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Genome analysis of the staphylococcal temperate phage DW2 and functional studies on the endolysin and tail hydrolase

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Abbreviations: MRSA, Methacillin resistant *Staphylococcus aureus*; VRSA, Vancomycin resistant *Staphylococcus aureus*; PGH, peptidoglycan hydrolase; VAPGH, virion-associated peptidoglycan hydrolase; ORF, open reading frame; Gp, gene product; PVL, Panton-Valentine leukocidin; SaPI, *Staphylococcus aureus* pathogenicity island; CHAP, cysteine, histidine-dependent amidohydrolases/peptidases; CDD, conserved domain database

This study describes the genome of temperate *Siphoviridae* phage DW2, which is routinely propagated on *Staphylococcus aureus* DPC5246. The 41941 bp genome revealed an open reading frame (ORF1) which has a high level of homology with members of the resolvase subfamily of site-specific serine recombinase, involved in chromosomal integration and excision. In contrast, the majority of staphylococcal phages reported to date encode tyrosine recombinases. Two putative genes encoded by phage DW2 (ORF15 and ORF24) were highly homologous to the NWMN0273 and NWMN0280 genes encoding virulence factors carried on the genome of ϕNM4, a prophage in the genome of *S. aureus* Newman. Phage DW2 also encodes proteins highly homologous to two well-characterized *Staphylococcus aureus* pathogenicity island derepressors encoded by the staphylococcal helper phage 80α indicating that it may similarly act as a helper phage for mobility of pathogenicity islands in *S. aureus*. This study also focused on the enzybiotic potential of phage DW2. The structure of the putative endolysin and tail hydrolase were investigated and used as the basis for a cloning strategy to create recombinant peptidoglycan hydrolyzing proteins. After overexpression in *E. coli,* four of these proteins (LysDW2, THDW2, $CHAP_{E1\text{-}153}$, and $CHAP_{E1\text{-}163}$) were demonstrated to have hydrolytic activity against peptidoglycan of *S. aureus* and thus represent novel candidates for exploitation as enzybiotics.

Introduction

One of the major dilemmas faced by modern medicine is the spread of antibiotic resistant genes among pathogenic bacteria, as is seen with methicillin and vancomycin resistance in the species *Staphylococcus aureus* (MRSA/VRSA).[1,2] Alternative therapies are urgently required. Bacteriophages (phages) are one such alternative[3,4] and have been used topically and systemically to successfully treat many bacterial infections including surgical wound infections,[5] urinary tract infections,[6] bacterial dysentry,[7] cholera,[8] neonatal sepsis,[9] staphylococcal lung infections,[10] *P. aeruginosa* infections in cystic fibrosis patients,[11] ocular infections,[12] and various types of skin infections.[13]

When a phage is considered for use in therapy it is essential that its genome has been completely sequenced and it has been demonstrated to be void of lysogenic capabilities, since temperate phage are known to play a primary role in the transfer of virulence determinants among bacteria.[14,15] This role can involve the phages acting as a vehicle of horizontal transfer of virulence genes between bacteria, by carrying such genes in their genome[14] or they may act as "helper" prophage which are involved in derepression of pathogenicity islands, as previously demonstrated for *Siphoviridae* staphylococcal phage 80α.[16] In addition to this, temperate phage can also influence the pathogenicity of their host by integrating into chromosomal virulence genes.[17] Considering that phage are the most numerous biological unit on earth[18] there have been relatively few phage genomes sequenced and there is a requirement for better understanding of the roles played by gene products whose function is as of yet undetermined. This information would help to consolidate the safety profile of phage therapy and strengthen the case for increased acceptance. In addition, this information will provide further insight into phage–host interactions and bacterial evolution, while potentially providing novel avenues for drug development.

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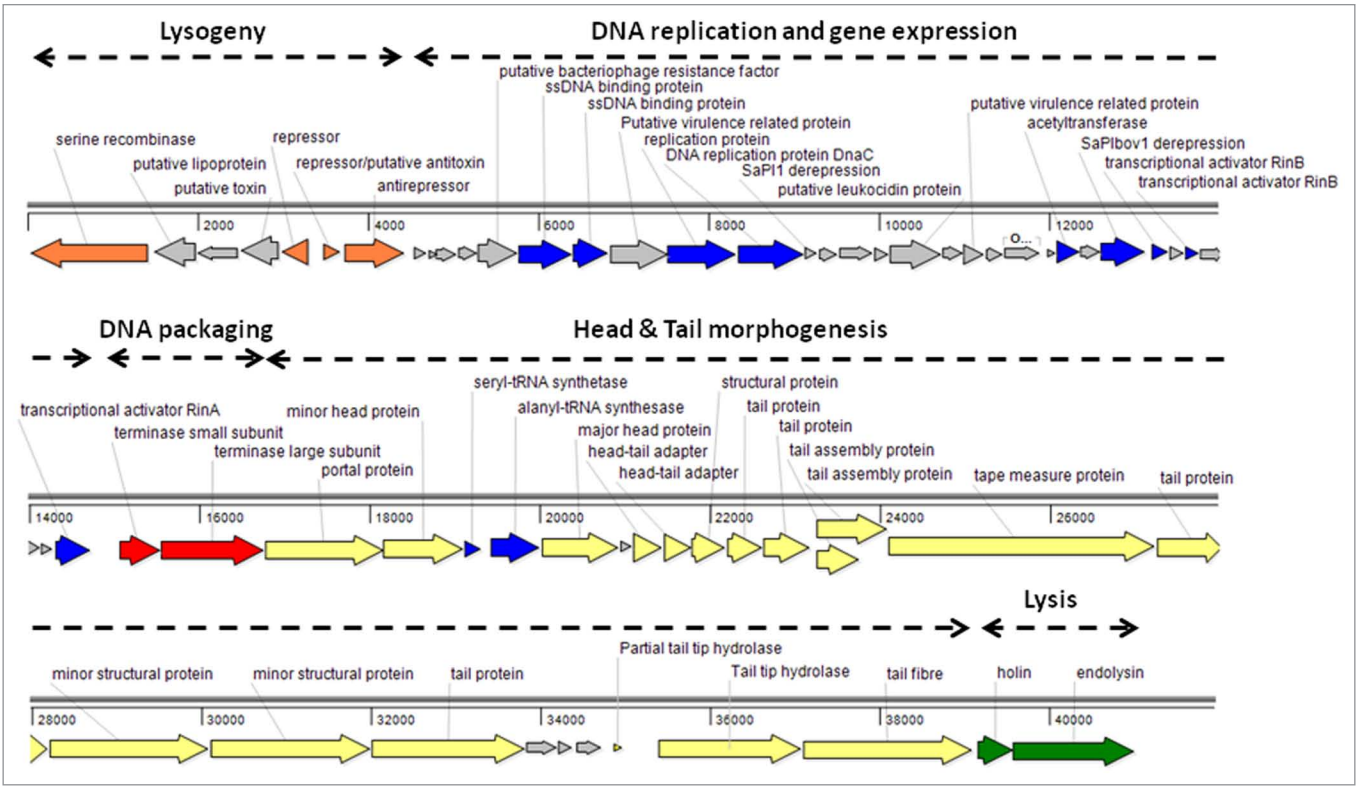

Figure 1. Map of the annotated genome of phage DW2. Functional modules are indicted by the dotted lines. The ORFs identified are indicated by arrows in the direction in which they are translated. The putative functions of the corresponding proteins are indicated. Any ORFs which are not labeled are hypothetical proteins which have no function assigned. ORFs are colored based on the predicted functions of their protein products. Orange: Lysogeny. Blue: DNA replication and gene expression. Red: DNA packaging. Yellow: Head and Tail morphogenesis. Green: Lysis.

