



Article PurN Is Involved in Antibiotic Tolerance and Virulence in Staphylococcus aureus

Qi Peng^{1,†}, Lu Guo^{1,†}, Yu Dong¹, Tingrui Bao¹, Huiyuan Wang¹, Tao Xu², Ying Zhang^{3,*} and Jian Han^{1,*}

- ¹ Department of Pathogenic Biology, School of Basic Medical Sciences, Lanzhou University, No. 199, Donggang West Rd., Lanzhou 730000, China
- ² Department of Infectious Diseases, Shanghai Key Laboratory of Infectious Diseases and Biosafety Emergency Response, National Medical Center for Infectious Diseases, Huashan Hospital, State Key Laboratory of Genetic Engineering, School of Life Science, Fudan University, Shanghai 200040, China
- ³ State Key Laboratory for the Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qingchun Rd., Hangzhou 310003, China
- * Correspondence: yzhang207@zju.edu.cn (Y.Z.); hanj@lzu.edu.cn (J.H.); Tel.: +86-571-87236450 (Y.Z.); +86-931-8915092 (J.H.)
- + These authors contributed equally to this work.

Abstract: *Staphylococcus aureus* can cause chronic infections which are closely related to persister formation. Purine metabolism is involved in *S. aureus* persister formation, and *purN*, encoding phosphoribosylglycinamide formyltransferase, is an important gene in the purine metabolism process. In this study, we generated a $\Delta purN$ mutant of the *S. aureus* Newman strain and assessed its roles in antibiotic tolerance and virulence. The $\Delta purN$ in the late exponential phase had a significant defect in persistence to antibiotics. Complementation of the $\Delta purN$ restored its tolerance to different antibiotics. PurN significantly affected virulence gene expression, hemolytic ability, and biofilm formation in *S. aureus*. Moreover, the LD₅₀ (3.28 × 10¹⁰ CFU/mL) of the $\Delta purN$ for BALB/c mice was significantly higher than that of the parental strain (2.81 × 10⁹ CFU/mL). Transcriptome analysis revealed that 58 genes that were involved in purine metabolism, alanine, aspartate, glutamate metabolism, and 2-oxocarboxylic acid metabolism, etc., were downregulated, while 24 genes involved in ABC transporter and transferase activity were upregulated in $\Delta purN$ vs. parental strain. Protein-protein interaction network showed that there was a close relationship between PurN and GltB, and SaeRS. The study demonstrated that PurN participates in the formation of the late exponential phase *S. aureus* persisters via GltB and regulates its virulence by activating the SaeRS two-component system.

Keywords: Staphylococcus aureus; purN; persister; virulence; purine metabolism

1. Introduction

Staphylococcus aureus is a common pathogen and usually resides asymptomatically on the skin and mucous membranes of humans and animals [1]. *S. aureus* can synthesize and produce various virulence factors, such as fibronectin-, fibrinogen-, and immunoglobulincell wall binding proteins and capsular polysaccharides, pore-forming toxins, enterotoxins, toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins, multiple tissue-damaging exoenzymes, etc. [2–6]. These virulence factors and the biofilm, which are established by attaching to medical implants and host tissues, are responsible for a variety of acute or chronic and relapsing suppurative infections such as impetigo, bacteremia, and endocarditis, pneumonia and empyema, osteomyelitis, infections of implanted devices, septic arthritis, etc. [7,8] and toxin-mediated diseases including scalded skin syndrome, food poisoning and toxic shock [6]. *S. aureus* has become a significant burden on the health care system and a major cause of nosocomial and community-acquired infections [8]. Due to the formation of persisters and the emerging resistance to antibiotics, the treatment of *S. aureus* infections, especially chronic and relapsing infections, has become quite challenging [9].



Citation: Peng, Q.; Guo, L.; Dong, Y.; Bao, T.; Wang, H.; Xu, T.; Zhang, Y.; Han, J. PurN Is Involved in Antibiotic Tolerance and Virulence in *Staphylococcus aureus. Antibiotics* **2022**, *11*, 1702. https://doi.org/10.3390/ antibiotics11121702

Academic Editor: Marc Maresca

Received: 17 October 2022 Accepted: 21 November 2022 Published: 25 November 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/).

colls in a genetically homogeneous

Persisters are a small subpopulation of bacterial cells in a genetically homogenous population that show tolerance to lethal doses of antibiotics without genetic mutations and present as phenotypic variants in a nongrowing dormant state [10]. Persister cells have been identified in every major pathogen [11,12], such as *Borrelia burgdorferi*, *My*-cobacterium tuberculosis, *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* typhimurium, etc. and are responsible for post-treatment relapse and can lead to chronic and recurrent infections [13–18].

Persisters are dormant cells [10,19]; however, there are similarities and differences in the mechanisms by which different bacteria form persisters. The mechanisms of persister formation and survival have been studied mainly in E. coli, and various genes and pathways have been confirmed to be involved in persister formation or survival [12]. The bestknown pathways include toxin-antitoxin modules (HipA/B) [20]; energy production (SucB, UbiF) [21]; the trans-translation mediated pathway (SsrA and SmpB) [22]; the stringent response (RelA) [23]; the phosphate and cellular metabolism PhoU-mediated pathway [24]; SOS response/DNA repair (LexA) [25], etc. However, the mechanisms of persistence in S. aureus are not well understood. Recent studies have identified several pathways involved in persister formation in *S. aureus*, such as biosynthesis of amino acids (Arg]) [26]; purine biosynthesis metabolism (PurF, PurB, and PurM) [27,28]; energy production (CtaB, SucA, SucB, SdhA, and SdhB) [29–31]; glycerol metabolism [32]; protein degradation (ClpX) [31]; and phosphate metabolism (PhoU) [33]. Numerous studies have demonstrated that persister formation in stationary phase bacteria is significantly higher than that of the bacteria in the exponential phase [10,12,34-37]. This indicates that there may be differences in the mechanisms of persister formation at different growth phases. Furthermore, multiple persistence-related genes such as argJ, lysR, phoU, and msaABCR [26,33,38,39] are involved in regulating S. aureus virulence, indicating that the persister formation mechanism is associated with virulence.

Previously, we found that purine metabolism plays a role in antibiotic tolerance and that PurB and PurM are involved in persister formation in *S. aureus* [27]. *purN*, encoding phosphoribosylglycinamide formyltransferase, is an important gene in the purine metabolism process. PurN catalyzes glycinamide ribonucleotide (GAR) to formylglycinamide ribonucleotide (fGAR), which is an important step to produce inosine monophosphate (IMP) [40]. In this study, we generated a purN mutant of the *S. aureus* Newman strain, and the effects of the *purN* deletion on bacterial growth, antibiotic tolerance, and virulence were investigated. Mutation analysis indicated that *purN* was important for persister formation and virulence in *S. aureus*. Our work provides new insights into the mechanisms of antibiotic tolerance and the factors affecting virulence in *S. aureus* and furnishes new therapeutic targets for improved treatment of *S. aureus* persistent infections.

2. Results

2.1. ΔpurN Had Significantly Decreased Antibiotic Tolerance

Based on our previous study, that PurB and PurM participated in purine metabolism and were involved in persister formation in *S. aureus* [27], we constructed a mutant strain of *purN* encoding phosphoribosylglycinamide formyltransferase in *S. aureus* Newman strain by homologous recombination to further explore the mechanisms by which purine metabolism regulates persister formation and virulence of *S. aureus* in this study.

