1 ***Correspondence: Spontaneous secondary mutations confound analysis of the essential two***

2 ***component system WalKR in*** **Staphylococcus aureus**

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10 Ji *et al*., [1] recently detailed the structure of the extracytoplasmic Per-Arnt-Sim (PAS) domain of

11 WalK (WalK^{EC-PAS}), the sensor kinase of the essential two-component system WalKR in

12 *Staphylococcus aureus*. The authors made two independent *walK* mutants in *S. aureus*, each with a

13 single amino acid alteration in WalK^{EC-PAS}. They postulated from comparative structural analysis

14 and primary sequence comparisons that these residues might be important for extra cellular signal

15 transduction. We have also been exploring the function of WalKR and were surprised by the

16 striking phenotypic impact of a single amino acid substitutions in the WalK sensor, which were

17 contrary to our own unpublished observations.

18

19 The authors subjected their WalK^{EC-PAS} mutants (WalK^{D119A} and WalK^{V149A}) to a series of phenotypic

20 screens to probe the function of this domain. Compared to the parental methicillin sensitive *S.*

21 *aureus* strain Newman [2], the mutants showed dramatic phenotype changes that included reduced

22 susceptibility to lysostaphin exposure, loss of haemolysis on sheep blood agar, reduced biofilm

23 formation and virulence in a mouse infection model. RNA-seq comparisons of mutants to wild

24 type showed substantial transcriptional changes, with 285 (111+174) differentially expressed

25 genes between the two WalK^{EC-PAS} mutants and wild type. They also used structure-based virtual

26 screening to identify 2, 4-dihydroxybenzophenone (DHBP) as a small molecule predicted to

27 interact with the WalK^{EC-PAS} domain. As DHBP stimulated lysostaphin induced lysis and biofilm

28 formation in strain Newman they postulated it as an activator of WalKR. They then compared

29 transcriptional responses of Newman against DHBP exposure and with their D119A mutant. The

30 authors reported that there were 41 genes differentially and inversely expressed in the WalK^{D119A}

31 mutant and DHBP-treated cells, and concluded that this supported a role for DHBP in activating

32 WalKR. No direct evidence to support activation of WalKR through enhanced phosphotransfer was

33 presented [1].

34

35 To investigate further, we were provided with *S. aureus* Newman wild type, WalK^{D119A} and

36 WalK^{V149A} by the senior author, Chuan He, University of Chicago (UoC) [1]. We first sequenced the

37 genomes of University of Melbourne (UoM) and UoC Newman strains and compared their

38 sequences to the published reference, with the two strains differing by only one synonymous

39 mutation in *sbnF* (NWMN_0065), showing that the strains were essentially identical. Then, using

40 allelic exchange, we recreated the WalK^{D119A} mutation in the Newman wild type and the USA300

41 lineage strain NRS384 [3]. We confirmed by genome sequencing that only the AT->CG substitution in

42 WalK^{EC-PAS} was introduced in both strains at position 25994 (Accession: PRJEB14381). We then

43 tested these mutants (UoM Newman WalK^{D119A} and NRS384 WalK^{D119A}) for some of the key

44 phenotype changes observed by Ji *et al.*, [1]. However, in contrast to Ji *et al.*, [1] we observed that

45 UoM WalK^{D119A} and NRS384 WalK^{D119A} were fully hemolytic (Fig. 1A) and exhibited identical

46 growth curve kinetics as the parental strains (optical density and CFU) (Fig. 1B). Concurrent

47 screening of UoC Newman WalK^{D119A} confirmed it was non-hemolytic (Fig. 1A). The mutant also

48 grew to an increased OD$_{600}$, as reported (Fig. 1B) [1], although CFU counts were identical to wild

49 type, suggesting that the increase in OD is not due to increased growth. Additionally, UoC

50 Newman WalK^{D119A} consistently exhibited larger colonies than Newman or UoM WalK^{D119A}. We

51 next measured the sensitivity of the strains to lysostaphin by cell viability (Fig. 1C). We observed

52 that the UoC WalK^{D119A} mutant was significantly more sensitive (not resistant) to lysostaphin than

53 wild type (3-log$_{10}$ reduction vs Newman), whereas the UoM WalK^{D119A} mutant showed no change.

