MICROBIOLOGY

SHORT COMMUNICATION

Altwiley *et al.*, *Microbiology* 2021;167:001108

DOI 10.1099/mic.0.001108

MICROBIOLOGY SOCIETY

OPEN ACCESS

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

Dina Altwiley[1,2], Tarcisio Brignoli[1], Andrew Edwards[3], Mario Recker[4], Jean C. Lee[5] and Ruth C. Massey[1,6,*]

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

Received 02 June 2021; Accepted 16 September 2021; Published 26 November 2021

Author affiliations: [1]School of Cellular and Molecular Medicine, University of Bristol, BS8 1TD, UK; [2]University of Jeddah, Saudi Arabia; [3]MRC Centre for Molecular Bacteriology and Infection, Imperial College London, London, SW7 2AZ, UK; [4]Centre for Ecology and Conservation, University of Exeter, Penryn Campus, TR10 9FE, UK; [5]Division of Infectious Diseases, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA; [6]Schools of Microbiology and Medicine, and APC Microbiome Ireland, University College Cork, Ireland.

***Correspondence:** Ruth C. Massey, ruth.massey@bristol.ac.uk

Keywords: capsule; menadione; persisters; SCVs; small colony variants; *Staphylococcus aureus*.

One supplementary table and one supplementary figure are available with the online version of this article.

001108 © 2021 The Authors

Table 1. Oligonucleotide primers used in this study

Primer	Sequence Sequence (5′ → 3′ end)
RT *capE* F	ACATTGGTGATGTGCGTGAT
RT *capE* R	TCACATGACGGCACTTGTTT
RT *gyrA* F	CCAGGTAAATTAGCCGATTGC
RT *gyrA* R	AAATCGCCTGCGTTCTAGAG

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.

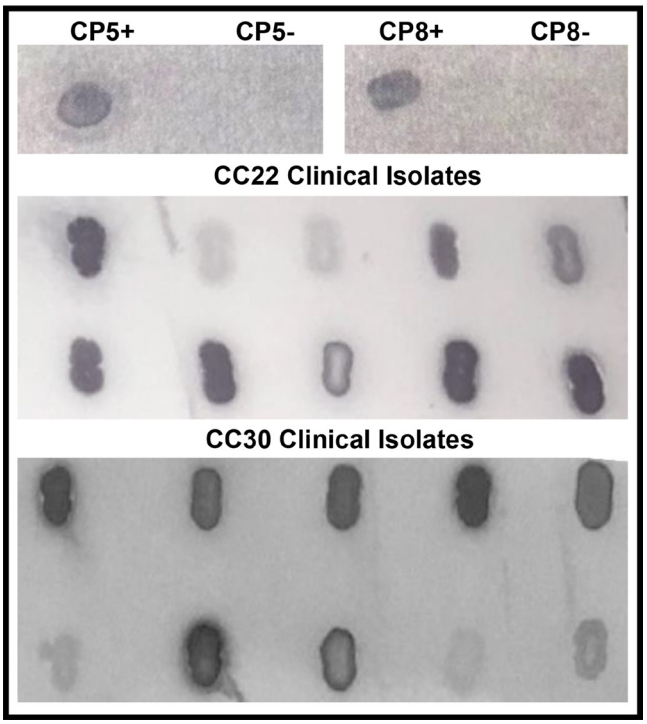

Fig. 1. Capsule production varies significantly across clinical bacteraemia isolates. Immunoblots of *S. aureus* isolates were performed with either anti-CP5 or anti-CP8 antiserum. Blots of isogenic CP5 and CP8 wild-type and cap- mutant were performed as controls (top row). Ten CC22 (rows 2 and 3) and 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.

