A horizontally gene transferred copper resistance locus confers hyper-resistance to antibacterial copper toxicity and enables survival of community acquired methicillin resistant *Staphylococcus aureus* USA300 in macrophages

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Summary

Excess copper is highly toxic and forms part of the host innate immune system’s antibacterial arsenal, accumulating at sites of infection and acting within macrophages to kill engulfed pathogens. We show for the first time that a novel, horizontally gene transferred copper resistance locus (*copXL*), uniquely associated with the SCCmec elements of the highly virulent, epidemic, community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) USA300, confers copper hyper-resistance. These genes are additional to existing core genome copper resistance mechanisms, and are not found in typical *S. aureus* lineages, but are increasingly identified in emerging pathogenic isolates. Our data show that CopX, a putative P1B-3-ATPase efflux transporter, and CopL, a novel lipoprotein, confer copper hyper-resistance compared to typical *S. aureus* strains. The *copXL* genes form an operon that is tightly repressed in low copper environments by the copper regulator CsoR. Significantly, CopX and CopL are important for *S. aureus* USA300 intracellular survival within macrophages. Therefore, the emergence of new *S. aureus* clones with the *copXL* locus has significant implications for public health because these genes confer increased resistance to antibacterial copper toxicity, enhancing bacterial fitness by altering *S. aureus* interaction with innate immunity.

Introduction

New methicillin resistant *Staphylococcus aureus* (MRSA) clones are emerging and circulating worldwide presenting a new threat to human health. The reasons for the emergence of these clones are currently unknown. *S. aureus* is an opportunistic pathogen responsible for a range of minor and life-threatening diseases (Sullivan *et al*., 1984; Tong *et al*., 2015) and is considered by the World Health Organisation to be one of the major multidrug resistant bacterial health threats globally (WHO, 2017). Hospital-associated (HA) MRSA are a leading cause of nosocomial infections worldwide, predominantly infecting patients with reduced immune function. However, the MRSA problem has been exacerbated by the emergence and spread of community-acquired (CA) MRSA, which can cause infections in healthy humans with no previous exposure to healthcare situations (Otto, 2013). CA-MRSA mainly causes severe skin and soft tissue infections (SSTI; Johnson *et al*., 2007) but can also cause invasive life-threatening infections, for example necrotizing pneumonia (Francis *et al*., 2005).
The CA-MRSA clone USA300 is epidemic in the United States (Mediavilla et al., 2012). The success of USA300 has been attributed in part to increased expression of virulence genes and the acquisition of several mobile genetic elements (MGE) encoding genes that enhance USA300 colonization, virulence and environmental survival (Diep et al., 2006). S. aureus USA300, and other CA-MRSA isolates, also show increased resistance to innate immunity and reduced clearance from healthy airways compared to other S. aureus clinical isolates (Yajjala et al., 2016). This has been attributed to elevated resistance to alveolar macrophage-mediated intracellular killing, although the exact mechanisms involved have not been established (Yajjala et al., 2016).

The North American USA300 clone (USA300-NAE) has spread globally (Glaser et al., 2016), and a Latin American variant (USA300-SAE) is epidemic in South America (Nimmo, 2012). Interestingly, phylogenetic analysis determined that USA300-NAE and USA300-SAE evolved from a common ancestor, with individual parallel epidemics emerging simultaneously (Planet et al., 2015). USA300- SAE cases are now being reported in North America and Europe (Dach et al., 2016; Planet et al., 2016) suggesting that this clone is also spreading globally.

Both USA300 variants cause the same range of diseases and have become the predominant epidemic clones. Acquisition by horizontal gene transfer (HGT) of the arginine catabolic mobile element (ACME; Thurlow et al., 2013) was suggested to be key to the success of USA300-NAE. However, USA300-SAE isolates do not encode ACME but instead carry a novel MGE encoding putative copper and mercury resistance (COMER) determinants (Fig. 1; Planet et al., 2015). Across the ACME and COMER elements of the USA300-NAE and USA300-SAE clades, there are only two genes that are conserved, SAUSA300_0078 and SAUSA300_0079, encoding a putative efflux transporter and a putative lipoprotein respectively (Fig. 1; Planet et al., 2015). Consequently we hypothesized that this novel locus may play a role in copper resistance, and potentially the enhanced fitness and success of the USA300 lineage by increasing survival against antimicrobial copper.

Copper is an essential nutrient as a cofactor for some essential enzymes, but excess copper is highly toxic to bacteria (Djoko et al., 2015). Copper accumulates at sites of infection, where it plays a major role in innate immunity, with the infected host using copper to kill invading microorganisms (Beveridge et al., 1985; Fones and Preston, 2013; Chaturvedi and Henderson, 2014; Fu et al., 2014; Johnson et al., 2015; Hyre et al., 2017). Importantly macrophages, which are critical to innate immunity, protecting the skin and respiratory tract (Malissen et al., 2014; Kopf et al., 2015), use copper as an antibacterial mechanism by actively importing it into the phagosome (White et al., 2009; Johnson et al., 2015). Consequently pathogens have mechanisms to counteract copper toxicity, mainly by limiting the copper concentration in their cytoplasm through efflux or sequestration, which are important for virulence in a diverse range of bacterial pathogens in a variety of infection models (Schwan et al., 2005; Ward et al., 2010; Shafeeq et al., 2011; Johnson et al., 2015; Hyre et al., 2017; Ladomersky et al., 2017).

All S. aureus possess a conserved operon encoding a \( P_{1 B} \)-type ATPase copper efflux transporter (copA) and a copper chaperone protein (copZ) (Sitthisak et al., 2007; Fig. 1A) encoded as part of the core genome. The role of S. aureus CopA in virulence is unknown. Some HA-MRSA (CC36 and CC22 lineages) also possess an additional copper exporting ATPase belonging to the \( P_{1 B} \)-3 subtype, designated copB, encoded on a plasmid that is either freely-replicating or integrated into the genome (Fig. 1B;
Baker et al., 2011). The copXL genes encoded in both the ACME and COMER elements are predicted to encode a novel copper resistance locus encoding a putative P$_{1B-3}$-type ATPase (SAUSA300_0078; herein designated copX) and a putative lipoprotein (SAUSA_0079; herein designated copL).

We have studied the function of this putative copper resistance locus (copXL) and found that it confers copper hyper-resistance and promotes survival of USA300 in macrophages. S. aureus isolates carrying the copXL locus showed significantly increased copper resistance relative to strains with only the conserved copAZ operon. Cells of isogenic mutants of S. aureus lacking copX showed decreased resistance to copper, and cells lacking either copX or copL showed increased copper accumulation, suggesting that both genes play a role in copper efflux, with CopX likely being a copper-specific efflux transporter. Transcriptional analysis demonstrated that expression of the copXL operon is induced by copper, and regulated by the copper sensor CsoR, which specifically binds to the promoters of both copAZ and copXL. Importantly, the copXL locus also plays a key role in survival against killing by macrophages, demonstrating the importance of copXL for increased resistance of S. aureus USA300 to innate immune defences.

