AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Genomic Sequencing of High-Efficiency Transducing Streptococcal Bacteriophage A25: Consequences of Escape from Lysogeny

Kimberly McCullor,[a] Brandon Postoak,[a]* Maliha Rahman,[a] Catherine King,[a] W. Michael McShan[a]

[a]Department of Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

ABSTRACT Lytic bacteriophage A25, which infects *Streptococcus pyogenes* and several related species, has been used to better understand phage-microbe interactions due to its ability to mediate high-efficiency transduction. Most of these studies, however, are decades old and were conducted prior to the advent of next-generation sequencing and bioinformatics. The aim of our study was to gain a better understanding of the mechanism of high-efficiency transduction through analysis of the A25 genome. We show here that phage A25 is related to a family of genome prophages and became a lytic phage following escape from lysogeny. A lambdoid-like residual lysogeny module consisting of an operator site with two promoters and a *cro*-like antirepressor gene was identified, but the genes for the cI-like repressor and integrase are missing. Additionally, the genetic organization of the A25 genome was found to be modular in nature and similar to that of many prophages of *S. pyogenes* as well as from other streptococcal species. A study of A25 homology to all annotated prophages within *S. pyogenes* revealed near identity within the remnant lysogeny module of the A25 phage genome to the corresponding regions in resident prophages of genome strains MGAS10270 (M2), MGAS315 (M3), MGAS10570 (M4), and STAB902 (M4). Host range studies of MGAS10270, MGAS315, and MGAS10750 demonstrated that these strains were resistant to A25 infection. The resistance mechanism of superinfection immunity was confirmed experimentally through complementation of the operator region and *cI*-like repressor from prophage MGAS10270.2 into susceptible strains SF370, CEM1Δ4 (SF370ΔSpyCIM1), and ATCC 12204, which rendered all three strains resistant to A25 infection. *In silico* prediction of packaging through homology analysis of the terminase large subunit from bacteriophages within the known packaging mechanism of Gram-positive bacteria as well as the evidence of terminally redundant and/or circularly permuted sequences suggested that A25 grouped with phages employing the less stringent *pac*-type packaging mechanisms, which likely explains the characteristic A25 high-efficiency transduction capabilities. Only a few examples of lytic phages appearing following loss of part or all of the lysogeny module have been reported previously, and the genetic mosaicism of A25 suggests that this event may not have been a recent one. However, the discovery that this lytic bacteriophage shares some of the genetic pool of *S. pyogenes* prophages emphasizes the importance of genetic and biological characterization of bacteriophages when selecting phages for therapeutics or disinfectants, as phage-phage and phage-microbe interactions can be complex, requiring more than just assessment of host range and carriage of toxoid or virulence genes.

IMPORTANCE Bacteriophages (bacterial viruses) play an important role in the shaping of bacterial populations as well as the dissemination of bacterial genetic material to new strains, resulting in the spread of virulence factors and antibiotic resistance

Received 14 June 2018 **Accepted** 12 September 2018

Accepted manuscript posted online 17 September 2018

Citation McCullor K, Postoak B, Rahman M, King C, McShan WM. 2018. Genomic sequencing of high-efficiency transducing streptococcal bacteriophage A25: consequences of escape from lysogeny. J Bacteriol 200:e00358-18. https://doi.org/10.1128/JB.00358-18.

Editor George O'Toole, Geisel School of Medicine at Dartmouth

Address correspondence to W. Michael McShan, William-Mcshan@ouhsc.edu.

* Present address: Brandon Postoak, Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma, USA.

genes. This study identified the genetic origins of *Streptococcus pyogenes* phage A25 and uncovered the molecular mechanism employed to promote horizontal transfer of DNA by transduction to new strains of this bacterium as well as identified the basis for its host range.

KEYWORDS bacteriophages, horizontal transfer, lysogeny, *Streptococcus pyogenes*, transduction

Bacteriophage A25 was isolated from Parisian sewers in the mid-twentieth century by Nicolai Bulgakov and has been used extensively to understand phage-microbe interactions and genetic transfer predominantly within group A streptococci (1, 2). Early studies described A25 as a lytic bacteriophage with an estimated burst size of 13 to 32 viral particles per bacterial cell (3–5), morphologically belonging to the *Siphoviridae* (3, 6, 7) and having a broad host range comprising streptococcal groups A, C, G, L, and H (8, 9). However, it was the ability of A25 to act as a high-efficiency transducing phage that received particular interest (10). Rates of transduction were found to vary among group A streptococcal strains, ranging from 10^{-6} to 10^{-9} transductants per PFU (11). Transduction via A25 has also been observed in group C and G streptococcal strains, although at lower frequency than for intragroup transduction within *Streptococcus pyogenes* (8, 12). A25 is able to transduce genes encoding antibiotic resistance, virulence genes, and intact lysogenic phages that potentially encode elements that alter the host phenotype (10, 11, 13–15).

