Identification of \textit{Staphylococcus aureus} genes involved in the formation of structured macrocolonies

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Abstract
The human pathogen \textit{Staphylococcus aureus} causes difficult-to-eradicate biofilm-associated infections that generally become chronic. Understanding the genetic regulation of biofilm formation in \textit{S. aureus} is central to a precise definition of the conditions and genes involved in development of chronic biofilm-associated infections. Biofilm-related genes have been detected by comparing mutants using the classical submerged biofilm formation assay, in which cells adhere to the bottom of a well containing culture medium. We recently developed an alternative biofilm formation model for \textit{S. aureus}, based on macrocolony formation on agar plates, comparable to an assay used to study biofilm formation in a few other bacterial species. As organism features are the result of environmental conditions as well as of genes, we used a genome-wide collection of transposon-mapped mutants in this macrocolony assay to seek \textit{S. aureus} developmental genes and pathways not identified by the classical biofilm formation assay. We identified routes related to glucose and purine metabolism and clarified their regulatory link to macrocolony formation. Our study demonstrates that formation of microbial communities must be correlated to specific growth conditions, and the role of metabolism must be considered in \textit{S. aureus} biofilm formation and thus, in the development of chronic infections.

INTRODUCTION
Populations of surface-associated bacteria are termed biofilms. The widespread recognition that biofilms affect numerous environments, from water pipes to indwelling devices in hospital patients, has increased interest in studying the molecular mechanisms that underlie the formation and maintenance of these communities [1]. The ability to form biofilms, once considered the domain of a few species, is now seen as an almost-universal attribute of microorganisms. Biofilm formation pathways are extremely diverse, as are the environmental conditions that promote them. A common feature is the capacity of bacteria to embed in a self-produced extracellular matrix (ECM) [2] that protects them from changing environmental conditions or external insults such as antibiotics or action of the immune system [3]. This ECM is composed of proteins, extracellular DNA (eDNA), and exopolysaccharides, and provides a physical barrier while acting as a substrate that attaches cells to submerged surfaces [4].

Biofilms have a profound impact in medicine, industry and agriculture. Much attention has been paid to their role in human disease. Biofilms that form on medical devices such as catheters, implants or contact lenses act as a reservoir for bacteria that can be shed in the body, leading to acute and chronic infections. Difficult-to-treat chronic infections are thought to result from bacterial colonization in biofilm form [5]. The main negative effect of biofilm-associated bacteria in clinical settings is they develop remarkably high levels of resistance to a wide variety of antimicrobial agents, in contrast to their planktonic counterparts [6]. Nosocomial pathogens can infect patients by forming biofilms on abiotic materials introduced into the body including implants or catheters, as well as on biotic surfaces such as burn wounds [7]. The ECM of these biofilms provides a diffusion barrier that protects the bacteria from antimicrobial treatment, generating hard-to-treat infections that become chronic. \textit{Staphylococcus aureus} is one pathogen that causes biofilm-associated hospital infections. Biofilm formation in \textit{S. aureus}...
requires activation of the icaADBC operon genes, responsible for generating poly-N-acetylglucosamine (PNAG or PIA), an extracellular polysaccharide matrix that protects cells within a biofilm. In addition to PNAG or PIA, S. aureus is able to form an ica-independent biofilm by expressing a large number of cell wall-anchored adhesion proteins (SpA and other MSCRAMM proteins) responsible for cell aggre-gation/attachment during biofilm formation, as well as via recycling cytoplasmic proteins and controlled release of eDNA [8–12].

To more precisely define conditions and genes involved in the development of chronic infections, much effort has been invested in understanding the genetic regulation of biofilm formation in S. aureus. The S. aureus agr quorum-sensing system downregulates its biofilm-associated lifestyle [9–11], and other signal transduction cascades upregulate biofilm formation. For instance, the alternative sigma factor σB inhibits the agr system [13]. σB is activated during early stationary phase [14] in response to distinct types of cell stress, which triggers a general stress response that affects expression of many virulence factors and indirectly represses agr [15]. σA thus antagonizes agr and induces biofilm formation [13, 16]. SarA is a master regulator that controls several biofilm-related pathways; it increases icaADBC transcription and simultaneously represses production of extracellular proteinases and nucleases that inhibit matrix production [17–20]. Repression of matrix-degrading enzymes is both direct and indirect (saeRS-dependent) [21]. The positive effects of SarA and SaeRS on biofilm formation can nonetheless be cancelled by constitutive SaeS activation, leading to biofilm defects [21–23].

This understanding of the genetic regulation of S. aureus biofilm formation was built on comparative analyses of clinical isolates and characterization of biofilm-defective mutants using the classical submerged biofilm formation assay [24], in which cells adhere to a polystyrene or glass surface and are quantitated using crystal violet. This assay is a standard approach used to compare results and consolidate conclusions. For instance, agr-defective isolates are frequently identified from chronic infections as these mutants usually develop robust biofilms [25–29], and agr dysfunction often correlates with chronic persistent S. aureus infections [30] by small colony variants, which have very low agr levels [31] and high expression of biofilm-related genes [32].

