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The identification of *Staphylococcus aureus* factors required for pathogenicity and growth in human blood.

John Connolly[a*~], Emma Boldock,[a,b,c~] Lynne R Prince,[c] Stephen A Renshaw,[b,c,d] Moira K Whyte,[^b,c] Simon J Foster[a,b#]

Department of Molecular Biology and Biotechnology, University of Sheffield, UK[a]; Florey Institute, University of Sheffield, UK[b]; Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, UK[c]; Bateson Centre, University of Sheffield, UK[d]

Running Head: Nucleotide salvage required for *S. aureus* pathogenicity

#Corresponding author s.foster@sheffield.ac.uk
[*]Present address: Institute of Infection, Immunity and Inflammation, University of Glasgow.
[^]Present address: MRC Centre for Inflammation Research, University of Edinburgh.

[~] J.C. and E.B. contributed equally to this work.

Abstract

Staphylococcus aureus is a human commensal but also has devastating potential as an opportunist pathogen. *S. aureus* bacteraemia is often associated with an adverse outcome. To identify potential targets for novel control approaches we have identified *S. aureus* components that are required for growth on human blood. An ordered transposon mutant library was screened, identifying 9 genes involved specifically in haemolysis or growth on human blood agar compared to the parental strain. Three genes (*purA*, *purB* and *pabA*) were subsequently found to be required for pathogenesis in the zebrafish embryo infection model. The *pabA* growth defect was specific to the red blood cell component of human blood, showing no growth difference compared to the parental strain on human serum, human plasma, sheep or horse blood. PabA is required in the tetrahydrofolate (THF) biosynthesis pathway. The *pabA* growth defect was found to be due to a combination of loss of THF-dependent dTMP production by the enzyme ThyA and an increased demand for pyrimidines in human blood. Our work highlights *pabA* and the pyrimidine salvage pathway as potential targets for novel therapeutics and suggests a previously undefined role for a human blood factor in the activity of sulphonamide antibiotics.

Introduction

Pathogenicity of the Gram-positive bacterium *Staphylococcus aureus* requires a multitude of virulence factors that are intricately co-ordinated and regulated (1, 2). In addition to the more 'classic' virulence factors such as pore-forming toxins and superantigens, fundamental metabolic processes of bacteria are also recognised as a prerequisite for disease. Indeed, the majority of antibiotics act by disrupting essential metabolic processes (3). However, pathogens

including *S. aureus*, have adapted to resist such insults by switching off, or severely reducing the activity of, aspects of metabolism in order to persist in the presence of antibiotics (4, 5).

Microbial fitness during pathogenesis requires efficient utilisation of available nutrients. Although the mammalian host is nutrient rich, many are sequestered as a means of inhibiting pathogen growth, a concept referred to as 'nutritional immunity' (6). Strategies to overcome the nutrient limited environment *in vivo* are well described in *S. aureus* and other bacteria, including the upregulation of peptide or amino acid transport mechanisms (7) and of proteins which enable the acquisition of nutrients sequestered by the host (8, 9). D*e novo* biosynthetic pathways are also required to produce essential products not readily available in the environment. Nucleotide biosynthetic pathways have been identified as critical for the proliferation of Gram-positive pathogens on human blood (10) yet detailed studies of the growth requirements of *S. aureus* are lacking.

To support studies on *S. aureus*, the Nebraska Transposon Mutant Library (NTML) was recently constructed in the CA-MRSA USA300 JE2 strain, deposited in the Network on Antimicrobial Resistance in *S. aureus* (NARSA) strain repository and made freely available to registered users (11). This library was created using the *mariner* based transposon (*bursa aurealis)* employing the same methodology as Bae and colleagues (12). To date, the NTML library has been used to carry out diverse screens to identify genes involved in *S. aureus* antibiotic persistence *in vitro* (13); altered haemolytic activity on rabbit blood agar (11, 14); polymicrobial interactions (15) and hyaluronidase activity (16).

A comprehensive approach to identify genes involved in the growth of *S. aureus* on human blood was undertaken using the NTML library. Genes were then further characterised to analyse their potential role in human infection. We show that purine biosynthesis is

indispensable for growth on human blood and *in vivo* pathogenicity using a zebrafish embryo model. In addition, a gene involved in tetrahydrofolate biosynthesis, *pabA*, was also identified as being required for virulence *in vivo* and was unable to grow specifically on human blood. The relationship between human blood, a folate poor environment and *S. aureus* pyrimidine salvage pathways was further elucidated.

Results

Screening of a *S. aureus* transposon library for growth defects on human blood. The NTML was screened to define gene disruptions leading to alterations in growth and/or haemolysis on agar containing human blood as the only nutrient source (see Materials and Methods). The library was also screened on bovine serum agar and 5% (v/v) sheep blood with Columbia agar base as comparators to determine human blood specific traits (data not shown). The Tn insert for each strain identified in the screen was transduced back into the parent strain (*S. aureus* JE2) and transductants were rescreened to establish that the mutant phenotype was associated with each Tn insertion. Fifteen transductants maintained the altered phenotype, nine of which (*purB*, *purA*, *pabA*, *atl*, *murQ*, *araC*, *mecA*, *odhB* and *lipA*) were taken forward for further study (Table 1). The remaining six strains had transposon disruptions in genes expected to produce an altered phenotype when grown on human blood agar (*agrA*, *agrB*, *agrC*, *hla*, *saeR* & *saeS*) confirming the ability of the screen to identify specific phenotypes.

Phenotypic characterisation of growth defective mutants *in vivo*. In order to define genes for further study, pathogenicity of the nine transduced strains in the JE2 background was assessed using the zebrafish embryo model of systemic *S. aureus* infection (17). *atl*, *murQ*, *araC*, *mecA*, *odhB* and *lipA* did not show altered killing in this model (Fig. S1a,b). However, three of

the strains, harbouring Tn inserts in the *purA*, *purB* and *pabA* genes (herein named JE2-*purA*, JE2-*purB* and JE2-*pabA*), showed significant attenuation in the zebrafish model ($P<0.0001$; Fig. 1a). To confirm that the reduced pathogenicity was not strain specific, Tn inserts containing the *purA*, *purB* and *pabA* genes were transduced into another strain background, *S. aureus* SH1000. These strains (herein named SH-*purA*, SH-*purB* and SH-*pabA*) also showed significant attenuation in the zebrafish embryo model ($P<0.0001$; Fig. 1b). *In vivo* growth analysis demonstrated that SH-*purA* and SH-*purB* were unable to replicate within zebrafish embryos and bacterial numbers recovered were lower than the inoculated dose (Fig. 1c,d). This is in stark contrast to the bacterial kinetics observed when parental *S. aureus* is injected at the same dose as previously published (Fig. S1c) (17). SH-*pabA* retained limited capacity to replicate and to cause host death in the zebrafish model (Fig. 1e). Using a knock-down approach to deplete zebrafish myeloid cells (*pu*.1 morpholino), SH-*pabA* was restored to similar virulence as the parental strain but with a slight temporal delay (Fig. 1f). By 20 hours post infection (hpi), all embryos injected with the parent strain, and 80% of SH-*pabA* injected embryos, had succumbed. The remaining SH-*pabA* injected embryos died over the following 24 hours. In myeloid depleted zebrafish, SH-*purA* and SH-*purB* caused death of approximately two thirds of subjects injected, significantly less than the parent strain ($P<0.0001$). The *pu*.1 knockdown approach causes a temporary delay in phagocytic cell development and as expected, no further host death was observed after 40 hours, a time point at which recovery of phagocyte production would occur (18, 19).