In order to investigate the effect of the *purN* knockout on the formation of *S. aureus* persisters, antibiotic exposure tests at different culture time points were performed to determine the survival of the wild-type and $\Delta purN$. Compared to the parental strain, $\Delta purN$ showed significantly increased susceptibility to ampicillin in 5-h cultures and was completely killed after 3 days of drug exposure, while the wild-type had approximately 10^6 CFU/ mL of viable cells remaining. Even on the 10th day of ampicillin treatment, the wild-type still had 10^2 CFU/mL of bacteria remaining (Figure 1A). There were no significant differences in the survival of the wild-type and $\Delta purN$ strains upon ampicillin



exposure when the bacteria were cultured for 9 and 18 h. Approximately 10³ CFU/mL of bacteria remained after 10 days of drug exposure (Figure 1B,C).

Figure 1. Exposure assay results of *S. aureus* wild-type, $\Delta purN$ to ampicillin (10 µg/mL, (**A–C**)), and levofloxacin (20 µg/mL, (**D–F**)) in cultures at different time points. 5 h point (**A**,**D**). 9 h point (**B**,**E**). 18 h point (**C**,**F**).

Similar results were observed for levofloxacin exposure. Compared with the parental strain, $\Delta purN$ showed increased sensitivity to levofloxacin when the bacteria were cultured for 5 and 9 h (Figure 1D,E). Among them, the most significant difference was observed in the 5-h cultures. After 3 days of levofloxacin exposure, $\Delta purN$ exhibited no surviving bacteria, whereas more than 10³ CFU/mL of bacteria remained for the parental strain. The persister level of *S. aureus* wild-type with levofloxacin exposure was similar to that of $\Delta purN$ in 18-h cultures (Figure 1F).

2.2. Complementation of the purN Restored Tolerance to Various Antibiotics

To further confirm the relationship between *purN* and *S. aureus* persister formation, the pRAB11 plasmid was used to complement $\Delta purN$ and the wild-type. Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRABpurN, and Newman::pRBpurN were successfully obtained. The growth curves for these four strains indicated no differences in either the log phase or stationary phases under non-stressed conditions (Supplementary Figure S1). Compared with the Newman::pRAB11 strain, RT-qPCR confirmed that the expression levels of *purN* in the complemented $\Delta purN$::pRABpurN strain (log₂ fold change: 5.58 ± 0.16) and Newman::pRBpurN strain (log₂ fold change: 6.48 ± 0.22) induced by anhydrotetracycline (Atc) were significantly higher than that of the wild-type with pRAB11 (*p* < 0.05).

An antibiotic exposure experiment was carried out for the constructed *S. aureus* strains. Due to the pRAB11 used in the complementation study being an Atc induced plasmid, all the complemented strains were cultured in TSB medium containing Atc (100 ng/mL) which can produce certain inhibition of *S. aureus* growth. The growth rates of each *S. aureus* complemented by pRAB11 or pRABpurN significantly decreased so that the numbers of live bacteria were still less than 10^8 CFU/mL after 9 h of culture, and they were still in the exponential phase. In 5-h culture, the antibiotics (e.g., ampicillin, vancomycin, gentamicin, and levofloxacin) exposure experiment demonstrated that $\Delta purN$::pRAB11 all died after 24 h of drug treatment, while Newman::pRAB11, $\Delta purN$::pRABpurN, and

Newman::pRBpurN had more than 10^2 CFU/mL of bacteria remaining. After 48 h of antibiotic exposure, the Newman::pRAB11, $\Delta purN$::pRABpurN, and Newman::pRBpurN strains had no viable bacteria (Figure 2A,C,E,G). Similar growth curves were observed in the 9-h cultures (Figure 2B,D,F,H). The *purN* complemented strain restored tolerance to antibiotics (e.g., vancomycin, gentamicin, and levofloxacin) except for ampicillin. However, for the 18-h cultures, except for the $\Delta purN$::pRAB11, which had less than 10^3 CFU/mL of bacteria remaining after 10 days of levofloxacin exposure, the other strains showed significant tolerance to ampicillin, vancomycin, gentamicin, and levofloxacin, with many viable bacteria remaining after 10 days of antibiotic exposure (Supplementary Figure S2A–D).



Figure 2. Drug exposure results of Newman::pRAB11, Δ*purN*::pRAB11, Δ*purN*::pRABpurN, and Newman::pRBpurN to ampicillin (**A**,**B**), vancomycin (**C**,**D**), gentamicin (**E**,**F**) and levofloxacin (**G**,**H**) at different culture times. 5-h culture (**A**,**C**,**E**,**G**); 9-h culture (**B**,**D**,**F**,**H**).

2.3. Knockout of purN Affected the Expression of Virulence Factors in S. aureus

To further investigate the effect of *purN* knockout on the expression of *S. aureus* virulence factors, RT-qPCR was used to compare the gene expression levels of the major virulence factors, including *hla*, *hlgA*, *hlgB*, *hlgC*, *lukS*, *lukF*, *eta*, *sea*, and *coa*, in the *S. aureus* Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRBpurN, and Newman::pRBpurN strains. The expression levels of the major virulence genes in the $\Delta purN$::pRAB11 strain were significantly lower than those in the Newman::pRAB11 strain (*p* < 0.05). The complemented strain, $\Delta purN$::pRBpurN, exhibited restored expression levels of virulence genes. Moreover, the expression levels of *hlgC* and *coa* in $\Delta purN$::pRBpurN were significantly higher than those for Newman::pRAB11 (*p* < 0.05). In addition, the expression levels of *hla*, *lukS*, *lukF*, and *coa* in Newman::pRBpurN were significantly higher than those in Newman::pRAB11 (*p* < 0.05) (Figure 3A).

2.4. The Ability of the $\Delta purN$ to Lyse Sheep Erythrocytes Was Significantly Reduced

To investigate the effect of the *purN* mutation on the hemolysis characteristics of *S. aureus*, the Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRBpurN, and Newman::pRBpurN strains were inoculated on sheep blood TSA plates containing Atc (100 ng/mL) at 37 °C for 10, 14 (images not shown), 24 and 48 h, respectively. The β -hemolytic rings around the colony of the Newman::pRAB11 colony (Figure 3(Ba,Be)) were larger and clearer than those of $\Delta purN$::pRAB11 (Figure 3(Bb,Bf)) at 24 and 48 h. In the 24- and 48-h cultures, the hemolytic rings of $\Delta purN$::pRBpurN (Figure 3(Bc,Bg)) and Newman::pRBpurN (Figure 3(Bd,Bh)) tended to be consistent with that of Newman::pRAB11. Hemolysis assays of each *S. aureus* culture indicated that at 10 and 14 h, the hemolyzing ability of Newman::pRAB11 cultures was significantly higher than that for $\Delta purN$::pRAB11 (p < 0.01, Figure 3(Bi,Bj)). With the prolongation of culture time and accumulation of hemolytic toxins, the differences in hemolytic ability between Newman::pRAB11 and $\Delta purN$::pRAB11 disappeared at 24 h and 48 h. However, when purN was overexpressed, compared with



Newman::pRAB11 and $\Delta purN$::pRAB11, the hemolytic ability of the $\Delta purN$::pRBpurN and Newman::pRBpurN strains was enhanced (*p* < 0.05, Figure 3(Bk,Bl)).

Figure 3. Comparison of the virulence of Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRABpurN, and Newman::pRBpurN in *S. aureus*. (**A**) The virulence gene expression levels detected by RT-qPCR. (**B**) Variation of hemolysis in different strains. Hemolysis status of Newman::pRAB11 (**a**,**e**), $\Delta purN$::pRAB11 (**b**,**f**), $\Delta purN$::pRABpurN (**c**,**g**), and Newman::pRBpurN (**d**,**h**) cultured for 24 h (**a**–**d**) and 48 h (**e**–**h**) on blood TSA plates. The hemolysis assay of the four strains was measured in different time points cultures. (**i**)10 h, (**j**) 14 h, (**k**) 24 h, and (**l**) 48 h. (**C**) The biofilm formation abilities of the four *S. aureus* strains in 96-well plates. Comparison of OD₅₅₀ and biofilm images in 96-well plate of different strains. * *p* < 0.05, ** *p* < 0.01.

