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Refutation: *Structure and mechanism of the essential two-component signal-transduction system WalKR in Staphylococcus aureus.*

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In a recent report in *Nature Communications*, Ji *et al*., (2016) [1] describe the structure of the extracytoplasmic Per-Arnt-Sim (PAS) domain of WalK ($WalK^{EC\text{-}PAS}$), the sensor kinase of the essential two-component regulator WalKR in *Staphylococcus aureus* [1]. The authors make two independent *walK S. aureus* mutants by changing two amino acid residues they postulate from structural analysis and comparisons might be important for signal transduction. We have also been exploring the function of WalKR and were surprised by the striking phenotypic impact of these single amino acid substitutions in the WalK sensor, which are contrary to our own (unpublished) observations.

Ji *et al*., subjected their $WalK^{EC\text{-}PAS}$ mutants (D119A and V149A) to a series of phenotypic screens to probe the function of this domain. Compared to the methicillin sensitive *S. aureus* Newman wild type, the mutants showed dramatic phenotype changes that included altered growth kinetics, reduced susceptibility to lysostaphin exposure, loss of haemolysis on sheep blood agar, reduced biofilm formation and reduced virulence in a mouse infection model. RNAseq comparisons of mutants and wild type showed substantial transcriptional changes, with 313 differentially expressed genes between the two $WalK^{EC\text{-}PAS}$ mutants and wild type. The authors also used structure-based virtual screening to identify 2, 4-dihydroxybenzophenone (DHBP) as a small molecule predicted to interact with the $WalK^{EC\text{-}PAS}$ domain. Under the assumption that DHBP is an activator of WalKR (no direct evidence for this was presented), they then compared transcriptional responses of *S. aureus* wild type against DHBP exposure and with their D119A mutant. The authors reported that there were 41 genes that were down-regulated in the D119A mutant and up-regulated in DHBP-treated cells, and concluded that this supported a role for DHBP in activating WalKR. No biochemical evidence to support activation of WalKR phosphotransfer activity was presented.

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To investigate further, we were provided with *S. aureus* Newman wild type, D119A and V149A by the senior author, Chuan He, University of Chicago (UoC) [1]. We first recreated the D119A mutation in two *S. aureus* backgrounds. We mutated methicillin sensitive *S. aureus* Newman wild type and methicillin resistant *S. aureus* NRS384 (USA300) using allelic exchange [2], and confirmed by whole genome sequencing, read-mapping and difference identification [3], that we had only introduced the A->C single nucleotide substitution in $WalK^{EC\text{-}PAS}$ (accession: PRJEB14381). We then tested these mutants for some of the key phenotype changes observed by Ji *et al.*, [1]. We established that D119A-UoC was non-hemolytic as published but also observed that both our D119A-UoM mutants (Newman and NRS384) were fully hemolytic (Fig. 1A) and exhibited identical growth curve kinetics as wild type Newman and NRS384 (Fig. 1B).

To resolve the discrepancies between our phenotypes and those of the apparently identical mutants published by Ji *et al.*, [1] we also subjected their three strains (*S. aureus* Newman wild type UoC, D119A-UoC and V149-UoC) to whole genome sequencing and analysis (accession: PRJEB14381) [3]. Relative to the *S. aureus* Newman UoC wild type, both mutants D119A and V149A had acquired at least four extra mutations in addition to their expected $walK^{EC\text{-}PAS}$ changes, most notably two independent loss-of-function mutations in *saeRS*, a major two-component regulator that controls expression of many genes involved in virulence and biofilm formation (Table 1) [4,5]. D119A-UoC had a TT insertion at chromosome position 757521 that introduced a frameshift to *saeS*. Strain V149A-UoC had a G->T substitution at chromosome position 757893 that introduced a premature stop codon to *saeR* (Table 1). It is these secondary mutations in *saeRS*, rather than the targeted mutations in $walK^{EC\text{-}PAS}$, that likely explain the phenotypes observed by Ji *et* al., (reduced biofilm, loss of hemolysis, reduced virulence) [5] . There is precedence for this specific phenomenon. Sun *et al.*, showed in detail that elevated temperature and antibiotic selection used during the mutagenesis process can aid in the selection of *saeRS* mutations [5]. How Ji *et al.*, managed to complement the mutations in D119A-UoC and V149A-UoC by phage integrase plasmid expression (pCL55) of *walKR* alleles remains to be explained [1]. Unfortunately, we have been unable thus far to obtain their complemented mutants for further analysis.

We also observed that D119A-UoC and V149A-UoC exhibited larger colonies compared to wild type, a phenotypic difference not discussed by Ji *et al.* [1]. This change in both mutants might be

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explained by the C>T substitution observed at 820318, leading to an A128V change in HprK (Table 1), a serine kinase known to be involved in catabolite repression and associated with a spreading colony phenotype [6].

