Competition between *Pseudomonas aeruginosa* and *Staphylococcus aureus* is dependent on intercellular signaling and regulated by the NtrBC two-component system

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*Pseudomonas aeruginosa* and *Staphylococcus aureus* are often comorbid human pathogens, isolated from expectorated sputum of cystic fibrosis patients and chronically infected wounds. Prior studies revealed a competitive advantage of *P. aeruginosa* over *S. aureus* in vitro that was slightly muted in vivo. Here, we demonstrated that the two-component regulatory system NtrBC influences the competitive advantage of *P. aeruginosa* over *S. aureus* in skin organoid and mouse models of co-infection. Expression of ntrBC was induced during co-culture of the two species and could be recapitulated in monoculture by the addition of the metabolite N-acetylglucosamine that is released from *S. aureus* following lysis. *P. aeruginosa* LESB58 WT, but not mutant (ΔntrC and ΔntrBC) strains, induced lysis of *S. aureus* USA300 LAC during planktonic growth and outcompeted *S. aureus* USA300 LAC during biofilm formation in vitro. We confirmed these findings in a murine abscess model of high-density infection. Accordingly, the secretory profile of *P. aeruginosa* LESB58 mutants revealed reduced production of anti-staphylococcal virulence factors including pyoverdine, pyocyanin and elastase. These phenotypes of LESB58 ΔntrBC could be at least partly complemented by overexpression of quorum sensing molecules including homoserine lactones or alkylquinolone signaling molecules. These data implicate the NtrBC two-component system in the complex regulatory cascade triggered by interspecies signaling that gives *P. aeruginosa* LESB58 a competitive edge over *S. aureus* USA300 LAC.

*Pseudomonas aeruginosa* is an opportunistic pathogen implicated in infections of different tissues that are increasingly difficult to treat due to the numerous intrinsic, acquired, and adaptive antibiotic resistance mechanisms it employs. *P. aeruginosa* is most commonly co-isolated with *Staphylococcus aureus* from chronic skin wound infections and the expectorated sputum of adults with cystic fibrosis (CF). Various studies have examined the relationship between *P. aeruginosa* and *S. aureus* in CF lung infection models. During early childhood, CF lungs are readily colonized by *S. aureus*, with a higher likelihood of colonization by *P. aeruginosa* in the mid- to late-teenage years. Once present, *P. aeruginosa* rapidly takes over, indicating a potential competitive exclusion of *S. aureus* in the context of CF.

Less is known about the relationship between these species in polymicrobial infections outside the context of CF. However, recent data indicates that the competitive advantage of *P. aeruginosa* over *S. aureus* is muted in the presence of certain host factors. For example, acute wound infection models that incorporate serum into the growth medium allowed *P. aeruginosa* and *S. aureus* to co-exist, at least in the early stages of infection. Thus, interspecific interactions appeared to be highly regulated and dependent on environmental conditions. Since

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expression of effectors important for adaptation. The NtrBC two-component system, comprised of the sensor kinase that is involved in the rapid adaptation to environmental conditions through signal transduction leading to the response regulators, are a class of regulatory element components, canonically comprising a sensor kinase and a response regulator, are a class of regulatory element components, canonically comprising a sensor kinase and a response regulator. Ammonium concentration in the medium was measured in parallel (Fig. 1B), since depletion of extracellular ammonium was correlated with low intracellular nitrogen availability and NtrC activation to the extent of other reference strains of P. aeruginosa. The luminescence (i.e., ntrBC promoter activity) detected from co-culture increased rapidly six h after inoculation, ntrBC promoter activity in the presence or absence of US300 LAC in competition assays in vitro, but LESB58 ΔntrC strains did not. The staphylococcal virulence factors. Importantly, the ntrBC-dependent competitive phenotypes were maintained, albeit somewhat muted, during biofilm formation in more complex human and mouse models of co-infection. Based on these data, we propose a model by which NtrBC activity could shape interspecies interactions between P. aeruginosa and S. aureus during the early stages of co-culture.

Results
To confirm the finding that P. aeruginosa ntrBC expression was stimulated in the early stages of co-culture with S. aureus, LESB58 ntrBC promoter activity was monitored by luminescence detection in the presence or absence of USA300 LAC (Fig. 1A). Ammonium concentration in the medium was measured in parallel (Fig. 1B), since depletion of extracellular ammonium was correlated with low intracellular nitrogen availability and NtrC activation of other reference strains of P. aeruginosa.

The luminescence (i.e., ntrBC promoter activity) detected from co-culture increased rapidly six h after inoculation. At six and seven h post-inoculation, ntrBC promoter activity in co-culture was 2.6- and 4.7-fold greater, respectively, than the promoter activity in monoculture (Fig. 1A). The maximum luminescence detected during co-culture was 5.1-fold greater than during monoculture at 12 h post-inoculation (7.2 versus 2.1). Ammonium depleted slowly and at similar rates during both mono- and co-culture of species (Fig. 1B), indicating that intercellular signaling impacts on the production of virulence factors, including pyoverdine, pyocyanin and elastases that are excreted from P. aeruginosa, interspecies interactions could represent a determinant of virulence.

