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# Functional analysis of the EsaB component of the *Staphylococcus aureus* Type VII secretion system

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### Abstract

Type VII secretion systems (T7SS) are found in many bacteria and secrete proteins involved in virulence and bacterial competition. In *Staphylococcus aureus* the small ubiquitin-like EsaB protein has been previously implicated as having a regulatory role in the production of the EsxC substrate. Here we show that in the *S. aureus* RN6390 strain, EsaB does not genetically regulate production of any T7 substrates or components, but is indispensable for secretion activity. Consistent with EsaB being an essential component of the T7SS, loss of either EsaB or EssC are associated with upregulation of a common set of iron acquisition genes. However, a further subset of genes were dysregulated only in the absence of EsaB. Quantitative western blotting indicates that EsaB is present at very low levels in cells. Substitution of a highly conserved threonine for alanine or arginine resulted in a loss of EsaB activity and destabilisation of the protein. Taken together our findings show that EsaB is essential for T7SS activity in RN6390.

# INTRODUCTION

Protein secretion systems are nanomachines employed by bacteria to transport protein substrates across their cell envelopes. Gram-negative bacteria produce a number of different secretion machineries that export proteins involved in a wide variety of processes including signalling, nutrient scavenging, host interaction and virulence [1]. The Type VII secretion system (T7SS) is found in some Gram-negative and many Gram-positive bacteria, and is particularly common among organisms of the actinobacteria and firmicutes phyla [2]. The T7SS was initially described in the pathogenic mycobacteria Mycobacterium tuberculosis and Mycobacterium bovis, where the ESX-1 T7SS was shown to be essential for virulence, due to the secretion of two major T-cell antigens EsxA (formerly known as ESAT-6) and EsxB (formerly known as CFP-10) [3-5]. EsxA and EsxB are founding members of the WXG100 protein family that appear to be exclusively linked to T7SSs, and all characterised T7 systems are associated with at least one family member. The presence of a membrane-bound ATPase of the SpoIIIE/FtsK family (termed EccC in actinobacteria and EssC in firmicutes) is another hallmark of all T7SSs [6]. In Mycobacteria, three further membrane proteins EccB, EccD and EccE assemble with EccC to form a large 1.5 MDa core complex [7, 8]. This complex further associates with a membrane-bound mycosin serine protease, MycP, that is essential for T7 protein secretion and for stability of the membrane complex [9].

Staphylococcus aureus, an opportunistic pathogen of humans and animals, also elaborates a T7SS that is distantly related to the T7SSs found in mycobacteria [10]. Mutational analysis has indicated that it plays an important role in persistence in mouse models of infection, intra-species competition and potentially iron homeostasis [10-15]. In commonly-studied strains of S. aureus such as Newman, USA300 and RN6390, the secretion system is encoded by the 12 gene ess locus [10, 12, 16]. The first six genes at this locus encode essential components of the secretion machinery, including the WXG100 protein EsxA and the SpoIIIE/ FtsK ATPase EssC (Fig. 1a, b). However, S. aureus and other firmicutes lack homologues of EccB, EccD, EccE and MycP and instead have an apparently unrelated set of membranebound secretion components (EsaA, EssA and EssB in S. aureus) [12, 17-19]. The sixth component of the S. aureus T7SS is EsaB, which is predicted to be a small cytoplasmic protein of 80 amino acids that is structurally related to ubiguitin [20]. In S. aureus strains Newman and USA300, a

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Keywords: Staphylococcus aureus; protein secretion; T7SS; regulation.

Abbreviations: LB, Luria Bertani medium; TSB, Tryptic Soy Broth; T7SS, type VII secretion system; YFP, yellow fluorescent protein.

The RNA-Seq data from this study is submitted to the European Nucleotide Archive with accession number ERP009279 and in Array express under accession number E-ERAD-362.

One supplementary table is available with the online version of this article.

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**Fig. 1.** EsaB is not a transcriptional regulator. (a) The *ess* locus in *S. aureus* RN6390. Genes encoding essential secretion components are shaded in grey, secreted proteins in blue and a cytoplasmic antitoxin in yellow. The regions analysed by RT-PCR are indicated. (b) Predicted subcellular locations of Ess-encoded components. cw–cell wall, cm–cytoplasmic membrane. (c) RT-PCR analysis of *esxA* (region 1) and *esxC/B* (region 2) from the RN6390 and isogenic *esaB* and *esxA* mutant strains. Shading is as for Fig. 1(a) with essential secretion components in grey, secreted proteins in blue and a cytoplasmic antitoxin in yellow. Equivalent amounts of mRNA from each strain were used to generate cDNA. RT: reverse transcriptase. (d) Total mRNA counts of *ess* genes from RNA-Seq analysis of RN6390 and the *esaB* mutant strain. RPKM – reads of transcript per kilobase per million of mapped reads.

transposon insertion in *esaB* does not abolish secretion of T7 substrates but is linked with an increase in RNA transcripts covering the gene encoding the substrate EsxC [11]. By contrast, in-frame deletion of *esaB* abolished EsxA and EsxC secretion in strain RN6390 but did not detectably affect production of these substrate proteins [12]. Similarly, inactivation of *yukD*, which encodes the *Bacillus subtilis esaB* homologue, also abolished T7 secretion [17, 18].

In this study, we have addressed the role of EsaB in *S. aureus* T7 secretion using strain RN6390. We show that EsaB does not regulate *esxC* transcripts or those of other *ess*-encoded genes. Instead our findings show that EsaB behaves as an essential component of the T7SS. Interestingly, however, RNA-Seq analysis identified a subset of genes from the AirSR regulon that showed altered regulation in the absence of EsaB. This suggests that loss of *esaB* has additional unexpected effects on *S. aureus* physiology.

## METHODS

### Bacterial strains and growth conditions

S. aureus strain RN6390 (NCTC8325 derivative, rbsU, tcaR, cured of  $\varphi$ 11,  $\varphi$ 12,  $\varphi$ 13; [21]) and the isogenic  $\Delta esaB$ ,  $\Delta essC$ and  $\Delta esx$  ( $\Delta esxA - esaG$ ) strains [12] were employed in this study, along with strain Newman [22]. The esaB deletion strain is an in-frame deletion of the gene that maintains the first ten and final three codons of *esaB* (as there is a 9 codon overlap between the end of essA and the start of esaB). S. aureus strains were cultured in Tryptic Soy Broth (TSB) at 37 °C with shaking unless otherwise stated. For calculation of cell numbers we estimated by dilution analysis that one unit at OD 600 nm corresponds to  $6 \times 10^8$  c.f.u. for strain. When required, chloramphenicol (Cml, final concentration  $10 \,\mu g \, m l^{-1}$ ) was added for plasmid selection. E. coli strain JM110 (Stratagene) was used for cloning purposes and BL21(DE3) [23] for EsaB overproduction and purification. E. coli was grown in Luria-Bertani (LB) medium at 37 °C with agitation. When appropriate, ampicillin was used for plasmid selection (final concentration  $125 \,\mu g \,ml^{-1}$ ).

### Genetic constructs

All plasmids used in this study are listed in Table 1. The *esaB* gene with its own RBS was PCR amplified from *S. aureus* RN6390 genomic DNA using primers EsaB-fw and EsaB-rev (Table S1, available with the online version of this article). The 0.3 kb *HpaI/Eco*RI restriction fragment was cloned into pRAB11 under control of the tetracycline inducible promoter, giving pRAB11-esaB. Clones were selected in *E. coli* and verified by DNA sequencing. Plasmid pRAB11-esaB-YFP was generated by cloning the 0.3 kb *HpaI/Eco*RI restriction fragment into pRAB11-YFP [15]. Clones were selected in *E. coli* and verified by DNA sequencing. Nucleotide variants of *esaB* were generated by the Quickchange site-directed mutagenesis protocol (Stratagene) using pRAB11-esaB or pRAB11-esaB-YFP as a template and primers listed in Table S1. Modified plasmids were digested using *DpnI* for at least 1 h at 37 °C and

transformed into *E. coli*. Single point mutations were verified by DNA sequencing.

### **RNA isolation and RT-PCR**

For RNA-Seq analysis, three biological repeats of the *S. aureus esaB* strain was grown aerobically in TSB up to an  $OD_{600}$  of 1 at which point mRNA was prepared (in three technical replicates). This experiment was carried out along-side the RN6390 and *essC* strains [15] and followed identical methodology.

For RT-PCR, the indicated S. aureus strains were grown aerobically in TSB and harvested at an OD<sub>600</sub> of 1. At this point, the mRNA was extracted using the SV total RNA Isolation Kit (Promega) with some minor modifications. Cell samples were stabilised in 5 % phenol/95 % ethanol on ice for at least 30 min and then centrifuged at 2770 g for 10 min. Cells were then resuspended in  $100\,\mu$ l of TE buffer containing 500  $\mu$ g ml<sup>-1</sup> lysostaphin and 50  $\mu$ g ml<sup>-1</sup> lysozyme and incubated at 37 °C for 30 min. Subsequently, the manufacturer's instructions were followed. Isolated RNA was subjected to a second DNase treatment using the DNAfree kit (Ambion). RNA was stored at -80 °C until use. RT-PCR to probe transcription of genes in the indicated strains was carried out using 500 ng of mRNA as template with the indicated primers (Table S1). PCR products were visualized on 1 % agarose gels.

# Purification of 6His-EsaB and generation of polyclonal antisera

The EsaB coding sequence (UniProt code ESAB STAAM) was PCR amplified from a synthetic gene (codon optimised for E. coli K12 (Genscript)) using the primers EsaB-pET1 and EsaB-pET2 (Table S1) and cloned into the NdeI/XhoI site of a modified pET15b vector (Novagen). The plasmid produces an N-terminal His<sub>6</sub>-tagged protein with a TEV (tobacco etch virus) protease cleavage site. The protein was expressed and purified as described previously [24], except the tag-free EsaB was not collected in the flow-through of the negative purification but required a 30 mM imidazole elution. The final size exclusion chromatography step used a 24 ml HR 30/100 GL Superdex75 column (GE healthcare), equilibrated with 20 mM Tris pH 7.8, 100 mM NaCl and was calibrated with molecular mass standards (thyroglobulin, 670 kDa;  $\gamma$ -globulin, 158 kDa; serum albumin, 67 kDa; ovalbumin; 44 kDa, myoglobin, 17 kDa; and vitamin B12, 1 kDa). Two mg purified EsaB (retaining a Gly-Ala-Ser-Thr sequence at the N-terminus after the cleavage step) was utilised as antigen to immunise rabbits for polyclonal antibody production in a standard three injections protocol (Seqlab).