Alternative biofilm models have been developed, based on the formation of ECM-encased macrocolony communities on agar dishes, widely recognized as a form of biofilm. Colony morphology varies widely, and highly structured colony morphology correlates well with strain ability to produce an ECM [33]. This assay is used in studies of Bacillus subtilis [34], Escherichia coli [35] and Pseudomonas aeruginosa [36] microbial communities. We recently described the genetic cascade that regulates macrocolony formation in S. aureus [37, 38]. In this model, magnesium ions from the medium bind S. aureus cell wall teichoic acids (TAs). Mg²⁺ preferentially binds the phosphate-rich TAs that decorate the cell wall and form a consolidated network that alleviates the electrostatic repulsive interactions between neighbouring TA phosphates; this leads to increased cell wall stability and rigidity [39–42]. This rigidity increase promotes expression of the cell wall stress factor sigB (σB), which in turn activates the stress regulon that induces agr downregulation. In magnesium-containing TSB (TSBMg), σB activation thus represses the quorum-sensing system agr, which in turn derepresses biofilm-related genes [43, 44]. The multicellular aggregates in this model have a characteristic three-dimensional morphology indicative of a high degree of organization within the macrocolony.

In the classical biofilm formation assay in liquid TSB, the model strain Newman has been catalogued as a poor biofilm former [22]. In contrast, in TSBMg plates or liquid medium, it develops robust and highly structured macrocolonies, respectively. This difference between the macrocolony assay and the classical biofilm formation assay indicates that these two developmental assays do not involve the same cellular processes in S. aureus. Since strain Newman was originally isolated from a long-term bone-associated infection that usually involves biofilm formation [45], and bone is an important Mg²⁺ reservoir in the body [46, 47], this strain might naturally develop strong biofilms in Mg²⁺-enriched growing conditions that would resemble the colonizing niches in which it develops biofilm-associated infections that recreate the TSBMg macrocolony assay.

As organism characteristics are a result of environmental as well as genetic conditions [48], we sought to identify the set of genes involved in macrocolony formation in these TSBMg conditions and to compare them to the genes associated with biofilm formation in the classical biofilm formation assay that entitles the formation of submerged pellicles in the bottom of a well plate with liquid culture. Since conditions differ in the two assays, identification of genes with functions important for biofilm formation might describe how a set of genes in distinct environments produce a phenotype [49]. Here we performed a genome-wide screen and established a macrocolony developmental assay to identify related genes, different from the biofilm-related genes identified in the several successful screens that have been performed using the classical biofilm formation assay [50, 51]. We define the response regulator of a two-component system (TCS) that affects macrocolony formation through the σB–agr pathway, and show that disruption of gluconeogenesis inhibits and of purine biosynthesis activates macrocolony formation. These findings contribute to our understanding of an alternative in vitro approach for the study of multicellular community development in the pathogen S. aureus.

RESULTS

Macrocolony formation in S. aureus is magnesium-dependent

We recently defined the genetic mechanism that triggers macrocolony formation in TSBMg medium [tryptic soy broth (TSB) with 100 mM MgCl₂] [37]. To further explore
machinery of macrocolony formation, we supplemented TSB individually with several salts (MgCl\(_2\), MgSO\(_4\), CaCl\(_2\), MnCl\(_2\), KCl, NaCl) and tested for development of macrocolonies on agar plates and also in submerged biofilms in liquid medium. In addition, we tested whether the conventional biofilm medium composition, TSB agar supplemented with 3 % NaCl and 0.5 % glucose (TSB-NaCl-glu) [52], triggered macrocolony formation. Macrocolonies formed in TSBMg, and to a lesser extent in TSB with 100 mM MgSO\(_4\), both on solid agar and in the submerged biofilm formation assay. We observed no macrocolony formation in TSB supplemented with other salts (CaCl\(_2\), MnCl\(_2\), KCl, NaCl) (Figs 1a and S1a, available in the online version of this article), which highlighted the Mg\(^{2+}\) requirement in the medium for macrocolony development. As growth curves were similar for \(S.\) \textit{aureus} cultured with MgCl\(_2\) or MgSO\(_4\) (Fig. S1b), the differences in macrocolony formation were unrelated to growth alterations.

Biofilm formation in \(S.\) \textit{aureus} requires production of the structural element PNAG and of surface adhesins such as protein A (SpA), as well as control by regulators including \textit{agr} and \(\sigma^B\) [53–57]. To determine the role of these factors in macrocolony formation, we assessed the colony morphology of mutants for the respective genes. An \textit{agr}-deficient strain formed a more robust and wrinkled macrocolony, whereas \(\sigma^B\) deletion reduced macrocolony complexity and wrinkling. A PNAG- and SpA-deficient strain formed more fragile macrocolonies with less pronounced wrinkling (Fig. S1c). The intensity of wrinkling on the macrocolony surface correlated positively with biofilm strength in conventional liquid assays. Since regulators of biofilm formation behaved as predicted in the regulation of macrocolony formation, we considered macrocolonies a suitable model for the study of multicellular community development in \(S.\) \textit{aureus}.