2.5. Knockout of purN Affected Biofilm Formation in S. aureus

The biofilm formation abilities of Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRABpurN, and Newman::pRBpurN were measured in 96-well plates. The results showed that the biofilm formation ability of Newman::pRAB11 was significantly higher than that of $\Delta purN$::pRAB11 (p < 0.01, Figure 3C). After complementation, the biofilm formation ability of $\Delta purN$::pRBpurN was restored. Furthermore, when *purN* was overexpressed, the biofilm formation ability of $\Delta purN$::pRBpurN was significantly higher than that of Newman::pRAB11 (p < 0.05, Figure 3C). In addition, there were no significant differences in biofilm formation between Newman::pRAB11 and Newman::pRBpurN.

2.6. The LD₅₀ Values of Δ purN in Mice Were Significantly Higher Than That of Wild-Type S. aureus

To further explore the effect of the purN mutation on the virulence of *S. aureus*, we determined the LD₅₀ of the *S. aureus* Newman strain and the $\Delta purN$ in BALB/c mice. Different doses of the wild-type and $\Delta purN$ bacterial suspensions were injected intraperitoneally. The LD₅₀ values for the wild-type and $\Delta purN$ in BALB/c mice were calculated according to the survival status of the mice, and the results showed that the LD₅₀ of the $\Delta purN$ mutant (3.28 × 10¹⁰ CFU/mL) was significantly higher than that of the wild-type (2.81 × 10⁹ CFU/mL).

2.7. Comparative Transcriptome Analysis of the $\Delta purN$ and the Wild-Type

To gain further insights into the molecular mechanisms by which PurN affects persister formation and virulence in *S. aureus*, the DEGs of the $\Delta purN$ mutant and the wild-type strain were profiled by RNA-seq. Compared with its parental strain, 58 genes were downregulated, and 24 genes were upregulated in the $\Delta purN$ mutant with a cutoff value of \log_2 fold change less than -2 or more than 2 (Supplementary Table S1). Thirteen DEGs were selected as target genes (e.g., saeS, saeR, ilvA, NWMN_1873, lukS, hla, hlgC, lukF, NWMN_2510, NWMN_2262, NWMN_2266, NWMN_0485, and NWMN_0845) for validation by RT-qPCR and the results confirmed the reliability of the transcriptome analysis (Supplementary Table S2). The DEGs were assigned to the following functional categories. KEGG pathway enrichment analysis suggested that these DEGs were mainly involved in purine metabolism, alanine, aspartate, and glutamate metabolism, 2-oxocarboxylic acid metabolism, histidine metabolism, biosynthesis of amino acids, ABC transporters, quorum sensing, etc. (Figure 4A). To evaluate the DEG associations, a PPI was constructed based on the STRING database, and the network showed that there were close relationships between *purN* and *gltB* and *saeR* and *saeS* (Figure 4B). Furthermore, compared with the wild-type, the transcription levels of virulence-related genes, including *lukS*, *lukF*, *hlgA*, *hlgB*, *hlgC*, and *hla*, were downregulated significantly in the $\Delta purN$ mutant (Supplementary Table S1).

To further explore the relationships between *purN* and *gltB* and *saeR* and *saeS*, RTqPCR was used to detect the *gltB*, *saeR*, and *saeS* expression in the Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRBpurN, and Newman::pRBpurN strains. Compared with Newman::pRAB11, the expression level of *gltB* in $\Delta purN$::pRAB11 was significantly lower (p < 0.05), whereas in Newman::pRBpurN, it was higher (p < 0.05), and there was no significant difference in $\Delta purN$::pRBpurN. Meanwhile, compared with Newman::pRAB11, the expression levels of *saeR* and *saeS* in $\Delta purN$::pRAB11 were significantly lower (p < 0.05), whereas in *purN* overexpressed strains, $\Delta purN$::pRBpurN and Newman::pRBpurN were significantly higher (p < 0.05) (Figure 4C). *purN* affected the expression of *gltB*, *saeR*, and *saeS* in *S. aureus* and was consistent with the PPI network (Figure 4B).



Figure 4. Comparative analyses of the transcriptomics of $\Delta purN$ and wild-type, and the *gltB, saeR*, and *saeS* expression in Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRBpurN, and Newman::pRBpurN strains. (**A**) DEGs and pathways involved in the comparison of $\Delta purN$ and wild-type. The genes in the green box and red box are downregulated and upregulated genes, respectively. (**B**) Protein-protein interaction network of DEGs between $\Delta purN$ and parental strain by STRING database. The line thickness of the network indicates the strength of association/binding. (**C**) Comparison of expression levels of the *gltB, saeR*, and *saeS* in the four *S. aureus* strains (* *p* < 0.05).

2.8. purN Affects the Persister Formation in S. aureus via gltB

To verify the PPI network based on the transcriptome analysis of the $\Delta purN$ mutant and the wild-type, $\Delta gltB$::pRAB11 and $\Delta gltB$::pRBpurN were constructed. Further experiments showed that $\Delta gltB$::pRAB11, $\Delta gltB$::pRBpurN, Newman::pRBpurN, and $\Delta purN$::pRABpurN had similar growth curves (Supplementary Figure S1). RT-qPCR confirmed that the expression level of *purN* in the $\Delta gltB$::pRABpurN strain (log₂fold change: 4.99 ± 0.016) was significantly higher than that in $\Delta gltB$::pRAB11 (*p* < 0.05). To further explore the association between *purN* and *gltB* in the formation of *S. aureus* persisters, four strains, Newman::pRAB11, $\Delta gltB$::pRAB11, $\Delta gltB$::pRBpurN, and Newman::pRBpurN, were incubated for 5, 9, and 18 h, respectively. Each strain was exposed to lethal concentrations of antibiotics, including ampicillin (10 µg/mL), levofloxacin (20 µg/mL), vancomycin (40 µg/mL), and gentamicin (100 µg/mL), to observe the differences in persister formation ability. The results showed that the four strains in 5-h cultures were completely killed after 1–2 days of drug exposure (Figure 5A,C,E,G). However, after 9 h of incubation, the changing

characteristics of the viable in $\Delta gltB$::pRBpurN and $\Delta gltB$::pRAB11 strains were similar and were completely killed after 2 days of antibiotic exposure, while the Newman::pRAB11 and Newman::pRBpurN strains retained more than 10² CFU/mL of viable bacteria. The Newman::pRAB11 was killed after 3 days of drug exposure, while Newman::pRBpurN was completely killed after approximately 4–5 days of antibiotic exposure (Figure 5B,D,F,H). In the 18-h cultures, the numbers of viable bacteria in $\Delta gltB$::pRAB11 and $\Delta gltB$::pRBpurN were less than those of Newman::pRAB11 and Newman::pRBpurN after 10 days of drug exposure (Supplementary Figure S3A–D). The results showed that when *gltB* was knocked out, overexpression of *purN* did not increase persister formation, indicating that *purN* affects persister formation in *S. aureus* via *gltB*.



Figure 5. Antibiotics exposure results of Newman::pRAB11, $\Delta gltB$::pRAB11, $\Delta gltB$::pRBpurN and Newman::pRBpurN to ampicillin (**A**,**B**), levofloxacin (**C**,**D**), gentamicin (**E**,**F**) and vancomycin (**G**,**H**) at different culture times. 5-h culture (**A**,**C**,**E**,**G**); 9-h culture (**B**,**D**,**F**,**H**).