We next re-examined the authors' RNAseq data (accession No: GSE75731) obtained from biological duplicate experiments of *S. aureus* Newman wild type, D119A-UoC, V149A-UoC and wild type with DHBP treatment). Using *Kallisto* [7] and the *S. aureus* Newman reference genome (NC_009641) we quantified transcript abundance for each of the four conditions. The output of *Kallisto* was analysed and visualized using Degust [8]. A dynamic link to this analysis is available [9] and the raw transcript abundance count data used in Degust is provided (Supplementary Table S1). Multi-dimensional scaling plots showed good consistency between the biological replicates. However wild type, DHBP and the two mutants each displayed clustering that suggested very distinct transcriptional profiles (Fig. 1C). Applying threshold cutoffs of 2-fold change and FDR <0.01, we then replicated the intersection analysis of Ji *et al.*, but found only nine genes (not the reported 41) that were down regulated in D119A-UoC and up regulated in DHBP-treated bacteria (Fig. 1D). Among the nine genes, only five overlapped with the 41 reported by Ji *et al.*,[1] and none of these included autolysins previously linked to the WalKR regulon (Table 2) [10,11]. In fact, five of the nine genes listed have all previously been reported to be under SaeRS control (Table 2) [4]. Given that D119A-UoC is a SaeS null mutant, these data suggest DHBP is as likely to be interacting with SaeS as it is with WalK.

We also noted that only 52 genes were differentially expressed upon exposure to DHBP, in comparison to the claimed 145 genes [1] . We think this discrepancy and the preceding difference were due to the absence of any filter for false discovery rate (FDR) applied by Ji *et al.*, [1]. For example, our analysis of their RNA-seq data without a FDR threshold resulted in 172 differentially expressed genes in DHBP-treated cells compared to WT) [9]. RNAseq expression data without threshold significance cutoffs are not meaningful [12].

We also mapped the authors' RNAseq reads for D119A-UoC and V149A-UoC (accession No: GSE75731) to the *S. aureus* Newman reference (NC_009641) [3] and readily detected the same *saeRS* mutations we observed from our independent sequencing of these mutants.

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Conclusion: Our analyses highlight two major issues with the study of Ji *et al.*[1] Firstly, the presence of unintended *saeRS* mutations in their *walK*$^{EC\text{-}PAS}$ mutants limit meaningful interpretation of their data and invalidate their conclusions with respect to role of the WalK extracytoplasmic domain in controlling WalKR function. Secondly, the RNAseq data appears to have been inadequately analysed, with no filtering for false discovery. Application of an appropriate threshold ($p<0.01$) to their data substantially changes the lists of differentially expressed genes. The authors' use the overlap between the D119A mutant and DHBP treatment to claim that DHBP is signaling through WalK$^{EC\text{-}PAS}$ when they state: *"Together, the transcriptome profiling supports the role of DHBP in activating WalKR TCS"*. However, this conclusion is not supported by the data at all. In fact, a more plausible conclusion is that DHBP is interacting with SaeRS (Table 2).

The discovery of small molecule inhibitors of WalKR function would represent a major advance in the fight against multidrug resistant *S. aureus*, and Ji *et al.*, have shown a promising approach through structural and functional analyses [1]. Unfortunately, based on the data presented, the authors' principal conclusions are not supported. This study is another example of the pitfalls associated with allelic exchange in *S. aureus* and the rigor that must applied to mutation validation [5]. Whole genome sequencing is now so affordable that it should be used routinely to verify targeted mutants and the complemented strains. In our own WalKR research we have observed a propensity for mutations introduced into this locus to yield secondary compensatory events [11,13]. These secondary changes can confound analysis of this essential two-component system, and highlight the extreme care needed when manipulating the *walKR* locus and then attributing specific phenotypes to particular mutational changes.

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158 **Table 1:** List of mutations identified by WGS in *S. aureus* Newman (University of Chicago)

Position *S. aureus* **Newman** (NC_009641)	**Sa_Newman-WT-UoC** (Acc: ERR1450026)	**Sa_Newman-D119A-UoC** (Acc: ERR1450027)	**Sa_Newman-V149A-UoC** (Acc: ERR1450028)	**Locus_tag** (NWMN_)	**Gene**	**Comment** (product, predicted consequence of mutation)
26001	A	C	A	0018	*walK*	Sensor kinase: missense_variant c.356A>C p.Asp119Ala
26091	T	T	C	0018	*walK*	Sensor kinase: missense_variant c.446T>C p.Val149Ala
757521	-	TT	-	0674	*saeS*	Sensor kinase: frameshift_variant c.152_153insAA p.Thr52fs
757893	C	C	A	0675	*saeR*	DNA-binding response regulator: stop_gained c.469G>T p.Glu157*
820318	C	T	T	0728	*hprK*	HPr kinase/phosphorylase: missense_variant c.383C>T p.Ala128Val
2370634	C	G	C	2142	*rplN*	50S ribosomal protein L14: missense_variant c.86G>C p.Gly29Ala

159

160

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Table 2: List of the nine CDS shown in Fig. 1D that were down regulated in D119A and up-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	2469	-1.25	1.45*

Notes: [#]FDR <0.01; [§]Numbers in red indicate previously reported as SaeRS regulated [4], *Asterisk indicates also reported as upregulated by Ji *et al.* [1].

