

# Pseudomonas aeruginosa CntM-Dependent Upregulation of Staphylococcus aureus cntA Enhances Fitness During Co-Infection

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## Research Article

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# Abstract

## Background

*Staphylococcus aureus* frequently occurs in polymicrobial infections, but how co-infecting species influence its in vivo fitness requirements remains poorly understood. Here, we investigated how *Pseudomonas aeruginosa* affects *S. aureus* fitness during co-infection.

## Results

We combined murine systemic mono-infection and co-infection models with transposon insertion sequencing (Tn-seq). Ninety genes met the fitness-gene criteria only during co-infection and were significantly enriched in the ATP-binding cassette (ABC) transporter pathway. Among genes assigned to this pathway, *cntA*, encoding the substrate-binding component of the Cnt metal acquisition system, showed the strongest depletion. Deletion of *cntA* caused no detectable fitness defect during mono-infection but reduced *S. aureus* fitness and tissue burdens during co-infection. In Zn-supplemented chemically defined medium,  $\Delta cntA$  also showed reduced growth relative to the wild-type strain during co-culture with *P. aeruginosa*. Co-culture with *P. aeruginosa* increased *S. aureus cntA* transcription, but co-culture with *Acinetobacter baumannii* or *Klebsiella pneumoniae* did not. Physical separation of *S. aureus* and *P. aeruginosa* in a Transwell system did not prevent the increase in *cntA* transcription, and cell-free co-culture supernatant retained this ability. Deletion of the *P. aeruginosa* pseudopaline biosynthetic gene *cntM* markedly reduced the *P. aeruginosa*-associated increase in *cntA* transcription.

## Conclusions

We identify *cntA* as a fitness determinant for *S. aureus* during co-infection with *P. aeruginosa*. *P. aeruginosa* upregulates *cntA* transcription through the CntM-dependent pseudopaline biosynthetic pathway, thereby enhancing the fitness of *S. aureus* in the coexistence environment. These findings link metal-acquisition responses to interspecies adaptation during polymicrobial infection and further suggest that this cross-species regulation of metal acquisition may offer a potential therapeutic target for polymicrobial infections.

## Background

Polymicrobial infections involve two or more microbial species and are common in clinical settings [1–3]. Interactions among co-infecting microorganisms can alter bacterial physiology, antimicrobial susceptibility, nutrient use and fitness, suggesting that phenotypes observed in single-species systems may not fully represent polymicrobial environments [1, 2, 4–6]. However, many studies of pathogenic microorganisms still rely on single-pathogen models. Therefore, models incorporating multiple pathogens are needed to examine interspecies interactions under polymicrobial conditions. For example,

*Klebsiella pneumoniae* can provide fermentation by-products that support the growth of *Acinetobacter baumannii*, whereas *A. baumannii* can protect *K. pneumoniae* from cefotaxime through secreted cephalosporinases [7]. These findings illustrate how interspecies interactions can modify nutrient utilization and antimicrobial responses during microbial coexistence.

*Staphylococcus aureus* is an important human pathogen frequently associated with polymicrobial infections [8]. Our previous clinical surveillance revealed that polymicrobial infections involving *S. aureus* and several clinically relevant bacterial species, including *A. baumannii* and *Pseudomonas aeruginosa*, are common in clinical settings [8]. Co-infection with *A. baumannii* altered the in vivo fitness requirements of *S. aureus*, with genes unique to co-infection significantly enriched in ABC transporter-associated pathways [8]. *S. aureus* and *P. aeruginosa* also coexist in several clinically relevant infection settings, including the airways of patients with cystic fibrosis and chronic wounds [9–11]. Extracellular molecules can mediate interactions between *S. aureus* and *P. aeruginosa*, including interactions involving metal acquisition [4, 12–17]. *S. aureus*-derived staphylopine (StP) can alter the metal homeostasis of *P. aeruginosa* and affect the pseudopaline/Cnt system [4]. PsP is a secreted opine metallophore involved in the acquisition of transition metals, including Zn and Ni [12–14]. Whether the *P. aeruginosa* PsP and its biosynthetic pathway influence *S. aureus* fitness during polymicrobial infection remains unclear.

To examine the effects of *P. aeruginosa* on *S. aureus*, we employed transposon insertion sequencing (Tn-seq). Tn-seq provides a genome-wide approach for examining bacterial fitness requirements under specific conditions [18–20]. It combines high-density transposon mutagenesis with high-throughput sequencing and estimates the contribution of individual genes to fitness from changes in mutant abundance between experimental conditions [18]. For example, previous Tn-seq studies showed that microbial partners can reshape bacterial fitness requirements in bacterial-fungal communities and murine polymicrobial infection models [21, 22]. These studies support the use of Tn-seq to examine how co-infecting species alter bacterial fitness requirements in vivo.

Here, we used Tn-seq in a murine systemic infection model to compare *S. aureus* fitness requirements during mono-infection and co-infection with *P. aeruginosa*. We subsequently focused on *cntA*, which encodes an extracytoplasmic substrate-binding protein of the Cnt transition-metal acquisition system [23, 24], and examined its contribution to *S. aureus* fitness during co-infection. We further assessed *cntA* transcription during co-culture, investigated whether the increase in *cntA* transcription required direct contact, and examined the contribution of the *P. aeruginosa* CntM-dependent PsP biosynthetic pathway.

## Methods

### Bacterial strains and culture conditions

All bacterial strains and plasmids used in this study are listed in Table S1. Unless otherwise specified, *S. aureus* Newman wild-type (WT) strain and its derivatives were cultured in brain heart infusion (BHI) broth. *P. aeruginosa* PAO1 and its derivatives, *A. baumannii*, *K. pneumoniae*, and *Escherichia coli* were cultured in Luria–Bertani (LB) broth. Cultures were incubated at 37 °C with shaking at 200 rpm.

For transition-metal experiments, chemically defined medium (CDM; Table S2) containing Na, K, Mg, and Ca as the only supplemented metal ions was used. For bacterial cultures,  $\text{NiCl}_2$ ,  $\text{CoCl}_2$ , or  $\text{ZnSO}_4$  was added at a final concentration of 1  $\mu\text{M}$  to establish specific transition-metal conditions. These media are referred to as CDM+Ni, CDM+Co, and CDM+Zn, respectively.

For physical separation of the two bacterial species, 12-mm Costar Transwell inserts with 0.4- $\mu\text{m}$ -pore polyester membranes (STEMCELL Technologies, Vancouver, BC, Canada) were used. The membrane physically separated the bacterial populations while allowing medium components and diffusible factors to pass between compartments.

### **Murine systemic infection**

Six- to eight-week-old female BALB/c mice were obtained from HUNAN SJA Laboratory Animal Co., Ltd. (Hunan, China) and maintained under a 12-h light/dark cycle. Systemic infection was established by intravenous tail-vein injection. All animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of Army Medical University (approval no. AMUWEC20223050) and were performed in accordance with the approved protocol and relevant institutional guidelines and regulations. Mice were euthanized by trained personnel using manual cervical dislocation before tissue collection.