Even though studies have estimated that recombination within *Streptococcus pyogenes* is high, with up to 35% of the genome estimated to have undergone recombination (16, 17), the predominant mechanism of horizontal transfer is unproven. Currently, only a single account of natural transformation exists within group A streptococcal strain MGAS315, occurring within biofilms (18), and no reports suggest that *S. pyogenes* employs conjugation for genomic transfer. Thus, phage transduction is probably an important mechanism of horizontal gene transfer among group A streptococci (19) and one that needs to be better understood. Indeed, this need is reflective of the larger problem of gene flow in prokaryotes that impacts many aspects of human society, including the spread of virulence and antimicrobial resistance genes. Within the literature, there is a growing appreciation of the role phages play in horizontal gene transfer, as phage-associated antibiotic resistance genes are being discovered everywhere from chicken meat and soil fertilizer containing dairy or municipal biosolid waste to human fecal samples and wastewater (20–23).

Genome sequencing has provided many examples of lysogens of *S. pyogenes* (24), but only scant genetic data concerning lytic phages of this bacterial pathogen are available. In order to better understand the role that lytic bacteriophages play in horizontal gene transfer within *S. pyogenes*, we present here the complete genome sequence of phage A25. Bioinformatic analysis reveals the genome mosaicism and complex origin of A25 and provides insights into the packaging mechanism and host range among strains of *S. pyogenes*. Unexpectedly, we discovered that A25 had escaped lysogeny at some past time through deletion of a portion of the associated module, providing insights into the host range of this now-lytic phage. These studies not only provide new information on the biology of A25 but also allow us to glean insights into horizontal gene transfer within *S. pyogenes*, as A25 is known to be a highly efficient transducing phage.

RESULTS

Organization of the phage A25 genome. Bacteriophage A25 has a genome of 33,900 bp with a GC content of 38.44%, very close to the predicted size (25) and GC content (7) obtained previously through physical mapping. Sequencing of the junction site confirmed the circular conformation of the A25 genome that has been previously described (6, 7). The A25 genome has 46 predicted genes (Table 1) that are organized in a characteristic modular fashion based upon function (Fig. 1A). These modules