Phage-encoded peptidoglycan hydrolases (PGH) are another possible alternative to antibiotics for elimination of infectious pathogens.[19,20] There are two types of bacteriophage PGH, namely endolysins, involved in phage progeny release from the bacterial cell and virion-associated peptidoglycan hydrolases (VAPGH), which are involved in the process of injecting phage DNA into the cell. The benefits of using phage-encoded lytic enzymes in place of whole phages as therapeutics to control Gram-positive pathogens include; a broader host range, standardization is simpler to achieve and there is no associated risk of horizontal gene transfer. In relation to pathogen control, another major advantage of using phage lytic enzymes over whole phages or antibiotics is that there have been no reports of bacteria developing resistance against these enzymes.[21] In relation to enzybiotics, endolysins are better characterized than VAPGHs.[22] They have frequently been purified and used exogenously to successfully eliminate Gram-positive bacterial infections in in-vivo studies.[19,21,23-33] Only a limited number of VAPGHs have been characterized for their antibacterial potential[20,34-39] and thus these enzymes warrant further investigation.

Staphylococcal phage DW2 is a *Siphoviridae* which was previously isolated from farmyard effluent.[40] Here we report the complete annotated genome, highlight several features which indicate the potential role played by phage DW2 in the evolution of virulence in its lysogenic host and pursue functional characterization of endolysin and tail hydrolase gene products and their truncated derivatives.

Results

Sequencing and general features of the phage DW2 genome

Pyrosequencing of the phage DW2 genome resulted in 93-fold genome coverage. The assembled genome was topologically circular. The sequence revealed that phage DW2 has a double stranded DNA genome of 41941 bp with a GC content of 35.98%. The size of the genome designates phage DW2 as a class II staphylococcal phage (~40 kb) based on the classification scheme by Kwan et al.[41] Sixty-four open reading frames (ORFs) were predicted using a nucleotide cut off of 100 bp (**Fig. 1**). The average gene product size was 206 amino acids. ORFs were found on five out of the six reading frames, with 59 coded on the forward strand and five on the reverse. Based on this predicted gene set, 93.2% of the genome was designated as coding sequence. No sequence encoding tRNA was identified.

Alignment of the phage DW2 genome against the NCBI nucleotide database, using the megablast algorithm, showed that a prophage sequence in the genome of *S. aureus* strain LGA251 (FR821779.1) has the highest level of similarity, at the nucleotide level, with 95% identity over 74% of the DW2 genome. This strain is a methicillin resistant bovine isolate sourced from bulk milk.[42] Other closely related nucleotide sequences include staphylococcal prophage ϕETA3 (AP008954.1) with 94% identity over 60% of the genome and staphylococcal prophage ϕNM4 (DQ530362.1) which shares 93% identity over 58% of

the phage DW2 genome. However, not all staphylococcal *Siphoviridae* phages demonstrated this high level of homology at the genome level with phage DW2. Phage 80α (DQ517338.1) was among the more distantly related staphylococcal phage with 88% identity over only 27% on the genome. An ideogram comparing the complete genome of staphylococcal phages DW2 to the phage mentioned can be seen in **Figure 2**. The most highly conserved region in the genome is located within the tail morphogenesis module between 25362–34938 bp, starting toward the center of the tape measure protein and ending in the partial tail hydrolase gene ORF60. In addition, this region contains two minor structural proteins, two-tail proteins and three significantly smaller hypothetical proteins.

Comparative analysis of the predicted ORFs and their gene products with sequences found on NCBI protein and nucleotide databases as well as the pfam database allowed allocation of putative functions to 44 of the 64 ORFs (**Table S1**). The gene products of the other ORFs were designated hypothetical proteins. This in silico functional analysis demonstrated that the genome has a modular structure, typical of staphylococcal *Siphoviridae*, with gene clusters representing each of the following functions: lysogeny, DNA replication, gene expression, DNA packaging, head morphogenesis, tail morphogenesis, and lysis. The complete annotated genome sequence of phage DW2 is shown in **Figure 1** in prophage orientation.

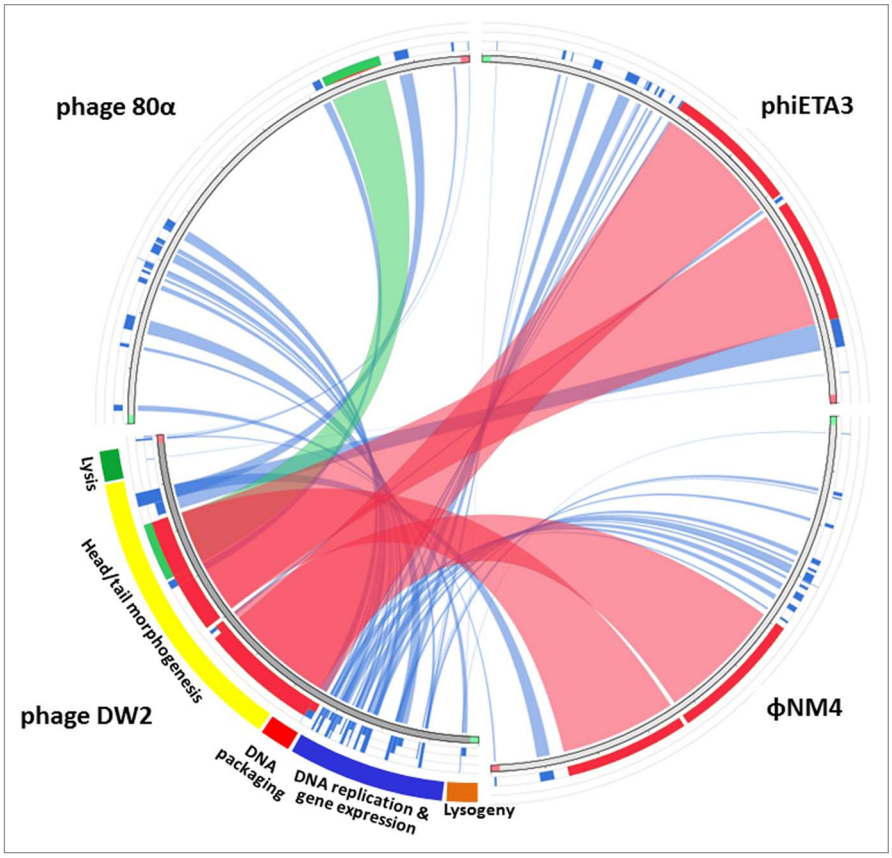

Figure 2. Ideogram built using the Circoletto program showing a comparison of phage DW2 genome with the genomes of staphylococcal *Siphoviridae* phages ϕNM4, ϕETA3, and phage 80α. Each quadrant represents an individual genome and the ribbons connecting genomes represent local alignments produced by BLAST. The ribbon colors blue, green, orange, and red respectively represent 25% blocks up to the maximum bitscore of 100%. The colored bars outside the phage DW2 reference genome, represent the locations of the functional modules.

