unlikely to be the cause of the increased silver tolerance. Instead, this observation could be due to the enzymes used to metabolize serine and threonine in *S. aureus*. These amino acids are generally the first to be metabolized during amino acid catabolism due to their easy conversion to pyruvate (44). Part of this conversion involves an L-serine dehydratase containing an iron-sulfur cluster (44). Targeting of iron-sulfur clusters by silver is one of the proposed mechanisms of toxicity (45). Thus, Ag attack on the dehydratase would lead to an excess of serine or threonine able to bind up the silver, protecting *S. aureus* from other detrimental effects.

Quorum sensing in *P. aeruginosa* is also involved as the spent media from gene deletion mutants for *lasR*, *lasI*, *rhlR*, and *rhlI* did not enhance silver tolerance in *S. aureus*. The *lasR* and *lasI* genes code for the transcriptional activator protein LasR, the regulator for the *las* system, and an acyl-homoserine-lactone synthase which produces the inducer molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3-*o*-C_{12}-HSL) (46). Similarly, *rhlR*'s protein product is the regulator for the *rhl* system, regulatory protein RhlR, and *rhlI*'s protein product is another acyl-homoserine-lactone synthase which produces *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone (HHL), both autoinducers for the *rhl* system (47, 48). The *las* and *rhl* systems regulate a large amount of *P. aeruginosa*'s genome and are highly interconnected (49). The absence of any these genes would cause a shift in the compounds produced, any of which could explain the reduction in provided tolerance.

The ability of PaS to provide silver tolerance to *S. aureus* is also associated with production of the *Pseudomonas* quinolone signal (PQS) but does not require the functional regulator, encoded by *mvfR*, in *P. aeruginosa*. Both spent medium from mutant *pqsH P. aeruginosa* grown in the presence of a lower concentration (50 μM) of PQS and that from PQS alone at a higher concentration (400 μM) were able to increase silver tolerance. This indicates that PQS can directly increase *S. aureus* silver tolerance at a high-enough concentration or signal within active *P. aeruginosa* causing production of another compound(s) which increases silver tolerance in *S. aureus* (49–53). PQS by itself is involved in both outer membrane vesicle generation and siderophore production in *P. aeruginosa* (53). An increase in either of these in the medium would reduce the bioavailability of silver through its binding, thus providing an increase to *S. aureus* tolerance. Additionally, PQS has been positively correlated with biofilm production in *S. aureus* (54). These outcomes of PQS-altered physiology increase tolerance to silver for *S. aureus*.

The ability of spent medium from *P. aeruginosa* to confer copper tolerance to *S. aureus* is directly linked to the presence of amino acids and Dha. Exogenous Dha provided an increase to copper tolerance in *S. aureus*. However, the disruption of *pchE*, coding for dihydroaeruginoic acid (Dha) synthetase responsible for synthesizing Dha from salicylic acid, does not change tolerance (55). This suggests that although Dha helps *S. aureus* copper tolerance, it is not a necessary component of *P. aeruginosa* spent medium to provide copper tolerance. Binding interactions of the various secreted compounds reducing copper bioavailability are the best explanation for the copper tolerance provided by amino acids or Dha.

The conferred silver and copper tolerances were also reduced when either *lasI* or *pqsL* was absent, but they could not be restored by addition of either 3-*o*-C_{12}-HSL or HQNO. Since the protein products of *lasI* and *pqsL* are responsible for the last step of 3-*o*-C_{12}-HSL and HQNO synthesis, respectively, if either 3-*o*-C_{12}-HSL or HQNO was directly involved in copper or silver tolerance, their exogenous addition would be expected to increase *S. aureus* tolerance. This was not the case, implying a more indirect mechanism of involvement for *lasI* and *pqsL*. When either *lasI* or *pqsL* is disrupted, there is likely a change to the yet-unidentified compounds secreted by *P. aeruginosa*. This

change in physiology then alters the ability of spent medium from either the *lasI* or *pqsL* mutants to increase silver or copper tolerance.