TABLE 1 A25 genome annotations

ORF[a]	Start site (bp)	Stop site (bp)	Protein with homology in NCBI	Accession no.	E value	% identity (% query cover)
1	233	451	Transcriptional regulator, Cro family (*S. pyogenes* phage 10270.2)	ABF33867.1	1.00E−44	100 (98)
2	546	797	Hypothetical protein (*S. pyogenes*)	WP_011528542.1	2.00E−53	100 (98)
3	978	1292	Transcriptional regulator (*S. pyogenes*)	WP_011528544.1	1.00E−69	100 (99)
4	1407	1517	Phage protein (*Streptococcus dysgalactiae* subsp. *dysgalactiae* ATCC 27957)	EFY03455.1	1.00E−11	83 (97)
5	1519	2001	Hypothetical protein (*S. pyogenes*)	WP_011528545.1	9.00E−109	100 (99)
6	2002	2682	DNA-binding protein (*S. pyogenes*)	WP_002995975.1	2.00E−165	100 (99)
7	2784	4013	ATP-dependent helicase (*S. pyogenes*)	WP_011528546.1	0	100 (99)
8	4029	4487	Hypothetical protein (*S. pyogenes*)	WP_002995969.1	9.00E−108	100 (99)
9	4490	5302	Hypothetical protein (*S. pyogenes*)	WP_030126642.1	0	99 (99)
10	5292	6773	DNA primase (*S. pyogenes*)	WP_020905118.1	0	99 (99)
11	7018	7338	VRR-NUC domain-containing protein (*S. pyogenes*)	WP_002995960.1	4.00E−71	100 (99)
12	7322	7678	Hypothetical protein (*S. pyogenes*)	WP_011018138.1	2.00E−79	100 (99)
13	7675	7926	Hypothetical protein (*S. pyogenes*)	WP_011528549.1	2.00E−54	100 (98)
14	7920	8204	Hypothetical protein (*S. pyogenes*)	WP_011017568.1	3.00E−64	100 (98)
15	8201	8470	Multispecies: hypothetical protein (*Streptococcus* sp.)	WP_002987593.1	8.00E−59	100 (98)
16	8480	8761	Phage protein (*S. pyogenes*)	WP_002987595.1	3.00E−44	100 (75)
17	8973	9323	Hypothetical protein STR01_48 (*Streptococcus* phage Str01)	APZ81912.1	5.00E−66	100 (95)
18	9323	9646	Hypothetical protein STR01_49 (*Streptococcus* phage Str01)	APZ81913.1	7.00E−49	99 (87)
19	9639	9869	Hypothetical protein AT55_00034 (*Streptococcus equi* subsp. *zooepidemicus* Sz4is)	KIS17986.1	7.00E−31	93 (74)
20	9866	10276	Endodeoxyribonuclease RusA (*Streptococcus dysgalactiae*)	WP_065359284.1	2.00E−94	98 (99)
21	10269	10649	Hypothetical protein STR01_52 (Streptococcus phage Str01)	APZ81908.1	6.00E−84	98 (99)
22	10704	11387	Hypothetical protein AT55_00030 (*Streptococcus equi* subsp. *zooepidemicus* Sz4is)	KIS17982.1	7.00E−165	99 (97)
23	11384	11767	Hypothetical protein (*Streptococcus dysgalactiae*)	WP_003058537.1	3.00E−77	100 (99)
24	11960	12439	Terminase small subunit (*Streptococcus dysgalactiae*)	WP_003058573.1	9.00E−97	100 (99)
25	12426	13739	Terminase (*Streptococcus dysgalactiae*)	WP_003058577.1	0	100 (99)
26	13751	15328	Capsid protein (*Streptococcus dysgalactiae*)	WP_003058556.1	0	100 (99)
27	15331	16479	Phage capsid protein (*Streptococcus dysgalactiae*)	WP_003058578.1	0	100 (99)
28	16626	17189	Scaffold protein (*Streptococcus dysgalactiae*)	WP_003058544.1	9.00E−99	99 (99)
29	17208	18086	Hypothetical protein (*Streptococcus dysgalactiae*)	WP_003058535.1	0	100 (95)
30	18097	18333	Hypothetical protein (*Streptococcus dysgalactiae*)	WP_003058596.1	2.00E−46	100 (98)
31	18341	18769	Hypothetical protein (*Streptococcus dysgalactiae*)	WP_003058548.1	2.00E−90	99 (90)
32	18753	19085	Minor capsid protein (*Streptococcus* phage Str01)	APZ81883.1	1.00E−66	94 (97)
33	19085	19444	Phage capsid protein (*Streptococcus hyovaginalis*)	WP_029691337.1	6.00E−77	92 (99)
34	19444	19863	Capsid protein (*Streptococcus cristatus*)	WP_048766652.1	8.00E−67	75 (96)
35	19856	20314	Major tail shaft protein (*Streptococcus equi* subsp. *zooepidemicus* Sz4is)	KIS17968.1	3.00E−73	89 (98)
36	20329	20697	Hypothetical protein (*Streptococcus anginosus*)	WP_049507620.1	4.00E−43	57 (99)
37	20697	21281	Hypothetical protein (*Streptococcus gallolyticus*)	WP_074628227.1	1.00E−94	71 (99)
38	21262	24969	Tail tape measure protein (*Streptococcus* phage Str01)	APZ81886.1	0	100 (93)
39	24966	26468	Tail protein (*Streptococcus dysgalactiae* and *Streptococcus equi* subsp. *zooepidemicus* Sz4is)	WP_003058481.1, KIS17964.1, WP_065359298.1	0	99 (99)
40	26472	28733	Tail endopeptidase (*Streptococcus* phage Str01)	APZ81887.1	0	99 (99)
41	28745	30610	Tail protein (*Streptococcus* phage Str01); hypothetical protein (*Streptococcus dysgalactiae*)	APZ81874.1, WP_003058489.1	0	99 (99)
42	30592	31089	Hypothetical protein STR01_19 (*Streptococcus* phage Str01)	APZ81904.1	2.00E−85	99 (85)
43	31055	31282	Hypothetical protein (*Streptococcus dysgalactiae*)	WP_037588167.1	3.00E−32	100 (75)
44	31284	31757	Holin (*Streptococcus* phage Str01)	APZ81878.1	3.00E−70	99 (91)
45	31750	32085	*N*-Acetylmuramoyl-L-alanine amidase (*Streptococcus dysgalactiae*)	WP_003058565.1	3.00E−74	100 (99)
46	32087	33532	Holin (*Streptococcus dysgalactiae*)	WP_065359301.1	0	99 (99)