The P. aeruginosa genome is well endowed with regulatory elements, comprising nearly 10% of all genes, including those involved in sensing and rapidly responding to dynamic environmental conditions. Two-component systems, canonically comprising a sensor kinase and a response regulator, are a class of regulatory element components, involved in the rapid adaptation to environmental conditions through signal transduction leading to the expression of effectors important for adaptation. The NtrBC two-component system, comprised of the sensor kinase NtrB and the response regulator NtrC, is important for regulating nitrogen metabolism during nutrient limitation. Some response regulators such as NtrC belong to a subclass known as bacterial enhancer binding proteins that promote transcription of genes from RpoN (σ54)-dependent promoters, though they may also regulate gene expression independent of σ54. Genes in the NtrC regulon are involved in surface colonization (e.g., muc operon), virulence in acute and chronic infections (e.g., algU, pvdD, psch, phuR) and scavenging of nutrients (e.g., nap, nas, nir operons), some of which have no annotated σ54 binding site. Accordingly, we previously demonstrated that NtrBC regulated several adaptive lifestyles of P. aeruginosa including biofilm formation in vitro, colonization in a subcutaneous infection model in vivo and expression of virulence factors.

Here, we aimed to elucidate the role of NtrBC signaling in interspecies competition between P. aeruginosa and S. aureus since expression of NtrC had been shown to be induced in the early stages of co-culture. It was confirmed that ntrBC promoter activity of the P. aeruginosa Liverpool Epidemic Strain (LES)B58 was induced in the presence of the community-acquired methicillin resistant S. aureus (MRSA) clinical isolate USA300 LAC, as well as the small molecule N-acetylglucosamine that is liberated from S. aureus. P. aeruginosa LESB58 wild-type (WT) and an isogenic ΔntrB mutant outgrew and induced lysis of S. aureus USA300 LAC in competition assays in vitro, but LESB58 ΔntrC and ΔntrBC strains did not. The staphylococcal virulence activity of LESB58 ΔntrC and ΔntrBC strains could be complemented by overexpression of genes encoding quorum sensing (QS) signaling molecules including lasI and pqsH but not rhlI, at least in part by restoring the production of Pseudomonas anti-staphylococcal virulence factors. Importantly, the ntrBC-dependent competitive phenotypes were maintained, albeit somewhat muted, during biofilm formation in more complex human and mouse models of co-infection. Based on these data, we propose a model by which NtrBC activity could shape interspecies interactions between P. aeruginosa and S. aureus during the early stages of co-culture.
LESB58 ntrBC promoter activity was independent of extracellular ammonium levels. Indeed, ammonium was only reduced by 32.6% during monoculture, from 0.92 μg/ml at the time of inoculation to 0.62 μg/ml 12 h post-inoculation. Similarly, ammonium was reduced by 31.9% during co-culture, from 0.91 μg/ml at the time of inoculation to 0.62 μg/ml 12 h post-inoculation.

N-acetylglucosamine is a component of peptidoglycan that can be liberated following bacterial (e.g., S. aureus) lysis, and D-ribose is an analogue of the autoinducer-2 QS molecule produced by Gram-positive pathogens (e.g., S. aureus). To determine whether these signaling molecules had a potential role in inducing lysis, and D-ribose is an analogue of the autoinducer-2 QS molecule produced by Gram-positive pathogens (e.g., S. aureus). Next, we confirmed the observation that P. aeruginosa competitively displaces S. aureus using clinical isolates in batch culture growth experiments (Fig. 3A). Since NtrC promoter activity was stimulated during co-culture with S. aureus USA300 LAC, it was hypothesized that NtrB and/or NtrC activity was important for the competitive advantage of P. aeruginosa over S. aureus. It was observed that P. aeruginosa LESB58 ΔntrC and ΔntrBC mutants were outcompeted by S. aureus USA300 LAC during batch culture in BM2 (Fig. 3C,D), whereas the WT and ΔntrB mutant maintained a competitive edge during co-culture (Fig. 3A,B).

During co-culture with either P. aeruginosa LESB58 WT or ΔntrB strains, S. aureus USA300 LAC grew steadily until six h post-inoculation (Fig. 3A,B). However, between six and 12 h, death of USA300 LAC was observed, since the number of USA300 LAC recovered from co-culture with LESB58 WT and ΔntrB at the 12 h time point was 21.7- and 26.0-fold less than at six h, respectively. During this period, the growth rate of LESB58 WT was exponential and constant, although LESB58 ΔntrB had a slight reduction in growth rate. This was reflected by the growth constants (μ) (Table S3) for LESB58 WT and ΔntrB, which were 0.43/h and 0.40/h during exponential growth, respectively. In contrast, the growth rate of the ΔntrC and ΔntrBC mutants never overtook USA300 LAC. S. aureus USA300 LAC grew to a total density of 1.4–1.6 × 10⁸ CFU/ml by 12 h post inoculum during co-culture with LESB58 ΔntrC and ΔntrBC mutants, respectively, whereas their density was reduced to 7.2 or 9.8 × 10⁷ CFU/ml during co-culture with WT and ΔntrB, respectively. Differences between the strains were ameliorated by complementation of the deleted gene (Fig. S1). This showed that interspecies inhibition of S. aureus USA300 LAC by P. aeruginosa LESB58 was dependent on NtrBC.

Figure 2. Induction in P. aeruginosa ntrBC promoter activity by S. aureus small molecules.

N-acetylglucosamine and to a lesser extent D-ribose caused the induction of P. aeruginosa LESB58 ntrBC promoter activity (ntrBC-pro). P. aeruginosa LESB58 strains were seeded at a density of ~ 5 × 10⁵ CFU/ml and treated with (A) N-acetylglucosamine (20 μM) or (B) D-ribose (20 μM) prior to luminescence detection for up to 12 h. Data are presented as mean ± standard error of the mean (SEM) for three independent experiments containing three technical replicates in each (n = 3).