3. Discussion

Persister formation in *S. aureus* is closely related to the growth phase of culture [10,12,35,36]. Previous studies have shown that purine biosynthesis plays an important role in persister formation in *S. aureus* [27]. *purN* is a crucial gene in the third step of purine biosynthesis. We analyzed the effect of the *purN* mutant of *S. aureus* and found that its mutation resulted in persister reduction in late exponential phase cultures, indicating PurN is important for persister formation.

purN participates in several important pathways, including purine metabolism, one carbon pool by folate, metabolic pathways, and biosynthesis of secondary metabolites. A large number of studies have confirmed that ATP production [16,41], alarmone ppGpp [10], amino acid synthesis, and metabolism in bacterial cells play important roles in the formation and regulation of persisters in bacteria [26,42,43].

It is well known that purine metabolism is crucial for ATP energy supply. PurN catalyzes GAR to fGAR, which is an important step in the purine metabolism process to produce IMP. IMP is converted to guanosine 5'-monophosphate (GMP) or adenosine 5'-monophosphate (AMP) by subsequent enzymes. In this process, both ribosylamine-5P produced by phosphoribosyl pyrophosphate (PRPP) and formylglycinamidine ribonucleotide (fGAM) produced by fGAR require glutamine to provide amido, and glutamate is also produced. At the same time, glycine is required to participate in the process of 5-amino-4-carboxyaminoimidazole ribonucleotide (CAIR) to generate N-succinylo-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR) [40] (Figure 6). The PPI network established by our data indicated that *purN* affected the persister formation in *S. aureus* via

gltB (Figure 4B). *gltB*, encoding the large subunit of glutamate synthase, is the key gene in glutamine and glutamate metabolism, which catalyzes L-glutamine and 2-oxoglutarate into two molecules of L-glutamate [44]. Transcriptome analysis found that *gltB* expression decreased in the $\Delta purN$ mutant (Figure 4A). Thus, glutamine and glutamate synthesis were reduced. The decrease of *gltB* resulted in an increase of 2-oxoglutarate, which has been shown to promote the TCA cycle and cause increased ATP production [45,46], which in turn would inhibit persister formation of the $\Delta purN$ mutant (Figure 6).



Figure 6. Pathways that indicate how *purN* is involved in persister formation and virulence in *S. aureus*.

Biofilm formation, a major virulence factor in *S. aureus* infections, accelerates bacterial colonization in host tissues and promotes persister formation and antimicrobial agents. Our data revealed that the *purN* mutant significantly decreased biofilm formation. In other previous studies, purine biosynthesis was shown to affect biofilm formation through the secondary messenger, cyclic di-AMP (c-di-AMP) [28,47]. PurN is involved in the third step of purine biosynthesis, which affects ATP production. c-di-AMP is synthesized by di-adenylate cyclase via the condensation of two ATPs, one of the final products of purine biosynthesis [48]. The $\Delta purN$ mutant may inhibit c-di-AMP synthesis from preventing bacterial biofilm formation in *S. aureus*. However, the underlying mechanisms deserve future detailed studies.

S. aureus has a complex regulatory network to control its virulence [49]. The regulatory systems include the accessory gene regulator (*agr*) quorum-sensing system [50], SarA protein family regulators [51], two-component system (TCS) of the SaeRS [52], SrrAB [53], ArlRS [54], and the alternative sigma factors (SigB and SigH) [51]. Transcriptome analyses of $\Delta purN$ and wild-type strains indicate that the expression levels of *saeR* and *saeS* encoding the SaeRS TCS were significantly decreased in the $\Delta purN$, and due to this, the expression levels of multiple virulence factors, including α -hemolysin, γ -hemolysin, PVL, and coagulase, were also significantly reduced. This is consistent with our mouse study, in which we found that the virulence of $\Delta purN$ was significantly reduced, as well as the results of the hemolysis assay (Figure 3B). The SaeRS TCS is an important regulatory system for the virulence of *S. aureus* [52]. SaeS is the sensor histidine kinase, which can sense signals in the environment and autophosphorylate at the His131 residue and then the phosphoryl group is transferred to Asp51 of SaeR, and the phosphorylated SaeR (SaeR-P) binds to the SaeR binding sequence (SBS) to activate the transcription of the target genes [52,55,56]. Several Sae target genes have been discovered, most of which are related to the virulence of S. aureus, including coa, fnbA, eap, sbi, efb, fib, saeP, hla, hlb, and hlgC [57,58]. The currently

reported signals of SaeRS TCS activation mainly include human neutrophil peptide 1, 2, and 3 (HNP1–3), calprotectin, hydrogen peroxide, etc. [59,60]. Our data showed that the expression levels of *saeR* and *saeS* were higher in the *purN* overexpressed strains (Figure 4C). The results confirmed the PPI networks (Figure 4B), which PurN may affect virulence through the SaeRS in *S. aureus* (Figure 6).

Our findings further suggest that there is a close relationship between persister formation and bacterial virulence. In addition to the reported multiple persistence-related genes, such as *argJ*, *lysR*, *phoU*, and *msaABCR*, which are involved in bacterial virulence [26,33,38,39], the PurN of *S. aureus* is another multifunctional factor that not only participates in persister formation but also participates in virulence regulation.

In summary, this study has demonstrated that PurN participates in the formation of the late exponential phase *S. aureus* persister formation via the key gene, *gltB*, in glutamate synthesis and regulates bacterial virulence by activating the SaeRS two-component system. Therefore, PurN can potentially serve as a novel therapeutic target to develop more effective treatments to control persistent *S. aureus* infections in the future.

4. Materials and Methods

4.1. Culture Media, Antibiotics, and Animals

Tryptic soy broth (TSB) and tryptic soy agar (TSA) were obtained from Becton Dickinson (BD). Luria-Bertani (LB) medium and anhydrotetracycline (Atc) were obtained from Solarbio (Beijing, China). The rationale for selecting the antibiotics used in antibiotics exposure experiments is based on clinically used antibiotics in treating *S. aureus* infections and three classes of bactericidal antibiotics commonly used for persister assays, i.e., cell wall inhibitors, aminoglycosides, and fluoroquinolones. Ampicillin, levofloxacin, rifampin, chloramphenicol, vancomycin, and gentamicin were obtained from Sangon Biotech (Shanghai, China), and their stock solutions were freshly prepared, filter-sterilized, and used at appropriate concentrations as indicated. BALB/c mice were purchased from Lanzhou University (China). The study was approved by the Ethics Committee of Lanzhou University.

4.2. Bacterial Strains and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All the *S. aureus* strains were cultivated in TSA and TSB. The *E. coli* DC10B strain was cultivated in LB. The shuttle vector, pRAB11, harbors a *tet* operator that is induced by Atc. In the process of inducing high expression of *purN*, *S. aureus* $\Delta purN$::pRBpurN, Newman::pRABpurN, and $\Delta gltB$::pRBpurN mutants, and the control strains, *S. aureus* $\Delta purN$::pRB11, Newman::pRAB11, and $\Delta gltB$::pRAB11 were all inoculated in TSB medium containing Atc (100 ng/mL). For the persister assays, antibiotics were used at the following concentrations: ampicillin, 10 µg/mL; levofloxacin, 20 µg/mL; vancomycin, 40 µg/mL; and gentamicin, 100 µg/mL.