Results

S. aureus USA300 encodes a novel copper resistance locus

The copXL locus shares 99% DNA sequence identity with a locus from coagulase negative Staphylococcus species, suggesting it has recently been acquired by S. aureus USA300 through HGT. In USA300-SAE, the copXL genes are found in COMER, associated with the staphylococcal cassette chromosome mec (SCCmec) element and a multi-copper oxidase (mco), whereas in USA300-NAE the copXL genes are directly adjacent to the SCCmec/ACME element (Fig. 1C). Genome searches confirmed that these genes are not found in typical S. aureus lineages such as methicillin sensitive S. aureus (MSSA). However, there is increasing evidence that some other clinical isolates have recently independently acquired copXL genes with 99% DNA sequence identity to the USA300 copXL genes, e.g., human and livestock-associated CC398 (Gómez-Sanz et al., 2014; Ward et al., 2014; Fig. 1C), Western Australia MRSA-59 (Monecke et al., 2015), and Irish healthcare associated ST779 (Kinnevey et al., 2013), suggesting that there is currently a strong selection for acquisition of these genes.

The CopX protein is a predicted P$_{1B-3}$-type ATPase efflux transporter (Fig. 2A; Odermatt et al., 1993; Mana-Capelli et al., 2003; Meloni et al., 2014). CopX shares 36% identity overall with the archetypical S. aureus CopA polypeptide (Sittisuk et al., 2007) but shares 83% overall identity with CopB, which is found only in the genomes of a subgroup of S. aureus lineages (Baker et al., 2011; Fig. 1B). All three proteins have the characteristic eight transmembrane domains including the key CPX motif, a conserved E1-E2 ATPase domain and a carboxyl terminal haloacid dehalogenase-like hydrolase domain. CopA has two amino terminal ferredoxin–like, heavy metal associated (HMA) domains and corresponding CxxC copper binding motifs, whereas both CopX and CopB lack these domains, instead possessing predicted intracellular amino-terminal histidine-rich metal binding domains common to the P$_{1B-3}$ Subtype of this protein family (Fig. 2A; Odermatt et al., 1993;
Mana-Capelli et al., 2003; Meloni et al., 2014). Alignment of CopX and the other S. aureus copper pumps that excluded the N-terminal domains showed similar levels of homology (37% identity to CopA, 86% identity to CopB), whereas an alignment of just the His-rich N-terminal domains of CopX and CopB showed just 59% identity (Fig. 2B).

The CopL polypeptide is predicted to be a surface-exposed lipoprotein that contains two novel tandem domains of unknown function (DUF1541). Homologues of CopL are highly conserved in Staphylococci spp. and are also present in other Gram-positive bacteria found in oceans, estuaries and soil but noCopL homologues have been functionally characterized, and thus the functions of these proteins and their domains are unknown. However, in silico analysis identified homology (51% identity) to an uncharacterized protein from Bacillus subtilis YdhK, whose structure has been determined by X-ray crystallography (PDB ID 4MDW). Modelling (SWISS-Model; Biasini et al., 2014) of the S. aureus CopL structure based on YdhK suggested that a number of conserved His and Met residues may form two spatial clusters that could form potential copper binding sites within the ‘horseshoe’ structure of the tandem DUF1541 domains (Fig. 2C).

To test the hypothesis that copXL is a novel copper resistance locus, we measured the growth of several S. aureus strains of various genotypes (copXL vs. copXL) in elevated concentrations of CuCl2 in the defined minimal medium RPMI-A. Our data show that S. aureus strains encoding a single chromosomal copy of the copXL locus, in addition to the core genome conserved copAZ operon, show copper hyper-resistance compared to typical S. aureus strains (p < 0.0001; Fig. 3A). The copXL strains, MRSA USA300 JE2, the CC398 MRSA porcine isolate 078588D (DP; Ward et al., 2014) and the CC398 MRSA human isolate 072736J (JH; Ward et al., 2014) all showed a significant increase in growth in high copper compared to S. aureus Newman (p < 0.0001; Fig. 3A). S. aureus Newman, which encodes only the core copAZ locus with no additional copper resistance elements, has copper resistance typical of the majority of S. aureus lineages (Baker et al., 2011).

EMRSA-16 and ATCC12600 both encode the additional copBmco locus, EMRSA-16 as a single copy on the chromosome, whereas ATCC12600 encodes the copBmco locus on a multicopy plasmid (Baker et al., 2011). The difference in gene copy number is reflected in copper resistance because ATCC12600, unlike EMRSA-16, shows a significantly increased growth in 300–500 μM copper compared to Newman (p < 0.0001; Fig. 3A). Interestingly, copper resistance of USA300 JE2, with its single chromosomal copy of the copXL locus, is comparable to ATCC12600, which possesses the copBmco locus on a multicopy plasmid. While it is possible that other factors may contribute to these phenotypes, these data suggest that the copXL locus may be a more effective copper resistance mechanism than a single copy of copA or copBmco.

It should be noted that all MRSA strains and the copper hyper-resistant S. aureus ATCC12600 showed a growth defect in low copper conditions compared to the methicillin sensitive strain, S. aureus Newman (p < 0.0001; Fig. 3A), although whether this fitness cost is due to the presence of these additional copper resistance loci or another, unrelated factor is unclear.

The copXL locus confers hyper-copper resistance

To investigate the role of copXL in copper resistance, S. aureus JE2 isogenic copX, copL, copA and copA insertion mutants were constructed using S. aureus JE2 transposon library mutants, as described previously (Bose et al., 2013). All mutations were transduced to a clean JE2 background and all copper-related phenotypes were maintained after transduction and were similar to the original transposon.

Our data show that mutation of the copX gene significantly increases S. aureus JE2 sensitivity to copper in the restricted medium RPMI-A, and in the rich complex medium BHI (p < 0.0001; Fig. 3B). Interestingly, the copL mutant showed a significant copper-dependent growth defect in RPMI-A (p < 0.0001; Fig. 3B) but showed only a minimal growth defect in BHI (p = 0.06; Fig. 3B). Together these demonstrate that CopX is not dependent on CopL to function. Unexpectedly, the S. aureus USA300 JE2 copA mutant did not show significantly increased copper sensitivity in either media. The double copAX mutant, however, showed an increased sensitivity to copper compared to the copX mutant demonstrating that CopA does play a role in copper resistance in USA300, which is masked by CopX activity. None of the mutants showed a growth defect in the absence of added copper (Supporting Information Fig. S1).