54 Interestingly, the WalK^{D119A} mutation in NRS384 caused an increase in lysostaphin sensitivity,

55 suggesting the mutation contributes to WalKR activation rather than repression, as proposed by Ji

56 *et al.*, [1,4].

57

58 To resolve the discrepancy between the published results [1] and our observations, we subjected

59 UoC WalK^{D119A} and UoC WalK^{V149} to whole genome sequencing and identified all DNA differences

60 (Accession: PRJEB14381). Relative to Newman - and in addition to their expected *walK^{EC-PAS}*

61 changes - both UoC mutants D119A and V149A had acquired six additional mutations (Table 1),

62 most notably two independent loss-of-function mutations in *saeRS*, a major two-component

63 regulator that controls expression of many genes involved in virulence and biofilm formation [5-8].

64   The UoC WalK^{D119A} had a TT insertion at chromosome position 757519 that introduced a

65   frameshift to *saeS*. The UoC WalK^{V149A} had a G→T substitution at chromosome position 757889

66   that introduced a premature stop codon to *saeR*. It is these secondary mutations in *saeRS*, rather

67   than the targeted mutations in *walK*$^{EC-PAS}$, that likely explain the phenotypes observed by Ji *et al.*,

68   (reduced biofilm, loss of haemolysis, reduced virulence) [1]. To confirm the predicted functional

69   consequences of the *saeRS* mutations, we used a P1 Sae red fluorescence reporter plasmid [5]. No

70   fluorescence activity was detected in the UoC WalK^{D119A} strain containing the P1 Sae reporter,

71   consistent with the predicted truncated histidine kinase preventing phosphorylation of SaeR.

72   While high level expression of P1 Sae from Newman and UoM WalK^{D119A} was observed leading to

73   red colonies (Fig. 1D) and high level fluorescence expression (data not shown). We then recreated

74   the mutated *saeS* allele from UoC WalK^{D119A} in both wild type Newman and UoM WalK^{D119A} (Fig.

75   1E). The mutation abolished haemolysis on sheep blood agar. We then repaired the *saeS* mutation

76   in UoC WalK^{D119A} and observed restoration of wild type haemolysis (Fig. 1E). These results

77   categorically show that the UoC WalK^{D119A} strain is an *sae* mutant with the majority of the

78   phenotypic changes reported in this strain (including the RNAseq changes discussed below) likely

79   associated with this mutation rather than the WalK^{D119A} mutation. These unintended secondary

80   mutations in a major *S. aureus* regulatory locus preclude analysis of the role of WalK^{EC-PAS} domain

81   in WalKR signal transduction.

82

83   There is precedence for this specific phenomenon. Sun *et al.*, showed in detail that elevated

84   temperature and antibiotic selection used during the *S. aureus* mutagenesis process can aid in the

85   selection of *saeRS* mutations [5]. How Ji et al., managed to complement the mutations (D119A and

86   V149A) by phage integrase plasmid expression (pCL55) of wild type *walKR* remains to be explained

87   [1]. We have been unable thus far to obtain the complemented mutants from the authors for

88   further analysis.

89

90   We also observed that UoC WalK^{D119A} and UoC WalK^{V149A} exhibited larger colonies compared to

91   wild type, a phenotypic difference not discussed by Ji *et al.* [1]. This change in both mutants might

92   be explained by the C→T substitution observed at 820314, leading to an A128V change in HprK, a

93   serine kinase known to be involved in catabolite repression and associated with a spreading

94   colony phenotype [9].

