De novo purine biosynthesis is required for intracellular growth of *Staphylococcus aureus* and for the hypervirulence phenotype of a purR mutant

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**ABSTRACT**

*Staphylococcus aureus* is a noted human and animal pathogen. Despite decades of research on this important bacterium, there are still many unanswered questions regarding the pathogenic mechanisms it uses to infect the mammalian host. This can be attributed to it possessing a plethora of virulence factors and complex virulence factor and metabolic regulation. PurR, the purine biosynthesis regulator, was recently also shown to regulate virulence factors in *S. aureus*, and mutations in *purR* result in derepression of fibronectin binding proteins (FnBPs) and extracellular toxins, required for a so-called hypervirulent phenotype. Here, we show that hypervirulent strains containing *purR* mutations can be attenuated with the addition of purine biosynthesis mutations, implicating the necessity for *de novo* purine biosynthesis in this phenotype and indicating that *S. aureus* in the mammalian host experiences purine limitation. Using cell culture, we show that while *purR* mutants are not altered in epithelial cell binding, compared to WT *S. aureus*, *purR* mutants have enhanced invasion of these non-professional phagocytes, consistent with the requirement of FnBPs for invasion of these cells. This correlates with *purR* mutants having both increased transcription of *fnb* genes, resulting in higher levels of surface-exposed FnBPs to promote invasion. These data provide important contributions to our understanding of how the pathogenesis of *S. aureus* is affected by sensing of purine levels during infection of the mammalian host.
INTRODUCTION

*Staphylococcus aureus* is a Gram-positive bacterium that is found as a commensal in about a third of the human population (1). However, *S. aureus* can also be pathogenic, causing a wide array of diseases, that range from mild skin and soft tissue infections, to life threatening infections such as endocarditis, pneumonia and bacteraemia (2). Data demonstrating that morbidity and mortality due to invasive *S. aureus* infection in the USA causes more deaths than HIV(3) lends further support to the burden that *S. aureus* infections place on society.

Purines are essential to life. All organisms, except for some parasitic worms, can synthesize purines de novo. In *S. aureus*, de novo purine biosynthesis is accomplished by the activity of 11 enzymes that convert phosphoribosyl pyrophosphate (PRPP) to inosine monophosphate (IMP) (Fig S1A). IMP can then be converted to ATP or GTP, by the PurA and PurB or the GuaA and GuaB proteins, respectively. Previous reports have shown that de novo purine biosynthesis is required for full virulence of *Francisella tularensis* (4), *Brucella abortus* (5), *Escherichia coli* (6) and many other pathogens. In *S. aureus* strain Newman, purA and purH mutants are attenuated in vivo (7). Furthermore, *S. aureus* mutants in guaA or guaB cannot grow in serum and fail to establish infection in a murine model (8). A purF mutant of USA300 was shown to have a modest defect in a rabbit endocarditis model, but the purF mutation did render the bacteria highly susceptible to vancomycin treatment (9).

Recently, it was demonstrated that inactivation of the transcriptional repressor of purine biosynthesis, PurR, results in a hypervirulent *S. aureus* in a mouse bacteraemia model (10, 11). In purR deficient *S. aureus*, transcription of purine biosynthesis genes and known virulence factor genes, including those encoding fibronectin binding proteins, is increased (10, 11). This purR mutant-dependent hypervirulent state was found to be mediated by aberrant upregulation of fibronectin binding proteins (FnBPs), whose expression is normally repressed by PurR. Since several known virulence factors, including exotoxins (11), are controlled by PurR, it is unclear whether FnBP expression alone is sufficient for hypervirulence of purR *S. aureus*, or whether the concurrent substantial increase in pur gene transcription is also required. Moreover, the specific events that occur in vivo that lead to increased virulence are unknown.

As FnBPs are required for the invasion of *S. aureus* into non-phagocytic cells (12–14), we sought to determine if purR mutants demonstrate increased invasion, which could in part account for their increased pathogenesis. Furthermore, we hypothesised that the increase in de novo purine biosynthesis may confer a growth advantage during intracellular replication in macrophages, allowing faster escape of purR mutant *S. aureus* from Kupffer
cells and quicker dissemination to other organs. In the present study we demonstrate purR deficient S. aureus have an increased capacity to invade epithelial cells and concurrently require de novo purine biosynthesis for intracellular replication in the absence of exogenous purines. Moreover, a systemic murine infection model mirrors these findings and demonstrates the ability to synthesize purines de novo is essential for the pathogenesis of purR deficient S. aureus, regardless of increased FnBP expression.

RESULTS

De novo purine biosynthesis is required for S. aureus replication and pathogenesis in vivo

Previously, we showed that FnBPs are essential for the hypervirulence of a purR mutant(10). However, it was not known whether FnBP expression is sufficient for this phenotype, or whether the concurrent increase in pur gene expression contributes to rapid lethality in mice. In an attempt to address this at the outset of this study, we assessed the virulence of a S. aureus USA300 purK::ΦNΔ purR mutant (15), which results in a block in the purine biosynthesis pathway (Fig S1), along with a purK::ΦNΔ purR double mutant (see below), in relation to WT and a ΔpurR mutant. To do this, we infected mice IV with each of the four strains using a well-established model of murine bacteraemia. While WT infected animals steadily lost weight over the course of the 4 days of infection, animals infected with the purR mutant required sacrifice at 24 hpi, as previously demonstrated (10) (Fig 1A), and this correlated with significant increases in bacterial burden, versus WT, in the heart and kidneys at 24 hpi (Fig 1B). In contrast, animals infected with the purK mutant did not lose weight (Fig 1A), or show outward signs of disease, even by 96 hpi, and had significantly lower bacterial burden in the heart and kidneys (Fig 1C). Most importantly, we found that including the purK::ΦNΔ mutation into the strain carrying ΔpurR converted what was a hypervirulent purR mutant strain into an attenuated strain (Fig 1ABC). In fact, not only was removal of de novo purine biosynthesis sufficient to eliminate the hypervirulence of a purR mutant, but it reduced bacterial virulence, as evidenced by weight loss and bacterial burdens, to levels lower than the WT. Altogether, these data demonstrate that de novo purine biosynthesis is required for the pathogenesis of S. aureus, as well as for the hypervirulence associated with purR inactivation during systemic disease.

