

Prophages of *Staphylococcus aureus* Newman and their contribution to virulence

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Summary

Four prophages (ϕ NM1–4) were identified in the genome of *Staphylococcus aureus* Newman, a human clinical isolate. ϕ NM1, ϕ NM2 and ϕ NM4, members of the siphoviridae family, insert at different sites (*poiA*, downstream of *isdB* and *geh*) in the staphylococcal chromosome. ϕ NM3, a β -haemolysin (*hlyB*) converting phage, encodes modulators of innate immune responses (*sea*, *sak*, *chp* and *scn*) in addition to other virulence genes. Replication of ϕ NM1, ϕ NM2 and ϕ NM4 occurs in culture and during animal infection, whereas ϕ NM3 prophage replication was not observed. Prophages were excised from the chromosome and *S. aureus* variants lacking ϕ NM3 or ϕ NM1, ϕ NM2 and ϕ NM4 displayed organ specific virulence defects in a murine model of abscess formation. *S. aureus* Newman lacking all four prophages was unable to cause disease, thereby revealing essential contributions of prophages to the pathogenesis of staphylococcal infections.

Introduction

Staphylococcus aureus is the leading cause of hospital acquired infections (Lowy, 1998). The spectrum of human diseases caused by staphylococci ranges from soft tissue infections, abscesses in organ tissues, osteomyelitis, endocarditis, toxic shock syndrome to necrotizing pneumonia (Archer, 1998; Lowy, 1998). *S. aureus* is a versatile pathogen and specific strains have been isolated from many different pathological-anatomical sites or disease entities (Kuehnert *et al.*, 2006). Such versatility is enabled by a long list of virulence factors, including cell wall anchored proteins, secreted toxins, capsular- and exo-

polysaccharides, iron-transport systems and modulators of host immune functions in addition to antibiotic-resistance genes (Archer, 1998; Dinges *et al.*, 2000; Novick, 2003a; Marraffini *et al.*, 2006). The vast majority of staphylococcal virulence functions (including surface proteins, sortase, α -haemolysin, exotoxins and *agr* regulon) are encoded on the bacterial chromosome (Kuroda *et al.*, 2001). These genes are not associated with mobile DNA elements and are found in all staphylococcal strains (Diep *et al.*, 2006). Because the pathogenesis of *S. aureus* infections is generally viewed as relying on a large array of factors, it is currently not possible to assign disease potential to staphylococcal strains strictly on the basis of their genome sequence.

Recent work identified *S. aureus* bacteriophages as important contributors to pathogenesis and the evolution of staphylococcal genomes (Novick, 2003b; van Wamel *et al.*, 2006). These conjectures are illustrated by the following observations: (i) Genome sequences of clinical *S. aureus* isolates harbour at least one or up to three prophages (Brussow *et al.*, 2004; Gill *et al.*, 2005; Diep *et al.*, 2006). (ii) The genomes of *S. aureus* strains vary for up to 22% of their DNA sequence (Fitzgerald *et al.*, 2001). Prophages are involved in genome variation, however, other mobile DNA elements, including pathogenicity islands (SaPIs), genomic islands, chromosome cassettes (SCC), transposons and conjugative plasmids, are also important contributors (Novick, 2003b). (iii) During the course of chronic lung infections in cystic fibrosis patients, phage excision or transduction are causes of genome variation (Goerke *et al.*, 2006a; Lindsay and Holden, 2006). (iv) Phages transfer SaPIs between strains, which is best exemplified by ϕ 80 α -mediated excision and transduction of SaPI1 (Ruzin *et al.*, 2001). (v) Prophages, e.g. ϕ 11 or ϕ 80 α , are thought to confer competence for genetic transformation (DNA uptake) (Rudin *et al.*, 1974; Thompson and Pattee, 1981). (vi) Prophage-encoded virulence factors may enable the pathogenesis of unique staphylococcal disease entities, involving secreted superantigens (SEA, SEG and SEK), Panton-Valentine leukocidins (PVL), exfoliative toxin type A (ETA) or the fibrinolytic enzyme staphylokinase (SAK) (Novick *et al.*, 2001). Recently, innate immune modulators such as chemotaxis inhibitory protein (CHIPS) and staphylococcal complement inhibitor (SCIN) were shown to be encoded by β -haemolysin

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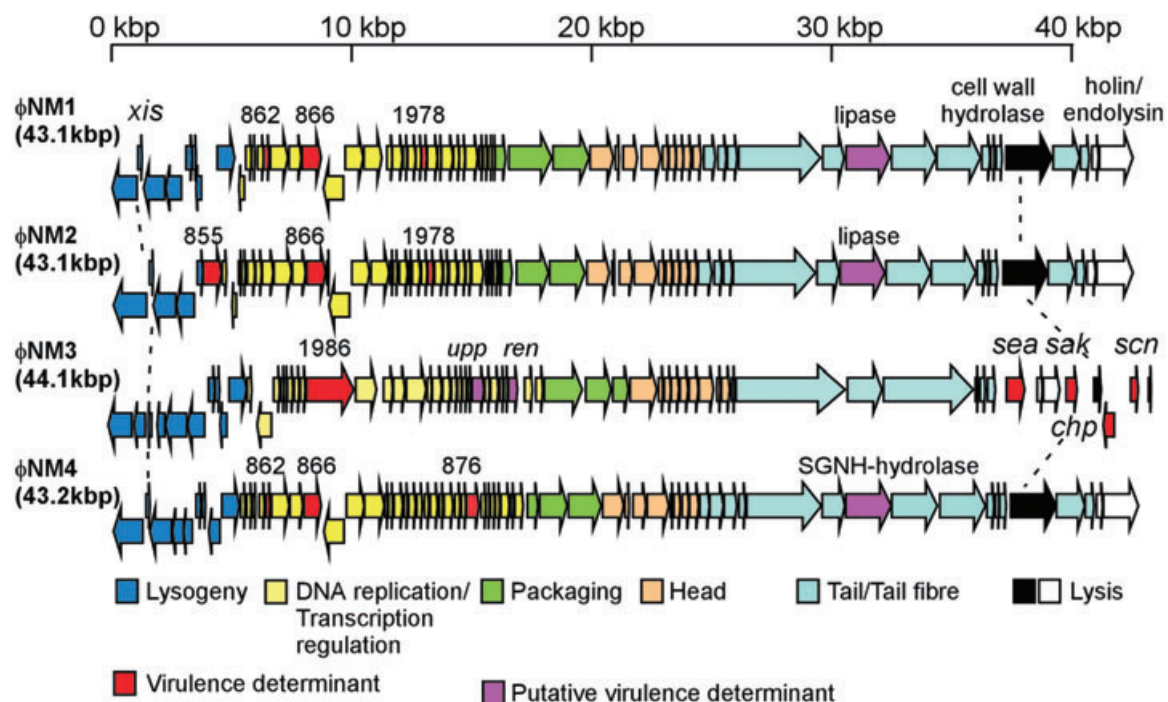


Fig. 1. Prophages of *S. aureus* Newman. Gene arrangement, sizes, direction of transcription (arrow), assigned function of four *S. aureus* Newman prophages: φNM1, φNM2, φNM3 and φNM4. The precise molecular function of phage genes is unknown, and their functional assignments are tentative. Numbers (SAV numbers) refer to nematode virulence genes of *S. aureus* Newman prophages that were identified as homologues of Mu50 prophages. The genome of φNM3, a β-haemolysin (*hlyB*)-converting phage, lacks excisionase (*xis*) and harbours a truncated cell wall hydrolase (dashed lines). Holin and endolysin genes are indicated with white arrows. *sea*, staphylococcal enterotoxin A; *sak*, staphylokinase; *chp*, chemotaxis inhibitory protein; *scn*, staphylococcal complement inhibitor; *upp*, dUTPase; *ren*, restriction endonuclease; SGNH-hydrolase, a lipolytic enzyme with invariant catalytic residues serine (S), glycine (G), asparagine (N) and histidine (H).

converting phages (van Wamel *et al.*, 2006) that integrate into the *hlyB* gene. β-Haemolysin converting phages are most frequently associated with clinical isolates of staphylococci and seven of eight sequenced *S. aureus* strains carry such phages (Carroll *et al.*, 1995). All currently sequenced *hlyB*-converting phages harbour one or more genes that modulate innate immune responses and transcription of these genes appears to be closely linked to phage replication (Sumbly and Waldor, 2003; van Wamel *et al.*, 2006).