95

96   We next re-examined the authors' RNAseq data (Accession: GSE75731) obtained from biological

97   duplicate experiments of *S. aureus* Newman wild type, WalK^{D119A}, WalK^{V149A} and Newman with 50

98   µM DHBP treatment. Using *Kallisto* [10] and the *S. aureus* Newman reference genome (Accession:

99   NC_009641) we quantified transcript abundance for each of the four conditions. The output of

100   *Kallisto* was analysed and visualized using Degust [11], the latter an interactive website to explore

101   the analysis [12]. Multi-dimensional scaling plots showed good consistency between the biological

102   replicates, with Newman wild type, Newman-treated-with-DHBP and the two mutants each

103   displayed clustering indicative of distinct transcriptional profiles (Fig. 2a). Applying the same

104   threshold cutoffs (2-fold change) and including a false discovery rate (FDR) of <0.01, we then

105   replicated the intersection analysis of Ji *et al*, but found only nine genes (not the reported 41) that

106   were down regulated in WalK^{D119A} and up regulated in Newman treated with DHBP (Fig. 1C).

107   Among the nine genes, only five overlapped with the 41 reported by Ji *et al.*, [1] and none of these

108   included autolysins previously linked to the WalKR regulon (Table 2) [13,14]. In fact, five of the nine

109   genes listed have all previously been reported to be under SaeRS control (Table 2). We repeated

110   our lysostaphin assay with and without the addition of 75 µM DHBP, however we did not observe

111   an impact on lysostaphin induced cell viability which is in direct contrast to the results of Ji *et al.* [1]

112   where they observed increased loss of turbidity in Newman pre-treated with DHBP upon

113   lysostaphin treatment.

114

115   We also noted that only 52 genes were differentially expressed upon exposure to DHBP, in

116   comparison to the reported 145 genes [1] . We think this discrepancy and the preceding difference

117   were due to the absence of any filter for false discovery rate (FDR) applied by Ji *et al.*, [1]. For

118   example, our analysis of their RNA-seq data without a FDR threshold resulted in 172 differentially

119   expressed genes in Newman treated with DHBP compared to Newman alone [12]. Transcriptome

120   data without statistical significance thresholds are not meaningful [15].

121

122   We also mapped the authors' RNAseq reads for WalK^{D119A} and WalK^{V149A} (Accession: GSE75731) to

123   the *S. aureus* Newman reference (Accession: NC_009641) and readily detected the same *saeRS*

124   mutations we observed from our independent sequencing of these mutants.

125

126   **Conclusion:** Our analyses highlight two major issues with the study of Ji *et al.* [1] Firstly, the

127   presence of unintended *saeRS* mutations in their *walK*$^{EC-PAS}$ mutants invalidate their conclusions

4

128 with respect to role of the WalK extracytoplasmic domain in controlling WalKR function. We

129 observed opposing results; with increased sensitivity to lysostaphin of the clean D119A mutant, a

130 phenotype previously linked with enhanced activity of the system [4]. Secondly, the RNAseq data

131 appears to have been inadequately analysed, with no filtering for false discovery. Application of an

132 appropriate threshold (p<0.01) to their data substantially changes the lists of differentially

133 expressed genes. The authors' use the overlap between the D119A mutant and DHBP treatment

134 to 'prove' that DHBP is signaling through WalK^{EC-PAS}, but this conclusion is not supported by the

135 data.

136

137 The discovery of small molecule inhibitors of WalKR function would represent a major advance in

138 the fight against multidrug resistant *S. aureus*, and Ji *et al.*, [1] have shown a potential approach

139 through establishing the structure of WalK^{EC-PAS}. Unfortunately, based on the data presented, the

140 authors' principal conclusions regarding WalK^{EC-PAS} domain function are not supported. This study

141 is another example of the pitfalls associated with allelic exchange in *S. aureus* and the rigor that

142 must applied to mutation validation [5]. Whole genome sequencing is now so affordable that it can

143 be readily used to verify targeted mutants and the complemented strains. In our own WalKR

144 research we have observed a propensity for mutations introduced into this locus to yield

145 secondary compensatory events [13,16]. These secondary changes can confound analysis of this

146 essential two-component system, and highlight the extreme care needed when manipulating this

147 locus and then attributing specific phenotypes to specific mutational changes.