4.3. Susceptibility of Mutants to Antibiotics

In order to assess the effects of *purN* knockout on persister formation, overnight cultures of the relevant *S. aureus* were diluted 1:1000 with TSB in bacterial culture tubes and cultured at 37 °C with shaking (180 rpm). At 5, 9, and 18 h of incubation, cultures were collected, and ampicillin (10 μ g/mL), levofloxacin (20 μ g/mL), vancomycin (40 μ g/mL), and gentamicin (100 μ g/mL) were added to assess persister survival. The cultures exposed to drugs were incubated without shaking at 37 °C for up to ten days. Aliquots of cultures exposed to antibiotics were taken at different time points and washed in TSB, and the number of viable cells was counted after serial dilutions.

Strains or Plasmid	Relevant Genotype and Property	Source or Reference
S. aureus Strains		
Newman	Clinical isolate, ATCC 25904	ATCC
$\Delta purN$	Newman with a deletion of <i>purN</i>	This study
Newman::pRAB11	Newman with pRAB11	This study
$\Delta purN::pRAB11$	$\Delta purN$ with pRAB11	This study
Δ <i>purN</i> ::pRBpurN	Δ <i>purN</i> with pRAB11-purN	This study
AgltB::pRAB11	$\Delta g l t B$ with pRAB11	This study
$\Delta gltB::pRBpurN$	$\Delta glt B$ with pRAB11-purN	This study
Escherichia coli strains		-
DC10B	Δdcm in the DH10B background; Dam methylation only	[33]
plasmids		
pMX10	A pKOR1 derivate for gene knockout, Cm ^R , Amp ^R	[29]
pRAB11	Atc inducible shuttle plasmid, Cm ^R , Amp ^R	[15]
pRAB11-purN	Overexpression plasmid for <i>purN</i>	This study

Table 1. Bacteria and plasmids used in this study.

 Cm^R : Chloramphenicol resistance; Amp^R: Ampicillin resistance. The antibiotics were used at the following concentrations: ampicillin at 100 µg/mL and chloramphenicol at 10 µg/mL to maintain the plasmids resistance.

4.4. Construction of Gene Knockout and Overexpression Strains

To construct *purN* knockout mutants of *S. aureus*, we followed the method described previously [15]. The plasmid, pMX10, was used for gene knockout in *S. aureus*. Q5 Master Mix PCR (New England BioLabs) was used for all PCR experiments, and restriction enzymes and T4 DNA Ligase (Thermo Fisher Scientific, Waltham, MA, USA) were used to construct the recombinant plasmids used in this study. The Primers used for *purN* of *S. aureus* gene knockout included purN-uf, purN-ur, purN-df, and purN-dr, and the primer sequences are listed in Supplementary Table S3.

To construct knockout mutants, upstream and downstream fragments of each gene were amplified with the corresponding primers using the genomic DNA of the *S. aureus* wild-type strain Newman as a template. Two fragments of each gene were then used as templates to amplify a fusion fragment with appropriate primers. The fusion fragment and pMX10 plasmid were digested with *Kpn* I and *Mlu* I, respectively, and ligated with T4 DNA ligase, and the recombinant plasmids were transformed into *E. coli* DC10B competent cells. The transformed DC10B was screened on LB agar plates containing ampicillin (100 μ g/mL), and the positive clones were verified by restriction digestion and DNA sequencing. The recombinant plasmid was electrotransformed into the *S. aureus* Newman strain, as we described previously [32]. Mutants selection was carried out following the previously published protocol [61]. Using the same method, we also obtained *gltB* knockout mutants of *S. aureus*.

The pRAB11 plasmid was used for inducible overexpression of *purN* in *S. aureus*. The full sequence of *purN* of wild-type *S. aureus* Newman was amplified with the primers OEpurN-f and OEpurN-r (Supplementary Table S3). After digestion with *Kpn*I and *EcoR*I, the fragment was inserted into pRAB11. The recombinant plasmid pRAB11-purN was transformed into *E. coli* DC10B competent cells. The recombinant plasmid pRAB11-purN, was verified by DNA sequencing and then electrotransformed into $\Delta purN$ and $\Delta gltB$ mutants and Newman wild-type to obtain $\Delta purN$::pRABpurN, $\Delta gltB$::pRBpurN and Newman::pRBpurN, while the empty pRAB11 was transformed into $\Delta purN$, $\Delta gltB$ mutants and Newman wild-type and $\Delta purN$::pRAB11, $\Delta gltB$::pRAB11 and Newman::pRAB11 were obtained.

4.5. RT-qPCR Detected Genes Expression

After the cultures of *S. aureus* were treated with lysostaphin (Shanghai Hi-tech Bioengineering Co., Ltd., Shanghai, China), total RNA was extracted using the Sangon RNeasy kit (Sangon Biotech, China), and the quality and concentration of the extracted RNA were analyzed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription was performed with SuperScript III First-Strand synthesis (Takara Bio, Japanese) using 1 μ g of total RNA that was isolated according to the manufacturer's instructions. RT-qPCR was performed using SYBR Green Supermix (Yeasen Biotech, Shanghai, China), and the relative fold changes in gene expression were calculated using 16S rRNA as an endogenous control gene [62]. The data represent the results from three independent experiments. The primers for each gene were designed using Primer Premier 5.0 software (PREMIER Biosoft International, San Francisco, USA), and the primer sequences are listed in Supplementary Table S3. All data were analyzed with GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) and compared using the independent-samples *t*-test. Differences with *p*-value < 0.05 were considered statistically significant.

4.6. Hemolysis Assay

S. aureus Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRABpurN, and Newman::pRBpurN were inoculated on TSA plates containing 10% sheep blood and Atc (100 ng/mL), incubated at 37 °C for 10, 14, 24 and 48 h, and the hemolysis that formed around the colonies were observed. The hemolysis analysis was conducted as described previously [63] with some modifications. Briefly, Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRABpurN, and Newman::pRBpurN were cultured in TSB medium with chloramphenicol (10 µg/mL) for 18 h at 180 rpm, diluted 1:1000 and cultured in 5 mL of TSB containing Atc (100 ng/mL) for 10, 14, 24 and 48 h. Each culture was centrifuged at 9000× *g* for 3 min. Then, 200 µL of supernatant was mixed with an equal volume of 4% (v/v) sheep red blood cells suspended in PBS buffer and incubated at 37 °C for 1.5 h with shaking at 180 rpm. Supernatants were collected after centrifugation (12,000× *g* for 1 min), and the optical density at 540 nm was measured with a spectrophotometer. All experiments were performed in triplicate.

4.7. Establishment of an In Vitro S. aureus Biofilm Model

The ability of the *S. aureus* strains to form biofilm was tested in a 96-well plate according to a previously published method [64]. Two hundred microliters of TSB with 0.25% glucose and Atc (100 ng/mL) were transferred to each of the wells on the microtiter plate. Two microliters of each overnight culture of *S. aureus* were transferred to the wells, except for the blank control. Each *S. aureus* strain was tested in three parallel wells. The 96-well plate was incubated at 37 °C for 24 h. The wells were then washed three times with 200 μ L of PBS and left to dry at 60 °C for 60 min. Then, 200 μ L of crystal violet (0.5% solution, Sigma Aldrich, St. Louis, MO, USA) was added and incubated at room temperature for 30 min. The wells were washed five times with 200 μ L of tap water. In order to extract the crystal violet from the biofilm, 200 μ L of 33% glacial acetic acid was added. The optical density of the solutions at 550 nm was measured.