Because of its stable *agr*⁺ *sigB*⁺ regulatory phenotype and robust virulence, *S. aureus* Newman, a human clinical isolate, is a prototype strain for the study of staphylococcal pathogenesis (Duthie and Lorenz, 1952; Mazmanian *et al.*, 2000). Previous work showed that the Newman strain harbours a *hlyB*-converting phage and expresses CHIPS (de Haas *et al.*, 2004). Using a nematode infection model, six prophage-encoded genes were shown to be required for staphylococcal killing of *Caenorhabditis elegans* and for *S. aureus* Newman virulence in a murine model of abscess formation (Bae *et al.*, 2004). Here we examined the prophages of *S. aureus* Newman and their contributions to the pathogenesis of murine infections.

Results

Staphylococcus aureus Newman harbours four prophages

Spontaneous induction or *S. aureus* Newman growth in the presence of mitomycin C were used to isolate phages from the filtrate of bacterial cultures. Phages were propagated by plating on *S. aureus* RN450 (8325-4) agar lawns. The RN450 strain, a variant of *S. aureus* 8325, is cured of its prophages and cannot induce plaque formation, even in the presence of mitomycin C, unless its cells have been newly infected with staphylococcal phage (Novick, 1967). Three *S. aureus* Newman phages, designated φNM1, φNM2 and φNM4, were plaque purified and their genomic DNA sequenced (Fig. 1). All three phages harbour double stranded DNA genomes, about 43 kb in size, with similar gene arrangements (Fig. 1). φNM1 and φNM2 are highly homologous in their central genome segment (97% identity between the 7.4–40.6 kb genome segments); each prophage harbours two virulence genes identified during nematode infection (homologues of Mu50 SAV0866 and SAV1978). Two other virulence genes, SAV0855 and SAV0862, are encoded by φNM2 and φNM1 respectively. The central genome segment of

ϕ NM4 (15.3–30.5 kb) is dissimilar to that of ϕ NM1 and ϕ NM2, whereas flanking sequences display a high degree of homology (Fig. S1). ϕ NM4 harbours three nematode virulence genes (SAV0862, SAV0866 and SAV0876), however, only one of them (SAV0876) is uniquely present in the genome of this prophage (Fig. 1).

The presence of ϕ NM3 prophage in *S. aureus* Newman was gleaned from DNA analysis during genome sequencing (Drs Tadashi Baba and Keiichi Hiramatsu). ϕ NM3 is a previously identified *hly*-converting phage (de Haas *et al.*, 2004); its genome sequence has no significant homology to that of the other three Newman phages (Fig. S1). ϕ NM3 carries genes that modulate host innate immune responses including enterotoxin A (*sea*), staphylokinase (*sak*), chemotaxis inhibitory protein (*chp*), complement inhibitor (*scn*) in addition to the nematode virulence gene SAV1986. ϕ NM3 prophage lacks an excisionase (*xis*) and its cell wall hydrolase gene specifies a prematurely truncated product (Fig. 1); this prophage does not appear to excise from the chromosome or replicate (see below).

Prophage integration sites

Inverse polymerase chain reaction (PCR) amplification and DNA sequencing were used to determine phage integration sites in the genome of *S. aureus* Newman (Fig. 2). ϕ NM1 integrates in a gene of unknown function [SAV1877, designated *poiA* – prophage ϕ NM1 (one) integration site A], which is also the target of integration for ϕ 11 and ϕ PV83-pro (Lee and landolo, 1988; Zou *et al.*, 2000). ϕ NM2 integrates at an intergenic region between *rpmF* (gene for 50S ribosomal protein L32) and *isdB*, encoding the staphylococcal haemoglobin receptor involved in haem-iron uptake (Mazmanian *et al.*, 2003). Integration of ϕ NM3 occurs at *hly* (β -haemolysin gene), whereas ϕ NM4 integrates into *geh* (glycerol ester hydrolase or lipase). Positions of prophage integration sites on the chromosome of *S. aureus* Newman are summarized in Fig. 2B. All prophages integrate with the same orientation, integrase left (L)–endolysin right (R), thereby positioning the integrase gene in closer proximity to the bidirectional origin of chromosome replication. Thus, integration of prophages into the staphylococcal genome may be informed by chromosome replication, a phenomenon that has also been reported for *Salmonella* phages (Bossi and Figueroa-Bossi, 2005). Attachment core sequences of all four phages ϕ NM1–4 are displayed in Fig. 3. ϕ NM1 harbours the same core sequence as ϕ 11 and ϕ P83-pro (Lee and landolo, 1988; Zou *et al.*, 2000). ϕ NM3 shares core sequence with other β -haemolysin converting phages (ϕ 13 and ϕ 42) (Coleman *et al.*, 1991; Carroll *et al.*, 1993), whereas ϕ NM4 core sequence is identical to that of ϕ L54a (Lee and landolo, 1986).

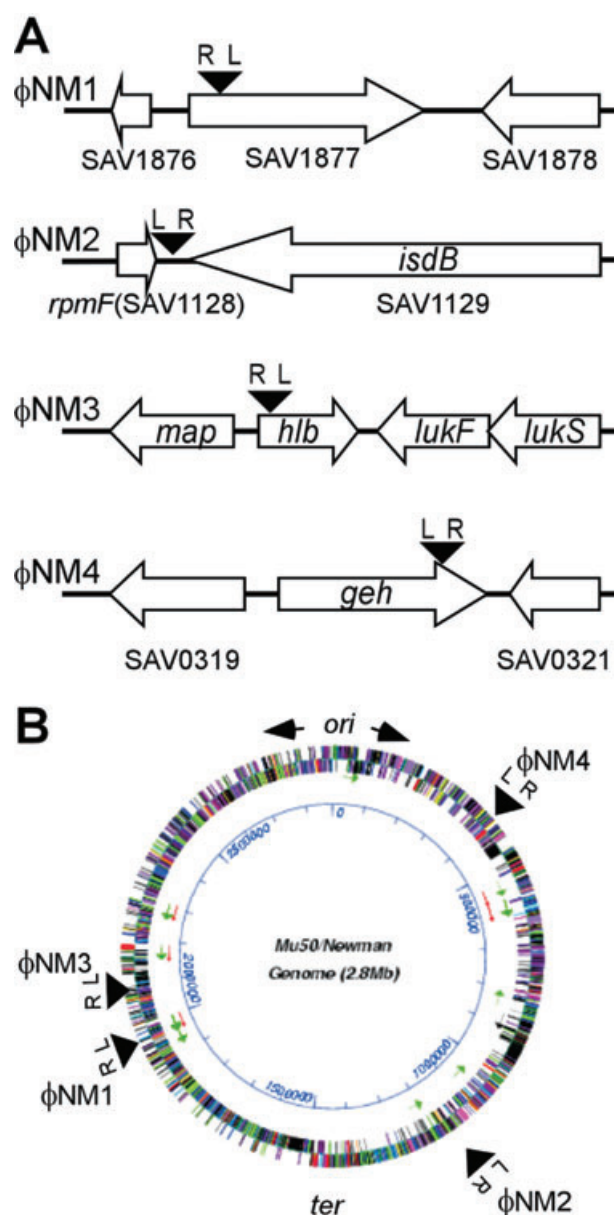


Fig. 2. Phage integration sites on the chromosome of *S. aureus* Newman.