The potential role of phage DW2 in the transfer of virulence genes

The identification of a putative serine recombinase (ORF1) gene strongly suggests that phage DW2 is a temperate phage, although lytic against *S. aureus* DPC5246, the propagating host. In silico analysis of the gene product of ORF1 (gp1) showed the presence of a N-terminal catalytic resolvase domain and a zinc ribbon domain toward the C-terminus, which is likely to be involved in DNA binding. In order to investigate the integration locus in a lysogenic host, a tblastn analysis was preformed against the NCBI collection of complete and draft staphylococcal genomes. Of the 81 hits identified, 56 of the corresponding contigs were sufficiently intact to identify the ORF downstream of the recombinase gene. Each of the 56 serine recombinases encoded by these contigs integrated into one of five integration loci, demonstrating the high level of integration specificity. Two of the loci identified were in non-coding regions downstream from the signal recognition particle protein and the 50S ribosomal protein L32. The other three were found within coding sequence and interrupted the genes for a lactose transporter subunit, a dicarboxylate ABC transporter and a mercuric reductase. Multiple sequence alignment and phylogenetic analysis classified these staphylococcal serine recombinases into four major clades (**Fig. S1**). Three of these clades clustered proteins which integrated in a single integration site, specific to each individual clade. Interestingly the remaining clade demonstrated a reduced level of integration specificity, such that recombinases with identical protein sequences integrated at two independant loci. The recombinase of phage DW2 clusters with proteins in clade 1 of which all members specifically integrate downstream of the signal recognition particle protein. All of the integrases found in this clade were encoded by strains of *S. aureus*.

The ORF15 and ORF24 of phage DW2 have high nucleotide sequence homology with two major virulence-related genes namely NWMN0273 and NWMN0280 encoded in the genome of staphylococcal phage ϕNM4.[43] The gene product of ORF15 (gp15) from phage DW2 shares 60.3% amino acid sequence identity with the gene product of NWMN0273, with a much higher level of homology toward the C-terminus. This may be due to the presence of a conserved functional domain. However, when analyzed against the pfam database no homologous conserved

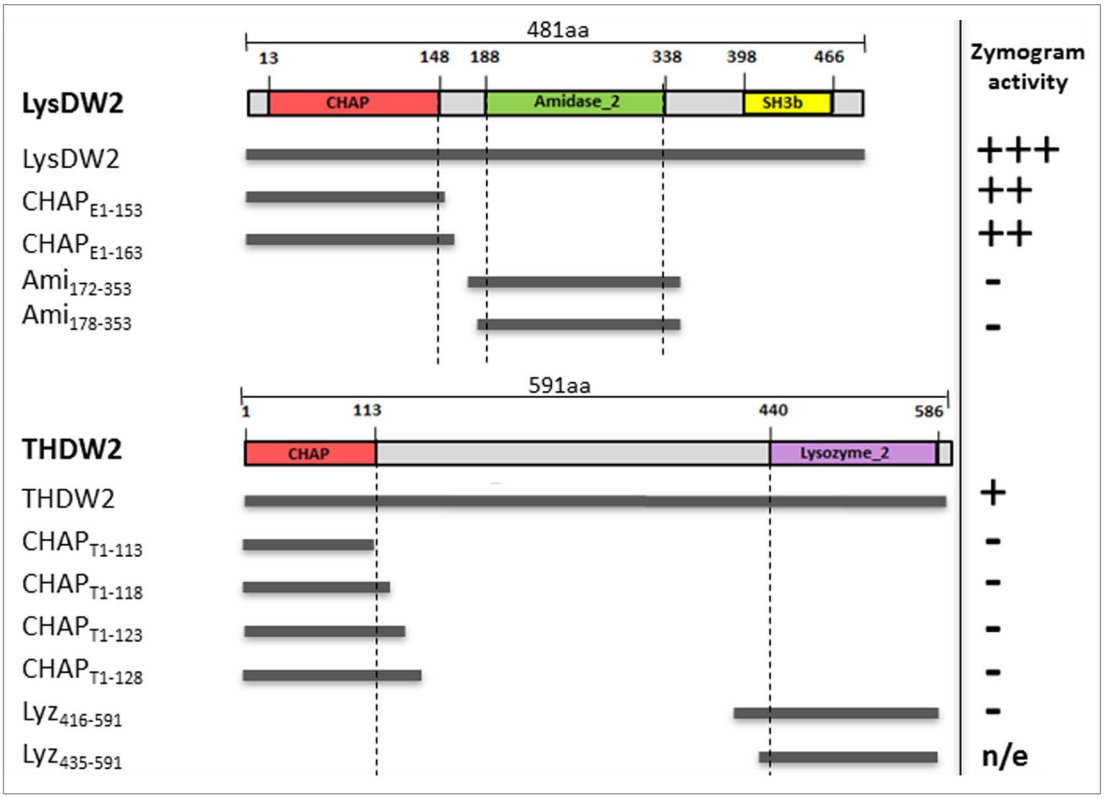

Figure 3. The modular structure of the recombinant endolysin LysDW2 and tail hydrolase THDW2 of phage DW2 as predicted by SMART online protein domain analysis software. The numbers above the schematic representation of each enzyme indicate the amino acid residues at the beginning and end of each functional domain. These numbers are based on the native sequence and do not include the additional Methionine codon added to the start of recombinant THDW2. The cloned constructs are represented by the grey lines below their respective protein with absence or presence of zymogram activity indicated to the right of each construct. +++: very strong clearing, ++: strong clearing, +: weak clearing, –: no clearing, n/e: not overexpressed.

domain was identified. Gp24 shares 91.4% identity with the gene product of NWMN0280. These results indicate that gp15 and gp24 are likely to be functional homologs of these virulence factors. Also in relation to virulence associated genes, comparison against the conserved domain database showed that gp22 from phage DW2 contains a conserved sequence of approximately 130 residues long which has also been found as part of the PVL (Panton-Valentine leukocidin) group of genes.

gp18 of phage DW2 shares 94.2% amnio acid sequence identity with gp22 of phage 80α, which has been experimentally demonstrated to be involved in derepression of SaPI1 pathogenicity island.[16] In addition, gp30 of phage DW2 shares 82.4% identity with gp32 of phage 80α, which is involved in derepression of the SaPIbov1 pathogenicity island.[16] gp15 from phage 80α has also been implicated in the derepression of a pathogenicity island.[16] However, when its nucleotide sequence was compared (using blastn) against the complete genome of phage DW2, no ORF homologous to gp15 of phage 80α was identified.