To confirm that copXL confers copper hyper-resistance in USA300, the copX, copL and copAX mutants were complemented by expression of a wild type copy of the copXL locus controlled by its native promoter encoded on pMK4 (pcoXL). Growth phenotype analyses showed significant complementation of the copX and copAX mutants in BHI, formally demonstrating that copXL does indeed confer copper hyper-resistance in USA300 (p < 0.01, Fig. 3C). However complementation of the copL mutant with pcoXL appeared to increase the sensitivity of the copL mutant to copper in BHI (Fig. 3C), and particularly RPMI-A (Fig. 3D, p < 0.0001), suggesting that pcoXL can inhibit growth. This growth inhibition is copper-dependent, because there are no differences in growth between any of the strains in BHI or RPMI-A in the absence of additional copper (Supporting Information Fig. S1). Interestingly, the pcoXL
plasmid also inhibited the growth of the copX and copAX mutants in the restrictive medium RPMI-A because both complemented mutants only showed a small increase in growth in the presence of copper in RPMI-A (copAX \( p \leq 0.04 \), Fig. 3D), unlike in BHI.

The copX and copL genes are important for copper efflux

To determine whether CopX functions as a copper efflux transporter, the whole cell metal content of wild type JE2 and the copA and copX mutant strains were compared by ICP-MS analysis of extracts from acid-digested cells after culture in RPMI-A medium in the presence or absence of subinhibitory 100 \( \mu \)M CuCl\(_2\), Fe\(_2\)(SO\(_4\))\(_3\) or ZnCl\(_2\) (Fig. 3E). As predicted the copX mutant contained significantly elevated copper content relative to the JE2 parent control, consistent with the hypothesis that \( S. \) aureus CopX is a copper efflux protein (\( p \leq 0.04 \); Fig. 3E). The cellular iron or zinc content was unchanged in the copX mutant strain (Fig. 3E), which correlates with our inability to detect any phenotype of this mutant strain during growth analyses in the presence of inhibitory concentrations of zinc, nickel, manganese and iron (1–3 mM) (data not shown), suggesting that CopX is a copper-specific resistance mechanism. Surprisingly, the copA mutant showed no change in cellular copper even though CopA is a known copper efflux protein, and has been shown to be essential for copper resistance for \( S. \) aureus strains that lack accessory copper resistance loci (Sitthisak et al., 2007; Fig. 3E). Thus, these data indicate that CopX is a highly effective efflux mechanism, and plays a major role in copper resistance in USA300.

ICP-MS was also used to determine whether the novel CopL protein has a role in copper efflux. Unexpectedly, considering that CopL is a predicted surface-bound lipoprotein, copper concentrations in the copL mutant cells were also elevated (\( p \leq 0.05 \), Fig. 3E) despite the CopA and CopX efflux proteins both being functional in the copL mutant, showing that CopL likely functions in copper resistance and that CopX and CopL can act independently.

The horizontally gene transferred copXL locus is regulated by copper and CsoR

Copper regulation of gene expression in \( S. \) aureus is mediated by the copper sensing transcriptional repressor CsoR (Baker et al., 2011). To determine whether the expression of copXL is regulated by copper and by CsoR in \( S. \) aureus, quantitative reverse transcriptase PCR (qRT-PCR) was used to compare the transcription of the copX and copA genes in \( S. \) aureus JE2 and the JE2 csoR::\( \phi \)NEI transposon mutant strain, grown in RPMI-A with and without subinhibitory 100 \( \mu \)M CuCl\(_2\) for 4 h. The JE2 csoR mutant strain showed no growth defect in elevated copper concentrations (data not shown), demonstrating that any impact on gene expression in the csoR mutant is due to a lack of CsoR repression. Analysis by qRT-PCR using intergenic copX and copL primers, showed that the two genes are transcribed as a single transcript, and copL expression paralleled copX expression when tested independently (Supporting Information Fig. S2), confirming that the copX and copL genes form a bi-cistronic operon.

The transcription of the copXL operon was regulated in a similar manner to the core genome copA gene. Transcription of copX was induced 17-fold, and copA 15-fold by copper (Fig. 4; \( p \leq 0.0001 \)), with both genes being repressed in low copper by CsoR, indicated by an 18-fold and 13-fold increase in expression in the csoR mutant in the absence of copper (Fig. 4; \( p \leq 0.0001 \)). Interestingly, both copA and copXL expression still showed some induction by copper in the csoR mutant, although only the copXL transcript showed a significant increase compared to low copper conditions (Fig. 4; \( p \leq 0.01 \)), suggesting that there is additional positive copper regulation in \( S. \) aureus USA300.

To investigate why the copA mutant strain showed negligible changes in copper efflux or growth, the transcription of the copA and copX genes in the reciprocal copA and copX mutants was assessed. Importantly, we detected a 40-fold copper-induced increase in copXL expression in the copA mutant (Fig. 4; \( p \leq 0.0001 \)) compared to a 15-fold induction in the JE2 wild type strain. However, there was no reciprocal increase in copA expression in the copX mutant (Fig. 4) relative to JE2 in the presence of copper. Induction of copXL expression in the copA mutant presumably
explains why the copA mutant showed no growth defect in high copper concentrations, because the increased expression of CopX could compensate for loss of CopA.

Transcription analysis showed that copL expression was found to be decreased in the copX insertion mutant, however, the gene is still expressed (Supporting Information Fig. S2). Additionally phenotypic analysis of a USA300 FPR3757 silent copX deletion mutation, which has a wild type level of copL transcription, had a similar phenotype to the copX insertion mutant, demonstrating that CopL is expressed at a sufficient level in the copX insertion mutant that its function is unaffected (Supporting Information Figs. S1 and S2).

To determine why the complemented strains showed growth inhibition in RPMI-A, transcription of copX was also assessed in the complemented pcopXL copX insertion mutant compared to wild type JE2. Surprisingly copX expression was induced 37-fold in low copper and 88-fold in this strain in the presence of copper compared to wild type JE2 in the absence of copper (Supporting Information Fig. S2). This substantial increase in copX expression could potentially explain the observed inhibition of growth in the complemented strains in high copper.