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Next, we determined whether the competitive advantage conferred on *P. aeruginosa* LESB58 by NtrBC depended on environmental conditions since, for example, it had been previously observed that interspecies competition was muted in the presence of host factors. Thus, interspecies competition was examined between LESB58 WT and mutant strains co-cultured with USA300 LAC in biofilm formation assays in vitro (Fig. 4A,B) and in a model of biofilm infection formed on a human skin organoid model (Fig. 4C,D).

The total amount of biomass that was formed by mixed species biofilms in polypropylene 96-well plates containing DMEM supplemented with FBS and glucose was not significantly different between strains (Fig. 4A), although the number (CFU/ml) of LESB58 or USA300 recovered from biofilms varied depending on which strain of LESB58 was co-inoculated (Fig. 4B). In the WT mixed species biofilms, there was much lower competition between *P. aeruginosa* LESB58 and *S. aureus* USA300 with only a 3.6-fold advantage for *P. aeruginosa* (Fig. 4), cf. the > 100-fold difference in broth co-culture (Fig. 3). More specifically, the number of LESB58 ΔntrBC was significantly reduced by 218-fold from $2.4 \times 10^8$ CFU/ml to $1.1 \times 10^7$ CFU/ml, on average. Accordingly, the number of USA300 LAC increased threefold from $6.2 \times 10^7$ CFU/ml (recovered from biofilms formed with LESB58 WT) to $1.9 \times 10^8$ CFU/ml (recovered from biofilms formed with LESB58 ΔntrBC). The *P. aeruginosa* LESB58 mutants might have exhibited different competition toward *S. aureus* USA300 LAC in biofilm or planktonic growth assays due to the reduced ability of ΔntrBC to form biofilms even in the absence of competition or due to the different composition of the medium that might impact on LESB58 ΔntrBC fitness.

Compared to biofilms formed on skin organoids with either USA300 LAC and LESB58 WT, mixed biofilms formed by USA300 LAC and either LESB58 ΔntrC or ΔntrBC caused 12.8% (23.1% cf. 35.9% relative to control) and 11.3% (24.6% cf. 35.9% relative to control) less cytotoxicity in a human skin organoid model (Fig. 4C). In contrast, mixed biofilms formed with USA300 LAC and LESB58 ΔntrB were comparable to that of WT (34.9% cf. 35.9% relative to control). *P. aeruginosa* LESB58 WT was recovered in 100-fold larger numbers than *S. aureus* USA300 LAC (Fig. 4D), similar to the observations in broth co-culture (Fig. 3). Recovery of LESB58 ΔntrC was significantly decreased by 2.8-fold from $1.9 \times 10^6$ CFU/ml to $6.9 \times 10^5$ CFU/ml, whereas recovery of LESB58 ΔntrBC was reduced even more by 232-fold to $8.2 \times 10^6$ CFU/ml (Fig. 4D). In co-culture with all mutant strains,
the number of *S. aureus* USA300 LAC was significantly increased by nearly 800-fold from 1.1 × 10⁶ CFU/ml to approximately 8 × 10⁸ CFU/ml (Fig. 4D). Thus, while in vitro biofilms showed somewhat different interspecies competition effects than those observed in batch culture, biofilms on skin organoids showed rather similar effects with modest differences.

To determine the importance of NtrBC on competition in vivo, the murine abscess model of high-density infection²¹ was modified by co-inoculating LESB58 strains and USA300 LAC (Fig. 5). The induction of *ntrBC* promoter activity was first assessed in vivo (Fig. 5A,B). Relative to 16S rRNA expression, there was a 2.9-fold greater *ntrBC* promoter activity observed during polymicrobial infection than monomicrobial infection at 24 h post-infection. Thereafter, the *ntrBC* promoter activity observed during polymicrobial infection declined, but was still higher than the activity observed during mono-species infection, although promoter activity during multi-species infection was only significantly greater than mono-species infection at the 48 h time-point (Fig. 5B).

The area of abscesses formed with USA300 LAC mixed with LESB58 WT were, on average, 58.3 mm², whereas polymicrobial abscesses formed with the mutants were only 31.1, 19.2 or 19.3 mm² (Fig. 5C), but were only statistically significant for Δ*ntrC* and Δ*ntrBC*. There were no statistically significant differences between the numbers of bacteria recovered from polymicrobial abscesses formed with LESB58 WT and Δ*ntrB* (Fig. 5D). However, in mixed infections the average numbers of LESB58 Δ*ntrC* were reduced threefold from 1.0 × 10⁹ CFU/ml to 3.4 × 10⁸ CFU/ml, and 5.3-fold for LESB58 Δ*ntrBC*, to 1.9 × 10⁸ CFU/ml (Fig. 5D).

To begin to unravel the mechanism(s) by which NtrBC conferred a competitive advantage on *P. aeruginosa* LESB58 over *S. aureus* USA300 LAC, the regions upstream of all coding sequences in *P. aeruginosa* were scanned for the NtrC binding motif²² (Fig. S2) using FIMO software²³. FIMO detected 259 binding targets (Table S4), some...
having more than one non-redundant binding site; 36 of the downstream genes were differentially expressed in PA14 ΔntrB or ΔntrC strains. A literature search identified strong possibilities from initial hits that were involved in the production of anti-Staphylococcal virulence factors and were expressed from RpoN-dependent promoters, and differential expression of these genes was confirmed using RT-qPCR (Table 1).