Macrocolony development of \(S.\) \textit{aureus} in TSBMg agar led us to search for genes with a role in staphylococcal multicellular development that were not detected in the classical biofilm formation assay using the formation of pellicles in liquid cultures. We performed a macrocolony formation screen using a genome-wide transposon-mapped collection of \(S.\) \textit{aureus} mutants (1920 mutants) (Nebraska Transposon Mutant Library, NTML) [58]. TSBMg induces formation of rigid, structured macrocolonies in the library USA300-JE2 parent strain (Fig. 1c). The TSBMg agar plates were spotted with 2 \(\mu\)l of a dense cell suspension, or cellular material was transferred directly from TSB plates. Although the shape of macrocolonies spotted from suspension was more regular due to more homogenous cell distribution, both methods led to very similar macrocolony phenotypes (Fig. S1d). For the screen, colonies were grown with an intermediate incubation step on TSB agar before incubation on TSBMg for up to 120 h. We examined the architecture of the screened macrocolonies visually, using a ZEISS Stemi 508 with 8:1 Zoom coupled to an Axiocam ERC 5 s and classified the

![Image](https://example.com/image1.png)

**Fig. 1.** Macrocolony biofilm assay in \(S.\) \textit{aureus}. (a) Macrocolonies of strain Newman on TSB supplemented with different ions. Only magnesium salts triggered the distinctive macrocolony wrinkling. (b) Quantitative biofilm assay of strain Newman in TSB with various ions. Only magnesium salts induced biofilm formation. (c) Macrocolony of USA300-JE2, the NTML \(S.\) \textit{aureus} parent strain, on TSB and TSBMg. (d) Scheme of the NTML screen used to identify modulators of macrocolony morphology. (a, c) All macrocolonies were imaged after 5 days. The graph shows means \pm SD of three independent experiments. \(P\)-values were calculated for unsupplemented medium as a reference using the unpaired Student’s t-test. *\(P\)\(\leq0.05\), **\(P\)\(\leq0.01\), ***\(P\)\(\leq0.001\). Scale bars, 2 mm.
mutants according to the number of wrinkles or the thickness of the macrocolony after 5 days of growth at 37°C.

**Genome-wide transposon library identifies novel phenotypes**

Based on the visual analyses of macrocolony morphology, we identified 28 transposon mutants with affected genes that were not previously associated with biofilm formation (Fig. 2b and Table 1). To validate the transposon insertion in the 28 genes that showed altered macrocolony phenotypes, we PCR-amplified and subsequently sequenced the chromosome and transposon junction site, showing that all transposon insertions were in the genes annotated (Fig. S2). The 28 mutants were then grouped into three categories based on macrocolony wrinkling and thickness (Fig. 2a). Mutants in which macrocolony surface wrinkling was minimal or absent but showed no alteration in macrocolony thickness were classified as category 1. The second group contained only two strains, whose macrocolony surface also lacked wrinkles but had a characteristic flattened appearance that contrasted with the thickened phenotype of the other two groups. Category 3 contained mutants in which surface wrinkling was more pronounced than that of WT. Other factors such as colony diameter or pigmentation varied among the 28 candidates and did not correlate with surface wrinkling (Fig. S3). According to these criteria, mutants of the biofilm regulators σ^B and agr are exemplary for the categories 1 and 3, respectively (Fig. 2a), whereas the category 2 phenotype is previously undescribed. The transposon mutants identified as candidates were affected in genes that encode cytoplasmic proteins (79%) and membrane proteins (21%) (Fig. 3b top and Table 1). Based on biological function, candidates with altered purine biosynthesis (25%) and carbohydrate metabolism (21%) were the largest functional

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**Fig. 2.** NTML collection screening identifies macrocolony morphology phenotypes. (a) Left, scheme showing macrocolony morphology and thickness of USA300-JE2 WT (see Fig. 1c) and the three candidate categories identified in the screen. Category 1, little or no surface wrinkling; category 2, thin macrocolony with few or no wrinkles; category 3, pronounced wrinkling of the macrocolony surface. Right, WT macrocolony; macrocolonies of mutants of known biofilm regulators σ^B and agr classified as categories 1 and 3. (b) Candidates identified in the NTML screen. Strains are sorted by category in the order in which they appear in Table 1. All macrocolonies were imaged after 5 days incubation. Scale bars, 2 mm.
categories among the mutants, and 18% were affected in cofactor and coenzyme biosynthesis. Biological functions represented by a maximum of two candidates (membrane transport, protein degradation, electron transport chain, redox homeostasis, oxygen sensing, fatty acid synthesis and antibiotic response) were clustered as ‘other’ and accounted for 25% of candidates. For 11% of the candidates, no known function could be assigned (Fig. 3b bottom and Table 1).

To discard mutants with altered biofilm formation due to growth defects, we measured the OD\textsubscript{600} of liquid shaking cultures after 10 h in TSB medium. Of the 28 candidates, 16 showed a defect relative to WT growth (Fig. 3a). The growth deficiency threshold was established at 80% of the WT OD\textsubscript{600} after 10 h (59), and growth-deficient mutants were excluded from further analysis. Despite not meeting growth criteria, the \(\Omega\)purK mutant was included as it developed highly structured macrocolonies (although colonies contained less biomass, this mutant generated architecturally more complex macrocolonies with a higher degree of wrinkles). We used crystal violet assays to determine biofilm formation by selected mutants in the submerged biofilm formation assay, in which we tested liquid TSBMg as well as standard TSB-NaCl-glu medium. Statistically significant changes were found for two candidates in TSB-NaCl-glu (Fig. 3c); the transposon mutant \(\Omega\)clpP showed increased biofilm formation and \(\Omega\)purK showed decreased biofilm formation in TSB-NaCl-glu. In TSBMg, \(\Omega\)tcaA and \(\Omega\)clpP showed statistically significantly increased biofilm formation. In both media tested in this assay, 11 of the 13 candidates did not differ from WT. The macrocolony assay thus allowed sensitive detection of developmental variations between mutants that were not possible to detect using the classic submerged biofilm formation assay.