The *S. aureus* Newman transposon mutant library was prepared as previously described [8]. Briefly, a 100- $\mu\text{L}$  aliquot of the frozen mutant library was inoculated into 100 mL antibiotic-free BHI and cultured overnight at 37 °C. The overnight culture was diluted 1:1000 into fresh BHI and grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.8.

For Tn-seq mono-infection, mice received  $1 \times 10^7$  CFU of the *S. aureus* transposon mutant library ( $n = 9$ ). For co-infection, mice received  $5 \times 10^6$  CFU of the *S. aureus* library together with  $5 \times 10^5$  CFU of *P. aeruginosa* PAO1 ( $n = 8$ ). The inoculation scheme was based on our previously established polymicrobial Tn-seq model, in which the co-infecting species was administered at a 10-fold lower inoculum than *S. aureus* to reduce the likelihood that observed effects were driven by an excessive co-infecting bacterial burden [8, 19]. Livers and kidneys were collected 5 days post-infection and snap-frozen in liquid nitrogen.

Frozen tissues were individually ground to a fine powder in liquid nitrogen. The tissue powder was resuspended in TE buffer in 2-mL Sarstedt microtubes containing 1 g of 0.1-mm zirconia beads (BioSpec) and homogenized using a Mini-Beadbeater (BioSpec). Genomic DNA was subsequently extracted using the TIANamp Bacteria DNA Kit (Tiangen).

### **Preparation of Tn-seq libraries for sequencing**

Genomic DNA extracted from liver and kidney samples from the mono-infection and co-infection groups was used to prepare Tn-seq libraries as described previously [8]. Briefly, genomic DNA was digested with MmeI (Thermo Scientific), treated with calf intestinal alkaline phosphatase (NEB), and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega).

A double-stranded adapter generated from 5'-phosphorylated oligonucleotides A and B (Table S3) was ligated to the purified fragments. The ligated fragments were amplified by PCR using primers P1-transposon-MmeI and P2-tn-seq-PCR (Table S3). This amplification step introduced barcodes for multiplex sequencing and generated a 103-bp product containing 20 bp of bacterial genomic sequence.

PCR products were separated on a 2% agarose gel. The target bands were excised and purified using a gel extraction kit (Promega). The resulting libraries were sequenced on an Illumina HiSeq platform by Novogene. Samples were subsequently demultiplexed according to barcode sequences, and the 20-bp bacterial genomic sequences were used to determine transposon insertion sites in the *S. aureus* Newman reference genome.

### **Tn-seq bioinformatics analysis**

Raw Tn-seq reads were processed using fastp (v0.20.1) for quality control and adapter trimming [25]. Samples were demultiplexed using SeqKit (v0.13.0) [26]. The 20-bp genomic sequences were extracted and mapped to the *S. aureus* Newman reference genome (GenBank accession AP009351.1). Uniquely mapped reads with no more than one mismatch were retained using in-house scripts, and gene-level transposon insertion counts were summarized using BEDTools (v2.29.2) [27]. Each output was compared independently with the input using DESeq2 (v1.30.1) [28].

The transposon library before infection was defined as the input. Libraries recovered after mono-infection and co-infection were defined as the mono-infection output and the co-infection output, respectively. Genes showing decreased mutant abundance relative to the input, with an adjusted  $P < 0.05$  and  $\log_2(\text{fold change}) < -1$  were classified as fitness genes for that infection condition.

Principal component analysis was performed using normalized gene-level transposon insertion counts.

To focus on in vivo infection-associated fitness requirements, 174 genes previously identified as in vitro fitness genes were excluded from downstream analyses [8]. For each infection condition, genes meeting the fitness-gene criteria in at least one of the two tissues were classified as fitness genes for that condition. Genes meeting the criteria under both infection conditions were classified as common fitness genes. Genes meeting the criteria only under one infection condition were defined as unique fitness genes.

KEGG pathway enrichment analysis was performed using clusterProfiler (v3.14.3) [29]. COG functional annotations were assigned by BLAST searches against the COG database [30].

### **Construction of *S. aureus* gene mutants**

To construct *S. aureus* Newman  $\Delta cntA$ ,  $\Delta fhuB$ , and  $\Delta fakA$  mutants, approximately 1000-bp fragments upstream and downstream of each target gene were amplified from Newman genomic DNA. The kanamycin-resistance cassette (Kan<sup>R</sup>) was amplified from plasmid MA15.

For each mutant, the upstream homology arm, Kan<sup>R</sup> cassette, and downstream homology arm were assembled into the temperature-sensitive plasmid pBT2 using Gibson Assembly (NEB). The resulting plasmid was used for allelic replacement by homologous recombination, in which the target gene was replaced by the Kan<sup>R</sup> cassette. Deletion mutants were confirmed by PCR and DNA sequencing. Primer sequences are provided in Table S3.

### **Construction of the *P. aeruginosa* gene mutant**

To construct the *P. aeruginosa* PAO1  $\Delta cntM$  mutant, approximately 900-bp fragments upstream and downstream of *cntM* were amplified from PAO1 genomic DNA, and the gentamicin-resistance cassette (Gm<sup>R</sup>) was amplified from plasmid pJQ200SK. The upstream fragment, Gm<sup>R</sup> cassette, and downstream fragment were assembled into Smal-digested plasmid pCVD442. The resulting plasmid was introduced into *E. coli* DH5 $\alpha$   $\lambda$ pir and verified by DNA sequencing, transferred into *E. coli* WM3064, and then introduced into PAO1 by conjugation. Gentamicin-resistant transconjugants were selected, followed by counterselection on LB agar containing 10% sucrose to obtain allelic replacement mutants. The target gene was replaced by the Gm<sup>R</sup> cassette. Deletion mutants were confirmed by PCR and DNA sequencing. Primer sequences are provided in Table S3.

### **Validation of mutant fitness using the in vivo systemic infection model**

Log-phase cultures of *S. aureus* Newman WT and the corresponding mutants were washed, normalized to the same OD600 in PBS, and mixed at a 1:1 ratio of mutant to WT. For mono-infection, mice (n = 5 per group) were inoculated via tail-vein injection with the WT-mutant mixture containing a total of  $1 \times 10^7$  CFU of *S. aureus*. For co-infection, mice were inoculated with the WT-mutant mixture containing a total of  $5 \times 10^6$  CFU of *S. aureus*, together with  $5 \times 10^5$  CFU of *P. aeruginosa* PAO1. At 5 days post-infection, tissues were collected, homogenized, serially diluted tenfold in PBS, and plated on BHI agar to quantify total *S. aureus* and on BHI agar supplemented with kanamycin to quantify the corresponding mutant. Under co-infection conditions, *S. aureus* and *P. aeruginosa* colonies on BHI agar were distinguished based on colony morphology. The competitive index (CI) for each mutant was calculated as the ratio of mutant to WT *S. aureus* recovered from each tissue:  $CI = [CFU \text{ of mutant } S. aureus / \text{tissue}] / [CFU \text{ of WT } S. aureus / \text{tissue}]$ . Each competition assay included five mice per group and was independently performed three times.

