A functional menadione biosynthesis pathway is required for capsule production by *Staphylococcus aureus*

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**Abstract**

*Staphylococcus aureus* is a major human pathogen that utilises a wide array of pathogenic and immune evasion strategies to cause disease. One immune evasion strategy, common to many bacterial pathogens, is the ability of *S. aureus* to produce a capsule that protects the bacteria from several aspects of the human immune system. To identify novel regulators of capsule production by *S. aureus*, we applied a genome wide association study (GWAS) to a collection of 300 bacteraemia isolates that represent the two major MRSA clones in UK and Irish hospitals: CC22 and CC30. One of the loci associated with capsule production, the *menD* gene, encodes an enzyme critical to the biosynthesis of menadione. Mutations in this gene that result in menadione auxotrophy induce the slow growing small-colony variant (SCV) form of *S. aureus* often associated with chronic infections due to their increased resistance to antibiotics and ability to survive inside phagocytes. Utilising such an SCV, we functionally verified this association between *menD* and capsule production. Although the clinical isolates with polymorphisms in the *menD* gene in our collections had no apparent growth defects, they were more resistant to gentamicin when compared to those with the wild-type *menD* gene. Our work suggests that menadione is involved in the production of the *S. aureus* capsule, and that amongst clinical isolates polymorphisms exist in the *menD* gene that confer the characteristic increased gentamicin resistance, but not the major growth defect associated with SCV phenotype.

**INTRODUCTION**

As a successful human pathogen, *Staphylococcus aureus* has evolved many mechanisms to evade host immunity, including the production of a polysaccharide capsule that protects the bacteria from uptake and killing by phagocytes [1–3]. The enzymes responsible for the biosynthesis of this capsule are encoded within a multi-gene locus (*cap*) that has both highly conserved and variable genes responsible for the capsule serotypes [2, 4]. The importance of capsule production to the ability of *S. aureus* to cause disease has been demonstrated in many animal models, and as a result it was a target of an anti-staphylococcal vaccine attempt, albeit unsuccessful in clinical trials in humans [5]. Recent population-based analyses of human isolates may partially explain the lack of success of this vaccine in clinical trials, as it found significant variability in the amount of capsule produced by clinical isolates [6, 7]. Although there are associations between the levels of capsule production and increased patient mortality [8], that capsule negative variants are frequently isolated from patients suggests that capsule production is not critical for survival in humans or the ability to cause disease.

Using traditional molecular approaches, several regulators of the expression of the *cap* locus have been identified such as Agr and MgrA [9, 10]. However, the existence of variability across a collection of isolates can facilitate alternative approaches to the identification of novel regulators through the use of genome wide association (GWAS) approaches [8, 11–14]. These have the added benefit of allowing a greater understanding of the role and relevance of these regulators in the natural environment of the human host [8, 11]. A previous application of this approach to a collection of community-acquired methicillin resistant *S. aureus* (MRSA) USA300 isolates identified several conserved mutations within the *cap* locus as responsible for variability in capsule production [6]. Here, we sought to extend this approach to a collection of healthcare-acquired MRSA representing the two major clones.
circulating in UK and Irish hospitals, clonal complexes 22 and 30 (CC22, CC30).

We observed a high level of variability with regards to capsule production within our collection of clinical isolates. Interestingly, no polymorphisms within the *cap* locus were identified as associated with this phenotype, although several loci distal to the *cap* locus were associated with capsule production. One of these genes, *menD*, encodes an enzyme critical to the biosynthesis of menadione [15]. Mutations in this gene have been shown in many studies to be responsible for an alternative means utilized by *S. aureus* to both resist the effect of antibiotics and evade clearance by phagocytes by switching to the slow growing small colony variant (SCV) or persister phenotype [15–17]. The expression of many virulence factors is reduced when the bacteria switch to SCVs, including the production of cytolytic toxins [18]; however, there are contradictory reports on what effect this switch has on capsule production [19–21]. In this study we explore the link between the SCV phenotype and capsule production and conclude that the link is dependent upon the specific pathway that becomes mutated during the switch to the SCV form.