We followed macrocolony progression of the 13 candidates used in the crystal violet assay between days 3 and 5, when the macrocolony is structured (compare Fig. S1d for structuring of the WT macrocolony). Macrocolony phenotypes of category 1 and 2 candidates differed from WT by day 3 (Fig. S4), whereas increased wrinkling developed in \(\Omega\)purK

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<th>Biological function</th>
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(category 3) only after day 4 (Fig. S4). Other category 3 candidates were characterized by accelerated macrocolony development; these macrocolonies were already clearly structured at day 3 (Fig. S4). To cover a broad range of macrocolony phenotypes, we selected the transposon mutants \( \text{C10srrA} \), \( \text{C10pckA} \) and \( \text{C10purK} \) as representatives of categories 1, 2 and 3, respectively. \( \text{pckA} \) and \( \text{purK} \) are metabolism-related genes, whereas \( \text{srrA} \) is involved in signalling. To rule out residual gene function in these mutants, we constructed deletion mutants of \( \text{pckA} \), \( \text{purK} \) and \( \text{srrA} \) in a plasmid-bearing clinical isolate of the USA300 lineage (USA300_TCH1516), which phenocopied the macrocolony morphology of the transposon mutants. All subsequent experiments were performed in USA300_TCH1516 that, unlike the USA300-JE2 parent strain, has not been manipulated for antibiotic susceptibility [60].

**Purine synthesis affects macrocolony formation**

\( \text{purK} \) and the remainder of the genes in the \( \text{purEKCSQLFMNHD} \) operon are needed for purine biosynthesis, necessary for virulence and rifampicin resistance in \( \text{S. aureus} \) [61, 62]. Two additional associated genes, \( \text{purA} \) and \( \text{purB} \), as well as the repressor \( \text{purR} \), are found at distinct loci in the chromosome (Fig. 4a). Compared to WT, the \( \text{C10purB} \), \( \text{C10purF} \), \( \text{C10purC} \), \( \text{C10purS} \) and \( \text{C10purL} \) mutants had a less wrinkled morphology, whereas \( \text{C10purA} \) and \( \text{C10purK} \) mutants showed a more wrinkled phenotype (Fig. 2b). Phenotypes of mutants lacking one of the remaining five genes or the repressor of the pathway (\( \text{purQ} \), \( \text{purM} \), \( \text{purN} \), \( \text{purH} \), \( \text{purD} \) and \( \text{purR} \)) resembled WT, and \( \text{purE} \) has been described as an essential gene (Fig. 4a) [58]. Based on our genome-wide transposon screening (Figs 2b and 4c), we selected the \( \text{DpurK} \) mutant to study purine biosynthesis in
macrocolony formation. In this mutant, macrocolony diameter was small and the core developed pronounced wrinkles (Figs 2b, 4c and S4). The macrocolonies were rigid, and dispersal with a blunt object led to breakage into small fragments (Fig. S5a).

PurK and PurE catalyse two reaction steps in the synthesis of CAIR (5'-phosphoribosyl-4-carboxy-5-aminoimidazole) from AIR (5-aminoimidizole ribonucleotide) (Fig. 4b). PurK binds bicarbonate to AIR in an ATP-dependent manner, to produce the unstable intermediate N5-carboxy-aminoimidazole ribonucleotide (NCAIR). NCAIR is converted to CAIR in a PurE-mediated mutase reaction [63]. Inosine monophosphate (IMP), the last precursor from the common pathway to adenine and guanine, is synthesized downstream of PurEK [64]. When we spotted ΔpurK macrocolonies on a dried drop of IMP solution on the agar, macrocolony diameter increased and wrinkling was reduced compared to ΔpurK on agar alone (Fig. 4c). When we complemented the ΔpurK mutant with a purK gene expressed in a neutral locus, WT-like biofilm formation was restored, with less wrinkling and increased colony diameter, (Fig. 4c).

As σB is a major positive regulator of macrocolony formation, we tested whether the ΔpurK hyperactive biofilm phenotype was σB-dependent. The ΔpurK ΔsigB double mutant also showed macrocolony hyperwrinkling (Fig. S6a), which indicated that the ΔpurK phenotype is not dependent on σB activation.

We used crystal violet staining to quantify ΔpurK and WT biofilm formation in the submerged biofilm formation assay in liquid TSB, TSB-NaCl-glu, and TSBMg media. In TSB and TSB-NaCl-glu, there was no difference in biofilm formation between the strains. TSBMg increased biofilm formation in both strains, but the mutant showed a twofold increase compared to WT (Fig. 4d). This contrasted to the phenotype in the ΔpurK transposon mutant on the USA300-JE2 background (Fig. 3c), in which we found reduced biofilm formation in TSB-NaCl-glu but no difference from WT in TSBMg medium.

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**Fig. 4. ΔpurK is a hyperactive biofilm former.** (a) Genetic organization of purine synthesis-related genes into a large operon and three monocistronic genes. All macrocolonies with a distinct phenotype are shown in Fig. 2(b). (b) The proteins PurE and PurK mediate the reaction from AIR to CAIR. AIR, 5-aminoimidizole ribonucleotide; CAIR, 5'-phosphoribosyl-4-carboxy-5-aminoimidazole; R5-P, ribose 5-phosphate; IMP, inosine monophosphate. (c) ΔpurK biofilms have a smaller diameter than WT macrocolonies, but wrinkling is more pronounced. purK expression from a neutral locus in the chromosome restores WT-like colony diameter and wrinkling. Exogenous supplementation with IMP restores colony diameter and wrinkling of ΔpurK biofilms to a WT-like state. Bar, 2 mm. (d) Quantification of biofilm formation in a crystal violet assay shows that biofilm overproduction in ΔpurK is Mg²⁺-dependent; the effect was not observed in alternative biofilm media without Mg²⁺. The graph shows mean±SD of three independent experiments. All biofilms were imaged after 5 days development. Statistical analysis was carried out using Student's t-test. *P<0.05.
SrrA is a response regulator that modulates macrocolony formation