According to RT-qPCR, the most significantly downregulated hit was phzA1, a phenazine biosynthesis protein that was 9.2- to 10.1-fold downregulated when compared to WT. Since phzA2 exhibited high percent identity (97.5%) with phzA1, it is possible that this result stemmed from dysregulation of either or both genes. The next most downregulated hit was pys2, a pyocin with antibacterial impacts on competitors, which was 6.3- to 6.8-fold downregulated. Other downregulated genes included transcriptional regulators such as algU, pvdS, and lasR, the last of which is a master regulator of QS in the hierarchical regulatory network of P. aeruginosa and has impacts on production of virulence factors with anti-Staphylococcal activity. In contrast, phospholipase C was repressed by ntrBC.

To validate whether dysregulated expression of QS systems contributed to NtrBC-dependent competitive exclusion of S. aureus USA300 LAC by P. aeruginosa LESB58, we investigated the competitive phenotype of LESB58 strains transformed with an overexpression vector containing the coding sequence of genes involved in the synthesis of QS molecules including lasI, rhlI or pqsH (Fig. 6).

As was observed for batch cultures seeded with LESB58 WT and USA300 LAC (Fig. 3A), the density of the complemented LESB58 ΔntrBC/ntrBC+ and USA300 increased from the time of inoculation to six h post-inoculation, when USA300 LAC numbers sharply declined from 8.2 × 10^6 CFU/ml to 5.3 × 10^5 CFU/ml (Fig. 6A),
much as had been observed for WT LESB58 (Fig. 3A). LESB58 ΔntrBC/lasI, when co-cultured with USA300 LAC, showed partial phenotypic complementation, in that the lasI overexpressing strain was able to outcompete USA300 at least partially by 12 h post-inoculation (Fig. 6B), with the number of USA300 declining beyond six h post-inoculation, but not to the same extent as observed for LESB58 ΔntrBC/ntrBC+.

In contrast, neither LESB58 ΔntrBC/rhlI (Fig. 6C) nor LESB58 ΔntrBC/pqsH+ (Fig. 6D) were able to outcompete USA300 at any time point. The growth rate of LESB58 ΔntrBC/pqsH+ was the lowest of all the strains examined in this mixed species growth experiment, reaching a maximum bacterial density of only 7.9 × 10^6 CFU/ml around six h post-inoculation, then remaining at this density until the experimental endpoint. Regardless of the LESB58 growth

### Table 1. Differential expression of selected genes involved in the production of anti-Staphylococcal virulence factors. Gene expression is shown as fold-change (FC) from WT using the ΔΔCt method. Dysregulated expression was considered meaningful when FC was greater than ±2. Data are shown as the mean from three independent experiments each containing three technical replicates.

| LESB58 Locus Tag | Gene Name | Annotation | Fold Change cf. WT | ntrB | ntrC | ntrBC |
|------------------|-----------|------------|--------------------|------|------|-------|
| PALES_07171      | phzA1     | Phenazine biosynthesis protein | −10.1 | −9.8 | −9.2 |
| PALES_41691      | pys2      | Pyocin S2 | −6.6 | −6.3 | −6.8 |
| PALES_45811      | algU      | RNA Polymerase sigma factor AlgU | −5.4 | −5.6 | −5.4 |
| PALES_28691      | pavS      | Extracytoplasmic function sigma factor | −4.5 | −4.1 | −4.3 |
| PALES_39841      | lasR      | Transcriptional regulator LasR | −3.9 | −4.1 | −4.0 |
| PALES_44741      | plcH      | Hemolytic phospholipase C precursor | 2.1 | 2.0 | 2.4 |

Figure 6. Competitive phenotype of *P. aeruginosa* LESB58 ΔntrBC could be phenotypically complemented. Phenotypic complementation by (A) ntrBC and (B) lasI, but not (C) rhlI or (D) pqsH+. *P. aeruginosa* LESB58 ΔntrBC strains were seeded with *S. aureus* USA300 at starting OD_{600}=0.1 in cultures that were sampled in 2-, 6- or 12 h intervals and plated on selective media for bacterial enumeration. Data are presented as mean ± standard error of the mean (SEM) for three independent experiments (n = 3).
rate, no staphylolytic activity by either of the latter two complemented strains was apparent, since the density of USA300 LAC did not decline at any point (Fig. 6C,D).

To confirm that the overexpression of specific QS determinants phenotypically complemented the decrease in *P. aeruginosa* virulence factors with known anti-Staphylococcal activity\(^{20}\), virulence factor secretion was examined in LESB58 strains (Fig. 7). We confirmed previous observations for PA14 mutant strains\(^{16}\), in showing that virulence factor production was significantly downregulated in strain LESB58 Δ*ntrBC* and/or Δ*ntrC* strains (Fig. 7A–C), depending on the virulence factor. While statistically significant, reduced production of pyoverdine by LESB58 Δ*ntrB* and Δ*ntrC* was not strong (~87% of WT). However, LESB58 Δ*ntrBC* produced only 22% of the level of pyoverdine cf. WT. Similarly, substantial reduction for pyocyanin (to 34% of WT levels) and elastase (19% of WT) was also observed for LESB58 Δ*ntrBC*. LESB58 Δ*ntrB* showed no significant changes in either of these virulence factors, whereas LESB58 Δ*ntrC* produced only 71% (\(P<0.01\)) and 17% (\(P<0.001\)) as much pyocyanin and elastase as WT. The production of virulence factors by LESB58 Δ*ntrBC* could be restored by overexpression of QS determinants, including *lasI*, *rhlI* and *pqsH* (Fig. 7D–F).