Challenges in this study arise from the variability of our data between experiments. This is a product of the challenge recovery assessment of the remaining fitness distribution of the cells in a culture after antimicrobial metal load. Differences in fitness possibilities of the community depend on the medium augmentation as well as other factors that would influence culture growth and density, including, but not limited to, number of phase variants, persister cells, or mutations that likely arise. Additionally, there can be remarkable differences in medium batches for antimicrobial testing (this is why clinical tests often use comparative breakpoints). This is increased when fetal bovine serum (FBS) is used as this medium component of SWF is undefined, leading to compositional differences between batches. These differences can result in slight changes to *S. aureus* physiological fitness and associated growth. The subtle differences in medium and cell composition can also lead to different metal ion speciation states (how the metal ion is coordinated and to what), altering the effective bioavailability and associated toxicity. There would also be slight genetic/phenotypic variation in the colonies picked between biological replicates. This is in part alleviated by comparing an average of technical replicates within a single biological replicate, yet there is still considerable variance at times between the magnitudes of the observed phenotype between replicates. These issues led to different metal tolerance levels during our study, and thus, the challenge concentrations used were altered in some experiments to ensure that inhibition occurred. Particularly, silver seemed to be more affected by these variations. All these considerations in combination with the cross signaling discovered in this study demonstrate how complex a mixed-species wound environment system is and the diversity of responses to antimicrobial treatment that is observed.

Overall, *S. aureus* is protected from both copper and silver toxicity in the presence of spent medium from *P. aeruginosa* when grown specifically in simulated wound fluid medium. The secretion of PQS or compounds controlled directly by PQS, as well as serine and threonine, all provides silver tolerance to *S. aureus*. These compounds likely act in combination to reduce the bioavailability of silver in the medium through binding (PQS) as well as affecting *S. aureus*' metabolism and physiology (PQS, serine, and threonine) to create a more tolerant phenotype. A similar mechanism occurs when protecting from copper; however, different compounds are involved. Compounds which bind copper to reduce its bioavailability include amino acids and Dha. While neither HQNO nor 3-*o*-C_{12}-HSL directly impacts silver or copper tolerance, the absence or dysfunction of the proteins which synthesize them leads to a loss of the *P. aeruginosa* causing spent media's ability to confer tolerance (summarized in Fig. 4). Our results suggest that further cell components are involved in this metal resistance communication and that even with a binary bacterial system there is remarkable complexity.

MATERIALS AND METHODS

Bacterial strains and culture maintenance. The strains used in this study include *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* ATCC 25923. Gene deletion mutants in *P. aeruginosa* PAO1 including *lasR*, *lasI*, *rhlR*, *rhlI*, *pilA*, and *fliC* were gifts from J. Harrison, and transposon mutants for *pchE*, *pchF*, *pvdD*, *pqsA*, *pqsH*, *pqsL*, and *mvfR* were obtained from I. Lewis, both at the University of Calgary. All strains were prepared by streaking from a −80°C glycerol stock onto a Luria-Bertani (LB) agar plate and growing overnight at 37°C. A single colony from this primary plate was then streaked onto a secondary LB agar plate and grown overnight at 37°C. Colonies from the secondary plate were then suspended in saline (0.9% NaCl in distilled-deionized H_2O [ddH_2O]) to match a 1.0 McFarland standard, creating a standardized inoculum. This suspension was diluted into simulated wound fluid (SWF; 50% fetal bovine serum, 50% peptone water [0.1 g/liter peptone in 0.85% NaCl]) for culturing.

Metal solutions. Metal stock solutions were prepared in distilled and deionized H_2O (ddH_2O), sterilized through an 0.2-μm filter, and stored at room temperature, except for $AgNO_3$, which was stored covered at 4°C. $CuSO_4$ was prepared at 1 M, $AgNO_3$ at 50 mM, and $NiSO_4$ and $AlSO_4$ at 100 mM. A working solution of metal was prepared by diluting the stock solution in sterile ddH_2O before addition to the medium to prevent precipitation.

