

RESEARCH ARTICLE

Diversity of bacteriophages encoding Panton-Valentine leukocidin in temporally and geographically related *Staphylococcus aureus*Geoffrey W. Coombs^{1,2*}, Sarah L. Baines³, Benjamin P. Howden⁴, Krister M. Swenson^{5,6}, Frances G. O'Brien^{7,8}

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Abstract

Production of the Panton-Valentine leukocidin (PVL) by *Staphylococcus aureus* is mediated via the genes *lukS-PV* and *lukF-PV* which are carried on bacteriophage ϕ Sa2. PVL is associated with *S. aureus* strains that cause serious infections and clones of community-associated methicillin-resistant *S. aureus* (CA-MRSA) that have additionally disseminated widely. In Western Australia (WA) the original CA-MRSA were PVL negative however, between 2005 and 2008, following the introduction of eight international PVL-positive CA-MRSA, PVL-positive WA CA-MRSA were found. There was concern that PVL bacteriophages from the international clones were transferring into the local clones, therefore a comparative study of PVL-carrying ϕ Sa2 prophage genomes from historic WA PVL-positive *S. aureus* and representatives of all PVL-positive CA-MRSA isolated in WA between 2005 and 2008 was performed. The prophages were classified into two genera and three PVL bacteriophage groups and had undergone many recombination events during their evolution. Comparative analysis of mosaic regions of selected bacteriophages using the Alignments of bacteriophage genomes (Alpha) aligner revealed novel recombinations and modules. There was heterogeneity in the chromosomal integration sites, the lysogeny regulation regions, the defence and DNA processing modules, the structural and packaging modules and the *lukSF-PV* genes. One WA CA-MRSA (WA5₁₈₇₅₁) and one international clone (Korean Clone) have probably acquired PVL-carrying ϕ Sa2 in WA, however these clones did not disseminate in the community. Genetic heterogeneity made it impossible to trace the source of the PVL prophages in the other WA clones. Against this background of PVL prophage diversity, the sequence of one group, the ϕ Sa2USA/ ϕ Sa2wa-st93 group, was remarkably stable

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over at least 20 years and associated with the highly virulent USA300 and ST93-IVa CA-MRSA lineages that have disseminated globally.

Introduction

Staphylococcus aureus is a pandemic pathogen that is also part of the human microbiota [1]. Paramount to the success of *S. aureus* has been its ability to utilize mobile elements to acquire and disseminate antibiotic resistance, virulence and adaptive mechanisms amongst staphylococcal populations. In methicillin-sensitive *S. aureus* (MSSA) and community-associated methicillin-resistant *S. aureus* (CA-MRSA) the Panton-Valentine leukocidin (PVL) is a virulence factor that is carried on a bacteriophage known as ϕ Sa2 which is integrated into the chromosome as a prophage [2]. PVL is a bi-component, pore-forming toxin produced by co-transcribed genes, *lukF-PV* and *lukS-PV*, that targets and lyses human macrophages, polymorphonuclear leukocytes and monocytes and also incites the human inflammatory immune response [3]. Strains encoding PVL are associated with skin and soft tissue infections and dangerous invasive infections however, the role that the toxin plays in virulence is controversial and as yet, no clear-cut selective advantage has been shown for CA-MRSA that produce PVL [4–8]. Many virulent strains of MSSA and CA-MRSA do not produce PVL, however, as the pathogen evolves it is evident that those that have disseminated to cause the greatest burden of infectious disease harbor the prophage [9].

The PVL bacteriophage genome is composed of functionally colinear main modules encoding genes for lysogeny, DNA processing, head morphogenesis and packaging, tail morphogenesis, and lysis, with the *lukSF-PV* genes encoded between the lysis and lysogeny modules in the circularly permuted bacteriophage [10]. The lysogeny, *lukSF-PV* and lysis regions are well conserved with minor polymorphisms. Most diversity occurs in the DNA processing module with the head and tail morphogenesis genes showing diversity depending on the genus and PVL group. ϕ Sa2 can be vertically transmitted with the chromosome during replication or it can enter the lytic cycle and transmit horizontally to another cell. It has been well documented that bacteriophages undergo high rates of recombination and both these forms of transmission allow opportunity for genetic exchange, the potential mechanisms being transposition, site-specific recombination, homing endonucleases and homologous and illegitimate recombination [11]. While it is believed that horizontal gene transfer between *S. aureus* of different lineages is rare due to a lineage-specific type1 restriction-modification system [12], an *in-vivo* study revealed that bacteriophage transferred frequently during co-colonization by *S. aureus* of the same lineage and recombination between different bacteriophage occurred [13]. An investigation of MRSA colonisation in remote WA revealed that 8% of screening swab sets with an MRSA were colonised with multiple lineages of MRSA and 51.7% were co-colonised with an MSSA [14]. This would provide ideal opportunities for bacteriophage transmission and recombination to occur.

Five lineages of CA-MRSA, ST1-IVa (WA1), ST78-IVa (WA2), ST5-IVa (WA3), ST45-V (WA4) and ST8-IVa (WA5) emerged in remote Western Australian (WA) communities and WA1, WA2 and WA3 eventually disseminated to the capital city Perth and the eastern states of Australia [15, 16]. Unlike CA-MRSA that were being reported outside of WA, the WA strains were PVL negative [17]. There were however, two lineages of PVL-positive MSSA in remote WA communities, ST93-MSSA and ST121-MSSA [14].

In 2005, a PVL-positive strain belonging to the same lineage as WA1 was isolated, followed in 2008, by WA2-, WA3- and WA5-like PVL positive clones. In WA, all MRSA are submitted

to a central facility for typing and epidemiological investigation [18] and between 2005 and 2008 eight international PVL-positive CA-MRSA were introduced into WA. The rise in the number of PVL-positive CA-MRSA in WA since the first was found in 2003 has been alarming. In 2003/2004 2.1% of CA-MRSA were PVL positive, however by 2015/2016 this had risen to 52.8%, with the predominant clones being ST93-IVa (Queensland clone, 63%), ST5-IVc (WA 121, 19.5%) and ST30-IVc (WSPP, 6.8%). WA1-, WA2- and WA3-like PVL-positive clones were still in the community in 2016 however, they had not thrived and formed lower percentages of 0.7%, 0.17% and 1.1% respectively while PVL-positive WA5 had disappeared [19].

The overall aims of this study were to investigate PVL prophages from lineages of PVL-positive MRSA isolated in WA between 2005 and 2008 firstly, to gain insights into the genetics of geographically and temporally related PVL prophages in WA and secondly, to determine if PVL bacteriophages from the international strains had horizontally transmitted into the local WA clones. A comparative analysis of the PVL prophages has been performed using conventional sequence analysis, and regions of selected prophages have been compared using the Alignments of bacteriophage genomes (Alpha) aligner, which is an application that creates a partial order of gapless alignments along the bacteriophage genomes, allowing the identification of common core sequences and modular segments [20, 21]. Heterogeneity between the bacteriophages has been investigated using Alpha aligner defined modules and coding sequence comparisons. PVL bacteriophages were induced from PVL-positive *S. aureus* from the WA community and attempts were made to lysogenise prototype PVL-negative WA CA-MRSA.