**METHODS**

**Bacterial strains and culture conditions**

Bacterial strains used are listed in Table S1 (available in the online version of this article). Bacterial strains were routinely stored at −80 °C in 15% glycerol/broth stocks until required. Unless stated otherwise, *S. aureus* strains were streaked onto Tryptic Soy agar (TSA) and single colonies transferred to 5 ml Tryptic Soy broth (TSB) in 50 ml tubes. All bacterial cultures were propagated in a shaking incubator for 18 h at 37 °C at 180 r.p.m.

**In vitro capsule production quantification**

To quantify capsule production, *S. aureus* strains were grown overnight on the surface of TSA plates at 37 °C. They were then transferred to nitrocellulose (NC) membranes and the membranes were placed bacteria side up in a clean petri dish and baked for 15 min at 60 °C. To remove excess bacteria from the filters, membranes were washed three times in PBS and the proteins removed by incubating the filters in trypsin solution for 1 h at 37 °C. Membranes were then rinsed and blocked in Bovine Serum Albumin (BSA) for 1 h, and washed three times in PBS with 0.05% Tween. The membranes were incubated for 1 h in diluted anti-cap antiserum 1 : 1000 - 1 : 3000 (5–15 µl:15 ml PBS) at room temperature with gentle agitation. Filters were washed three times for 3 min each with PBS/Tween. Protein G-HRP conjugate was diluted in PBS/Tween to a 1 : 5000 dilution and incubated for 1 h at room temperature with gentle agitation. The membranes were washed three times for 3 min each with PBS. Finally, the reactivity of the colonies was detected using the Opti-4CN Substrate Kit (BIORAD), according to manufacturer instructions. The clinical isolates were scored visually by three individuals as 0, 1 or 2, where 0 indicated no capsule detection, 1 a medium level of capsule detection and 2 a high level of capsule detection (Table S1).

**GWAS**

Genome-wide association mapping was conducted using a generalized linear model, with capsule production as the quantitative response variable. We accounted for bacterial population substructure by adding to the regression model the first two components from a principal component decomposition of SNP data for each set of clinical samples (CC22 and CC30). The first two components accounted for 32 and 40% of the total variance for CC22 and CC30, respectively. In both cases, three distinct clusters were identified. We further considered a third model where we used cluster membership as covariates in our regression model, where clusters were

### Table 1. Oligonucleotide primers used in this study

| Primer | Sequence (5’ → 3’ end) |
|--------|------------------------|
| RT capE F | ACATTGGTGATGTGCGTGAT |
| RT capE R | TCACATGACGGGACTTGTTT |
| RT gyrA F | CCAGGTAATTAGCGGATTGC |
| RT gyrA R | AAATCGCCTGGTTCTAGAG |

**Fig. 1.** Capsule production varies significantly across clinical bacteraemia isolates. Immunoblots of *S. aureus* isolates were performed with either anti-CP5 or anti-CP8 antiserum. Blots of isogenic CP5 and CP8 wild-type and cap- mutant were performed as controls (top row). Ten CC22 (rows 2 and 3) and 10 CC30 (rows 4 and 5) isolates representative of the variability in intensity of anti-capsule anti-serum binding are presented. The CC22s were probed with the anti-CP5 antiserum and the CC30s with the anti-CP8 antiserum.
defined using K-means clustering analysis (setting K=3); this, however, yielded identical results to the one based on PCA components. In total, 2066 (CC22) and 3189 (CC30) unique SNPs were analysed, the majority of which were subsequently filtered out for exhibiting a minor allele frequency (maf) of <0.03, reducing the data to 378 and 1124 SNPs, respectively. Reported P-values are not corrected for multiple comparisons; Sidak corrected significance thresholds are indicated in the Manhattan plots.

mRNA extraction

The bacteria were grown in TSB at 37 °C in a shaking incubator for 18 h. RNA was extracted by Quick-RNA Fungal/Bacterial Miniprep Kit (Zymo Research) according to the manufacturer’s instructions. RNA integrity was checked by running a 5 µl aliquot of the RNA on a 1% agarose gel and observing the intensity of the ribosomal RNA (rRNA). RNA samples were treated by TURBO DNase (Invitrogen) to eliminate any genomic DNA contamination. To verify that the samples were free from any DNA contamination, RNA samples were subjected to RT-qPCR with a no template control (NTC) and 2.5 ng of a known genomic DNA, and threshold rates were compared.