Spent medium preparation. Spent media, or the sterile secreted compounds, from either *P. aeruginosa* or *S. aureus* were prepared identically. The standardized inoculum for either *P. aeruginosa* or *S. aureus* was diluted 30-fold in a 96-well plate containing SWF. The culture was then grown for 24 h with

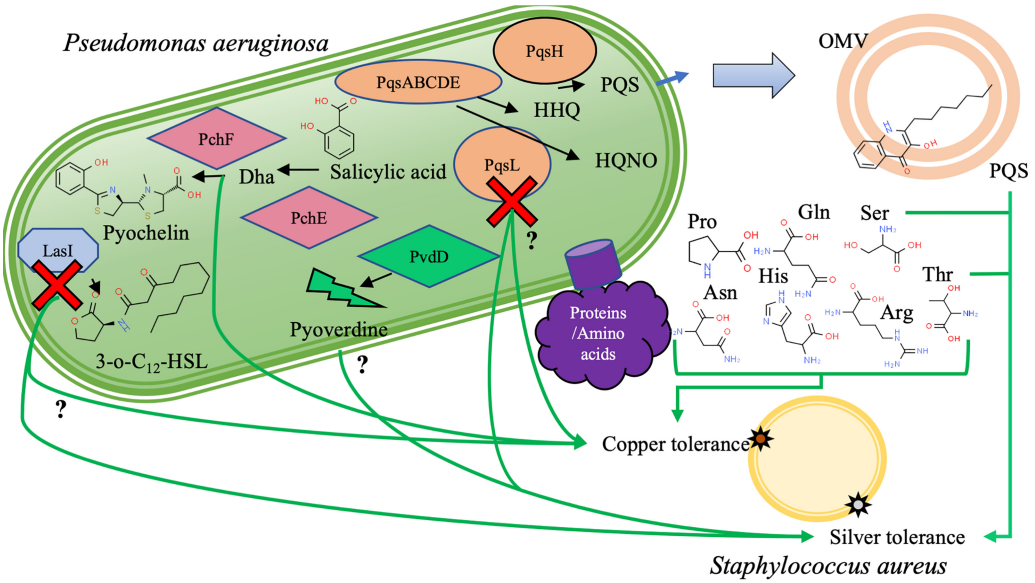

FIG 4 Summary of the compounds or genes involved in providing either copper or silver tolerance to *S. aureus*. Green arrows represent compounds influencing copper or silver tolerance, and the question mark indicates a minor impact that was not confirmed by addition of the individual compound. The red "x" represents disruption of either *pqsL*, coding for a probable flavin adenine dinucleotide (FAD)-dependent monooxygenase which produces HQNO, or *lasI*, coding for an acyl-homoserine-lactone synthase which produces 3-*o*-C_{12}-HSL. While this disruption reduces the provided tolerance from *P. aeruginosa* spent medium, loss of HQNO or 3-*o*-C_{12}-HSL is not the cause. Other compounds involved with either copper or silver tolerance enhancement include dihydroaeruginoic acid (Dha), the *Pseudomonas* quinolone signal (PQS) likely secreted through outer membrane vesicles (OMV), serine, threonine, histidine, arginine, asparagine, proline, and glutamine.

150-rpm shaking at 37°C until an optical density at 600 nm (OD_{600}) of ~1.0 was reached. The culture was collected in microcentrifuge tubes and centrifuged at 8,000 × *g* for 10 min. Spent medium was collected by filter sterilizing the resulting supernatant through an 0.2-μm syringe filter. Spent medium obtained from *P. aeruginosa* culture is referred to as PaS while spent medium from *S. aureus* culture is referred to as SaS. Further separation of spent medium was performed by filtration through molecular-weight-cutoff filters of 50, 30, 10, and 3 kDa in size. The temperature stability was also determined by heating at 95°C for 30 min. The hydrophobic and hydrophilic compounds were extracted from the spent medium using a 2:1 chloroform-methanol extraction.