Structural analysis of phage DW2 endolysin

ORF64 encodes the endolysin of DW2 which will be referred to in this paper as LysDW2. This enzyme was calculated to have a molecular weight of 53.9 kDa and a pI of 8.48. Functional domain analysis of LysDW2 using SMART online software revealed a C-terminal SH3b cell wall binding domain (398–466aa) and two peptidoglycan hydrolyzing (PGH) domains. A schematic representation of the modular structure of LysDW2 can be seen in **Figure 3**. The PGH domains identified were a centrally located N-acetylmuramoyl-L-alanine amidase (ami_2) domain (188–338aa), which cleaves the peptidoglycan bond between the N-acetylmuramoyl residues and L-amino acid residues and an N-terminal cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) domain (13–148aa). CHAP domains are specifically found in phage targeting Gram positive bacteria and have been shown to have functional diversity with CHAP domains from different phages specifically cleaving different bonds in the peptidoglycan structure. The CHAP domain produced by streptococcal phage B30 acts as a D-alanyl-L-alanyl endopeptidase,[44] while streptococcal phage C1 CHAP was shown to have N-acetylmuramoyl-L-analine amidase activity.[45] The CHAP domain of LytA endolysin from staphylococcal phage ϕ11 has been demonstrated to have D-alanyl-glycine endopeptidase activity.[46] LytA CHAP domain shares 136/148 residues with that of LysDW2 thus strongly indicating that LysDW2 is a functional homolog and also has D-alanyl-glycyl endopeptidase activity. This pattern of functional domains is common among the endolysins of staphylococcal phages, even those of varying morphology and genome size.

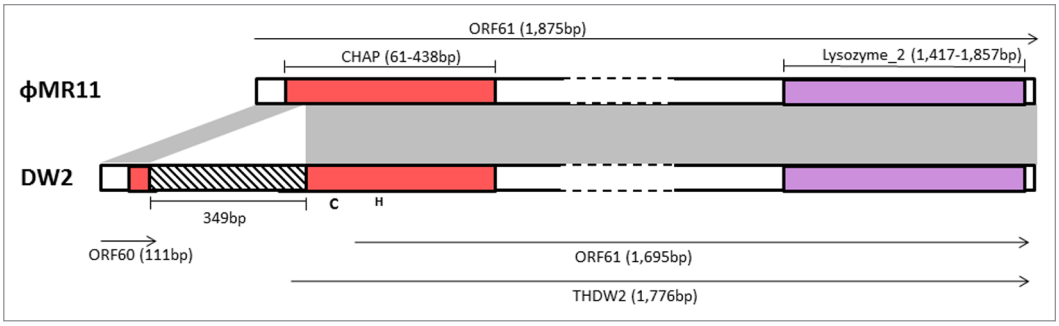

Figure 4. Schematic representation of a ClustalW alignment between the tail hydrolase gene from phage ϕMR11 and the 34,850–37,073 bp region of the phage DW2 genome. The alignment shows an additional 349 bp (box with diagonal lines) in the phage DW2 sequence which contains an in-frame stop codon resulting in two bioinformatically predicted partial tail hydrolase genes (ORF60 and ORF61). This insertion sequence is a putative group I intron in the tail hydrolase gene of phage DW2. The location of the critical cysteine (C) and histidine (H) residues of the of the CHAP domain are indicated below the phage DW2 sequence.

Structural analysis of DW2 tail hydrolase

The phage DW2 tail hydrolase gene (ORF61) predicted by Glimmer is 564 residues long with a calculated molecular weight of 63.8 kDa and pI of 9.5. Analysis of the protein sequence with SMART online software identified a C-terminal lysozyme 2 (Lyz_2) subfamily domain (413–559aa) and a truncated N-terminal CHAP (2–86aa). The Lyz_2 domain is found in eubacterial enzymes distantly related to eukaryotic lysozymes and has mannosyl-glycoprotein endo-β-N-acetylglucosaminidase activity which cleaves the bond between the glycan backbone of the peptidoglycan structure and the L-alanine of the tetrapeptide moiety. The N-terminus had high homology with CHAP domains from other staphylococcal phage VAPGHs but it was 60 amino acids shorter than most of these VAPGHs and it did not contain the conserved cysteine residue required for the catalytic mechanism of the CHAP domain. Prodigal ORF prediction program predicted ORF60 (111 bp) upstream from ORF61 which had homology to the sequence encoding the N-terminus of other VAPGH homologs which was missing in ORF61. When the sequence including and between ORF60 and ORF61, was aligned with the nucleotide sequence encoding the tail hydrolase gp61 (ORF61) from phage ϕMR11, it was seen that all of the ϕMR11 VAPGH aligned with the DW2 sequence (**Fig. S2**). However, the homologous sequence encoded by DW2 was separated by an additional 349 bp, which did not have a corresponding region in ϕMR11 and contained an in-frame stop codon 4 bp downstream from the start of this non homologous region (**Fig. 4**; **Fig. S2**). When compared against the NCBI nucleotide database using the blastn algorithm the 349 bp insert had 81% identity over 30% of its sequence with the nrdE-12 intron (1087 bp) found in the large subunit ribonucleotide reductase gene of staphylococcal Myoviridae phage Twort.[47] It also shared 83% identity over 24% of its sequence with an intron found in a putative RecA gene of the staphylococcal Myoviridae phage ϕ812b.[48] When the nucleotide sequence upstream from ORF61 was translated as far as the previous stop codon, alignment with the conserved domain database (CDD) indicated that this peptide had a complete CHAP domain and contained the conserved cysteine residue necessary for its catalytic mechanism. The protein encoded by this nucleotide sequence is referred to from here on as THDW2. The other *Siphoviridae* staphylococcal VAPGHs that have been cloned have the same modular structure as that of THW2 (CHAP and Lyz_2). However, the *Myoviridae* phage K and *Podoviridae* phage P68 VAPGHs did not have a similar modular structure. The tail hydrolase (gp56) of phage K gp56, had a single C-terminal CHAP domain predicted by SMART analysis. Interestingly no functional domains could be identified for the VAPGH of the *Podoviridae* P68, by comparison against sequences in the PFAM and CDD databases.

Comparative analysis of LysDW2

The endolysin of bacteriophage DW2, LysDW2, was aligned using ClustalW to the native amino acid sequence of all known staphylococcal phage endolysins that have been cloned and characterized and a phylogenetic tree was generated (**Fig. 5**). From this group of endolysins, the closest homolog to LysDW2 was LysH5 from the phage ϕH5 with 94.8% amino acid sequence identity. Endolysins LytA and MV-L from phage ϕ11 and ϕMR11 respectively also had > 90% sequence identity with LysDW2. The remaining eight endolysin sequences showed clear divergence from the afore-mentioned set of four endolysin sequences but could be defined as functional homologs due to sharing 27–48% sequence identity with LysDW2. Interestingly the LysWMY from the *S. warneri* phage ϕWMY showed 39.9% amino acid sequence identity with DW2 which was greater than some of the other *S. aureus* phage endolysins. This could potentially be an indication of gene transfer between phages targeting different staphylococcal species.