Lack of de novo purine biosynthesis is without effect on serum- and FnBP-dependent hyperclumping of S. aureus
Given that the purK mutation completely abrogated the hypervirulence of the purR mutation, we next investigated whether this may be due simply to effects on growth in the absence of purines, or whether the inability to synthesize purines affected FnBP-dependent bacterial clumping in serum, which we previously correlated with hypervirulence (10). The purK mutant, irrespective of whether or not it also contained a purR mutation, demonstrated attenuated growth in tryptic soy broth (TSB) (Fig 2A). Provision of purK in trans partially restored the growth defect of the single purK mutant and we attribute this to the fact that purK is the second gene in the 11-gene operon, and the transposon insertion exerts a polar effect on downstream gene transcription. Consistent with this notion, when the double mutant was complemented with the same purK expression plasmid, we observed full restoration of growth, ostensibly because the purR mutation results in significantly increased transcription of the complete pur operon (10).

To further investigate the requirement of de novo purine biosynthesis for S. aureus growth, we analyzed growth of WT and four different purine biosynthesis gene mutants, obtained from the Nebraska transposon library (15), in a chemically defined medium lacking purines. While no differences in end point growth were evident between the WT and a purR mutant, none of the mutants that are deficient for purine biosynthesis were able to grow under identical conditions (Fig 2B). In agreement with the idea that disruption of the purine biosynthesis pathway was the only reason for significantly diminished growth, provision of IMP restored growth of each mutant to WT levels, in a dose dependent manner (Fig 2C). The only exception to this was the purA mutant, which has a defect in the biosynthetic pathway at a step after IMP (Fig S1), and therefore should not be supported by the presence of the metabolite. Overall, these findings demonstrate the importance of the pur operon for S. aureus growth. Moreover, these results demonstrate that the purK mutant, which is complemented by both purK in trans and by the metabolite IMP, serves as a useful mutant to study the role of purine biosynthesis in further detail, especially in the context of a purR mutation. As such, this mutant is used throughout the remainder of this study.

We next assessed whether the inability to synthesize purines inhibited the serum- and FnBP-dependent clumping phenotype of a purR mutant (10). As demonstrated in Fig 2D, purR S. aureus displayed the characteristic hyper-clumping phenotype when cultured in the presence of serum; this is characterized by a drop in culture OD$_{600}$ as bacteria settle to the bottom of the culture tube within minutes. Cultures of WT and the purK mutant in serum resulted in modest clumping that is characteristic of S. aureus with WT FnBP expression. In contrast, the purR purK S. aureus displayed archetypal purR-dependent hyper-clumping, despite displaying an overall reduction in growth (Fig 2D). This phenotype was strictly due to inactivation of purR, as provision of purR in trans eliminated hyper-clumping, whereas
provision of *purK* in trans did not (Fig 2D). Taken together, these data show that, in the context of a *purR* mutation, disruption of purine biosynthesis does not abrogate FnBP-dependent hyper-clumping, indicating that elevated FnBP expression due to loss of *purR* is without effect.

A *S. aureus* *purR* mutant demonstrates enhanced invasion of non-professional phagocytes

As a way to explain the hypervirulence, we hypothesized that *purR* mutants have an increased capacity to invade non-professional phagocytes, since it is well established that *S. aureus* uses FnBPs as a means to invade these cells (12–14). We have demonstrated *purR* mutants overexpress FnBPs, at least transiently, in early stages of growth(10). To define this, we performed an invasion assay using the human lung epithelial cell line A549.

Using this system, we assessed the ability of WT or *purR* deficient *S. aureus* to adhere to and invade A549 cells. Bacteria were grown to two different growth phases – OD 600 of 0.6, where FnBP expression is elevated in the *purR* mutant, compared to WT, and OD 600 of 2.0, where no transcriptional differences in fnb genes were previously reported (10). We observed no obvious trends in bacterial adhesion outside of a small decrease in the adhesive capacity of strains lacking FnBPs (Fig 3A). When bacteria were grown to OD 600 of 0.6, mutants lacking FnBPs showed no invasion, confirming that entry into epithelial cells absolutely depends on FnBP expression. At this growth phase, we observed no significant increase in the ability of the *purR* mutant to invade epithelial cell (Fig 3B). Interestingly, we also observed similar levels of invasion between the WT, and each of the *purK* and *purR* *purK* mutants, further supporting our contention that the inability to synthesize purines does not affect levels of FnBP expression and, consequently, the invasive capacity of these bacteria. In contrast, when the bacteria were grown to OD 600 of 2.0, *purR* mutants, as compared to WT, demonstrated a significantly higher capacity to invade host cells (Fig 3B, right panel). Furthermore, even though *purK* mutants invaded host cells similarly to WT, the double mutant lacking both *purR* and *purK* showed increased invasion, akin to the *purR* mutant alone. To confirm these data, we visualized infected A549 cells by fluorescence microscopy, using bacteria expressing GFP from a plasmid. We were able to determine the frequency with which each of the aforementioned strains were found inside epithelial cells (Figs 3C S2), using antibody-based staining to differentiate intracellular from extracellular bacteria. This analysis corroborated the results from bacterial counts, with WT and *purK* mutants demonstrating similar levels of invasion (Fig 3D). In contrast, bacteria lacking *purR*, irrespective of the *purK* mutation, display enhanced invasion of A549 cells. Moreover, this
analysis revealed purR mutants are more invasive at both growth phases tested (Fig 3D), likely because this technique is more sensitive than counting for CFUs. There is an apparent discordance between the growth-phase dependent transcriptional upregulation of FnBPs in purR deficient S. aureus and the growth phase during which we saw increased invasion. This could be due to the effect of purR deficiency on other proteins that may affect FnBP expression. In S. aureus FnBPs have been shown to be post-translationally targeted and removed from the bacterial cell surface by the action of secreted proteases, including aureolysin (16) and V8 (17). Interestingly, secreted proteases have decreased transcription in a purR mutant of S. aureus (10, 11), indicating differential post-translational regulation of surface-exposed FnBPs may occur in purR mutants. Altogether, these data demonstrate the hyper-invasive phenotype of a purR mutant is due to an early transcriptional increase in FnBP expression, followed by an effect on proteins that decrease the levels of FnBPs on the bacterial cell surface.