CsoR directly binds to the copXL promoter to regulate copper-dependent expression

Direct regulation of the copX and copL putative promoters by binding of CsoR was tested by electrophoretic mobility shift analysis (EMSA) (Fig. 5). Putative CsoR binding sequences were identified in the promoters of copA (ATACCtataGGGGGTAC) and copX (ATACCGggGTGGGTAT), but there is no obvious binding motif in the 20 bp intergenic region between copX and copL. Recombinant CsoR protein was produced in Escherichia coli, purified to homogeneity by liquid chromatography, and used in anaerobic assays to characterize its binding to ~200 bp DNA fragments containing the sequences upstream of copA (P_{copA}), copX (P_{copX}) or copL (P_{copL}). As expected (Liu et al., 2007; Smaldone and Helmann, 2007), metal-free CsoR showed strong binding to the PCR product encompassing the copA promoter, strongly retarding the
migration of PcopA in a dose-dependent manner (Fig. 5B). In contrast, this DNA binding was absent after anaerobic incubation of CsoR with one equivalent of Cu(I) prior to addition of DNA (Fig. 5B). A PCR product of the sequence upstream of copX was also strongly retarded in its gel migration by apo-CsoR (Fig. 5C), demonstrating specific binding of CsoR to the copX promoter. Conversely, the migration of a PCR product of the sequence upstream of copL (encompassing the 3’ end of the copX gene and the short intergenic region) showed no retardation (Fig. 5C), demonstrating no binding of CsoR to a putative copL promoter, confirming the qRT-PCR data (Supporting Information Fig. S2) that the two genes are co-transcribed. Therefore, expression of the copXL operon is repressed by direct binding of CsoR to the binding motif in the promoter upstream of copX under low copper conditions, and becomes de-repressed under elevated copper conditions.

CopX plays a major role in USA300 macrophage survival

S. aureus can survive phagocytic killing by macrophages (Kubica et al., 2008) but a role for copper resistance in the intracellular survival of S. aureus has not been described. A mechanism of copper-enhanced bactericidal activity in macrophages was previously reported by White and colleagues (2009) whereby IFN-γ-activated upregulation of the copper importer, CTR1, and intracellular trafficking of the ATP7A transporter leads to enhanced killing of copper sensitive E. coli mutants by macrophages. To investigate the relevance of copXL-mediated copper hyper-resistance to the ability of S. aureus to survive in macrophages, IFN-γ-activated macrophage survival experiments were performed. The levels of survival (CFU ml⁻¹) of intracellular USA300 JE2 and the mutant strains lacking copA, copX or copL were compared 3 h after phagocytosis (Fig. 6). There was no difference in uptake of the mutants by macrophages as assessed by CFU at T0 (Supporting Information Fig. S3). Intracellular numbers of the wild type remained high at this time point contrasting with all of the copper sensitive mutants, which showed reduced survival. Both the copX and copL mutants showed a statistically significant decrease in survival (Fig. 6; p ≤ 0.001), suggesting an important role of the CopX copper exporter and CopL lipoprotein in overcoming copper toxicity following macrophage phagocytosis (Fig. 6).

Discussion

In this study we demonstrate that the horizontally transferred copX and copL genes, confer copper hyper-resistance, are repressed in low copper concentrations via the core genome copper-dependent regulator CsoR, and play a key role in S. aureus survival in macrophages.
Interestingly, unlike CopX, the similar \textit{S. aureus} CopB \textit{P}_{1B-3} type transporter only confers copper-hyper resistance when expressed at very high levels due to there being multiple copies on free-replicating plasmids (Baker et al., 2011); unpublished data not shown). A single copy of \textit{copXL} in \textit{S. aureus} JE2 shows equivalent copper hyper-resistance to \textit{S. aureus} ATCC12600, which has \textit{copB} on a multicopy plasmid. Our transcription data do not suggest that expression of \textit{copXL} is notably higher than that of the single copy chromosomally encoded \textit{copA} in wild type USA300. Therefore, together our data suggest that \textit{S. aureus} CopX is likely a highly effective transporter, or there are other copper-dependent factors involved.

The novel CopL lipoprotein is also important for copper hyper-resistance, copper efflux and survival in macrophages. Homologues of this protein are found in many commensals and environmental bacteria and yet its function is unknown. It is predicted that CopL is surface bound and may bind copper via the tandem DUF1541 domains, though this remains to be tested. Thus, CopL could act as a surface-bound copper sink that interacts with CopX or CopA. Alternatively CopL could act as a copper sensor and regulate copper-dependent gene expression, thus increasing resistance to copper, analogous to the \textit{E. coli} NlpE/CutF lipoprotein, which is proposed to induce expression of other genes involved in the adaptation to copper through interaction with the CpxA-CpxR two-component regulator system (Gupta et al., 1995).

Our data show that \textit{S. aureus} strains that possess the \textit{copXL} genes show copper hyper-resistance compared to \textit{S. aureus} strains that do not. Importantly CopX and CopL enhance intracellular survival in macrophages. This could explain why USA300 shows increased fitness in lower lung infection compared to hospital-associated (HA)-MRSA and improved survival in alveolar macrophages (Yajjala et al., 2016).

Importantly possession of the \textit{copXL} locus is predicted to confer a strong selective advantage during infection, because copper is used as an antibacterial by the host and plays an essential role in innate immunity, virulence and colonization of the nasopharynx (Shafeeq et al., 2011; Chatterjee and Otto, 2013; Fu et al., 2014; Johnson et al., 2015; Hyre et al., 2017). Copper hyper-resistance will also confer increased fitness clinically (Marcus et al., 2017) and in the community because copper toxicity is increasingly being exploited therapeutically as an antimicrobial. Therefore, acquisition of the \textit{copXL} genes may in part explain the success of the \textit{S. aureus} USA300 epidemic strain in both North and South America.

In conclusion, this study shows that acquisition of the \textit{copXL} locus enhances \textit{S. aureus} USA300 fitness due to increased resistance to innate immunity. This has worrying implications because \textit{S. aureus} USA300 clones are spreading globally (Glaser et al., 2016; Dach et al., 2016; Planet et al., 2016), with emergences in Europe, Africa and India (Schaumburg et al., 2014; Egyir et al., 2015; Rajan et al., 2015). Consequently, our study shows the importance of the impact of acquisition of additional copper resistance mechanisms for pathogenic bacteria and the subsequent risks to our health and well-being, and demonstrates the need for surveillance and efforts to combat copper resistance mechanisms.

**Experimental procedures**

**Bacterial strains**

All bacterial strains and \textit{Staphylococcus aureus} mutants are listed in Supporting Information Table S1. \textit{E. coli} was cultured in Luria broth (LB) and was handled and transformed using standard protocols. \textit{S. aureus} strains were plated onto Luria agar (LA) and cultured statically at 37°C in 5% (v/v) CO$_2$, in the chemically-defined growth medium RPMI 1640 (Sigma-Aldrich) supplemented with an amino acids solution (Sigma-Aldrich) to a final concentration of 2% (v/v), term RPMI-A or Brain Heart Infusion (BHI) or Tryptic soy agar (TSA). Media were supplemented with tetracycline (5 \text{\mu}g \text{ml}^{-1}), chloramphenicol (10 \text{\mu}g \text{ml}^{-1}), ampicillin (100 \text{\mu}g \text{ml}^{-1}), kanamycin (75 \text{\mu}g \text{ml}^{-1}), spectinomycin (1250 \text{\mu}g \text{ml}^{-1}) or erythromycin (10 \text{\mu}g \text{ml}^{-1}) when required. For blue/white screening, 40 \text{\mu}l of 50 mg \text{ml}^{-1} X-Gal was spread onto agar plates before inoculation with bacteria.