# Secretion assays, subcellular fractionation and western blotting

The indicated strains were grown overnight in TSB, diluted 1/100 in fresh medium and grown up to mid-log phase, at which point whole cells and supernatant fractions were harvested as described previously [12]. Briefly, cells and supernatant were separated by a 10 min centrifugation step at

Table 1. Plasmids used in this stuc
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Plasmid	Relevant genotype or description	Source or reference
pRAB11	E. coli/S. aureus shuttle vector, inducible protein expression, Amp <sup>r</sup> , Cml <sup>r</sup>	[35]
pRAB11-esaB	pRAB11 producing EsaB	This study
pRAB11-esaB-T8A	pRAB11 producing T8A-substituted EsaB	This study
pRAB11-esaB-T8E	pRAB11 producing T8E-substituted EsaB	This study
pRAB11-esaB-T8R	pRAB11 producing T8R-substituted EsaB	This study
pRAB11-esaB-T8H	pRAB11 producing T8H-substituted EsaB	This study
pRAB11-esaB-T8K	pRAB11 producing T8K-substituted EsaB	This study
pRAB11-esaB-T8S	pRAB11 producing T8S -substituted EsaB	This study
pRAB11-esaB-D10A	pRAB11 producing D10A-substituted EsaB	This study
pRAB11-esaB-D20A	pRAB11 producing D20A-substituted EsaB	This study
pRAB11-esaB-L21A	pRAB11 producing L21A-substituted EsaB	This study
pRAB11-esaB-K30A	pRAB11 producing K30A-substituted EsaB	This study
pRAB11-esaB-K52A	pRAB11 producing K52A-substituted EsaB	This study
pRAB11-esaB-K56A	pRAB11 producing K56A-substituted EsaB	This study
pRAB11-esaB-L66A	pRAB11 producing L66A-substituted EsaB	This study
pRAB11-esaB-G74A	pRAB11 producing G74A-substituted EsaB	This study
pRAB11-esaB-D75A	pRAB11 producing D75A-substituted EsaB	This study
pRAB11-esaB-YFP	pRAB11 producing EsaB-YFP	This study
pRAB11-esaB-T8A-YFP	pRAB11 producing T8A-substituted EsaB-YFP	This study
pRAB11-esaB-T8R-YFP	pRAB11 producing T8R-substituted EsaB-YFP	This study
pET15b-HISEsaB	pET15b expressing 6XHis-tagged EsaB	This study

2770 g. Cells were washed twice with PBS, adjusted to and  $OD_{600}$  of 1 and digested using 50 µg ml<sup>-1</sup> of lysostaphin by incubation at 37 °C for 30 min. Supernatants were filtered using a 0.22 µm filter and TCA-precipitated in the presence of 50  $\mu$ g ml<sup>-1</sup> deoxycholate, as described. For *S. aureus* subcellular fractionation, cells were grown to mid-log phase with shaking and treated as previously described [12]. Briefly, cells were harvested by centrifugation and resuspended in TSM buffer (50 mM Tris-HCl pH 7.6, 0.5 M sucrose, 10 mM MgCl<sub>2</sub>). Lysostaphin was added to a final concentration of  $50 \,\mu g$  ml<sup>-1</sup> and cells were incubated at 37 °C for 30 min to digest the cell wall. At this point, protoplasts were sedimented to recover the cell wall (supernatant fraction). Protoplasts were disrupted by sonication and the membrane was obtained after an ultracentrifugation step at 227 000 g for 30 min and at 4 °C. The supernatant was retained as the cytoplasmic fraction. Samples were boiled for 10 min prior to separation in bis-Tris gels and subsequent western blotting.

Polyclonal antisera were used at the following dilutions:  $\alpha$ -EsxA 1:2500 [12],  $\alpha$ -EsxB 1:1000 [15],  $\alpha$ -EsxC 1:2000 [12],  $\alpha$ -EsaB 1:500,  $\alpha$ -TrxA 1:20000 [25] and  $\alpha$ -SrtA (Abcam) 1:3000. Anti-GFP antibody was obtained from Roche and used according to manufacturer's instructions.

### RESULTS

# EsaB does not regulate the level of *esxC* transcripts in strain RN6390

A previous study has shown that a transposon insertion in the esaB gene results in an increase in esxC transcripts in

the Newman and USA300 strain backgrounds, and a concomitant increase in the EsxC polypeptide, implicating it as a regulator [11]. To investigate whether loss of *esaB* by inframe deletion affects the level of *esxC* mRNA in strain RN6390, we isolated mRNA from the parental strain and the isogenic *esaB* mutant, prepared cDNA and undertook reverse transcriptase PCR with primers covering either *esxA* (the first gene at the *ess* locus, included as a negative control) or *esxC* (Fig. 1a). It can be seen (Fig. 1c) that the level of transcripts for each of these genes was qualitatively similar in the wild-type and *esaB* backgrounds.

To examine this quantitatively, we undertook RNA-Seq analysis on RNA prepared from three biological repeats of the RN6390 and *esaB* strains grown aerobically in TSB to an  $OD_{600}$  of 1. Note that these experiments were performed at the same time as the RN6390 versus *essC* RNA-Seq analysis described in [15] and used the same RN6390 dataset. Fig. 1 (d) shows that the level of *esxC* transcripts were indistinguishable between the wild-type and *esaB* strains. Analysis of the transcript levels of the other genes at the *ess* locus indicates that in general they were also not significantly altered by the loss of *esaB* although there was a small increase in the level of *essE*. We conclude that there is no evidence that *esaB* regulates the level of *esxC* transcripts in RN6390.

We next examined the entire transcript profile of the *esaB* mutant to investigate the transcriptional/post-transcriptional response to the loss of this small protein. We found 101 genes de-regulated in the *esaB* mutant compared to the parental strain (using a cut off of logFC >2 or <-2 and

qvalue <0.05, as applied previously [15]), Fig. 2(a). Of these, 43 were upregulated by the loss of esaB whereas 58 were downregulated when esaB was absent - these genes are listed in Table 2. Interestingly, almost all of the genes that were differentially regulated in the essC mutant [15] were also similarly regulated in the esaB strain (Fig. 3b), although there was a substantive subset of genes that were differentially expressed in the esaB mutant but not the essC strain (Table 2). It can be seen that almost all of the iron acquisition genes, including those for heme acquisition, staphyloferrin synthesis and uptake and ferrichrome import were commonly upregulated by loss of either esaB or essC (Table 2). Furthermore six of the eight downregulated genes from the essC strain were also down regulated in the esaB strain (note that one of the two genes unaffected in the esaB dataset is essC itself, which appears downregulated in the essC dataset because it has been deleted). The finding that almost the entire subset of genes differentially regulated in the absence of essC is also similarly altered by loss of esaB strongly suggests that EsaB is, like EssC, a component that is essential for activity of the secretion machinery in strain RN6390.

As mentioned above, a subset of transcripts were differentially expressed in the *esaB* but not the *essC* strain. These include downregulated genes required for anaerobic nitrate respiration (*narGHJ/narK*), some secreted proteases (*sspA/ B/C*, *aur*), capsular polysaccharide synthesis (*capG/F/hysA*), lactose metabolism (*lacB/C/D*) and antimicrobial peptide synthesis (*epiA/C/D/P*). Many of these genes are under control of the essential two component regulatory system AirSR (formerly YhcSR) [26–29]. This observation indicates that EsaB has additional effects on *S. aureus* physiology. This could be indirect and arising from its role in T7 secretion, for example through altered membrane permeability when EsaB is absent. Alternatively, EsaB may have additional roles in the cell in addition to its requirement for T7 protein secretion.

# EsaB is present at low amounts in cells of *S. aureus* RN6390

To explore the biological role of EsaB in T7 secretion, we overproduced recombinant EsaB with a cleavable His-tag in E. coli. The purified protein eluted from gel filtration as a monomer, in agreement with structural analysis of the B. subtilis EsaB homologue, YukD, which also appears to be monomeric [20]. Polyclonal antisera were raised against purified EsaB and the antibody was affinity purified against the EsaB antigen, before being used to detect the protein in whole cells of S. aureus. Fig. 4(a) shows that although the purified antiserum could clearly recognise purified EsaB, it did not detect a band of the expected size of EsaB in whole cells. We have shown previously that expression of the T7SS genes in RN6390 is upregulated approximately 2-3-fold in the presence of exogenous hemin, and fourfold by hemin in a  $\Delta essC$  background [15]. However, supplementation of either of these strains with hemin did not result in detectable EsaB in the cellular fraction (Fig. 4b) and it could also not be detected in cells of strain Newman (Fig. 4b). Probing a dilution series of purified EsaB indicated that the antibody was able to cross-react with as little as 25 ng of protein (Fig. 4a), which is equivalent to  $1.6 \times 10^{11}$  EsaB molecules. Since the antibody was unable to detect EsaB in whole cells of RN6390 from  $9.6 \times 10^8$  colony forming units that were loaded onto the SDS gel, we conclude that are less than 170 molecules of EsaB per cell.

Since we were unable to detect native EsaB in S. aureus cell extracts, we constructed a series of tagged variants for which commercial antisera were available. To this end we introduced His<sub>6</sub>, Myc, hemagglutinin (HA) and Strep epitopes onto the N-terminus of EsaB, and His<sub>6</sub>, Myc, HA, mCherry or FLAG epitopes onto the C-terminus, but in each case were unable to detect the tagged protein (not shown). We also introduced His<sub>6</sub> and His<sub>9</sub> epitopes into two predicted loop regions internal to the EsaB sequence but again were unable to detect tagged EsaB (not shown). The only tag we introduced that allowed detection of EsaB was a C-terminal yellow fluorescent protein (YFP) tag. Fig. 4(c) shows that basal production of either native (untagged) EsaB or EsaB-YFP from plasmid vector pRAB11 was sufficient to restore secretion of the T7SS extracellular protein EsxA and of substrates EsxB and EsxC to the culture supernatant. Blotting the same cell samples for the presence of the YFP fusion protein (Fig. 4d) showed that it migrated at close to the predicted mass (37 kDa) of the EsaB fusion. There was no evidence for degradation of the fusion protein even after prolonged exposure of the immunoblot (Fig. 4d). We conclude that the YFP-tagged variant of EsaB probably retains functionality.

### EsaB-YFP partially localises to the cell membrane

EsaB is predicted to be a soluble cytoplasmic protein [10], and is known to share structural homology with ubiquitin [20]. Interestingly, a domain sharing the same fold is also associated with the actinobacterial T7SS, being found at the cytoplasmic N-terminus of EccD [30], indicating that such proteins may be essential features of all T7SSs. To determine the subcellular location of EsaB-YFP, we blotted secreted and whole cell samples of the *esaB* mutant strain producing plasmid-encoded EsaB-YFP with the YFP antiserum. Fig. 3(a) shows that EsaB-YFP was associated exclusively with the cellular fraction.

