

1 **Correspondence: Spontaneous secondary mutations confound analysis of the essential two**
2 **component system WalkR in *Staphylococcus aureus***

3 Ian R. Monk¹, Benjamin P. Howden¹, Torsten Seemann² and Timothy P. Stinear¹

4

5 **Affiliations:**

6 ¹Department of Microbiology and Immunology, at the Peter Doherty Institute for Infection and Immunity,
7 University of Melbourne, Melbourne, 3000, Victoria, Australia.

8 ²Victorian Life Sciences Computation Initiative, University of Melbourne, Carlton, 3010, Victoria, Australia

9

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17 contrary to our own unpublished observations.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 Newman², 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 OD₆₀₀, 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-log₁₀ 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^{V149A} 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 μ M 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 Degust¹¹, the latter an interactive website to explore
101 the analysis¹². 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 μ M 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 genes¹. 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 alone¹². Transcriptome
120 data without statistical significance thresholds are not meaningful¹⁵.

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

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 system⁴. 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 validation⁵. 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.](http://www.idtdna.com)
154 [idtdna.com](http://www.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 10³ 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-F¹⁷ 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
169¹⁸, creating pIMC8-RFP. To clone into pIMAY-Z² 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 IM08B², 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* strain².

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 CellLytic 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 reference².

192

193 **References:**

- 194 1 Ji, Q. *et al.* Structure and mechanism of the essential two-component signal-transduction
195 system WalkR in *Staphylococcus aureus*. *Nat Commun* **7**, 11000, (2016).
- 196 2 Baba, T., Bae, T., Schneewind, O., Takeuchi, F. & Hiramatsu, K. Genome sequence of
197 *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal
198 genomes: polymorphism and evolution of two major pathogenicity islands. *J Bacteriol* **190**,
199 300-310, (2008).
- 200 3 Monk, I. R., Tree, J. J., Howden, B. P., Stinear, T. P. & Foster, T. J. Complete Bypass of
201 Restriction Systems for Major *Staphylococcus aureus* Lineages. *mBio* **6**, e00308-00315,
202 (2015).
- 203 4 Dubrac, S., Boneca, I. G., Poupel, O. & Msadek, T. New insights into the Walk/WalR
204 (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell
205 wall metabolism and biofilm formation in *Staphylococcus aureus*. *J Bacteriol* **189**, 8257-
206 8269, (2007).
- 207 5 Sun, F. *et al.* Aureusimines in *Staphylococcus aureus* are not involved in virulence. *PLoS One*
208 **5**, e15703, (2010).
- 209 6 Nygaard, T. K. *et al.* SaeR binds a consensus sequence within virulence gene promoters to
210 advance USA300 pathogenesis. *J Infect Dis* **201**, 241-254, (2010).
- 211 7 Liang, X. *et al.* Inactivation of a two-component signal transduction system, SaeRS,
212 eliminates adherence and attenuates virulence of *Staphylococcus aureus*. *Infect Immun* **74**,
213 4655-4665, (2006).
- 214 8 Cue, D. *et al.* SaeRS-dependent inhibition of biofilm formation in *Staphylococcus aureus*
215 Newman. *PLoS One* **10**, e0123027, (2015).
- 216 9 Ueda, T., Kaito, C., Omae, Y. & Sekimizu, K. Sugar-responsive gene expression and the agr
217 system are required for colony spreading in *Staphylococcus aureus*. *Microb Pathog* **51**, 178-
218 185, (2011).
- 219 10 Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq
220 quantification. *Nature biotechnology* **34**, 525-527, (2016).
- 221 11 Powell, D. R. *Degust*, <<https://github.com/Victorian-Bioinformatics-Consortium/degust>>
222 (2013).
- 223 12 Monk, I. R., Seemann, T. & Stinear, T. P. *Degust: RNAseq analysis*,
224 <[http://www.vicbioinformatics.com/degust/compare.html?code=d2ac966d9f90624bd366](http://www.vicbioinformatics.com/degust/compare.html?code=d2ac966d9f90624bd3663687475d71c2)
225 [3687475d71c2](http://www.vicbioinformatics.com/degust/compare.html?code=d2ac966d9f90624bd3663687475d71c2)> (2016).
- 226 13 Howden, B. P. *et al.* Evolution of multidrug resistance during *Staphylococcus aureus*
227 infection involves mutation of the essential two component regulator WalkR. *PLoS Pathog*
228 **7**, e1002359, (2011).
- 229 14 Dubrac, S. & Msadek, T. Identification of genes controlled by the essential YycG/YycF two-
230 component system of *Staphylococcus aureus*. *J Bacteriol* **186**, 1175-1181 (2004).
- 231 15 Conesa, A. *et al.* A survey of best practices for RNA-seq data analysis. *Genome Biol* **17**, 13,
232 (2016).
- 233 16 McEvoy, C. R. *et al.* Decreased vancomycin susceptibility in *Staphylococcus aureus* caused
234 by IS256 tempering of WalkR expression. *Antimicrob Agents Chemother* **57**, 3240-3249,
235 (2013).
- 236 17 Bose, J. L., Fey, P. D. & Bayles, K. W. Genetic tools to enhance the study of gene function
237 and regulation in *Staphylococcus aureus*. *App Environ Microbiol* **79**, 2218-2224, (2013).