Comparative analysis of THDW2

THDW2 was compared by ClustalW, to the amino acid sequence of all VAPGHs which have been cloned and experimentally demonstrated to have peptidoglycan hydrolyzing activity. This alignment was used to create a phylogenetic tree (**Fig. 5**). The closest homolog to THDW2 was gp61 from *S. aureus* phage ϕMR11 with 94.8% amino acid sequence identity. It was also a functional homolog to the VAPGHs from the other staphylococcal *Siphoviridae* phage vB_SauS-phiIPLA88 and phage 187, with 62.1% and 41.7% sequence identity respectively. In contrast to what was observed for the cloned staphylococcal endolysin sequences, pairwise ClustalW alignment with all the cloned staphylococcal VAPGHs divided the sequences in a way that correlated with phage classification based on genome size and morphology. In contrast to the relatively high homology seen among the *Siphoviridae* VAPGHs, THDW2 only exhibited 6.5% and

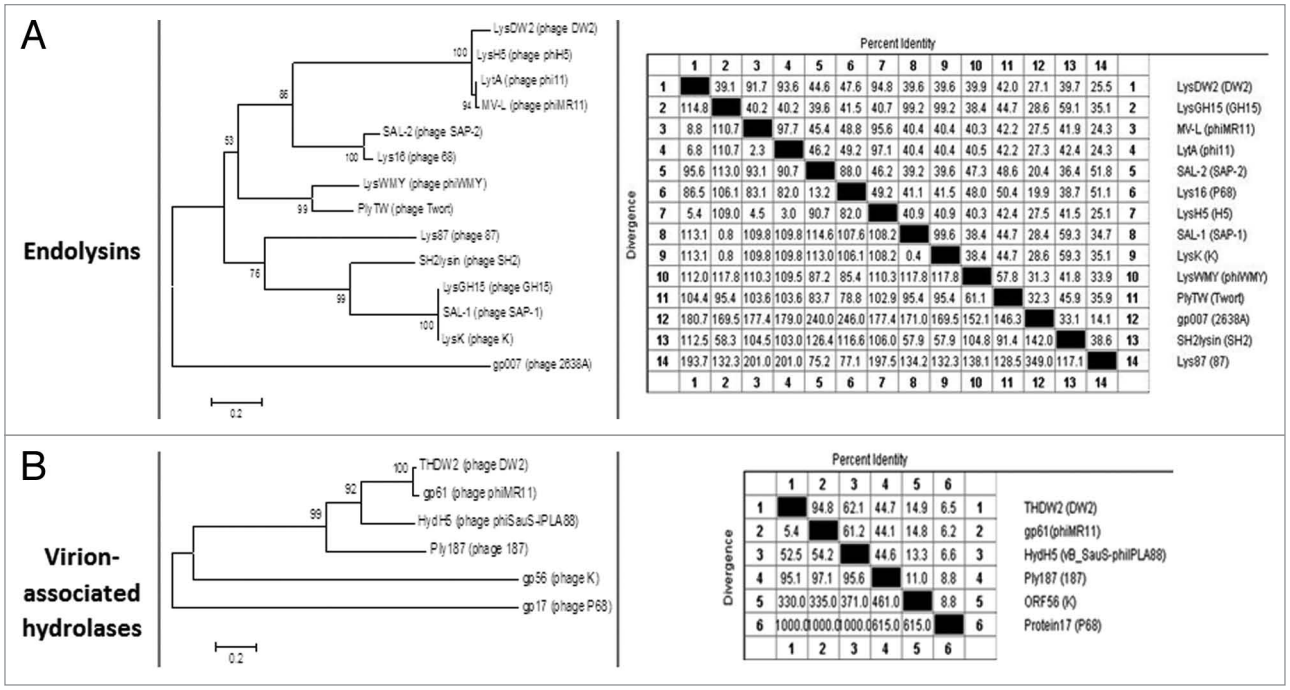

Figure 5. Phylogenetic tree and percent identity and divergence matrix of the native amino acid sequence of staphylococcal phage endolysins (**A**) and staphylococcal phage virion hydrolases (**B**), which have been cloned and characterized.

14.9% identity with protein 17 of the staphylococcal *Podoviridae* phage P68 and gp56 of the *Myoviridae* phage K respectively. In addition, neither of these two phages had greater than 15% similarity with any of the other cloned staphylococcal *Siphoviridae* VAPGHs.

Cloning of LysDW2 and THDW2 and truncated derivatives

To identify and analyze the function of LysDW2 and THDW2, their complete nucleotide sequence and various truncated derivatives were amplified by PCR and cloned into the *E. coli* expression vector pQE60 (**Fig. 3**). All inserts contained a stop codon directly after the coding sequence in order to prevent the expression of the His-tag. Nucleotide sequencing across the multiple cloning site confirmed that all inserts were in the correct orientation and showed that a single base mismatch had been incorporated in the LysDW2 clone during the amplification, causing a silent mutation, with a change from T to C at position 657. The recombinant plasmids were successfully transformed into XL1-Blue *E. coli*.

Overexpression of LysDW2, THDW2 and their truncated derivatives

SDS-PAGE analysis of cultures induced for 5 h showed that all proteins were successfully overexpressed with the exception of $Lyz_{435-591}$. The proteins containing just the indiviudal CHAP domain from the endolysin, namely $CHAP_{E1-153}$ and $CHAP_{E1-163}$ were present in the soluble fraction of the expression culture lysate while all other overexpressed proteins were found to be insoluble. Induction of protein expression at 26 °C for 14 h did not improve solubility of any of the proteins nor did it improve expression levels.

Zymogram activity test

Zymogram gels containing autoclaved *S. aureus* cells were used to investigate the peptidoglycan hydrolyzing activity of recombinant LysDW2, THDW2, and truncated derivatives. Crude lysate of induced cultures of LysDW2, $CHAP_{E1-153}$, and

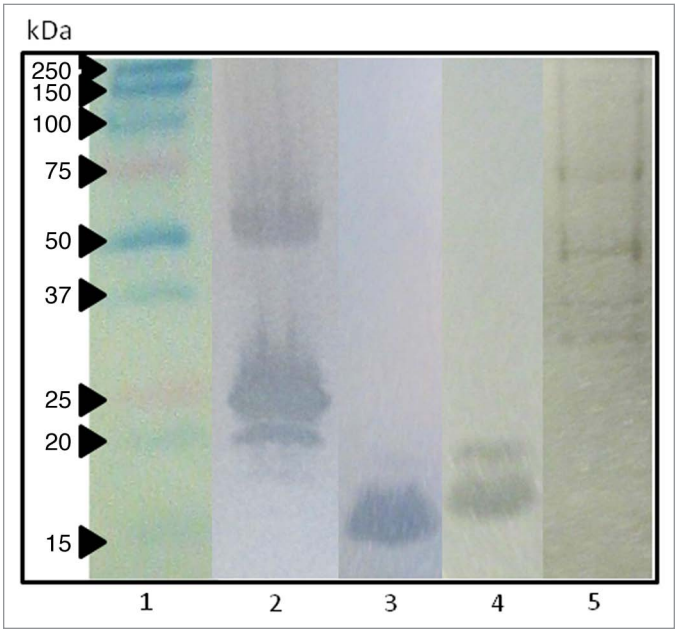

Figure 6. Composite zymogram showing clearing resulting from the lytic activity of the crude lysate of an induced *E. coli* culture expressing recombinant LysDW2 (lane 2), $CHAP_{E1-153}$ (lane 3), $CHAP_{E1-163}$ (lane 4), and THDW2 (lane 5) on a zymogram gel seeded with *S. aureus* DPC5246. Lane 1 contains Precision Plus Protein Dual Extra standard (Biorad).