We next fractionated these cells to obtain cytoplasm, cell wall and membrane fractions. Immunoblotting with antisera to control proteins known to localize to the cell membrane (SrtA) and cytoplasm (TrxA) indicated that the fractionation had been largely successful, although some SrtA was found in the cell wall fraction (Fig. 3b). Blotting these same fractions for the presence of EsaB-YFP showed that the protein localised to both the cytoplasm and membrane fractions. Some degradation of the fusion protein was also noted in these experiments which may result from the activation of proteases during fractionation. Indeed, in the membrane fraction it appears that EsaB-YFP migrated as a doublet band of around 37 kDa, indicating some probable truncation of the protein. When unfused YFP was produced



**Fig. 2.** RNA-Seq analysis of differentially regulated genes in the *esaB* mutant strain. (a) Volcano plot representation of the differentially expressed genes in RN6390 strain compared to the isogenic *esaB* mutant. The orange and grey spots represent, respectively, genes that are up- or down-regulated in the *esaB* mutant relative to the parental strain. (b). Overlap between up- and down-regulated genes in the *esaB* and *essC* datasets.

in the wild-type strain it did not localise to the membrane (Fig. 3c), indicating that membrane binding was unlikely to be mediated through the YFP portion of the fusion.

Next we tested whether EsaB-YFP localised to the membrane through interactions with membrane components of the T7SS. To this end we repeated the fractionation in a Table 2. Genes differentially regulated (>log 2 fold) in the RN6390 esaB deletion mutant, sorted by ascending fold change (FC)

Genes highlighted in grey are also differentially regulated in the *essC* deletion strain. The column on the right shows the fold change (FC) of the same gene in the *essC* dataset where NS indicates no statistically significant change in expression level relative to the same gene in the wild-type dataset.

Locus ID	Gene name	FC in esaB mutant	Proposed function	FC in essC mutant
Downregulated genes				
SAOUHSC_00986	sspC	-23.7	Cysteine protease	NS
SAOUHSC_00988	sspA	-22.3	Glutamyl endopeptidase	NS
SAOUHSC 00987	sspB	-20.8	Cysteine protease	NS
SAOUHSC 01573	_	-19.0	Unknown, hypothetical protein	NS
SAOUHSC 01941	splB	-18.8	Serine protease SplB	-4.3
SAOUHSC 02971	aur	-17.1	Zinc metalloproteinase aureolysin	NS
SAOUHSC 01942	splA	-16.4	Highly specific serine protease specific to S. aureus	-5.4
SAOUHSC 02680	narH	-15.7	Nitrate reductase subunit beta	NS
SAOUHSC 01944	-	-14.3	Unknown, hypothetical protein	-4.5
SAOUHSC 02681	narG	-14.3	Nitrate reductase subunit alpha	NS
SAOUHSC 01121	hla	-13.5	α-hemolysin	-4.1
SAOUHSC 02241	lukF	-13.0	Unknown, hypothetical protein	-3.3
SAOUHSC 02163	hlb	-12.3	β-hemolysin	NS
SAOUHSC 01938	splD	-12.2	Serine protease SplD	-4.3
SAOUHSC 02679	narl	-12.2	Nitrate reductase subunit delta	NS
SAOUHSC 02671	narK	-11.6	Putative nitrate transporter	NS
SAOUHSC 02455	lacA	-11.0	Galactose-6-phosphate isomerase subunit LacA	ND
SAOUHSC 01530	_	-10.9	Hypothetical phage protein	NS
SAOUHSC 01542	_	-10.9	Unknown SNF2 family protein	NS
SAOUHSC 01535	_	-10.9	Phage cansid protein	NS
SAQUHSC 02240	hlb	-10.5	Truncated <i>B</i> -hemolysin	NS
SAOUHSC 02243	lukG	-10.4	Leukocidin like toxin	-4.5
SAOUHSC 02685	nirR	-10.3	Unknown, hypothetical protein	NS
SAOUHSC 01939	splC	-10.3	Serine protease SplC	-3.2
SAOUHSC 01937	-	-10.3	Unknown, hypothetical protein	-2.8
SAOUHSC 02970	arøR	-8.8	Arginine repressor family protein	NS
SAOUHSC 00113	adhE	-86	Bifunctional acetaldebyde-CoA/alcohol debydrogenase	NS
SAQUHSC 00051	plc	-8.1	1-phosphatidylinositol phosphodiesterase	-2.5
SAQUHSC 00898	aroH	-67	Argininosuccinate lvase	NS
SAOUHSC 02684	nasD	-66	Assimilatory nitrite reductase [NAD(P)H] large subunit	NS
SAOUHSC 02709	hløC	-6.5	$\gamma$ -hemolysin component C precursor	-1.8
SAOUHSC 02682	nasF	-6.4	Uroporphyrin-III C-methyltransferase	NS
SAOUHSC 02462	_	-6.4	Unknown, hypothetical protein	NS
SAOUHSC 00401	_	-6.3	Putative exported protein	-1.6
SAOUHSC 01950	epiD	-6.3	Flavoprotein	NS
SAOUHSC 01936	splE	-63	Serine protease SpIE	-3.3
SAOUHSC 02454	lacB	-63	Galactose-6-phosphate isomerase subunit LacB	-3.4
SAOUHSC 00899	arøG	-6.2	Argininosuccinate synthase	NS
SAOUHSC 02108	ftn	-6.1	Ferritin	NS
SAOUHSC 00368	-	-6.1	Unknown, hypothetical protein	NS
SAOUHSC 00411	_	-5.9	Unknown, hypothetical protein	-2.2
SAOUHSC 01951	epiC	-5.8	Epidermin biosynthesis protein EpiC	NS
SAOUHSC 02683	nasE	-5.6	Assimilatory nitrite reductase [NAD(P)H] small subunit	NS
SAOUHSC 01935	stlF	-5.3	Serine protease SplF	-2.7
SAOUHSC 02452	lacD	-5.2	Tagatose 1,6-diphosphate aldolase	-2.6
SAOUHSC 01953	epiA	-5.2	Gallidermin superfamily EpiA protein	NS
SAOUHSC 02941	nrdG	-4.9	Anaerobic ribonucleotide reductase activating protein	NS
SAOUHSC 00717	saeP	-4.7	Putative lipoprotein	-1.4
SAOUHSC 01990	ølnO	-4.6	Glutamine transport ATP-binding protein	NS
SAOUHSC_02557	yut	-4.5	Putative urea transporter	NS

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### Table 2. cont.