Materials and methods

Bacterial strains

Genotypes and year of isolation of PVL-positive clones and their PVL prophage sizes are presented in Table 1. All MRSA except WA2_{RNSH95} and USA300 FPR3757 were from cases of infection or colonization in the WA community [18]. WA2_{RNSH95} was a WA2 clone from Sydney, Australia. The USA300 clone was present in WA [22] and the prophage ϕ Sa2USA from FPR3757 (Genbank: NC_007793) was used for genetic comparison. MSSA isolates W17S and K25S were colonizing isolates from remote WA communities [14]. ST772-V was previously sequenced [23]. MW2 (Genbank: BA000033) was used as a *lukSF-PV* gene-sequencing and prophage induction control. ϕ SLT (Genbank: AB045978) and ϕ Sa2958 (Genbank: AP009363) were *lukSF-PV* gene sequencing controls.

PVL-negative WA1_{WBG8287}, WA2_{WBG8366}, WA3_{WBG8378}, WA4_{WBG8404} and WA5_{WBG7583} are historic prototype clones from the WA community [24]. Bacteriophage indicator and propagating strains were RN4220, WBG248, WBG356, WBG696 and WBG286.

Sequencing of bacterial and PVL-prophage genomes and genetic analysis

Twelve bacterial genomes were sequenced using Illumina NextSeq sequence chemistry (Illumina Australia, Scoresby, Victoria 3179) and assembled with SPAdes, v3.9.0. The PVL-prophage reads were extracted and analysed using MacVector with Assembler, v15.5.3 (Accelrys, Cambridge, UK). The sequences of ϕ Sa2wa-st1, -st8, -st30, -st72 and -st93mssa were on single contigs, the remainder were assembled by overlapping contigs utilising the MacVector Assembler bowtie and phrap algorithms. Bacteriophage were designated as phi Sa2 Western Australia-host sequence type (ϕ Sa2wa-st). Except for prophages ϕ Sa2USA and ϕ Sa2wa-st772, National Centre for Biotechnology Information (NCBI) homology searches used only whole bacteriophage genome sequences for comparisons.

Table 1. WA PVL-positive bacteriophages and lysogens.

| Bacteriophage | Size (bp) | Lysogen genotype CC, ST-SCCmec | Clone ^{Strain} | Year of isolation | Reference |
|------------------------|-----------|-----------------------------------|-----------------------------------|-------------------|------------|
| WA PVL-positive Clones | | | | | |
| φSa2wa-st1 | 45,585 | 1, ST1-IVa | WA1 ₁₅₇₉₈ | 2005 | This study |
| φSa2wa-st5 | 44,823 | 5, ST5-IVa | WA3 ₁₈₇₉₀ | 2008 | This study |
| φSa2wa-st8 | 45,914 | 8, ST8-IVa | WA5 ₁₈₇₅₁ | 2008 | This study |
| φSa2wa-st78 | 45,878 | 88, ST78-IVa | WA2 _{RNSH95} | 2008 | This study |
| φSa2wa-st93mssa | 45,913 | Singleton, ST93 | W17S | 1995 | [14] |
| φSa2wa-st121mssa | 45,621 | 121, ST121 | K25S | 1995 | [14] |
| International Clones | | | | | |
| φSa2wa-st22 | 38,576 | 22, ST22-IVc | 16386 | 2007 | [18] |
| φSa2wa-st30 | 45,780 | 30, ST30-IVc | WSPP ₁₆₆₆₃ | 2002 | [25] |
| φSa2wa-st59 | 42,133 | 59, ST59-V | Taiwan clone ₁₆₆₇₂ | 2003 | [26] |
| φSa2wa-st72 | 47,213 | 72, ST72-IVa | Korean clone ₁₅₈₀₃ | 2006 | [18] |
| φSa2wa-st80 | 45,164 | 80, ST80-IVc | European clone ₁₅₃₉₅ | 2004 | [27] |
| φSa2wa-st93 | 45,913 | ST93-IVa | Qld clone ₁₆₇₉₀ | 2003 | [28] |
| φSa2wa-st772 | 42,402 | 1, ST772-V | Bengal Bay clone ₁₇₀₄₈ | 2007 | [23] |
| φSa2USA | 45,914 | 8, ST8-IVa | USA300_FPR3757 | 2003 | [29] |
| WA PVL-negative Clones | | | | | |
| NA | NA | 1, ST1-IVa | WA1 _{WBG8287} | 1995 | [24] |
| NA | NA | 88, ST255-IVa | WA2 _{WBG8366} | 1995 | [24] |
| NA | NA | 5, ST5-IVa | WA3 _{WBG8378} | 1995 | [24] |
| NA | NA | 45, ST45-V | WA4 _{WBG8404} | 1995 | [24] |
| NA | NA | 8, ST8-IVa | WA5 _{WBG7583} | 1989 | [30] |

Abbreviations: bp, base pairs; NA, Not applicable; WA, Western Australian, Qld, Queensland; WSPP, Western Samoan Phage Pattern

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lukSF-PV sequencing

Isolates were cultured on brain heart infusion agar (BHIA) (Gibco Diagnostics, Gaithersburg, MD, USA), incubated at 37°C, grown in trypticase soy broth (Gibco Diagnostics, Gaithersburg, MD, USA) and incubated overnight at 37°C. DNA was extracted using the Invitrogen PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer with lysostaphin (Sigma-Aldrich, St. Louis, MO, USA) used to lyse the *S. aureus* cell wall. *lukSF-PV* was amplified as previously described [31]. Amplicons were purified using the Ultraclean DNA PCR Clean Up Kit (MoBio Laboratories, GeneWorks, Thebarton, SA, Australia) and sequences were compared with the *lukSF-PV* genes from φSLT (Genbank: AB045978).

Bacteriophage induction and hybridisation

Bacteriophage were induced using Mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) as previously described [10]. Plaques were transferred onto nylon membranes using standard techniques [32] and DNA was cross-linked to the membrane (Amersham Biosciences, Little Chalfont, Bucks, England) using a GS Gene Linker UV Chamber (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were treated with 2 mg/mL Proteinase K (Roche Diagnostics, Mannheim, Germany). The hybridisation probe was obtained by PCR amplification of *lukSF-PV* using previously described primers [33]. PCR products were purified using the MoBio PCR Cleanup Kit. Probes were prepared using the DIG DNA Labelling and Detection

Kit according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Plaque hybridisation was performed as directed by the manufacturer (Boehringer Mannheim, Mannheim, Germany).

PVL-bacteriophage propagation and lysogenisation of PVL-negative WA CA-MRSA

To propagate the mitomycin C-induced PVL-positive bacteriophages the plaques were extracted and crushed with 3 drops of BHIB, and the mixture left to stand for 10 minutes. This suspension was added to 100 μ L of an overnight culture of the indicator strain, 3 mL of molten 3% BHIA was added and the mixture poured onto a BHIA plus 0.004M Ca^{2+} base plate which was incubated overnight at 30°C. The overlay containing the bacteriophage and indicator strain was scraped off and filtered. Each of the PVL-negative strains of WA CA-MRSA were grown overnight in BHIB and lawn-inoculated onto BHIA plus 0.004M CaCl_2 . A drop of each PVL-bacteriophage lysate was placed on the lawn and incubated at 30°C overnight. Isolated colonies growing in the centre of plaques present on lawns of PVL-negative WA clones were picked, their total DNA was isolated and lysogeny was detected using previously described primers [33].