[a]ORF, open reading frame.

included genes involved in regulation, DNA replication, packaging, structure, and bacterial lysis. Although a previous study reported low-level A25 hyaluronidase activity (26), no specific hyaluronidase gene was identified in the tail fiber cluster, consistent with the required supplementation of hyaluronidase to optimize A25 infection (1). The lysis module consisted of two holin genes and a putative peptidoglycan hydrolase; the first holin was predicted to be a type III with one predicted transmembrane domain, while the second was predicted to be a type I holin with three predicted transmembrane domain regions. The employment of two holin genes is not typical, having been reported previously in only a few phage genomes (27–31). The overall organization of the genome was found to be similar to that of many group A streptococcal prophages and differed significantly from the group C streptococcal lytic phage C1 (32).

The beginning portion of the A25 genome consisted of genes that are involved with regulation, DNA replication, and DNA processing. This region of the genome surprisingly was found to have a genetic sequence with near identity (99%) to the

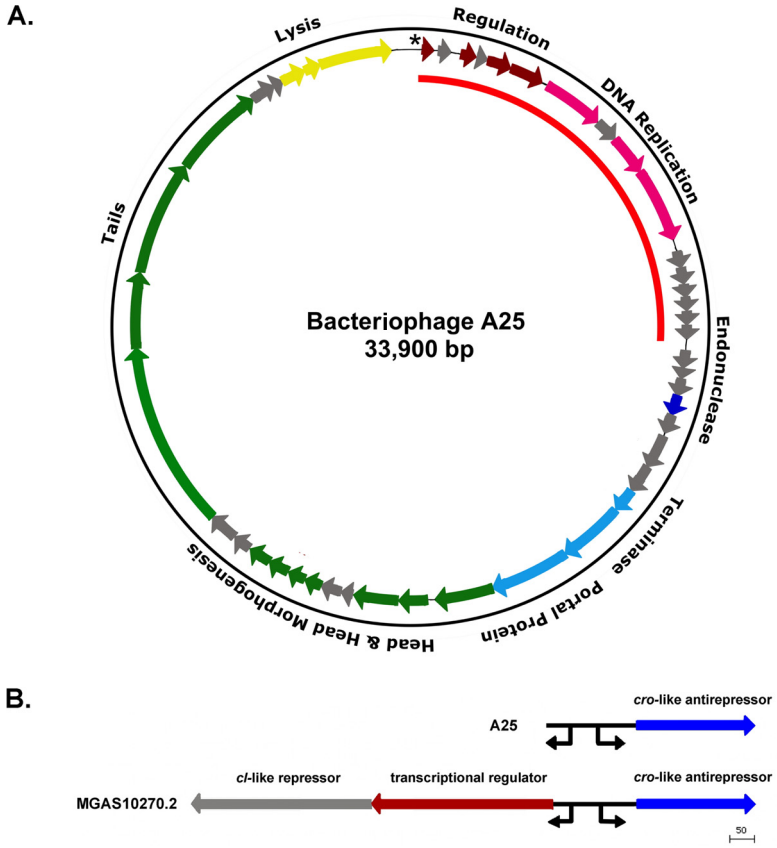

FIG 1 (A) Graphical representation of the A25 genome comprised of a predicted 46 genes that have modular organization characteristic of bacteriophages. The organizations of these functional modules were found to closely resemble those of lysogenic phages from group A streptococci. The coloring indicates the category of function with which the gene was found to have homology. The modules included those involved in regulation (dark red), DNA replication (pink), encoding of endonucleases (dark blue), genome packaging (light blue), structure (green), and lysis (yellow). An extremely interesting and unexpected find was the presence of a lysogenic regulatory *cro*-like antirepressor gene and the operator site (starred). Additionally, nearly identical homology of A25 and several lysogenic phages from genomic strains of group A streptococci occurred within the region indicated by the arced red line. (B) Graphical depiction of the residual *cro*-like antirepressor and operator region of A25 (upper line). This region has nearly identical homology of A25 to some group A streptococcal lysogenic phages, such as MGAS10270.2. As depicted, the two promoters (black arrows), i.e., the *cI*-like repressor promoter (gene colored red in the depicted portion of MGAS10270.2) and the *cro*-like antirepressor promoter as well as the *cro*-like antirepressor gene (blue) are all that remain. The *cI*-like repressor has been lost, however, indicating the escape from lysogeny undergone by A25.