$CHAP_{E1-163}$ demonstrated strong lytic activity after 1h incubation in renaturation buffer at 37 °C (**Fig. 6**, lanes 2–4). LysDW2 showed a band of clearing at ~54 kDa which corresponded with the size of the complete LysDW2 protein. A further two bands of clearing could be seen which corresponded to molecular weights of approximately 24 kDa and 21 kDa. $CHAP_{E1-153}$ and $CHAP_{E1-163}$ showed single bands of clearing at ~17 kDa and ~18 kDa respectively which correlates closely with their predicted molecular weights of 17.4 kDa and 18.4 kDa. The other endolysin derivatives $Ami_{172-353}$ and $Ami_{178-353}$ did not show any clearing after 1 h incubation nor did any of the THDW2 derivatives. Nevertheless after 72 h in renaturation buffer the crude lysate of an induced culture of the THDW2 clone produced four distinct bands of clearing (**Fig. 6**, lane 5). The largest of these bands corresponded to a band of ~67 kDa which matches the molecular weight of the complete THDW2 protein. The other bands were seen at molecular weights of approximately 45 kDa, 37 kDa, and 31 kDa. The endolysin derivatives containing the amidase_2 domain, the tail hydrolase derivaties containing the CHAP domain and the Lyz_2 domain (which successfully overexpressed) did not show any clearing even after incubation for 72 h. All of the recombinant proteins which did not demonstrate activity when analyzed by zymogram were found in the insoluble fraction. Thus the lack of peptidoglycan hydrolyzing activity maybe due to insolubility or misfolding of the proteins.

Discussion

Staphylococcus aureus is commonly found as an asymptomatic commensal organism on the skin and mucosal membranes of humans and animals.[49] However this species can also be responsible for a diverse range of diseases including, but not limited to; skin infections, such as mastitis and dermatitis, food poisoning, septicemia, osteomyelitis, necrotizing pneumonia, and endocarditis.[50] The diverse virulence profile of this species is made possible by the high level of genomic plasticity. Rearrangements in the genome are due to mobile genetic elements including; plasmids, pathogenicity islands, insertion sequences, transposons and temperate phages.[51] As well as contributing to the virulence of the species, such lateral gene transfer allows the movement of genetic material encoding genes for antibiotic-resistance. Analysis of the staphylococcal phage DW2 genome revealed several key features which point to its potential role in the molecular evolution of the staphylococcal genus. Annotation of the genome identified putative genes encoding a serine recombinase (ORF1), a repressor (ORF6) and an anti-repressor (ORF7) indicating that the phage is temperate and thus has significant potential for genetic exchange with its host. The presence of a putative serine recombinase in the phage DW2 genome is interesting because the majority of staphylococcal phages reported to date have a tyrosine recombinase.[51] Serine recombinases are of interest as molecular tools in fields such as gene therapy and cell line manipulation due to their ability to conduct unidirectional, site-specific recombination between sequences which are relatively short and unique from one another.[52] The phage encoded serine recombinases from streptomyces phages ϕC31[53] and R4[54] and lactococcal phage TP901[55] have been well characterized and demonstrated to work efficiently in mammalian cells without the requirement for co-factors. Phylogenetic analysis of the phage DW2 serine recombinase indicated that it is likely to have a high level of integration specificity, as all recombinases in this clade integrated at the same locus downstream from the signal recognition particle protein. The specificity of these recombinases means that there is a requirement for a comprehensive bank targeting various sequences in order to improve the utility of such a tool. Therefore it would be worth investigating further the specific mechanism of the phage DW2 serine recombinase for use in similar applications.

The mosaic nature of bacteriophage genomes makes them difficult to classify. In relation to staphylococcal *Siphoviridae*, two studies have used phage integrase polymorphism alone or in conjunction with other factors as a means of phage classification.[51,56] The study by Deghorain et al.[56] classified staphylococcal phage serine recombinases into three major clades, each with one specific integration locus. Here, we identified the PTS lactose transporter subunit and the dicarboxylate ABC transporter as two additional integration loci for staphylococcal serine recombinases. Previous reports indicated that staphylococcal serine recombinases were completely specific in their integration loci. Interestingly however our study demonstrated a redundancy in specificity in one of the major clades of staphylococcal serine recombinases, where enzymes with identical amino acid sequences integrated at two independent loci; downstream from the 50S ribosomal protein L32 and in the coding sequence of the dicarboxlyate ABC transporter. It is worth noting that all serine recombinases which clustered in the same clade as the serine recombinase from phage DW2 were found exclusively in *S. aureus* as opposed to two of the major clades which contained serine recombinases from various species of staphylococci. This suggests that phage DW2 cannot integrate into any staphylococcal species other than *S. aureus*. Thus although phage DW2 has the potential to act as a vector of genes between *S. aureus* strains it seems likely to be limited to this staphylococcal species and thus would not promote interspecies virulence gene transfer in this manner.

From the staphylococcal phage genomes which have been reported, that of staphylococcal phage ϕNM4, a prophage of *S. aureus* Newman, has the closest homology to phage DW2. *S. aureus* Newman is a prototype for the study of staphylococcal pathogenesis because of its ability to stably maintain an agr^+ phenotye and to cause animal disease.[57] This strain contains four prophages, and using a murine model of abscess formation it was demonstrated that when the prophages were deleted from the host genome, it was no longer able to cause disease.[58] *Bursa aurealis* transposon mutagenesis was used to screen the *S. aureus* Newman genome for virulence genes and identified 6 groups of virulence determinants present in the prophage genomes.[59] The prophage ϕNM4 contained four major virulence genes of unknown function. The fact that ORF15 and ORF24 of phage DW2 are close homologs to two of these genes (NWMN0273 and NWMN0280) indicates that phage DW2 may also act to augment the virulence of *S. aureus* strains infected by phage DW2. In addition gp22 of phage DW2 contains a conserved

region, which in some cases is found as part of the PVL group of genes. These genes are encoded by staphylococcal prophage and produce a toxic protein which forms pores in the membrane of leukocytes killing them and causing tissue necrosis.[60] PVL has been implicated as having a major role in pathogenicity and staphylococcal strains encoding PVL have been shown to cause severe skin and soft tissue infections,[61] necrotizing pneumonia, and sepsis.[62,63]