**Copper locus mutagenesis**

The Nebraska Transposon Mutant Library (NTML) consists of 1920 defined mutants of non-essential genes in \textit{S. aureus} USA300 JE2 (Bose et al., 2013). JE2 transposon mutants NE590, NE191, NE561 and NE1750 (\textit{copX}, \textit{copL}, \textit{copA} and \textit{csoR} respectively) were transduced, using phage, into a clean USA300 JE2 background to eliminate any secondary mutations that may have been generated during library construction. The large transposon insertion in each mutant can be replaced with either an antibiotic selective marker or a markerless cassette using a set of molecular tools, as described by Bose and colleagues (2013). The transposon in \textit{copX} was replaced with a markerless cassette, in \textit{copL} with an \textit{aphA}-3 kanamycin resistance gene, and in \textit{copA} with an \textit{aad9} spectinomycin resistance gene using the temperature sensitive vectors pTNT, pKAN and pSPC respectively (Bose et al., 2013), resulting in the production of new JE2 mutant strains designated \textit{copX::TnT}, \textit{copL::kanR} and \textit{copA::specR}. The \textit{copA::specR} mutation was also transduced into the \textit{copX} mutant to create the double \textit{copAX} mutant. The \textit{copXL} complementation plasmid was constructed by cloning the complete \textit{copXL} coding sequence alongside 551 bp upstream of \textit{copX}, allowing transcription from the native promoter, into the \textit{E. coli} \textit{S. aureus} shuttle vector pMK4 (Sullivan et al., 1984). Briefly, the \textit{copXL} sequence was PCR amplified from USA300 JE2 wildtype DNA using primers \textit{copX4} and \textit{copL4}, which contained EcoRI and BamHI restriction sites respectively. The resulting PCR product was digested and ligated into pMK4 at these sites, transformed into \textit{S. aureus}
strain RN4220 and subsequently phage transduced into each recipient strain.

For construction of a clean deletion mutant of \textit{copX}, the temperature sensitive plasmid pMAY that utilizes a \textit{secY} antisense counter-selection determinant for allelic replacement was used (Monk et al., 2012; Monk and Foster, 2012). Briefly, flanking regions of \textit{copX} were amplified by PCR using primer pairs OL961F/962R and OL963F/964R with \textit{S. aureus} FPR3757 genomic DNA as the template and PrimeStar Max DNA polymerase (Takara). A ‘crossover’ PCR was done with the primers OL961F and OL964R using the upstream and downstream PCR products as templates. After subcloning into pCR2.1 (Invitrogen), the fragment was cloned into pMAY using NotI and EcoRI restriction sites and the recombinant plasmid transformed into chemically competent \textit{E. coli} SA08B (Lucigen) (Monk et al., 2012). The plasmid was isolated from \textit{E. coli}, verified by sequencing and then electroporated into \textit{S. aureus} FPR3757, which was plated onto TSA plus Cm 10 µg ml\(^{-1}\) and incubated at 28°C. Transformants were incubated on TSA plus Cm 10 µg ml\(^{-1}\) at 37°C to stimulate pMAY chromosomal integration. Colonies free of replicating plasmid were grown at 28°C without Cm and then plated onto TSA containing hydrodextratocycline 1 µg ml\(^{-1}\) for counter-selection. Resulting colonies that showed loss of Cm resistance were screened by colony PCR for the absence of \textit{copX}. Putative mutants were validated by PCR amplification of genomic DNA flanking the deletion and DNA sequencing (Monk et al., 2012).

The pcopX complementation plasmid was constructed by cloning a 2.2 kb fragment PCR product using primers 944F and 947R, that contained \textit{copX} and its predicted promoter (−196 bp upstream from start codon) into the \textit{E. coli}/ \textit{S. aureus} shuttle vector pOS1, provided by Dr. V.J. Torres at New York University School of Medicine. The amplified fragment was cloned into the EcoRI and BamHI restriction sites of pOS and then transformed into \textit{E. coli} SA08B (Lucigen). The plasmid was isolated from \textit{E. coli}, verified by sequencing and electroporated into the \textit{S. aureus} FRP3757, which was plated onto TSA containing Cm 1 µg ml\(^{-1}\) and incubated at 37°C. Transformants were incubated on TSA plus Cm 1 µg ml\(^{-1}\) at 37°C to stimulate pMAY chromosomal integration. Colonies free of replicating plasmid were grown at 28°C without Cm and then plated onto TSA containing hydrodextratocycline 1 µg ml\(^{-1}\) for counter-selection. Resulting colonies that showed loss of Cm resistance were screened by colony PCR for the absence of \textit{copX}. Putative mutants were validated by PCR amplification of genomic DNA flanking the deletion and DNA sequencing (Monk et al., 2012).

Copper growth assay

Strains were incubated in 20 ml RPMI-A or BHI for 16 h at 37°C and 5% (v/v) CO\(_2\). Cells were pelletted and re-suspended in 1 ml of fresh RPMI-A or BHI and used to inoculate fresh, prewarmed RPMI-A or BHI to an OD\(_{600nm}\) of 0.05. Aliquots (10 ml) were taken and supplemented CuCl\(_2\) and grown for 24 h. Subinhibitory and toxic copper concentrations were determined using OD\(_{600}\) as a measure of growth after 24 h. For copper toxicity growth assays, RPMI-A was supplemented with 500 µM CuCl\(_2\), while BHI was supplemented with 2 mM CuCl\(_2\). Control conditions without additional copper were included in all experiments. Supplementation with 100 µM was determined to be subinhibitory in RPMI-A and used for subsequent experiments were subinhibitory conditions were required.