**Discussion**

We examined here the importance of NtrBC in interspecies competition between clinical isolates of *S. aureus* and *P. aeruginosa*, pathogens that can be comorbid in upper respiratory tract infections of CF patients, as well as in skin wound infections\(^{64}\). Induction of the *P. aeruginosa* Liverpool epidemic strain LESB58 ntrBC promoter

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**Figure 7.** Virulence factor production was restored in *P. aeruginosa* LESB58 Δ*ntrBC* by overexpression of ntrBC, *lasI*, *rhlI* and *pqsH*. Levels of (A,D) pyoverdine, (B,E) pyocyanin and (C,F) elastase produced by mutants (A–C) or complements (D–F) were quantified using well established methods. Data are presented as mean ± standard error of the mean (SEM) for three independent experiments containing three biological replicates in each (\(n=9\)). *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\) different from control according to one-way ANOVA followed by Dunnett’s post-hoc analysis.
activity was greater in the presence of *S. aureus* USA300 LAC than in monoculture, and this was independent of the ammonium concentration of the supernatant (Fig. 1). Extracellular ammonium is usually correlated with intracellular nitrogen availability of laboratory reference strains of *S. aureus* and *P. aeruginosa* since ammonium is their preferred source of nitrogen. However, clinical isolates may exhibit autonomy for essential amino acids, which limits protein synthesis, adaptation and growth, unless that amino acid is abundant in the environment. Thus, the extracellular concentration of ammonium may not be the best indicator of intracellular nitrogen status for the strains of *S. aureus* and *P. aeruginosa* used in this study (Fig. 1). However, ntrBC can be regulated by other means than through sensing of intracellular nitrogen. Indeed, induction of ntrBC promoter activity could be recapitulated by addition of specific *S. aureus* extracellular signaling molecules (Fig. 2), including N-acetylglutcosamine, a component of peptidoglycan that can be liberated following bacterial lysis, and D-ribose, an analogue of the autoinducer-2 QS molecule. Future studies could focus on comprehensively examining the secretome of *S. aureus* USA300 LAC during competition and defining the molecular mechanism(s) by which secreted molecules might induce ntrBC promoter activity. Interestingly, D-ribose only slightly stimulated ntrBC promoter activity in LESB58 WT, but not any mutant strains (LESB58 ΔntrB, ΔntrC, or ΔntrBC) (Fig. 2). This indicated that NtrB is required for ntrBC promoter activity following stimulation by D-ribose during coculture. While N-acetylgltosamine did not apparently induce ntrBC promoter activity in mutant strains (since the amount of luminescence detected at t = 0 h was not significantly different from the luminescence detected at later time points in mutant strains), ntrBC promoter activity was detected (Fig. 2). This provided evidence that NtrB was not essential for ntrBC promoter activity following stimulation by N-acetylgltosamine. Still, induction of ntrBC promoter activity of LESB58 WT was (at its peak) ~ sixfold greater than at the point of inoculation. Taken together, these data indicate that self amplification of NtrB and/or amplification by exogenous molecules released in co-culture may play a role in NtrB signaling downstream of interspecies competition with *S. aureus* USA300 LAC. Overall, the data presented (Figs. 1-2) supported the hypothesis that NtrB was important for conferring a competitive advantage on LESB58 over USA300 LAC, and that NtrB self amplification of promoter activity, dependent in part on molecules released into the environment by *S. aureus*, may be needed for full responsiveness to interspecies signaling molecules.

*P. aeruginosa* LESB58 WT and ΔntrB strains outcompeted *S. aureus* USA300 LAC in a planktonic competition assay, whereas ΔntrC and ΔntrBC strains did not (Fig. 3). This might be due to crosstalk between NtrC and another sensor kinase, which might activate the regulatory activity of NtrC independently of NtrB, compensating for its deletion. Crosstalk between NtrC and other sensor kinases has been suggested in other bacterial species, including *Escherichia coli* and *Rhodobacter capsulatus*. Further, NtrC can autophosphorylate in the presence of selected metabolites, bypassing NtrB-mediated activation. Following its activation, NtrC could then regulate the production of anti-Staphylococcal molecules by enhancing RpoN-mediated transcription or through a mechanism independent of RpoN. The anti-Staphylococcal activity of *P. aeruginosa* in cell–culture systems has been described previously and attributed to the production of various molecules including 4-hydroxy-2-heptylquinolone N-oxide (HQNO), which is regulated by *Pseudomonas* quinolone signal (PQS), as well as other virulence factors with anti-Staphylococcal activity. Other molecules produced by *P. aeruginosa* that are not typically considered to be virulence factors, such as the acyl homoserine lactone (AHL) molecules involved in the LasRI and RhlRI QS signaling systems, can also interfere with the fitness of *S. aureus* by inhibiting respiratory (electron transport chain) activity and preventing planktonic growth. The anti-Staphylococcal activity of *P. aeruginosa* is known to be influenced by environmental factors, including the presence of host factors such as serum or mediators of immune signaling. Accordingly, it has been observed that competitive inhibition by *P. aeruginosa* of *S. aureus* can be muted in host-like conditions characteristic of, for example, animal models of disease and biofilm formation in host-mimicking media. This could partially explain why different patterns of competitive exclusion were exhibited by strains of *P. aeruginosa* LESB58 (WT, ΔntrB, ΔntrC, or ΔntrBC) in biofilm assays in vitro (Fig. 4A–B) and in an air–liquid interface skin organoid model (Fig. 4C–D). Generally, LESB58 and USA300 LAC co-existed better during in vitro biofilm growth, suggesting that either *S. aureus* USA300 LAC was producing fewer molecules that primed *P. aeruginosa* LESB58 strains and/or the latter demonstrated muted production of anti-Staphylococcal molecules.