In addition to prophages, SaPIs represent another group of mobile genetic elements, which are commonly associated with horizontal transfer of genes encoding virulence factors and superantigens, between *S. aureus* strains.[64] This family of mobile genetic elements is dependent on "helper phage" for their transduction. SOS induction of certain prophage can result in expression of proteins, which cause derepression of SaPI proteins thus initiating their transduction. In addition, SaPIs are dependent on encapsidation by phage-encoded proteins for their mobilization. The gene products of the *sri* (ORF22) and *dut* (ORF32) genes from phage 80α have been reported to be involved in derepression of the SaPI1 and SaPIbov1 respectively, by binding to the SaPI-encoded master repressor Stl protein.[16] This derepression is the initial step of the SaPI mobilization process. Phage DW2 contains two putative proteins, gp18 and gp30, which have significant sequence identity with the gene products of the *sri* and *dut* genes respectively. Relatively few studies have reported on the involvement of helper phage genes in SaPI mobilization. It has however been shown that phage encoded derepressors are specific in the SaPIs that they mobilize.[16] Gp18 and gp30 of phage DW2 present novel candidates for further investigation of the mechanisms involved in SaPI mobilization among *S. aureus* strains.

Due to the increasing prevalence of antibiotic resistant pathogens, including staphylococcal strains such as MRSA and VRSA, there is an urgent need for development of novel antibacterial agents. In relation to *S. aureus*, a number of phage endolysins have been cloned and characterized to gain greater understanding of the mechanism of their targeted peptidoglycan hydrolysis and to validate their utility as novel antibacterial agents.

In this study we exploited the genome of phage DW2 to create PGH enzymes lytic against *S. aureus*, based not only on the endolysin but also the tail hydrolase of phage DW2. Four of these recombinant proteins (LysDW2, THDW2, $CHAP_{E1\text{-}153}$, and $CHAP_{E1\text{-}163}$) were shown to be lytic against a bovine mastitis associated isolate *S. aureus* DPC5246 which is resistant to several antibiotics.[65] The presence of several distinct bands resulting from zymograms analysis of both LysDW2 and THDW2 expression cultures suggests that both proteins are undergoing autoproteolysis at specific sites. This phenomenon has been reported previously for the tail associated lysin of the lactococcal phage Tuc2009.[66]

In recent years, protein engineering has taken advantage of the modular structure of phage lysins to create chimeric proteins by fusing domains from different endolysins or domains from endolysins with domains from other proteins. Such domain swapping and fusion of complete lysins with other peptide moities has been successfully used to improve activity,[35,67] alter substrate specificity,[68] and improve solubility[69] when compared with the complete native lysin from which they were derived. Due to their high level of solubility the recombinant endolysin CHAP domain proteins created in this study are attractive candidates to be used alone or fused with other functional modules to create chimeras for development of novel antimicrobial therapeutics.

VAPGH are relatively uncharacterized when compared with endolysins. However, due to the demonstrated successes of recombinant phage endolysins in controlling Gram positive pathogens, VAPGHs have recently become a focus of research interest in terms of enzybiotics. Analysis of the bioinformatically predicted DW2 tail hydrolase genes (ORF60 and ORF61) gives an insight into the gene structure and mechanism of action of this VAPGH. The presence of a highly homologous region (9577 bp) which terminates at the end of ORF60 indicates that the tail tip hydrolase gene may be an area of site specific recombination and this may have resulted in a shortened tail hydrolase void of a functional CHAP domain. This hypothesis complements a recent study by Rodríguez-Rubio et al.[70] which demonstrated that the VAPGH was not necessary for phage replication in *S. aureus* phage ϕ11. However, when the VAPGH was not present it did significantly reduce the structural viability of the phage particle. The fact that phage DW2 was routinely propagated to high titers indicates that the tail hydrolase of phage DW2 is structurally functional. A more likely explanation for the inserted sequence seen in the phage DW2 tail hydrolase is the presence of a group I intron. There have been numerous reports of phage introns and they include those present in staphylococcal phage tail genes.[71] However, to date there have been no reports in the published literature of an intron in a tail hydrolase gene of a staphylococcal phage. The 349 bp size of the insert is compatible with the hypothesis that the insert in an intron, as group I introns found in phages are typically 250–500 bp in length.[71] In addition, the 349b insertion has homology, albeit at a low level, with the nrdE-I2 intron identified in the ribonucleotide reductase gene of staphylococcal phage Twort[47] and an intron found in a putative RecA gene identified in spontaneous host range mutants of phage 812.[48] However, unlike these introns the DW2 insertion does not carry an endonuclease gene. Although the THDW2 recombinant protein contains the conserved cysteine and histidine residues believed to be involved in the catalytic mechanism,[34] it demonstrated a low level of activity when analyzed by zymogram. This may be due to the insolubility of the protein but it may also be consequence of the missing 35 residues at the N-terminus relative to its homolog, the tail hydrolase from phage ϕMR11. This low level of activity demonstrated by the recombinant tail hydrolase of phage DW2 further suggests that the functional tail hydrolase gene expressed by the phage may be a longer gene derived by excision of a group I intron. Further characterization of the 349 bp insert in the tail hydrolase gene of phage DW2 is currently being pursued.

In conclusion sequencing of the phage DW2 genome shows that while the whole phage is not applicable for anti-staphylococcal therapy due to its temperate nature its peptidoglycan hydrolases present alternative candidates for enzybiotic applications.

Materials and Methods

Bacterial strains, bacteriophage and media

The host strain used in this study was *S. aureus* bovine mastitis isolate DPC5246 from the Dairy Products Research Centre culture collection.[72] The phage under investigation was *Siphoviridae S. aureus* phage DW2 previously isolated from farmyard effluent.[40] Brain heart infusion (BHI) agar (1.5%) and 3 ml overlays of BHI agar (0.75%) were used for plaque assays. BHI broth was used for routine growth of bacteria and phage propagation. *E. coli* transformants were grown in super broth containing 32 g/L tryptone, 20 g/L yeast extract, 5 g/L NaCl, and selected with 200 mg/L ampicillin.

Phage amplification

Phage DW2 was propagated on *S. aureus* DPC5246 as follows: 100 μl of an overnight culture of DPC5246, 20 μl of 1 M $CaCl_2$ and 1 ml of phage stock at a concentration between 1×10^7 pfu/ml and 1×10^{10} pfu/ml were added to 10 ml BHI broth. The sample was then incubated at 37 °C in a shaking incubator at 90 rpm for 18–24 h. The propagation sample was spun down at 6000 g for 15 min, subsequently filter sterilized using a 0.22 μm filter, and finally stored at 4 °C.