Purine biosynthesis is required for growth in blood. Analysis of the *purA* and *purB* genes (20, 21) demonstrated that *purA* and *purB* code for enzymes in the purine biosynthesis pathways (adenylosuccinate synthase and adenylosuccinate lyase respectively) (Fig. S2). *In vitro*, JE2-*purA* and JE2-*purB* showed reduced growth on human blood and bovine serum agar

plates, but growth similar to the parent strain on 5% (v/v) sheep blood which contains a rich nutrient base (data not shown). Growth assays of JE2-*purA* and JE2-*purB* in liquid media were also conducted (BHI, bovine serum or human serum) (Fig. 2a-c). Growth was comparable to the parent only in nutrient rich BHI media, matching that seen in the initial NTML screen. This suggested that the reduced growth phenotype was due to a nutrient requirement not readily available in human blood or human/bovine serum. Analysis of the purine biosynthesis pathway suggested that both strains should require adenine for growth, whilst in addition to adenine, JE2-*purB* should also require guanine (or inosine). Chemically defined media (CDM) analysis confirmed that *purA* growth was dependent on the presence of adenine and *purB* growth was dependent on adenine and guanine (Table 2; Fig. 2d). Addition of 20 μg ml^{-1} adenine and 20 μg ml^{-1} inosine restored growth of each *pur* mutant, to similar levels obtained for the parent strain (data not shown). Biochemical complementation of *purA* and *purB* was not successful in the zebrafish infection model, likely due to poor diffusion of nucleobases into zebrafish embryos (data not shown). The importance of purine biosynthesis pathway enzymes in disease has been well characterised (22, 23).

***pabA* is required for virulence in the murine sepsis model and growth in human blood.** In a mouse sepsis model, mice injected with *S. aureus* SH-*pabA* (4 x 10^7 CFU) lost significantly less weight compared to those receiving the parent strain (2 x 10^7 CFU). Bacterial numbers were also significantly lower in kidneys harvested from mice injected with SH-*pabA* (Fig. 3a,b; $P<0.01$).

The *pabA* Tn mutant was found to have a unique growth phenotype in the initial screen. Growth was highly reduced on 30% (v/v) human blood but had only slightly reduced growth on 30% (v/v) rabbit blood (Table 1). However, *pabA* grew well on both sheep and horse blood agar

(30% v/v) demonstrating that the phenotype was species specific. In addition, *pabA* demonstrated good growth on 50% (v/v) human serum or plasma agar (Fig. S3). To ascertain if the amount of human plasma in 30% (v/v) whole blood agar was too low to support growth, *pabA* was compared to the parent strain on agar increasing in plasma concentration up to 50% (v/v). At the lower concentrations of 10% (v/v) and 15% (v/v) (15% being the approximate plasma concentration in 30% (v/v) blood agar) growth of *pabA* was poor, but comparable to JE2 which also displayed poor growth at this concentration. Therefore, the reduced *pabA* growth on human blood was not a result of lower plasma levels in human blood agar (data not shown).

PabA is an enzyme required for tetrahydrofolate (THF) synthesis (para-aminobenzoate synthetase component II) (20) and *pabA* is found in an operon with *pabB* and *pabC*, which is responsible for the synthesis of the folate pathway intermediate, 4-aminobenzoic acid (PABA) (20). Strains from the NTML harbouring a Tn disrupting *pabB* or *pabC* were transduced into the SH1000 background and also found to be attenuated in the zebrafish infection model (Fig. 3c,d; $P<0.001$). Genetic complementation of the *pab* operon restored JE2-*pabA* growth on human blood (Fig. 3e) and SH-*pabA* virulence in the zebrafish model (Fig. 3f).

Reduced growth on human blood could be due to lack of nutrients that are required by a THF-lacking strain. The end-product of the folate pathway, THF, acts as single-carbon donor/acceptor in glycine/serine interconversion, vitamin B_5 synthesis, methionine synthesis, purine synthesis, N-formylmethionine-tRNA charging, glycine cleavage and deoxythymidine monophosphate (dTMP) synthesis (Fig. S4). To further characterise *pabA*, different media were used to interrogate the mechanism underpinning the lack of growth on human blood. In liquid culture, *pabA* growth was comparable to that of the wildtype in both BHI, bovine and human serum (data not shown) suggesting that the reduced growth phenotype was specific to blood.

154 Using CDM base media lacking purines, serine and glycine, only the addition of purines, serine
155 and glycine together could restore growth yield of the mutant to parental levels (as measured by
156 maximum OD_{600} reached) (data not shown). Biochemical complementation with the same
157 supplements did not restore growth of *pabA* on human blood, nor did addition of folic acid.
158 However, addition of PABA fully complemented growth (Fig. S5a) as would be expected based
159 on similar work done in *Lactococcus lactis* (24). Immersion of zebrafish embryos injected with
160 SH-*pabA* into E3 medium containing PABA, restored virulence *in vivo* (Fig. 3g; $P<0.0001$).

161 **Pyrimidine salvage pathways are required to bypass *pabA*.** Synthesis of dTMP is
162 achieved via a THF-dependent route, or via an alternative nucleotide salvage pathway requiring
163 thymine or thymidine (Fig. 4a). A combination of glycine, serine and purines could not restore
164 growth of *pabA* on human blood, however, the addition of pyrimidines (thymine) supported
165 growth to the extent of the parent strain, JE2 (Fig. S5a). The crucial role of pyrimidines in
166 bacterial survival under folate deprived conditions has been reported previously (25, 26). Neither
167 pyrimidines, nor folic acid, could restore pathogenicity of *pabA* in the zebrafish embryo model
168 (data not shown).

169 Double mutants defective in *pabA* and one of the pyrimidine nucleotide salvage pathway
170 genes *pdp*, *tdk* or the thymidine transporter gene, *nupC,* were constructed to assess their role in
171 *pabA* growth. Growth of all three double mutants was reduced on human blood but could be
172 complemented with PABA (Fig. S5b). Thymine and thymidine addition to blood could
173 complement all mutants except for the *pabA tdk* double mutant. This highlighted that pyrimidine
174 salvage pathways are required to bypass the deficit of *pabA* and if an inhibitory factor in blood
175 was responsible for preventing *pabA* growth, Tdk is the likely target. Unexpectedly, the *pabA*
176 *pdp* double mutant was complemented by thymine and the *pabA nupC* mutant was

complemented by thymidine. This suggests that conversion of thymine to thymidine can be achieved independently of Pdp and that an alternative thymidine transporter to NupC is available in *S. aureus.* Two remaining putative pyrimidine transporters have been identified in *S. aureus* and not yet investigated (27).

Investigating a nucleotide salvage pathway inhibitory component in human blood. The nucleotide salvage pathway appears to provide enough dTMP (later converted to dTTP) for DNA synthesis and growth of *pabA* on human plasma/serum, but not on human blood, unless thymine/thymidine is added. This suggested that a factor in whole blood either competitively inhibits the nucleotide salvage pathway enzymes, or that growth on human blood leads to an increased requirement for dTMP, which cannot be met without increasing the thymine/thymidine concentration (Fig. 4a). To hone in on an inhibitory factor, different components of blood were assessed for their ability to replicate the *pabA* poor growth phenotype seen on whole human blood. JE2-*pabA* growth was comparable to JE2 on platelet rich plasma (PRP) and on PRP that had been vortexed to disrupt platelets (data not shown). Similarly, parent and mutant growth were equivalent when white blood cells (WBCs), either intact or lysed, were added to platelet poor plasma (PPP). Vortexing of whole human blood followed by centrifugation produces red, rather than straw coloured, plasma, indicating RBC lysis. Plasma from vortexed blood was mixed with PPP to give a 9:1 ratio of non-vortexed to vortexed plasma, decreasing incrementally to a ratio of 1:9. At the lowest ratio of non-vortexed to vortexed plasma the growth of JE2-*pabA* was highly reduced (Fig. 4b). This suggested that there is a potent inhibitor of *pabA* growth in the red blood cell (RBC) component of human blood.