SrrAB in *S. aureus* was initially detected as a TCS similar to the ResDE system in *B. subtilis* [65, 66], which is responsible for differential gene expression in response to anaerobiosis. SrrB is a transmembrane histidine kinase that phosphorylates its cognate regulator SrrA following activation of the system. SrrAB regulates the response to variations in oxygen concentration [65, 66]. The stimulus for the histidine kinase is suggested to be menaquinone-dependent sensing of the redox state of the cell [67]. Activation of the SrrAB regulon is strain-, growth phase- and oxygen concentration-dependent [68–71]. SrrAB is thought to induce its own expression and to repress the *agrBDCA* operon [68], and thus represses *agr*-dependent virulence factors in microaerobic or anaerobic conditions (Fig. 5a). A role for SrrAB was recently described in biofilm formation [67, 72]; its activation in anaerobic conditions drives programmed cell lysis and eDNA accumulation in the biofilm matrix [67].

The transposon mutant ΔsrrA and knockout mutant ΔsrrA macrocolonies did not develop characteristic wrinkles, but remained flat (Figs 2b, 5b and S4). ΔsrrA macrocolonies nevertheless formed a rigid surface that resisted cell dispersal with blunt objects (Fig. S5a). Expression of *srrA* from a neutral locus in the ΔsrrA mutant restored the macrocolony wrinkling phenotype (Fig. 5b). Staphyloxanthin production is increased in TSBMg compared to TSB medium as a result of *σ^B* activation in response to magnesium [37]. As *σ^B* directly activates the *crt* operon, responsible for staphyloxanthin production, pigment quantification can serve as a readout of *σ^B* activation [73]. Staphyloxanthin was methanol-extracted from the macrocolonies and quantified by measuring absorption at 463 nm and normalized to cell density at 600 nm. In ΔsrrA macrocolonies, staphyloxanthin production increased by 64 and 73% compared to WT after 3 and 5 days, respectively (Fig. 5c). There was an 11% increase in staphyloxanthin production between the two time points in ΔsrrA in contrast with a 6% increase in WT, which indicated stronger *σ^B* activation in the absence of the SrrAB system.

The *agr* system is the major *σ^B* antagonist in macrocolony aggregate formation. Its activity can be estimated by determining bacterial lysis of erythrocytes on blood agar plates, as the system controls haemolytic toxin expression [74, 75]. To account for the regulation that environmental conditions causes on *agr* activity [76, 77], we used experimental conditions that resemble those of our macrocolonies and measured *agr* activity in biofilms submerged in TSBMg. Haemolytic activity in TSBMg-submerged biofilms was unaffected by *srrA* deletion (Fig. 5d). The haemolytic activity of the ΔsrrA ΔsigB double mutant resembled that of the WT; hence, *srrA* deletion neutralized *agr* system hyperactivation in the ΔsigB strain (Fig. 5d). We analysed the macrocolony morphology of the ΔsrrA ΔsigB and ΔsrrA Δagr double mutants. ΔsrrA ΔsigB showed greater attenuation of macrocolony architecture than the single mutants (Fig.

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**Fig. 5.** ΔsrrA strain has an *agr*-dependent biofilm defect. (a) Organization of the srrAB operon. Model of SrrAB menaquinone-dependent activation and activity in micro- or anaerobic conditions. (b) ΔsrrA strain forms a macrocolony biofilm with no wrinkles after 5 days. srrA expression from a neutral locus in the genome restored biofilm wrinkling. The Δagr ΔsrrA strain had an intermediate phenotype with restoration of light wrinkling. Bar, 2 mm. (c) ΔsrrA is hyperpigmented with staphyloxanthin in mid- and late-stage biofilm development, which indicates *σ^B* hyperactivation. Data shown as mean±SD of 15 macrocolonies from three independent experiments. (d) Haemolysis of submerged biofilms in TSBMg after 24 h. Mean±SD of six samples from three independent experiments. Statistical analysis was carried out using the unpaired Student’s *t*-test. *P*≤0.05, ***P*≤0.001, n.s., not significant (P>0.05).
S6b), indicated by a lack of resistance to macrocolony dispersal, which remained intact in the single mutants (Figs S5a and S6d). ΔsrrA Δagr mutants showed partial restoration of the wrinkled macrocolony surface (Fig. 5b).

Disruption of the \textit{S. aureus} oxygen sensing system thus reduced structural organization in the macrocolony model, and was reversible by \textit{agr} deletion. It also had an effect on \(\sigma^B\) and on \textit{agr} activity on the \(\Delta\text{sigB}\) background. SrrAB might thus fine-tune the \(\sigma^B\)-\textit{agr} pathway in \textit{S. aureus} macrocolony formation.