same region in a group of resident *S. pyogenes* prophages: MGAS10270.2, MGAS10750.2, MGAS315.5, SPsP2, and unannotated phages from genome strains M3-b and STAB902 (indicated by the red arc in Fig. 1A). Within the beginning portion of this high-homology section, the A25 genome contains a residual operator site for controlling lysogeny in prophages (region indicated by an asterisk in Fig. 1A), including two predicted promoters. The first was found on the negative strand (base numbers 43 to 77) and would likely have served as the promoter for the now absent *cI*-like repressor, sharing 100% genetic homology to lysogenic phages MGAS10270.2, MGAS10750.2, SPsP2, and MGAS315.5 (Fig. 1B). On the positive DNA strand, the second promoter (base numbers 147 to 176) likely controls expression of the predicted *cro*-like antirepressor (first dark red arrow of the A25 genome in Fig. 1A). This genetic remnant argues that A25 is an escaped lysogen that is no longer able to integrate and excise from the bacterial chromosome, as the integrase and *cI*-like repressor (needed to silence the lytic portion of the phage genome) are no longer present. The escape from lysogeny by Gram-positive prophages has been described previously only in *Lactococcus*

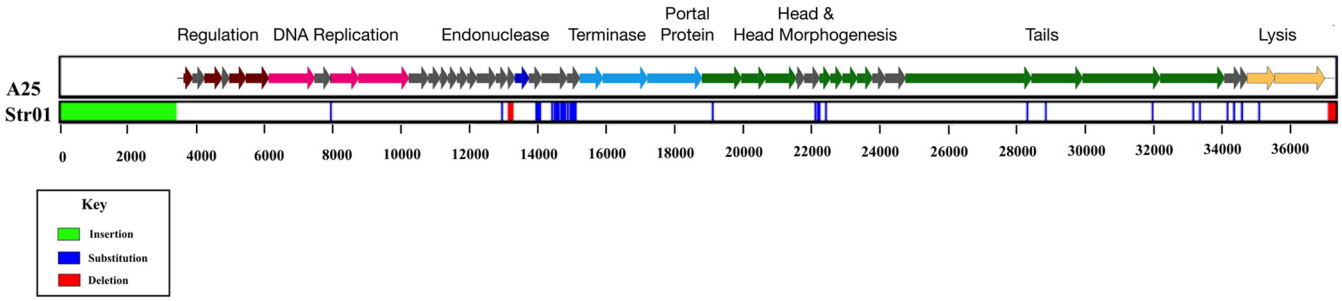

FIG 2 A25 and streptococcal phage Str01 homology. This base-by-base chart compares the Str01 genome (bottom box) to A25 (upper box). As shown, the large portion of differences between the two phages is within the beginning portion of the genome where Str01, a lysogenic phage, contains a module to facilitate lysogeny (depicted in the lower box as green). The majority of the two genomes are highly homologous (as shown in white in the bottom box), as there is only a small portion of substitutions (shown in the lower box as blue) or deletion events (shown in the lower box as red) occurring throughout the genomes. A clustering of substitutions was found within the module encoding endonuclease and terminase activity. An accumulation of deletions within Str01 was found at the very end of the genome around the lysis module.

lactis bacteriophage 31, in which the integrase gene was deleted (33) and in *Streptococcus thermophilus* (34, 35). Bacteriophage Str01 (GenBank accession no. KY349816.1), a recent addition to GenBank, was found to have high homology to A25, the two being colinear through most of their genomes (Fig. 2). Interestingly, this phage still contains the intact lysogenic regulatory elements, including integrase, although the Str01 integrase gene is distinct from the one present in the *S. pyogenes* genome prophages.

Phylogenetic analysis of the A25 genome reveals genetic mosaicism. Phage A25 is the first lytic phage genome of *S. pyogenes* to be reported, and due to the observed genetic organization and homology to genome prophages from *S. pyogenes*, we investigated the homology of the A25 genome to currently available annotated prophage genomes of various M-type *S. pyogenes* strains (Fig. 3). The majority of bacteriophages studied held some degree of homology to the A25 genome, with only a few lacking any homology to A25. The greatest homologies were found to cluster around prophages from M2, M3, and M4 strains, specifically prophages MGAS10270.2, MGAS315.5, SPsP2, and MGAS10750.2. All of the high-homology prophages were found to have homology within the beginning regions of the A25 genome, as described earlier, specifically within the regions encoding the residual lysogenic operator site, *cro*-like antirepressor, and genes involved in regulation, DNA replication, and endonuclease activity (Fig. 1A; region depicted as a red line).