Results

Sequence analysis and bacteriophage classification reveal diversity amongst the prophages

Fourteen prophage genomes between flanking direct 21 base pair (bp) repeats of 5'-AGGGCAAAAAAGGGCg/aGATT-3' termed *attL* and *attR* were analysed (Table 1). The 12 new prophage sequences from this study have been deposited in the NCBI database under accession numbers MF580410, MK940809 and MG029509 to MG029518.

The prophages were between 38,576 and 47,213 bp in size with between 41.4 and 100% nucleotide (nt) identity, GC compositions of 31 to 33.4% and 52 to 75 protein-coding sequences of 25 or more amino acids (aa).

The prophage genomes had the organisation of *Siphoviridae* family Sfi21-like PVL viruses of the *Caudovirales* order and, according to the most recent staphylococcal bacteriophage classification criteria, were placed into two genera and three PVL bacteriophage groups (Table 2) [34–36]. ϕ Sa2wa-st22, -st59 and -st772 (76.1–76.7% nt identity) were placed into the 77like-virus genus of icosahedral-headed bacteriophage. ϕ Sa2wa-st22 and -st772 were group 1 PVL bacteriophage with 74.4% nt identity and ϕ Sa2wa-st59 was group 3. ϕ Sa2wa-st1, -st5, -st8, -st30, -st72, -st78, -st80, -st93, -st93mssa, -st121mssa and ϕ Sa2USA (74.2–100% nt identity) were 3alikevirus genus, prolate-headed group 2 PVL bacteriophage. ϕ Sa2wa-st5 was unusual in that it encoded type C DNA polymerase (Genbank: AUM57702) rather than type A (exemplified by ϕ Sa2wa-st93 Genbank: AUM58245) (Fig 1).

ϕ Sa2wa-st93, -st93mssa (100% nt identity) and -st8 (99.97% nt identity) were considered to be the same bacteriophage as the international ϕ Sa2USA (99.97% nt identity), with ϕ Sa2wa-st72 (96.6% nt identity) very closely related. These will be known as the ϕ Sa2USA/ ϕ Sa2wa-st93 group in this study. The prophages found in the WA clones, WA1₁₅₇₉₈ (ϕ Sa2wa-st1), WA2_{RNSH95} (ϕ Sa2wa-st78) and WA3₁₈₇₉₀ (ϕ Sa2wa-st5) had identities of 80.8 to 93.8% and, although related, they were not identical to each other or any PVL bacteriophage in this study or in the NCBI database while ϕ Sa2wa-st8 from WA5₁₈₇₅₁ had only 1 bp difference with ϕ Sa2USA and will be included in the ϕ Sa2USA/ ϕ Sa2wa-st93 group.

Table 2. WA PVL prophage *lukSF-PV* polymorphisms, prophage classifications and lysogen lineages.

| Prophage | Lysogen CC, ST | Lysogen Genus/PVL gp. | SNPs | | | | | | | | |
|------------------|----------------|-----------------------|----------------|-----|-----|-----|-----|-----|----------------|------|------|
| | | | <i>lukS-PV</i> | | | | | | <i>lukF-PV</i> | | |
| | | | 33 | 105 | 345 | 443 | 527 | 663 | 1186 | 1396 | 1729 |
| φSLT | 30, ST30 | 3alikevirus/2 | G | T | C | G | A | G | C | A | A |
| φSa2wa-st30 | 30, ST30 | 3alikevirus/2 | G | T | C | G | A | G | C | A | A |
| φSa2wa-st772 | 1, ST772 | 77likevirus/1 | G | T | C | G | A | G | C | A | A |
| φSa2958 | 5, ST5 | 3alikevirus/2 | G | T | C | G | A | G | C | G | A |
| φSa2wa-st1 | 1, ST1 | 3alikevirus/2 | G | T | C | G | A | G | C | G | A |
| φSa2wa-st22 | 22, ST22 | 77likevirus/1 | G | T | C | G | A | G | C | G | A |
| φSa2wa-st59 | 59, ST59 | 77likevirus/3 | G | T | C | G | A | G | C | G | A |
| φSa2wa-st8 | 8, ST8 | 3alikevirus/2 | G | T | C | G | G | T | C | A | G |
| φSa2wa-st72 | 72, ST72 | 3alikevirus/2 | G | T | C | G | G | T | C | A | G |
| φSa2wa-st93 | S, ST93 | 3alikevirus/2 | G | T | C | G | G | T | C | A | G |
| φSa2wa-st93mssa | S, ST93 | 3alikevirus/2 | G | T | C | G | G | T | C | A | G |
| φSa2USA | 8, ST8 | 3alikevirus/2 | G | T | C | G | G | T | C | A | G |
| φSa2wa-st5 | 5, ST5 | 3alikevirus/2 | G | T | C | A | A | G | C | G | A |
| φSa2wa-st78 | 88, ST78 | 3alikevirus/2 | G | C | C | G | A | G | C | G | A |
| φSa2wa-st121mssa | 121, ST121 | 3alikevirus/2 | G | T | C | G | A | G | T | A | A |
| φSa2wa-st80 | 80, ST80 | 3alikevirus/2 | A | T | T | G | A | G | C | A | A |
| φSa2mw | 1, ST1 | 3alikevirus/2 | G | T | C | G | G | T | C | A | A |

Nucleotides differing from those of φSLT are shaded. Abbreviations: gp., group

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The φSa2 chromosomal integration site was heterogenous

Chromosomal sequences proximal to the prophage terminals encoded the hybrid *attLi* and *attRi* sites of the *attB* and *attP* sites on the chromosome and a circularly permuted form of the bacteriophage. They consist of a 29-bp central core and 25-bp left-hand (LH) and right-hand (RH) arms (Fig 2) [37]. There were nine single nucleotide polymorphism (SNP) profiles for *attLi* and seven for *attRi* (Fig 2). Two groups of prophages shared identical *attLi* and *attRi* sites; international prophage φSa2USA and φSa2wa-st72 with φSa2wa-st8, and Australian international prophage φSa2wa-st93 with φSa2wa-st93mssa. Of the prophage in the WA CA-MRSA-like strains, φSa2wa-st8 shared *attRi* with the φSa2USA/φSa2wa-st93 group and φSa2wa-st772; φSa2wa-st1 had a unique *attLi* and φSa2wa-st8 shared *attLi* with φSa2wa-st72 and φSa2USA. φSa2wa-st5 and φSa2wa-st78 had unique integration-site sequences. *attLi* of φSa2wa-st78 could not be identified, however its *attRi* was reasonably similar to the φSa2wa prophages over the LH arm and the first 17 bp of the common core (3 bp difference) while 32 of the remaining 37 bp were different (Fig 2). *attLi* of φSa2wa-st30 was absent due to a 268 bp deletion (detected by comparison with the intact “preferred integration site” of WA2_{RNSH95}).