Whole cell metal analysis by ICP-MS

Strains were incubated in RPMI-A for 16 h and then inoculated into RPMI-A to an OD\(_{600nm}\) of 0.05. Aliquots were made from these subcultures and supplemented with 100 µM CuCl\(_2\), Fe\(_2\)(SO\(_4\))\(_3\) or ZnCl\(_2\) with a fourth aliquot prepared with no metal supplementation. Samples were normalized to the same OD\(_{600nm}\), harvested by centrifugation, and washed twice in 50mM Tris, pH 7.5, 100 mM NaCl, 10 mM EDTA at 4°C. Washed pellets were stored at −80°C until use, then thawed and digested with concentrated nitric acid (Merck) for 48 h. Digests were centrifuged at 21 000 g for 20 min at 4°C and the supernatants were analysed for total metal content using inductively coupled plasma mass spectrometry (ICP-MS; Thermo x-series). Samples were diluted 10-fold in 2% nitric acid containing 20 µg ml\(^{-1}\) platinum and silver as internal standards, and analysed (100 reads, 30 ms dwell, 3–5 channels, 0.02 atomic mass unit separation, in triplicate) for \(^{56}\)Fe, \(^{60}\)Cu, \(^{66}\)Zn, \(^{107}\)Ag and \(^{195}\)Pt in collision cell mode (3 ml min\(^{-1}\) 8% H\(_2\) in He collision gas), and metal concentrations determined by comparison to matrix-matched elemental standard solutions.

RNA extraction

Strains were grown to mid-exponential phase in RPMI-A, with and without 100 µM CuCl\(_2\). Bacteria were pelleted and re-suspended in 1 ml PBS, and 200 µl of 19:1 ethanol/phenol was added to preserve the RNA during storage. Bacteria were pelleted and lysed for 30 min at 37°C in 200 µl TE buffer containing 10 µg ml\(^{-1}\) lysozyme and 20 µg ml\(^{-1}\) proteinase K, before being homogenized in RLT buffer (Qiagen) containing 10 µl ml\(^{-1}\) β-mercaptoethanol, using a MP Biomedical Fast Prep Instrument and Lysing Matrix B tubes (MP Biomedicals). RNA was purified from this lysate using a Qiagen RNaseasy kit, with both on column DNase treatment (Qiagen) and a subsequent TurboDNase step (Thermo Fisher) to ensure RNA purity. All RNA samples were confirmed to be DNA-free using qPCR.

qRT-PCR

Total RNA (1 µg) was converted into cDNA using Superscript IV Vilo Master Mix reverse transcriptase (Invitrogen), and from this 0.5 ng of cDNA was used per qPCR reaction. qPCR was carried out using SYBR Green Master mix (Applied Biosystems) in a 7300 Fast System (Applied Biosystems) following manufacturer's instructions. Relative gene expression for each of the sample genes (for primers and concentrations used see Supporting Information Table S2) was normalized to the expression of the endogenous control gene \textit{gyrB}, and calculated in relation to expression of a calibrator strain, JE2, without copper, using the \(\Delta\Delta CT\) method to calculate \(RQ\) (Livak and Schmittgen, 2001). Results are the product of 3 independent biological replicates, and samples were run in technical triplicate within each experiment. Statistical significance was calculated using 2-way ANOVA with Dunnett’s multiple comparison test.

Cloning, expression and purification of recombinant CsoR

The wild type \textit{csoR} gene was amplified from \textit{S. aureus} genomic DNA by PCR using primers CsoR_F and CsoR_R.
EMSA

DNA regions (~200 bp) upstream of copX, copL and copA were amplified from S. aureus genomic DNA by PCR using the relevant primer pairs (see Supporting Information Table S2), cloned into pGEM-T and confirmed by sequencing. These (and empty pGEM-T for the control DNA) were used in PCRs using M13 primers to produce DNA samples for each operator/promoter region, which were purified (PCR clean-up kit; Sigma). DTT was removed from recombinant CsoR in an anaerobic ([O2] < 5 ppm) chamber (Belle Technology) by binding to a 1 ml AEX column and washing with 10 CV anaerobic 20 mM Hepes, pH 7.5, then eluting in this buffer with 1 M NaCl. CsoR was quantified from absorbance at 280 nm using an experimentally-determined (by amino acid analysis – Alta Biosciences) extinction coefficient, (ε280nm=1490 M⁻¹ cm⁻¹), and the 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) (Sigma) assay for free thiols (Vita et al., 2015). Stock solutions (100 mM) of Cu(I) were prepared as previously described (Vita et al., 2015), and 1 mole equivalent was added to CsoR anaerobically and incubated for 30 min at room temperature. EMSA reactions contained 100 ng µl⁻¹ poly(dI-dC) (Sigma), 1 mM DTT, 0.4 mg ml⁻¹ bovine serum albumin (BSA), 100 nM target DNA and 100 nM control DNA in 20 mM Hepes, 100 mM NaCl, pH 7.0. Apo- or Cu(I)-CsoR was added (0–100 µM), and the mixture equilibrated anaerobically for 30 minutes at room temperature, before resolution on 6% (w/v) native polyacrylamide gels (pre-electrophoresed for 15 min at 42 V) for 60–80 min at 82 V. Gels were stained in 10% (v/v) SYBR Safe (Invitrogen).

Macrophage survival assays

The RAW264.7 mouse macrophage cell-line was cultured in DMEM 10% (v/v) FBS. Monolayers were prepared by seeding 2 × 10⁶ cells per ml (500 µl per well) in 24-well plates, which were then cultured for 24 h in serum-free DMEM supplemented with copper sulfate (20–40 µM) and mouse IFNγ (50 µg ml⁻¹, Gibco) for 18 h at 37°C, 5% CO₂. Immediately before infection, RAW264.7 cells were washed with DMEM alone. S. aureus strains were cultured statically in RPMI-1640 supplemented with 100 µM copper sulfate for 18 h at 37°C, 5% CO₂. Immediately before the experiment bacteria were washed twice with DMEM and adjusted to an OD₆₀₀ ~ 0.05 (ca. 2 × 10⁷ CFU ml⁻¹) in DMEM and co-incubated with monolayers for 30 min. The monolayers were washed and extracellular bacteria killed by treatment with gentamicin (200 µg ml⁻¹) & lysostaphin (100 µg ml⁻¹) for 30 min. Monolayers were washed with DMEM and then lysed with ice-cold water at time point 0 (T0) and after additional 3 h incubation (T3) to determine the survival rates (CFU ml⁻¹), lysates plated on agar, and CFUs counted.

Statistical analysis

Data are summarized as standard error of the mean (SEM) and were analysed using GraphPad Prism version 6.04 (GraphPad Software, La Jolla, CA). ANOVA or t tests were used to determine significance as appropriate.

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References

Baker, J., Sengupta, M., Jayaswal, R.K., and Morrissey, J.A. (2011) The Staphylococcus aureus CsoR regulates both chromosomal and plasmid-encoded copper resistance mechanisms. Environ Microbiol 13: 2495–2507.
Beveridge, S.J., Garrett, I.R., Whitehouse, M.I., Vernon-Roberts, B., and Brooks, P.M. (1985) Biodistribution of ⁶⁴Cu in inflamed rats following administration of two anti-inflammatory copper complexes. Agents Actions 17: 104.
Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., et al. (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res 42: W252–W258.