SACURSC.0009	Locus ID	Gene name	FC in esaB mutant	Proposed function	FC in <i>essC</i> mutant
SACURSC012	SAOUHSC_01949	epiP	-4.4	Intracellular serine protease	NS
SACUTISC_0092é.ad-1.4Landiac regionalis injortion prote EpilSiSACUTISC_0095M.GZ-1.41Capular prisocialistic singulatory subaticSiSACUTISC_0285M.AA-1.3Bylaconia byleSiSACUTISC_0286and-1.41Tagance Spenphare littar:SiSACUTISC_0286and-1.41Tagance Spenphare littar:SiSACUTISC_0287agp I.A-4.3Distance Mylenphare littar:SiSACUTISC_0287agp I.A-4.3Distance Mylenphare littar:SiSACUTISC_0287agp I.A-4.3Distance Mylenphare littar:SiSACUTISC_0287add-4.3Distance Mylenphare littar:SiSACUTISC_0287add-4.3Distance Mylenphare littar:SiSACUTISC_0287add-4.3Distance Mylenphare littar:SiSACUTISC_0287add-4.3Distance Mylenphare littar:SiSACUTISC_0287add-4.3Distance Mylenphare littar:SiSACUTISC_0287add-4.3Distance Mylentari lyttar:-4.3SACUTISC_0287add-4.3Distance Mylentari lyttar:-4.3SACUTISC_0287add-4.3Distance Mylentari lyttar:-4.3SACUTISC_0287add-4.3Distance Mylentari lyttar:-4.3SACUTISC_0287add-4.3Distance Mylentari lyttar:-4.3SACUTISC_0287add-4.3Distance Mylentari lyttar:-4.3SACUTISC_0287add-4.3<	SAOUHSC_00120	capG	-4.4	UDP-N-acetylglucosamine 2-epimerase	NS
SAOUBS_0015no.244AT polyschwischmeiner explaiory selout of ano.SAOUBS_00150.4013Hydronick havie howyschust protein Cap10.51SAOUBS_00150.4014Hydronick havie howyschust protein Cap10.51SAOUBS_00150.4014Harber chydrophar havae0.51SAOUBS_00150.60141.620.62SAOUBS_00150.67441.620.62SAOUBS_00150.7441.680.62SAOUBS_0175451.680.62SAOUBS_0175451.680.62SAOUBS_0175451.680.62SAOUBS_0175451.680.62SAOUBS_0175451.680.62SAOUBS_0275451.680.62SAOUBS_0275451.680.62SAOUBS_0275451.680.68SAOUBS_0275451.680.68SAOUBS_0275451.680.68SAOUBS_0276451.680.68SAOUBS_0277461.680.68SAOUBS_0278451.690.61SAOUBS_0277471.680.68SAOUBS_0277461.690.61SAOUBS_0278471.680.68SAOUBS_02798471.680.61SAOUBS_027	SAOUHSC_01952	bsaB	-4.4	Lantibiotic epidermin biosynthesis protein EpiB	NS
SAOUBS_0019opf-1.4Clupular polyackanik basyshes proton Capf98SAOUBS_02053hyte-1.3Hytaromat hyte-2.2SAOUBS_02064ahl-1.1Taginos + pholyak kinas-2.2SAOUBS_02067ap.1.44.0Pather All ryanperta andrata- binding protein2.6SAOUBS_02078ap.1.44.0Pather All ryanperta andrata- binding protein2.6SAOUBS_02078ap.1.44.0Pather Division System Call3.7SAOUBS_02078ap.1.44.1Pather Division Capf3.7SAOUBS_02078ap.1.44.1Pather Division Capf3.7SAOUBS_02079ap.1.44.1Pather Capperta Pather3.7SAOUBS_02079ap.1.44.1Pather Capperta Pather3.7SAOUBS_02071ap.1.44.1Pather Capperta Pather3.7SAOUBS_02071ap.1.44.3Pather Capperta Pather3.7SAOUBS_02071ap.1.43.3Pather Capperta Pather3.7SAOUBS_02071ap.1.43.3Pather Capperta Pather3.7SAOUBS_02071ap.1.4ap.3Pather Capperta Pather3.7SAOUBS_02071ap.1.4ap.3Pather Capperta Pather3.7SAOUBS_02071ap.1.4ap.3Pather Capperta Pather3.7SAOUBS_02071ap.1.4ap.3Pather Capperta Pather3.7SAOUBS_02071ap.1.4ap.3Pather Capperta Pather3.7SAOUBS_02071ap.1.4ap.3Pather Capperta Pather	SAOUHSC_03015	hisZ	-4.4	ATP phosphoribosyltransferase regulatory subunit	NS
SAOUBSC,0853japa-1.3Higtmanic logarsetSAOUBSC,0853laft-1.4Akola diphrpensaakolaSAOUBSC,0853off4.3Akola diphrpensaakolaSAOUBSC,0853-4.3Papida ABC transporter substrate-londing protein5.4SAOUBSC,0853-4.4Henrei-degrafing menoscoperate-londing protein6.3SAOUBSC,0853-4.4Henrei-degrafing menoscoperate-londing protein6.3SAOUBSC,0853-4.5Hatter DAVA forget protein6.7SAOUBSC,0853-4.6Staphyloccol tacceser regulator6.7SAOUBSC,0853-4.6Staphyloccol tacceser regulator6.7SAOUBSC,0852-4.8Staphyloccol tacceser regulator6.7SAOUBSC,0852-4.8Staphyloccol tacceser regulator6.7SAOUBSC,0852-4.8Diatones hystopheral target protein6.7SAOUBSC,0852-4.8Diatones hystopheral target protein6.7SAOUBSC,0852-4.8Diatones hystopheral target protein6.7SAOUBSC,08526.3Horis transportsin6.7SAOUBSC,08536.3Horis transportsin6.7SAOUBSC,08546.3Horis transportsin6.7SAOUBSC,08546.3Horis transport and heit stypidant7.7SAOUBSC,08546.3Horis transport and heit stypidant7.8SAOUBSC,0854 <td>SAOUHSC_00119</td> <td>capF</td> <td>-4.4</td> <td>Capsular polysaccharide biosynthesis protein CapF</td> <td>NS</td>	SAOUHSC_00119	capF	-4.4	Capsular polysaccharide biosynthesis protein CapF	NS
SACURSC, 2000def-14Tagone - phopelate kinane-22SADURSC, 2007aft-14Alcohal dolytrigense	SAOUHSC_02463	hysA	-4.3	Hyaluronate lyase	NS
SAUCHSC_0008abl11Alobal dalylargensessUpreplate Alleopplabe Alle transporter substrate binding protein6.3SAUCHSC_0207oppl.A4.4Parture DAA bahang protein6.3SAUCHSC_02084.44Parture DAA bahang protein6.3SAUCHSC_02084.54Parture DAA bahang protein6.3SAUCHSC_02084.54Parture DAA bahang protein6.3SAUCHSC_02084.54Parture DAA bahang protein6.3SAUCHSC_02084.84Uhonen, Nynothtical protein6.3SAUCHSC_02084.84Uhonen, Nynothtical protein6.3SAUCHSC_02084.84Uhonen, Nynothtical protein6.3SAUCHSC_02184.34Uhonen, Nynothtical protein6.3SAUCHSC_02196.3Parture mombrace protein6.3SAUCHSC_02196.4Parture mombrace protein6.3SAUCHSC_02196.4Parture mombrace protein6.3SAUCHSC_02196.4Parture mombrace protein6.3SAUCHSC_02196.4Parture Moreaneat HaB6.3SAUCHSC_02196.4Parture Moreaneat HaB6.3SAUCHSC_02196.4Parture Moreaneat HaB6.3SAUCHSC_02196.4Parture Moreaneat HaB6.3SAUCHSC_02196.4Parture Moreaneat HaB6.3SAUCHSC_0218/-/-7.4 <td>SAOUHSC_02453</td> <td>lacC</td> <td>-4.1</td> <td>Tagatose-6-phosphate kinase</td> <td>-2.2</td>	SAOUHSC_02453	lacC	-4.1	Tagatose-6-phosphate kinase	-2.2
Uppealand gene         Uppeala	SAOUHSC_00608	adh1	-4.1	Alcohol dehydrogenase	NS
SNOUHS_0057         0.97-A         4.9         Peptek ABC transporter abstrate-binding protein         2.6           SNOUHS_0055         -         4.2         Unknown, hynothatial protein         6.3           SNOUHS_0052         -         4.4         Pentave DNA binding protein         8.7           SNOUHS_0057         -         4.5         Database inding protein         8.7           SNOUHS_0057         -         4.5         Database inding protein         8.7           SNOUHS_0052         iftA         4.5         Database interprotein         6.7           SNOUHS_0052         iftA         4.5         Unknown, hynothatial protein         6.2           SNOUHS_0052         iftA         5.1         Pentative membrane protein         6.2           SNOUHS_0052         iftA         5.1         Pentative membrane protein         6.1           SNOUHS_0051         iftA         5.1         Pentative membrane protein         8.1           SNOUHS_01071         -         6.2         ACE transporter ATP-binding protein flab         5.4           SNOUHS_01071         -         6.1         Unknown, hynothetial protein         8.1           SNOUHS_01071         -         6.1         Unknown, hynothetial protein         8.1	Upregulated genes				
SAOUHSC.0835         -         4.2         Unknown. hypothetical protein         6.3           SAOUHSC.0132         -         4.4         Pataive DAK binding protein         8.5           SAOUHSC.01016_01176         -         4.5         Extracolular robust-holding protein         8.7           SAOUHSC.00135_01176         -         4.5         Statuse transporter         8.7           SAOUHSC.00135_0126_01779         ast         1.6         Statuse transporter         8.7           SAOUHSC.00135_0126_0179         ast         1.6         Statuse transporter         8.7           SAOUHSC.00152_0126_0171         -         4.8         Unknown, hypothetical protein         6.5           SAOUHSC.00252 <i>fload</i> 5.1         Periodrem membrane protein         6.5           SAOUHSC.00271         arC         5.3         Putative membrane protein         6.5           SAOUHSC.00271         arC         5.3         Putative insporter ATP-binding protein         5.5           SAOUHSC.00271         arC         6.3         Putative insporter ATP-binding protein         6.3           SAOUHSC.00271         arC         6.3         Putative insporter ATP-binding protein         6.3           SAOUHSC.00272         arC         6.4         P	SAOUHSC_02767	opp-1A	4.0	Peptide ABC transporter substrate-binding protein	2.6
SAOUHSC,0132      4.4     Patrice DNA-binding protein     ss       SAOUHSC,0130     iull     4.4     Henci digrafing monocorganas Iull     5.7       SAOUHSC,0130     iull     4.4     Henci digrafing monocorganas Iull     5.7       SAOUHSC,0215     iull     4.5     Diatoro, hypothetial protein     6.2       SAOUHSC,0215     -     4.8     Diatoro, hypothetial protein     6.2       SAOUHSC,0242     -     4.8     Diatoro, hypothetical protein     6.3       SAOUHSC,0211     -     5.3     Patriter membrare protein     6.1       SAOUHSC,0221     -     6.4     Diatoro membrare protein     8.3       SAOUHSC,02328     fuld     6.3     Patriter membrare protein     8.5       SAOUHSC,0022     inf     6.5     Inon regulard here: ion inding protein     5.4       SAOUHSC,0023     inf     6.5     Inon regulard here: ion inding protein     8.5       SAOUHSC,0024     inf     6.5     Inon regulard here: ion inding protein     8.5	SAOUHSC_02655	-	4.2	Unknown, hypothetical protein	6.3
SAOUHSC,0019         indf         4.4         Heme-degrading noncoxygenase liel         5.7           SAOUHSC,00175         -         4.5         Extracellular solar-binding protein         8.5           SAOUHSC,00279         sar7         4.6         Staphylocsccal accessory regulator         8.5           SAOUHSC,00279         -         4.8         Unknown, hypothetical protein         6.5           SAOUHSC,00271         -         4.9         Unknown, hypothetical protein         6.5           SAOUHSC,00271         afrC         5.3         Involved in staphyloferin B transport in the cyteplaam         4.6           SAOUHSC,00271         -         5.8         Putative membrare protein         6.1           SAOUHSC,00271         -         6.2         ABC transporter ATP-binding protein         8.5           SAOUHSC,00279         -         6.3         Putative membrare protein         8.5           SAOUHSC,0028         Abd         6.3         Heme transport system permoser Hill         5.4           SAOUHSC,0029         -         6.4         Unknown, hypothetical protein         8.5           SAOUHSC,0020         sird         6.5         Herne transport system permoser Hills         5.4           SAOUHSC,00971         -         6.4	SAOUHSC_01292	-	4.4	Putative DNA-binding protein	NS
SAUCHSC,00176     -     4.5     Extracellular solute bunding protein     so       SAUCHSC,0245     66.4     4.5     Putative transporter     6.7       SAUCHSC,0279     ar7     4.6     Suphyloceccal accessory regulator     so       SAUCHSC,0215     -     4.8     Unknown, hypothetical protein     6.5       SAUCHSC,0652     /fucl     5.1     Pertechnome ARC transporter ATP-binding protein     6.1       SAUCHSC,0071     arC     5.3     Putative membrane protein     6.1       SAUCHSC,0071     -     6.2     ABC transporter ATP-binding protein     6.1       SAUCHSC,0071     -     6.3     Putative membrane protein     6.1       SAUCHSC,0719     -     6.2     ABC transporter ATP-binding protein     5.5       SAUCHSC,0719     -     6.3     Putative Importants     5.6       SAUCHSC,0719     -     6.4     Unknown, hypothetical protein     5.4       SAUCHSC,0721     airB     6.5     Involved in staphyloferrin B transport into the cytoplasm     7.4       SAUCHSC,0721     airB     6.5     Involved in staphyloferrin B transport into the cytoplasm     7.4       SAUCHSC,0721     airB     6.5     Involved in staphyloferrin B transport into the cytoplasm     7.4       SAUCHSC,07097     -     6.7	SAOUHSC_00130	isdI	4.4	Heme-degrading monooxygenase IsdI	5.7
SAOUHSC 02435         gloA         4.5         Patative transporter         6.7           SAOUHSC 0279         arT         4.6         Staphylocccal accessory regulator         ws           SAOUHSC 0232         -         4.8         Unknown, hypothetical protein         6.2           SAOUHSC 0622         fbiA         5.1         Ferrichrone AdC transporter ATF-binding protein FluA         7.0           SAOUHSC 0001         arG         5.3         Laview and a staphylocccal actory bindering protein FluA         7.0           SAOUHSC 0001         arG         5.3         Patative membrane protein         6.1           SAOUHSC 0013         -         5.8         Patative membrane protein         8.1           SAOUHSC 0013         -         6.3         Patative lipoprotein         8.5           SAOUHSC 0024         -         6.4         Unknown, hypothetical protein         8.6           SAOUHSC 0021         arB         6.5         Ineviewal instaphyloferring transmit         5.4           SAOUHSC 0022         arB         6.5         Ineviewal instaphyloferring transmit         5.4           SAOUHSC 0023         arB         6.5         Ineviewal instaphyloferring transmit         6.1           SAOUHSC 00190         -         6.7         U	SAOUHSC_00176	-	4.5	Extracellular solute-binding protein	NS