With the exception of φSa2wa-st78, the bacteriophages had inserted into a gene within a cluster of three or four open reading frames (ORFs) encoding a putative domain of unknown function (DUF)1672 lipoprotein [38], one downstream of the integration site and two or three upstream. The four DUF1672 domain-containing proteins of φSa2wa-st72 had amino acid similarity scores of 61.1–79.7% indicating they were paralogues. There was variability in the truncated ORF. φSa2wa-st1, -st5, -st59, -st772 and the φSa2USA/φSa2wa-st93 group had truncated the 3' end of an ORF encoding a lipoprotein_7 superfamily domain-containing protein (54.5–100% nt identity and 55.6–100% amino acid similarity) which variably also encoded a structural maintenance of the chromosome SMC_N domain (φSa2wa-st1, -st5, -st59 and -st72). φSa2wa-

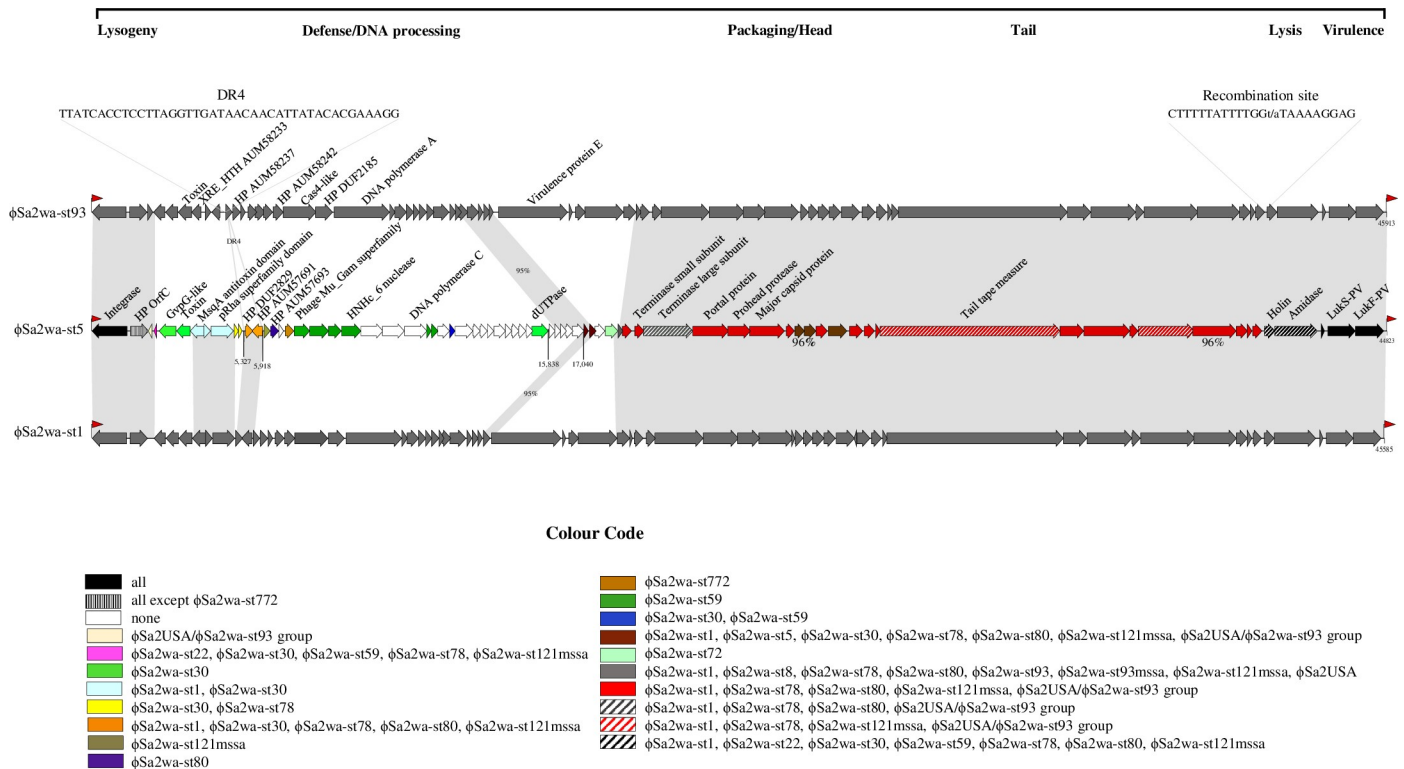


Fig 1. Diagrammatic comparison of phiSa2wa-st5 with phiSa2wa-st1 and phiSa2wa-st93. phiSa2wa-st5 ORFs are represented as arrows indicating the direction of transcription and coloured according to the PVL prophage or groups of PVL prophages from PVL-positive *S. aureus* in WA that share 97 to 100% nucleotide identity. Where identity is less than 97% this is indicated under the ORFs. Regions of the genomes with 97 to 100% identity with phiSa2wa-st5 are shaded. Where identity is less than 97% this is indicated in the shaded region. The main functional modules are indicated on a line above the genomes. Red flags indicate *attL* and *attR* sites. Genome size is indicated at the right-hand end. Proteins encoded by ORFs relevant to this study and structural proteins are indicated. Hypothetical proteins are identified by their Genbank accession number. The positions and sequences of DR4 and a widely-shared recombination site are presented. Abbreviations: DR, direct repeat; HP, hypothetical protein.

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st22, -st30, -st80 and -st121mssa, had truncated an ORF encoding a hypothetical protein (HP) which was in the same position as the lipoprotein_7 domain ORF but lacked the lipoprotein_7 domain. ORFs truncated by phiSa2wa-st22, -st30 and -st80 had 82.2–94% nt identity however,

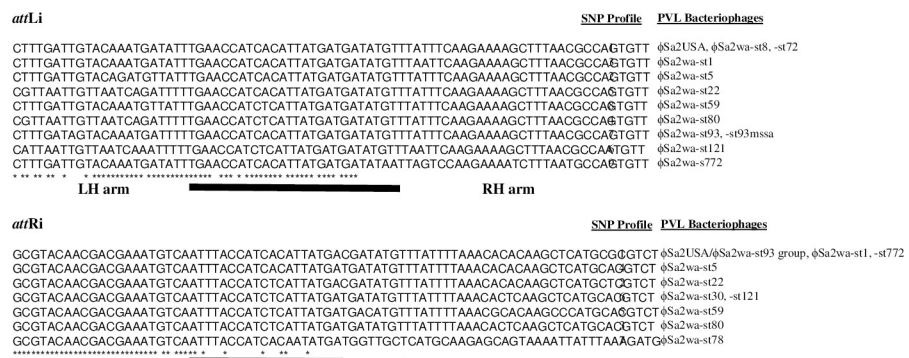


Fig 2. Integration-site sequences proximal to the terminals of PVL prophages from PVL-positive *S. aureus* in WA (2005 to 2008). Sequences have been aligned using ClustalW. The central core sequence is underlined with a thick black line. Left-hand and right-hand arms are indicated. SNP profiles are numbered alongside their respective bacteriophages. Identical nucleotides are indicated by an asterisk, absence of an asterisk indicates a polymorphic site. Abbreviations: LH, left hand; RH, right hand; *attLi*, left-hand integration site; *attRi*, right-hand integration site.