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

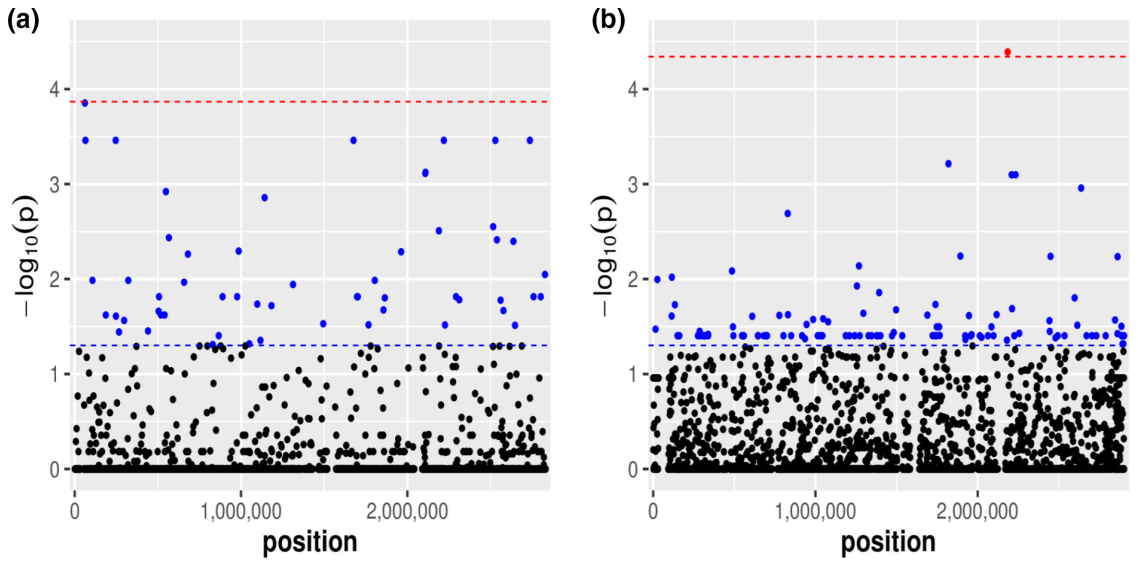

Fig. 2. *S. aureus* loci associated with capsule production. Manhattan plots representing the results of a GWAS analysis identifying polymorphic loci associated with the level of capsule produced by (a) 136 CC22 and (b) 159 CC30 bacteraemia isolates. The *x*-axes represent the genomic position of the 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 ($P<1.3\times10^{-4}$, for CC22; and $P<4.5\times10^{-5}$ for CC30s) significance thresholds are indicated as blue and red lines, respectively.

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 µl 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 $2^{-(\Delta Ct\ ply\ -\ \Delta Ct\ recA)}$ 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

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 *clfB*		0.00034659
SAEMRSA15_RS11275	thiol-disulfide oxidoreductase DCC family protein	0.00034659
SAEMRSA15_RS12900	NAD(P)-dependent oxidoreductase	0.00034659
SAEMRSA15_RS01030	type 1 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
graR	response regulator transcription factor GraR/ApsR	0.00546077
rsmG	16S rRNA (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 *guaC* 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

Table 2. Continued

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-butanone-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

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

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
leuB	3-isopropylmalate dehydrogenase	0.00079868
SAR2555	conserved hypothetical protein	0.001102934
Intergenic between *sstD* and SAR0791		0.002038995
ccpA	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	0.008232546
SAR2533	putative ketopantoate reductase	0.008799502
SAR0109	putative transporter protein	0.009570567
yycG	Two-component regulatory system family, sensor kinase protein.	0.010100134
thiE	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
SAR0122	putative transport protein	0.018645214
SAR1668	conserved hypothetical protein	0.019293181
ilvA	threonine dehydratase biosynthetic	0.020495129
dfrB	dihydrofolate reductase type I	0.021075917
Intergenic between *polS* and *proC*		0.022922752
SAR2025	putative ABC transporter ATP-binding protein	0.02367155
SAR0793	hypothetical protein	0.023724266
SAR1619	putative exported protein	0.023978842
SAR0743	putative sodium:sulphate symporter protein	0.02413635
arsR2	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	0.026988447
ureC	urease alpha subunit	0.027434322
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