Quantitative reverse transcriptase (RT-qPCR)

To quantify the expression of the capE gene of the wild-type and the mutants, RT-qPCR was performed using gyrB as a reference gene. Complementary DNA (cDNA) was generated from mRNA using a qScript cDNA Synthesis Kit following the manufacture’s (Quantabio) protocol, and the cDNA was used as a template for the qPCR reaction. Primers used are listed in Table 1. The reverse-transcriptase PCR (RT-PCR) was performed as follows: 10 µl 2x SensiFAST SYBR Mix, 0.8 µl of 10 µM forward primer, 0.8 µl of 10 µM reverse primer, 1 µl cDNA and RNase-free water up to a total of 20 µl volume. The PCR cycles consisted of initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 60 s and extension at 72 °C for 10 s. RT-PCR was carried out in triplicate for each sample and ≥3 biological repeats using the primers listed in Table 1. The ratio of capE and gyrB transcript number was calculated using the using the $2^{-\Delta\Delta CT}$ method.

Selection and verification of SCV strains

S. aureus strain Newman was grown in TSB at 37 °C in a shaking incubator overnight. The culture was diluted 1/10 into TSB with 2 µg ml$^{-1}$ gentamicin and incubated for 8 h. The resulting culture was then plated on blood agar containing 2 µg ml$^{-1}$ gentamicin. Pin-prick sized colonies were further isolated by streaking onto fresh agar plates with 2 µg ml$^{-1}$ gentamicin. Auxotrophy to both menadione and hemin was examined by placing a filter disc saturated in these growth reagents onto a freshly inoculated lawn of the purified SCV colonies, and enhanced growth surrounding the disc visually examined.

RESULTS AND DISCUSSION

Capsule production varies across closely related S. aureus bacteraemia isolates

Recent work has suggested that there is significant variability amongst clinical S. aureus isolates in the amount of

![Fig. 2. S. aureus loci associated with capsule production. Manhattan plots representing the results of a GWAS analysis identifying polymorphic loci associated with the level of capsule produced by (a) 136 CC22 and (b) 159 CC30 bacteraemia isolates. The x-axes represent the genomic position of the polymorphisms relative to the origin of replication and the y-axes represent the strength of the association with capsule production. Uncorrected (P<0.05) and multiple tests corrected (P<1.3x10^{-4}, for CC22; and P<4.5x10^{-5} for CC30s) significance thresholds are indicated as blue and red lines, respectively.](image-url)
Table 2. Loci associated with capsule production in the CC22 collection of *S. aureus* isolates