### **Time-course analysis of *S. aureus* burden during co-infection**

To investigate the contribution of *cntA* at different time points during co-infection, mice were intravenously inoculated with either *S. aureus* WT or  $\Delta cntA$  together with *P. aeruginosa* PAO1.

*S. aureus* WT,  $\Delta cntA$ , and *P. aeruginosa* PAO1 were grown to mid-logarithmic phase, washed, and adjusted to the required concentrations in PBS. BALB/c mice (n = 5 per group) received  $1 \times 10^7$  CFU of *S. aureus* WT or  $\Delta cntA$  for mono-infection, or  $5 \times 10^5$  CFU of *P. aeruginosa* PAO1 plus  $5 \times 10^6$  CFU of *S. aureus* WT or  $\Delta cntA$  for co-infection. At 1, 3, 5, and 7 days post-infection, mice were euthanized, and

livers were aseptically collected. Liver homogenates were serially diluted and plated on BHI agar for bacterial enumeration. Bacterial burdens were expressed as CFU per liver. Each group included five mice per time point in each experiment, and three independent experiments were performed.

### **Histopathological analysis**

*S. aureus* WT,  $\Delta cntA$ , and *P. aeruginosa* PAO1 were grown to mid-logarithmic phase, washed, and adjusted to the required concentrations in PBS. BALB/c mice (n = 5 per group) received  $1 \times 10^7$  CFU of *S. aureus* WT or  $\Delta cntA$  for mono-infection, or  $5 \times 10^5$  CFU of *P. aeruginosa* PAO1 plus  $5 \times 10^6$  CFU of *S. aureus* WT or  $\Delta cntA$  for co-infection.

At 5 days post-infection, livers were collected and fixed in neutral-buffered formalin for 24 h. Tissues were dehydrated, paraffin-embedded, and sectioned at a thickness of 4  $\mu$ m. Sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy for histopathological features, including inflammatory cell infiltration and changes in tissue architecture.

### ***S. aureus* growth assays under mono- and co-culture conditions**

*S. aureus* growth was assessed by viable-cell counting over 12 h. Overnight cultures of *S. aureus* WT,  $\Delta cntA$ , and *P. aeruginosa* PAO1 were washed, resuspended in PBS, and normalized to the same OD600.

For mono-culture, *S. aureus* WT or  $\Delta cntA$  was inoculated at a 1:100 dilution into CDM+Zn, CDM+Co or CDM+Ni.

For direct co-culture, *P. aeruginosa* was first diluted tenfold; *S. aureus* WT or  $\Delta cntA$  and the diluted *P. aeruginosa* suspension were then each inoculated 1:100 into CDM+Zn, CDM+Co, or CDM+Ni.

For Transwell co-culture, *P. aeruginosa* was first diluted tenfold and then inoculated 1:100 into the lower chamber, while *S. aureus* WT or  $\Delta cntA$  was inoculated 1:100 into the insert chamber.

Transwell cultures were incubated at 37 °C with gentle orbital shaking. All other cultures were incubated at 37 °C with shaking at 200 rpm. Samples were collected at 0, 2, 4, 6, 8, 10, and 12 h, serially diluted, and plated on BHI agar for CFU enumeration. *S. aureus* was distinguished from *P. aeruginosa* based on colony morphology, including its characteristic yellow staphyloxanthin pigmentation.

### **RT-qPCR analysis**

#### **Bacterial culture and RNA preparation**

For direct co-culture, overnight cultures of *S. aureus* WT and *P. aeruginosa* PAO1 were washed, resuspended in PBS, and normalized to the same OD600. *P. aeruginosa* was diluted tenfold and then co-cultured with *S. aureus* WT at a 1:100 dilution into CDM+Zn. Cells were collected at 8, 16, and 24 h after inoculation.

For species comparisons, *S. aureus* WT was mono-cultured or co-cultured with *A. baumannii* or *K. pneumoniae* under the conditions described above, and cells were collected 8 h after inoculation.

For Transwell co-culture, *S. aureus* WT was inoculated into the upper insert and the tenfold-diluted *P. aeruginosa* PAO1 into the lower chamber. For the mono-culture control, fresh medium replaced *P. aeruginosa* in the lower chamber. *S. aureus* cells were collected from the upper insert at 16 and 24 h. The 8-h time point was not included in the Transwell experiments because the limited culture volume of the upper insert did not yield sufficient total RNA for reliable downstream cDNA synthesis and RT-qPCR analysis.

For candidate screening, *S. aureus* WT was co-cultured with *P. aeruginosa* PAO1 WT or each PAO1 mutant listed in Table S4 under the same conditions. Cells were collected after 8 h for RNA extraction.

Cell pellets were resuspended in TE buffer and transferred to a 2-mL Sarstedt tube containing 0.1-mm zirconia beads, then mechanically disrupted using a Mini-Beadbeater (BioSpec). Total RNA was extracted with the RNeasy Pure Cell/Bacteria Kit (Qiagen) according to the manufacturer's instructions. Residual genomic DNA was removed with gDNA Eraser (TaKaRa), and cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa).

### **Supernatant pretreatment assay**

After 16 h of *S. aureus* WT/*P. aeruginosa* PAO1 co-culture, co-culture supernatant was collected by centrifugation and passed through a 0.22- $\mu$ m filter (Sangon Biotech) to obtain cell-free supernatant.

Cell-free supernatant was divided into aliquots that were left untreated, boiled for 20 min, or treated with DNase I at room temperature for 30 min. Cell-free supernatant was mixed 1:1 with 2 $\times$  CDM+Zn and used to culture *S. aureus* WT for 8 h. Fresh medium mixed 1:1 with 2 $\times$  CDM+Zn served as the control. RNA was then extracted as described above.

### **Quantitative PCR**

Quantitative PCR was performed using BeyoFast™ SYBR Green qPCR Mix (Beyotime) in a total reaction volume of 10  $\mu$ L. cDNA templates were diluted 100-fold before amplification. Relative *cntA* transcript levels were calculated using the  $2^{-\Delta\Delta C_t}$  method [31], with *gyrA* as the reference gene and the corresponding *S. aureus* mono-culture condition as the control. Three independent biological experiments were performed. Primer sequences are provided in Table S3.

### **Statistical analysis**

Unless otherwise specified, statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, Inc.). Data are presented as the mean  $\pm$  standard deviation (SD). In vitro experiments were performed using three independent biological replicates unless otherwise specified. Comparisons between two independent groups were performed using unpaired two-tailed Student's *t*-

tests. Comparisons among three or more independent groups were performed using one-way ANOVA followed by Tukey's multiple-comparisons test. Growth curves were analyzed using two-way ANOVA followed by Tukey's multiple-comparisons test. The statistical tests used for individual experiments are specified in the corresponding figure legends. A two-sided  $P$  value  $< 0.05$  was considered statistically significant.