Table 3. 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 UreE	0.035624064
odhA	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	0.039515902
SAR0987	conserved hypothetical protein	0.039515902
SAR1868	aldo/keto reductase family protein	0.039515902
SAR0466	MutT domain containing protein	0.039515902
SAR0278	putative exported protein	0.039515902
Intergenic between *rsbU* and SAR2156		0.039515902
ldh1	L-lactate dehydrogenase 1	0.039515902
mvaD	mevalonate diphosphate decarboxylase	0.039515902
SAR0969	conserved hypothetical protein	0.039515902
SAR1973	putative membrane protein	0.039515902
SAR0247	putative zinc-binding dehydrogenase	0.039515902
SAR0770	conserved hypothetical protein	0.039515902
SAR2619	thiamine pyrophosphate enzyme	0.039515902
SAR1281	conserved hypothetical protein	0.039515902
SAR0655	putative Na +dependent nucleoside transporter	0.039515902
SAR1332	response regulator	0.039515902
SAR2588	putative membrane protein	0.039515902
SAR1165	hypothetical protein	0.039515902
SAR1221	putative CoA synthetase protein	0.039515902
Intergenic between SAR0994 and tRNA-Ser		0.039515902
SAR2006	conserved hypothetical protein	0.039515902
SAR0636	putative membrane protein (pseudogene)	0.039515902
SAR2780	putative membrane protein	0.039515902
SAR1141	Similar to Staphylococcus aureus exotoxin	0.039515902
SAR1670	conserved hypothetical protein	0.039515902
SAR1685	putative biotin carboxylase subunit of acetyl-CoA carboxylase	0.039515902
lysS	lysyl-tRNA synthetase	0.039515902
Intergenic between *lysS* and SAR1413		0.039515902

Continued

Table 3. Continued

Gene or locus tag	Protein function	*P* value
Intergenic between SAR1326 and SAR1327		0.039515902
pbp4	penicillin-binding protein 4	0.039515902
SAR0880	conserved hypothetical protein	0.039515902
Intergenic between *ehb* and SAR1448		0.039515902
vraD	ABC transporter ATP-binding protein	0.039515902
SAR1265	putative pyruvate flavodoxin/ferredoxin oxidoreductase	0.039515902
SAR0147	putative nucleotidase	0.039515902
Intergenic between *sodM* and *sasG*		0.039515902
SAR1193	hypothetical protein	0.039515902
SAR0509	putative RNA binding protein	0.039515902
Intergenic between SAR1932 and SAR1933		0.039515902
SAR0810	putative phosphohydrolase	0.039515902
Intergenic between *rpmH* and *dnaA*		0.039515902
bglA	6-phospho-beta-glucosidase	0.039515902
Intergenic between SAR1870 and a SAM riboswitch		0.039515902
cysJ	putative sulfite reductase [NADPH] flavoprotein alpha-component	0.039515902
SAR2424	putative aldose 1-epimerase	0.039515902
pta	putative phosphate acetyltransferase	0.039515902
SAR0269a	hypothetical protein	0.039515902
tRNA-Thr	tRNA Thr anticodon TGT, Cove score 85.17	0.039515902
SAR1352	putative transketolase	0.039515902
SAR0290	hypothetical protein	0.039515902
crtN	squalene synthase	0.039515902
SAR1840	putative exported protein	0.039630048
SAR1941	RNA pseudouridylate synthase	0.041094881
SAR2411	putative transport protein	0.041580291
SAR0900	putative pyridine nucleotide-disulphide oxidoreductase	0.042472995
folC	putative folylpolyglutamate synthase	0.042664521
sasC	putative surface anchored protein	0.043300254
SAR2119	membrane anchored protein	0.044028342
SAR1279	conserved hypothetical protein	0.047982904
gidB	putative glucose inhibited division protein B	0.048056799
SAR2787	hypothetical protein	0.048327331