During genome annotation, it became clear that the A25 genome was mosaic in nature (Fig. 4A). In addition to the above-mentioned homology to *S. pyogenes* prophages, homology was found to occur in lysogenic phages of other streptococcal species such as *Streptococcus pneumoniae* bacteriophage MM1 and *Streptococcus suis* bacteriophage SMP (Fig. 4). More specifically, A25 was found to have homology with MM1 within the modular regions involved in DNA packaging and head morphogenesis (Fig. 4B). SMP was determined to share a short amount of homology with A25 within the lysis module (Fig. 4A), more specifically within the lysin gene predicted to encode peptidoglycan hydrolase. Homology to the identified A25 holins previously described was also observed with phages from *S. dysgalactiae* subsp. *equisimilis* genomic strains SK1249 and UT 4234 DH, *S. iniae* genomic strain UEL-Sil, and *S. equi* subsp. *zooepidemicus* genomic strain Sz4is. The mosaic nature of the A25 genome suggests that escape from lysogeny was a more distant event with multiple rounds of recombination occurring with genetic material from phages infecting other streptococcal species.

Host range of A25 group A streptococci. The high degree of homology within the lysogeny modules of A25 and a number of the *S. pyogenes* genome prophages suggested that superinfection immunity might play a role in determining the A25 host range. While the majority of the strains tested (Table 2) were found to be susceptible to A25 infection (9 of 14 strains tested), the degree of susceptibility varied (Fig. 5). Among susceptible strains, the M12 strain K56 was observed to be the most susceptible