148

149 **Methods:**

150 **Bacterial strains, primers, plasmids and growth conditions.**

151 The *S. aureus* UoM Newman was obtained from Prof. Tim Foster (Trinity College Dublin), NRS384

152 was obtained from BEI resources (www.beiresources.org). *S. aureus* was routinely grown in Tryptic

153 Soy Broth (TSB-Oxoid) at 37 ˚C with aeration at 200 rpm. Primers were purchased from IDT (www.

154 idtdna.com) with primer sequences detailed in Table 3. Restriction enzymes, Phusion DNA

155 polymerase and T4 DNA ligase were purchased from New England Biolabs. Genomic DNA was

156 isolated from 1 ml of an overnight culture (DNeasy blood and tissue kit – Qiagen) pretreated with

157 100 μg of lysostaphin (Sigma). DHBP was purchased from Sigma (cat no. 126217-100g).

158

159 **Lysostaphin sensitivity assay.**

160   Overnight cultures of *S. aureus* were diluted 1:100 in fresh, pre-warmed TSB in the presence of 0.2

161   µg/ml of lysostaphin (AMBI) with or without 75 µM DHBP (100 mM stock in methanol). Broths

162   were incubated statically at 37˚C. Colony forming units were determined by spot plate dilution on

163   Brain heart infusion agar at 0 and 90 min. Limit of detection for the assay was 10^3 CFU/ml.

164

165   **Construction of pIMC8-RFP and SLiCE cloning**

166   The *S. aureus* codon optimized DsRED red fluorescent protein and upstream TIR sequence from

167   pRFP-F [17] was PCR amplified with primers IM314/IM315. The product was digested with KpnI/SacI

168   and cloned into the complementary digested pIMC8 (non-temperature sensitive version of pIMC5

169   [18], creating pIMC8-RFP. To clone into pIMAY-Z [2] and pIMC8-RFP primers were tailed with 30 nt of

170   complementary sequence to the plasmid. Amplimers were inserted with seamless ligation cloning

171   extract (SLiCE) [19] into the vector (pIMAY-Z: *walRK*^{D119A}, *sae*^{STOP}, *sae*^{FIX}; pIMC8-RFP: P1 sae). Either

172   vector was linearized with KpnI, gel extracted and PCR amplified with primers IM1/IM2 (pIMAY-Z)

173   or IM1/IM385 (pIMC8-RFP). Both amplimers (vector and insert) were combined in a 10 µl reaction

174   containing 1×T4 ligase buffer, with 1 µl of SLiCE extract. The reaction was incubated at 37 for 1h

175   and then transformed into *Escherichia coli* strain IM08B [2], with selection on Luria agar plates

176   containing chloramphenicol 10 µg/ml. Plasmids were extracted and directly transformed by

177   electroporation into the target *S. aureus* strain [2].

178

179   **Production of SLiCE extract.**

180   The SLiCE was isolated from DY380 grown in 50 ml 2xYT at 30°C after a 1:100 dilution of the

181   overnight culture and a 42°C heat shock (50 ml of 42°C 2xYT added) for 25 min once the culture

182   reached OD600=2.5. Cells were processed as described by Zhang et al., [19] with the pellet lysed in

183   500 ul of CelLytic B cell lysis reagent (C87040-10ml-Sigma).

184

185   **Whole genome sequencing and data analysis**

186   Whole genome sequencing was performed using the Illumina NextSeq (2x150 bp chemistry), with

187   library preparation using Nextera XT (Illumina). Resulting reads were mapped to the *S. aureus*

188   Newman reference (Accession: NC_009641) using *Snippy* v3.1

189   (https://github.com/tseemann/snippy). Note that 89 substitutions, 20 deletions and 25 insertions

190   were shared between UoC and UoM Newman strains compared to the NC_009641 reference

191   sequence, representing likely sequencing errors in the 2008 published reference [2].

192

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247 **Table 1:** Whole genome sequencing to identify the introduction of unintended polymorphisms

248 within the *S. aureus* Newman WalK mutants.