**Purine biosynthesis mutants demonstrate decreased replication in epithelial cells**

The invasive capacity of S. aureus is strictly dependent on FnBPs but the ability to replicate intracellularly is a multi-factorial process. We speculated the increased pool of purines in a purR mutant may provide an advantage in the restricted intracellular environment. Therefore, we sought to determine the ability of S. aureus to replicate in epithelial cells. Examination of bacterial burden at different times post infection demonstrated replication began by approximately 8hpi and, thus, we routinely measured intracellular replication at 10 hpi for the most reliable data (Fig S3). Replication of the purR mutant, when compared to the WT, showed similar trends as observed for invasion, with purR deficient bacteria showing increased levels of replication when the inoculum was grown to OD$_{600}$ of 2.0 (Fig 4A). To test the dependence of intracellular replication on de novo purine biosynthesis, we also examined the replication of purK and purR purK mutants. We observed a marked defect in intracellular replication for both the purK and the purR purK mutant (Fig 4A), demonstrating a strict reliance on purine biosynthesis for this intracellular replication.

We sought to examine this in further detail using fluorescence microscopy. Our lab has established a fluorescence-based proliferation assay (18), where bacteria are surface-labelled with eFluor™ 670 at the outset of infection, and lose the dye as they undergo replication. We used this system to examine intracellular replication at a single cell level, where bacteria that have proliferated will be GFP positive and eFluor™ 670 negative (Figs 4B S4). Analysis of images acquired at 10hpi demonstrated that more replication occurred in
purR infected cells, when compared to the WT (Fig 4C). Furthermore, despite the low level increase in CFUs, purK and purR purK mutants were still capable of some intracellular replication (Fig 4C). In fact, the number of cells containing replicating bacteria was not very different (Fig 4C), suggesting that replication occurs for the purK mutant bacteria, but to levels significantly lower than those seen with the WT. Overall, these data demonstrate that mutations in the pur pathway hinder, but do not prevent the ability of S. aureus to replicate intracellularly and a purR mutant shows improved intracellular replication, when compared to the WT.

The effect of a purR mutation on intracellular replication is due to increased invasion

In addition to increased invasion of purR mutants, we also saw increased intracellular replication in epithelial cells. To determine whether purR bacteria truly proliferate more efficiently within host cells or whether this is simply due to increased host cell invasion, we devised an experiment to circumvent FnBP-dependent bacterial uptake. To do this we employed COS7 fibroblast cells, stably expressing human FcγIIa receptor (COSIIA cells), which engenders these cells with the ability to phagocytose IgG bearing targets (19) (Fig 5A). As this cellular model requires opsonization (Fig 5A), the bacteria employed here carried deletions of the spa and sbi genes, to eliminate non-specific IgG binding. Furthermore, to eliminate any confounding effect of FnBP expression, the bacterial strains also carry deletions of the fnbAB genes.

At the outset, we showed that the COSIIA cells were indeed capable of phagocytosing IgG coated beds, verifying their ability to phagocytose in an IgG-dependent manner (Fig 5B). In order to opsonise bacteria with IgG, we performed a biotinylation step to coat the bacteria, and followed that with opsonization using an anti-biotin antibody. We were able to confirm biotinylation of the bacterial cell surface through fluorescent avidin staining, and IgG opsonization was confirmed via staining with a specific fluorescent secondary antibody (data not shown). Having verified opsonisation of the bacteria, we then sought to examine the ability of these strains to grow intracellularly. Examination of the replicative capacity of S. aureus in these cells demonstrated that there was no appreciable difference between the replication of WT and purR mutant cells (Fig 5C). In contrast, little to no replication was observed for the purK or purR purK double mutant, in agreement with the findings in epithelial cells (Fig 5C). Taken together, these data demonstrate that purR mutants do not grow at an accelerated rate within host cells, but rather suggest that the replicative advantage displayed by purR S. aureus within epithelia is due to their enhanced ability to invade more host cells.
De novo purine biosynthesis is required for S. aureus replication in macrophages

The above findings demonstrate a defect of pur biosynthesis mutants in their ability to replicate in non-professional phagocytes. However, it is well established that S. aureus must replicate in Kupffer cells, the resident liver macrophages, in order to establish infection in murine systemic models (20, 21). Therefore, as we have seen a growth defect of pur pathway mutants in vitro and in epithelial cells, we were interested to study their replication in macrophage cells, which are the bottleneck to systemic infection in mice. Our lab has a well-established gentamicin protection assay in RAW 264.7 macrophages (22), which we utilized to interrogate the ability of purK and purR bacteria to grow in macrophages.