A. Phage integration sites are indicated with inverted triangles. L denotes the position of *attL* and integrase gene, whereas R denotes *attR* and the endolysin gene. ORFs are identified by locus tags (SAV number) of the *S. aureus* Mu50 genome sequence. B. Position of ϕ NM1, ϕ NM2, ϕ NM3 and ϕ NM4 prophages on the *S. aureus* Newman/Mu50 genome. Strain Newman genome sequence has not yet been completed, and the Mu50 strain genome was therefore used as a reference. Circular map displays colour coded ORFs in the staphylococcal genome. Small arrows indicate rRNA and tRNA loci. Chromosomal origin of replication (*ori*, replication origin for bidirectional DNA synthesis) and replication termination site (*ter*) are displayed.

Phage morphologies

Infectious particles of ϕ NM1, ϕ NM2 and ϕ NM4 were purified and viewed by electron microscopy (Fig. 4A). The

φNM1 (SAV1877)

attP TGTATATAAATTTCA**CTTCCC**ATGGGTCATTATGAATTCT
 attL taacatcgttatatg**CTTCCC**ATGGGTCATTATGAATTCT
 attR TGTATATAAATTTCA**CTTCCC**ATGGatcattgtaaagtct
 attB taacatcgttatatg**CTTCCC**ATGGatcattgtaaagtct
 V D N Y A E W P D N Y I S

φNM2

attP GTTGGAAAGTGTGTATC**AGGTATCTG**CATAGTTATTCCGAA
 attL aaataattggcgaacg**AGGTA**ACTGCATAGTTATTCCGAA
 attR GTTGGAAAGTGTGTATC**AGGTATCTG**gatacctcatccgcc
 attB aaataattggcgaacg**AGGTA**ACTGgatacctcatccgcc

φNM3 (h1b)

attP TATGTTAAAAGTCT**CCAGTTTGG**ATACATAGAAACCTTGT
 attL cgtttatattgccc**CCAGTTTGG**ATACATAGAAACCTTGT
 attR TATGTTAAAAGTCT**CCAGTTTGG**ATACaaaacggtcgata
 attB cgtttatattgccc**CCAGTTTGG**ATACaaaacggtcgata
 K Y Q G W N P Y L V T S

φNM4 (geh)

attP ACCGGTTTACC**ATCATACAAGGATGGG**ATTAACCTTGTGTT
 attL aagttaaacca**ATCATACAAGGATGGG**ATTAACCTTGTGTT
 attR ACCGGTTTACC**ATCATACAAGGATGGG**ATcatgtcgattt
 attB aagttaaacca**ATCATACAAGGATGGG**ATcatgtcgattt
 V K P I I Q G W D H V D F

Fig. 3. Core sequences of phage attachment sites. Attachment sites of φNM1, φNM2, φNM3 and φNM4 are aligned and core sequences indicated with bold print. Prophage sequences are shown in capital characters, whereas staphylococcal sequences are printed in small characters. ORF locus tags or gene names are printed at the top. Predicted amino acid sequences of *S. aureus* ORFs are printed beneath the DNA sequence. Italicized amino acid sequences denote ORFs that are specified by complementary DNA.

three phages assemble into nearly identical structures with hexagonal heads (approx. 50 nm diameter) and non-contractile, flexible tails. These features are shared with bacteriophages of the siphoviridae family (morphotype B1). φNM1, φNM2 and φNM4 assemble globular adhesion organelles at the tip of their tails. Similar structures have been observed in many other staphylococcal phages (Yamaguchi *et al.*, 2000; Resch *et al.*, 2005). One notable difference between these phage particles is the length of their tails, which is more extended for φNM2 (200 nm) as compared with φNM1 and φNM4 (167 nm). Despite many attempts, we failed to isolate plaques (spontaneous or mitomycin C-induced) or infectious particles for strains harbouring φNM3 prophage (see below).

Spontaneous induction of prophages

During growth of bacterial phage lysogens in culture, small numbers of cells release phage particles even without DNA damaging stimuli such as mitomycin C or ultra-violet light (Fig. 5A). Such lytic induction in coliphage λ requires proteolytic properties of RecA (Roberts and Devoret, 1983). To test whether spontaneous induction of staphylococcal prophages also requires RecA, wild-type *S. aureus* Newman (lysogen for φNM1–4) as well as an isogenic *recA* variant were grown to late-log phase and

culture filtrates were plated on *S. aureus* RN450 lawns. Spontaneous induction occurred in *S. aureus* Newman cultures, as plating of culture filtrates with recipient cells led to the formation of phage plaques. In contrast, culture filtrates of *recA* variants failed to produce plaques, consistent with the hypothesis that RecA-mediated cleavage of prophage repressors is required for replication of *S. aureus* φNM1, φNM2 and φNM4 (Fig. S2).

Wall teichoic acid receptors of φNM1, φNM2 and φNM4

Phage titres decline during staphylococcal end-logarithmic or stationary phase growth (Resch *et al.*, 2005) and this can also be observed for the released bacteriophages of *S. aureus* Newman (Fig. 5A). We wondered whether the binding of phages to teichoic acids, the surface receptors for all staphylococcal viruses, may be responsible for this phenomenon (Coyette and Ghuysen, 1968; Umeda *et al.*, 1980). Phage adsorption to teichoic acid receptor may be accompanied by non-productive DNA injection (into bacterial lysogens that are immune to super-infection), thereby precipitating loss of infectivity and decline in phage titres. If so, one would predict that phage titres cannot decline in cultures of *S. aureus* variants lacking the teichoic acid receptors of phages.