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the ORF truncated by ϕ Sa2wa-st121mssa had only 26.4–36.2% nt identity. Immediately upstream of all prophages except ϕ Sa2wa-st78 was an ORF encoding a 62-aa HP whose sequence indicated that it was the 3' terminal of the truncated lipoprotein_7 protein ORF, when compared with the intact lipoprotein_7 domain-encoding ORF of WA2_{RNSH95} (not shown).

ϕ Sa2wa-st78 had truncated the 3' end of a 6-phospho-beta-galactosidase gene and inserted upstream of a galactose-6-phosphate degradation enzyme. The ϕ Sa2wa-st78 host genome, WA2_{RNSH95}, encoded an intact lipoprotein_7 domain-encoding ORF that contained an *attLi* site which was homologous over the LH arm and central core (1 bp difference) with the consensus *attLi* but had 14 bp differences in the RH arm. This may have prevented insertion of ϕ Sa2wa-st78 into what appears to be a preferred site for ϕ Sa2.

Lysogeny regulation and modular recombination sequences

The intergenic region between the divergently transcribed integrase gene *int* and its associated HP ORF, originally called *orfC* [39] (Fig 1), contained structures indicative of involvement in regulation and lysogeny in all prophages (Fig 3). There were SNPs between the prophages, however all except ϕ Sa2wa-st772 had the same secondary structure which consisted of a consensus sigma factor H (SigH) binding-site [40] and a downstream inverted repeat (IR) of 5' – GAACGTAC/tGTTTC–3'. Overlapping the SigH binding-site was an inverted repeat that could form a possible stem-loop structure of 5' –GGGTAGgtgggCTACCC–3' (stem-loop 1) (Fig 3). The first two nucleotides of the loop could be GT, TC or GC (ϕ Sa2wa-st772). There was then a previously identified and highly conserved stem-loop putative regulatory site, stem-loop 2 [41]. Both stem-loops were flanked by heptanucleotide direct repeats (DR) of 5' – AAAATAA–3' (DR1) the first of which comprised 7 bp of the SigH binding site.

ϕ Sa2wa-st772, which has previously been predicted to be a recombinant bacteriophage [23] had a regulation region that was somewhat different. The intergenic region was between *int* and a different HP ORF (exemplified by YP_00910342) transcribed on the same strand. The regulatory features however, included the SigH binding-site with its downstream IR and stem-loop 1; stem-loop 2 was absent and there was only one copy of DR1, which occurs from 24 to 33 times in the prophage genomes.

Of the prophages in the WA CA-MRSA the ϕ Sa2wa-st1 regulation region was identical with that of ϕ Sa2wa-st80 while ϕ Sa2wa-st5, -st8 and -st78 were identical with ϕ Sa2USA.

A previously described 23-bp recombination site that has been found in unrelated staphylococcal bacteriophage [42] was found downstream of the holin gene in all prophages (Fig 1). ϕ Sa2wa-st772 encoded the enterotoxin A gene flanked by direct repeats (DRs) of 5' – CTTTTTATTTTG–3' immediately downstream of this site thus implicating the site in the acquisition of an extra virulence factor, probably from an unrelated family ϕ 3 beta haemolysin-converting bacteriophage.

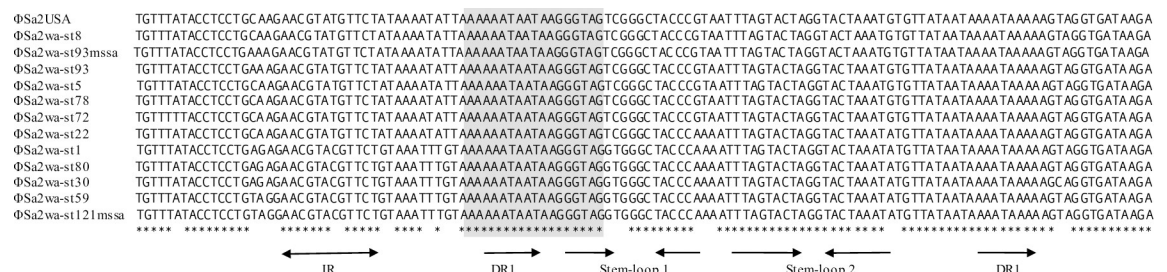


Fig 3. Regulation regions of PVL prophages from PVL-positive *S. aureus* in WA (2005 to 2008). Sequences have been aligned using ClustalW. The SigH binding site is shaded. Identical nucleotides in the alignment are indicated by an asterisk. Repeats are indicated by arrows. Abbreviations: IR = inverted repeat; DR = direct repeat.

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ϕ Sa2wa-st5 has a module of 592 bp (bp 5,327–5,918) which encodes a DUF2829 protein and a HP ORF (Fig 1, Genbank: AUM57690 and AUM57691) flanked by 42-bp direct repeats (Fig 1, DR4). This module is present in ϕ Sa2wa-st1, -st30, -st78, -st80 and -st121mssa however, ϕ Sa2wa-st1, -st80 and -st121mssa lack a LH copy of DR4. Furthermore, this repeat is present as a similarly positioned single copy in all other study prophage except ϕ Sa2wa-st22 and -st772, indicating that the module has disseminated horizontally between bacteriophage of the same genera and the repeat is a conserved sequence that could mediate recombination, integration and excision.

lukS/F-PV sequence SNPs were not specific for PVL bacteriophage genera or lysogen genotype

A single copy of a DR that has been implicated in the deletion of the *lukSF-PV* and integrase module [43] was found downstream of the *lukSF-PV* genes in all prophages however, a second copy was not found in any of the genomes. Nine *lukSF-PV* SNPs were found, six in *lukS-PV* and three in *lukF-PV* and there were eight SNP profiles (Table 2). All except the A→G (histidine→arginine) substitution at position 527 in the ϕ Sa2USA/ ϕ Sa2wa-st93 group and ϕ Sa2MW, were synonymous. The ϕ Sa2USA/ ϕ Sa2wa-st93 group SNPs were identical. ϕ Sa2wa-st30 and -st772 were identical to ϕ SLT. ϕ Sa2wa-st22, -st59 and -st1 were identical to the CC5 control ϕ 2958. ϕ Sa2wa-st78, -st80 and -st121mssa had individual *lukSF-PV* SNPs reported previously for their respective genetic lineages [44, 45] and ϕ Sa2wa-st5 had a unique *lukSF-PV* SNP profile.