## Results

### Identification of *S. aureus* fitness genes during mono-infection and co-infection with *P. aeruginosa*

To identify genes contributing to *S. aureus* fitness during mono-infection and co-infection with *P. aeruginosa*, we performed genome-wide Tn-seq using a previously constructed *S. aureus* Newman transposon library [8]. For mono-infection, BALB/c mice received  $1 \times 10^7$  CFU of the *S. aureus* library. For co-infection, mice received  $5 \times 10^6$  CFU of the *S. aureus* library together with  $5 \times 10^5$  CFU of *P. aeruginosa*. Consistent with previously established polymicrobial Tn-seq models, *P. aeruginosa* was administered at a 10-fold lower inoculum than *S. aureus* to minimize potential effects attributable to an increased bacterial inoculum [8, 19]. At 5 days post-infection, tissue samples were collected for Tn-seq analysis (Fig. 1A).

To focus on genes contributing to fitness during in vivo infection, we excluded 174 genes previously classified as in vitro fitness genes from subsequent analyses [8]. The pre-infection transposon library was designated as the input, whereas libraries recovered after mono-infection and co-infection were designated as the mono-infection output and the co-infection output. Comparison of each output with the input identified changes in mutant abundance during infection (Fig. S1 and Table S5). Genes showing decreased abundance relative to the input, with  $\log_2(\text{fold change}) < -1$  and adjusted  $P < 0.05$  were defined as fitness genes under the corresponding infection condition.

Principal component analysis revealed that the input showed different clustering patterns compared to the mono-infection and co-infection outputs in the two tissues. The mono-infection and co-infection outputs also showed different distributions in both tissues (Fig. 1B, C). We identified 646 fitness genes during mono-infection and 462 during co-infection (Fig. 1D and Table S6). Among these, 372 met the fitness criteria in both comparisons and were defined as common fitness genes. In addition, 274 genes met the criteria only during mono-infection and were classified as Unique to Mono-infection, whereas 90 met the criteria only during co-infection and were classified as Unique to Co-infection.

COG classification showed different functional distributions among genes unique to the two infection conditions. Compared with mono-infection, transport-related categories were relatively abundant among genes unique to co-infection (Fig. 1E). KEGG analysis showed enrichment of nicotinate and nicotinamide metabolism, riboflavin metabolism, and alanine, aspartate and glutamate metabolism among genes unique to mono-infection (Fig. 1F). In contrast, the genes unique to co-infection were enriched in histidine metabolism and ABC transporters (Fig. 1G). Together, the COG and KEGG analyses highlighted

the importance of transport-related functions for *S. aureus* during co-infection, particularly ABC transporter-associated functions. Among genes assigned to the ABC transporter pathway, *cntA* (NWMN\_2364) showed the most negative log<sub>2</sub>(fold change) (-3.40; Table 1) and was selected for further analysis.

### ***cntA* contributes to *S. aureus* fitness and persistence during co-infection with *P. aeruginosa***

To validate the Tn-seq results, we selected three representative genes for further analysis: *cntA* (NWMN\_2364), identified as a fitness gene unique to co-infection; *fhuB* (NWMN\_0617), identified as a fitness gene unique to mono-infection; and *fakA* (NWMN\_1136), which was not identified as a fitness gene under either infection condition. The corresponding deletion mutants ( $\Delta cntA$ ,  $\Delta fhuB$ , and  $\Delta fakA$ ) were generated. WT and mutant strains showed comparable growth profiles when mono-cultured in BHI broth (Fig. 2A), with no obvious growth defect under the tested conditions.

We next assessed the competitive fitness of these mutants in murine mono-infection and co-infection models. Consistent with the Tn-seq results,  $\Delta fakA$  showed no detectable fitness defect under either infection condition.  $\Delta fhuB$  exhibited reduced fitness during mono-infection but not during co-infection. In contrast,  $\Delta cntA$  showed a pronounced fitness defect during co-infection with *P. aeruginosa* but no defect during mono-infection (Fig. 2B). These data validated the Tn-seq results and supported a contribution of *cntA* to *S. aureus* fitness during co-infection with *P. aeruginosa*.

Furthermore, we examined whether *cntA* contributed to maintaining the *S. aureus* population during co-infection. *S. aureus* burdens were determined at successive time points in mice co-infected with *P. aeruginosa* and either WT or  $\Delta cntA$ .  $\Delta cntA$  burdens were significantly lower than WT burdens at each examined time point (Fig. 2C). WT bacterial burdens increased during the early stage of co-infection and subsequently remained relatively stable, whereas  $\Delta cntA$  burdens progressively declined. These findings indicate that *cntA* contributes to the persistence of *S. aureus* during co-infection.

Finally, we examined tissue pathology. During mono-infection, tissues from mice infected with WT or  $\Delta cntA$  showed comparable histopathological features. In contrast, during co-infection with *P. aeruginosa*, tissues from the  $\Delta cntA$  group showed less pronounced inflammatory cell infiltration and tissue damage than the WT group (Fig. 2D).

Together, these results indicate that *cntA* contributes to *S. aureus* fitness and persistence during co-infection.

### ***cntA* contributes to *S. aureus* growth during co-culture with *P. aeruginosa***

The Cnt system mediates transition-metal acquisition in *S. aureus* through the metallophore StP, and CntA is the substrate-binding component that recognizes StP-metal complexes containing transition metals such as Zn<sup>2+</sup>, Ni<sup>2+</sup>, or Co<sup>2+</sup> [23, 24]. To determine whether the *cntA*-dependent phenotype observed during co-infection was also detected in vitro, we compared the growth of *S. aureus* WT and  $\Delta cntA$  during mono-culture and co-culture with *P. aeruginosa*. The experiments were performed in

chemically defined medium (CDM) supplemented with 1  $\mu$ M ZnSO<sub>4</sub> (CDM+Zn). WT and  $\Delta cntA$  showed comparable growth during mono-culture (Fig. 3A). However, the growth of WT and  $\Delta cntA$  differed during co-culture, with  $\Delta cntA$  reaching a lower abundance than WT during the later stage of co-culture (Fig. 3B).

We next tested whether this phenotype was also observed under other transition-metal conditions. WT and  $\Delta cntA$  were examined in CDM supplemented with 1  $\mu$ M NiCl<sub>2</sub> or CoCl<sub>2</sub> (CDM+Ni or CDM+Co). As in CDM+Zn, WT and  $\Delta cntA$  grew similarly during mono-culture, whereas  $\Delta cntA$  reached lower abundance than WT during co-culture with *P. aeruginosa* (Fig. S2). Together, these results indicate that *cntA* contributes to *S. aureus* growth during co-culture with *P. aeruginosa*.