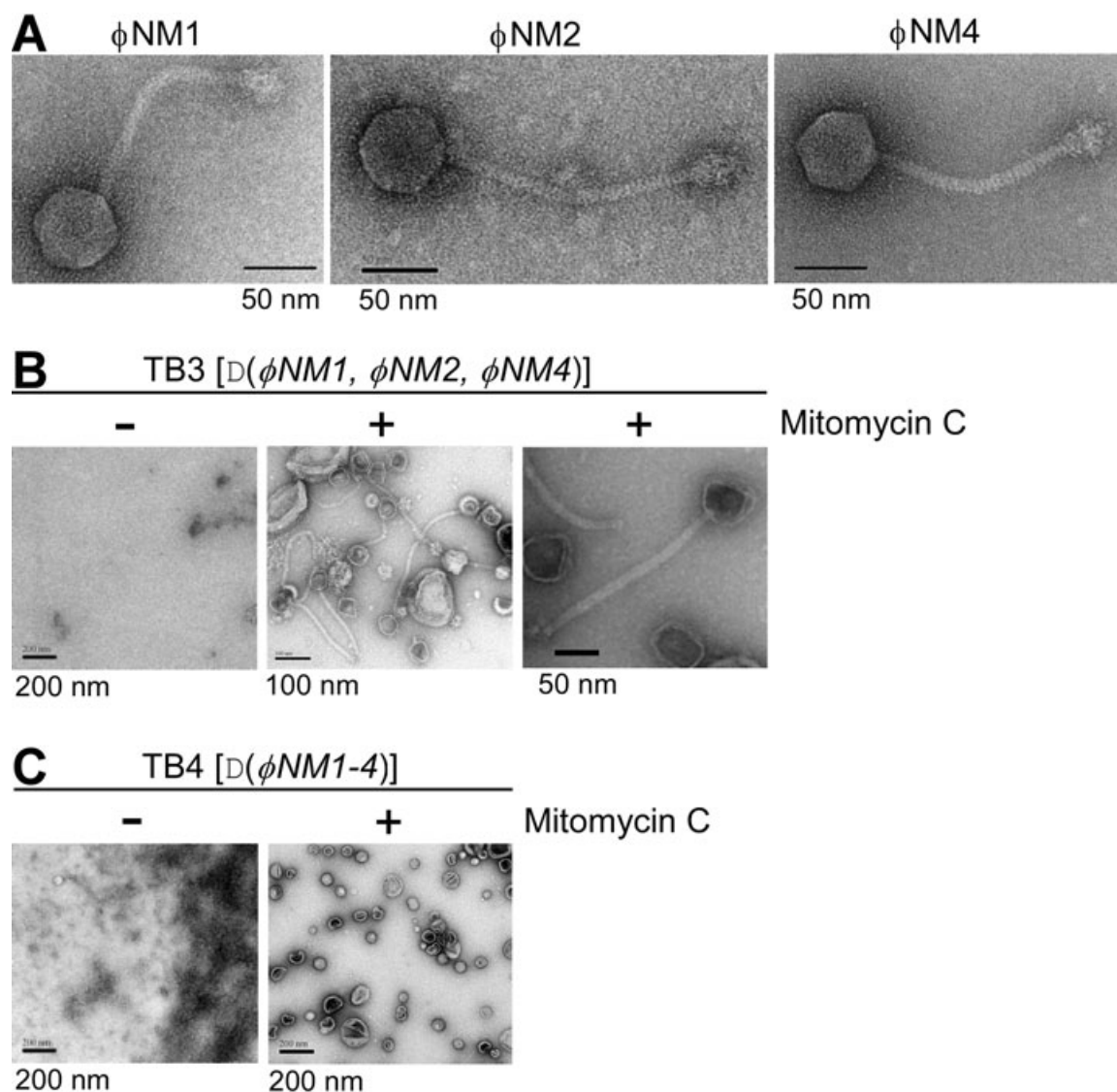


Fig. 4. Morphology of phage particles.

A. Transmission electron microscopy images of bacteriophage ϕ NM1, ϕ NM2 and ϕ NM4 infectious particles. Black bars represent size calibration markers.

B. Phage-like particles released from *S. aureus* TB3 [$\Delta(\phi$ NM124)]. Culture supernatants from mitomycin C-treated cultures were concentrated and images of ϕ NM3 phage-like particles captured by transmission electron microscopy.

C. *S. aureus* TB4 [$\Delta(\phi$ NM1-4)] cultures were treated with mitomycin C or left untreated and culture supernatants were viewed by transmission electron microscopy. Strains are indicated above the images.

TagO catalyses the first step in teichoic acid biosynthesis, transferring *N*-acetylglucosamine to bactoprenol (Soldo *et al.*, 2002; Weidenmaier *et al.*, 2004). As expected, *tagO* mutants failed to propagate ϕ NM1, ϕ NM2 or ϕ NM4 and did not support plaque formation on agar lawns (data not shown). Both wild-type strain Newman and its isogenic *tagO* mutant were grown as rotating cultures and phage titres in culture filtrates were determined in timed intervals via plaque formation on RN450 lawns. Even though phage titres in the filtrates of *S. aureus* Newman cultures declined over time, this phenomenon

did not occur in *tagO* mutant strains (Fig. 5A). We assumed that spontaneously induced phage particles may be located in bacterial cells as well as in the culture medium. To test this, the staphylococcal cell wall envelope was removed with muralytic enzymes. Bacterial lysates were then filtered and analysed for phage particles. Mid-log cultures of *S. aureus* Newman and its phage receptor mutant (Δ *tagO*) harboured about 10^5 pfu in the media and 10^5 pfu inside bacterial cells. Stationary phase cultures of the wild type, but not of Δ *tagO* mutants, displayed a decline in phage titre in the culture medium, while the

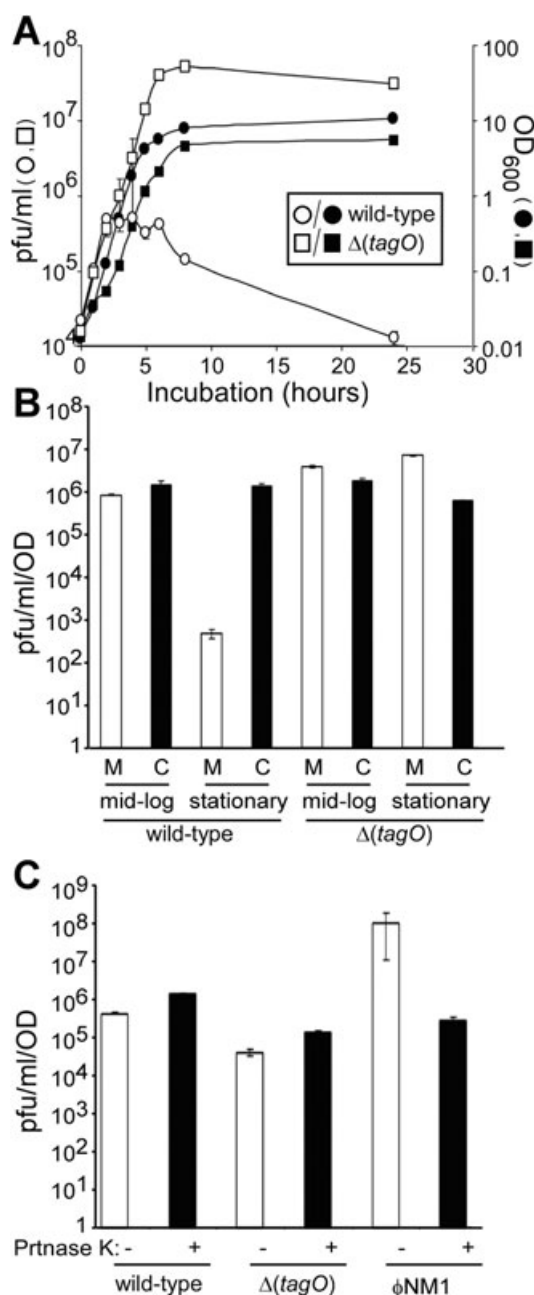


Fig. 5. Spontaneous induction of phage replication. A. Release of phage particles in cultures of *S. aureus* Newman (wt, wild type) and ANG260 [Δ (tagO)] mutant that were incubated at 37°C. Culture aliquots were withdrawn, optical density (OD₆₀₀) measured, and phage titres in filtrate measured by plaque formation on *S. aureus* RN450 lawns. B. Infectious phage particles in culture media (M) and within cells (C) of *S. aureus* Newman wild type (wt) or isogenic Δ (tagO) mutants. To release phage particles from cells, staphylococci were sedimented by centrifugation and peptidoglycan removed with muralytic enzymes. Following protoplast lysis, filtrate of released phage particles as well as culture media was analysed for plaque forming units and normalized for absorbance of culture aliquots (OD₆₀₀). C. Proteinase K sensitivity (+) and mock treated controls (–) of isolated bacteriophage ϕ NM1 and of phages associated with staphylococcal cells, either wild type or Δ (tagO) mutant.

levels of spontaneously induced phages associated with cells remained constant (Fig. 5B).