SAOUHSC 02799arr?4.6Supplexcal accessory regulatorissSAOUHSC 02432-4.8Urknown, hypothetical protein6.2SAOUHSC 0052//bud5.1Ferrichtome AIC transporter ATP-binding protein FluxA7.0SAOUHSC 0051 <i>atrC</i> 5.3Pattive membrase protein6.1SAOUHSC 02211-5.5Pattive membrase protein6.1SAOUHSC 022121-6.3Pattive membrase protein8.1SAOUHSC 02213-6.3Pattive membrase protein8.1SAOUHSC 02228IntaB6.3Hence transport system permease HtsB5.4SAOUHSC 02248IntaB6.3Hence transport system permease HtsB5.4SAOUHSC 02248IntaB6.5Iroobed in stapholetrain B transport into the cytoplasm7.4SAOUHSC 0022 <i>atrB</i> 6.5Iroobed in stapholetrain B transport into the cytoplasm7.4SAOUHSC 0022 <i>atrB</i> 6.5Iroobed in stapholetrain B transport into the cytoplasm7.4SAOUHSC 0002 <i>atrB</i> 6.5Iroobed in stapholetrain B transport into the cytoplasm7.4SAOUHSC 0003-6.7Urknown, hypothetical protein8.9SAOUHSC 00045 <i>indE</i> 8.6Hence head pathole more system in the cytoplasm6.3SAOUHSC 00052 <i>indE</i> 8.6Hence head pathole more system in the cytoplasm7.4SAOUHSC 00053 <i>indE</i> 8.6Hence head pathole more system in the cytoplasm7.4SAOUHSC 00054 <i>indE</i> 8.5ABC	SAOUHSC_02435	sfaA	4.5	Putative transporter	6.7
SAOUHSC,02432-4.8Unknown, hypothetical protein6.2SAOUHSC,0032JinA5.1Performer ARC transporter ATP-binding protein BhuA7.0SAOUHSC,00031airC5.3Involved in staphyloferrin B transport into the cytoplasm4.6SAOUHSC,00011airC5.3Pattitive membrane protein8.1SAOUHSC,00011-5.3Pattitive membrane protein8.1SAOUHSC,02211-6.2ABC transport a TP-binding protein8.5SAOUHSC,02219-6.3Pattitive (hepprotein8.5SAOUHSC,02428AirB6.3Hence transport syme permase HuB5.4SAOUHSC,02428AirB6.3Hence transport syme permase HuB5.4SAOUHSC,02021airB6.5Involved in staphyloferrin B transport into the cytoplasm7.4SAOUHSC,00012airB6.5Involved in staphyloferrin B transport into the cytoplasm7.4SAOUHSC,00012airB6.5Involved in staphyloferrin B transport into the cytoplasm7.4SAOUHSC,00012airB6.5Involved in staphyloferrin B transport into the cytoplasm7.4SAOUHSC,00013-7.9Uchnown, hypothetical protein8.5SAOUHSC,0003airB8.5ABC permase Inff6.1SAOUHSC,0004airG8.7Hence cytopic Inportein IndE5.6SAOUHSC,0018airG8.7Hence cytopic Inportein IndE5.6SAOUHSC,0018airG8.6Henc receipic Inportein IndE5.6	SAOUHSC_02799	sarT	4.6	Staphylococcal accessory regulator	NS
SAOUHSC,02215-4.9Unknown, hypothetical protein6.5SAOUHSC,0052 <i>flud.</i> 5.3Ferrichrome ABC transport FATP-Inding protein FluA7.0SAOUHSC,0011 <i>i</i> .a5.3Involved in a taphyloferrin B transport into the cytoplasm6.6SAOUHSC,0021 <i>i</i> .a5.3Putative membrane protein8.1SAOUHSC,02210-6.2ABC transport FATP-binding protein8.8SAOUHSC,02282 <i>i</i> .a6.3Putative membrane protein8.1SAOUHSC,02282 <i>i</i> .a6.4Unknown, hypothetical protein8.4SAOUHSC,02072 <i>i</i> .a6.4Unknown, hypothetical protein8.1SAOUHSC,0072 <i>i</i> .a6.5Ferric hydoxamate receptor 1 Pha1026.8SAOUHSC,0072 <i>i</i> .a6.5Ferric hydoxamate receptor 1 Pha1026.8SAOUHSC,0073-7.9Unknown, hypothetical protein8.4SAOUHSC,0085 <i>i</i> .aff6.5Ferric hydoxamate receptor 1 Pha1026.6SAOUHSC,0085 <i>i</i> .aff6.5Ferric hydoxamate receptor 1 Pha1026.6SAOUHSC,0085 <i>i</i> .aff6.5Ferrichrome.ABC transport AIC7.1SAOUHSC,0085 <i>i</i> .aff6.5Ferrichrome.ABC transport Infall6.5SAOUHSC,0085 <i>i</i> .aff6.5Ferrichrome.ABC transport AIC7.1SAOUHSC,0085 <i>i</i> .aff6.5Ferrichrome.ABC transport AIC7.1SAOUHSC,0085 <i>i</i> .aff6.3Ferrichrome.ABC transport Protein8.0SAOUHSC,0084 <i>i</i> .aff <t< td=""><td>SAOUHSC_02432</td><td>-</td><td>4.8</td><td>Unknown, hypothetical protein</td><td>6.2</td></t<>	SAOUHSC_02432	-	4.8	Unknown, hypothetical protein	6.2
SAOUHSC,00652 <i>fmA</i> 5.1         Ferrichrome ABC transporter ATP-binding protein FunA         7.0           SAOUHSC,0071 <i>iii</i> C         5.3         Involved in staphyloferin B transport into the cytoplasm         4.6           SAOUHSC,0071 <i>iii</i> C         5.3         Patative membrane protein         8.8           SAOUHSC,0191         -         6.2         ABC transporter ATP-binding protein         8.8           SAOUHSC,02028 <i>iii</i> B         6.3         Patative improvem protein         8.8           SAOUHSC,0074         -         6.4         Unknown, hypothetical protein         8.8           SAOUHSC,0081 <i>iii</i> A         6.5         Incorregulated heme-transport system permases         16.8           SAOUHSC,0087 <i>iii</i> A         6.5         Incorregulated heme-transport system permases         16.8           SAOUHSC,0087 <i>iii</i> B         6.5         Incoregulated heme-transport system permases         16.8           SAOUHSC,0087 <i>iii</i> B         6.5         Incoregulated heme-transport system permases         16.9           SAOUHSC,0086 <i>iii</i> B         6.5         Incoregulated heme-transport system permases         16.9           SAOUHSC,0087         -         6.7         Unknown, hypothetical protein         15.	SAOUHSC_02245	_	4.9	Unknown, hypothetical protein	6.5
SAOUHSC_00071 <i>i</i> rfC5.3Involved in staphyloferin B transport into the cytoplasm4.6SAOUHSC_00131-5.3Putative membrane protein6.1SAOUHSC_00132-6.2ABC transport ATP binding protein5.5SAOUHSC_00132-6.3Putative incerbrane protein8.1SAOUHSC_00132hBB6.3Herne transport system permetse HIsB5.4SAOUHSC_00132ind6.3Herne transport system permetse HisB5.4SAOUHSC_00132ind6.5Iron-regulated herne-iron binding protein5.4SAOUHSC_00132ind6.5Iron-regulated herne-iron binding protein5.4SAOUHSC_00152ind6.5Iron-regulated herne-iron binding protein5.4SAOUHSC_00154 <i>fluD2</i> 6.5Iron-regulated herne-iron binding protein5.4SAOUHSC_00154 <i>fluD2</i> 6.7Unknown, hypothetical protein5.9SAOUHSC_00155 <i>indF</i> 8.5ABC permease half6.1SAOUHSC_00185 <i>indG</i> 8.7Herne-edegrading monocovgenase kalf6.3SAOUHSC_00182 <i>indG</i> 8.7Herne-edegrading monocovgenase kalf6.3SAOUHSC_00182 <i>indG</i> 8.9Herne-transporter kolf8.9SAOUHSC_00182 <i>indG</i> 8.9Herne transporter kolf8.9SAOUHSC_00182 <i>indG</i> 8.9Herne transporter kolf8.9SAOUHSC_00183 <i>indG</i> 8.9Herne transporter kolf8.9SAOUHSC_00184 <i>indG</i> 8.9Herne-	SAOUHSC_00652	fhuA	5.1	Ferrichrome ABC transporter ATP-binding protein FhuA	7.0
SAOUHSC, 00131-5.3Putative membrane protein6.1SAOUHSC, 0321-5.8Putative membrane proteinNoSAOUHSC, 0321-6.2ABC transporter ATP-binding proteinSoSAOUHSC, 0329-6.3Putative lipoproteinNoSAOUHSC, 03428ht/B6.3Heme transport system permease Ht/B5.4SAOUHSC, 00974-6.4Unknown, hypothetical proteinSoSAOUHSC, 00071si/A6.5Iron-regulated heme iron binding protein5.4SAOUHSC, 00072si/B6.5Forric hydroxanate receptor 1 FhaD26.8SAOUHSC, 00073-6.7Unknown, hypothetical protein3.9SAOUHSC, 01086is/B8.5ABC permease Is/B6.1SAOUHSC, 01086is/B8.5ABC permease Is/B6.1SAOUHSC, 01086is/B8.6Heme degrading monoxygenase Is/G4.7SAOUHSC, 01086is/B8.6Heme degrading monoxygenase Is/G4.7SAOUHSC, 01082is/C8.9Heme degrading monoxygenase Is/G5.5SAOUHSC, 01082is/C8.9Heme degrading monoxygenase Is/G8.7SAOUHSC, 01082is/C8.9Heme degrading monoxygenase Is/G5.5SAOUHSC, 01082is/C8.9Heme degrading monoxygenase Is/G8.7SAOUHSC, 01082is/C8.9Heme degrading monoxygenase Is/G8.7SAOUHSC, 01082is/C8.9Heme degrading monoxygenase Is/G8.7SAOUHSC, 0	SAOUHSC 00071	sirC	5.3	Involved in staphyloferrin B transport into the cytoplasm	4.6
SAOUHSC.02821         -         5.8         Putative membrane protein         NS           SAOUHSC.02719         -         6.2         ARC transporter ATP-binding protein         5.5           SAOUHSC.02719         -         6.3         Putative lipoprotein         5.5           SAOUHSC.00974         -         6.4         Unknown, hypothetical protein         5.4           SAOUHSC.00072 <i>inB</i> 6.5         Iron-regulated heme-iron binding protein         5.4           SAOUHSC.00072 <i>inB</i> 6.5         Iron-regulated heme-iron binding protein         5.4           SAOUHSC.00072 <i>inB</i> 6.5         Iron-regulated heme-iron binding protein         7.4           SAOUHSC.00073         -         6.7         Unknown, hypothetical protein         8.8           SAOUHSC.00085 <i>indF</i> 8.5         ABC permess idf         6.1           SAOUHSC.00086 <i>indF</i> 8.8         ABC permess idf         6.3           SAOUHSC.00085 <i>indF</i> 8.8         ABC permess idf         6.1           SAOUHSC.00086 <i>indF</i> 8.9         Heme deptral inporter permesse         6.3           SAOUHSC.00085 <i>indF</i> 8.9         Heme deptral inporter permesse	SAOUHSC 00131	-	5.3	Putative membrane protein	6.1
SAOUHSC_02719-6.2ABC transporter ATP-binding protein5.5SAOUHSC_02230-6.3Putative lipoprotein88SAOUHSC_02428ht/b6.3Hene transport system permease HisB5.4SAOUHSC_0074-6.4Ucharom, hypothetical protein88SAOUHSC_0072sir/B6.5Iron-regulated hene-iron binding protein5.4SAOUHSC_0072sir/B6.5Iron-regulated hene-iron binding protein7.4SAOUHSC_0072sir/B6.5Ferric hydroxamate receptor 1 FhuD26.8SAOUHSC_0080-6.7Ucharom, hypothetical protein3.9SAOUHSC_0080is/B8.5ABC permease Is/B6.1SAOUHSC_0086is/B8.6Hene-receptor lipopton Is/B5.5SAOUHSC_0086is/B8.6Hene-receptor lipopton Is/B5.5SAOUHSC_0087-8.9Iron compound ABC transporter permease6.3SAOUHSC_0084is/B8.9Hene transporter Is/C9.1SAOUHSC_0048sidC8.9Hene transporter ATP-binding protein SatC9.1SAOUHSC_0048sidC9.6Ferrichrome ABC transporter ATP-binding protein SatC9.1SAOUHSC_0048sidD10.0Iron compound ABC transporter ATP-binding protein SatC9.1SAOUHSC_0048sidD10.0Iron compound ABC transporter ATP-binding protein SatC9.1SAOUHSC_0045sidD10.0Iron compound ABC transporter Permease SatB9.0SAOUHSC_0046sidA <td< td=""><td>SAOUHSC 02821</td><td>-</td><td>5.8</td><td>Putative membrane protein</td><td>NS</td></td<>	SAOUHSC 02821	-	5.8	Putative membrane protein	NS
SAOUHSC_01920         -         6.3         Puttive Topportin         No           SAOUHSC_01920         -         6.3         Henne transport system pernease HisB         5.4           SAOUHSC_00974         -         6.4         Unknown, hypothetical protein         85           SAOUHSC_00973         isdA         6.5         Ironvolating in staphyloferrin B transport into the cytoplasm         7.4           SAOUHSC_00072 <i>siB</i> 6.5         Ferric hydroxamate receptor 1 FhuD2         6.8           SAOUHSC_00073         -         6.7         Unknown, hypothetical protein         3.9           SAOUHSC_00084 <i>isdB</i> 8.5         ABC proteinase IsdF         6.1           SAOUHSC_00085 <i>isdB</i> 8.5         ABC pernose IsdF         6.1           SAOUHSC_00082 <i>isdG</i> 8.7         Heme-degrading monoxygenase IsdG         4.7           SAOUHSC_00082 <i>isdG</i> 8.7         Heme-degrading monoxygenase IsdG         4.7           SAOUHSC_00082 <i>isdG</i> 8.7         Heme-degrading monoxygenase IsdG         5.5           SAOUHSC_0082 <i>isdG</i> 8.7         Heme-degrading monoxygenase IsdG         5.5           SAOUHSC_0082 <i>isdG</i> 8.7	SAOUHSC 02719	-	6.2	ABC transporter ATP-binding protein	5.5
And United Control         And         Hermit Deprint         And           SAOUHSC_02428         hink         6.3         Hermit trapport system permease Hisk         5.4           SAOUHSC_00974         -         6.4         Unknown, hypothetical protein         85           SAOUHSC_00972         sirB         6.5         Iron-regulated heme-iron binding protein         5.4           SAOUHSC_00900         sirB         6.5         Iron-regulated heme-iron binding protein         7.4           SAOUHSC_00900         -         6.7         Unknown, hypothetical protein         3.9           SAOUHSC_00901         -         6.7         Unknown, hypothetical protein         85           SAOUHSC_00903         -         7.9         Unknown, hypothetical protein         85           SAOUHSC_0086         iddF         8.5         ABC permease IsdF         6.1           SAOUHSC_0089         iddG         8.7         Heme-receptor lipoprotein IsdE         5.6           SAOUHSC_0089         iddC         8.9         Horn compound ABC transporter Parmease         6.3           SAOUHSC_01087         -         8.9         Horn compound ABC transporter ParD1         8.0           SAOUHSC_02076         shdD         10.0         Forindorne ABC transporter ParD1 <td>SAOUHSC 01920</td> <td>-</td> <td>6.3</td> <td>Putative lipoprotein</td> <td>NS</td>	SAOUHSC 01920	-	6.3	Putative lipoprotein	NS