At 18hpi intracellular replication of both WT and purR mutant bacteria could be seen, with no obvious differences between the two strains (Fig 6A). In contrast, purK and purR purK mutants showed no replication even at 24 hpi, a time where WT and purR S. aureus had killed the host cells and were freely replicating in the culture medium (Fig 6A). These data suggest mutants unable to synthesize purines are severely restricted within professional phagocytes.

We chose the intermediate point of 18hpi, where intracellular replication of the WT and purR mutant could be reliably detected and investigated the behaviour of the purine biosynthesis mutants in more detail. To determine whether this growth impairment is specific to purK, or if it is a general response of mutants of the pur pathway, we performed similar infections using purA and purF mutants. As demonstrated in Figure 6B, intracellular growth of purF and purA mutants was also inhibited, indicating general defects in purine biosynthesis compromise bacterial growth within macrophages. This conclusion was further supported by fluorescence imaging, which revealed that the purK and purR purK mutants failed to grow despite being phagocytosed (Figs 6C-D, S5).

Our findings indicate that the growth defect of purine biosynthesis mutants can be restored through the addition of exogenous purines (Fig 2C). As we detected no replication of pur mutants in macrophages we chose to assess if intracellular growth could also be rescued by supplying IMP exogenously. Indeed, the addition of 100μM IMP to the culture medium fully restored the growth of purK, purR purK and purF mutants, but not of the purA mutant (Fig 7A); the purA mutation affects the pathway after the IMP step (Fig S1). Analysis of fluorescence images supported these data, with replication of purK and purR purK mutants being readily detectable in RAW macrophages in the presence of IMP (Figs 7 and S6). Importantly, the addition of IMP did not compromise RAW cell viability (Fig S7) and therefore the observed replication was due to availability of purines, not sudden macrophage...
death. Indeed, exogenous IMP allowed purK and purR purK mutants to replicate to levels higher than the WT (Figs 7B and 7C). Altogether, these findings demonstrate de novo purine biosynthesis is required for replication of S. aureus in macrophages.

Discussion

The ability of pathogenic bacteria to synthesize and/or acquire nutrients is integral to their survival and capacity to cause disease. Purines are essential components of life, and the importance of these macromolecules is highlighted by the fact most free-living organisms are capable of de novo purine biosynthesis. Here, we demonstrate that, in S. aureus, de novo purine biosynthesis is essential for in vitro and in vivo virulence, including hypervirulence associated with inactivation of the purine biosynthesis repressor PurR.

We report the inability of a number of pur mutants to grow in a chemically defined medium, which can be alleviated by the addition of exogenous purines (Fig 2). This is consistent with previous reports for S. aureus (23), but we were interested to note that exogenous purines, while required for pur mutants, did not enhance the growth of WT S. aureus. Dependence on purines has also been demonstrated for Francisella tularensis (4), Brucella abortus (5) and E. coli (6), among other bacteria. S. aureus has been shown to replicate in macrophages (22, 24) and osteoclasts (25), but little is known about the nutritional requirements of S. aureus in the intracellular environment. Studies in S. aureus metabolism have identified that mutants in glycolysis (pfkA and pyk)(26) and lactate dehydrogenase (27) have reduced survival in RAW 264.7 macrophages, but no data is available on nucleotide biosynthesis. We observed decreased intracellular replication of S. aureus pur biosynthesis mutants in epithelial cells (Fig 4) and virtually no replication in macrophages (Fig 6). Interestingly, WT S. aureus has been shown to inhibit nucleotide biosynthesis of A549 cells (28), but it is presently unclear if that can influence the levels of purines in the phagosome. This is the first report demonstrating intracellular growth of S. aureus requires purine biosynthesis, although similar findings have been reported for a number of intracellular pathogens. Indeed, purL, purH and purE mutants of B. abortus have been shown to be attenuated in macrophages (5), and purD and purF mutants of the same bacterium have reported defects during replication in RAW 264.7 macrophages and HeLa cells (29). All these findings suggest the requirement for purine synthesis to grow within the intracellular environment and indicate that purines are unavailable for bacterial utilization.

Mutations in purR lead to hypervirulence through overexpression of the FnBPs. Nevertheless, these mutants also display upregulation of a number of other genes, including the purine biosynthesis operon (10, 11). In order to gain a better appreciation of S. aureus...
pathogenesis, it is critical to know whether upregulation of FnBPs alone is sufficient for this hypervirulence or whether concurrent upregulation of purine biosynthesis is also needed. We observed severe attenuation of individual purine biosynthesis mutants during murine infections, congruent with previous reports (7, 30). However, we also demonstrated similar findings for a purR purK mutant, which behaved like an attenuated pur mutant, rather than the hypervirulent purR mutant (Fig 1). This was further reflected in disease progression, where a purR purK mutant did not have improved bacterial replication later in disease.

To date, only one study has examined S. aureus with mutations in the purine biosynthetic operon and purR. The authors demonstrated purR purA and purR purH mutants were more virulent than the purA and purH single mutants, respectively (11). Nevertheless, in both cases, the purR pur mutants were still attenuated, when compared to WT bacteria, indicating an overall reduction in bacterial pathogenesis. Moreover, that study only examined the bacterial burden in the kidneys of infected mice at 20 hpi. We saw a similar trend in kidney samples at 24 hpi (Fig 1B) and 96 hpi (Fig 1C) although our data did not reach statistical significance. Therefore, our data consistently show de novo purine biosynthesis is required for the establishment and progression of S. aureus bacteraemia. Indeed, this fits well with our findings from macrophage infections, as replication in the resident liver macrophages (Kupffer cells) is required for the systemic spread of S. aureus from the liver (20, 21).