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

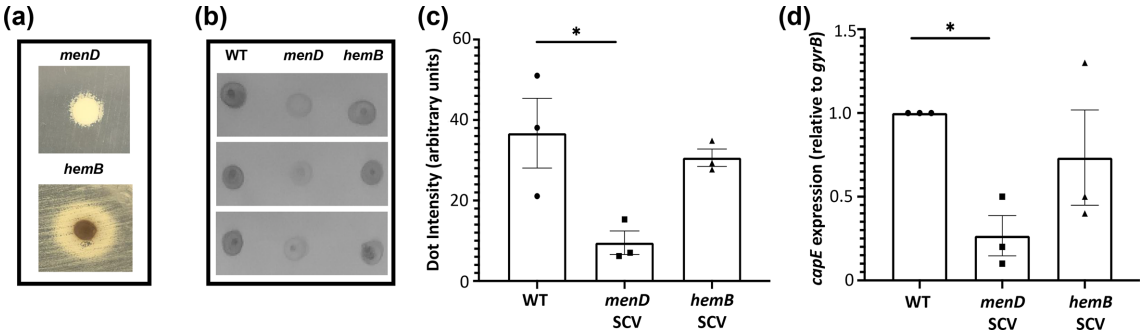

Fig. 3. Capsule production is affected in a menadione auxotrophic SCV. (a) *menD* and *hemB* SCVs of *S. aureus* strain Newman were selected, and auxotrophy to menadione and hemin determined by examining enhanced growth of the SCV when the medium was supplemented with a disc containing the respective 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.

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).

```
                                 G
MGNHKAALTKQVFTFASELYAYGVREVVISPGSRSTPLALAFEAHPNIKTWIHPDERSAA

FFAVGLIKGSERPVAILCTSGTAAANYTPAIAESQISRIPLIVLTSDRPHELRSVGAPQA

                                                        S
INQVNMFNNYVSYEFDMPIADDSKETIDAIYYQMQIASQYLYGPHKGPIHFNLPFRDPLT

                                                          M
PDLNATELLTSEMKILPHYQKSIDASALRHILNKKKGLIIVGDMQHQEVDQILTYSTIYD
          STOP       T       G
LPILADPLSHLRKFDHPNVICTYDLLFRSGLDLNVDFVIRVGKPVISKKLNQWLKKTDAF
        Y                   V
QILVQNNDKIDVFPIAPDISYEISANDFFRSLMEDTTINRVSWLEKWQRLEKKGRKEIKC

YLEQATDESAFVGELIKKTSEKDALFISNSMPIRDVDNLLLNKNIDVYANRGANGIDGIV

STALGMAVHKRITLLIGDLSFYHDMNGLLMSKLNNIQMNIVLLNNDGGGIFSYLPQKESA
         C                      V
TDYFERLFGTPTGLDFEYTAKLYQFDFKRFNSVSEFKNATLLSETSTIYELITNREDNFK

QHQILYQKLSEMIHDTL
```

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).