Antimicrobial susceptibility determination. The standardized inoculum from either *P. aeruginosa* or *S. aureus* was diluted 300-fold into a 96-well plate containing 2-fold serial dilutions of either $CuSO_4$ (63 to 4,000 μM), $NiSO_4$ (63 to 4,000 μM), $AgNO_3$ (16 to 500 μM), $AlSO_4$ (500 to 8,000 μM), K_2TeO_3 (130 to 8,000 μM), Na_2SeO_3 (130 to 8,000 μM), $NaAsO_2$ (130 to 8,000 μM), nalidixic acid (6.25 to 200 μg/ml), tetracycline (6.25 to 200 μg/ml), benzalkonium chloride (0.78 to 50 μg/ml), or H_2O_2 (1.6 to 8,000 μM). To determine the influence of spent medium on antimicrobial susceptibility, 16% of either PaS or SaS was also added to the previous plate. The 96-well plate containing the standardized inoculum, metal challenge, and spent medium was grown for 24 h at 37°C with 150-rpm shaking. The optical density at 600 nm was then recorded with a PerkinElmer 2030 Victor X4 microplate reader. The MIC was determined by the initial concentration in which there was a significant reduction in visible cell growth as read by optical density at 600 nm (OD_{600}). Range finding experiments explored other concentrations of spent medium, time endpoints, and medium choice for this assay; the described assay method provided the clearest phenotype. The above protocol is based on a previous protocol with minor alterations for accommodating spent medium (56).

Normalization. The OD_{600} readings during some trials of the antimicrobial susceptibility determinations were lower for unexposed *S. aureus* alone than *S. aureus* with PaS. Due to this variance in optical density, a normalization was performed on the optical density readings to correct for the difference. The OD_{600} values for *S. aureus* both alone and with PaS were normalized between 0 and 100%. The optical density of both *S. aureus* alone and with PaS without metal exposure was set to 100% and an optical density reading of 0% to 0%. The optical density of *S. aureus* alone or with PaS during metal exposure was then set within the appropriate range whether PaS was present or not. This normalization allows for accurate comparison of susceptibility changes for *S. aureus* alone or with PaS.

Optical density bioassay (BA^{OD}). The increased tolerance of *S. aureus* to $AgNO_3$ and $CuSO_4$ with spent medium from *P. aeruginosa* was tested using an optical density bioassay (BA^{OD}). A 96-well plate was prepared with wells containing either SWF alone, SWF with 500 or 600 μM $CuSO_4$, SWF with 25, 40, or 50 μM $AgNO_3$, SWF with 500 or 600 μM $CuSO_4$ and 16% spent medium, or SWF with 25, 40, or 50 μM $AgNO_3$ and 16% spent media. Multiple spent medium types were tested in each bioassay always at the same concentration (16%). The plate was then inoculated with a 300-fold dilution of the standardized inoculum of *S. aureus* and incubated at 37°C for 24 h with 150-rpm shaking.

The optical density at 600 nm was then determined with a PerkinElmer 2030 Victor X4 microplate reader. The optical density was normalized and plotted as normalized tolerance (%) where the optical density of *S. aureus* without metal exposure was set to 100% and an optical density reading of 0% to 0%.

Fraction separation using reverse-phase chromatography (RPC) and fraction testing with the bioassay. A μRPC C_2-C_{18} ST 4.6/100 column (Pharmacia Biotech) was used during all RPC separation and stored in 70% methanol until use. Into the column, 100 μl of either PaS, Pa10Aq, or Pa3Aq was injected, and the column was then equilibrated with eluent A (Milli-Q H_2O with 0.065% trifluoroacetic acid [TFA]) for 1 column volume, followed by a gradient from 0 to 100% eluent B (acetonitrile with 0.05% TFA) for 10 column volumes with an 0.5-ml/minute flow rate, collecting 0.5-ml fractions during flowthrough and 2-ml fractions during the gradient. The resulting fractions were dried under cold N_2 gas and suspended in 100 μl phosphate-buffered saline (PBS) and then autoclaved prior to addition to the bioassay at a 16% concentration.