Staphylococcal phages are sensitive to protease treatment (Resch *et al.*, 2005) and a three log reduction in ϕ NM1 titre was observed upon treatment with proteinase K (Fig. 5C). As a test whether staphylococci harboured infectious phage particles bound to wall teichoic acid receptors on the bacterial surface, cells were harvested from *S. aureus* Newman or Δ tagO mutant cultures. Cells were treated with proteinase K or left untreated. After protease quenching, filtrates of bacterial lysates were examined for phage titres. The results in Fig. 5C show that proteinase K treatment did not reduce the titre of phages associated with staphylococcal cells, indicating that these phages must reside intracellularly and not on the bacterial surface. In sum, stationary phase cultures likely harbour fewer phages because some of their infectious particles bind to staphylococcal wall teichoic acid receptors and inject their DNA into cells that are already immune to infection because they harbour both prophage and repressor (Fig. 5C). This phenomenon cannot occur in Δ tagO mutants. Although the mutants harbour prophage and undergo spontaneous induction, replication and lysis, released phage particles cannot bind to phage receptors and inject their DNA into the staphylococcal population. *S. aureus* srtA mutants, lacking the sortase A transpeptidase that immobilizes surface proteins in the cell wall envelope (Mazmanian *et al.*, 2000), displayed no defect in phage absorption or plaque formation. Thus, unlike the surface protein receptor (GamR) of Gamma phage in the gram-positive organism *Bacillus anthracis* (Davison *et al.*, 2005), sortase A-anchored surface proteins are not required for ϕ NM1, ϕ NM2 or ϕ NM4 infection of staphylococci.

Prophage excision from the chromosome of *S. aureus* Newman

Analysis of the contribution of prophages to staphylococcal physiology or pathogenesis requires pairwise comparison of lysogen and non-lysogen. DNA damaging agents induce phage excision from the bacterial chromosome, and variants generated in this manner likely carry additional mutations. ϕ NM3 excision cannot be induced with mitomycin C, prompting us to employ a new technology for the removal of prophages from staphylococcal genomes. Plasmid pKOR1 replicates in staphylococci at 30°C, but not at or above 42°C (Bae and Schneewind, 2005). pKOR1 recombinants, carrying bacteriophage att sites and approximately 1 kb flanking DNA sequence, were introduced into *S. aureus* Newman or its variants by electroporation. Plasmid integration into the chromosome was selected at non-permissive temperature. Anhydro-tetracycline driven expression of secY antisense (encoded by pKOR1) pre-

cipitated plasmid excision and double crossover excision of prophage genomes. Using this technology, we successively deleted ϕ NM1, ϕ NM2, ϕ NM3 and ϕ NM4. Infection of *S. aureus* TB4, lacking all four prophages, with plaque purified ϕ NM1, ϕ NM2 or ϕ NM4 particles caused lysis of bacterial cultures, indicating that prophage excision from the chromosome is accompanied by loss of immunity to bacteriophage infection. Excision of ϕ NM3 prophage in strain TB4 restored the integrity and function of *hly* and, similarly, excision of ϕ NM4 restored the integrity and function of *geh* (Fig. 6).

Prophages of *S. aureus* Newman contribute to pathogenesis

BALB/c mice were infected with *S. aureus* Newman or isogenic variants lacking prophages by retro-orbital injection. Infected animals were killed after 4 days, livers and kidneys removed, and homogenized tissues were plated on agar to enumerate staphylococcal replication in organ abscesses (Albus *et al.*, 1991). When compared with the wild-type parent, staphylococcal variants lacking ϕ NM3 or ϕ NM1, ϕ NM2 and ϕ NM4 prophages displayed no defect in kidney abscess formation, whereas replication of mutant staphylococci in liver abscesses was significantly impaired. *S. aureus* Newman variants lacking all four prophages lost the ability to form abscesses in liver or kidney tissue. This defect is at least equal to or greater than those observed for *agr/sarA* or *srtA* (sortase A) mutants, which cannot secrete toxins or display proteins on the staphylococcal surface (Cheung *et al.*, 1994; Mazmanian *et al.*, 2000) (Fig. 7).

These results prompted us to investigate whether organ tissues infected with *S. aureus* Newman harboured phage particles. Liver homogenate obtained from mice infected with *S. aureus* Newman harboured on average of 10^7 staphylococcal colony-forming units (cfu) and 10^5 plaque forming units (Fig. 7C). Thus, spontaneous phage replication does occur in infected tissues and this phenomenon may contribute to the pathogenesis of staphylococcal infections. Alternatively, phage genes may contribute secreted virulence factors or cell wall envelope factors that aid in the establishment of animal infections.

We also investigated individual genes of prophage ϕ NM3 for their contribution to staphylococcal virulence. Three genes, SAV1986, *sak* and *sea* were previously shown to play a role in the pathogenesis of staphylococcal diseases, but were not yet examined during *S. aureus* infections of mice. Staphylokinase (SAK), a 136 residue secreted polypeptide, binds plasminogen in human blood, thereby generating fibrinolytic plasmin and promoting bacterial spread beyond fibrin deposits (Parry *et al.*, 1998). Staphylokinase also neutralizes α -defensins, antibacterial peptides released by human polymorphonuclear

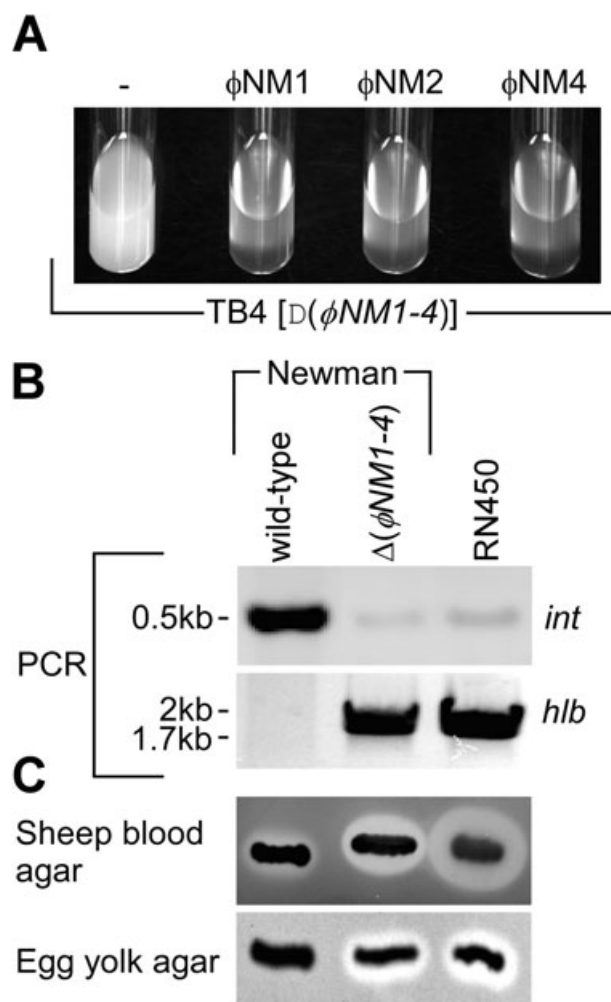


Fig. 6. *S. aureus* Newman variants lacking prophages.

A. *S. aureus* TB4 [$\Delta(\phi$ NM1-4)] is a mutant of wild-type strain Newman in which the four prophages have been excised from the genome by homologous recombination. *S. aureus* TB4 was either mock infected or mixed with infectious particles of phage ϕ NM1, ϕ NM2 and ϕ NM4 as indicated. Phage-mediated lysis of *S. aureus* TB4 demonstrates that prophage excision is accompanied by loss of immunity to phage infection.

B. DNA coding sequence of ϕ NM3 integrase (*int*) or *S. aureus* β -haemolysin (*hly*), encompassing the ϕ NM3 *attB* site, were PCR-amplified from wild-type parent *S. aureus* Newman (NM), the prophage-free variant TB4 as well as *S. aureus* RN450, a prophage-free variant of strain 8325. Electrophoretic positions of amplified fragments is compared with DNA size markers (kb, kilobase pairs).