4.8. Median Lethal Dose Determination

Seventy-five female BALB/c mice weighing approximately 18–22 g were randomly divided into 15 groups to measure the median lethal dose (LD₅₀) of the *S. aureus* Newman wild-type strain and the $\Delta purN$ mutant. The overnight *S. aureus* Newman wild-type and the $\Delta purN$ were diluted 1:100 in 100 mL TSB and shaken overnight at 37 °C. The cultures were centrifuged at 12,000 rpm for 3 min, and the pellets were washed twice with sterile PBS. After the removal of the supernatant, the pellets were resuspended in 10 mL PBS, and the viable bacteria in the suspension were counted by serial dilution. Then, the suspensions were diluted to form 7 concentration gradients using a double dilution method. Each mouse in each group was injected intraperitoneally with 0.6 mL of a bacterial suspension at doses ranging from 10^8-10^{10} CFU/mL. After 5 days of observation, the LD₅₀ value of each strain was calculated by the Reed-Muench method [65].

4.9. Transcriptome Analysis

To identify the key genes regulating the differential responses between the parental Newman strain and $\Delta purN$ mutant, triplicate samples cultured for 5 h in TSB after di-

lution of 1:1000 were collected and subjected to high-throughput mRNA transcriptome sequencing. Total RNA was extracted as mentioned above. Sequencing libraries were generated according to the manufacturer's protocol (NEBNext[®]UltraTM RNA Library Prep Kit for Illumina[®]) [66]. Cleaved RNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers. The cDNA library preferentially selected fragments of 200–250 bp in length, which were prepared by the AMPure XP system. Then, the fragment products were amplified by Illumina cBot and sequenced on an Illumina HiSeq 2500 system (Illumina, San Diego, CA USA). Library construction and sequencing were performed at the Shanghai Applied Protein Technology Co., Ltd. By using Hisat2 (v2.0.5) (https://daehwankimlab.github.io/hisat2/manual/ (accessed on 19 August 2020)), paired-end clean reads were aligned to the reference genome of *S. aureus* Newman on the NCBI website. The number of reads corresponding to each gene was calculated using Feature Counts v1.5.0-p3. Then, each gene fragment per kilobase million (FPKM) was calculated based on the gene lengths and read counts mapped to this gene. In order to control the false discovery rate, Benjamini and Hochberg's approach was used to adjust the *p*-values to compare FPKM values between the mutant and wild-type groups. Genes with $P_{adi} < 0.05$ and \log_2 fold change >2 or <-2 were defined as differentially expressed genes (DEGs). RT-qPCR, which was performed in triplicate, was used to confirm the RNA expression levels, and the primer sequences are listed in Supplementary Table S2.

4.10. Protein-Protein Interaction Network

In order to explore the interactive relationships among DEGs, the web portal for the STRING database (http://www.string-db.org/ (accessed on 1 April 2021)) was used for protein–protein interaction (PPI) network analysis. The following two criteria were applied to detect the important nodes: (1) medium confidence equal to 0.4 and (2) network clustering by K-means clustering.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/antibiotics11121702/s1, Table S1: DEGs between $\Delta purN$ and its parental strain (log₂ fold change greater than 2 or less than -2); Table S2: Oligonucleotide sequences of RT-qPCR primers used in this study. RT-qPCR verified DEGs between $\Delta purN$ and its parental strain from transcriptome analysis. Results were normalized using 16S rRNA and expressed as fold change (mean \pm SD, p < 0.05); Table S3: Primers and oligonucleotides used in this study; Figure S1: The growth curves for *S. aureus* Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRABpurN, $\Delta gltB$::pRAB11, $\Delta gltB$::pRABpurN and Newman::pRAB11, $\Delta purN$::pRAB11, $\Delta purN$::pRBpurN to ampicillin (A), vancomycin (B), gentamicin (C) and levofloxacin (D); Figure S3: Drug exposure results of 18-h culture of Newman::pRAB11, $\Delta gltB$::pRAB11, $\Delta gltB$::pRAB11,

Author Contributions: Conceptualization, J.H. and Y.Z.; methodology, Q.P., L.G., Y.D., T.B., H.W. and T.X.; formal analysis, Q.P., L.G. and Y.D.; writing—original draft preparation, L.G.; writing—review & editing, J.H. and Y.Z.; supervision, J.H. and Y.Z.; funding Acquisition, L.G., J.H., and Y.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Natural Science Foundation of China (Grant Numbers: 81902098, 81571952, 81772231).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Lanzhou University, China (protocol code jcyxy20190209b and 25 February 2019 approval).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Dayan, G.H.; Mohamed, N.; Scully, I.L.; Cooper, D.; Begier, E.; Eiden, J.; Jansen, K.U.; Gurtman, A.; Anderson, A.S. *Staphylococcus aureus*: The current state of disease, pathophysiology and strategies for prevention. *Expert. Rev. Vaccines* 2016, *15*, 1373–1392.
 [CrossRef] [PubMed]
- 2. Vandenesch, F.; Lina, G.; Henry, T. *Staphylococcus aureus* hemolysins, bi-component leukocidins, and cytolytic peptides: A redundant arsenal of membrane-damaging virulence factors? *Front. Cell. Infect. Microbiol.* **2012**, *2*, 12. [CrossRef] [PubMed]
- Gordon, C.P.; Williams, P.; Chan, W.C. Attenuating *Staphylococcus aureus* virulence gene regulation: A medicinal chemistry perspective. *J. Med. Chem.* 2013, 56, 1389–1404. [CrossRef] [PubMed]
- 4. Bronner, S.; Monteil, H.; Prevost, G. Regulation of virulence determinants in *Staphylococcus aureus*: Complexity and applications. *FEMS Microbiol. Rev.* **2004**, *28*, 183–200. [CrossRef] [PubMed]
- 5. Cheung, A.L.; Bayer, A.S.; Zhang, G.; Gresham, H.; Xiong, Y.Q. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunol. Med. Microbiol.* **2004**, *40*, 1–9. [CrossRef] [PubMed]
- 6. Murray, P.R.; Rosenthal, K.S.; Pfaller, M.A. Medical Microbiology, 8th ed.; ELSEVIER Inc.: Philadelphia, PA, USA, 2016; pp. 173–175.
- Conlon, B.P. *Staphylococcus aureus* chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in *S. aureus* disease. *Bioessays* 2014, 36, 991–996. [CrossRef]
- Lister, J.L.; Horswill, A.R. Staphylococcus aureus biofilms: Recent developments in biofilm dispersal. Front. Cell. Infect. Microbiol. 2014, 4, 178. [CrossRef]
- 9. Fisher, R.A.; Gollan, B.; Helaine, S. Persistent bacterial infections and persister cells. *Nat. Rev. Microbiol.* 2017, 15, 453–464. [CrossRef]
- 10. Harms, A.; Maisonneuve, E.; Gerdes, K. Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science* **2016**, 354, aaf4268. [CrossRef]
- 11. Defraine, V.; Fauvart, M.; Michiels, J. Fighting bacterial persistence: Current and emerging anti-persister strategies and therapeutics. *Drug Resist. Updat.* **2018**, *38*, 12–26. [CrossRef]
- 12. Zhang, Y. Persisters, persistent infections and the Yin-Yang model. Emerg. Microbes. Infect. 2014, 3, e3. [CrossRef] [PubMed]
- 13. Feng, J.; Li, T.; Yee, R.; Yuan, Y.; Bai, C.; Cai, M.; Shi, W.; Embers, M.; Brayton, C.; Saeki, H.; et al. Stationary phase persister/biofilm microcolony of *Borrelia burgdorferi* causes more severe disease in a mouse model of Lyme arthritis: Implications for understanding persistence, Post-treatment Lyme Disease Syndrome (PTLDS), and treatment failure. *Discov. Med.* **2019**, *27*, 125–138. [PubMed]
- 14. Shi, W.; Zhang, X.; Jiang, X.; Yuan, H.; Lee, J.S.; Barry, C.E., 3rd; Wang, H.; Zhang, W.; Zhang, Y. Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis. Science.* **2011**, 333, 1630–1632. [CrossRef] [PubMed]
- 15. Xu, T.; Wang, X.Y.; Cui, P.; Zhang, Y.M.; Zhang, W.H.; Zhang, Y. The Agr Quorum Sensing System Represses Persister Formation through Regulation of Phenol Soluble Modulins in *Staphylococcus aureus*. *Front. Microbiol.* **2017**, *8*, 2189. [CrossRef]
- 16. Shan, Y.; Brown Gandt, A.; Rowe, S.E.; Deisinger, J.P.; Conlon, B.P.; Lewis, K. ATP-Dependent Persister Formation in *Escherichia coli. mBio* **2017**, *8*, e02267-16. [CrossRef]
- Hazan, R.; Maura, D.; Que, Y.A.; Rahme, L.G. Assessing *Pseudomonas aeruginosa* Persister/antibiotic tolerant cells. *Methods Mol. Biol.* 2014, 1149, 699–707. [CrossRef]
- 18. Stapels, D.A.C.; Hill, P.W.S.; Westermann, A.J.; Fisher, R.A.; Thurston, T.L.; Saliba, A.E.; Blommestein, I.; Vogel, J.; Helaine, S. Salmonella persisters undermine host immune defenses during antibiotic treatment. *Science* **2018**, *362*, 1156–1160. [CrossRef]
- 19. Lewis, K. Persister cells. Annu. Rev. Microbiol. 2010, 64, 357–372. [CrossRef]
- 20. Moyed, H.S.; Bertrand, K.P. hipA, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J. Bacteriol.* **1983**, 155, 768–775. [CrossRef]
- 21. Ma, C.; Sim, S.; Shi, W.; Du, L.; Xing, D.; Zhang, Y. Energy production genes *sucB* and *ubiF* are involved in persister survival and tolerance to multiple antibiotics and stresses in *Escherichia coli*. *FEMS Microbiol*. *Lett.* **2010**, *303*, 33–40. [CrossRef]
- Li, J.; Ji, L.; Shi, W.; Xie, J.; Zhang, Y. Trans-translation mediates tolerance to multiple antibiotics and stresses in *Escherichia coli*. J. *Antimicrob. Chemother.* 2013, 68, 2477–2481. [CrossRef] [PubMed]
- Korch, S.B.; Henderson, T.A.; Hill, T.M. Characterization of the *hipA7* allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. *Mol. Microbiol.* 2003, *50*, 1199–1213. [CrossRef] [PubMed]
- 24. Li, Y.; Zhang, Y. PhoU is a persistence switch involved in persister formation and tolerance to multiple antibiotics and stresses in *Escherichia coli. Antimicrob. Agents Chemother.* **2007**, *51*, 2092–2099. [CrossRef] [PubMed]
- Debbia, E.A.; Roveta, S.; Schito, A.M.; Gualco, L.; Marchese, A. Antibiotic persistence: The role of spontaneous DNA repair response. *Microb. Drug Resist.* 2001, 7, 335–342. [CrossRef]
- Yee, R.; Cui, P.; Shi, W.; Feng, J.; Wang, J.; Zhang, Y. Identification of a novel gene argJ involved in arginine biosynthesis critical for persister formation in *Staphylococcus aureus*. *Discov. Med.* 2020, 29, 65–77.
- 27. Yee, R.; Cui, P.; Shi, W.; Feng, J.; Zhang, Y. Genetic Screen Reveals the Role of Purine Metabolism in *Staphylococcus aureus* Persistence to Rifampicin. *Antibiotics* **2015**, *4*, 627–642. [CrossRef]
- Li, L.; Li, Y.; Zhu, F.; Cheung, A.L.; Wang, G.; Bai, G.; Proctor, R.A.; Yeaman, M.R.; Bayer, A.S.; Xiong, Y.Q. New Mechanistic Insights into Purine Biosynthesis with Second Messenger c-di-AMP in Relation to Biofilm-Related Persistent Methicillin-Resistant *Staphylococcus aureus* Infections. *mBio* 2021, 12, e0208121. [CrossRef]