| Gene or locus tag | Protein function | *P* value |
|------------------|------------------|-----------|
| SAEMRSA15_RS00260 | type I restriction endonuclease subunit R | 0.00014021 |
| Intergenic between SAEMRSA15_RS13970 and cjB | 0.00034659 |
| SAEMRSA15_RS11275 | thiol-disulfide oxidoreductase DCC family protein | 0.00034659 |
| SAEMRSA15_RS12900 | NAD(P)-dependent oxidoreductase | 0.00034659 |
| SAEMRSA15_RS01030 | type I glutamine amidotransferase | 0.00034659 |
| SAEMRSA15_RS00265 | hypothetical protein | 0.00034659 |
| SAEMRSA15_RS08245 | acetyl-CoA carboxylase biotin carboxylase subunit | 0.00034659 |
| SAEMRSA15_RS10695 | LytTR family DNA-binding domain-containing protein | 0.00075253 |
| SAEMRSA15_RS10690 | GHKL domain-containing protein | 0.00077225 |
| SAEMRSA15_RS02555 | RNA polymerase sigma factor | 0.00120048 |
| ileS | isoleucine--tRNA ligase | 0.00139006 |
| SAEMRSA15_RS12840 | glycerate kinase | 0.00280998 |
| SAEMRSA15_RS11120 | ATP synthase subunit I | 0.0030996 |
| SAEMRSA15_RS02630 | amidohydrolase | 0.00366703 |
| SAEMRSA15_RS12955 | APC family permease | 0.00386528 |
| SAEMRSA15_RS13455 | LrgB family protein | 0.00400753 |
| menD | 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid synthase | 0.00507518 |
| SAEMRSA15_RS09800 | metal-dependent hydrolase | 0.00516556 |
| gvrR | response regulator transcription factor GraR/ApsR | 0.00546077 |
| rsmG | 16S RNA (guanine(527)-N(7))-methyltransferase RsmG | 0.00895798 |
| Intergenic between SAEMRSA15_RS08855 and SAEMRSA15_RS08860 | 0.01032749 |
| Intergenic between SAEMRSA15_RS01390 and brnQ | 0.01032749 |
| sbnC | staphyloferrin B biosynthesis protein SbnC | 0.01032749 |
| SAEMRSA15_RS03060 | WecB/TagA/CpsF family glycosyltransferase | 0.0108 |
| Intergenic between gnuC and SAEMRSA15_RS06385 | 0.01141554 |
| SAEMRSA15_RS04275 | Glu/Leu/Phe/Val dehydrogenase | 0.01534142 |
| SAEMRSA15_RS14050 | amidase domain-containing protein | 0.01534142 |
| SAEMRSA15_RS04670 | ATP-binding cassette domain-containing protein | 0.01534142 |
| SAEMRSA15_RS08370 | histidine--tRNA ligase | 0.01534142 |
| SAEMRSA15_RS08350 | replication-associated recombination protein A | 0.01534142 |
| Intergenic between SAEMRSA15_RS02335 and tilS | 0.01534142 |
| SAEMRSA15_RS14225 | ATP phosphoribosyltransferase | 0.01534142 |
| SAEMRSA15_RS11600 | hypothetical protein | 0.01534142 |
| SAEMRSA15_RS09105 | MFS transporter | 0.01577408 |
| SAEMRSA15_RS11705 | energy-coupling factor transporter ATPase | 0.01652847 |

Continued
capsule they produce [6, 7]. Given the importance of capsule in protecting the bacteria from many aspects of the human immune system, we sought to examine the variability of this in isolates from invasive disease, where the anti-bacterial effects of the immune system should be the most stringent. We focussed on a collection of isolates from 300 cases of bacteraemia, representing both the two major clones of MRSA strains circulating in UK and Irish hospitals (CC22 and CC30), as well as the two major capsule serotypes of *S. aureus* that cause disease in humans (capsule type 5 (CP5) and type 8 (CP8)). We performed a semi-quantification of capsule production by each isolate using anti-CP5 (for the CC22 isolates) and anti-CP8 (for the CC30 isolates) antiserum. The reactivity of the antisera was demonstrated using a pair of wild-type and capsule negative isogenic mutants (Fig. 1). Across the clinical bacteraemia isolates there was significant variability in capsule production, with the majority of isolates producing abundant capsule (57%), 20% producing moderate levels of capsule and with no detectable capsule being produced by 23% of the isolates (Fig. 1, Table S1).

The genetic basis of the variability in capsule production is multifactorial

As the genome sequence for each of the 300 clinical *S. aureus* isolates was available, we performed a GWAS (genome wide association study) to identify polymorphic loci that associated with the level of capsule produced by the isolates. For this, the data from the two distinct clones were analysed independently, with population structure within the clones being accounted for (Fig. 2, Tables 2 and 3). We applied both uncorrected and corrected (for multiple comparisons) significance thresholds to this analysis, as our previous work has demonstrated that the stringency of multiple correction approaches increases the likelihood of type II errors or false negative results. Only one locus was found associated at the multiple test corrected significance threshold: the *agrC* gene, which is part of a well-established regulatory system of many virulence factors including capsule [8, 9, 11], providing good proof of concept for this approach. A further 169 loci were found associated with capsule production at the $P<0.05$ significance threshold, including two genes, in which mutations result in the