### ***P. aeruginosa* co-culture increases *cntA* transcription in *S. aureus***

We next investigated whether co-culture with *P. aeruginosa* altered *cntA* transcription in *S. aureus*. RT-qPCR showed that *S. aureus cntA* transcription during co-culture with *P. aeruginosa* was significantly higher than during mono-culture at 8, 16, and 24 h (Fig. 3C).

We next examined whether similar changes occurred during co-culture with other bacterial species. Co-culture with *A. baumannii* or *K. pneumoniae* did not increase *S. aureus cntA* transcript levels (Fig. 3D). Thus, across the three co-culture conditions tested, the increase in *cntA* transcription was specific to the co-culture partner.

### **A diffusible *P. aeruginosa*-associated factor contributes to increased *cntA* transcription in *S. aureus***

To determine whether this *P. aeruginosa*-specific phenotype required direct physical contact between the two bacterial species, we established a Transwell system that physically separated *S. aureus* and *P. aeruginosa* while allowing diffusible molecules to pass between compartments. For co-culture, *S. aureus* was inoculated into the upper insert and *P. aeruginosa* into the lower chamber. For mono-culture, *S. aureus* was also inoculated into the upper insert, while fresh medium replaced *P. aeruginosa* in the lower chamber (Fig. 4A).

After 16 or 24 h of culture in CDM+Zn, *S. aureus* cells were collected for RT-qPCR analysis. At 8 h of Transwell co-culture, an insufficient number of bacterial cells could be recovered from the limited volume of the upper insert to obtain the amount of RNA required for reliable RT-qPCR analysis; therefore, we analyzed only the 16- and 24-h time points. At both time points, *cntA* transcript levels in *S. aureus* were significantly higher during Transwell co-culture with *P. aeruginosa* than during mono-culture (Fig. 4B). Thus, the *P. aeruginosa*-associated increase in *cntA* transcription did not require direct cell–cell contact under the tested conditions.

We next compared the growth of *S. aureus* WT and  $\Delta cntA$  during Transwell co-culture with *P. aeruginosa* in CDM+Zn. Consistent with the direct co-culture experiments, *S. aureus* WT showed greater growth than  $\Delta cntA$  during Transwell co-culture (Fig. 4C). Similar differences were observed in CDM+Ni or CDM+Co (Fig. S3). These data show that *cntA* contributes to *S. aureus* growth in the presence of *P. aeruginosa* even when the two species are physically separated.

To determine whether the extracellular co-culture environment was sufficient to increase *cntA* transcription, *S. aureus* was exposed to cell-free supernatants collected from 16-h *S. aureus*/*P. aeruginosa* co-cultures. The supernatants were left untreated, boiled, or treated with DNase I. Untreated supernatant increased *cntA* transcript levels. This activity was retained after DNase I treatment but was abolished after boiling (Fig. 4D). Together, the co-culture supernatant may contain a *P. aeruginosa*-associated diffusible factor that increases *cntA* transcription in *S. aureus*.

### **The CntM-dependent PsP biosynthetic pathway contributes to increased *cntA* transcription in *S. aureus***

To identify *P. aeruginosa* factors associated with increased *cntA* transcription in *S. aureus*, we screened 15 PAO1 mutants affecting candidate extracellular factors or related biosynthetic pathways (Table S4). Most mutants retained the ability to increase *cntA* transcript levels during co-culture (Fig. S4 and Table S7). In contrast,  $\Delta cntM$  showed a marked loss of this activity, prompting further analysis. *cntM* encodes an enzyme required for the biosynthesis of *P. aeruginosa* PsP, an extracellular opine metallophore that contributes to transition-metal acquisition [12, 13].

We first compared the growth of *P. aeruginosa* WT and  $\Delta cntM$  during mono-culture in CDM+Zn. The two strains showed similar growth over 24 h (Fig. 5A), indicating that deletion of *cntM* did not cause an evident growth defect during the tested mono-culture conditions.

We next measured *cntA* transcript levels in *S. aureus* during mono-culture or co-culture with *P. aeruginosa* WT or  $\Delta cntM$  by RT-qPCR. *cntA* transcript levels were significantly higher during co-culture with *P. aeruginosa* WT than during *S. aureus* mono-culture. In contrast, *cntA* transcript levels during co-culture with  $\Delta cntM$  were significantly lower than during co-culture with *P. aeruginosa* WT and did not differ significantly from levels observed during *S. aureus* mono-culture (Fig. 5B). These findings support the involvement of the CntM-dependent PsP biosynthetic pathway in the *P. aeruginosa*-associated increase in *cntA* transcription.

## **Discussion**

Polymicrobial infections are common in clinical settings, and their biological effects are not simply the sum of those caused by individual pathogens. Microorganisms coexisting within the same infection environment can interact through resource competition, metabolic exchange, and extracellular molecules, thereby influencing bacterial physiology, resource utilization, antimicrobial susceptibility, and adaptation to infection environments [1, 2, 5]. Such interactions can alter the selective pressures encountered by individual pathogens and thereby change their genetic requirements for fitness during co-infection. For example, our previous study showed that co-infection with *A. baumannii* reshaped the in vivo fitness requirements of *S. aureus*, with ABC transporter-associated pathways significantly enriched among fitness genes unique to co-infection [8]. Similarly, co-infection has been reported to alter the colonization capacity and metabolic fitness of *Streptococcus gordonii* in vivo [22]. Consistent with these studies, our study demonstrates that the presence of *P. aeruginosa* significantly alters the fitness requirements of *S. aureus* during co-infection, and fitness genes unique to co-infection were enriched in

transport-related functions, particularly ABC transporter-associated pathways. ABC transporters comprise diverse uptake and efflux systems involved in the transport of nutrients, metals, and other substrates, and some members also contribute to resistance against environmental stresses [15, 32, 33]. These findings suggest that altered transporter requirements may represent a recurring feature of *S. aureus* adaptation to polymicrobial environments. However, the specific transport systems and fitness determinants involved are likely to depend on the identity of the co-infecting microorganism and the infection context.

Among the ABC transporter-associated genes identified as unique fitness genes during co-infection, *cntA* showed the strongest depletion and was further identified as a key fitness gene of *S. aureus* during co-infection with *P. aeruginosa* in subsequent studies. The StP/Cnt system is an important transition-metal acquisition pathway in *S. aureus*. StP can bind transition metals, including  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Co}^{2+}$ , while CntA functions as an extracytoplasmic solute-binding protein that recognizes StP-metal complexes and facilitates their subsequent uptake [23, 24, 34]. Previous studies have primarily characterized the role of the StP/Cnt system in overcoming host-imposed metal limitation during infection, in which the host restricts metal bioavailability through mechanisms such as calprotectin to defend against *S. aureus* invasion, whereas *S. aureus* utilizes StP/Cnt-mediated metal acquisition to counteract nutritional immunity [15, 23, 35, 36]. In this study, we demonstrated that *cntA* contributes to *S. aureus* fitness during co-infection. Deletion of *cntA* did not result in an apparent fitness defect during mono-infection or mono-culture, consistent with previous findings showing that *cntA* deletion did not significantly reduce *S. aureus* bacterial burdens in the liver or kidneys during systemic infection [23]. In contrast,  $\Delta cntA$  exhibited reduced fitness and impaired persistence during co-infection with *P. aeruginosa*, and also showed a growth disadvantage during co-culture with *P. aeruginosa*. These findings suggest that the physiological contribution of metal acquisition systems is context dependent, with specific pathways becoming important fitness determinants in the presence of particular co-infecting microorganisms.