LC-HR MS/MS analysis. Three biological replicates were prepared from fraction 3 of Pa3Aq separated using the previous RPC protocol. However, after the fraction was dried it was suspended in 50% methanol. The sample was processed at the Calgary Metabolomics Research Facility (CMRF) at the University of Calgary for metabolite separation and analysis using high-resolution liquid chromatography (LC-HR) MS. A Vanquish ultrahigh-performance LC (UHPLC) system (Thermo-Fisher) was used for sample injection and separation on a Syncronis HILIC column (Thermo-Fisher).The following conditions were used for separation: solvent A (20 mM ammonium formate, pH 3.0, in MS-grade H_2O), solvent B (0.1% formic acid in MS-grade acetonitrile), and a gradient of 2 min at 100% B, 5 min to 80% B, 3 min to 5% B, 2 min at 5% B, and 1 min to 100% B at a flow rate of 0.6 ml/minute. A Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo-Fisher) was then used with the following parameters: HESI source parameters were sheath gas flow rate 25, auxiliary gas flow rate 10, sweep gas flow rate 2, spray voltage 2.5 kV, capillary temperature 275°C, S-lens radio frequency level 60, and auxiliary gas heater temperature 325°C. MS scan parameters were runtime 15 min, negative polarity, full MS scan type, 240,000 resolution, automatic gain control (AGC) target of 3e6, interaction time maximum of 200 ms, and scan range between 50 and 750 *m/z*. The resulting files were analyzed using the MAVEN software program to detect compounds comparing to a list of known standards that had been previously separated using the same instrument (57, 58).

Metabolite addition for bioassay. The effect of individual metabolites on *S. aureus* tolerance to metals was determined following a similar protocol as the previous bioassay. However, instead of addition of spent medium, a 10 mM concentration of either glutamine, citrate, succinate, mannitol, glucose, histidine, asparagine, serine, arginine, threonine, or proline was added to the 96-well plate described in the bioassay. With these data, no normalization was performed to display the effect of metabolites on *S. aureus* fitness without metal exposure.

Gene deletion and disruption mutant spent medium preparation and bioassay. Stock solutions of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-*o*-C_{12}-HSL), dihydroaeruginoic acid (Dha), 2-heptyl-4-hydroxyquinoline *n*-oxide (HQNO), and *Pseudomonas* quinolone signal (PQS) were prepared at 10 mM in dimethyl sulfoxide (DMSO). Spent medium for Δ*lasI* was prepared as described previously but with 50 μM 3-*o*-C_{12}-HSL added to the culture before and after growth. Spent media from the *pchE*, *pqsL*, and *pqsH* Tn mutants were prepared similarly to Δ*lasI* with 3-*o*-C_{12}-HSL but using their corresponding compound (Dha to *pchE*, HQNO to *pqsL*, and PQS to *pqsH*). This spent medium as well as each compound alone at a 400 μM concentration was added to *S. aureus* exposed to either 40 μM $AgNO_3$ or 500 μM $CuSO_4$ in the bioassay described previously.

***S. aureus* cyanide exposure bioassay.** A stock solution of 1 M KCN was prepared in Milli-Q H_2O and filter sterilized with an 0.2-μm filter syringe system. The stock KCN was subsequently diluted to a working concentration in SWF prior to use in the previously described bioassay. Three 96-well plates were prepared containing either no KCN, 10 μM KCN, or 500 μM KCN. Each of the plates also contained wells with SWF without KCN or metal exposure, 40 μM $AgNO_3$ with appropriate KCN addition, and 500 μM $CuSO_4$ with appropriate KCN addition. *S. aureus* was then inoculated into each plate, cultured, and processed as described in the bioassay.

Statistics. Statistical differences were determined using a Welch *t* test, one-way analysis of variance (ANOVA) or a two-way ANOVA with Dunnett's multiple comparisons using the GraphPad Prism version 8.2 for Mac, GraphPad Software, La Jolla, CA, USA.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.3 MB.

FIG S2, TIF file, 2.3 MB.

FIG S3, TIF file, 1.2 MB.

FIG S4, TIF file, 1.1 MB.

FIG S5, TIF file, 1.3 MB.

FIG S6, TIF file, 1.1 MB.

FIG S7, TIF file, 1 MB.

FIG S8, TIF file, 1.7 MB.

TABLE S1, DOCX file, 0.1 MB.

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