To further explore this issue, the mechanism(s) possibly underlying inhibition of *S. aureus* USA300 LAC by different strains of *P. aeruginosa* LESB58 were interrogated in the context of planktonic or biofilm competition assays. Upstream regions of coding sequences of *P. aeruginosa* LESB58 were searched for NtrC binding motifs (Fig. S2), by inputting a prior defined position weight matrix to FIMO software, identifying the potential binding locations (Table S4). The number of hits identified was likely an underestimate, since the binding motif of NtrC is not well conserved, and since NtrC is known to bind to RpoN directly from solution. Inputting a prior defined position weight matrix to FIMO software, identifying the potential binding locations (Table S4). The number of hits identified was likely an underestimate, since the binding motif of NtrC is not well conserved, and since NtrC is known to bind to RpoN directly from solution. Consequently, it would be challenging to identify members of the NtrBC regulon by this approach. Leads identified by FIMO included the alternative sigma factor PvdS, implicated in iron scavenging and pyoverdine synthesis for iron acquisition as well as in exotoxin A production, and expression of the transcriptional regulator LasR, the master regulator of the hierarchical QS regulatory network of *P. aeruginosa*. Differential expression of strong leads was confirmed by RT-qPCR (Table 1). Although *P. aeruginosa* LESB58 mutants exhibited different competitive phenotypes when co-cultured with *S. aureus* USA300 LAC (Fig. 3), differential expression of selected genes encoding anti-Staphylococcal molecules was similar across strains (Table 1). This could be due to the different experimental conditions used to examine competition and genetic regulation, or could indicate that expression of other anti-Staphylococcal molecules that might be impacted during co-culture and may have contributed to the observed phenotypes. Additionally, downregulated expression of *phzA1* (Table 1), which encodes a phenazine biosynthetic protein, did not always correlate with lesser production of the phenazine pyocyanin (Fig. 7). Since PhzA1 is involved in the synthesis of phenazine-1-carboxylic acid, which is further oxidized to pyocyanin or one of three other phenazines, and there are two functionally redundant operons for this process encoded in *P. aeruginosa*, it is difficult to determine exactly why this was. Nonetheless, LasR was considered a strong lead due to its expression
from an RpoN-dependent promoter\(^3\), global regulation of processes including synthesis of virulence factors with anti-Staphylococcal activity\(^9\), and differential expression in PA14 \(\Delta ntrB\) and \(\Delta ntrC\) mutants\(^15\). Thus, the impact of overexpression of QS molecules on competitive and virulence phenotypes of LESB58 \(\Delta ntrB\), \(\Delta ntrC\) and \(\Delta ntrBC\) mutants was examined (Figs. 6 and 7). Overexpression of lasI in the LESB58 \(\Delta ntrBC\) genetic background restored competition with USA300 (Fig. 6), as well as reflected by the restoration of pyoverdine, pyocyanin and elastase production (Fig. 7). However, \(rhl\) and \(pgsH\) did not restore the competitive advantage of LESB58 \(\Delta ntrBC\) (Fig. 6), despite improving pyoverdine, pyocyanin and elastase production (Fig. 7). This indicated that other anti-Staphylococcal molecules, such as N-dodecanoyl-L-homoserine lactone, might be regulated by something downstream of the LasRI QS system, but not other QS systems lower in the hierarchical QS regulatory network.

## Materials and methods

### Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are described in Table S1. Overnight cultures were routinely maintained in Luria–Bertani (LB) broth or 2× yeast extract tryptone (2×YT) prepared according to the manufacturer’s specifications (Thermo Scientific). Overnight and subcultures were incubated for no longer than 18 h at 37 °C with shaking (250 rpm). Modified forms of basal medium (BM2; containing 62 mM potassium phosphate buffer (pH = 7.0), 0.1% casamino acids (CAA) and/or 7 mM (NH\(_4\))\(_2\)SO\(_4\), 2 mM MgSO\(_4\), 10 μM FeSO\(_4\), 20 mM glucose) were used for promoter induction assays, competition assays and biofilm induction assays in vitro. Other media used in specific assays are described elsewhere. Gentamicin (500 μg/ml) was added to growth media for plasmid selection in \(P.\ aeruginosa\) LESB58 strains. Kanamycin (30 μg/ml) or gentamicin (15 μg/ml) was added to growth media for plasmid selection in \(E.\ coli\) DH5a. Bacterial growth was monitored by measuring optical density (OD\(_{600}\)) with a spectrophotometer (Eppendorf, Missisauga, Canada).