Phage DNA preparation

In order to remove contaminating host RNA and DNA from a filter sterilized lysate of phage DW2, prior to phage DNA extraction, DNase I was added to a final concentration of 3.3 U/μl (Fisher Scientific, 11873795) and RNase to a final concentration of 6.7 U/ml (Roche, 10109142001). The sample was then incubated at 37 °C for 10 min. To lyse the protein capsid 150 μl of lysis buffer (0.4 M EDTA, 1% SDS and 0.05 M TRIS-HCl pH 8) and 10 μl of 10 mg/ml proteinase K (Roche, 3115879001) was added to 750 μl aliquots of the phage lysate sample and they were incubated at 65 °C for 30 min. Proteins were removed by two chloroform: isoamylalcohol: phenol (24:1:25) and a chloroform: isoamylalcohol steps. DNA was precipitated from the sample using isopropanol and sodium acetate at final concentrations of 40% and 0.3 M respectively. The DNA was washed twice with 70% ethanol. For complete genome sequencing the DNA was resuspended in sterile water and for cloning the DNA was resuspended in TE buffer pH 7.4. The concentration of DNA was estimated using a Nanodrop spectrophotometer (Nanodrop ND 1000).

Genome sequencing and analysis

Twenty micrograms of phage DW2 DNA was sent to Beckman Coulter Genomics for 454 pyrosequencing on a GS-FLX Next generation sequencing platform. Mira assembler was used for de novo assembly. The complete genome was compared against the NCBI nucleotide database using the megablast algorithm. The online visualization tool Circoletto[73] (http://tools.bat.infspire.org/circoletto/) was used to create an ideogram providing an overview of the sequence similarity between the complete genome of DW2 and that of *Siphoviridae* staphylococcal phages ϕNM4, ϕETA3, and 80α (**Fig. 2**). Predicted ORFs were assigned to the phage DW2 genome using Glimmer online server[74] (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi) and further verified by Prodigal online server v1.20[75] (http://prodigal.ornl.gov/server.html) and manual curation. Functional annotation was performed using the blastp algorithm against the NCBI database of non-redundant protein sequences and the conserved domain database and further refined by manual curation. The genome was analyzed for the presence of genetic sequence encoding tRNA using tRNA SCAN.

Nucleotide sequence accession number

The genome sequence of phage DW2 has been deposited in the GenBank database under accession number KJ140076.

Identification of DW2 integration loci

The recombinase of phage DW2 was aligned using the tblastn algorithm against the complete and draft staphylococcal genomes on the NCBI database and sequences with a minimum of 80% query coverage were selected. Recombinases for which the integration loci could be identified were aligned using the MUSCLE algorithm. A phylogenetic tree was created using the maximum-likelihood method preformed in the MEGA v5.2[76] with the WAG matrix and a bootstrap consensus setting of 1,000 replicates. A discrete Gamma distribution was used to model evolutionary rate differences among sites and a gamma correction for variable evolutionary rates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated the tyrosine recombinase from staphylococcal phage ϕNM4 was used to root the tree.

In silico analysis of DW2 endolysin and tail hydrolase

The predicted endolysin (ORF64) and tail hydrolase (ORF61) gene products, gp64 and gp61 respectively, from phage DW2 were analyzed for functional domains using SMART online software, employing a PFAM domain search and default HMMER analysis. Gp64 (LysDW2) and all staphylococcal phage endolysins which have been cloned and demonstrated to be functionally active were aligned by ClustalW.[77] Similarly, gp61 with an additional 27 amino acids encoded by the sequence just upstream from ORF61, referred to here as THDW2, was aligned by ClustalW with all the staphylococcal phage VAPGHs which have been cloned and demonstrated to have peptidoglycan hydrolyzing activity. Through the MEGAlign program of the DNAstar suite both alignments were used to infer amino acid percentage identities. A phylogenetic tree was generated for both LysDW2 and THDW2 using the method previously outlined for the DW2 serine recombinase.

Cloning of putative endolysin and tail hydrolase genes and truncated derivatives

LysDW2 and various derivatives containing the individual CHAP and amidase_2 peptidoglycan hydrolase domains were amplified by PCR using Expand HiFi Plus System (Roche, 03300242001) (**Fig. 3**). THDW2 and derivatives containing the individual CHAP and Lyz_2 domain were also amplified. The specific primers used for amplification are listed in **Table S2**. All the amplicons were cloned into the pQE-60 plasmid (Qiagen, 32169) and transformed into chemically competent XL1-Blue *E. coli* (Stratagene, 200249). Transformants were selected for on LB agar containing 200 mg/L ampicillin. Successful transformants were screened for inserts of the desired length by PCR and conventional gel analysis. Inserts of the correct size

were subsequently sequenced to confirm their sequence and orientation.

Overexpression of LysDW2, THDW2, and truncated derivatives

LysDW2, THDW2, and truncated derivatives were overexpressed in the pQE-60/XL1 Blue expression system as per the Qiagen QIAexpressionist manual with minor changes. Twenty milliliters of superbroth containing 200 μg/ml was inoculated with 2% inoculum of overnight cultures of the DW2 peptidoglycan hydrolase clones and the empty pQE-60/XL1Blue which was used as a negative control. The cultures were incubated at 170 rpm at 37 °C until they reached an OD_{590nm} of 0.4–0.6. After cooling the samples on ice for 1 h, 1 mM of IPTG (MP Biomedicals, 114064112) was added. The cultures were then further incubated 37 °C for 5 h before being spun at 6000 g for 20 min and the supernatant was discarded. In a separate experiment clones were induced at 26 °C for 14 h. The cell pellets were washed with 25 mM Tris pH 8 and resuspended in bugbuster protein extraction reagent (Novagen, 70584-3) and shaken at 120 rpm for 20 min. The cells were then spun at 6000 g for 20 min and the soluble fraction was separated from the insoluble fraction which was subsequently resuspended in bugbuster.

SDS-PAGE and Zymogram analysis

The soluble and insoluble fractions of the crude lysate of the induced clones were visualized by SDS-PAGE on a 12% gel as described by Henry et al. with minor changes.[78] Briefly 30 μl of the enzyme solution was added to 20 μl of sample buffer. Directly prior to loading onto the gel the sample was heated to 95 °C for 5 min. Fifteen microliters of each sample was loaded onto the gel and 8 μl of Precision Plus Protein Dual Extra Standard (BioRad, 161-0374). The gels were run for 45 min at 180 V and were stained for 1 h in staining buffer (0.05% brilliant Blue R, 25% isopropanol, 10% acetic acid) and destained for 1 h (40% ethanol, 7% acetic acid) before visualization.

Proteins that were identified by SDS-PAGE analysis to be expressed in the soluble or insoluble fractions of the induced lysate were used for zymogram analysis. Zymograms were performed using a similar method to the SDS-PAGE method outlined above but with the following changes. Heat treated *S. aureus* DPC5246 was resuspended in the 12% polyacrylimide. β-mercaptoethanol was not added to the sample buffer and the samples were not boiled before loading them onto the gel. After running the zymogram gel for 50 min at 180 V the gel was rinsed in distilled water for 30 min at room temperature before being transferred to renaturation buffer (25 mM TRIS-HCl, pH 8.0 with 1% triton X) and incubated shaking 30 rpm at 37 °C for and checked for clearing after 30 min, 1 h, 24 h, 48 h, and 72 h.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/bacteriophage/article/28451

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