Haemoglobin/haem was deemed a likely candidate for the inhibitory factor. Haemoglobin, a complex of four heme groups, is the most abundant hemoprotein in humans.

Heme is an iron containing ring structure and usage of heme as an iron source can be toxic to bacteria due to its active redox potential (28). Though the mechanisms underlying this are not fully understood, it has been reported that heme induced monooxygenase like activity can cause direct DNA damage (28, 29). In *S. aureus*, haem is extracted from haemoglobin and transported into the cell by the iron regulated surface determinant system (Isd system) (30). Toxicity induced by liberation of iron from heme by *S. aureus* is reduced by the two component heme-regulated transporter (*hrtAB*). Haem is also transported into *S. aureus* by the ABC transporter HtsABC, which requires haem extraction from haemoglobin by the Isd system (30). Both transport systems are upregulated in low Fe by alleviation of the negative regulator Fur. However, supplementing human blood agar with an alternative Fe source (ammonium ferrous sulphate) did not support growth of JE2-*pabA* on human blood (data not shown). In addition, lyophilised bovine haemin, bovine haemoglobin and human haemoglobin did not prevent *pabA* growth on plasma (data not shown).

***S. aureus* growth in human blood requires an increased demand for pyrimidines.** Rather than an inhibitory factor in blood preventing *pabA* growth, it is possible that human blood leads to an increased requirement for dTMP, which cannot be met in a folate-deficient mutant reliant solely on the pyrimidine salvage pathway (Fig. 4a). Thymidylate synthase (*thyA*) is highly conserved, requiring THF as a cofactor for conversion of dUMP to dTMP, an essential step in DNA synthesis. To maintain viability, *thyA* mutants can utilise extracellular thymidine, via pyrimidine salvage pathways (31) and thus cannot grow *in-vitro* on media lacking pyrimidines such as Mueller-Hinton (MH) agar or human blood (27). To determine if human blood increases the demand for pyrimidines, a minimal permissive concentration of thymidine to allow *thyA* growth (500 ng ml^{-1}) was added to MH agar (Fig. 5a). As the added concentration of human

blood increased, ranging from 1-50% (v/v), *thyA* growth became increasingly inhibited, suggesting that as for *pabA*, pyrimidine requirements are elevated by human blood. This was further confirmed by addition of higher concentration thymidine (400 μg ml^{-1}) which allowed biochemical complementation of *thyA* (Fig. 5b).

In the host environment, when innate immune cells encounter bacteria, reactive oxygen species (ROS) such as superoxide and nitric oxide are generated (32). Bacteria have developed sophisticated mechanisms to resist such oxidative stress. Although heme acquisition is a necessity for *S. aureus* survival *in vivo*, we hypothesised that heme causes bacterial oxidative stress requiring increased dTTP requirements for DNA repair and *pabA* would be less able to compensate, compared to the parent strain. To test this, the *pabA* mutation was transduced into a strain unable to acquire heme due to a disrupted Isd and haem transport systems, LS1Δ*isdE*Δ*htsA* (33). The triple mutant (LS1Δ*isdE*Δ*htsApabA*) was inoculated onto human blood agar to determine if the removal of potential heme toxicity would restore *pabA* growth. No growth was observed for *pabA* or Δ*isdE*Δ*htsApabA* on unsupplemented blood agar but both strains displayed good growth in the presence of exogenous pyrimidines (data not shown). However, it has been demonstrated that in the absence of functional haem transport and Isd systems, *S. aureus* can still acquire haem, by a 3rd, as yet unknown, haem transport mechanism (33).

In the presence of sulphonamide antibiotics, nucleotide salvage pathways are required for *S. aureus* growth in blood. The effect of folate antagonistic, sulphonamide antibiotics, such as trimethoprim (TMP), on *S. aureus*, leads to loss of THF synthesis and similar to *pabA*, a dependence on the pyrimidine nucleotide salvage pathway for dTMP synthesis. The activity of this class of antibiotics can be reversed by providing enough thymine to bypass the requirement for the THF-dependent dTMP synthesis pathway (34). Pyrimidine reversal of TMP

246 activity for JE2, JE2-*pabA* and JE2-*tdk* was assessed for growth on human, sheep or horse blood
247 agar. On human blood, thymidine reversed TMP activity against JE2, and JE2-*pabA* growth was
248 restored in the presence of thymidine; however, TMP was active against JE2-*tdk* in the presence
249 or absence of thymidine (Table 3). Similar results on horse blood to those seen on human blood
250 were found. On sheep blood TMP antibiotics were inactive against JE2 and JE2-*pabA*, likely due
251 to a higher pyrimidine concentration in sheep blood (35) demonstrating that the JE2-*pabA*
252 phenotype on human blood may also be due to differences in blood pyrimidine content. As with
253 human and horse blood, JE2-*tdk* was inhibited by TMP on sheep blood and addition of thymidine
254 could not reverse this, as the nucleotide salvage pathways are prohibited.
255
256 **Discussion**
257 In order to identify novel pathogenicity determinants, an ordered library of transposon
258 mutants was screened for gene disruptions causing growth and haemolysis defects on agar
259 containing human blood as the only nutrient source. This identified *purA*, *purB* and *pabA* as
260 being required for growth on human blood. The *purA* and *purB* genes are part of the *de novo*
261 biosynthetic pathway for purines and *pabA* is involved in folate synthesis. Confirming an
262 important role in pathogenesis, all three mutations were found to lead to significant attenuation
263 in the zebrafish systemic model of infection.
264 In a study detailing the non-essential genes involved in growth of *Escherichia coli*,
265 *Salmonella enterica* and *Bacillus anthracis* in human serum using a microarray-based system,
266 the majority of mutants identified were involved in purine or pyrimidine biosynthesis (10). This
267 suggests a scarcity of nucleotides *in vivo*, which bacteria counteract by being equipped with
268 energy costly metabolic pathways permitting *de novo* synthesis. Similarly, in our study, the

ability of purine biosynthesis mutants to grow in nutrient rich media suggested that *purA* and *purB* have a requirement for nutrients not readily available in human serum, whole blood and the live zebrafish.

The reduced growth of *S. aureus pabA in vitro* was intriguing as it was specific to human blood, with normal growth seen on blood components (serum, plasma), horse and sheep blood. PabA is required for production of PABA, an essential intermediate in the synthesis of THF. A *pabB* mutant of *Streptococcus pneumoniae* has been used as an attenuated strain for vaccine research highlighting the importance of this pathway in the development of prophylactic strategies (36). Using CDM liquid and solid agar, purines, glycine and serine were required for growth by the *pabA* mutant in excess of that required by the parental strain. However, when assessed on human blood agar, growth inhibition could not be rescued with any compound except pyrimidines suggesting that all other necessary factors to bypass the lack of THF, are present in serum, plasma and whole blood. The concept of 'thymine-less' death has been previously noted and demonstrates the fundamental importance of pyrimidines in bacterial survival, over and above the other downstream effectors of THF (25). The addition of thymidine to human blood permitted *pabA* growth (Fig. S5a). Human blood is known to have a low thymidine content compared to other animals (35). However, growth of the mutant on other thymidine poor media (e.g. CDM, horse blood) suggested that thymidine deficiency per se was not solely responsible for the growth phenotype.