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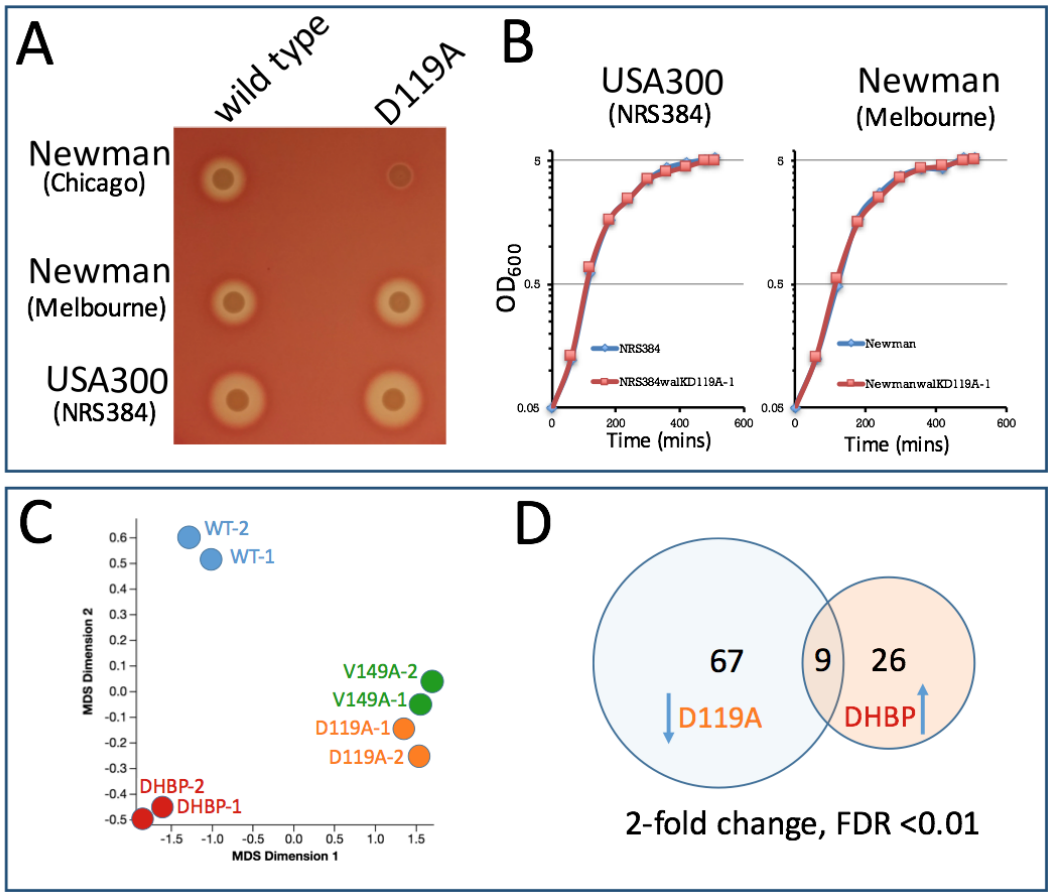

Fig. 1

Fig. 1: Comparisons of *S. aureus walK*$^{EC\text{-}PAS}$ mutants. (A) Hemolysis pattern of D119A mutants on sheep blood agar versus wild type for *S. aureus* Newman (University of Chicago), *S. aureus* Newman (University of Melbourne) and *S. aureus* NRS384 (USA300). (B) Growth curve analysis of *S. aureus* Newman (University of Melbourne) and *S. aureus* USA300 NRS384 D119A mutants, showing no difference to wild type. (C) Multidimensional scaling plot based on re-analysis of published RNAseq data from Ji *et al.*, [1] showing similar transcriptional profiles for the *walK*$^{EC\text{-}PAS}$ mutants but distinct from wild type treated with 2, 4-dihydroxybenzophenone (DHBP) (2-fold change, FDR <0.01). (D) Venn diagram based on published RNAseq data from Ji *et al.*, [1] showing nine CDS that are down regulated in the D119A mutant and upregulated on exposure to DHBP.