SAOUHSC,00974         -         6.4         Unknown, hypothetical protein         83           SAOUHSC,00974         -         6.4         Unknown, hypothetical protein         5.4           SAOUHSC,00972         sirB         6.5         Iron-regulated heme-iron binding protein         6.4           SAOUHSC,00972         sirB         6.5         Iron-regulated heme-iron binding protein         6.8           SAOUHSC,00973         -         6.7         Unknown, hypothetical protein         3.9           SAOUHSC,00973         -         7.9         Unknown, hypothetical protein         3.9           SAOUHSC,0086         isdF         8.5         ABC permease IsdF         6.1           SAOUHSC,01086         isdF         8.5         Heme-receptri Iportein IsdE         5.6           SAOUHSC,01087         -         8.9         Iron compound ABC transporter permease IsdG         4.7           SAOUHSC,01082         isdC         8.9         Heme transporter IsdC         5.5         5.5           SAOUHSC,0087         -         8.9         Heme transporter IsdC         5.1         5.3           SAOUHSC,0082         isdC         9.6         Ferrichrome ABC transporter ParD1         8.0         6.2           SAOUHSC,00747         stdB	SAOUHSC 02428	htsB	6.3	Heme transport system permease HtsB	5.4
SAOUHSC_01081isdA6.5Iron-regulated heme-iron binding protein5.4SAOUHSC_00072sirB6.5Involved in staphyloferrin B transport into the cytoplasm7.4SAOUHSC_02554 <i>fhuD2</i> 6.5Ferric hydroxanate receptor 1 FhuD26.8SAOUHSC_00973-6.7Unknown, hypothetical protein3.9SAOUHSC_00973-7.9Unknown, hypothetical protein8.8SAOUHSC_01086isdF8.5ABC permase IsdF6.1SAOUHSC_01085isdE8.6Heme-receptor Ipoprotein IsdE5.6SAOUHSC_01087-8.9Iron compound ABC transporter permease6.3SAOUHSC_01087-8.9Heme receptor Ipoprotein IsdE5.5SAOUHSC_01087-8.9Iron compound ABC transporter permease6.3SAOUHSC_01087-8.9Herme transporter IslC9.1SAOUHSC_01087isdC8.9Herme transporter IslC9.1SAOUHSC_00748stdC8.6Ferrichroner, ABC transporter ATP-binding protein SatC9.1SAOUHSC_00745stdrD10.0Iron compound ABC transporter PhuD18.0SAOUHSC_0074stdL10.0Iron compound ABC transporter PhuD18.0SAOUHSC_0074stdL10.4Ferrichroner ABC transporter PhuD18.0SAOUHSC_0074stdL10.4Ferrichroner ABC transporter permease SatB9.0SAOUHSC_0074stdL11.2Unknown, hypothetical protein18.5SAOUHSC_0074stdL11.2	SAOUHSC 00974	-	6.4	Unknown, hypothetical protein	NS
SAOUHSC_0072 <i>internal and the set of the</i>	SAOUHSC 01081	isdA	6.5	Iron-regulated heme-iron binding protein	5.4
SAOUHSC, 02554         fmuD2         6.5         Ferric hydroxamate receptor 1 FhuD2         6.8           SAOUHSC, 01090         -         6.7         Unknown, hypothetical protein         3.9           SAOUHSC, 0073         -         7.9         Unknown, hypothetical protein         85           SAOUHSC, 01086         iidf         8.5         ABC permease IsdF         6.1           SAOUHSC, 01085         iidf         8.6         Heme-degrading monoxygenase IsdG         4.7           SAOUHSC, 01089         iidG         8.7         Heme-degrading monoxygenase IsdG         4.7           SAOUHSC, 01087         -         8.9         Iron compound ABC transporter permease         6.3           SAOUHSC, 01082         iidG         8.9         Heme transporter ATP-binding protein SstC         9.1           SAOUHSC, 00748         sstC         9.6         Ferrichrome ABC transporter PhuD1         80           SAOUHSC, 00745         sdrD         10.0         Iron compound ABC transporter PhuD1         80           SAOUHSC, 00747         sdrB         10.2         Sortase StrB         6.2           SAOUHSC, 00747         sdrB         10.4         Ferrichrome ABC transporter permease StrA         10.5           SAOUHSC, 00746         stdA         11.2 <td>SAOUHSC 00072</td> <td>sirB</td> <td>6.5</td> <td>Involved in staphyloferrin B transport into the cytoplasm</td> <td>7.4</td>	SAOUHSC 00072	sirB	6.5	Involved in staphyloferrin B transport into the cytoplasm	7.4
SACUHSC_01090-67Unknown, hypothetical protein10SACUHSC_01096isdF8.5ABC permease IsdF6.1SAOUHSC_01085isdE8.6Heme-receptor lipoprotein IsdE5.6SAOUHSC_01089isdG8.7Heme-degrading monooxygenase IsdG4.7SAOUHSC_01087-8.9Iron compound ABC transporter permease6.3SAOUHSC_01087-8.9Heme degrading monooxygenase IsdG9.1SAOUHSC_01082isdC8.9Heme transporter IsdC5.1SAOUHSC_00748sstC9.6Ferrichrome ABC transporter ATP-binding protein SstC9.1SAOUHSC_0055sdrD10.0Florinogen-binding protein SdrDNSSAOUHSC_00742-10.1Unknown, hypothetical proteinNSSAOUHSC_00772-10.1Unknown, hypothetical proteinNSSAOUHSC_00747sstB10.2Sortase StrB6.2SAOUHSC_00748strH11.2Unknown, hypothetical proteinNSSAOUHSC_00740strH111.2Unknown, hypothetical proteinNSSAOUHSC_00741strA11.9Ferrichrome ABC transporter permease SatB9.0SAOUHSC_00744strA11.2Heme transport permease SatA10.9SAOUHSC_00744strA11.3ATP-hydrolysing and heme-binding protein IsdD6.2SAOUHSC_00744strA11.6Receptor component of staphyloferrin B16.3SAOUHSC_00744strA11.6Nakown, hypothetical proteinNS <td>SAOUHSC 02554</td> <td>fhuD2</td> <td>6.5</td> <td>Ferric hydroxamate receptor 1 FhuD2</td> <td>6.8</td>	SAOUHSC 02554	fhuD2	6.5	Ferric hydroxamate receptor 1 FhuD2	6.8
SAOUHSC_00973-7.9Unknown, hypothetical proteinBSSAOUHSC_01085isdF8.5ABC permease IsdF6.1SAOUHSC_01085isdE8.6Heme-receptor lipoprotein IsdE5.6SAOUHSC_01089isdG8.7Heme-degrading monooxygenase IsdG4.7SAOUHSC_01087-8.9Iron compound ABC transporter permease6.3SAOUHSC_00748isdC8.9Heme transporter IsdC5.5SAOUHSC_00748isdC8.9Heme transporter ATP-binding protein SstC9.1SAOUHSC_00748isdC9.6Ferrichrome ABC transporter FMD180SAOUHSC_00742isdC9.6Ferrichrome ABC transporter FMD180SAOUHSC_00742isdB10.0Iron compound ABC transporter FMD180SAOUHSC_00772-10.1Unknown, hypothetical protein81SAOUHSC_00747stfB10.2Sortase StrB6.2SAOUHSC_00740strfH11.2Unknown, hypothetical regulatory-like proteinNSSAOUHSC_00746stA11.9Ferrichrome ABC transporter permease StA10.9SAOUHSC_00746isdD13.3ATP-hydrolysing and heme-binding protein IsdD6.2SAOUHSC_00741isdD13.6Receptor component of staphyloferrin B16.3SAOUHSC_00741isdD13.6Receptor component of staphyloferrin B16.3SAOUHSC_00741isrA13.6Receptor component of staphyloferrin B16.3SAOUHSC_00744-15.6Unknown,	SAOUHSC 01090	_	67	Unknown, hypothetical protein	3.9
SAOUHSC_01086isdF8.5ABC permease IsdF6.1SAOUHSC_01085isdE8.6Heme-receptor lipoprotein IsdE5.6SAOUHSC_01089isdG8.7Heme-degrading monooxygenase IsdG4.7SAOUHSC_01087-8.9Iron compound ABC transporter permease6.3SAOUHSC_01082isdC8.9Heme transporter IsdC5.5SAOUHSC_00748sstC9.6Ferrichrome ABC transporter ATP-binding protein SstC9.1SAOUHSC_00748sstC9.6Ferrichrome ABC transporter ATP-binding protein SstC9.1SAOUHSC_00747ssdD10.0Florinogen-binding protein SdrDNSSAOUHSC_00792-10.1Unknown, hypothetical proteinNSSAOUHSC_00747sstB10.2Sortase StB6.2SAOUHSC_00747sstB10.4Ferrichrome ABC transporter permease SstB9.0SAOUHSC_00740hitsA11.2Heme transport sperm lipoprotein HsA10.5SAOUHSC_00746stA11.9Ferrichrome ABC transporter permease SstA10.9SAOUHSC_0074sirA13.3ATP-hydrolysing and heme-binding protein HsA10.5SAOUHSC_01084isdD13.3ATP-hydrolysing and heme-binding protein IsdD6.2SAOUHSC_01084isdD13.3ATP-hydrolysing and heme-binding protein IsdD6.2SAOUHSC_021514-15.6Unknown, hypothetical proteinNSSAOUHSC_02222-16.7Hypothetical proteinNSSAOUHSC_021514- <t< td=""><td>SAOUHSC 00973</td><td>_</td><td>79</td><td>Unknown, hypothetical protein</td><td>NS</td></t<>	SAOUHSC 00973	_	79	Unknown, hypothetical protein	NS
And the second	SAOUHSC 01086	isdF	85	ABC permease IsdF	61
SAOUHSC_01089iside8.7Heme-degrading monoxygenase Isde4.7SAOUHSC_01087-8.9Iron compound ABC transporter permease6.3SAOUHSC_01082isidC8.9Heme transporter IsdC5.5SAOUHSC_00748stfC9.6Ferrichrome ABC transporter ATP-binding protein StfC9.1SAOUHSC_00545sdrD10.0Fibrinogen-binding protein SdrDNSSAOUHSC_00972-10.1Unknown, hypothetical proteinNSSAOUHSC_00972-10.1Unknown, hypothetical proteinNSSAOUHSC_00074sstB10.2Sortase StrB6.2SAOUHSC_00747sstB10.4Ferrichrome ABC transporter permease SstB9.0SAOUHSC_00747sstB11.2Unknown, hypothetical regulatory-like proteinNSSAOUHSC_00746sstA11.2Heme transport system lipoprotein HtsA10.5SAOUHSC_0074sirA13.6Receptor component of staphyloferrin B6.2SAOUHSC_0074sirA13.6Receptor component of staphyloferrin B6.3SAOUHSC_0074sirA13.6Receptor component of staphyloferrin B6.3SAOUHSC_01514-15.6Unknown, hypothetical proteinNSSAOUHSC_0232-16.7Hypothetical proteinNSSAOUHSC_0238-15.6Unknown, hypothetical proteinNSSAOUHSC_0238-16.7Phypothetical proteinNSSAOUHSC_0238-16.7Phypothetical proteinNS	SAOUHSC 01085	isdF	86	Heme-receptor linoprotein IsdF	5.6
ANOTHSC_01087-8.9Iron compound ABC transporter permease6.3SAOUHSC_01082isdC8.9Heme transporter IsdC5.5SAOUHSC_00748sstC9.6Ferrichrome ABC transporter ATP-binding protein SstC9.1SAOUHSC_00545sdrD10.0Fibrinogen-binding protein SdrD88SAOUHSC_0072-10.1Unknown, hypothetical protein80SAOUHSC_00772-10.1Unknown, hypothetical protein80SAOUHSC_00772-10.1Unknown, hypothetical protein80SAOUHSC_00772-10.1Unknown, hypothetical protein80SAOUHSC_00774sstB10.2Sortase StrB6.2SAOUHSC_0070sarH111.2Unknown, hypothetical regulatory-like protein85SAOUHSC_00746sstA11.2Heme transporter permease SstB10.9SAOUHSC_00746sstA11.2Heme transport system lipoprotein HtsA10.5SAOUHSC_00746sstA11.3ATP-hydrolysing and heme-binding protein IsdD6.2SAOUHSC_0074irA13.6Receptor component of staphyloferrin B16.3SAOUHSC_0074irA13.6Receptor component of staphyloferrin B16.3SAOUHSC_01514-15.6Unknown, hypothetical protein85SAOUHSC_0218-16.7Hypothetical protein85SAOUHSC_0218-15.5Dexisin A15SAOUHSC_02218-51.5Dexisin A15	SAOUHSC 01089	isdG	87	Heme-degrading monooxygenase IsdG	47
SAOUHSC_01082isdC8.9Here transporter JsdC5.5SAOUHSC_00748sstC9.6Ferrichrome ABC transporter ATP-binding protein SstC9.1SAOUHSC_00545sdrD10.0Fibrinogen-binding protein SdrDssSAOUHSC_00246fhuD110.0Iron compound ABC transporter FhuD18.0SAOUHSC_0072-10.1Unknown, hypothetical proteinssSAOUHSC_00772-10.1Unknown, hypothetical proteinssSAOUHSC_00772-10.1Unknown, hypothetical proteinssSAOUHSC_00774sstB10.2Sortase StrB6.2SAOUHSC_00770sarH111.2Unknown, hypothetical regulatory-like proteinssSAOUHSC_00766sstA11.9Ferrichrome ABC transporter permease StA10.9SAOUHSC_00741isdD13.3ATP-hydrolysing and heme-binding protein IsdD6.2SAOUHSC_00741isdD13.3ATP-hydrolysing and heme-binding protein IsdD6.2SAOUHSC_00741isdD13.3ATP-hydrolysing and heme-binding protein IsdD6.2SAOUHSC_00741isdA11.6Unknown, hypothetical proteinssSAOUHSC_00741isdA13.6Receptor component of staphyloferrin B16.3SAOUHSC_00222-16.7Hypothetical proteinssSAOUHSC_02232-16.7Phage repressor proteinssSAOUHSC_02218-51.5Pertain AssSAOUHSC_02218-51.5Pertain Ass	SAOUHSC 01087	_	89	Iron compound ABC transporter permease	63
SAOUHSC_00748std9.6Ferrichrome ABC transporter ATP-binding protein SstC9.1SAOUHSC_00748sstC9.6Ferrichrome ABC transporter ATP-binding protein SstC9.1SAOUHSC_00246fhuD110.0Fibrinogen-binding protein SdrDNSSAOUHSC_02246fhuD110.0Iron compound ABC transporter FhuD18.0SAOUHSC_00972-10.1Unknown, hypothetical proteinNSSAOUHSC_00747sstB10.2Sortase StrB6.2SAOUHSC_00747sstB10.4Ferrichrome ABC transporter permease SstB9.0SAOUHSC_00700sarH111.2Unknown, hypothetical regulatory-like proteinNSSAOUHSC_00746sstA11.9Ferrichrome ABC transporter permease SstA10.9SAOUHSC_00746sstA11.2Heme transport system lipoprotein HtsA10.5SAOUHSC_00746sstA11.9Ferrichrome ABC transporter permease SstA10.9SAOUHSC_0074sirA13.6Receptor component of staphyloferrin B6.2SAOUHSC_0074sirA13.6Receptor component of staphyloferrin B16.3SAOUHSC_01514-15.6Unknown, hypothetical proteinNSSAOUHSC_02223-16.7Hypothetical proteinNSSAOUHSC_02218-17.7Phage repressor proteinNSSAOUHSC_02218-25.9Unknown, hypothetical proteinNSSAOUHSC_02218-51.5Pertrin ATransporter ProteinNS	SAOUHSC 01082	isdC	89	Heme transporter IsdC	5.5
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All CHR C_LOLJunctInternational protein that prior tha	SAOUHSC 02246	fhuD1	10.0	Iron compound ABC transporter FhuD1	8.0
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**Fig. 3.** EsaB-YFP localises to the cytoplasm and membrane. (a) EsaB-YFP is not secreted in *S. aureus* strain RN6390. RN6390 harbouring empty pRAB11 and the isogenic  $\Delta esaB$  strain harbouring empty pRAB11 or pRAB11 encoding EsaB-YFP were cultured in TSB medium until mid-log phase and separated into cellular and supernatant fractions (sn). For each gel, 10 µl of  $OD_{600}$ 1 adjusted cells and 15 µl of TCA-precipitated culture supernatant were loaded. Blots were probed with anti-EsxA, anti-TrxA (cytoplasmic control) and anti-GFP antisera. Cell and supernatant samples have been blotted on the same gel but intervening lanes have been spliced out. Subcellular localisation of (b) EsaB-YFP in RN6390 and an isogenic  $\Delta esx$  ( $\Delta (esxA-esaG)$ ) strain or (c) YFP in RN6390. Cells were grown aerobically in TSB to mid-log phase and fractionated as indicated in the Methods. Equivalent amounts of each fraction was probed with anti-TrxA (cytoplasmic control), anti-TrxA (cytoplasmic control), anti-EsxA and anti-GFP antisera.