Further analysis using a Transwell system demonstrated that *P. aeruginosa* increased *S. aureus cntA* transcription during co-culture in a manner independent of direct bacterial contact. This finding suggests that the regulation of *cntA* expression may be associated with a diffusible extracellular factor produced by *P. aeruginosa*. Screening of a panel of *P. aeruginosa* mutants defective in candidate extracellular factors or associated biosynthetic pathways revealed that deletion of *cntM*, a gene involved in PsP biosynthesis, significantly reduced the increase in *S. aureus cntA* transcription mediated by *P. aeruginosa* during co-culture. PsP is an opine metallophore synthesized through the CntL/CntM pathway and secreted into the extracellular environment, where it contributes to *P. aeruginosa* acquisition of transition metals, including Zn and Ni [12, 14]. Therefore, we propose that PsP produced by *P. aeruginosa* may serve as the extracellular factor mediating *P. aeruginosa*-associated upregulation of *cntA* in *S. aureus* and influencing the fitness of *S. aureus* under coexistence conditions. These findings support a working model describing the interaction between *S. aureus* and *P. aeruginosa* during mono- and polymicrobial conditions (Fig. 6). Under mono-species conditions, *S. aureus* acquires environmental transition metals through the StP/Cnt system. When *P. aeruginosa* is present, the CntM-dependent PsP biosynthetic pathway may contribute to the increase in *S. aureus cntA* transcription, which may enhance CntA-

dependent transition-metal acquisition and contribute to *S. aureus* fitness during coexistence with *P. aeruginosa* (Fig. 6).

Our study links the *P. aeruginosa* CntM-dependent PsP biosynthetic pathway with increased *S. aureus* *cntA* transcription during co-culture. Previous studies have reported that *P. aeruginosa* can sense StP secreted by *S. aureus*, subsequently altering its metal homeostasis and the transcription of related genes, and affecting the PsP/Cnt system and biofilm formation [4]. Based on these findings, we propose that under co-infection conditions, bacterial metal acquisition systems not only function in self-directed metal acquisition but also act as interspecies communication molecules that regulate metal acquisition responses in coexisting microorganisms, thereby contributing to interspecies interactions and coordinated adaptation. Recent studies have also highlighted the role of metal acquisition and homeostasis in shaping pathogen adaptation and interspecies interactions within polymicrobial infection environments [4, 15, 37]. However, this study did not directly measure PsP production in the co-culture system or perform genetic complementation experiments for  $\Delta cntM$ ; therefore, it remains unclear whether PsP is the direct molecular regulator of *cntA* transcription. Future studies should perform complementation experiments, PsP quantification, and purified PsP restoration assays to establish the direct causal relationship between PsP and *cntA* transcriptional regulation.

Overall, this study identified *cntA* as a key gene contributing to the fitness of *S. aureus* during co-infection with *P. aeruginosa* through in vivo fitness screening, and linked this phenotype to the *P. aeruginosa* CntM-dependent PsP biosynthetic pathway. Our findings suggest that metal acquisition pathways can contribute not only to nutrient acquisition but also to interspecies responses in polymicrobial environments. They further suggest that pathogen metal homeostasis may be influenced by both host-imposed metal restriction and coexisting microorganisms. These findings highlight interspecies metal acquisition as a relevant component of *S. aureus*-*P. aeruginosa* coexistence. These findings not only provide new insights into *S. aureus*-*P. aeruginosa* coexistence but also suggest that metal acquisition pathways involved in interspecies interactions may represent potential therapeutic targets for polymicrobial infections.

## Conclusions

In conclusion, genome-wide Tn-seq screening and subsequent functional validation demonstrated that the presence of *P. aeruginosa* alters the fitness requirements of *S. aureus* during co-infection and identified the metal acquisition-associated gene *cntA* as a key fitness determinant during co-infection with *P. aeruginosa*. In vivo and in vitro experiments further demonstrated that *cntA* contributes to the maintenance of *S. aureus* fitness in the presence of *P. aeruginosa*. The increase in *cntA* transcription in *S. aureus* induced by *P. aeruginosa* was specific to the co-cultured microorganism and did not require direct contact between the two bacterial species. The *P. aeruginosa*-associated increase in *cntA* transcription was linked to the *P. aeruginosa* CntM-dependent PsP biosynthetic pathway. Together with previous evidence that *S. aureus*-derived StP influences *P. aeruginosa* metal homeostasis and the PsP/Cnt system [4], our study further connects the *S. aureus* StP/Cnt system and the *P. aeruginosa* PsP/Cnt system with

interspecies interactions between these two pathogens. This study expands our understanding of pathogen adaptation in polymicrobial infections by demonstrating that coexisting microorganisms can shape bacterial fitness requirements through metal acquisition-associated responses. Further investigation of the CntM-dependent pathway and the interactions between the StP/Cnt and PsP/Cnt systems during in vivo co-infection may provide insights into *S. aureus*/*P. aeruginosa* co-infection adaptation and reveal potential targets for future intervention in polymicrobial infections.

## Declarations

### Ethics approval and consent to participate

All animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of Army Medical University (approval no. AMUWEC20223050) and were performed in accordance with institutional guidelines and relevant regulations. Consent to participate was not applicable because this study did not involve human participants.

Clinical trial number: not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The raw Tn-seq sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA1344585. All data generated or analysed during this study are included in this published article and its supplementary information files.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

X.R., Y.Z., and M.L. conceived and designed the study. Y.Z. and T.H. performed the Tn-seq analysis. T.H., Y.T., and J.Z. performed the in vitro experiments and analyzed the data. T.H., G.L., and Y.R. performed the in vivo animal experiments and analyzed the data. T.H., Y.Y., W.S., H.P., and Z.H. contributed to manuscript preparation and revision. All authors read and approved the final manuscript.

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Not applicable.