S. aureus is not alone in the requirement for de novo purine biosynthesis for full pathogenesis. There are reports of purine biosynthetic mutants of Salmonella enterica serovar Typhimurium, E. coli and Bacillus anthracis having decreased growth in human serum (31), and S. aureus purA and purB mutants have been shown to have the same defect (23). Furthermore, animal models have demonstrated purF and purA mutants of F. tularensis are also severely attenuated in mice (4), purD and purF mutants of B. abortus have decreased persistence (29) and a purF mutant of uropathogenic E. coli was attenuated in a mouse bladder colonisation model (6). Overall, this suggests purine availability is significantly limited during infection, and purine biosynthesis inhibitors could potentially be used in combination therapy with antibiotics to increase bacterial clearance. This is of particular importance in bacteria such as S. aureus, where antibiotic resistance is rampant.

The ability of S. aureus to acquire nutrients is paramount to its replication and subsequent success as a pathogen. Here, we further demonstrate the essential role that de novo purine biosynthesis plays in the pathogenesis of S. aureus. Furthermore, we de-couple the elevated expression of FnBPs and pur biosynthesis genes in a purR mutant and demonstrate their individual roles in virulence. These findings represent an important step
towards the understanding of *S. aureus* biology during infection, and the interplay between nutrient acquisition, virulence factor expression and disease severity.

**Materials and methods**

**Tissue culture**

Human lung epithelial A549 cells were purchased from the ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) at 37˚C, 5% CO_2_ and passaged twice a week. RAW 264.7 macrophages were purchased from the ATCC and maintained in Roswell Park Memorial Institute (RPMI-1640) medium with 5% (v/v) FBS at 37˚C, 5% CO_2_ and passaged every 2 days. COSIIA cells (19), stably transfected with FcγIIa receptor, were a gift from Dr. Sergio Grinstein and were maintained in DMEM with 10% (v/v) FBS and 500µg/mL G418 at 37˚C, 5% CO_2_ and passaged twice a week.

**Bacterial growth**

Bacterial strains used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) broth and *S. aureus* was grown in tryptic soy broth (TSB) at 37˚C, shaken at 200 rpm, unless otherwise stated. Where appropriate, media were supplemented with erythromycin (3 µg/mL), chloramphenicol (12 µg/mL), lincomycin (10 µg/mL), ampicillin (100 µg/mL) or tetracycline (3 µg/mL). Solid media were supplemented with 1.5% (w/v) Bacto agar. For induction of complementation plasmids, bacteria were grown in TSB to OD<sub>600</sub> of 0.3 and induced with 300ng/µL tetracycline overnight. For growth in DMEM, bacteria were grown O/N in TSB, diluted to an OD<sub>600</sub> equivalent of 0.01 and grown in 2 mL of Dulbecco’s modified Eagle’s medium (DMEM) in a 13mL snap cap tube O/N.

**Invasion of epithelial cells**

All invasion and infection experiments were performed at a multiplicity of infection of 10. For invasion, confluent A549 cells in 12 well tissue culture plates were used. Cells were maintained in DMEM + 10% (v/v) FBS until the day of infection. On the day of infection, cells were washed with PBS and maintained in serum free (SF) DMEM at least 1h prior to infection. Bacterial strains of interest were grown O/N in TSB, with appropriate antibiotics. Bacteria were then sub-cultured at OD<sub>600</sub> of 0.1 and grown in TSB, with appropriate antibiotics, to the desired density, as indicated in the text. Where necessary, bacteria were incubated with eFluor<sup>TM</sup>-670 dye (0.5µg/mL) in PBS for 5 min, followed by the addition of TSB (18). Cells were then pelleted, washed twice with PBS and re-suspended in PBS to a density of 2x10<sup>7</sup> CFU/mL. 50 µL of that suspension was added to a well of confluent A549...
cells, containing 700 µL of SF DMEM. Plates were pelleted at 1000 rpm for 1 min and incubated at 37˚C, 5% CO₂ for 15 min. Cells were then washed once with PBS and fresh SFM added for a further 15 min at 37˚C, 5% CO₂. Cells were then treated with 150 µg/mL gentamicin for 30 min at 37˚C, 5% CO₂, extensively washed to remove the gentamicin, and kept in SF DMEM for the desired duration of the infection, as indicated in the text. At specific times post infection, media was removed, and cells lysed in PBS + 0.1% (v/v) Triton-X100, scraped from the well, and plated for CFU. For fluorescence analysis, extracellular bacteria were stained with a rabbit anti-sheep IgG conjugated to TRITC (Jackson Immunoresearch) (0.75 µg/mL) for 5 min, washed with PBS and fixed with 4% (v/v) paraformaldehyde (PFA) for 20 min.

For infection of COSIIA cells, cells in 12 well tissue culture plates were maintained in DMEM+ 10% (v/v) FBS in the absence of antibiotics. On the day of the infection, cells were washed with PBS and maintained in SFM DMEM at least 1h prior to infection. *S. aureus* lacking *spa*, *sbi* and *fnbAB* were grown in TSB O/N, 500 µL were pelleted and washed 4 times with PBS, pH 8.0. Bacteria were then resuspended in PBS pH 8.0 with succinimidyl ester biotin and incubated at RT for 45 min. Cells were washed twice with PBS and incubated with mouse anti-biotin antibody (Jackson Immunoresearch) (5 µg/ml) at RT for 30 min. Cells were pelleted, washed twice with PBS and normalized to a density of 2x10⁷ CFU/mL. 50 µL of that suspension was added to a well of confluent COSIIA cells, containing 700 µL of serum free DMEM. Plates were pelleted at 1000 rpm for 1 min and incubated at 37˚C, 5% CO₂ for 30 min. Cells were then treated with 150 µg/mL gentamicin for 30 min at 37˚C, 5% CO₂, extensively washed to remove the gentamicin, and kept in SFM DMEM for the desired duration of the infection, as indicated in the text. At desired times, media was removed, cells lysed in PBS + 0.1% (v/v) Triton-X100, scraped of the well, and plated for CFU determination.