C. *S. aureus* strains were streaked either on sheep blood or egg yolk agar plates and incubated at 37°C overnight. The sheep blood agar plate was incubated at 4°C for another 6 h. Images show that *S. aureus* TB4 [$\Delta(\phi$ NM1-4)] and RN450 secrete haemolysin and lipase, whereas the wild-type clinical isolate *S. aureus* Newman does not.

leukocytes, an innate immune response against invading pathogens. *sea* encodes staphylococcal enterotoxin A (SEA), a causative agent of staphylococcal food poisoning. When injected into rabbits, purified SEA causes hypotension and death due to its superantigen

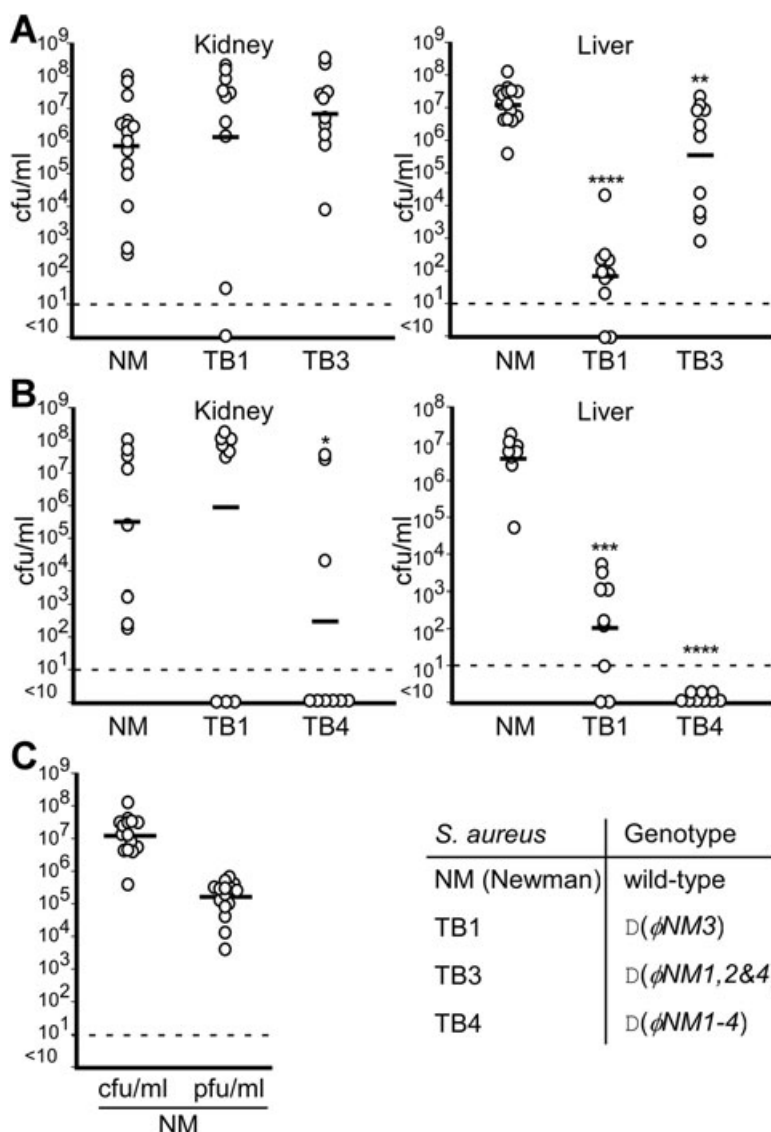


Fig. 7. Prophages are required for the pathogenesis of *S. aureus* Newman infections. BALB/c mice were infected by retro-orbital injection of staphylococcal suspensions. Four days after infection, animals were killed, kidneys and livers removed and bacterial load in homogenized tissues was enumerated by plating and colony formation on agar media (cfu, colony-forming unit).

A and B. Inocula of (A) 1×10^7 cfu or (B) 0.5×10^7 cfu staphylococci per mouse were used. Each circle represents data from one experimental animal. Dashed lines mark limits of detection and horizontal bars indicate observation means. Asterisks denote statistical significance: * $P < 0.05$; ** $P < 0.005$, *** $P < 10^{-6}$, **** $P < 10^{-12}$. C. Bacterial load and phage titres were determined in liver homogenates during murine infection with *S. aureus* Newman. cfu, colony-forming unit; pfu, plaque forming unit.

properties with MHC class II receptors (Schad *et al.*, 1995; Tiedemann *et al.*, 1995; Dinges *et al.*, 2000). Lastly, SAV1986 is required for staphylococcal killing of nematodes (Bae *et al.*, 2004). *Bursa aurealis* insertions in three ϕ NM3 genes, SAV1986, *sak* or *sea*, were transduced into the parent strain *S. aureus* Newman. Newly isolated variants were analysed for their ability to establish murine abscesses. As compared with wild type or ϕ NM3 deletion strain TB1, which displays a dramatic defect in the ability to cause liver abscesses (vide supra), the variants TB6 (SAV1986), TB7 (*sak*) and TB8 (*sea*) showed no significant reduction in virulence (Fig. S3). Together these data suggest that although each of the three virulence genes of ϕ NM3 alone was not required for staphylococci to cause invasive disease in mice, the sum of all genes encoded by ϕ NM3 prophages does play an important role in the establishment of staphylococcal infections.

ϕ NM3 particles

To test whether a ϕ NM3 lysogen can produce phage particles, *S. aureus* TB3 (lacking all prophages except ϕ NM3) cultures were treated with mitomycin C. Unlike *S. aureus* Newman cultures, where mitomycin C-induction precipitated bacterial lysis, treatment of TB3 cultures with mitomycin C did not cause complete cell lysis (data not shown). Moreover, filtrates of TB3 cultures did not form plaques on RN450 lawns (Fig. S2). Filtrate of mitomycin C-induced TB3 cultures was analysed by electron microscopy. Figure 4B demonstrates the presence of phage-like particles encompassing phage heads and tail fibres in TB3 filtrate. Of note, ϕ NM3 head structures appeared empty and not filled with DNA. As a control, mitomycin C-induced phage-like particles were not observed in culture filtrates of strain TB4, lacking all four

prophages (ϕ NM1–4), albeit that mitomycin C treatment seemed to precipitate formation of cell wall envelope fragments (ghosts) (Fig. 4C). Because phage heads with genomes and globular adhesion organelles are missing in these phage particles, we presume that intact ϕ NM3 prophage cannot be released or transduced without helper phages.

Discussion

There have been extensive studies on secreted toxins and immune modulators encoded by staphylococcal prophages (Novick, 2003b; van Wamel *et al.*, 2006). Nevertheless, due to technical limitations, the overall contribution of prophages to the pathogenesis of *S. aureus* infections could not be examined. These obstacles have been overcome here, as pKOR1 technology permits excision of more than 40 kb prophage DNA from the staphylococcal chromosome.

We report that the human clinical isolate *S. aureus* Newman harbours four prophages, ϕ NM1–4 and determined their DNA sequence. Staphylococcal growth in liquid culture or infected tissues promotes spontaneous excision and replication of ϕ NM1, ϕ NM2 and ϕ NM4. Electron microscopy studies indicate that these phages are members of the siphoviridae family that insert into the genome at specific sites and orientation that is informed by attachment core sequences and the overall direction of chromosome replication. Previous work identified six prophage genes that are required for *S. aureus* nematode killing and murine pathogenesis (Bae *et al.*, 2004). Phage virulence genes are distributed between all four prophages and some of them are found in the genomes of more than one prophage. ϕ NM3 prophage was not observed to generate infectious particles even when staphylococci were treated with the DNA damaging agent mitomycin C. We excised prophages from the chromosome of *S. aureus* Newman and generated isogenic mutants for pairwise comparison of virulence properties. Our results suggest that the presence of prophages, ϕ NM1, ϕ NM2, ϕ NM3 or ϕ NM4, is essential for the pathogenesis of *S. aureus* infections. These results form the technological base whereupon genetic requirements of individual prophage genes for the pathogenesis of staphylococcal disease can now be interrogated.