The distribution of the SNP profiles was heterogeneous. (i) Highly similar PVL prophage with the same *lukSF-PV* SNPs lysogenised *S. aureus* of three different lineages, indicating horizontal dissemination of a successful PVL bacteriophage between *S. aureus* of three lineages; the ϕ Sa2USA/ ϕ Sa2wa-st93 group lysogenised ST8-IVa, ST72-IVa, ST93, and ST93-IVa. (ii) Different PVL prophages with different *lukSF-PV* SNP profiles lysogenised *S. aureus* of the same lineage, indicating horizontal transmission of different PVL bacteriophage into *S. aureus* of the same lineage; 77likevirus, PVL group 1 (ϕ Sa2wa-st772) and 3alikevirus, PVL group 2 (ϕ Sa2wa-st1 and ϕ Sa2mw) prophages lysogenised CC1 strains, ST772-V and ST1-IVa respectively. (iii) Different genera of PVL prophage with the same *lukSF-PV* SNP profile lysogenised different *S. aureus* lineages, indicating that different PVL bacteriophage can carry the same *lukSF-PV* module and that either the *lukSF-PV* genes disseminate horizontally between different genera of PVL bacteriophage or random substitutions occur during replication and the fit-test permutations prevail regardless of the genus of PVL prophage; 77likevirus, PVL groups 1 (ϕ Sa2wa-st22) and 3 (ϕ Sa2wa-st59) lysogenised ST22-IVc and ST59-V respectively and 3alikevirus, PVL group 2 prophage (ϕ 2958 and ϕ Sa2wa-st1) lysogenised ST5-II and ST1-IVa respectively.

Alpha alignment of colinear regions of ϕ Sa2wa-st1, -st5, -st59 and -st93 identified novel modules and heterogeneous genes

The DNA processing main module is the most variable region in PVL bacteriophages and Fig 4 presents Alpha alignments of colinear sections of ϕ Sa2wa-st1, -st5, -st59 and -st93 from the 5' end of the bacteriophages. The region encodes conserved genes associated with lysogeny, and a variable region of early transcribed genes associated with lysogeny, bacteriophage defence and regulation. Variable genes and different colinear modules with similar functions can be detected in this graphical representation of heterogeneity.

Fig 4A has 15 alignment nodes. The anchor sequences are nodes 1 and 14 which encode *int* with the 5' end of *orfC* (exemplified by ϕ Sa2wa-st5, Genbank: AUM57679 and AUM57680)

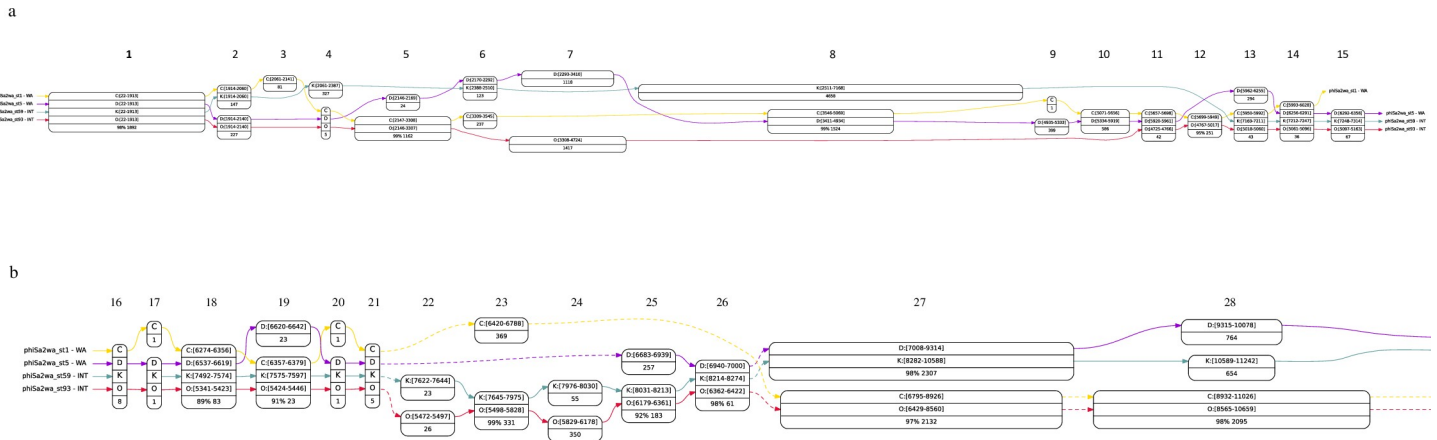


Fig 4. A and B. Alpha alignment of colinear sections of ϕ Sa2wa-st1, ϕ Sa2wa-st5, ϕ Sa2wa-st59 and ϕ Sa2wa-st93. Each genome in the alignment is assigned an uppercase letter. Alignment positions for the corresponding genome are indicated in parentheses alongside the letter. Anchor sequences are similar segments of significant length shared by all genomes in the alignment. Nodes are gapless alignments specific for individual genomes in the alignment; they display the length and percent identity of the aligned region; unless otherwise indicated identity is 100%. Anchors and nodes are connected by color-coded arrows, one color for each genome and numbered sequentially on the figure. Dotted arrows replace nodes of less than 20 bp. Abbreviations: C, ϕ Sa2wa-st1; D, ϕ Sa2wa-st5; K, ϕ Sa2wa-st59; O, ϕ Sa2wa-st93; WA, Western Australian; INT, International.

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and the 5' terminal of a helix-turn-helix (HTH) domain-encoding ORF (Genbank: AUM57693) respectively (Fig 1). The genomes diverge at bp 1,913 (Alpha alignment node 1). Following two or three HPs of unknown function ϕ Sa2wa-st1 (c3081-3542), -st5 (c2946-3416) and -st93 (c3080-3550) encode a putative toxin gene, (Genbank: AUM57684, 72–89% nt identity, 82.8–94.9% aa similarity) (Fig 1). The ϕ Sa2wa-st1 and -st93 putative toxin sequences (77.3% nt identity) diverge at bp 3,308 and 3,307 respectively (Alpha alignment node 5). All polymorphisms in these ORFs are in the 5' ends and most (27/38) are non-synonymous. Non-random distribution of polymorphisms such as this indicates homologous recombination between divergent genes has probably occurred. ϕ Sa2wa-st5 has a gas vesicle protein G (GvpG) domain-encoding ORF (Genbank: AUM57683) downstream of the toxin gene (Genbank: AUM57684, Alpha alignment node 7) which has 89% nt identity with the ϕ Sa2wa-st1 toxin gene. The ϕ Sa2wa-st1 and -st5 toxin genes are followed by a xenobiotic response element (XRE)-HTH putative transcriptional regulator ORF which also encodes a MqsA antitoxin superfamily domain (Genbank: AUM57685, Alpha alignment node 8, 99.4% nt identity), while ϕ Sa2wa-st93 encodes a XRE-HTH transcriptional regulator (Genbank: AUM58233, Alpha alignment node 7). ϕ Sa2wa-st1 and -st5 then encode a XRE_HTH family regulator followed by a bacteriophage pRha superfamily domain-containing protein that interferes with infection of strains that lack integration host factor (Genbank: AUM57686 and AUM57687, Alpha alignment node 8).

When compared with all the WA-PVL prophages the node 8 module of ϕ Sa2wa-st1 and -st5 is shared with only ϕ Sa2wa-st30, and the node 7 module of ϕ Sa2wa-st93 is shared with only the ϕ Sa2USA/ ϕ Sa2wa-st93 group. ϕ Sa2wa-st59 encodes a unique 4,658 bp section (bp 2511–7168), which encodes two XRE family transcriptional regulators, a bacteriophage anti-repressor and 10 putative HPs (AUM57878 to AUM57891, Alpha alignment node 8). Nodes 13, 14 and 15 encode the 5' end of a HP ORF (Genbank: AUM58237) with a common core (Alpha alignment node 14) and divergence by ϕ Sa2wa-st5 and ϕ Sa2wa-st1 indicated by the graph.