Position *S. aureus* Newman (NC_009641)	UoC Newman (Acc: ERR1450026)	UoC WalK^{D119A} (Acc: ERR1450027)	UoC WalK^{V149A} (Acc: ERR1450028)	Locus_tag (NWMN_)	Gene	Comment (product, predicted consequence of mutation) c.= codon p. = amino acid position.
25994	A	C	A	0018	*walK*	Sensor kinase: missense_variant c.356A>C p.Asp119Ala
26084	T	T	C	0018	*walK*	Sensor kinase: missense_variant c.446T>C p.Val149Ala
87710	A	A	A	0065	*sbnF*	Siderophore biosynthesis Synomous c. 425G>A
757519	-	TT	-	0674	*saeS*	Sensor kinase: frameshift variant c.152_153ins AA p.Thr52fs
757889	C	C	A	0675	*saeR*	DNA-binding response regulator stop_gained c.469G>T p.Glu157*
820314	C	T	T	0728	*hprK*	HPr kinase/phosphorylase: missense_variant c.383C >T p.Ala128Val
897870	-	TG	TG	0810		Truncated hypothetical Created full length gene. c.98_99ins TG p. 50 - 119
1914999	T	T	C			Intragenic between NWMN_1716/1717
2370632	C	G	C	2142	*rplN*	50S ribosomal protein L14: missense_variant c.86G>C p.Gly29Ala

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253 **Table 2:** List of the nine CDS shown in Fig. 1B that were down regulated in D119A and up-

254 regulated on exposure to DHBP (both treatments compared to *S. aureus* Newman wild type).

No.	Product	Locus_tag NWMN_	Log_2FC WT vs D119A	$Log_2FC^{\#}$ WT vs DHBP
1[§]	Staphylocoagulase precursor (*coa*)	0166	-2.92	2.40*
2	Superantigen-like protein 7 (*ssl7*)	0394	-1.64	2.46*
3	Hypothetical protein	0401	-1.94	2.56
4	Sodium-dependent symporter protein	0423	-1.18	1.47
5	Secreted VWF-binding protein precursor	0757	-1.88	2.40*
6	Cytochrome D ubiquinol oxidase, subunit I	0952	-2.05	1.32
7	Cytochrome D ubiquinol oxidase, subunit II	0953	-1.88	1.39
8	Fibronectin binding protein B (*fnbB*)	2397	-4.02	1.53*
9	Immunodominant antigen A (*isaA*)	2469	-1.25	1.45*

255 Notes: [#]FDR <0.01; [§]Numbers in bold typeface indicate previously reported as SaeRS regulated [5,6]. *Asterisk indicates

256 also reported as upregulated by Ji *et al.* [1].

257

258

259 **Table 3:** Oligonucleotides used in the study.

	Oligonucleotide sequence (5'-3')
Allelic Exchange	
WalK D119A	
IM107 walR AF	**CCTCACTAAAGGGAACAAAAGCTGGGTACC**ATGGCTAGAAAAGTTGTTGTAG
IM548 D119A walK BR	gcCATTCGCTTTTTGATTGATTAGACTACG
IM549 D119A walK CF	CCGTAGTCTAATCAATCAAAAAGCGAATGcgAGTTCTGTCCAAAAAGCACTATCAC
IM10 walK DR	**CGACTCACTATAGGGCGAATTGGAGCTC**CTCCTTATTATTCATCCCAATCACCGTC
IM552 D119A con F	CTAATCAATCAAAAAGCGAATGCG
IM1 pIMAY-Z F	GGTACCCAGCTTTTGTTCCCTTTAGTGAGG
IM2 pIMAY-Z R	GAGCTCCAATTCGCCCTATAGTGAGTCG
SaeS	
IM488 saeS AF	**CCTCACTAAAGGGAACAAAAGCTGGGTACC**TGATATCATGGTACTTGATATCATGATGC
IM488 saeS DR	**CGACTCACTATAGGGCGAATTGGAGCTC**TTGTAGAAGAAGTACAATTTGATGATGG
P1sae reporter	
IM494 P1 Sae F	**CCTCACTAAAGGGAACAAAAGCTGGGTACC**TTGGTACTTGTATTTAATCGTCTATC
IM495 P1 Sae R	**ATGTTTTTCCTCCTTATAAAGTTAATCA**TGTTGTGATAACAGCACCAGCTGC
IM314 TIR-RFP(KpnI) F	ATAT<u>GGTACC</u>GGTGATTAACTTTATAAGGAGGAAAAACATATG
IM315 RFP(SacI) R	ATAT<u>GAGCTC</u>AACATCTGTGGTATGGCGCTAGG
IM385 pIMC8-RFP R	TGATTAACTTTATAAGGAGGAAAAACATATG