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**Fig. 4.** EsaB is present in cells at low amounts. (a) Titration of  $\alpha$ -EsaB antibodies. The indicated amounts of purified EsaB, alongside 30 µl of OD<sub>600</sub>5 adjusted cells were loaded on a SDS-PAGE as indicated and blotted using  $\alpha$ -EsaB antibodies. Two exposures of the blot are shown. (b) RN6390 and the isogenic  $\Delta essC$  strain (top) or strain Newman (bottom panel) were grown aerobically in TSB medium with or without hemin, as indicated, until an OD<sub>600</sub> of 2 was reached, at which point cells were harvested as described in Methods. In each case, for detection of EsaB, 25 µl of OD<sub>600</sub>2 adjusted cells were loaded and 25 ng of purified EsaB protein was loaded as a positive control. 5 µl of OD<sub>600</sub>2 adjusted cells were probed against anti-TrxA antisera as a cytoplasmic control. (c) RN6390 harbouring empty pRAB11 (labelled RN6390), and the isogenic *esaB* deletion strain harbouring pRAB11 (labelled  $\Delta esaB$ ), or pRAB11 encoding native EsaB or EsaB-YFP was cultured aerobically in TSB medium until an OD<sub>600</sub> of 2 was reached. Samples were fractionated to give cells and supernatant (sn), and supernatant proteins were precipitated using TCA. For each gel, 10 µl of OD<sub>600</sub>1 adjusted cells and 15 µl of culture supernatant samples have been blotted on the same gel but intervening lanes have been spliced out. (d) EsaB-YFP can be detected in whole cells. RN6390 harbouring empty pRAB11 (labelled  $\Delta esaB$ ), or pRAB11 encoding EsaB-YFP was cultured aerobically in TSB medium until an OD<sub>600</sub> of 2 was reached. Supernatant samples have been blotted on the same gel but intervening lanes have been spliced out. (d) EsaB-YFP can be detected in whole cells. RN6390 harbouring empty pRAB11 (labelled RN6390), and the isogenic *esaB* deletion strain harbouring pRAB11 (labelled  $\Delta esaB$ ), or pRAB11 encoding EsaB-YFP was cultured aerobically in TSB medium until an OD<sub>600</sub> of 2 was reached. Whole cell samples (20 µl of OD<sub>600</sub>2 adjusted cells) were loaded and blots were probed with anti GFP antibodies. Two exposures of the blot are shown.