Following two to four heterogenous colinear HP ORFs the Fig 4B Alpha alignment node 27 reveals two unrelated colinear modules. ϕ Sa2wa-st5 and -st59 encode ORFs for a bacteriophage Mu Gam-like protein which protects double stranded DNA from exonuclease degradation, two

overlapping single-stranded binding proteins and a putative HNHc_6 superfamily nuclease (Genbank: AUM57696 to AUM57698) which are not shared by any other WA PVL prophage (Fig 1). ϕ Sa2wa-st1 and -st93 encode three overlapping ORFs encoding a HP, a Cas4-like protein and a DUF2185 protein (Genbank: AUM58242, AUM58243 and AUM58244). This module may be a defence system or part thereof against the bacterial CRISPR-Cas system and it is shared by all study prophage (97–100% nt identity) except ϕ Sa2wa-st5, -st22, -st59 and -st772. ϕ Sa2wa-st1 and -st93 then encode DNA polymerase A (Alpha alignment node 28) while ϕ Sa2wa-st5 (Fig 1) and -st59 encode DNA polymerase C.

Alpha aligner-defined nodes reveal extensive mosaicism and recombination in PVL bacteriophage from WA

The singleton ST93 genome is stable and well-adapted in the geographical region. To further investigate the mosaicism in the prophages Table 3 presents the nodes of ϕ Sa2wa-st93 as determined by the Alpha aligner in Fig 4A and 4B, identifies the putative proteins or protein

Table 3. Alpha aligner defined nodes of ϕ Sa2wa-st93 and node-associated ORFs or intergenic regions having 97–100% sequence identity with other WA PVL prophages.

| ϕ Sa2wa-st93 Position/Node* | Protein(s) accession no's, HPs or regions | WA PVL bacteriophages with 97–100% sequence identity |
|----------------------------------|---|--|
| 22-1913/1 | Integrase, AUM58227; Split HP, AUM58228 | ϕ Sa2wa-st1, -st5, -st22, -st30, -st59, -st72, -st78, -st80 -st121mssa |
| 1914-2140/2 | Split HP, AUM58228; split HP AUM58229 | ϕ Sa2wa-st5, -st72, -st78 |
| 2146-3307/5 | Split HP, AUM58229; HP, AUM58230; HP, AUM58231; split Toxin, AUM58232 | ϕ Sa2wa-st1, -st72 |
| 3308-4724/7 | Split Toxin, AUM58232; HP, AUM58233; HP, AUM58234; HP, AUM58235 | ϕ Sa2wa-st72 |
| 4725-4766/11 | Intergenic region | ϕ Sa2wa-st1, -st5, -st30, -st59, -st72, -st78, -st80, -st121mssa |
| 4767-5017/12 | HP, AUM58236 | ϕ Sa2wa-st72 |
| 5018-5060/13 | Split HP, AUM58237 | ϕ Sa2wa-st1, -st30, -st59, -st72, -st78 |
| 5061-5096/14 | Split HP, AUM58237 | ϕ Sa2wa-st1, -st5, -st30, -st59, -st72, -st78, -st80, ϕ -st121mssa |
| 5097-5163/15 | Split HP, AUM58237 | ϕ Sa2wa-st5, -st30, -st59, -st72, -st78, -st80, -st121mssa |
| 5341-5423/18 | Split DUF1270, AUM58238 | ϕ Sa2wa-st72, -st78 |
| 5424-5446/19 | Split DUF1270, AUM58238 | ϕ Sa2wa-st72, -st78 |
| 5472-5497/22 | Intergenic region | ϕ Sa2wa-st72 |
| 5498-5828/23 | HP, AUM58239; split DUF2482 HP, AUM58240 | ϕ Sa2wa-st59, -st72 |
| 5829-6178/24 | Split DUF2482 HP, AUM58240; split DUF1108 HP, AUM58241 | ϕ Sa2wa-st72 |
| 6179-6361/25 | Split DUF1108 HP, AUM58241 | ϕ Sa2wa-st72 |
| 6362-6422/26 | Split DUF1108 HP, AUM58241 | ϕ Sa2wa-st5, -st59, -st72 |
| 6429-8560/27 | HP, AUM58242; Cas4-like, AUM58243; DUF2815 HP, AUM58244 | ϕ Sa2wa-st1, ϕ -st30, -st72, -st78, -st80, -st121mssa |
| 8565-10659/28 | DNA polymerase A, AUM58245; split DUF3113 HP, AUM58246 | Sa2wa-st1, -st30, -st72, -st78, -st80, -st121mssa |

Proteins and hypothetical proteins are indicated by their Genbank protein-id number. Genbank domains of unknown function are indicated; Split proteins represent split open reading frames. Abbreviations: DUF, domain of unknown function; HP, hypothetical protein; no's, numbers

* As presented in Fig 4

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Table 4. Bacteriophage induction and lysogenisation of historic PVL-negative WA CA-MRSA.

| Lysogen | Lysogenised recipients Total pfu/mL | | PVL positive plaques | Induced PVL bacteriophage | PVL-negative CA-MRSA lysogenised |
|----------------------------|--|-------------------|----------------------|---------------------------|----------------------------------|
| | RN4220 | WBG286 | | | |
| MW2 | >1x10 ⁵ | 0 | >100 | φSa2mw | WA5 _{WBG7583} |
| WA1 ₁₅₇₉₈ | 0 | 0 | 0 | 0 | NA |
| WA2 _{RNSH95} | 0 | 1x10 ³ | 0 | 0 | NA |
| WA3 ₁₈₇₉₀ | 2x10 ³ | 0 | 20 | φSa2wa-st5 | None |
| W17S | 0 | 1x10 ² | 1 | φSa2wa-st93mssa | Not tested |
| K25S | 3x10 ² | 0 | 3 | φSa2wa-st121mssa | Not tested |
| Qld Clone ₁₆₇₉₀ | 0 | 1x10 ² | 1 | φSa2wa-st93 | WA5 _{WBG7583} |

Abbreviations: NA, not applicable; pfu, plaque forming units.

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sections and shows the local prophages that encode the same sequence with 97 to 100% nt identity. φSa2wa-st8, -st93mssa and φSa2USA are almost identical to φSa2wa-st93 and have been excluded from the Table. There is evidence of extensive recombination. The only prophage that was identical in this region was the closely related φSa2wa-st72 and the only prophage not to share any module was φSa2wa-st772. Most of the shared sequence involved the prolate-headed 3alikevirus prophages however, there was also evidence of recombination with the 77likevirus icosahedral-headed φSa2wa-st59. The Alpha aligner defined modules consist of split genes, single genes, groups of genes and intergenic regions, some shared by several prophages and others by only one or two. At 96.6% nt identity φSa2wa-st72 is a member of the φSa2USA/φSa2wa-st93 group in this study and the Table 3 modules with homology only with φSa2wa-st72 represent modules encoding functions that, amongst the prophages in this study, are unique to this successful bacteriophage.