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Bose, J.L., Fey, P.D., and Bayles, K.W. (2013) Genetic tools to enhance the study of gene function and regulation in \textit{Staphylococcus aureus}. \textit{Appl Environ Microbiol} \textbf{79}: 2218–2224.

Chatterjee, S.S., and Otto, M. (2013) Improved understanding of factors driving methicillin-resistant \textit{Staphylococcus aureus} epidemic waves. \textit{Clin Epidemiol} \textbf{5}: 205–217.

Chaturvedi, K.S., and Henderson, J.P. (2014) Pathogenic adaptations to host-derived antibacterial copper. \textit{Front Cellular Infect Microbiol} \textbf{4}: 3.

Diep, B.A., Gill, S.R., Chang, R.F., Phan, T.H.V., Chen, J.H., Davidson, M.G., et al. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant \textit{Staphylococcus aureus}. \textit{Lancet} \textbf{367}: 731–739.

Djoko, K.Y., Ong, C.-L.Y., Walker, M.J., and McEwan, A.G. (2015) The role of copper and zinc toxicity in innate immune defense against bacterial pathogens. \textit{J Biol Chem} \textbf{290}: 18954–18961.

Egyir, B., Guardabassi, L., Monecke, S., Addo, K.K., Newman, M.J., and Larsen, A.R. (2015) Methicillin-resistant \textit{Staphylococcus aureus} strains from Ghana include USA300. \textit{J Glob Antimicrob Resist} \textbf{3}: 26–30.

Fones, H., and Preston, G.M. (2013) The impact of transition metals on bacterial plant disease. \textit{FEMS Microbiol Rev} \textbf{37}: 495–519.

Francis, J.S., Doherty, M.C., Lopatin, U., Johnston, C.P., Sinha, G., Ross, T., et al. (2005) Severe community-onset pneumonia in healthy adults caused by methicillin-resistant \textit{Staphylococcus aureus} carrying the Panton-Valentine leukocidin genes. \textit{Clin Infect Dis} \textbf{40}: 100–107.

Fu, Y., Chang, F.-M.J., and Giedroc, D.P. (2014) Copper transport and trafficking at the host-bacterial pathogen interface. \textit{Acc Chem Res} \textbf{47}: 3605–3613.

Glaser, P., Martins-Simoes, P., Villain, A., Barbier, M., Tristan, A., Bouchier, C., et al. (2016) Demography and intercontinental spread of the USA300 community-acquired methicillin-resistant \textit{Staphylococcus aureus} lineage. \textit{mBio} \textbf{7}: e02183-15–e02115.

Gómez-Sanz, E., Simón, C., Ortega, C., Gómez, P., Lozano, C., Zarazaga, M., and Torres, C. (2014) First detection of methicillin-resistant \textit{Staphylococcus aureus} ST398 and \textit{Staphylococcus pseudintermedius} ST68 from hospitalized equines in Spain. \textit{Zoonoses Public Health} \textbf{61}: 192–201.

Gupta, S.D., Lee, B.T., Camakaris, J., and Wu, H.C. (1995) Identification of \textit{cutC} and \textit{cutF} (\textit{nlpE}) genes involved in copper tolerance in \textit{Escherichia coli}. \textit{J Bacteriol} \textbf{177}: 4207–4215.

Hyre, A.N., Kavanagh, K., Kock, N.D., Donati, G.L., and Subashchandrabose, S. (2017) Copper is a host effector mobilized to urine during urinary tract infection to impair bacterial colonization. \textit{Infect Immun} \textbf{85}: e01410-16.

Johnson, J.K., Khoe, T., Shurland, S., Kreisel, K., Stine, O.C., and Roghmann, M.-C. (2007) Skin and soft tissue infections caused by methicillin-resistant \textit{Staphylococcus aureus} USA300 clone. \textit{Emerg Infect Dis} \textbf{13}: 1195–1200.

Johnson, M.D.L., Kehl-Fie, T.E., Klein, R., Kelly, J., Burnham, C., Mann, B., and Rosch, J.W. (2015) Role of copper efflux in pneumococcal pathogenesis and resistance to macrophage-mediated immune clearance. \textit{Infect Immun} \textbf{83}: 1684–1694.

Kinnevey, P.M., Shore, A.C., Brennan, G.I., Sullivan, D.J., Ehrlich, R., Monecke, S., et al. (2013) Emergence of sequence type 779 methicillin-resistant \textit{Staphylococcus aureus} harboring a novel pseudo staphylococcal cassette chromosome \textit{mec} (SCCmec)-SCC-SCC\textsubscript{CRISPR} composite element in Irish hospitals. \textit{Antimicrob Agents Chemother} \textbf{57}: 524–531.

Kopf, M., Schneider, C., and Nobs, S.P. (2015) The development and function of lung-resident macrophages and dendritic cells. \textit{Nat Immunol} \textbf{16}: 36–44.

Kubicz, M., Guzik, K., Koziel, J., Zarebski, M., and Richter, W. (2008) A potential new pathway for \textit{Staphylococcus aureus} dissemination: the silent survival of \textit{S. aureus} phagocyted by human monocyte-derived macrophages. \textit{PLoS One} \textbf{3}: e1409.

Ladomersky, E., Khan, A., Shanbhag, V., Cavet, J.S., Chan, J., Weisman, G.A., and Petris, M.J. (2017) Host and pathogen copper-transporting P-Type ATPases function antagonistically during salmonella infection. \textit{Infect Immun} \textbf{85}: e00351-17.

Liu, T., Ramesh, A., Ma, Z., Ward, S.K., Zhang, L., George, G.N., et al. (2007) CsoR is a novel \textit{Mycobacterium tuberculosis} copper-sensing transcriptional regulator. \textit{Nat Chem Biol} \textbf{3}: 60–68.

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. \textit{Methods} \textbf{25}: 402–408.

Malissen, B., Tamoutounour, S., and Henri, S. (2014) The origins and functions of dendritic cells and macrophages in the skin. \textit{Nat Rev Immunol} \textbf{14}: 417–428.

Mena-Capelli, S., Mandal, A.K., and Arguillo, J.M. (2003) Archaeoglobus fulgidus CopB is a thermophilic Cu(2+)-ATPase: functional role of its histidine-rich-N-terminal metal binding domain. \textit{J Biol Chem} \textbf{278}: 40534–40541.

Marcus, E.-L., Yosef, H., Borkow, G., Caine, Y., Sasson, A., and Moses, A.E. (2017) Reduction of health care-associated infection indicators by copper oxide-impregnated textiles: crossover, double-blind controlled study in chronic ventilator-dependent patients. \textit{Am J Infect Control} \textbf{45}: 401–403.