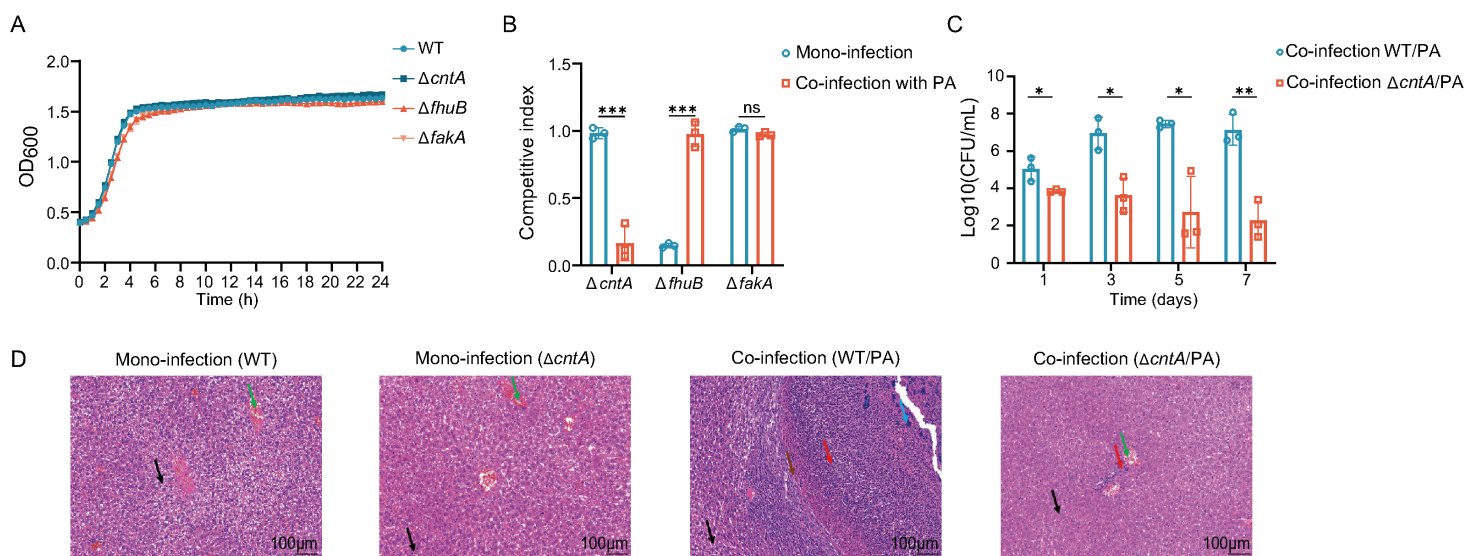
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## Figures



## Figure 2

### ***cntA* contributes to *S. aureus* fitness and persistence during co-infection with *P. aeruginosa*.**

(A) Growth of *S. aureus* WT and  $\Delta cntA$ ,  $\Delta fhuB$ , and  $\Delta fakA$  mono-cultured in BHI broth. Data are from three independent biological replicates. Growth curves were analyzed using two-way ANOVA followed by Tukey's multiple-comparisons test. (B) Competitive indices of the indicated *S. aureus* mutants during murine mono-infection and co-infection with *P. aeruginosa*. WT and mutant strains were co-inoculated at a 1:1 ratio, and the CI was calculated as the ratio of mutant to WT CFU recovered from each tissue. Each point represents the mean CI obtained from five mice in one independent experiment; three independent experiments were performed. CIs under mono-infection and co-infection conditions were compared using unpaired two-tailed Student's *t*-tests. (C) Time-course analysis of *S. aureus* burdens during co-infection with *P. aeruginosa*. Mice were co-infected with *P. aeruginosa* and either *S. aureus* WT or  $\Delta cntA$ , and liver samples were collected at 1, 3, 5, and 7 days post-infection. Each point represents the mean bacterial burden obtained from five mice in one independent experiment; three independent experiments were performed. Comparisons at each time point were performed using an unpaired two-tailed Student's *t*-test. (D) Representative H&E-stained liver sections from mono-infection and co-infection groups. Pathological features are indicated by arrows: inflammatory cell infiltration and leukocyte accumulation (red arrows), necrosis (brown arrows), steatosis (black arrows), venous congestion (green arrows), and bacterial microcolonies (blue arrows). Scale bars, 100  $\mu$ m. Data are presented as mean  $\pm$  SD. ns, not significant; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. PA, *P. aeruginosa* WT.