- 238 18 Monk, I. R., Shah, I. M., Xu, M., Tan, M. W. & Foster, T. J. Transforming the
239 untransformable: application of direct transformation to manipulate genetically
240 *Staphylococcus aureus* and *Staphylococcus epidermidis*. *mBio* **3**, (2012).
241 19 Zhang, Y., Werling, U. & Edelmann, W. SLiCE: a novel bacterial cell extract-based DNA
242 cloning method. *Nucleic Acids Res* **40**, e55, (2012).
243
244
245
246

247 **Table 1:** Whole genome sequencing to identify the introduction of unintended polymorphisms
 248 within the *S. aureus* Newman Walk mutants.

Position <i>S. aureus</i> 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	<i>walk</i>	Sensor kinase: missense_variant c.356A>C p.Asp119Ala
26084	T	T	C	0018	<i>walk</i>	Sensor kinase: missense_variant c.446T>C p.Val149Ala
87710	A	A	A	0065	<i>sbnF</i>	Siderophore biosynthesis Synomous c. 425G>A
757519	-	TT	-	0674	<i>saeS</i>	Sensor kinase: frameshift variant c.152_153ins AA p.Thr52fs
757889	C	C	A	0675	<i>saeR</i>	DNA-binding response regulator stop_gained c.469G>T p.Glu157*
820314	C	T	T	0728	<i>hprK</i>	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	<i>rpIN</i>	50S ribosomal protein L14: missense_variant c.86G>C p.Gly29Ala

249

250

251

252

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	Log ₂ FC	Log ₂ FC [#]
		NWMN_	WT vs D119A	WT vs DHBP
1[§]	Staphylocoagulase precursor (<i>coa</i>)	0166	-2.92	2.40*
2	Superantigen-like protein 7 (<i>ssl7</i>)	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 (<i>fnbB</i>)	2397	-4.02	1.53*
9	Immunodominant antigen A (<i>isaA</i>)	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.