Glucogenesis is crucial for matrix production in macrocolonies

Of the genes identified in the screen, 21\% are involved in carbohydrate metabolism, especially in glucogenesis and the tricarboxylic acid (TCA) cycle. Glucogenesis is the process of generating glucose from TCA intermediates or from glycolysis-derived pyruvate \cite{78, 79}. Generation of the first intermediate – the phosphoenolpyruvate (PEP) – is the rate-limiting step in the pathway. To generate PEP, oxaloacetate is phosphorylated and decarboxylated by the enzyme PckA (Fig. 6b) \cite{80}. The \textit{pckA} gene is transcribed monocistronically from the \textit{S. aureus} chromosome (Fig. 6a). In our screen, PckA-lacking mutants had a severe macrocolony defect; macrocolonies spread broadly, but were thin and developed no 3D structure (Figs 2b, 6c and S4); despite which \textit{DpckA} mutants resisted macrocolony dispersal with blunt objects (Fig. S5a). \textit{pckA} expression from a neutral locus rescued the WT phenotype (Fig. 6c). The \textit{DpckA} \(\Delta\text{sigB}\) mutant had a flat, parchment-like appearance, similar to the \textit{DpckA} strain; this indicated that \(\sigma^B\)-activation has no role in the \textit{DpckA} macrocolony phenotype (Fig. S6c).

As the \textit{DpckA} mutant was unable to build 3D-structured macrocolonies, we studied whether this phenotype was due to production of an ECM that provides less rigidity to the biofilm. Using NaCl treatment, we extracted the ECM from mature macrocolonies and quantified the macromolecular matrix components \cite{81}. The matrix was isolated from 5-day-old macrocolonies of WT and \textit{DpckA} and analysed quantitatively. Concentrations of eDNA, protein and saccharides in the matrix extract were determined spectrophotometrically. In the mutant, eDNA, protein and saccharide levels were respectively 71, 67 and 66\% lower than in WT (Fig. 6d), which might explain its weaker phenotype. Macrococlnies cracked spontaneously with mild stress such as heat exposure from a light source; within 15 s the macrocolony showed distinct cracks that over the next 30 s expanded, fragments detached from the agar and curled (Fig. S5b, top). WT macrocolonies were unaffected by heat exposure (Fig. S5b, bottom).

To determine whether the inability of \textit{DpckA} to build a sturdy ECM was related to biofilm conditions or to a general fitness defect, we tested WT and \textit{DpckA} in various growth conditions. TSB used in biofilm formation assays has \(\approx 0.25\%\) glucose as a carbon source, a difference from lysogeny broth (LB), another complex medium used to culture \textit{S. aureus}, which has no glucose. We supplemented LB with various carbon sources to test \textit{DpckA} ability to grow in conditions that require glucogenesis. In TSB and LB supplemented with 0.5\% glucose, \textit{DpckA} grew similarly or more rapidly than WT during exponential and stationary phases, respectively (Fig. 7a, c). In LB with no glucose, \textit{DpckA} growth lagged behind that of WT in both phases (Fig. 7b); the effect was similar with sodium pyruvate as a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{\textit{DpckA} strain macrocolony shows compromised integrity. (a) The \textit{pckA} gene is transcribed monocistronically. (b) Oxaloacetate is derived from pyruvate carboxylation or is donated from the TCA cycle. PckA mediates the oxaloacetate reaction to the energy-rich intermediate PEP. (c) The \textit{DpckA} macrocolony is flat, thin and brittle at day 5. \textit{pckA} expression from a neutral locus in the chromosome restored WT-like wrinkling, thickness and stability. Scale bar, 2 mm. (d) \textit{DpckA} has less eDNA, protein and saccharide in its ECM than WT. Mean±SD of five independent experiments. Quantification at day 5. Statistical analysis was carried out using the unpaired Student’s \(t\)-test. ***\(P<0.001\).}
\end{figure}
Due to their relatively large size (compared to all other invertebrates and mammals [83, 86]. Galleria mellonella depletion from the medium [82]. ΔpckA thus has a fitness defect in long-term growth (6 h), which is likely to contribute to the macrocolony and matrix defects observed.

Decreased virulence in ΔsrrA but not in ΔpckA and ΔpurK

We used the non-vertebrate infection model Galleria mellonella to compare the pathogenic potential of the ΔpckA, ΔpurK and ΔsrrA mutants to the WT [83, 84]. This model is frequently used to identify virulence factors in S. aureus and other pathogens, and to test the effectiveness of antibacterial compounds [85–87]. Galleria mellonella is a particularly suitable non-vertebrate model for mammalian infection, as the larvae can be held at temperatures up to 37 °C, the body temperature in most mammalian hosts [86]. These larvae have a complex innate immune system, with antimicrobial peptides and phagocytosing cells [88, 89]. Similar virulence factors, including cell adhesion, toxin production and cell invasion, are needed for infection in G. mellonella and mammals [83, 86].

Due to their relatively large size (~250 mg), larvae can be inoculated manually with defined infection doses [83]. Cohorts of 15 larvae received 1.5 × 10⁶ c.f.u. in the last proleg in three independent experiments; the control group received an injection of saline solution. Surviving larvae were counted after 24 and 48 h, and survival rates determined. Of larvae infected with the WT strain, ~40 and ~15 % survived at 24 and 48 h, respectively (Fig. 8). Infection with both metabolism-related genes, ΔpckA and ΔpurK, resulted in survival rates of ~30 and ~10 % at these times, similar to infection with the WT strain. In a murine abscess model, a study of the role of purine biosynthesis in virulence used a purA mutant defective in both de novo purine synthesis and the purine salvage pathway [62], the latter of which is intact in ΔpurK. In the G. mellonella model, 80 % of larvae were alive at 24 h post-ΔsrrA infection, and 40 % survived at 48 h. Virulence was statistically significantly attenuated at 24 h, but not at 48 h (P > 0.05) (Fig. 8). This finding is consistent with reports that SrrAB truncation decreases S. aureus virulence [69, 70, 90]. The strength of macrocolonies did not correlate directly with strain virulence in this non-vertebrate infection model.