| Gene or locus tag   | Protein function                          | $P$ value |
|--------------------|-------------------------------------------|-----------|
| SAEMRSA15_RS13060  | hypothetical protein                      | 0.01671052|
| *sdhA*             | succinate dehydrogenase flavoprotein subunit | 0.01836527|
| *fabD*             | ACP S-malonyltransferase                  | 0.01905729|
| *ribB*             | 3,4-dihydroxy-2-butane-4-phosphate synthase | 0.02115675|
| SAEMRSA15_RS13160  | DUF3427 domain-containing protein         | 0.02147993|
| SAEMRSA15_RS02320  | nucleotide pyrophosphohydrolase           | 0.02182662|
| Intergenic between brnQ and SAEMRSA15_RS00800 |                           | 0.02388376|
| *radA*             | DNA repair protein RadA                   | 0.02388376|
| SAEMRSA15_RS02405  | tRNA-Lys                                  | 0.02388376|
| SAEMRSA15_RS01035  | PrsW family intramembrane metalloprotease | 0.0245801 |
| SAEMRSA15_RS01275  | ABC transporter permease                  | 0.02728306|
| SAEMRSA15_RS07140  | hypothetical protein                      | 0.02954242|
| *pyk*              | pyruvate kinase                           | 0.0303263 |
| SAEMRSA15_RS11310  | hypothetical protein                      | 0.03044146|
| *feoB*             | ferrous iron transport protein B          | 0.03071363|
| SAEMRSA15_RS02010  | YbcC family protein                       | 0.03520898|
| SAEMRSA15_RS01135  | CDP-glycerol glycerophosphotransferase family protein | 0.03607997|
| *dltB*             | PG:teichoic acid d-alanyltransferase DltB | 0.03944867|
| SAEMRSA15_RS05405  | YfcC family protein                       | 0.04425032|
| SAEMRSA15_RS05040  | DUF4064 domain-containing protein         | 0.04807582|
| SAEMRSA15_RS03910  | thermonuclease family protein             | 0.04901599|
### Table 3. Loci associated with capsule production in the CC30 collection of S. aureus isolates

| Gene or locus tag | Protein function                                                                 | \(P\) value          |
|------------------|----------------------------------------------------------------------------------|-----------------------|
| agrC             | autoinducer sensor protein                                                       | 4.06×10\(^{-5}\)      |
| SAR1756          | hypothetical protein                                                             | 0.000610781           |
| kdpA             | putative potassium-transporting ATPase a chain                                    | 0.00079868            |
| locB             | 3-isopropylmalate dehydrogenase                                                  | 0.00079868            |
| SAR2555          | conserved hypothetical protein                                                    | 0.001102934           |
| Intergenic between sarD and SAR0791 |                                                                              | 0.002038995         |
| cpbA             | catabolite control protein A                                                     | 0.005730767           |
| SAR2382          | putative transcriptional regulator                                               | 0.005772766           |
| SAR2759          | putative aminotransferase-putative imidazoleglycerol-phosphate dehydratase       | 0.005813137           |
| SAR1218          | putative membrane protein                                                        | 0.007268775           |
| SAR0457a         | hypothetical protein                                                             | 0.008232346           |
| SAR2533          | putative ketopantoate reductase                                                   | 0.008799302           |
| SAR0109          | putative transporter protein                                                      | 0.009570567           |
| jycG             | Two-component regulatory system family, sensor kinase protein.                   | 0.010100134           |
| thiK             | putative thiamine-phosphate pyrophosphorylase                                    | 0.010702445           |
| fabD             | ACP S-malonyltransferase                                                         | 0.011824195           |
| SAR1343          | amino acid permease                                                             | 0.01386841            |
| SAR2522          | putative glycerate kinase                                                        | 0.01579356            |
| SAR1674          | putative GTPase                                                                  | 0.018539766           |
| SAR0112          | putative transport protein                                                       | 0.018645214           |
| SAR1668          | conserved hypothetical protein                                                    | 0.019293181           |
| ilvA             | threonine dehydratase biosynthetic                                               | 0.020495129           |
| dfrB             | dihydrofolate reductase type 1                                                   | 0.021075917           |
| Intergenic between polS and proC |                                                                              | 0.022922752         |
| SAR2025          | putative ABC transporter ATP-binding protein                                     | 0.02367155            |
| SAR0573          | hypothetical protein                                                             | 0.023724286           |
| SAR1619          | putative exported protein                                                        | 0.023978842           |
| SAR0743          | putative sodium-sulphate symporter protein                                       | 0.02413635            |
| atsR2            | arsenical resistance operon repressor 2                                          | 0.024274209           |
| SAR0108          | putative peptidase                                                              | 0.024560717           |
| SAR0559          | putative aminotransferase                                                        | 0.02466874            |
| SAR1002          | putative membrane protein                                                        | 0.02622901            |
| SAR0942          | putative membrane protein                                                        | 0.026651981           |
| SAR2740          | conserved hypothetical protein                                                    | 0.026988847           |
| ureC             | urease alpha subunit                                                            | 0.027343322           |
| qoxB             | putative quinol oxidase polypeptide I                                            | 0.028188487           |
| mnhD             | Na+/H+antiporter subunit                                                        | 0.030064463           |
| SAR2534          | putative transport protein                                                       | 0.030662069           |
| SAR2779          | putative N-acetyltransferase                                                     | 0.031463295           |
| SAR1684          | conserved hypothetical protein                                                   | 0.031847864           |