260 **Notes:** Bold: Tails for recombination into the vector; Lower case: Mutation introduction; Underlined: Restriction site
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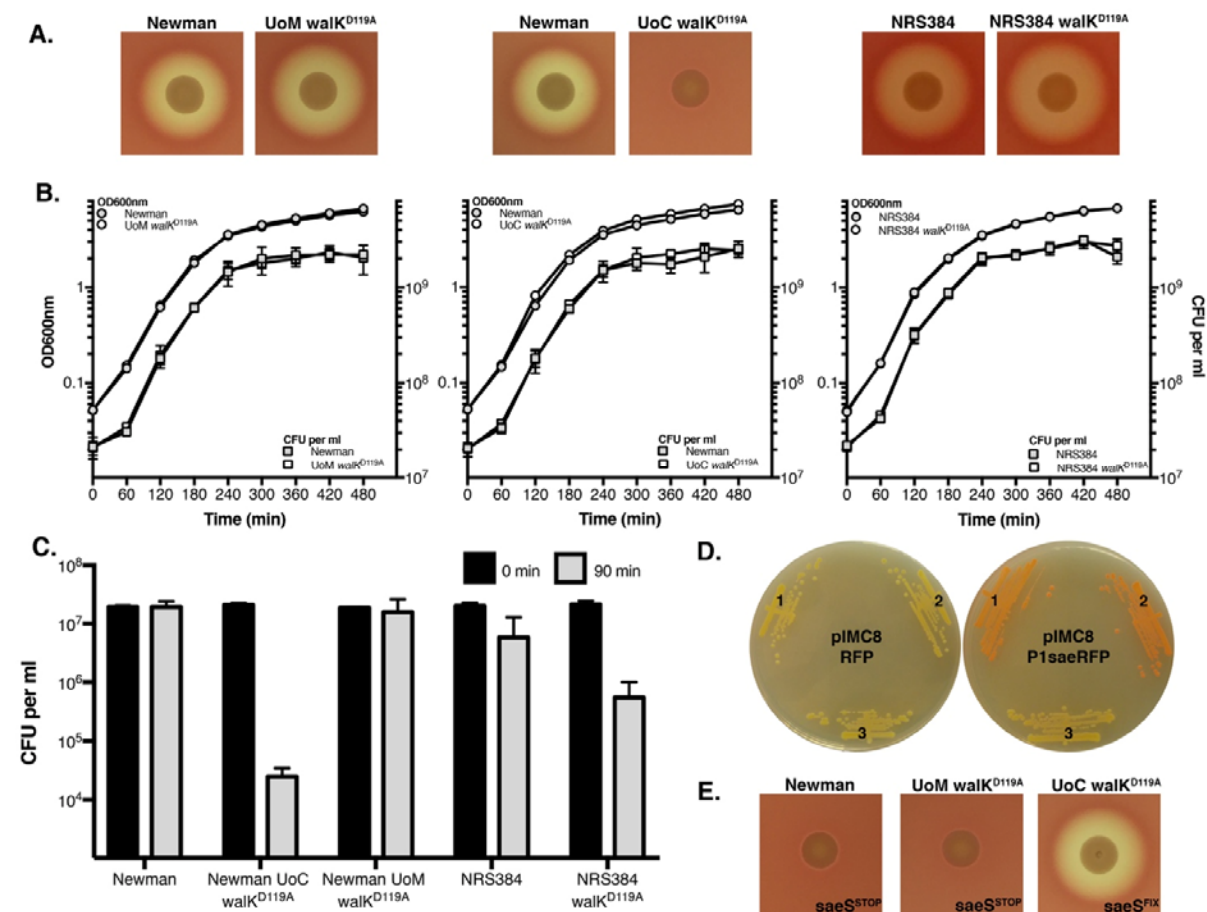263 **Fig.1**