DISCUSSION

Phenol-soluble modulins (PSM) are one of the main structural components of S. aureus biofilms [91]; PSM also affect macrocolony structure, but are not the sole actors in this process [37]. Here we performed a genome-wide screen of S. aureus to identify factors that strongly affect multicellular structure on a biofilm-inducing medium. The majority of candidates are involved in carbohydrate and nucleotide metabolism. Disruption of nucleotide synthesis and gluconeogenesis had antagonistic effects on macrocolony morphology. By linking SrrAB activity to macrocolony morphology, we also identified a signalling pathway involved in shaping the macrocolonies. This finding is of particular interest, as we associated it to the main determinants of macrocolony formation, σ⁶ and agr [37]. The
of evolution and cell differentiation in community-living models, formation; similar to our findings in the macrocolony of SrrAB in biofilm formation. The study of the other laboratories and those reported here highlight the role of the regulon of this TCS have been hampered by different independent experiments). Statistical analysis was carried out using the unpaired Student's t-test; *P<0.05; n.s., not significant (P>0.05).

\[ Fig. 8. \Delta srrA is attenuated in virulence. Galleria mellonella larvae were inoculated with various S. aureus strains (1.5x10^7 c.f.u.); percent survival is shown. Control larvae received saline solution. Larvae were incubated (37°C, 48 h), and surviving larvae counted after 24 and 48 h. Strain \Delta srrA virulence was attenuated compared to WT after 24 h; the difference was not significant after 48 h. ΔpckA and ΔpurK virulence was similar to WT. Data shown as mean±SD (n=15 larvae/group; three independent experiments). Statistical analysis was carried out using the unpaired Student’s t-test; *P<0.05; n.s., not significant (P>0.05). \]

\( \omega^{B}-agr \) axis is thus subject to modulation by SrrAB, which integrates other external signals into the pathway. None of the macrocolony-defective candidate mutants identified in the screen showed alterations in S. aureus surface or adhesion proteins [57]; the functions of these proteins might be largely redundant or their ability to adhere might have a minor role in the macrocolony model environment.

This study identifies a connection between S. aureus macrocolony formation and purine biosynthesis or gluconeogenesis. In Saccharomyces cerevisiae, a >500-fold upregulation of the pckA homologue was reported during the attachment stage of biofilm formation [92]. Whereas purK is essential for S. aureus growth in chronic wounds, where it readily forms biofilms, this is not strictly linked to biofilm formation [93, 94]. In contrast, in Pseudomonas and Burkholderia species, disruption of purine biosynthesis reduces biofilm formation [95, 96]. SrrAB is considered a driving force for anaerobic [67] and in vivo biofilm formation [72]. Studies of the regulon of this TCS have been hampered by differences in growth phases and models [68, 97]. Results from other laboratories and those reported here highlight the role of SrrAB in biofilm formation. The study of the \( \Omega fakA \) mutant from the NTML showed a role for fakA in biofilm formation; similar to our findings in the macrocolony model, \( \Omega fakA \) formed a stronger biofilm in a dynamic flow model [98].

The S. aureus macrocolony model is suitable for the study of evolution and cell differentiation in community-living organisms [37, 38]. The model simulates clinically relevant conditions in magnesium-rich infection niches. With the identification of factors involved in macrocolony formation, our study lays the groundwork for further characterization of the underlying processes. We previously demonstrated that macrocolonies are a differentiation site and yield distinct cell lineages [37]. It will be of interest to determine whether the modulators identified here also affect the distribution of other cell types, which could affect virulence; this would signal an importance beyond the field of microbial communities.

**METHODS**

**Strains, media and culture conditions**

Experiments were performed in the S. aureus strains Newman [45], USA300-JE2 [58] and USA300_TCH1516 [60]. S. aureus RN4220 [99] and E. coli DH5α [100] were used for cloning. S. aureus and E. coli were propagated in TSB and LB, respectively. Agar plates were prepared with 1.5% (w/v) agar. Selective media were prepared with ampicillin (100 µg ml\(^{-1}\)) for E. coli. For S. aureus, selective media were prepared with erythromycin (2 µg ml\(^{-1}\)) for RN4220, Newman and USA300-JE2, and 125 µg ml\(^{-1}\) for USA300_TCH1516), tetracycline (15 µg ml\(^{-1}\)) or spectinomycin (600 µg ml\(^{-1}\)). X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside, 50 µg ml\(^{-1}\)) was added to plates for blue/white screenings.

**Strain construction**

All strains and primers used are listed in Tables S1 and S2. Deletion strains were generated using the pMAD vector system [101]; cassettes conferring resistance to tetracycline or spectinomycin were cloned into the multiple cloning sites to generate pMAD-AB. Gene flanking regions were cloned into pMAD-AB using suitable restriction enzymes. Expression was reconstituted in deletion strains using the pMAD derivative pAmy [102]. For the native promoter, a fragment 500 bp upstream of the gene was amplified with the coding DNA sequence (CDS) and cloned into pAmy using appropriate restriction enzymes. When necessary, the promoter fragment was fused to the CDS using long flanking homology PCR [103]. pMAD and pAmy plasmids were constructed in E. coli and transformed into S. aureus RN4220 by electroporation. The plasmid was integrated into the genome by growth at 42°C (first recombination). To eliminate the plasmid backbone, cells were grown at 30°C and shifted to 42°C. Colonies without the plasmid backbone were identified in blue/white screening and the presence of the construct was confirmed by colony PCR (second recombination). Constructs were shuttled from RN4220 to USA300_TCH1516 by φ11 phage transduction.