ϕ NM3 prophage carries innate immune modulatory genes *sea*, *sak*, *chp* and *scn* and is a member of the *hly*-converting bacteriophage family that is thought to play important roles during *S. aureus* infections of human hosts (Coleman *et al.*, 1991; de Haas *et al.*, 2004). These innate immune modulators are thought to be specific for human complement or antigen presentation strategies and have either no or significantly reduced impact on the immune system of mice. For example, SEA is 1000-fold

less effective in activating murine T cells, when compared with human T cells (Dohlsten *et al.*, 1993). SAK activates human but not murine plasminogen (Gladysheva *et al.*, 2003). CHIPS displays 30-fold lower efficacy inhibiting C5a-induced calcium mobilization in murine neutrophils, when compared with the human counterpart (de Haas *et al.*, 2004). Finally, murine serum does not support SCIN-mediated inhibition of haemolysis (Rooijackers *et al.*, 2005). Considering previous work in the field and our observations for mutants with *Bursa aurealis* insertions in *sea* and *sak*, we did not expect to observe differences in the ability to generate murine infections for strains with or without ϕ NM3 prophage. Surprisingly, ϕ NM3 excision triggered a significant reduction in the ability of mutant strains to replicate in liver tissues. This finding points to the possibility that ϕ NM3 prophage may harbour additional virulence genes that modulate innate immune functions of murine hosts.

Electron microscopy studies of mitomycin C-treated staphylococci harbouring ϕ NM3 suggest that phage replication is defective and that infectious particles may not be formed (Fig. 4B and Fig. S2). Two interesting features of ϕ NM3 are the absence of *xis* (excisionase) and truncation of its cell wall hydrolase (Fig. 1). ϕ 13, another *hly*-converting prophage isolated from NCTC8325 has similar properties (no *xis* and a truncated cell wall hydrolase). Nevertheless, ϕ 13 prophage replicates, forms plaques and lysogenizes *S. aureus* strains that otherwise lack *hly*-converting prophages (Coleman *et al.*, 1986; Goerke *et al.*, 2006a). ϕ SLT (staphylococcal leukocytolytic toxin) generates infectious phage particles despite the fact that the prophage genome also does not carry *xis* (Narita *et al.*, 2001). In fact, all of these staphylococcal *cos* phages, ϕ 13, ϕ PVL, ϕ PV83-pro and ϕ SLT – which cut at *cos* sites to package DNA – lack *xis* (Kaneko *et al.*, 1998; Zou *et al.*, 2000; Narita *et al.*, 2001). Their excision from the chromosome and subsequent replication is obviously distinct from that of *xis* containing *pac* prophages, whose particle assembly involves head-full loading of DNA into phage capsids. Recent work on human clinical *S. aureus* isolates suggests that *hly*-converting prophages can replicate during infection as prophage genomes were observed at different chromosomal sites (Goerke *et al.*, 2006b).

There is, however, ample precedent for replication defects of *hly*-converting prophages. Mitomycin C cannot induce the formation of infectious particles for ϕ SA3ms, the *hly*-converting phage of MSSA476 (Sumbly and Waldor, 2003). Further, 63% (29 out of 46 strains examined) of clinical *S. aureus* isolates carrying *hly*-converting prophages failed to produce infectious particles or plaques (van Wamel *et al.*, 2006). In sum, we do not know why some, but not all, *hly*-converting prophages have lost their ability to excise and replicate and whether such

Table 1. Bacterial strains used in this study.

Strains	Relevant characteristics	Source
<i>E. coli</i> DH5 α		Stratagene
<i>S. aureus</i> RN4220	restriction deficient, prophage-cured	Kreiswirth <i>et al.</i> (1983)
RN450	prophage-cured <i>S. aureus</i> strain 8325	Novick (1967)
ANG342	<i>tagO</i> deletion mutant RN4220	Gründling and Schneewind (2006)
Newman	wild type, human clinical isolate	Duthie and Lorenz (1952)
TB4	<i>S. aureus</i> Newman with all four prophages deleted $\Delta(\phi\text{NM1-4})$	This study
TB3	<i>S. aureus</i> Newman with three prophages deleted $\Delta(\phi\text{NM124})$	This study
TB1	<i>S. aureus</i> Newman with ϕNM3 prophage deleted $\Delta(\phi\text{NM3})$	This study
TB5	<i>S. aureus</i> Newman with <i>Bursa aurealis</i> insertion in <i>recA</i> (SAV1285), transduced from strain $\Phi\text{H}\Xi\text{17482}$	This study, Bae <i>et al.</i> (2004)
TB6	<i>S. aureus</i> Newman with <i>Bursa aurealis</i> insertion in ϕNM3 prophage gene SAV1986, transduced from strain $\Phi\text{H}\Xi\text{3200}$	This study, Bae <i>et al.</i> (2004)
TB7	<i>S. aureus</i> Newman with <i>Bursa aurealis</i> insertion in ϕNM3 prophage gene <i>sak</i> , transduced from strain $\Phi\text{H}\Xi\text{2464}$	This study, Bae <i>et al.</i> (2004)
TB8	<i>S. aureus</i> Newman with <i>Bursa aurealis</i> insertion in ϕNM3 prophage gene <i>sea</i> , transduced from strain $\Phi\text{H}\Xi\text{3552}$	This study, Bae <i>et al.</i> (2004)
ANG260	<i>tagO</i> deletion mutant of <i>S. aureus</i> Newman	Gründling and Schneewind (2006)

features are important for staphylococcal pathogenesis. Future work should therefore address the genetic requirements of ϕNM3 and other *hlyB*-converting prophages for pathogenesis, excision and replication.

Experimental procedures

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. For phage infection experiments, *S. aureus* was grown in heart infusion broth (HIB) supplemented with 5 mM CaCl_2 (HIB_{Cas}). For molecular biology experiments, staphylococci were grown in tryptic soy broth (TSB) and *Escherichia coli* strains were grown in Luria–Bertani broth (LB). Antibiotics were used at the following concentrations: carbenicillin, 50 $\mu\text{g ml}^{-1}$; chloramphenicol, 10 $\mu\text{g ml}^{-1}$.

Phage isolation, sequencing and electron microscopy

Staphylococcus aureus Newman was grown to mid-log phase and 1 $\mu\text{g ml}^{-1}$ mitomycin C was added. Cultures were incubated with shaking until staphylococcal lysis occurred. Cell lysate was passed through 0.22 μm filters and used to infect prophage-free *S. aureus* strains RN450 or RN4220 (Kreiswirth *et al.*, 1983). Small plaques and large plaques were picked and re-inoculated into *S. aureus* RN4220 cultures to amplify phages. From 1 ml of the amplified phage lysate, phage DNA was purified by phenol/chloroform extraction followed by ethanol precipitation. Isolated phage DNA was subjected to PCR amplification with primers Ndel-SAV0862-N and BamHI-SAV0866-C (Table S1) to identify phage carrying SAV0862 and SAV0866. A 2.3 kb product was identified from four phage isolates, one of which (ϕNM1) was chosen and subjected to genome DNA sequencing. One of the two phage picks that did not amplify the 2.3 kb product (ϕNM2) was also subjected to genomic DNA sequencing. Phage DNA was large scale purified and