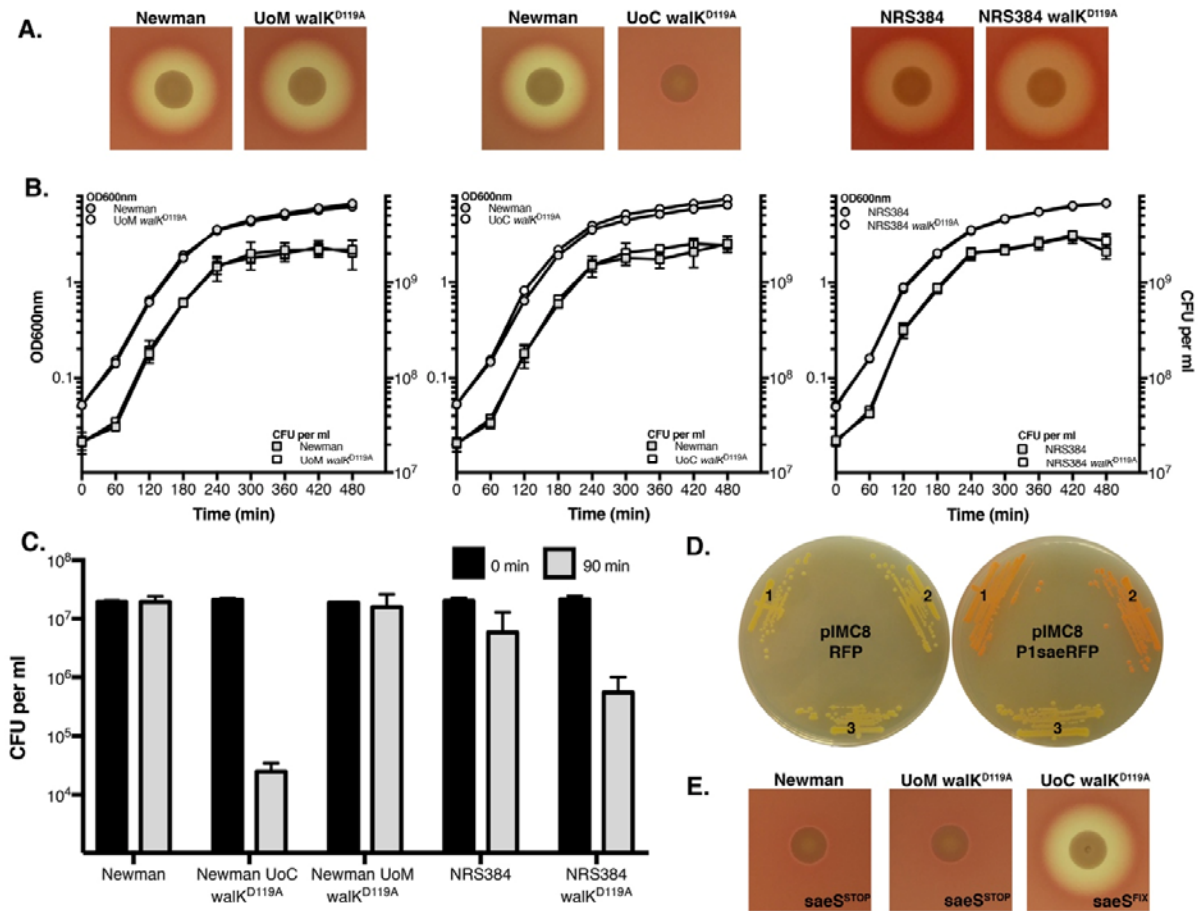
	Oligonucleotide sequence (5'-3')
Allelic Exchange	
WalK D119A	
IM107 walR AF	CCTCACTAAAGGGAACAAAAGCTGGGTACCATGGCTAGAAAAGTTGTTGTAG
IM548 D119A walK BR	gcCATTTCGCTTTTTGATTGATTAGACTACG
IM549 D119A walK CF	CCGTAGTCTAATCAATCAAAAAGCGAATGcgAGTTCTGTCCAAAAGCACTATCAC
IM10 walK DR	CGACTCACTATAGGGCGAATTGGAGCTCCTCCTTATTATTCATCCAATCACCGTC
IM552 D119A con F	CTAATCAATCAAAAAGCGAATGCG
IM1 pIMAY-Z F	GGTACCCAGCTTTTGTCCCTTTAGTGAGG
IM2 pIMAY-Z R	GAGCTCCAATTCGCCCTATAGTGAGTCG
SaeS	
IM488 saeS AF	CCTCACTAAAGGGAACAAAAGCTGGGTACCTGATATCATGGTACTTGATATCATGATGC
IM488 saeS DR	CGACTCACTATAGGGCGAATTGGAGCTCTTGTAGAAGAAGTACAATTTGATGATGG
P1sae reporter	
IM494 P1 Sae F	CCTCACTAAAGGGAACAAAAGCTGGGTACCTGGTACTTGATTTAATCGTCTATC
IM495 P1 Sae R	ATGTTTTCTCCTTATAAAGTTAATCATGTTGTGATAACAGCACCAGCTGC
IM314 TIR-RFP(KpnI) F	ATAT <u>GGTACC</u> GGTGATTAACTTTATAAGGAGGAAAAACATATG
IM315 RFP(SacI) R	ATAT <u>GAGCTCA</u> ACATCTGTGGTATGGCGCTAGG
IM385 pIMC8-RFP R	TGATTAACTTTATAAGGAGGAAAAACATATG

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

261

262

263 Fig.1



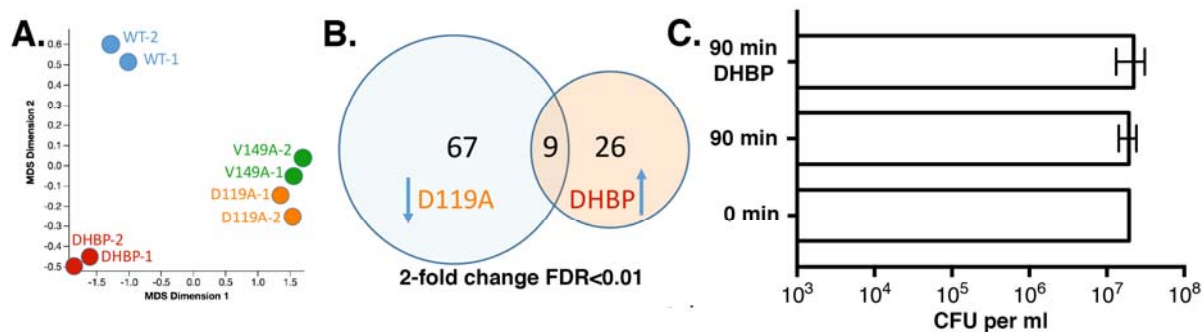
264

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 (○) 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) UoC *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 μ M DHBP.

289