In the absence of THF dependent *thyA* activity, pyrimidine salvage pathways are essential to convert thymidine to dTMP (via Tdk) which is necessary for DNA replication. Both *pabA* and *thyA* rely on these salvage pathways to provide a permissive amount of thymidine and therefore, dTTP, to remain viable. It is difficult to tease apart exactly how human blood subverts this

process and we hypothesised that Tdk was the target of competitive inhibition in the *pabA* mutant, given that supplemental thymidine restored *pabA* growth and a genetic knockout of *pabA tdk* eliminated this biochemical complementation. Double knockouts of *pabA* with the gene responsible for conversion of thymine to thymidine (*pabA pdp*) or a pyrimidine transporter (*pabA nupC*) had no effect on biochemical complementation. Furthermore, *pabA* growth was reduced on human plasma supplemented with lysed RBC products. As excess haem is toxic to *S. aureus* (28), haem and related molecules were ruled out as Tdk inhibitory factors. Tdk is an enzyme requiring zinc which is purported to be required for transcriptional regulation (37) and zinc sequestration by human blood and other potential inhibitory factors should be further investigated in future work (38).

Although the exact mechanism is yet to be elucidated, it is clear however that human blood, or a component therein, leads to an increased demand for dTMP, which cannot be met in a THF-deficient mutant, hence why exogenous thymidine/thymine is necessary to support growth of the mutant specifically on human blood.

Finally, little is known about the clinical prevalence or relevance of *pabA* mutations. Trimethoprim is used in the control of *S. aureus* infections and long-standing treatment can lead to failure due to development of antibiotic resistance (39). In this context, *thyA* mutations are usually observed in the resistant subpopulation and such mutations cause formation of thymidine dependent small colony variants (SCVs) which rely on pyrimidine salvage pathways (via Pdp and Tdk) (40). However, these antibiotics remain bacteriocidal, unless a thymidine rich environment exists, such as damaged host tissues, which allow *S. aureus* to utilise pyrimidine salvage pathways and thus survive (41). The work presented here suggests that the activity of sulphonamide drugs is the result of inhibition of THF coupled with reduced activity of the

pyrimidine salvage pathways and/or an increased demand for dTMP imparted by human blood. The identification of metabolic pathways important for host:pathogen interactions provides novel avenues to be explored to combat antibiotic resistant pathogens.

Materials and methods

Ethics statement. Zebrafish embryos less than 5 days post fertilization (dpf) are not protected under the Animals (Scientific Procedures) Act 1986 but all zebrafish work was carried out according to the details set out in Project License PPL 40/3574. Murine work was carried out according to UK law in the Animals (Scientific Procedures) Act 1986, under Project License PPL 40/3699. Human blood was obtained from healthy volunteers in compliance with the guidelines of the South Sheffield Research Ethics Committee (STH13927).

Bacterial strains, plasmids and growth conditions. The Nebraska transposon mutagenesis library (11) was acquired from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) strain repository, now available from BEI Resources (www.beiresources.org/) and used for screening experiments. Originally in the USA300 LAC JE2 background, mutations were transduced back into JE2 or SH1000 as required. All other strains and the list of plasmids used in this study are given in Table 4. *S. aureus* strains were routinely grown in Brain Heart Infusion (BHI) media at 37^{o}C with aeration at 250 rpm, unless otherwise stated. Mueller- Hinton agar (Oxoid) was as a thymidine poor media where stated. *E. coli* strains were grown in Luria Bertani at 37°C with aeration at 250 rpm. Agar at 1.5% (w/v) was added for solid media. Antibiotics were added as required. For MIC determination, a bacterial colony was inoculated into 2 ml sterile dH_2O and spread onto an agar plate using a sterile swab (Oxoid). Trimethoprim E-tests® (bioMérieu) were applied to the solid media surface using tweezers and incubated overnight at 37°C.

The chemically defined media used in this study has been previously described (42). The following components were dissolved into 1 litre of H_2O: $Na_2HPO_4.2H_2O$, 7g; KH_2PO_4, 3g; L-Aspartic Acid, 0.15g; L-Alanine, 0.1g; L-Arginine, 0.1g; L-Cysteine, 0.05g; Glycine, 0.1g; L-

Glutamic Acid, 0.15g; L-Histidine, 0.1g; L-Isoleucine, 0.15g; L-Lysine, 0.1g; L-Leucine, 0.15g; L-Methionine, 0.1g; L-Phenylalanine, 0.1g L-Proline, 0.15g; L-Serine, 0.1g; L-Threonine, 0.15g; L-Tryptophan, 0.1g; L-Tyrosine, 0.1g; L-Valine, 0.15g; Biotin, 0.02g; Pyridoxal HCl, 0.8g; Nicotinic Acid, 0.4g; Pyridoxamine di-HCl, 0.8g; D-Pantothenic Acid, 0.4g; Riboflavin, 0.4g; Thiamine HCl, 0.4g; Adenine Sulphate, 0.02g; Guanine HCl, 0.02g; $CaCl_2.6H_2O$, 0.01g; $(NH_4)_2Fe(SO_4)_2.6H_2O$, 0.006g; Glucose, 10g $MgSO_4.7H_2O$, 0.5g. Inosine was used as described.

Human, or other animal, blood or blood components were added to agar at varying concentrations, as required. Venous blood was collected from healthy volunteers following informed consent. For plasma preparation, blood was centrifuged at 270 g for 20 min in 50 ml Falcon tubes. The upper platelet rich phase was collected and used directly as platelet rich plasma (PRP), or centrifuged again at 1155 g for 30 min to give platelet poor plasma (PPP). Plasma was stored at -20^{o}C. Animal blood and blood products were purchased from Thermoscientific or Sigma and stored at 4^{o}C. Bovine haemin/haemoglobin, human haemoglobin, thymine, thymidine, glycine, serine, vitamin B_5, methionine, PABA, folic acid or methyl viologen (Sigma) was added to media as and when required at the stated concentrations.

Genetic manipulation. Electroporation was used to transform *S. aureus* RN4220 and *E. coli* using previously published methods (43, 44). All *S. aureus* transduction experiments were carried out with φ11 as described previously (45).

For genetic complementation of SH-*pabA* and JE2-*pabA*, Phusion polymerase (NEB) was used to amplify the *pab* operon from *S. aureus* SH1000 genomic DNA, using primers containing appropriate restriction sites (forward, ATAATAGGGCCCATTGTA-CTGTCTTGACCACCACT; reverse, ATAATACTCGAGATACGTATACAAGAATTAA-CAACAGCA). The PCR product was inserted into pKASBAR (46), a plasmid encoding an *attP*

site. Using this *attP* site, bacteriophage DNA can integrate into the *S. aureus* genome at the *attB* site, in the presence of an integrase (47). The *attB* site is located at the glycerol ester hydrolase (*geh*) gene so integration can be verified by loss of lipase activity. For such genetic manipulation, the integrase is provided by an additional helper plasmid, pYL112Δ19, propagated in the *S. aureus* recipient strain, RN4220. The insert was then transduced from RN4220 into *pabA* and control strains.

To prepare double mutants within Tn insertions, the "toolkit" for switching antibiotic resistance within NTML strains was used as published previously (48). Tn inserts in *pdp*, *nupC* and *tdk* genes, with alternate antibiotic resistance markers, were transduced into *pabA* as listed in Table 4.

Strains LS1 and LS1Δ*isdE*Δ*htsA* were kindly provided by Dr Sean Nair (University College London). *pabA* was transduced into both strains and successful transductants were confirmed by PCR.