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265

266 **Fig. 1 Phenotypic screening of *walK^{EC-PAS}* mutants.** (A) No impact on haemolysis was observed for

267 the WalK^{D119A} mutation in either Newman or NRS384 on sheep blood agar. (B) Growth kinetics

268 were identical for the newly created WalK^{D119A} mutants in TSB at 37°C with aeration (200 rpm)

269 when compared to the parental strain. Optical density (O) and colony forming units (□) were

270 enumerated. (C) In a lysostaphin growth sensitivity assay the UoC WalK^{D119A} exhibited a loss of

271 viability, while the parent or UoM WalK^{D119A} did not. The WalK^{D119A} mutation in the NRS384

272 background enhanced sensitivity to lysostaphin when compared to the parental strain. Error bars

273 depict the standard deviation of the mean from three independent experiments. (D) P1 sae

274 promoter activity with a DsRED reporter. Left: no promoter. Right: P1 sae driving RFP expression;

275 (1) Newman (2) UoM WalK^{D119A} (3) UoM WalK^{D119A}. No expression of *sae* was observed in UoC

276 WalK^{D119A}. (E) By allelic exchange the UoC *saeS* mutation was introduced into Newman or UoM

277 WalK^{D119A} (*saeS^{STOP}*) abolished haemolysis and the introduction of the wild type *saeS* gene into

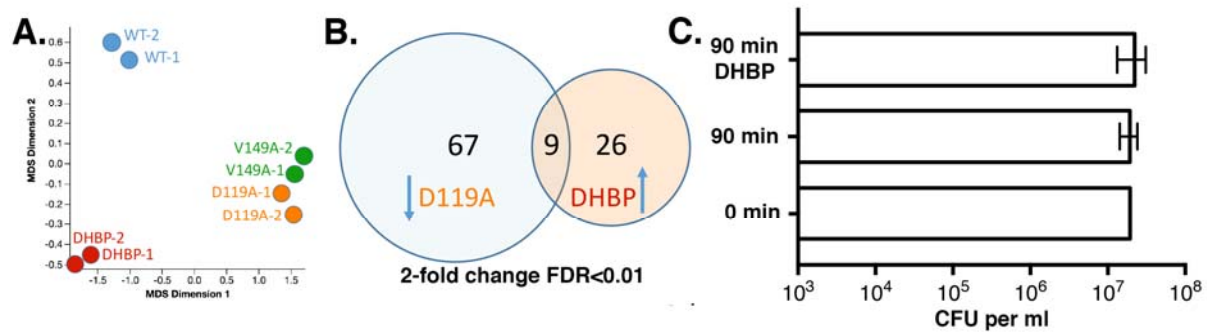278 UoC WalK^{D119A} (*saeS^{FIX}*) restored haemolysis.

279

280 **Fig. 2**

281

282

283 **Fig. 2: Comparisons of *S. aureus* walK^{EC-PAS} mutants.** Multidimensional scaling plot based on

284 published RNAseq data from Ji et al [1], showing similar transcriptional profiles for the *walK^{EC-PAS}*

285 mutants but distinct from wild type treated with 2, 4-dihydroxybenzophenone (DHBP). (B) Venn

286 diagram showing nine CDS that are down regulated in the D119A mutant and upregulated on

287 exposure to DHBP. (C) No enhanced impact on lysostaphin induced lysis in strain Newman was

288 observed when grown in the presence of 75 μM DHBP.

289