**Biofilm assays**

Macrocolonies were cultured on TSB+100 mM MgCl\(_2\) (TSBMg) at 37°C. Cell suspension (2 µl) was spotted onto agar plates and dried briefly. Plates were inverted for incubation (up to 5 days). Macrocolonies were imaged on a stereomicroscope. NTML mutants were screened on 12×12 cm
TSBMg agar plates in batches of 96 strains in three biological replicates.

Macrocolonies were visually inspected using a stereomicroscope (ZEISS Stemi 508 with 8:1 Zoom coupled to an AxioCam ERC 5 s) and compared to macrocolonies of the WT strain. The parameters surface structure (i.e. the number of wrinkles) and the thickness of the macrocolonies were assessed to assign the transposon mutants to one of three phenotypic categories (Fig. 2a).

Robustness of macrocolonies was judged by testing their resistance to mechanical dispersal. A blunt object (e.g. pipette tip or scalpel) was touched onto the surface of the core of the macrocolony. The object was slowly moved laterally until fragmentation of the macrocolony occurred.

Submerged biofilms of *S. aureus* were grown based on a standard protocol [104]. Briefly, overnight cultures were diluted 1:100, grown for 4 h to exponential phase and the starting OD$_{600}$ of biofilms adjusted to 0.05. Cultures were inoculated in multiwell plates and incubated statically at 37°C for 24–48 h. Biofilm formation was quantitated using crystal violet dye. Medium was discarded and plates gently washed twice with PBS. Biofilms were heat-fixed (65°C, 30 min), stained with 0.1% (w/v) crystal violet solution (5 min), and the wells washed thoroughly to remove unbound dye. The stain was solubilized using 33% (v/v) acetic acid, and absorption at 595 nm determined to quantify biofilm formation.

**Extracellular matrix extraction and quantification**

Extraction of the ECM of multicellular communities was based on a protocol using high NaCl concentrations [81]. Briefly, macrocolonies were scraped off the agar, washed in PBS, resuspended in 1.5 M NaCl, sonicated to disperse aggregates, and normalized by adjusting the OD$_{600}$. Extracellular and cellular fractions were separated by centrifugation (10 000 g, 5 min, room temperature), and the supernatant recovered as the ECM fraction.

Matrix extract protein and eDNA content was quantified by NanoDrop (Thermo Fisher). The saccharide concentration was determined colorimetrically by the phenol–sulphuric acid method [105]. Briefly, 143 µl matrix extract was mixed with an equal volume of 5% phenol and 715 µl sulphuric acid, and incubated (25°C, 10 min). Absorption was measured at 492 nm using glucose as a standard.

**Staphyloxanthin extraction**

To extract staphyloxanthin from macrocolonies, the colonies were resuspended in PBS and dispersed mechanically. The OD$_{600}$ was recorded for normalization. Cells were collected by centrifugation, resuspended in 250 µl methanol, incubated (55°C, 3 min) and the supernatant collected. This procedure was repeated twice and supernatants pooled. Finally, the volume of the staphyloxanthin extract was adjusted to 1 ml and absorption at 463 nm measured. The OD$_{463}$/OD$_{600}$ ratio was calculated to determine relative staphyloxanthin production.

**Haemolysis assay**

Haemolysis was assessed in submerged biofilms in TSBMg after 24 h. An equal volume of 4% (v/v) sheep erythrocytes was added and incubated (37°C, 4 h). Cell components were removed by centrifugation of the plate (500 g, 15 min). Haem release from lysed blood cells was determined by quantifying supernatant absorption at OD$_{405}$.

**Galleria infection model**

Larvae of the wax moth *Galleria mellonella* were used as a non-vertebrate infection model [84]. Cultures of *S. aureus* were grown overnight in brain-heart infusion, diluted to OD$_{600}=0.05$, grown to OD$_{600}=0.6$ and washed twice with 10 mM MgSO$_4$. Optical density at 600 nm was adjusted to 0.3 and larvae were inoculated with 20 µl *S. aureus* using insulin pens. A control group received the same volume of buffer. The infection dose corresponded to 1.5×10$^6$ c.f.u., as determined by cell plating. Larvae were incubated (37°C) and survival was assessed after 24 and 48 h. Survival was scored based on melanization of the larvae and response to gentle tapping. Experiments were performed in triplicate with cohorts of 15 larvae.

**Statistical analysis**

Statistical analyses were performed using the software GraphPad Prism (Version 7). All graphs represent data from at least three independent experiments. Error bars represent the SD of the data (mean±SD). Pairwise statistical analyses were performed using unpaired Student’s *t*-test. Differences were considered statistically significant when the *P*-value was equal to or smaller than 0.05. Statistical significance: *P*≤0.05, **P*≤0.01, ***P*≤0.001.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


58. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ et al. A genetic resource for rapid and comprehensive phenotype screening of nonessential Staphylococcus aureus genes. MBio 2013;4:e00337-12.


95. Yoshioka S, Newell PD. Disruption of *de novo* purine biosynthesis in *Pseudomonas fluorescens* P1O-1 leads to reduced biofilm formation and a reduction in cell size of surface-attached but not planktonic cells. *PeerJ* 2016;4:e1543.


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