Continued
| Gene or locus tag | Protein function | P value     |
|------------------|------------------|-------------|
| SAR1699          | conserved hypothetical protein | 0.031847864 |
| SAR1995          | putative lipoprotein | 0.031847864 |
| SAR0463          | putative lipoprotein | 0.031847864 |
| SAR0010          | putative membrane protein | 0.033712843 |
| SAR0245          | putative zinc-binding dehydrogenase | 0.035487178 |
| ureE             | urease accessory protein | 0.035624064 |
| adlA             | 2-oxoglutarate dehydrogenase E1 component | 0.036566931 |
| SAR2186          | conserved hypothetical protein | 0.037233673 |
| hisB             | putative imidazoleglycerol-phosphate dehydratase | 0.037571061 |
| SAR0291          | putative membrane protein | 0.038246027 |
| SAR1876          | hypothetical protein | 0.038551026 |
| SAR1703          | putative oxygenase | 0.039218922 |
| SAR1655          | putative methyltransferase | 0.039424074 |
| SAR2464          | TetR family regulatory protein | 0.039519902 |
| SAR0987          | conserved hypothetical protein | 0.039519902 |
| SAR1868          | aldo/keto reductase family protein | 0.039519902 |
| SAR0466          | MutT domain containing protein | 0.039519902 |
| SAR0278          | putative exported protein | 0.039519902 |
|Intergenic between rsbU and SAR2156 |                     | 0.039519902 |
| ldh1             | l-lactate dehydrogenase 1 | 0.039519902 |
| mvaD             | mevalonate diphosphate decarboxylase | 0.039519902 |
| SAR0699          | conserved hypothetical protein | 0.039519902 |
| SAR1973          | putative membrane protein | 0.039519902 |
| SAR2427          | putative zinc-binding dehydrogenase | 0.039519902 |
| SAR0770          | conserved hypothetical protein | 0.039519902 |
| SAR2619          | thiamine pyrophosphate enzyme | 0.039519902 |
| SAR1281          | conserved hypothetical protein | 0.039519902 |
| SAR0655          | putative Na + dependent nucleoside transporter | 0.039519902 |
| SAR1332          | response regulator | 0.039519902 |
| SAR2588          | putative membrane protein | 0.039519902 |
| SAR1165          | hypothetical protein | 0.039519902 |
| SAR1221          | putative CoA synthetase protein | 0.039519902 |
|Intergenic between SAR0994 and tRNA-Ser |                     | 0.039519902 |
| SAR2006          | conserved hypothetical protein | 0.039519902 |
| SAR0836          | putative membrane protein (pseudogene) | 0.039519902 |
| SAR2780          | putative membrane protein | 0.039519902 |
| SAR1141          | Similar to Staphylococcus aureus exotoxin | 0.039519902 |
| SAR1670          | conserved hypothetical protein | 0.039519902 |
| SAR1685          | putative biotin carboxylase subunit of acetyl-CoA carboxylase | 0.039519902 |
| lysS             | lysyl-tRNA synthetase | 0.039519902 |