**IgG bead opsonisation**

Silica beads (3.14 µm, Bangs Laboratories) were opsonized with human IgG (0.8 mg/mL) for 1h. IgG-opsonized beads were added to individual wells containing COSIIA cells and then centrifuged at 277 x g for 1 min to synchronize binding of targets to the cells. After phagocytosis of 30 min, cells were washed vigorously to remove unbound silica beads. Beads remaining extracellular were detected by staining for 3 min with anti-human fluorophore-conjugated secondary antibodies (0.75 µg/mL) prior to fixation with 4% (v/v) PFA.

**Macrophage infections**
For infection of RAW 264.7 macrophages, the protocol as established by Flannagan et al. (22) was used. Where necessary, cells were supplemented with IMP after the removal of gentamicin. For propidium iodide (PI) stains, cells were treated with 1 μg/mL of PI in RPMI for 5 min, prior to live cell imaging (22). Where necessary, cells were incubated with eFluor™-670 dye (0.5 μg/mL) in PBS for 5 min, followed by the addition of TSB (18). For fluorescence analysis, cells were stained with a tetramethylrhodamine conjugated wheat germ agglutinin (WGA) (1 μg/mL) for 5 min, washed with PBS and fixed with 4% (v/v) PFA for 20 min.

**Fluorescence microscopy**

Widefield fluorescence and DIC microscopy was performed on a Leica DMi6000 B inverted microscope equipped with 40× (NA 1.3), 63× (NA 1.4) and 100× (NA 1.4) oil immersion PL-Apo objectives, a Leica 100 W Hg high pressure light source and the Hamamatsu Orca Flash 4.0 and Photometrics Evolve 512 Delta EM-CCD cameras. All images were analyzed and contrast enhanced using Image J (National Institutes of Health, Bethesda, MD).

**Clumping assays**

Clumping assay were performed as previously described (10). Briefly, O/N cultures grown in TSB were diluted to an OD600 equivalent of 0.01 and grown in TSB or TSB with 10% (v/v) heat inactivated horse serum for 3.5 h at 37˚C. Tubes were then allowed to sit for 5 min on the bench and optical density of the centre of the culture was measured.

**PCR and construct generation**

*S. aureus* strain USA300 LAC, cured of the 27-kb plasmid that confers antibiotic resistance, was used as the WT strain for mutant generation, unless otherwise stated. Primers used in this study are listed in Table 2. For mobilizing transposon insertion mutations into various genetic backgrounds, phage transduction was performed according to standard techniques. Phage lysate was prepared from the donor strain using phage 80α, recipient strains were infected and transductants selected using appropriate antibiotics (10). Insertions were confirmed by PCR. Markerless deletions were constructed using the pIMAY system, as previously described (32). For complementation, the full-length genes were amplified using primers, ligated into pALC2073 and transformed into *E.coli*. All plasmids were passaged through RN4220, prior to transfer to the strain of interest.

**Mouse infections**

All animal experiments were performed in compliance with guidelines set out by the Canadian Council on Animal Care. All animal protocols (protocol 2017-028) were reviewed.
and approved by the University of Western Ontario Animal Use Subcommittee, a subcommittee of the University Council on Animal Care. 6-8-week-old female BALB/c mice (Charles River laboratories) were injected via tail vein with 100 µL of bacterial culture, containing 1x10^7 CFU of bacteria, as described in the text. To prepare the bacteria, strains were grown to OD_{600} 2-2.5 in TSB, washed twice with PBS and re-suspended to the desired numbers in PBS. Infections were allowed to proceed for up to 96 h before animals were euthanized, or when they met guidelines for early euthanasia. Organs were harvested in PBS + 0.1% (v/v) Triton X-100, homogenized in a Bullet Blender Storm (Next Advance, Troy, NY), using 2 runs of 5 min at setting 10, and metal beads. Dilutions of organ homogenates were plated on TSA for CFU enumeration.

Table 1 – Bacterial strains used in this study.

| Strain               | Description                                                                 | Source            |
|----------------------|-----------------------------------------------------------------------------|-------------------|
| USA300 WT            | USA300 LAC, cured of resistance plasmids                                    | Lab stock         |
| WT pGFP              | USA300 carrying superfolder GFP in plasmid pCM29 (pGFP)                     | This study        |
| ΔpurR                | USA300 with a deletion of purR                                              | This study        |
| ΔfnbAB               | USA300 with a deletion of fnbAB                                             | (10)              |
| ΔpurR ΔfnbAB         | USA300 with deletions of purR and fnbAB                                     | This study        |
| ΔpurR pGFP           | USA300 with a deletion of purR carrying pGFP                                 | This study        |
| ΔfnbAB pGFP          | USA300 with a deletion of fnbAB carrying pGFP                               | This study        |
| ΔpurR ΔfnbAB pGFP    | USA300 with deletions of purR and fnbAB carrying pGFP                       | This study        |
| purK::ΦΝΣ            | USA300 with a transposon insertion in purK                                   | This study        |
| purK::ΦΝΣ pGFP       | USA300 with a transposon insertion in purK carrying superfolder GFP in plasmid pCM29 | This study        |
| ΔpurR purK::ΦΝΣ      | USA300 with a deletion of purR and a transposon insertion in purK            | This study        |
| ΔpurR purK::ΦΝΣ pGFP | USA300 with a deletion of purR and a transposon insertion in purK carrying pGFP | This study        |
| WT pALC              | WT carrying pALC2073                                                       | (10)              |
| ΔpurR pALC           | USA300 with a deletion of purR carrying pALC2073                            | This study        |
| purK::ΦΝΣ pALC       | USA300 with a transposon insertion in purK carrying pALC2073                 | This study        |
| ΔpurR purK::ΦΝΣ pALC | USA300 with a deletion of purR and a transposon insertion in purK carrying pALC2073 | This study        |
| ΔpurR ppurR          | USA300 with a deletion of purR carrying a copy of purR in pALC2073           | This study        |
Table 2 List of primers used in this study.