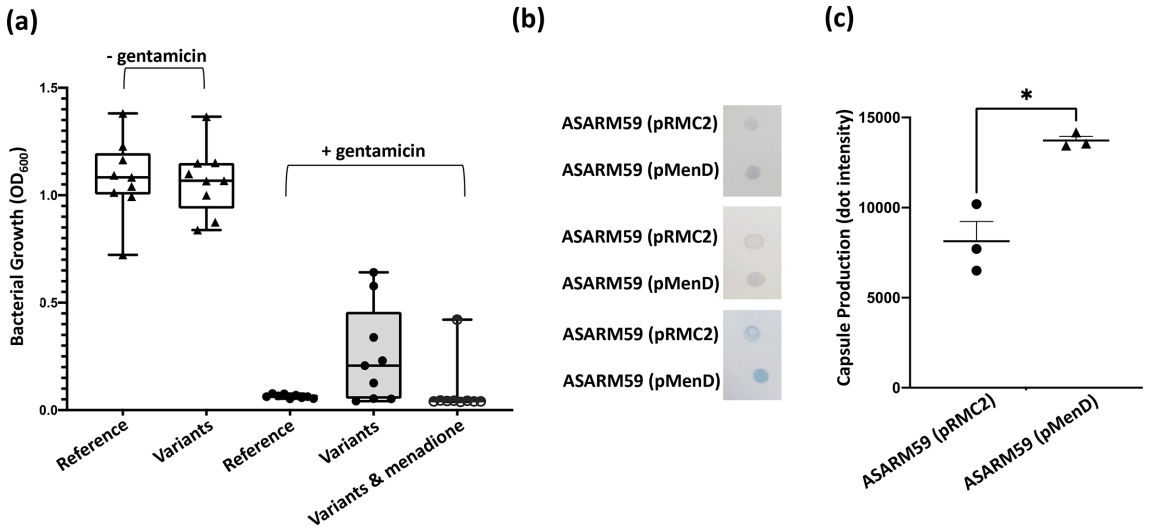

Fig. 5. Clinical isolates with *menD* polymorphisms have no growth defect but are more resistant to gentamicin. (a) The growth of the nine clinical isolates containing non-synonomous polymorphism in the *menD* gene was compared to that of nine randomly selected isolates with the wild-type or reference *menD* gene. In TSB we observed no effect on growth associated with the polymorphic *menD* gene, however in a concentration of 2 µg ml^{-1} of gentamicin, the *menD* variants grew significantly better. The ability of the variants to grow in gentamicin was lost for all but one isolate when menadione was added to the growth medium. (b and c) The clinical isolate ASARM59 was complemented by introducing the *menD* gene on the expression plasmid pRMC2, and this resulted in an increase in capsule production.

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-synonomous 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 around 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 cost 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.

Funding information
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).

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. A.E. and J.L., provided resources and contributed to writing the manuscript. M.R., analysed data and contributed to writing the manuscript. R.C.M., conceptualized the projects, provided supervisory support, analysed data and contributed to writing the manuscript.