Intergenic between lysS and SAR1413 | 0.039519902 |
switching of *S. aureus* to the small colony variant (SCV) or persister phenotype: *fabD* and *menD*. SCVs auxotrophic for fatty acids that are more resistant to FAS-II inhibitors, such as triclosan, are associated with mutations in the *fabD* and *fabI* genes [22, 23]. SCVs auxotrophic for menadione are more resistant to aminoglycoside antibiotics, such as gentamicin, and are associated with mutations in the *menD* gene [15, 16].

### Functional verification of the role of *menD* in capsule production

There are contradictory reports in the literature on the effect the switch to SCV has on capsule production [19–21], and as such we sought to resolve these contradictions by verifying our GWAS findings with a focus on the *menD* gene. The *menD* gene encodes an enzyme involved in the biosynthesis of menadione, which is a vitamin K2 precursor that is
synthesised by *S. aureus* [15]. The importance of menadione for efficient respiration by the bacteria is such that inactivation of the gene results in a slow-growing small colony variant (SCV) phenotype [15, 16]. There are other metabolic pathways that can mutate and result in an SCV phenotype such as in the hemin biosynthesis pathway [18], and collectively the SCV phenotype is associated with significant changes in *S. aureus* virulence, in particular with regards to reduced toxin production [18].

Given the association between polymorphisms in the *menD* gene and capsule production, we sought to examine this in further detail. SCVs were selected from a culture *S. aureus* strain Newman by overnight growth in gentamicin (2 µg ml⁻¹), on the basis of their enhanced resistance to the aminoglycoside class of antibiotics. Of these SCVs we identified a menadione auxotrophic SCV, as well as a hemin auxotrophic SCV as a comparator, by restoring the growth defect through the addition of either menadione or hemin on a disc (Fig. 3a).

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**Fig. 3.** Capsule production is affected in a menadione auxotrophic SCV. (a) *menD* and *hemB* SCVs of *S. aureus* strain Newman were selected, and auxotrophy to menadione and hemin determined by examining enhanced growth of the SCV when the medium was supplemented with a disc containing the respective growth reagent. (b and c) Immunoblotting of the wild-type Newman and the *menD* and *hemB* SCVs demonstrate that the capsule production is only affected in the menadione auxotrophic SCV. (d) Transcription of the *capE* gene is lower in the menadione-auxotrophic SCV relative wild-type Newman, but not in the hemin auxotroph.

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**Fig. 4.** The *S. aureus* MenD amino acid sequence. The effect of the non-synonomous polymorphism present in the CC30 (indicated in blue font) and CC22 (in red font) collection of isolates studied here are indicated. The mutation responsible for the menadione auxotrophic SCV phenotype of strain Newman is highlighted in yellow (K253:STOP).