| Primer name | Function | Primer sequence |
|-------------|----------|-----------------|
| purK F      | Amplifying whole length purK for complementation | GGGGAGCTCATAAAATGGGAGACATGCAAATG |
| purK R      | Generating an upstream fragment for purR deletion | GGGGTCGACTTCTTTGATATAGGGGCGAGTT |
| purR Up F   | Generating an upstream fragment for purR deletion | CTTTCAACCCTCTATCCTA |
| purR Up R   | Generating a downstream fragment for purR deletion | TAGGATAGAAGGTTGAAAAGAAGGAGTTTAT |
| purR down F | Generating a downstream fragment for purR deletion | ATTATGA |
| purR down R | Generating a downstream fragment for purR deletion | GGGGGTACCGTATATATCTCTGTTTAT |

Figure legends

Figure 1 – *De novo* purine biosynthesis is required for *S. aureus* pathogenesis *in vivo.*

Groups of 6-8 8 week old female Balb/c mice were infected with 1x10^7 CFU *S. aureus* via tail vein injection. A – Animal weight was recorded daily and is shown as percentage of weight loss from initial weight. Data shown are mean ± SEM of 2 experiments, to a total of 14 animals. B – at 24 hpi animals were sacrificed, organs harvested and CFU per organ
determined. Data shown are mean ± SEM of 8 animals per group. The dotted line represents the limit of accurate detection. C – at 96 hpi animals were sacrificed, organs harvested and CFU per organ determined. Data shown are mean ± SEM of 2 experiments, to a total of 14 animals. The dotted line represents the limit of accurate detection. Animals infected with ΔpurR met early euthanasia criteria at 24hpi. * indicates a p value < 0.05, ** < 0.01, **** < 0.0001, based on a one-way ANOVA, with Bonferroni post-test.

Figure 2 – Lack of de novo purine biosynthesis limits growth of S. aureus, but not FnBP-dependent serum clumping of ΔpurR S. aureus. A - S. aureus strains were grown in TSB with 200 ng/mL tetracycline O/N at 37°C. End point growth was determined by measuring OD\textsubscript{600}. Data shown are mean ± SEM of 3 independent experiments, with 3 biological replicates per experiment. B – Strains were grown in TSB O/N, diluted to OD\textsubscript{600} of 0.01 and grown in DMEM for 24h. Data shown are mean ± SEM of 3 independent experiments, with 3 biological replicates per experiment. Vertical dotted line represents the limit of accurate detection. C - Strains were grown in TSB O/N, diluted to OD\textsubscript{600} of 0.01 and grown in DMEM, supplemented with various concentrations of IMP for 24h. Data shown are mean ± SEM of 3 independent experiments, with 3 biological replicates per experiment. Vertical dotted line represents the limit of accurate detection. D – Strains were grown in TSB (white bars) or TSB with 10% (v/v) horse serum (grey bars) for 3.5h at 37°C. Optical density of the centre of the tube was measured after static incubation for 5 min. Data shown are mean ± SEM of 3 independent experiments, with 3 biological samples per experiment. * indicates a p value < 0.05, ** < 0.01, **** < 0.0001, based on a one-way ANOVA, with Bonferroni post-test.

Figure 3 – A purR mutant demonstrates enhanced invasion of epithelial cells. A – Bacteria were grown to different OD, as indicated and used in gentamicin protection assays. Adhesion is shown as the total number of bacteria recovered at 30 min post infection, prior to gentamicin treatment. Data shown are mean ± SEM of 5-8 independent experiments, with 2 biological replicates per experiment. B – invasion is shown as the total number of bacteria recovered at 1h post infection, immediately after the removal of gentamicin. Data shown are mean ± SEM of 5-8 independent experiments, with 2 biological replicates per experiment. C - Coverslips of cells were infected with bacteria as in A and stained after gentamicin treatment. At onset of infection, cells were stained with eFluor\textsuperscript{TM} 670 dye and prior to fixing were incubated with a Cy3 conjugated rabbit anti sheep IgG, to detect extracellular bacteria. Representative images of WT bacteria are shown. White arrows indicate intracellular bacteria, yellow arrows indicate extracellular bacteria. Scale bar equals 20 µm. D – cells in images from C were analysed, and the number of epithelial cells containing bacteria was counted. Cells with extracellular bacteria (yellow arrows) were excluded. The percentage of
cells with intracellular bacteria per field of view was calculated. 35-40 fields of view of 3-4 independent experiments were analysed per group. Data shown are mean ± SEM. * indicates a p value < 0.05, *** < 0.001, **** < 0.0001, based on a one-way ANOVA, with Bonferroni post-test.