### Generation of bioluminescence reporter strains.

High-fidelity polymerase chain reaction (PCR) was carried out using the Thusion DNA Polymerase (Thermo Scientific) in accordance with the manufacturer’s specifications and optimized annealing temperatures. Oligomer sequences were based on the genome of \(P.\ aeruginosa\) LESB58 (GenBank: NC_002516.2) available from NCBI. For colony PCR reactions performed on LESB58, cells were boiled at 98 °C with shaking (1,000 rpm) for 10 min and pelleted by centrifugation at 14,500 rpm for 3 min. Restriction digests were performed using FastDigest restriction enzymes according to the manufacturer’s specifications (Thermo Scientific). All ligation reactions were carried out at room temperature using T4 DNA ligase (Invitrogen). DNA purifications were performed using the GeneJET PCR purification kit or the GeneJET Gel extraction kit following the manufacturer’s instructions (Thermo Scientific).

To generate recombinant strains, the coding sequences of LESB58 \(rhl\), \(lasI\) and \(pgsH\) were PCR amplified, gel purified and digested with restriction enzymes EcoRI and BamHI. PCR products were subsequently cloned into EcoRl/BamHI digested pBBR1MCS-5. LESB58 were scraped from an agar plate and resuspended in 300 mM sucrose. After washing twice, pelleted cells were resuspended in 100 μl of 300 mM sucrose and mixed with 500 ng of plasmid. Cells were transformed via electroporation (2.5 kV, 25 μF, 200 Ω). All steps were carried out at room temperature. Cells were recovered for 3 h at 37 °C in 2×YT broth with shaking that 220 rpm after electroporation.

Plasmid pUC18T-min-Tn7T-lux\(^38\) was modified by cloning the EcoRI/BamHI digested \(ntrBC\) promoter into the multiple cloning site. The derivative pUC-Tn7T-lux-\(ntrBC\) was co-electroporated with helper plasmid pTNS2\(^38\) into electrocompetent \(P.\ aeruginosa\) LESB58 strains, as described above. Positive clones, showing strong bioluminescence, were selected on LB agar plates containing gentamicin and further verified for correct chromosomal insertion via PCR of the flanking regions with transposon- and chromosome-specific primers as described previously\(^38,59\).

### Promoter induction assays in vitro.

Luminescence tagged bacteria were seeded at a density of ~1.5×10\(^6\) CFU/ml in flat-bottomed 96-well white plates (Corning) containing BM2 with or without signaling molecules. Plates were incubated at 37 °C with continuous shaking (250 rpm). OD\(_{600}\) and luminescence measurements were taken in one h increments for 20 h (Synergy H1, BioTek). Experiments were performed three times with at least three technical replicates. The ammonium concentration in the medium was measured, in parallel, using an ammonia assay kit (Sigma) on centrifuged (8000 rpm for 5 min) and filtered (0.2 μm pore size) cell supernatants, according to the manufacturer’s specifications.

### Competition assays.

Each species of bacteria was seeded at an adjusted OD\(_{600}\)=0.1 in batch cultures. Competition assays with LESB58 and USA300 LAC were grown for 24 h, with shaking (250 rpm) at 37 °C. Samples were taken for serial dilution and bacterial enumeration on selective media (mannitol salt agar (MSA) and \(Pseudomonas\) isolation agar (PIA)) prepared according to the manufacturer’s specifications) after 18–34 h incubation. Experiments were performed three times.

### Biofilm formation in vitro.

Biofilm assays were performed as previously described\(^41\), with minor modifications. Briefly, bacteria were scraped from a plate, resuspended in phosphate buffered saline (PBS) (pH=7.4, Gibco) and mixed at OD\(_{600}\)=0.1. Polymicrobial cultures were seeded into round-bottomed 96-well polypyrrole plates (Corning) and incubated at 37 °C for 24–28 h. Planktonic cells were removed and biofilms were washed prior to staining with crystal violet (0.1%) or resuspension and serial dilution for bacterial enumeration on selective media (MSA and PIA) after overnight incubation at 37 °C. Experiments were performed three times with three technical replicates in each.
**Biofilm formation on a human skin organoid model.** A human air liquid interface organoid model was modified by using Ker-CT human keratinocytes (ATCC CRL_4048) that were routinely cultured in Keratinocyte-SFM medium (Gibco) at 37 °C, 5% CO₂. Human skin-equivalent organoids were formed by seeding cells on Transwell filter inserts (0.4 μm pore size) in deep 12-well ThinCert™ plates containing Dermalife K Keratinocyte Complete Medium (Lifeline Cell Technology) prepared according to the manufacturer’s specifications. After growth to confluence the medium in the Transwells above the keratinocyte layer was removed for 2–3 days to initiate multi-structured skin formation. Prior to infection, K0 medium (Dulbecco’s Modified Eagle Medium supplemented with Ham’s F-12, hydrocortisone, isoproterenol, insulin, selenious acid, L-serine and L-carnitine; Gibco) was added to the wells.

Bacteria from overnight cultures were sub-cultured in BM2 with 0.1% CAA to mid-log phase (OD₆₀₀ = 0.4–0.6) prior to infection. Bacteria were then washed twice in PBS and resuspended to an OD₆₀₀ = 0.1 for each species. Polymicrobial cultures were then added to the apical surface of the human skin organoid model for biofilm formation. Infected skin organoids were incubated at 37 °C, 5% CO₂ for 24 h. Uninfected controls were treated with Triton X-100 (Sigma) and skins were incubated for an additional one h. Transfer inserts were removed from wells and skins were extracted for homogenization followed by serial dilution and enumeration of colony forming units (CFU/ml) on selective media (MSA and PIA) after overnight incubation at 37 °C. Supernatants were tested for lactate dehydrogenase (LDH) release due to cell lysis using an LDH assay kit as previously described. Experiments were performed three times with one or two technical replicates.