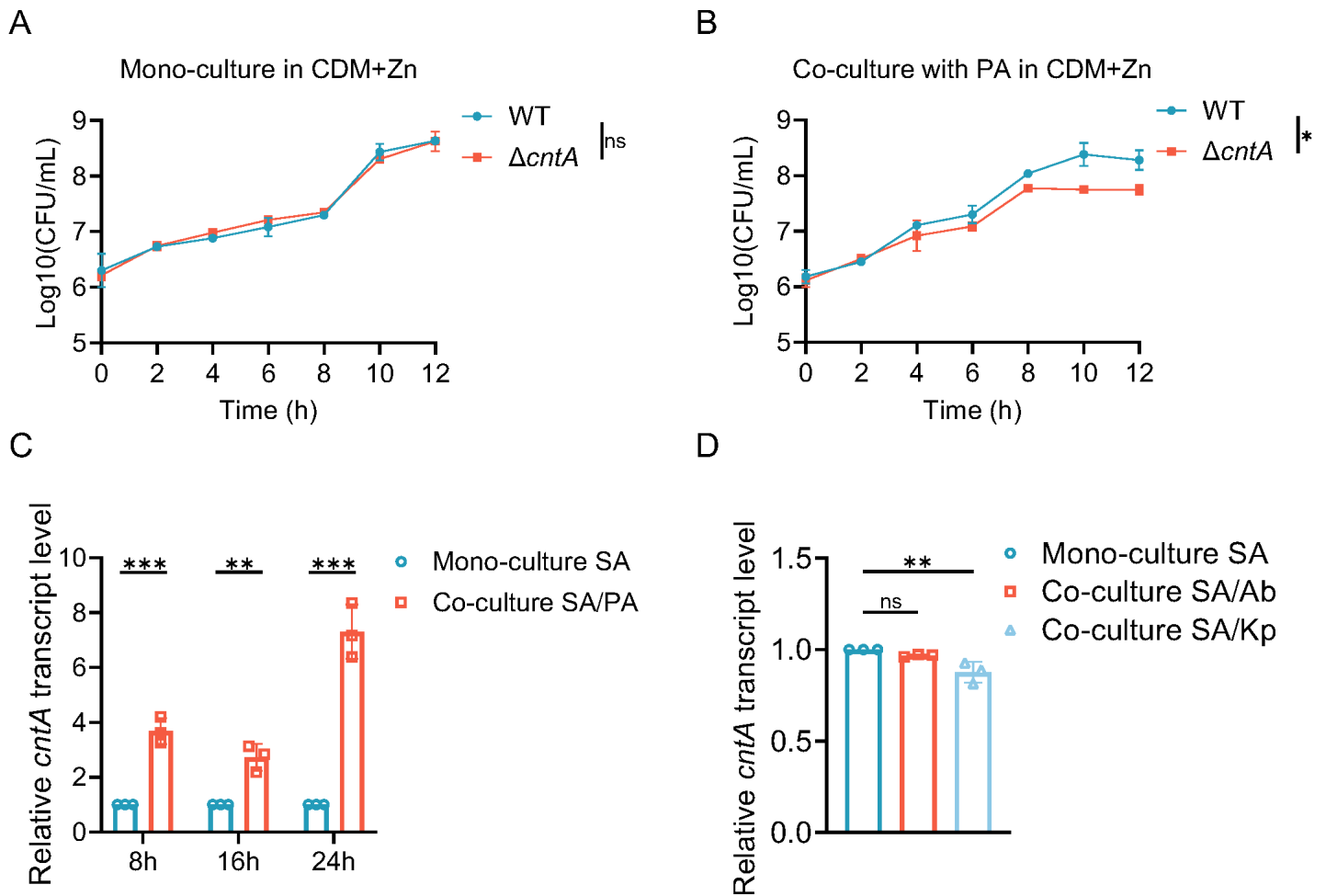


**Figure 3**

***cntA* contributes to *S. aureus* fitness during co-culture with *P. aeruginosa*.**

(A) Growth of *S. aureus* WT and  $\Delta cntA$  under mono-culture conditions in chemically defined medium (CDM) supplemented with 1  $\mu$ M ZnSO<sub>4</sub> (CDM+Zn). (B) Growth of *S. aureus* WT and  $\Delta cntA$  during co-culture with *P. aeruginosa* in CDM+Zn. Bacterial abundance was determined by CFU enumeration, and *S. aureus* colonies were distinguished from *P. aeruginosa* by colony morphology. Growth curves were analyzed using two-way ANOVA followed by Tukey's multiple-comparisons test. (C) Relative *cntA* transcript levels in *S. aureus* during mono-culture or co-culture with *P. aeruginosa* at 8, 16, and 24 h. Statistical significance was assessed using an unpaired two-tailed Student's *t*-test for each time point. (D) Relative *cntA* transcript levels in *S. aureus* during mono-culture or co-culture with *A. baumannii* or *K. pneumoniae* after 8 h of culture in CDM+Zn. Relative transcript levels were normalized to *gyrA*. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple-comparisons test. Data are presented as mean  $\pm$  SD from three independent experiments. ns, not significant; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. SA, *S. aureus* WT; PA, *P. aeruginosa* WT; Ab, *A. baumannii* WT; Kp, *K. pneumoniae* WT.

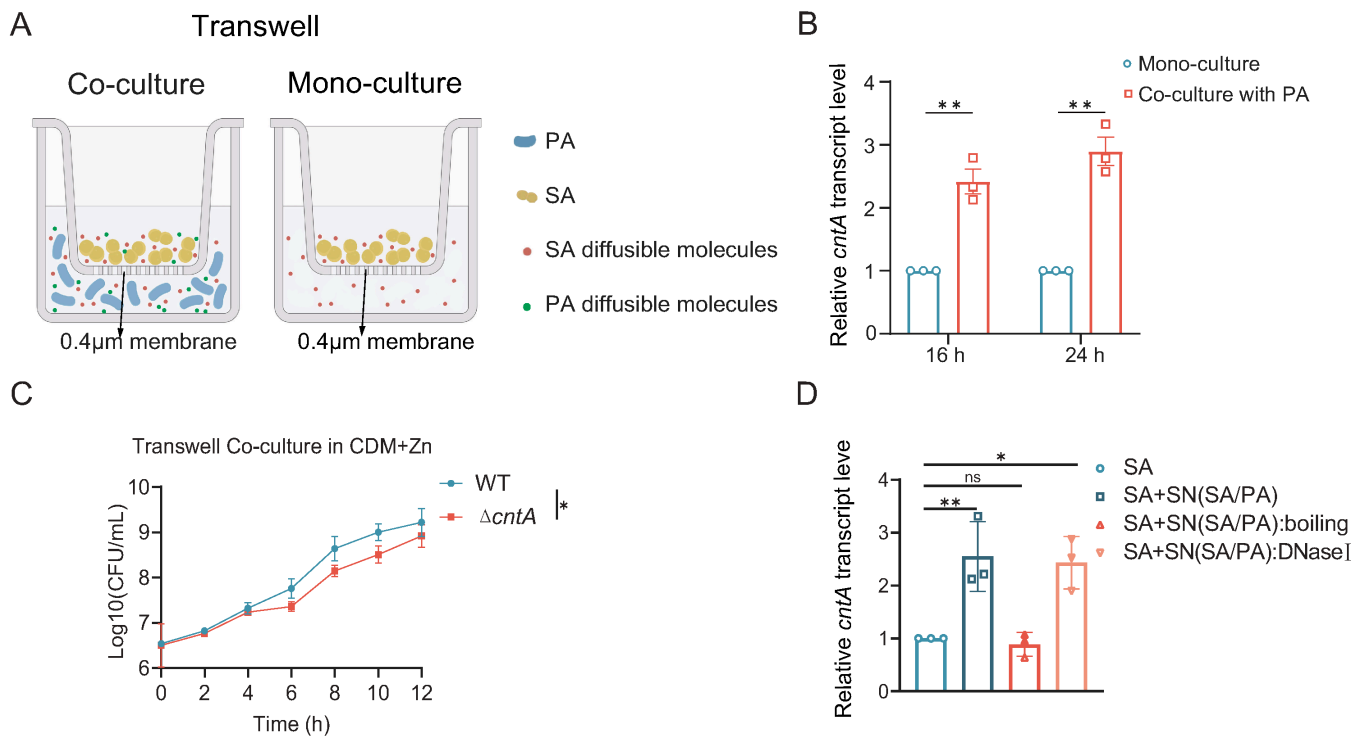


**Figure 4**

**A diffusible *P. aeruginosa*-associated factor increases *cntA* transcription in *S. aureus*.**

(A) Schematic representation of the Transwell co-culture and mono-culture system. For co-culture, *S. aureus* was inoculated into the upper insert and *P. aeruginosa* into the lower chamber. The chambers were separated by a membrane with 0.4-µm pores that allowed the passage of diffusible molecules. For mono-culture, the same *S. aureus* inoculum was added to the upper insert, and fresh medium was added to the lower chamber. (B) Relative *cntA* transcript levels in *S. aureus* during mono-culture or Transwell co-culture with *P. aeruginosa* at 16 and 24 h. Statistical significance was assessed using an unpaired two-tailed Student's *t*-test for each time point. (C) Growth of *S. aureus* WT and  $\Delta cntA$  during Transwell co-culture with *P. aeruginosa* in CDM+Zn. Growth curves were analyzed using two-way ANOVA followed by Tukey's multiple-comparisons test. (D) Relative *cntA* transcript levels in *S. aureus* after 8 h of exposure to untreated or treated cell-free supernatants collected from 16-h *S. aureus*/*P. aeruginosa* co-cultures. Supernatants were left untreated, boiled, or treated with DNase I before exposure to *S. aureus*. Fresh medium served as the control. Relative transcript levels were normalized to *gyrA*. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple-comparisons test. Data are presented as mean  $\pm$  SD from three independent experiments. ns, not significant; \**P* < 0.05, \*\**P* < 0.01. SA, *S. aureus* WT; PA, *P. aeruginosa* WT; SN, *S. aureus*/*P. aeruginosa* co-culture cell-free supernatant.

## Supplementary Files

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