With between 77% and 82.9% nt identity φSa2wa-st5 from WA3 was the most distantly related of the 3alikevirus group and was successfully induced and therefore probably transmissible (Table 4). It had highest homology with φSa2958 (Genbank: AP009363; 99% nt identity over 72% of the genome) however, this was essentially in the structural morphology and lysis, virulence and lysogeny modules. Fig 1 presents a diagrammatic comparison of φSa2wa-st5, -st93 and -st1 with the φSa2wa-st5 ORFs coloured according to the WA PVL-prophages that shared 97–100% sequence identity. With the exception of the bp 15,838 to 17,040 module which was homologous with non-PVL bacteriophage 53 (Genbank: AY954952; 100% nt identity) the unshared regions of φSa2wa-st5 encoding multiple ORFs were unique. As well as random mutations that occur during chromosomal replication this heterogeneity indicates that horizontal recombination has occurred between the bacteriophage during their evolution.

***in-vitro* induction of PVL-positive φSa2 from Australian *S. aureus* and lysogenisation of historic PVL-negative WA CA-MRSA**

To test the transmissibility of PVL-positive φSa2 lysogenising the Australian *S. aureus* and the lysogenic capabilities of the historic PVL-negative WA CA-MRSA (Table 1), *in-vitro* induction, propagation and lysogenisation experiments were performed (Table 4).

Bacteriophage were induced from all isolates tested except WA1₁₅₇₉₈. Overall, only two of the five indicator strains, RN4220 and WBG286 were lysogenised and specifically, only one in each induction experiment, demonstrating some specificity of lysogenisation (Table 4). Hybridisation of the plaques revealed that φSa2wa-st5, -st93mssa, -st93, -st121mssa and the control, φSa2mw, were induced out of their lysogens. φSa2wa-st78 may not have been induced because

it lacked an evident *attLi* integration site (Fig 1) however, the reason why ϕ Sa2wa-st1 was not induced is currently unclear.

ϕ Sa2wa-st121mssa could not be propagated to a sufficiently high titre *in-vitro* however, ϕ Sa2wa-st5, -st93 and the control, ϕ Sa2mw, were tested for their ability to lysogenise all of the historic PVL-negative WA CA-MRSA. WA5_{WBG7583} was lysogenised with ϕ Sa2wa-st93 and the control, ϕ Sa2mw, but not ϕ Sa2wa-st5. None of the other PVL-negative WA CA-MRSA were lysogenised *in-vitro* with any of the induced and propagated bacteriophages.

Discussion

The genomes of PVL prophages from temporally and geographically related *S. aureus* of local and international origin have revealed an unexpected amount of diversity that has made it difficult to trace their origins. There has been recombination between bacteriophage of the same and different genera as well as genetic diversity in the chromosomal integration sites, the regulation regions, the defence, DNA-processing, structural and packaging modules and the *lukSF-PV* genes. There was no evidence that the icosahedral-headed prophages from international clones of CA-MRSA had transferred to the WA clones. The prolate-headed prophage formed the largest group however, with the exception of ϕ Sa2wa-st8, they were so diverse it was not possible to determine if there had been horizontal transmission of whole bacteriophages. There has been recombination between the international and local prolate-headed bacteriophage and, to a lesser extent, also between prolate- and icosahedral-headed bacteriophage that are present in WA at some stage during their evolution.

With 99.97% sequence identity, it is evident that WA5₁₈₇₅₁ has probably acquired ϕ Sa2wa-st8 in the WA community from either a ST93 *S. aureus* or USA300. ϕ Sa2wa-st93 was induced *in-vitro* and then it lysogenised PVL-negative WA5_{WBG7583} demonstrating that this clone can accept the bacteriophage. On-the-other-hand, WA5₁₈₇₅₁ and USA300 had identical ϕ Sa2 integration-site sequences indicating that the bacteriophage could also have been horizontally transmitted from USA300.

ϕ Sa2wa-st8, -st72, -st93, -st93mssa and ϕ Sa2USA probably represent a single bacteriophage that has transmitted between lineages of *S. aureus*. USA300 and the Queensland clone are two of the most virulent and widely disseminated CA-MRSA and in this and a previous study [46] it is evident that there has been horizontal transmission of a ϕ Sa2USA/ ϕ Sa2wa-st93-type bacteriophage between their CC8 and Singleton 93 ancestors, however there is no indication of when or where this occurred. USA300 acquired ϕ Sa2USA in North America following importation of its ancestor in the early 20th century [47]. ϕ Sa2wa-st93mssa was present in ST93-MSSA, the most prevalent colonizer in remote WA in 1995, and this clone was the ancestor of the Queensland clone that emerged in Queensland, Australia in the early 2000's [14, 48, 49]. ϕ Sa2wa-st93mssa was well adapted in ST93-MSSA and Australia before the clone acquired the *SCCmec* and before USA300 was imported into Australia [22]. Against the background of PVL prophage diversity revealed in this study it is extraordinary that the ϕ Sa2USA/ ϕ Sa2wa-st93 bacteriophage has remained stable over at least 20 years in different geographic and genetic environments. To gain insights into the success of this bacteriophage it would be informative to investigate the putative proteins of unknown function encoded by the unique modules of ϕ Sa2wa-st93 revealed in Table 3.

The international Korean CA-MRSA clone is characteristically PVL-negative [50] and has probably acquired ϕ Sa2wa-st72 in the WA community. This may represent a recent acquisition of a ϕ Sa2USA/ ϕ Sa2wa-st93 bacteriophage with the prophage undergoing gradual changes as it adapts to a CC72 background and different geographical conditions. As with the WA

PVL-positive CA-MRSA the PVL-positive Korean clone has not thrived and forms only 0.02% of CA-MRSA in the WA community [19].

The diversity in shared genes such as the hypothetical proteins that have been split in Table 3 according to their homologies with all the prophage in the study is interesting. In the putative toxin genes, all of the nucleotide differences were in the 5' end of the ORF and most resulted in different amino acids. This may be a defence strategy that has involved recombination within genes resulting in proteins with the same function but different antigenic profiles.

With the exception of two pairs of prophage all had distinct integration-site sequences however, the impact of this on the specificity of lysogenisation is currently unknown. The effect of lysogeny by ϕ Sa2 on host fitness could not be determined. The preferred insertion site was within a lipoprotein_7 domain-encoding ORF within a paralogous cluster of three or four ORFs that encoded a DUF1672 lipoprotein. As has been previously reported, the ORFs truncated by ϕ Sa2wa-st22, -st30 and -st80 type bacteriophage [37] and now ϕ Sa2wa-st121, were different, however they were similarly positioned within the same DUF1672 lipoprotein cluster. Lipoproteins serve as transporters of nutrients and contribute to virulence and fitness in *S. aureus* and increased complements have been associated with particularly pathogenic strains [38]. The impact of truncation of the ϕ Sa2 target genes on host fitness requires further investigation.

Prophage lysogenising 11 lineages of *S. aureus* have been investigated in this study and adaptation to different genetic backgrounds is undoubtedly one of the reasons for the diversity observed. Investigation of more genomes of PVL prophage from *S. aureus* belonging to the same genetic lineage is now required. The low occurrence of PVL-positive variants of established PVL-negative CA-MRSA in the WA community suggests that the clones may not have adapted well to the acquisition of PVL-positive ϕ Sa2.

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