Transposon library screen. The NTML was grown for 18 h at 37°C in 96-well microtiter dishes. Using a 96-pin replicator (Boekel Industries), the contents of each well were transferred to BHI agar, BHI + erythromycin (10 µg/ml)/lincomycin (25 µg/ml) agar, 30% (v/v) human blood agar, 50% (v/v) bovine serum agar and 5% (v/v) sheep blood, plus Columbia agar base in rectangular OmniTray plates (Nunc). Human blood and bovine serum plates were incubated for 48 h at 37°C, all other plates were incubated for 18 h at 37°C, with an additional 4 h at 4°C for sheep blood plates, to ensure efficient haemolysis. Phenotypes were determined by comparison of each spot (colony size and haemolysis zone) to the surrounding spots on the plate.

Zebrafish model. Zebrafish embryos, strain London wild-type (LWT), were maintained in E3 medium at 28°C, following standard protocols (17). Embryos were bred in the aquarium

facilities at the University of Sheffield. Microinjection of embryos was performed as described previously (17). Individual infected embryos were kept in 100 µl E3 media and survival was assessed over 90 h. For *in vivo* complementation experiments, compounds were dissolved in E3 medium and buffered to a pH of 6.5-7.5. Immediately following injections, embryos were placed in compound solutions at the stated concentrations. Further compound solution was added in the embryo washing step. 96-well microtitre plates were placed in a plastic box, with damp paper, to reduce evaporation during incubation.

pu.1-antisense morpholino-modified oligos (49) were injected into zebrafish embryos using the method described previously (17). Bacteria were recovered from infected embryos at 12 h time intervals. Individual embryos were transferred to microcentrifuge tubes and homogenised using a PreCellys 24-dual (Peqlab). Bacterial numbers were then determined by serial dilution in phosphate buffered saline (PBS) and plating onto BHI agar.

Murine infection model. Female BALB/c mice were purchased from Charles River Laboratories (Margate, UK) and maintained by standard husbandry techniques at the University of Sheffield (Biological Services). Bacteria were washed in endotoxin free PBS (Sigma) and 100 µl (2-4 x 10^7 CFU) was injected i.v. into the tail vein. Serial dilutions of culture were prepared to confirm injection CFU. Mice were monitored and sacrificed at 72 hpi. Mouse organs were individually homogenised in PBS and after serial dilution, plated onto BHI agar supplemented with antibiotics as needed for bacterial number enumeration.

Statistical analysis. Sample sizes were predetermined for mouse (n=10) and zebrafish experiments (n=20) based on previous experimental data (50). All zebrafish experiments are representative of n=2 unless otherwise stated. For zebrafish embryo survival experiments, the Kaplan-Meier method was employed. Comparison between survival curves was made using the

log-rank (Mantel Cox) test. For bacterial count comparison in murine experiments, the Mann-Whitney U test was used. Statistical analysis was performed using Prism version 6.0 (GraphPad) and $P < 0.05$ was considered significant.

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Author contributions for this study were as follows: J.C. and E.B. performed and analysed the experiments. L.R.P., S.A.R., M.K.W. and S.J.F. contributed to study design and data analysis. J.C., E.B. and S.J.F. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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568

569

Category	Protein ID	NARSA ID	Protein Name	Growth Phenotype		Haemolysis Phenotype	
				Human Blood	Rabbit Blood	5% Human Blood + Columbia Agar	5% Sheep Blood + Columbia Agar
A1	SAUSA300_1889	NE522	adenylosuccinate lyase, PurB	Reduced Growth	Reduced Growth	Increased Haemolysis	Reduced Haemolysis
	SAUSA300_0017	NE529	adenylosuccinate synthetase, PurA	Reduced Growth	Reduced Growth	Increased Haemolysis	Slightly Increased Haemolysis
	SAUSA300_0698	NE821	para-aminobenzoate synthase, glutamine amidotransferase, component II, PabA	Highly Reduced Growth	Slightly Reduced Growth	------	------
A2	SAUSA300_0955	NE460	autolysin, Atl	Opaque Colony	Opaque Colony	Increased Haemolysis	------
	SAUSA300_0193	NE1253	N-acetylmuramic acid-6-phosphate etheraur, MurQ	------	------	Increased Haemolysis	Increased Haemolysis
	SAUSA300_2326	NE1304	transcription regulatory protein, AraC	------	------	Reduced Haemolysis	------
	SAUSA300_0899	NE1315	adaptor protein, MecA	------	------	Reduced Haemolysis	Slightly Reduced Haemolysis
	SAUSA300_1305	NE1391	dihydrolipoamide succinyltransferase, OdhB	Slightly Reduced Growth	Slightly Reduced Growth	Increased Haemolysis	Increased Haemolysis
	SAUSA300_0320	NE1775	triacylglycerol lipase, LipA	Slightly Reduced Growth	------	Increased Haemolysis	------
B	SAUSA300_1989	NE95	accessory gene regulator protein B, AgrB	------	------	------	Reduced Haemolysis
	SAUSA300_1991	NE873	accessory gene regulator protein C, AgrC	------	------	Slightly Reduced	No Haemolysis
	SAUSA300_0690	NE1296	sensor histidine kinase, SaeS	------	------	------	No Haemolysis
	SAUSA300_1058	NE1354	alpha-hemolysin, Hla	------	------	------	No Haemolysis
	SAUSA300_1992	NE1532	accessory gene regulator protein A, AgrA	------	------	Reduced Haemolysis	No Haemolysis
	SAUSA300_0691	NE1622	DNA-binding response regulator, SaeR	------	------	------	No Haemolysis

Table 1 Tn library mutants identified as having altered phenotype on human blood agar

A1 - Strains with a defect in growth on human blood agar which were investigated further; A2 - strains with altered haemolysis on human blood agar which were investigated further; B - strains expected to show a haemolysis phenotype and not explored further. ---, No difference from the JE2 control.

575

Strain Name	Chemically Defined Media			
	+Adenine +Guanine	**-Adenine -Guanine**	**+Adenine -Guanine**	**-Adenine +Guanine**
JE2	+	+	+	+
purB	+	-	-	-
purA	+	-	+	-

576

577 **Table 2 Growth analysis on solid media of JE2-*purA* and JE2-*purB* in the presence or**

578 **absence of adenine and guanine**

579 Adenine 20 μg ml^{-1}; guanine 20 μg ml^{-1}; + growth; - no growth
580

581

582

TMP MIC (mg/L)	BHI		Human blood		Sheep blood		Horse blood	
	- T	+ T	- T	+ T	- T	+ T	- T	+ T
parent	1	>32	0.75	>32	>32	>32	1	>32
pabA	1	>32	-	>32	>32	>32	>0.002	>32
tdk	0.25	0.25	0.75	0.75	0.5	0.5	1	1

Table 3 Minimum inhibitory concentration (MIC, mg/L) of trimethoprim (TMP) of parent, JE2-*pabA* or JE2-*tdk* *S. aureus* strains on various media

–T no exogenous thymidine added, +T thymidine (400 μg ml^{-1}) added

Strain	Relevant genotype/markers	Source/reference
***S. aureus* strains**		
SH1000	Functional *rsbU+* derivative of 8325-4	(51)
RN4220	Restriction negative, modification positive strain	(52)
USA300 JE2	USA300 LAC strain cured of plasmids p01 and p03	(11)
SJF4669	*SH-pabA::spc*, *pdp::ery*	This study
SJF4670	*SH-pabA::spc*, *nupC::ery*	This study
SJF 4671	*SH-thyA::ery*	(27)
SJF4678	*pabA::spc*, *tdk::ery*	This study
JC006	JE2-*pabA*, pJC002 inserted at lipase – *pabA*$^{+}$ Ery^{R} Lin^{R} Tet^{R}	This study
JC007	JE2-*pabA*, pKASBAR inserted at lipase Ery^{R} Lin^{R} Tet^{R}	This study
JC010	SH-*pabA*, pJC002 inserted at lipase – *pabA*$^{+}$ Ery^{R} Lin^{R} Tet^{R}	This study
JC011	SH-*pabA*, pKASBAR inserted at lipase Ery^{R} Lin^{R} Tet^{R}	This study
LS1	Spontaneous murine arthritis isolate	(53)
LS1Δ*isdE*Δ*htsA*	LS1 derivative, Δ*isdE* Δ*htsA*	(33)
***E. coli* strains**		
TOP10	F- *mcr Δ* (*mrr-hsdRMS-mcrBC*) Φ 80 *lacZ ΔM15 ΔlacX74 recA1 deoR araD139 Δ*(*ara-leu*) *7697 galK rpsL* (Str^{R}) *endA1 nupG*	Invitrogen
Plasmids		
pKASBAR	Hybrid vector of pCL84 and pUC18 for integration into *S. aureus* lipase gene (*geh*), *attP*; Tet^{R} (*S. aureus*), Spec^{R} (*E. coli*)	(46)
pJC002	pKASBAR containing the *pab* operon, *pabA*, *pabB* and *pabC* and upstream control elements; Tet^{R}	This study