strain carrying a chromosomal deletion in all twelve genes at the *ess* locus (Fig. 1a). However this did not alter the localisation of EsaB-YFP, which was still detected in both cytoplasm and membrane fractions (Fig. 3b). It is possible that EsaB-YFP localises to the membrane through interaction with additional membrane proteins. Alternatively, it may be that the membrane localisation arises as an artefact of the C-terminal YFP tag, since this tag is known to influence protein behaviour (e.g. [31]).

### Mutagenesis of conserved residues in EsaB

An alignment of EsaB homologues encoded across firmicutes (Fig. 5a) identifies a number of highly conserved amino acids. Many of these are hydrophilic and fall on one face of the predicted structure of EsaB including T8 (*S. aureus* numbering) which is highly conserved as either threonine or serine, and the invariant K56. The presence of an invariant lysine is intriguing since there are a number of highly conserved lysine residues on the structurally-related protein ubiquitin, which are used to assemble polyubiquitin chains [32]. To probe potential roles of these conserved residues we mutated each of T8, D10, L21, K30, K52, K56, L66, G74 and D75 to alanine on plasmid-encoded EsaB and assessed whether the variant EsaB proteins were able to restore T7 secretion activity to the *esaB* deletion strain.

Fig. 5(c) shows that alanine substitutions of each of these conserved residues was tolerated by EsaB with the exception

of T8A, which completely abolished EsaB activity. To test whether other side chain substitutions were permissive at T8, we subsequently constructed EsaB T8S, T8E, T8H, T8K and T8R substitutions. As seen in Fig. 5(d), in addition to T8A the T8R substitution also abolished EsaB activity, but the other substitutions resulted in active protein. Finally we attempted to assess whether the T8A and T8R inactivating substitutions altered the subcellular location of EsaB-YFP. However, when we introduced these into EsaB-YFP we found that they destabilised the protein as it was almost undetectable in whole cells (Fig. 5e), precluding further analysis. We are therefore unable to determine whether substitution of T8 directly alters EsaB function or has an indirect effect by disrupting folding.

### DISCUSSION

In this work we have investigated the role of EsaB in Type VII secretion. EsaB proteins are conserved in firmicutes that produce the T7SS and are encoded at the same loci. Previous work had implicated EsaB in the regulation of *esxC* transcripts [11], although this cannot be a conserved role for EsaB proteins as they are found in all *S. aureus* strains, including the subset that do not encode *esxC* [16]. Here we show that EsaB does not regulate *esxC* in strain RN6390, nor any of the other genes encoded at the *ess* locus. Instead, deletion of *esaB* is associated with upregulation of genes involved in iron acquisition, mirroring the upregulation of



**Fig. 5.** Site-directed mutagenesis of conserved residues of EsaB. (a) Sequence alignment of EsaB homologues from: Sau-*Staphylococcus aureus*; Slu-*Staphylococcus lugdunensis*; Lmo - *Listeria monocytogenes*; Lgr-*Listeria grayi*; Bce - *Bacillus cereus*; Bam-*Bacillus amyloliquefaciens*; Bsu-*Bacillus subtilis*; Bli-*Bacillus licheniformis*; Bhc-*Bhargavaea cecembensis*; Ssi-*Solibacillus silvestris*; Sor-*Streptococcus oralis*; Sga-*Streptococcus gallolyticus*. \* indicate conserved residues and † indicates residues forming a potential hydrophobic patch that were mutated in this work. (b) Model of *S. aureus* EsaB with positions of conserved residues targeted for mutagenesis highlighted. The N- and C-termini are also indicated. (c) and (d) RN6390 harbouring empty pRAB11, and the isogenic *esaB* deletion strain harbouring pRAB11, or pRAB11 encoding native, the indicated variants of EsaB were cultured aerobically in TSB medium until an OD<sub>600</sub> of 2 was reached. Samples were fractionated to give cells and supernatant (sn), and supernatant proteins were precipitated using TCA. For each gel, 10 µl of OD<sub>600</sub>1 adjusted cells and 15 µl of culture supernatant were loaded. Blots were probed with anti-EsxA, and anti-TrxA (cytoplasmic control) antisera. (e) The  $\Delta esaB$  strain harbouring pRAB11 encoding EsaB-YFP (WT-YFP) or the T8A or T8R amino acid-

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substituted variants were cultured in TSB medium until mid-log phase and separated into cellular and supernatant fractions (sn). For each sample,  $10 \,\mu$ l of OD<sub>600</sub>1 adjusted cells and  $15 \,\mu$ l of culture supernatant were loaded and blots were probed with anti-EsxA, anti-TrxA or anti-GFP antisera. The cell samples and supernatant samples have been blotted on the same gels but intervening lanes have been spliced out.

iron-acquisition genes seen when the core T7 component, EssC, is absent [15]. This supports the notion that EsaB is an essential component of the secretion machinery in RN6390 that is necessary for activity, and in agreement with this, deletion of *esaB* prevented export of the T7-dependent extracellular proteins EsxA, EsxB and EsxC. This conclusion is also in agreement with related studies in *B. subtilis*, where the EsaB homologue YukD was shown to be essential for secretion of the WXG100 protein YukE [17, 18].

The precise role of EsaB in T7 secretion is unclear. Structural analysis of B. subtilis YukD shows that it shares a very similar fold to ubiquitin but that it lacks the ability to be conjugated with other proteins [20]. Interestingly, a structurally-related domain is associated with the actinobacterial T7SS, being found at the cytoplasmic N-terminus of the polytopic EccD membrane component [30], suggesting that EsaB-like domains may be essential features of all T7SSs. Given its small size and the observation that highly conserved residues fall primarily on one face of the protein, we reasoned that EsaB may interact with one or more components of the S. aureus T7SS, potentially regulating activity. Post-translational regulation of the S. aureus T7SS has been suggested because in some growth conditions the secretion machinery is present but there is no or very little substrate secretion [12, 19]. Other protein secretion systems are also post-translationally regulated, for example the flagellar Type III secretion system is regulated through interaction of the FliI component with the second messenger cyclic di-GMP [33], and Type VI secretion systems are regulated by phosphorylation [34]. In this context, EsaB proteins contain a highly conserved threonine (or serine) residue close to their N-termini which we considered as a potential site for phosphorylation. Intriguingly, substitution of EsaB T8 for alanine abolished the function of EsaB, although introduction of either the phospho-mimetic glutamate at this position or a positively charged lysine did not affect EsaB activity.

RNA-Seq analysis of the *esaB* mutant strain showed that in addition to a common set of genes showing similar regulation in the *esaB* and *essC* strains, a further subset of genes were uniquely deregulated in the *esaB* mutant. Many of the genes in this EsaB-specific subset are part of the AirSR regulon [26–29]. The AirSR two component system responds to oxidation signals via a redox-active [2Fe-2S] cluster in the sensor kinase AirS to regulate diverse sets of genes involved in anaerobic respiration, lactose metabolism and capsule biosynthesis. It is possible that these genes are dysregulated indirectly, for example in the absence of EsaB the T7 machinery may be in a state that causes ion leakage or membrane stress. Alternatively it is possible that EsaB interacts directly with a component of the Air system. In future it will be interesting to further decipher the roles of EsaB in T7 protein secretion and *S. aureus* physiology.

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### Conflicts of interest

The authors declare that there are no conflicts of interest.

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