**Bacterial colonization in mice.** Animal experiments were performed in accordance with the Canadian Council on Animal Care (CCAC) guidelines and were approved by the University of British Columbia Animal Care Committee (protocol A19-0064). The study is reported in accordance with ARRIVE guidelines 2.0. Mice used in this study were inbred CD-1 mice (female, aged 5–7 weeks). No mice were excluded from analysis. All animals were purchased from Charles River Laboratories, Inc. (Wilmingtom, MA) and underwent a one-week acclimatization period in the Modified Barrier Facility at UBC. CD-1 mice weighed 25 ± 5 g at the time of experiment and were group housed in cohorts of 4–5 littermates exposed to the same bacterial strains. Otherwise, to minimise potential confounders, order of treatment and examination of mice was done randomly. Blinding was not used at any step of data collection due to isolated working conditions under COVID-19 safety protocols in the animal facility. Standard animal husbandry protocols were employed.

Bacterial colonization in vivo was assessed using a subcutaneous abscess model, as previously described. Briefly, luminescently-tagged LESB58 and non-luminescent USA300 LAC subcultures were grown to stationary phase, then washed twice with sterile PBS and resuspended at an OD₆₀₀ = 1.0. For monoculture assays, only LESB58 (50 μl) was inoculated. For co-culture assays, species were mixed and 50 μl were injected subcutaneously into the right dorsal of mice. Bacterial density of inocula were constant. Abscesses were formed for 72 h prior to measurement of visible dermonecrosis, a primary outcome. Luminescence, another primary outcome, was monitored in 24 h increments using an in vivo imaging system (IVIS; Perkin Elmer, Waltham, MA, USA). Luminescence from mice inoculated with mixed species was compared to luminescence from mice inoculated with LESB58 only. Abscesses were harvested for bacterial enumeration on selective media (MSA and PIA) following homogenization and serial dilution. Number of bacteria recovered and size of abscesses from mixed species abscesses were compared to WT. Experiments were repeated three times with two replicates in each, and a total of 36 mice were used.

**Virulence factor production assays.** Pyoverdine was assessed as previously described. Briefly, bacteria were incubated in Casamino acid medium (0.5% CAA, 0.1 mM MgSO₄, 0.4% glucose, 7 mM potassium phosphate buffer, pH = 7.0) at 37 °C (250 rpm). Turbid cultures were pelleted, and the supernatant was collected in a fresh microfuge tube. Five μl of supernatant was mixed with 995 μl 10 mM Tris–HCl (pH = 6.8). Pyoverdine was quantified based on intrinsic fluorescence at an excitation wavelength of 400 nm and emission 460 nm using a microplate reader (Synergy H1, Biotek). Pyocyanin concentrations were determined spectrophotometrically after extraction with chloroform and 0.2 M HCl as described elsewhere. Absorbance at 520 nm was read (Synergy H1, Biotek). Elastase was determined by proteolysis of Elastin-Congo red complex (Sigma) as described elsewhere. Fifty hundred μl of supernatant from cultures grown for 18–24 h at 37 °C was collected and heated for 10 min at 100 °C. The clear supernatant was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and silver-stained as described elsewhere. The protein band was excised from the gel and subjected to matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described elsewhere.

**Transcriptomic studies.** LESB58 strains were sub-cultured to an OD₆₀₀ = 0.4–0.6 and spot cultured onto BM2 glucose agar plates for 18–24 h at 37 °C. Surface colonized cells were harvested from the plate in PBS containing RNAProtect (at a 1:2 ratio) reagent (Qiagen). RNA extraction from three biological replicates was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s specifications. Deoxyribonucleic acids were removed using the TURBO DNA-free kit (Thermo Fisher). RT-qPCR was used to validate expression of selected dysregulated genes previously identified in the mutants using RNA-Seq. Reaction samples were prepared using qScript one-step SYBR green RT-qPCR Kit (QuantaBio) with 0.2 ng/μl RNA. Amplification was performed using a LightCycler 96 instrument (Roche, Indianapolis, IN). Gene expression was quantified by the ΔΔCt method with normalization to reference genes. Primers used for qRT-PCR are listed in Table S1.

**Binding site analysis.** To predict sites where NtrC or RpoN directly bind to DNA, a position weight matrix (PWM) model was generated from available ChIP-Seq or HT-SELEX data using Autoseed software and
manual refinement. Sites upstream of coding sequences in the LESB58 genome were scanned for binding sites using the Find Individual Motif Occurrences (FIMO) software that returned significant hits with P < 10−4.

Statistical analysis. Statistics were performed using GraphPad Prism 9.0 (La Jolla, CA, USA). P values were calculated using One-Way or Two-Way analysis of variance (ANOVA) with post-hoc analysis as indicated in the Figure captions. Statistical significance was established when P < 0.05.

Data availability

Datasets discussed in this manuscript are publicly accessible in the NCBI Gene Expression Omnibus (GEO) database under the accession number GSE145591. Received: 15 March 2022; Accepted: 12 May 2022 Published online: 30 May 2022

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Author contributions
M.A.A. conceived the project and performed experiments, analyzed data, presented data, and drafted the manuscript. S.M. and N.A. performed experiments and assisted with troubleshooting assays. R.E.W.H. provided materials, conceived the project, assisted with troubleshooting assays, and revised the manuscript.

Competing interests
The authors declare no competing interests.

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