596 **Table 4 Strains and plasmids used in this study**

597

598 **Figure Legends**

599

600 **Figure 1 *In vivo* characterisation of *S. aureus* strains in the zebrafish embryo model of**
601 **infection with reduced growth on human blood *in vitro***

602 **a,** Survival curves of fish injected with *S. aureus* JE2 (1500 CFU, JE2), *S. aureus* JE2 *purA,*
603 *purB or pabA* (1500 CFU). **b,** Survival curves of fish injected with *S. aureus* SH1000 (1500
604 CFU, SH), *S. aureus* SH1000 *purA, purB or pabA* (1500 CFU). **c-e,** Growth of *S. aureus* mutants
605 within embryos after injection with 1500 CFU of *purA* **(c)**, *purB* **(d)** or *pabA* **(e)**. Open circles,
606 live and filled circles, dead embryos. **f,** Survival curves of *pu.1* knockdown fish injected with *S.*
607 *aureus* SH1000 (1500 CFU, SH), *S. aureus* SH1000 *purA, purB or pabA* (1500 CFU).

608

609 **Figure 2 The *purA* and *purB S. aureus* mutants require exogenous purines for growth**

610 **a-c,** Strains were grown in BHI **(a)**, bovine serum **(b)** or human serum **(c)**. Data are from three
611 independent repeats, error bars represent standard errors. ●= JE2, ■= JE2-*purB* and ⑩= JE2-
612 *purA*. **d,** Growth of strains on CDM agar plates with or without adenine (20 μg ml^{-1})/guanine (20
613 μg ml^{-1}) after 24 h incubation aerobically at 37°C.

614

615 **Figure 3 The *pabABC* operon is required for pathogenesis**

616 **a,b,** Female BALB/c mice (n = 10) were injected i.v. with $2x10^7$ CFU *S. aureus* SH1000 or
617 $4x10^7$ CFU *S. aureus* SH1000 *pabA*. Weight loss **(a)** and kidney **(b)** CFU were measured after 3
618 days. **c,** Survival curves of fish injected with *S. aureus* SH1000 (1500 CFU, SH) or *S. aureus*
619 SH1000 *pabB*. **d,** Survival curves of fish injected with *S. aureus* SH1000 (1500 CFU, SH) or *S.*
620 *aureus* SH1000 *pabC*. **e,** Growth of parent (JE2), *pabA*, genetically complemented *pabA*

(integration of pJC002, JC006) or control integrated strain (pKASBAR empty plasmid in *pabA* mutant, JC007) on unsupplemented human blood agar (30% v/v). Plates were incubated aerobically at 37°C for 48 h. **f,** Survival curves of fish injected with *S. aureus* SH1000 (1500 CFU, SH), *S. aureus* SH1000 *pabA* (1500 CFU), *S. aureus* SH1000 *pabA* + *pabABC* operon (pJC002, 1500 CFU, JC010) or *S. aureus* SH1000 *pabA* with empty plasmid only (pKASBAR, 1500 CFU, JC011). **g,** Survival curves of fish injected with *S. aureus* SH1000 (1500 CFU, SH) or *S. aureus* SH1000 *pabA* (1500 CFU, SH-*pabA*) followed by immediate immersion in either unsupplemented E3 medium (red) or supplemented with 7 μg ml^{-1} PABA (black). Uninjected fish were included as controls under each condition.

Figure 4 Folate biosynthesis pathway and effect of lysed RBCs on *S. aureus pabA* growth

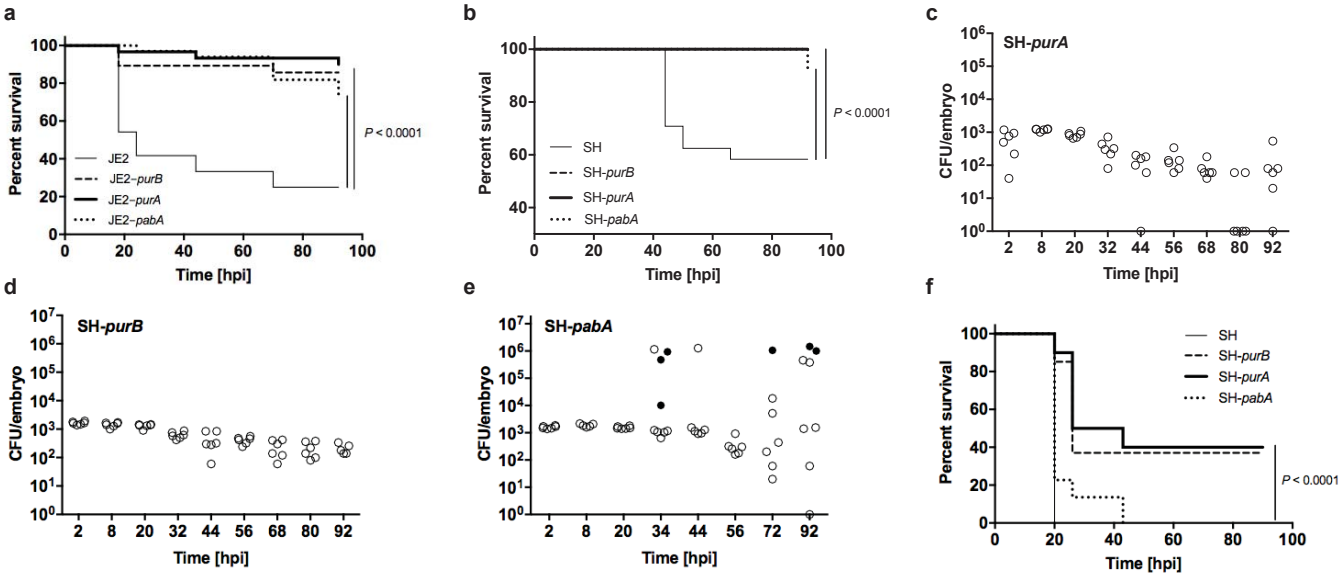
a, The folate biosynthesis pathway and pyrimidine nucleotide salvage pathway (20, 21). Possible hypotheses for poor *pabA* growth on human blood are shown as (1) *S. aureus* Tdk is the target of competitive inhibition by human blood or (2) increased dTMP demand necessitates supplemental thymidine in *S. aureus*. **b,** Growth of *S. aureus* JE2 or JE2-*pabA* on non-vortexed human PPP or a decreasing ratio of vortexed:non-vortexed agar. Plates were incubated aerobically at 37°C for 48 h.