Mediavilla, J.R., Chen, L., Mathema, B., and Kreiswirth, B.N. (2012) Global epidemiology of community-associated methicillin resistant \textit{Staphylococcus aureus} (CA-MRSA). \textit{Curr Opin Microbiol} \textbf{15}: 588–595.

Meloni, G., Zhang, L., and Rees, D.C. (2014) Transmembrane type-2-like Cu2+ site in the P1B-3-type ATPase CopB: implications for metal selectivity. \textit{ACS Chem Biol} \textbf{9}: 116–121.

Monecke, S., Coombs, G.W., Pearson, J., Hotzel, H., Slickers, P., and Ehrlich, R. (2015) A clonal complex 12 methicillin-resistant \textit{Staphylococcus aureus} strain, West Australian MRSA-59, harbors a novel pseudo-SCCmec element. \textit{Antimicrob Agents Chemother} \textbf{59}: 7142–7144.

Monk, I.R., and Foster, T.J. (2012) Genetic manipulation of Staphylococci-breaking through the barrier. \textit{Front Cellular Infect Microbiol} \textbf{2}: 49.

Monk, I.R., Shah, I.M., Xu, M., Tan, M.-W., and Foster, T.J. (2012) Transforming the untransformable: application of direct transformation to manipulate genetically \textit{Staphylococcus aureus} and \textit{Staphylococcus epidermidis}. \textit{mBio} \textbf{3}: e00277-11.

Nimmo, G.R. (2012) USA300 abroad: global spread of a virulent strain of community-associated meticillin-resistant \textit{Staphylococcus aureus}. \textit{Clin Microbiol Infect} \textbf{18}: 725–734.
Odermatt, A., Suter, H., Krapf, R., and Solioz, M. (1993) Primary structure of two P-type ATPases involved in copper homeostasis in Enterococcus hirae. J Biol Chem 268: 12775–12779.

Otto, M. (2013) Community-associated MRSA: what makes them special?. Int J Med Microbiol 303: 324–330.

Planet, P.J., Diaz, L., Kolokotronis, S.-O., Narechania, A., Reyes, J., Xing, G., et al. (2015) Parallel epidemics of community-associated methicillin-resistant Staphylococcus aureus USA300 infection in North and South America. J Infect Dis 212: 1874–1882.

Planet, P.J., Diaz, L., Rios, R., and Arias, C.A. (2016) Global spread of the community-associated methicillin-resistant Staphylococcus aureus USA300 Latin American variant. J Infect Dis 214: 1609–1610.

Rajan, V., Schoenfelder, S.M.K., Ziebuhr, W., and Gopal, S. (2015) Genotyping of community-associated methicillin resistant Staphylococcus aureus (CA-MRSA) in a tertiary care centre in Mysore, South India: ST2371-SCCmec IV emerges as the major clone. Infect Genet Evol 34: 230–235.

Schaumburg, F., Alabi, A.S., Peters, G., and Becker, K. (2014) New epidemiology of Staphylococcus aureus infection in Africa. Clin Microbiol Infect 20: 589–596.

Schwan, W.R., Warrener, P., Keunz, E., Stover, C.K. (2005) Mutations in the cueA gene encoding a copper homeostasis P-type ATPase reduce the pathogenicity of Pseudomonas aeruginosa in mice. Int J Med Microbiol 295: 237–242.

Shafieeq, S., Yesilkaya, H., Kloosterman, T.G., Narayanan, G., Wandel, M., Andrew, P.W., et al. (2011) The cop operon is required for copper homeostasis and contributes to virulence in Streptococcus pneumoniae. Mol Microbiol 81: 1255–1270.

Sitthisak, S., Knutsson, L., Webb, J.W., and Jayaswal, R.K. (2007) Molecular characterization of the copper transport system in Staphylococcus aureus. Microbiology 153: 4274–4283.

Smaldone, G.T., and Helmann, J.D. (2007) CsoR regulates the copper efflux operon copZA in Bacillus subtilis. Microbiology 153: 4123–4128.

Sullivan, M.A., Yasbin, R.E., and Young, F.E. (1984) New shuttle vectors for Bacillus subtilis and Escherichia coli which allow rapid detection of inserted fragments. Gene 29: 21–26.

Thurlow, L.R., Joshi, G.S., Clark, J.R., Spontak, J.S., Neely, C.J., Maille, R., and Richardson, A.R. (2013) Functional modularity of the arginine catabolic mobile element contributes to the success of USA300 methicillin-resistant Staphylococcus aureus. Cell Host Microbe 13: 100–107.

Tong, S.Y.C., Davis, J.S., Eichenberger, E., Holland, T.L., and Fowler, V.G. Jr (2015) Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev 28: 603–661.

Vita, N., Platsaki, S., Basil, A., Allen, S.J., Paterson, N.G., Crombie, A.T., et al. (2015) A four-helix bundle stores copper for methane oxidation. Nature 525: 140–143.

Von Dach, E., Diene, S.M., Fankhauser, C., Schrenzel, J., Harbarth, S., and Francois, P. (2016) Comparative genomics of community-associated methicillin-resistant Staphylococcus aureus shows the emergence of clone ST8-USA300 in Geneva, Switzerland. J Infect Dis 213: 1370–1379.

Ward, M.J., Gibbons, C.L., McAdam, P.R., van Bunnik, B.A.D., Girvan, E.K., Edwards, G.F., et al. (2014) Time-scaled evolutionary analysis of the transmission and antibiotic resistance dynamics of Staphylococcus aureus clonal complex 398. Appl Environ Microbiol 80: 7275–7282.

White, C., Lee, J., Kambe, T., Fritsche, K., and Petris, M.J. (2009) A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem 284: 33949–33956.
represents the baseline level of expression for the calibrator strain JE2 grown without copper. Data are presented on a log10 scale, and the dashed line at \( y = 1 \) indicates the expression level in the reference strain. Significance of expression in each strain compared to the reference was determined by two-way ANOVA with Dunnett's multiple comparison test \( * = p \leq 0.0001 \).

**Fig. S3.** Macrophage intracellular bacterial survival CFU data. Mouse RAW264.7 macrophages were seeded at \( 2 \times 10^6 \) per ml in DMEM into 24-well plates and activated with mouse IFN-\( \gamma \) and Cu2SO4 for 18 h. Bacteria were added at a MOI of 10 in DMEM and co-incubated with monolayers for 30 min. The monolayers were subsequently washed and extracellular bacteria were killed by treatment with gentamicin (200 \( \mu \)g ml\(^{-1}\)) and lysostaphin (100 \( \mu \)g ml\(^{-1}\)) for 30 min. To quantify intracellular bacteria, monolayers were washed and lysed with ice-cold water at time point 0 (T0) and after 3 h (T3). CFUs were counted to determine numbers of viable bacteria. Bars represent the mean CFU/ml \( \pm \) SD for three independent experiments.