**Figure 4 – Purine biosynthesis mutants are defective for intracellular replication.** A549 cells were infected as in Figure 3 and the infection was allowed to proceed for 9h after gentamicin removal. A – Fold increase in bacterial numbers was calculated by dividing the number of bacteria recovered at 10h post infection by the number of bacteria recovered at 1h (post gentamicin). Data shown are mean ± SEM of 5-8 independent experiments, with 2 biological replicates per experiment. B - Coverslips of cells were infected with bacteria as in Figure 2 and were stained at 10 hpi. At onset of infection, cells were stained with eFluoTM 670 dye and prior to fixing were incubated with a TRITC conjugated rabbit anti sheep IgG, to detect extracellular bacteria. Representative images of WT bacteria are shown. White arrows indicate bacteria that have not replicated, yellow arrows indicated bacteria that have replicated intracellularly (extracellular bacteria are stain red). Scale bar equals 20µm. C – cells in images from B were analysed, and the number of epithelial cells containing bacteria was counted. Cells with eFluoTM 670 negative bacteria (white arrows), indicating intracellular replication, were also counted, and the percentage of cells with intracellular replicating bacteria per field of view was calculated. 35-40 fields of view of 3-4 independent experiments were analysed per group. Data shown are mean ± SEM. * indicates a p value < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, based on a one-way ANOVA, with Bonferroni post-test.

**Figure 5 – The intracellular replication of a purR mutant is equivalent to WT, when differences in cell invasion capacity are removed.** A – schematic representation of the experiments performed with COSIIA cells. B – 0.3µm beads were opsonised with human IgG and added to COSIIA cells for 30 min. Extracellular beads were then stained with a Cy5 conjugated anti-human IgG, cells were fixed and imaged on a widefield microscope. Representative images are shown. Scale bar equals 20µm. C – Indicated strains were treated according to the schematic shown in A, and added to confluent COSIIA cells for 30 mins, followed by treatment with gentamicin for 30 min. At 9h post gentamicin, cells were lysed, plated and CFU counted. Data shown is fold increase in CFU at 10h, compared to 1h (immediately after gentamicin removal). Data shown are mean ± SEM of 5 experiments, with 2-3 biological replicates per experiment. **** indicates a p value < 0.0001, based on a one-way ANOVA, with Bonferroni post-test.
Figure 6 – Purine biosynthesis mutants are completely attenuated in macrophages. A – RAW 264.7 macrophages were infected with the indicated strains for 30 min, treated with gentamicin for 1 h and maintained in RPMI+5% FBS. At indicated times, cells were lysed and CFU determined. Data shown are fold increase in CFU over values recovered at 1.5 h (immediately after gentamicin treatment). Data shown are mean ± SEM of 5-6 experiments, with 2-3 biological replicates per experiment. B – RAW 264.7 macrophages were infected as in A, lysed at 18 hpi and CFU determined. Data shown are fold increase in CFU at 18 h over values recovered at 1.5 h. Data shown are mean ± SEM of 4-6 experiments, with 2-3 biological replicates per experiment. C – Bacteria were labelled with eFluor™ 670 and used to infect cells, as in A. At 18 hpi the macrophage cell membrane was labelled with TMR wheat germ agglutinin (WGA) for 5 min and the cells were fixed. Coverslips were imaged on a widefield microscope. Representative images are shown. Yellow arrows indicate bacteria that have not replicated, white arrows indicated bacteria that have replicated intracellularly (extracellular bacteria stain red). Scale bar equals 20µm. D – cells in images from C were analysed, and the number of cells containing bacteria and replicating bacteria were counted. The percentage of cells containing replicating bacteria was calculated by dividing the number of cells containing replicating bacteria by the number of cells containing bacteria. At least 10 fields of view of 3 independent experiments were analysed. Data shown are mean ± SEM. * indicates a p value < 0.05, ** < 0.01, **** < 0.0001, based on a one-way ANOVA, with Bonferroni post-test.

Figure 7 – Intracellular growth defects of purine biosynthesis mutants are restored by the addition of exogenous purines. A – RAW 264.7 macrophages were infected with the indicated strains for 30 min, treated with gentamicin for 1 h and maintained in RPMI+5% FBS, with or without indicated concentrations of IMP. At 18 hpi, cells were lysed and CFU determined. Data shown are fold increase in CFU over values recovered at 1.5 h. Data shown are mean ± SEM of 4-6 experiments, with 2-3 biological replicates per experiment. B – Bacteria were labelled with eFluor™ 670 and used to infect cells, as in A. At 18 hpi the macrophage cell membrane was labelled with TMR wheat germ agglutinin (WGA) for 5 min and the cells were fixed. Coverslips were imaged on a widefield microscope. Representative images are shown. Yellow arrows indicate bacteria that have not replicated, white arrows indicated bacteria that have replicated intracellularly (extracellular bacteria stain red). Scale bar equals 20µm. C – cells in images from B were analysed, and the number of cells containing bacteria and replicating bacteria were counted. The percentage of cells containing replicating bacteria was calculated by dividing the number of cells containing replicating bacteria by the number of cells containing bacteria. At least 10 fields of view of 3 independent
experiments were analysed. Data shown are mean ± SEM. * indicates a p value < 0.05, ** < 0.01, *** < 0.001 **** < 0.0001, based on a one-way ANOVA, with Bonferroni post-test.

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Fold increase in CFU (1h vs 10h)

- 0
- 10^4
- 10^5
- 10^6
- 10^7
- 10^8
- 10^9
- 10^10

Figure a: Diagram of the process.

Figure b: DIC images of extracellular beads.

Figure c: Graph showing fold increase in CFU.
a) 

![Graph showing fold increase in CFU (1.5h vs 18h) with different IMP concentrations and bacterial strains.](image)

b) 

![Images of DIC, S. aureus, eFluor™, Extracellular S. aureus, and Merge with yellow triangles indicating specific areas.](image)

c) 

![Graph showing percent of bacteria containing cells with replicating bacteria with different IMP concentrations and bacterial strains.](image)