ϕNM1 DNA was digested with Sau3A overnight at 37°C, whereas ϕNM2 DNA was sheared with hydroshear (GeneMachines, 20 cycles at speed setting 6). Phage DNA fragments were cloned into pUC18 digested with BamHI (ϕNM1) or SmaI (ϕNM2) and electroporated into *E. coli* DH5 α . Transformants were plated on an LB agar containing Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactoside, 160 $\mu\text{g ml}^{-1}$), IPTG (isopropylthio- β -D-galactoside, 1 mM) and carbenicillin (50 $\mu\text{g ml}^{-1}$). After overnight incubation at 37°C, 250–300 white colonies were each inoculated in 5 ml of LB medium containing 50 $\mu\text{g ml}^{-1}$ of carbenicillin. Following plasmid purification, the nucleotide sequence of cloned phage inserts was determined at the DNA sequencing facility of the University of Chicago. Phage DNA inserts were sequenced with the primers pUC-forward and pUC-reverse primers (Table S1). Sequences were analysed with BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) and assembled. Sequence gaps were filled by PCR amplification and sequencing. To identify *attB* sites for ϕNM1 and ϕNM2 , inverse PCR was performed with primers: N-1-iPCR-F/N-1-iPCR-R (ϕNM1) and iPCR-NM2-1F/iPCR-NM2-1R (ϕNM2) (Table S1). PCR products were purified and sequenced.

Genome sequences of ϕNM3 and ϕNM4 were extracted from *S. aureus* Newman genome sequence, which is being generated by Drs Tadashi Baba and Keiichi Hiramatsu. Phage particles of ϕNM4 were identified by PCR screening of purified phage DNAs with primers ct-253- ϕNM4 -F and ct-253- ϕNM4 -R (Table S1). For electron microscopy, phage lysates were treated with lysostaphin (40 $\mu\text{g ml}^{-1}$), mutanolysin (100 U ml^{-1}), DNase (1 $\mu\text{g ml}^{-1}$) and RNase (4 $\mu\text{g ml}^{-1}$), in the presence of 2 mM PMSF (phenylmethylsulphonyl fluoride) at 37°C for 2 h. Phage particles were collected by ultracentrifugation at 50 000 *g* in an SW41 rotor (Beckman) and suspended in 0.5 ml of TM buffer (10 mM Tris-HCl, 10 mM MgCl_2 , pH 8.0). Images were captured by transmission electron microscopy after staining phages with 1% (w/v) uranyl acetate. Phage sequences were submitted to GenBank [Accession numbers DQ530359 (ϕNM1), DQ530360 (ϕNM2), DQ530361 (ϕNM3), DQ530362 (ϕNM4)].

Prophage excision

For excision of ϕ NM1, ϕ NM2 and ϕ NM4, a 2 kb insert DNA encompassing 1 kb flanking sequences of phage attachment sites was generated by PCR using chromosomal DNA of *S. aureus* RN450, a prophage-free strain, as template (Table S1). To generate template for deletion of ϕ NM3, 1 kb DNA fragments were amplified on both flanks of the phage attachment site with primer pairs attB1- ϕ hNM3-1/hlb-att-R and attB2- ϕ hNM3-2/hlb-att-F. PCR products were ligated with T4 DNA ligase, and product inserted by recombination into pKOR1 (Bae and Schneewind, 2005). pKOR1 recombinants were electroporated first into *E. coli* DH5 α , then *S. aureus* RN4220 and finally *S. aureus* Newman. The ϕ NM2-deletion construct was electroporated directly into the *S. aureus* strains RN4220 and Newman, because stable *E. coli* transformants could not be obtained (Bae and Schneewind, 2005). Immunity phenotypes were examined by infecting mutants with ϕ NM1, ϕ NM2 or ϕ NM4. ϕ NM3 and ϕ NM4 integrate into β -haemolysin (*hly*) and glycerol-ester hydrolase (*geh*) respectively. Mutant candidates were spread on TSA plates containing either 5% sheep red blood cells (Beckton Dickinson) or 5% sterile egg yolk emulsion and incubated for phenotypic analysis. Prophage excision was confirmed by PCR amplification with the primers: ct-890- ϕ hNM3-F/ct-890- ϕ hNM3-R (ϕ NM3) and ct-253- ϕ hNM4-F/ct-253- ϕ hNM4-R (ϕ NM4).

Spontaneous induction

Staphylococcus aureus Newman (wild type) or isogenic Δ tagO mutants were grown overnight in 5 ml of HIB_{Ca5}. Staphylococci were collected by centrifugation from 1.5 ml (wild type) or 3 ml (tagO mutant) of cultures and washed five times with 1 ml of phosphate-buffered saline (PBS) containing 10 mM EDTA (ethylenediamine tetraacetic acid). Bacteria were suspended in 1 ml of HIB_{Ca5} and optical density at 600 nm (OD₆₀₀) was determined. Staphylococci were added to 100 ml of HIB_{Ca5} (prewarmed at 37°C) to OD₆₀₀ 0.01 and then incubated at 37°C at 200 r.p.m. for 24 h. During incubation, 5 ml aliquots were sampled at timed intervals. Following OD₆₀₀ measurement, 1 ml of the sample was centrifuged and supernatant sterilized by filtration. Filtrate, 10 μ l, was mixed with 200 μ l of RN450 mid-log phase HIB_{Ca5} cultures and 200 μ l of phage buffer (50 mM Tris-HCl, pH 7.8, 1 mM MgSO₄, 4 mM CaCl₂, 100 mM NaCl, 0.1% gelatin) (Novick, 1991). After 10 min at room temperature, 5 ml of top agar (0.8% agar, 0.8% NaCl) was added and suspensions were plated on HIA_{Ca5} agar. For measurement of phage titres in bacterial cells, staphylococci were suspended in 100 μ l of sterile TSM (50 mM Tris HCl, pH 7.5, 0.5 M sucrose, 10 mM MgCl₂). Lysostaphin and mutanolysin were added to final concentrations of 100 μ g ml⁻¹ and 250 units ml⁻¹ respectively. After incubation at 37°C for 15 min, HIB_{Ca5} was added to a final volume of 1 ml and phage titres in filtrate were determined.

Murine infection and abscess formation

Staphylococcus aureus strains were grown overnight in TSB at 37°C diluted 100-fold into 5 ml of fresh media and incu-

bated at 37°C until cultures reached OD₆₀₀ 1.0. Staphylococci were collected by centrifugation, washed and suspended in sterile PBS. Bacterial suspensions were further diluted to OD₆₀₀ 0.2 or 0.4. Viable staphylococci were enumerated by colony formation on TSA plates to quantify infectious doses. One hundred μ l of bacterial suspension ($0.5\text{--}1 \times 10^7$ cfu) was administered intravenously *via* retro-orbital injection into each of 10 7-week-old BALB/c mice. Four days after the injection, the mice were killed by CO₂ asphyxiation and kidneys and liver were removed. Organs were homogenized in 1 ml of 1% Triton X-100 and dilutions of homogenates with sterile PBS were plated on TSA. Student's *t*-test was performed for statistical analysis using the software Analyse-it™.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Phage genome sequence comparison illustrated with dot plotting.

Fig. S2. Spontaneous induction of ϕ NM1, ϕ NM2 and ϕ NM4 replication requires *recA*. *S. aureus* Newman or isogenic variants, TB3 [$\Delta(\phi$ NM1, 2 and 4)], lacking all prophages (ϕ NM1, ϕ NM2 and ϕ NM4) except ϕ NM3, and TB5, a *recA* mutant, were grown in heart infusion broth with 5 mM CaCl₂ until late-log phase (OD at 2.0).

Fig. S3. Contribution of prophage ϕ NM3 and ϕ NM3-encoded genes to the pathogenesis of liver abscesses following *S. aureus* infection of mice.

Table S1. Primers used in this study.

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