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

References
1. Lowy FD. *Staphylococcus aureus* infections. *N Engl J Med* 1998;339:520–532.
2. O'Riordan K, Lee JC. *Staphylococcus aureus* capsular polysaccharides. *Clin Microbiol Rev* 2004;17:218–234.
3. Thakker M, Park JS, Carey V, Lee JC. *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. *Infect Immun* 1998;66:5183–5189.
4. Keinhörster D, Salzer A, Duque-Jaramillo A, George SE, Marincola G, *et al*. Revisiting the regulation of the capsular polysaccharide biosynthesis gene cluster in *Staphylococcus aureus*. *Mol Microbiol* 2019;112:1083–1099.
5. Proctor RA. Challenges for a universal *Staphylococcus aureus* vaccine. *Clin Infect Dis* 2012;54:1179–1186.
6. Boyle-Vavra S, Li X, Alam MT, Read TD, Sieth J, *et al*. USA300 and USA500 clonal lineages of *Staphylococcus aureus* do not produce a capsular polysaccharide due to conserved mutations in the cap5 locus. *mBio* 2015;6::e02585-14.
7. Mohamed N, Timofeyeva Y, Jamrozy D, Rojas E, Hao L, *et al*. Molecular epidemiology and expression of capsular polysaccharides in *Staphylococcus aureus* clinical isolates in the United States. *PLoS One* 2019;14:e0208356.
8. Recker M, Laabei M, Toleman MS, Reuter S, Saunderson RB, *et al*. Clonal differences in *Staphylococcus aureus* bacteraemia-associated mortality. *Nat Microbiol* 2017;2:1381–1388.
9. George SE, Nguyen T, Geiger T, Weidenmaier C, Lee JC, *et al*. Phenotypic heterogeneity and temporal expression of the capsular polysaccharide in *Staphylococcus aureus*. *Mol Microbiol* 2015;98:1073–1088.
10. Lei MG, Lee CY. MgrA activates staphylococcal capsule via SigA-dependent promoter. *J Bacteriol* 2020;203:e00495-20.
11. Laabei M, Uhlemann A-C, Lowy FD, Austin ED, Yokoyama M, *et al*. Evolutionary trade-offs underlie the multi-faceted virulence of *Staphylococcus aureus*. *PLoS Biol* 2015;13:e1002229.
12. Laabei M, Recker M, Rudkin JK, Aldeljawi M, Gulay Z, *et al*. Predicting the virulence of MRSA from its genome sequence. *Genome Res* 2014;24:839–849.
13. Méric G, Mageiros L, Pensar J, Laabei M, Yahara K, *et al*. Disease-associated genotypes of the commensal skin bacterium *Staphylococcus epidermidis*. *Nat Commun* 2018;9:5034.
14. Das S, Lindemann C, Young BC, Muller J, Österreich B, *et al*. Natural mutations in a *Staphylococcus aureus* virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation. *Proc Natl Acad Sci U S A* 2016;113:E3101-10.
15. Kohler C, von Eiff C, Liebeke M, McNamara PJ, Lalk M, *et al*. A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*. *J Bacteriol* 2008;190:6351–6364.
16. Lannergård J, von Eiff C, Sander G, Cordes T, Seggewiss J, *et al*. Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2008;52:4017–4022.
17. Kahl BC. Small colony variants (SCVs) of Staphylococcus aureus--a bacterial survival strategy. *Infect Genet Evol* 2014;21:515–522.
18. von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G, *et al*. A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly. *J Bacteriol* 1997;179:4706–4712.
19. Seggewiss J, Becker K, Kotte O, Eisenacher M, Yazdi MRK, *et al*. Reporter metabolite analysis of transcriptional profiles of a *Staphylococcus aureus* strain with normal phenotype and its isogenic hemB mutant displaying the small-colony-variant phenotype. *J Bacteriol* 2006;188:7765–7777.
20. Bui LMG, Hoffmann P, Turnidge JD, Zilm PS, Kidd SP. Prolonged growth of a clinical *Staphylococcus aureus* strain selects for a stable small-colony-variant cell type. *Infect Immun* 2015;83:470–481.
21. Pandey S, Sahukhal GS, Elasri MO. The msaABCR operon regulates persister formation by modulating energy metabolism in *Staphylococcus aureus*. *Front Microbiol* 2021;12:657753.
22. Bazaid AS, Forbes S, Humphreys GJ, Ledder RG, O'Cualain R, *et al*. Fatty acid supplementation reverses the small colony variant phenotype in triclosan-adapted *Staphylococcus aureus*: genetic, proteomic and phenotypic analyses. *Sci Rep* 2018;8:3876.
23. Schleimer N, Kaspar U, Ballhausen B, Fotiadis SA, Streu JM, *et al*. Adaption of an episomal antisense silencing approach for investigation of the phenotype switch of *Staphylococcus aureus* small-colony variants. *Front Microbiol* 2019;10:2044.
24. Holden MTG, Feil EJ, Lindsay JA, Peacock SJ, Day NPJ, *et al*. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A* 2004;101:9786–9791.
25. Holden MTG, Hsu L-Y, Kurt K, Weinert LA, Mather AE, *et al*. A genomic portrait of the emergence, evolution, and global spread of a methicillin-resistant *Staphylococcus aureus* pandemic. *Genome Res* 2013;23:653–664.
26. Cao S, Huseby DL, Brandis G, Hughes D. Alternative evolutionary pathways for drug-resistant small colony variant mutants in *Staphylococcus aureus*. *mBio* 2017;8:e00358-17.