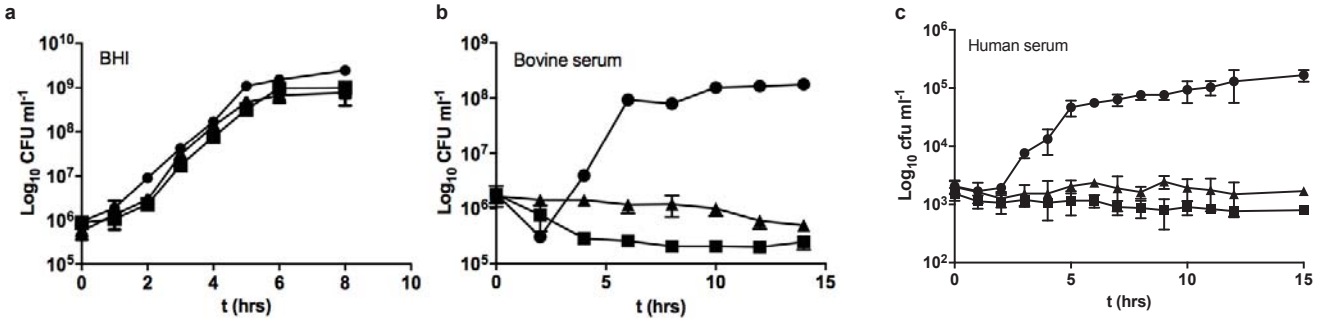
Figure 5 An increased demand for thymidine is required for *S. aureus* growth on human blood

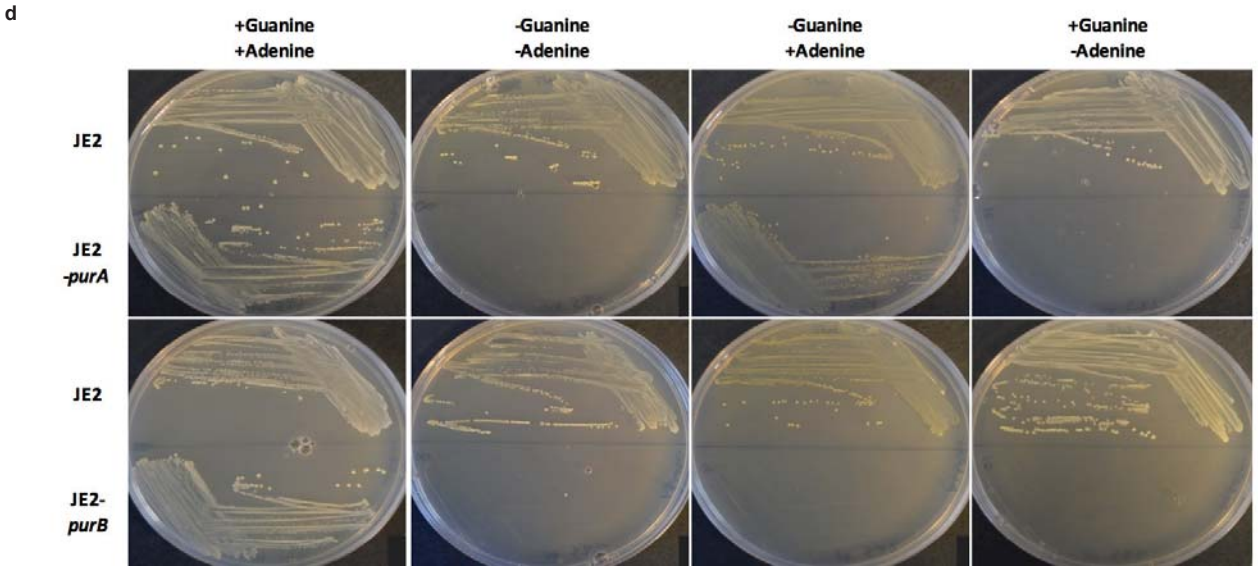
a, Growth of *S. aureus* SH-*thyA* on MH agar. Media was either unsupplemented (top right box only) or contained a permissive amount of thymidine (500 ng ml^{-1}). Increasing concentrations of human blood was added ranging from 1-50% (v/v) with MH agar base, containing thymidine

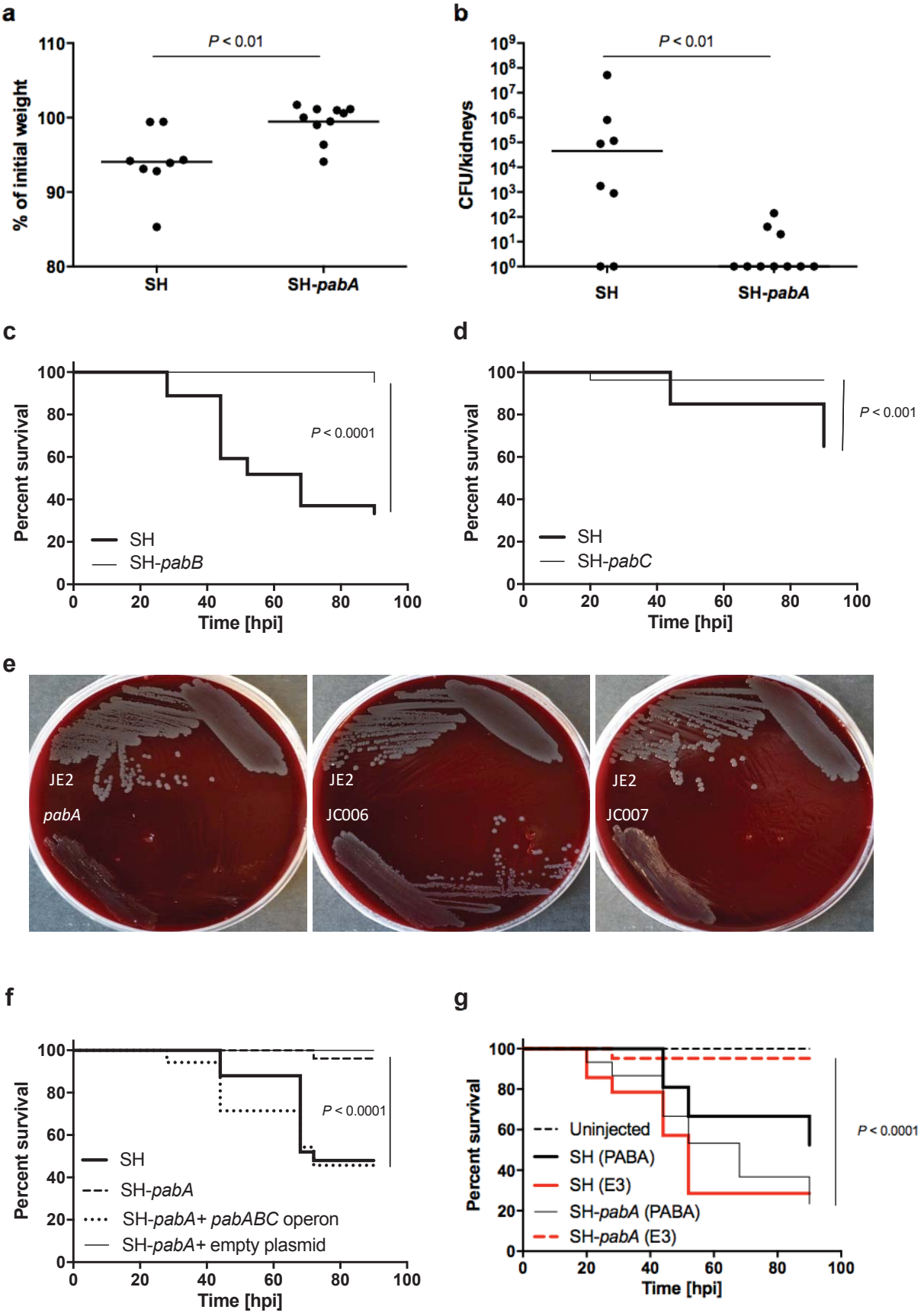
644 (500 ng ml^{-1}). Plates were incubated aerobically at 37°C for 24 h. **b,** At concentrations of human
645 blood causing reduced *thyA* growth, biochemical complementation was achieved by addition of
646 400 μg ml^{-1} thymidine. Plates were incubated aerobically at 37°C for 24 h.