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G
MGNHKAALTKQVFTFASELYAYGVREVVISPGSRTPLALAFEAHPNIKTWIHDPERSAA
FFAVGLIKGSRPVAILCTSGTAAANYTPAIAESQISRIPLIVLTSDRPHELRSGAPQA
S
INQVNMFNNYVSYEFDMPIADDSKETIDAIYYQMIAQYLYGPKGPLHFNLPFRDPLT
M
PDLNATELTTSEMKLPHYKQSIDASALRHINKKGLIIIVGDQMHEVDQILTYSTIYD
STOP
T
G
LPILADPLSRLRKFDPHNVICTYDLFRRSGDLNVDFVIRVGKPVISKLNLQWLKKTDAF
Y
QILVQNNNDKIVFPIAPDISYEISANDFRRSLMEDTTINRVSWLEKQREKGRKEIKC
Y
YLEQTADSAFVGELIKKTSEKDALFISNSMIPRDVDNLLLNKIDVYANRGANGIDGIV
STALGMAVHRKRTLLIGDLFSFYHDMNGLLLMSKNNIQMIVLLNNDDGGGFYSYLQPKE
C
A
TDYFERLFGTPTGLDFEYAKLYQFDKRFNSVSEFKNATLLSETSTIYELITNREDNFK
QHQLYQKLSEMIHDTL
The menD gene in the menadione auxotrophic SCV was sequenced where we found a K253STOP substitution to be responsible for the SCV phenotype (Fig. 4). We performed immunoblots of the wild-type strain Newman and the SCVs, where there was a significant effect on capsule production for the menadione auxotrophic SCV but not the hemin auxotrophic SCV (Fig. 3b, c). The effect on capsule production by the menD SCV was restored by growing the bacteria in the presence of exogenous menadione (Fig. S1). To further examine the effect on capsule production, we quantified the transcription of the capE gene, where we found this to be significantly reduced in the menadione auxotroph, but not the hemin auxotroph (Fig. 3d). While further work is underway to examine the effect mutations in fabD and triclosan resistance has on capsule production, here we have verified the observed association between the menD gene and capsule production. The discrepancy between the levels of capsule production by the hemB and menD SCVs may also explain some of the discrepancy in the literature in relation to capsule production by SCVs, in that the effect is dependent upon the pathway that becomes mutated.

The menD polymorphisms in the clinical isolates do not affect growth but do increase resistance to gentamicin

Having demonstrated that capsule production is affected in the menadione auxotrophic SCVs, we examined whether the isolates with polymorphisms within our collections of bacteraemia isolates also had the SCV phenotype. There were nine isolates with non-synonymous polymorphism in the menD gene, and the position and effect of the SNPs on the amino acids sequence are illustrated in Fig. 4. We selected at random nine isolates from the collection with the non-polymorphic menD gene (i.e. identical to the respective reference strains MRSA252 [24] and HO 5096 0412 [25]). These isolates were grown in TSB with and without 2 µg ml⁻¹ of gentamicin to examine the two main features of SCVs, slow growth and increased resistance to gentamicin. We found that the clinical menD variants grew as well as those with the reference menD gene in TSB, demonstrating that they have no growth defect in vitro, perhaps as a result of compensatory mutations [26]. However, in the presence of gentamicin we found that the variants had a growth advantage, which suggests they have a partial SCV phenotype, at least with respect to their enhanced resistance to this antibiotic (Fig. 5a). The addition of menadione restored the sensitivity of the menD variants to gentamicin. To further verify the association between the variant menD gene and capsule production in the clinical isolates, we cloned the gene into the pRMC2 expression plasmid and introduced this into the clinical isolate ASARM59. This had the effect of increasing capsule production in this isolate (Fig. 5b, c).

In summary, in this study we have identified novel putative effectors of capsule production by S. aureus, including the menadione biosynthesis pathway. In doing so, we have resolved an apparent contradiction in the literature with respect to the effect that the switch from normal growth to the SCV form has on capsule production. We found that this crucially depends on which metabolic pathway has been mutated to result in the switch. What is intriguing is that all isolates studied here were from cases of bacteraemia, and despite the importance of capsule production to the...
protection of the bacteria from many aspects of the human immune system, we found that among one in five isolates do not express capsule to any detectable levels. It is possible that the loss of capsule coincides with enhanced antibiotic resistance, as we have observed here for mutations in menD. With further investigation we may find that mutations of the other associated loci also confer advantages to the bacteria that over-ride the costs associated with the loss of capsule. But what is clear is that even within a clone, S. aureus is highly adaptable and diverse in its means of causing disease, which may explain our lack of success in producing an effective vaccine using capsule as its major target.

Author contributions
D.A., developed the methodology, performed experiments, analysed data and contributed to writing the manuscript. T.B., provided supervisory support, analysed data and contributed to writing the manuscript. R.C.M. is a Wellcome Trust funded Investigator (grant reference number: 212258/Z/18/Z). This work was funded by a PhD studentship to D.A. funded by the Saudi Arabian Cultural Bureau. R.C.M. is a Wellcome Trust funded Investigator (grant reference number: 212258/Z/18/Z).

Conflicts of interest
The authors declare that there are no conflicts of interest.

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