- Xu, T.; Han, J.; Zhang, J.; Chen, J.; Wu, N.; Zhang, W.; Zhang, Y. Absence of Protoheme IX Farnesyltransferase CtaB Causes Virulence Attenuation but Enhances Pigment Production and Persister Survival in MRSA. *Front. Microbiol.* 2016, 7, 1625. [CrossRef]
- 30. Wang, Y.; Bojer, M.S.; George, S.E.; Wang, Z.; Jensen, P.R.; Wolz, C.; Ingmer, H. Inactivation of TCA cycle enhances *Staphylococcus aureus* persister cell formation in stationary phase. *Sci. Rep.* **2018**, *8*, 10849. [CrossRef]
- Wang, W.; Chen, J.; Chen, G.; Du, X.; Cui, P.; Wu, J.; Zhao, J.; Wu, N.; Zhang, W.; Li, M.; et al. Transposon Mutagenesis Identifies Novel Genes Associated with *Staphylococcus aureus* Persister Formation. *Front. Microbiol.* 2015, 6, 1437. [CrossRef]
- Han, J.; He, L.; Shi, W.; Xu, X.; Wang, S.; Zhang, S.; Zhang, Y. Glycerol uptake is important for L-form formation and persistence in *Staphylococcus aureus*. *PLoS ONE* 2014, 9, e108325. [CrossRef] [PubMed]
- 33. Shang, Y.; Wang, X.; Chen, Z.; Lyu, Z.; Lin, Z.; Zheng, J.; Wu, Y.; Deng, Q.; Yu, Z.; Zhang, Y.; et al. *Staphylococcus aureus* PhoU Homologs Regulate Persister Formation and Virulence. *Front. Microbiol.* **2020**, *11*, 865. [CrossRef] [PubMed]
- Xu, T.; Wang, X.; Meng, L.; Zhu, M.; Wu, J.; Xu, Y.; Zhang, Y.; Zhang, W. Magnesium Links Starvation-Mediated Antibiotic Persistence to ATP. *mSphere* 2020, 5, e00862-19. [CrossRef]
- Mechler, L.; Herbig, A.; Paprotka, K.; Fraunholz, M.; Nieselt, K.; Bertram, R. A novel point mutation promotes growth phasedependent daptomycin tolerance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 2015, 59, 5366–5376. [CrossRef]
- 36. Keren, I.; Kaldalu, N.; Spoering, A.; Wang, Y.; Lewis, K. Persister cells and tolerance to antimicrobials. *FEMS Microbiol. Lett.* **2004**, 230, 13–18. [CrossRef] [PubMed]
- 37. Kamble, E.; Pardesi, K. Antibiotic Tolerance in Biofilm and Stationary-Phase Planktonic Cells of *Staphylococcus aureus*. *Microb. Drug Resist.* **2021**, 27, 3–12. [CrossRef]
- Han, J.; Liu, Z.; Xu, T.; Shi, W.; Zhang, Y. A Novel LysR-Type Global Regulator RpvA Controls Persister Formation and Virulence in *Staphylococcus aureus*. *bioRxiv* 2019, 12, 861500.
- Sahukhal, G.S.; Pandey, S.; Elasri, M.O. msaABCR operon is involved in persister cell formation in *Staphylococcus aureus*. *BMC Microbiol.* 2017, 17, 218. [CrossRef]
- 40. Nygaard, P.; Smith, J.M. Evidence for a novel glycinamide ribonucleotide transformylase in *Escherichia coli*. J. Bacteriol. **1993**, 175, 3591–3597. [CrossRef]
- 41. Conlon, B.P.; Rowe, S.E.; Gandt, A.B.; Nuxoll, A.S.; Donegan, N.P.; Zalis, E.A.; Clair, G.; Adkins, J.N.; Cheung, A.L.; Lewis, K. Persister formation in *Staphylococcus aureus* is associated with ATP depletion. *Nat. Microbiol.* **2016**, *1*, 16051. [CrossRef]
- 42. Shan, Y.; Lazinski, D.; Rowe, S.; Camilli, A.; Lewis, K. Genetic basis of persister tolerance to aminoglycosides in *Escherichia coli*. *mBio* **2015**, *6*, e00078-15. [CrossRef] [PubMed]
- 43. Yan, D.; Zhang, Q.; Fu, Q.; Sun, M.; Huang, X. Disruption of Fis reduces bacterial persister formation by regulating glutamate metabolism in Salmonella. *Microb. Pathog.* **2021**, 152, 104651. [CrossRef] [PubMed]
- 44. Castaño, I.; Bastarrachea, F.; Covarrubias, A.A. gltBDF operon of Escherichia coli. J. Bacteriol. 1988, 170, 821–827. [CrossRef] [PubMed]
- Joseph, A.; Aikawa, S.; Sasaki, K.; Teramura, H.; Hasunuma, T.; Matsuda, F.; Osanai, T.; Hirai, M.Y.; Kondo, A. Rre37 stimulates accumulation of 2-oxoglutarate and glycogen under nitrogen starvation in Synechocystis sp. PCC 6803. FEBS Lett. 2014, 588, 466–471. [CrossRef]
- Kim, J.N.; Méndez-García, C.; Geier, R.R.; Iakiviak, M.; Chang, J.; Cann, I.; Mackie, R.I. Metabolic networks for nitrogen utilization in Prevotella ruminicola 23. Sci. Rep. 2017, 7, 7851. [CrossRef]
- Zeden, M.S.; Kviatkovski, I.; Schuster, C.F.; Thomas, V.C.; Fey, P.D.; Gründling, A. Identification of the main glutamine and glutamate transporters in *Staphylococcus aureus* and their impact on c-di-AMP production. *Mol. Microbiol.* 2020, 113, 1085–1100. [CrossRef]
- Bowman, L.; Zeden, M.S.; Schuster, C.F.; Kaever, V.; Gründling, A. New Insights into the Cyclic Di-adenosine Monophosphate (c-di-AMP) Degradation Pathway and the Requirement of the Cyclic Dinucleotide for Acid Stress Resistance in *Staphylococcus aureus*. J. Biol. Chem. 2016, 291, 26970–26986. [CrossRef]
- 49. Jenul, C.; Horswill, A.R. Regulation of Staphylococcus aureus Virulence. Microbiol. Spectr. 2019, 7, 29. [CrossRef]
- Dunman, P.M.; Murphy, E.; Haney, S.; Palacios, D.; Tucker-Kellogg, G.; Wu, S.; Brown, E.L.; Zagursky, R.J.; Shlaes, D.; Projan, S.J. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the agr and / or sarA loci. *J. Bacteriol.* 2001, 183, 7341–7353. [CrossRef]
- Andrey, D.O.; Jousselin, A.; Villanueva, M.; Renzoni, A.; Monod, A.; Barras, C.; Rodriguez, N.; Kelley, W.L. Impact of the Regulators SigB, Rot, SarA and sarS on the Toxic Shock Tst Promoter and TSST-1 Expression in Staphylococcus aureus. *PLoS ONE* 2015, 10, e0135579. [CrossRef]
- 52. Liu, Q.; Yeo, W.S.; Bae, T. The SaeRS Two-Component System of Staphylococcus aureus. Genes 2016, 7, 81. [CrossRef] [PubMed]
- Tiwari, N.; López-Redondo, M.; Miguel-Romero, L.; Kulhankova, K.; Cahill, M.P.; Tran, P.M.; Kinney, K.J.; Kilgore, S.H.; Al-Tameemi, H.; Herfst, C.A.; et al. The SrrAB two-component system regulates *Staphylococcus aureus* pathogenicity through redox sensitive cysteines. *Proc. Natl. Acad. Sci. USA* 2020, 117, 10989–10999. [CrossRef] [PubMed]
- Walker, J.N.; Crosby, H.A.; Spaulding, A.R.; Salgado-Pabón, W.; Malone, C.L.; Rosenthal, C.B.; Schlievert, P.M.; Boyd, J.M.; Horswill, A.R. The *Staphylococcus aureus* ArlRS two-component system is a novel regulator of agglutination and pathogenesis. *PLoS Pathog.* 2013, 9, e1003819. [CrossRef] [PubMed]
- Giraudo, A.T.; Calzolari, A.; Cataldi, A.A.; Bogni, C.; Nagel, R. The sae locus of *Staphylococcus aureus* encodes a two-component regulatory system. *FEMS Microbiol. Lett.* 1999, 177, 15–22. [CrossRef] [PubMed]