647









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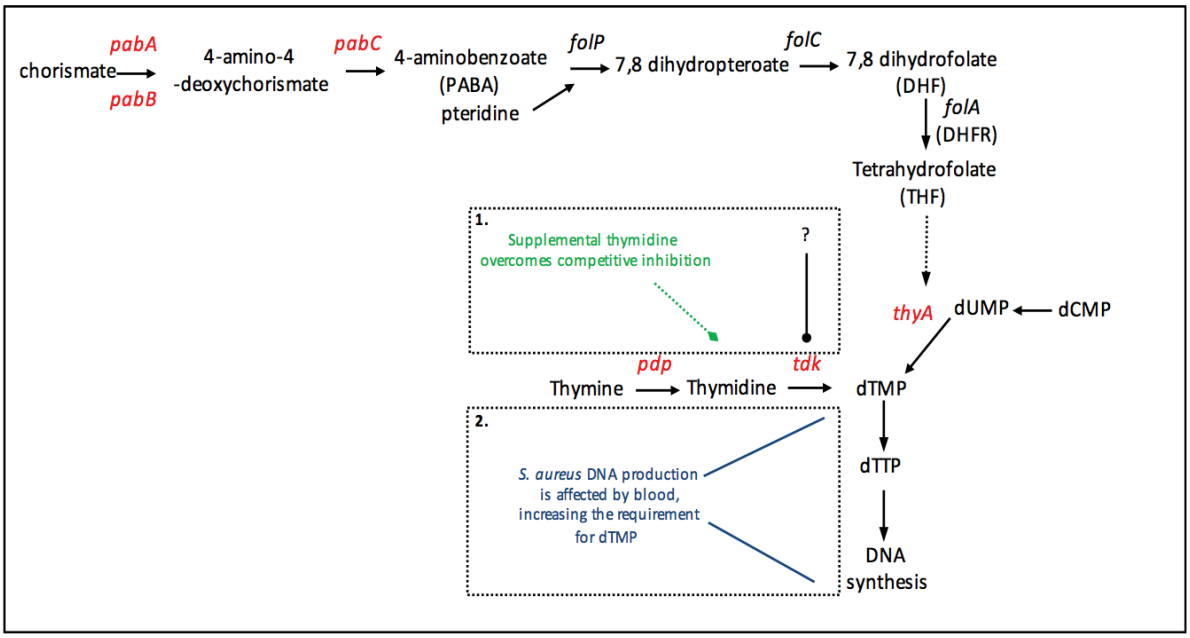

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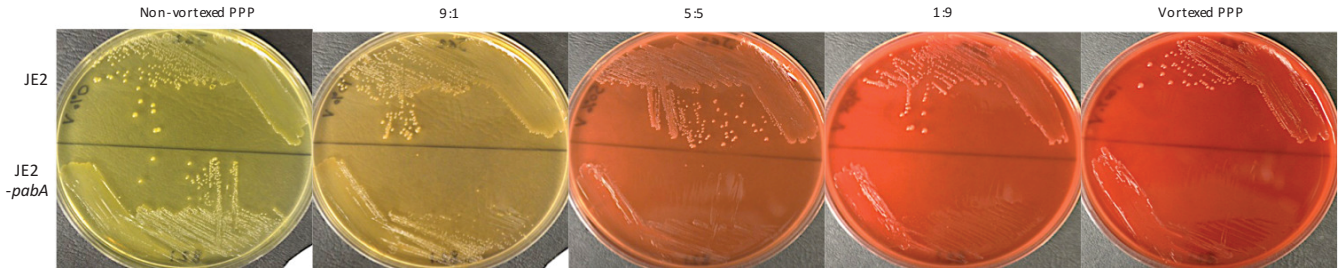
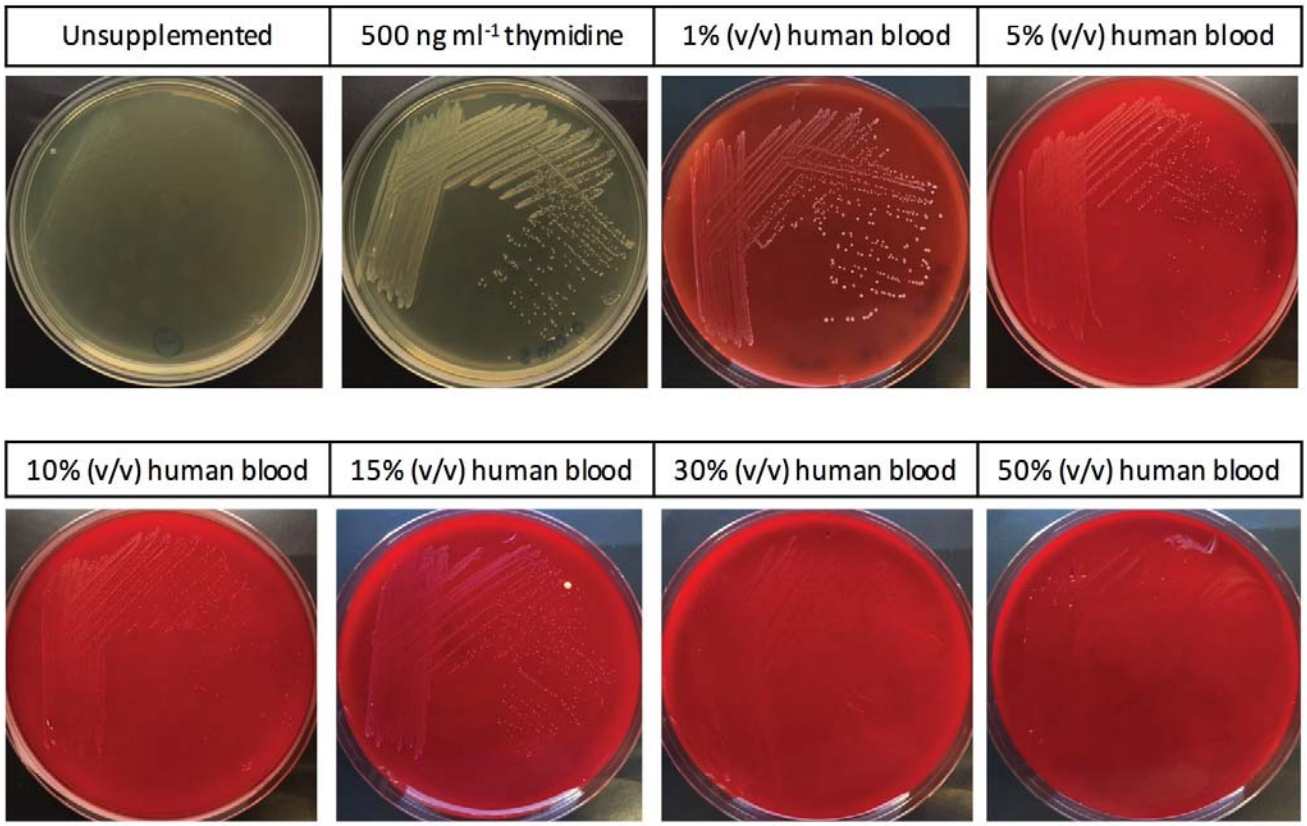


a



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