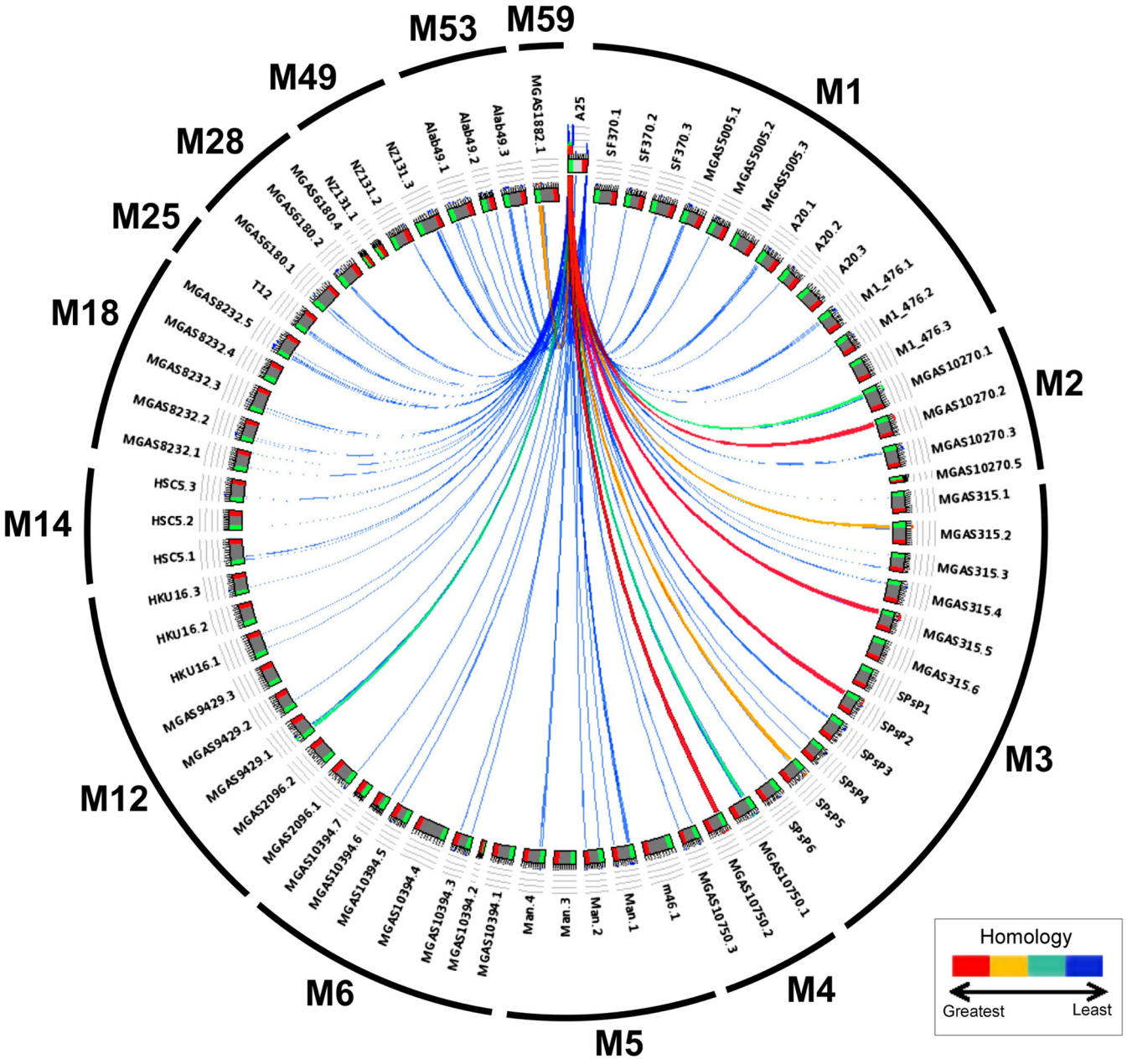

FIG 3 Phylogenetic analysis of A25 to prophages from genomic group A streptococcal strains. Lines connecting A25 (apex of the circle) to listed prophage genomes indicate homology. The degree of homology to A25 is indicated by the color of the line. The M type of the genome strain that contains the prophage listed is indicated on the outermost line of the circle. The beginning (green), middle (gray), and end (red) portions of each of the genomes are shown. The site where the line connects the A25 to the prophage indicates which portion of the genome shares homology with A25.

and, being the host strain for propagation of A25, served as a positive control. The two M6 strains (MGAS10395 and its isogenic pair CEM6Δ8, which has the *Streptococcus pyogenes* phage-like chromosomal island [SpyCI] removed from the genome) were the least susceptible. Interestingly, an increased susceptibility to A25 within SpyCI knockout strains CEM6Δ8 and CEM1Δ4 (SF370ΔSpyCIM1) was observed compared to their isogenic wild-type SpyCI-containing genomic strains MGAS10395 and SF370, respectively. Complete resistance was observed in a few strains (5 of the 14 tested). These included strains MGAS10270, MGAS10750, MGAS315, and the two M-type 49 strains NZ131 and CS101.

Mechanisms of resistance to A25. The A25 genome was compared to the clustered regularly interspaced short palindromic repeat(s) (CRISPR) database (36) to iden-