- Sun, F.; Li, C.; Jeong, D.; Sohn, C.; He, C.; Bae, T. In the *Staphylococcus aureus* two-component system sae, the response regulator SaeR binds to a direct repeat sequence and DNA binding requires phosphorylation by the sensor kinase SaeS. *J. Bacteriol.* 2010, 192, 2111–2127. [CrossRef]
- 57. Mainiero, M.; Goerke, C.; Geiger, T.; Gonser, C.; Herbert, S.; Wolz, C. Differential target gene activation by the *Staphylococcus aureus* two-component system saeRS. *J. Bacteriol.* **2010**, 192, 613–623. [CrossRef]
- Liang, X.; Yu, C.; Sun, J.; Liu, H.; Landwehr, C.; Holmes, D.; Ji, Y. Inactivation of a two-component signal transduction system, SaeRS, eliminates adherence and attenuates virulence of *Staphylococcus aureus*. *Infect. Immun.* 2006, 74, 4655–4665. [CrossRef]
- 59. Geiger, T.; Goerke, C.; Mainiero, M.; Kraus, D.; Wolz, C. The virulence regulator Sae of *Staphylococcus aureus*: Promoter activities and response to phagocytosis-related signals. *J. Bacteriol.* **2008**, *190*, 3419–3428. [CrossRef]
- Cho, H.; Jeong, D.W.; Liu, Q.; Yeo, W.S.; Vogl, T.; Skaar, E.P.; Chazin, W.J.; Bae, T. Calprotectin Increases the Activity of the SaeRS Two Component System and Murine Mortality during *Staphylococcus aureus* Infections. *PLoS Pathog.* 2015, 11, e1005026. [CrossRef]
- 61. Bae, T.; Schneewind, O. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid* 2006, 55, 58–63. [CrossRef]
- 62. Han, J.; Shi, W.; Xu, X.; Wang, S.; Zhang, S.; He, L.; Sun, X.; Zhang, Y. Conditions and mutations affecting *Staphylococcus aureus* L-form formation. *Microbiology* **2015**, *161*, 57–66. [CrossRef] [PubMed]
- 63. Larzábal, M.; Mercado, E.C.; Vilte, D.A.; Salazar-González, H.; Cataldi, A.; Navarro-Garcia, F. Designed coiled-coil peptides inhibit the type three secretion system of enteropathogenic *Escherichia coli*. *PLoS ONE* **2010**, *5*, e9046. [CrossRef] [PubMed]
- 64. Stepanović, S.; Vuković, D.; Hola, V.; Di Bonaventura, G.; Djukić, S.; Cirković, I.; Ruzicka, F. Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS* **2007**, *115*, 891–899. [CrossRef] [PubMed]
- 65. Reed, L.J.M.H. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 1932, 27, 493–497.
- Mortazavi, A.; Williams, B.A.; McCue, K.; Schaeffer, L.; Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods* 2008, *5*, 621–628. [CrossRef] [PubMed]