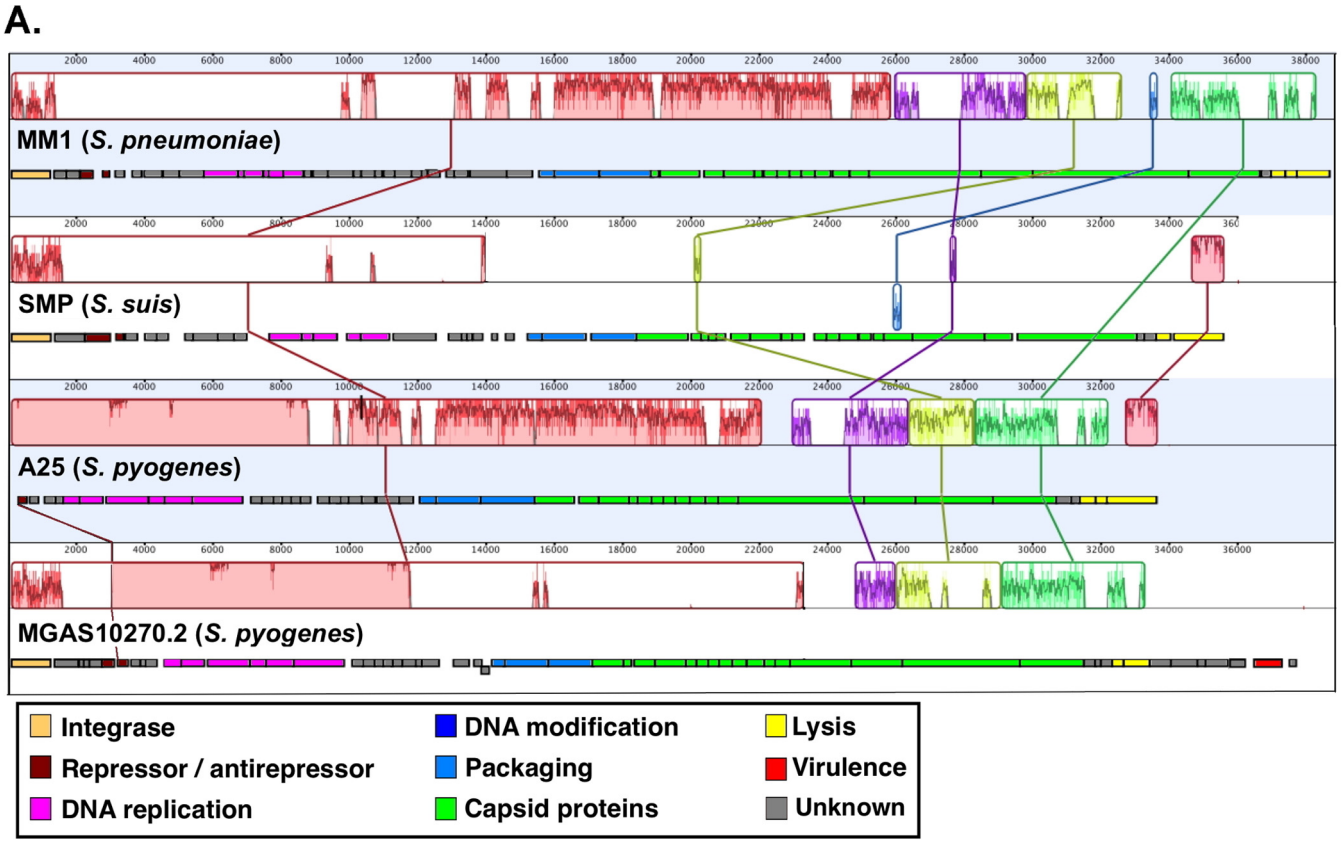

FIG 4 (A) Mauve alignment of the A25 genome demonstrates mosaicism. The A25 genome was found to contain homology with other bacteriophages from *S. pyogenes* and other streptococcal species. This mosaicism suggests that escape from lysogeny was not a recent event. Regions of homology are depicted by matching colors. The genome of each bacteriophage can be found below the mauve alignment diagram. The genes are colored by function as indicated. (B) Clustal W alignment of the A25 genome depicts the amount of homology with the MM1 and MGAS10270.2 genomes. The red shading depicts the location of homology among the compared genomes. The loss of lysogenic regulatory genes allowing for escape in A25 is shown as the white region in the A25 genome. MGAS10270.2 was found to contain close homology with A25 within the region of DNA replication and regulation, whereas MM1 was found to contain homology within the regions encoding the terminases, portal proteins, and head construction.

tify matching spacer sequences within the five resistant *S. pyogenes* strains; however, no CRISPR sequences were identified. Interestingly, matches were found to a CRISPR spacer occurring in several *emm1* strains, including susceptible strains SF370 and MGAS5005 (Table 3). This CRISPR spacer matched a sequence in the predicted promoter for gene A25_03, a Cro/cI family transcriptional regulator. The sensitivity to A25 in SF370 and MGAS5005 in spite of this spacer suggests that targeting this sequence by the CRISPR-Cas system may not be sufficiently protective. The A25-resistant strain NZ131 had no matches to the CRISPR database, but we were unable to rule out this mechanism of resistance in M49 strain CS101, as its genome sequence, to date, has not been reported.

Three of the five resistant strains, however, did share a trait: a prophage with high homology to A25 within the beginning portion of the phage sequence (red arc in Fig. 1). From this observation, we decided to test the hypothesis that the *cI*-like repressors from

TABLE 2 *Streptococcus pyogenes* strains used in this study

Strain	M type	Isolate source	Reference or source
SF370	1	Wound	64
CEM1Δ4[a]	1	Isogenic derivative of SF370	50
MGAS5005	1	CSF	65
MGAS10270	2	Pharyngitis	66
MGAS315	3	Toxic shock	67
MGAS10750	4	Pharyngitis	66
MGAS10394	6	Pharyngitis	68
CEM6Δ8[a]	6	Pharyngitis	C. W. Euler
K56	12	Pharyngitis	69
Rosenbach	25		ATCC 12204
MGAS6180	28	Invasive infection	70
CS101	49	Glomerulonephritis	71
NZ131	49	Glomerulonephritis	72
ALAB49	53	Impetigo lesion	73

[a]Isogenic derivative of genome strain cured of SpyCI.

these resident high-homology prophages are suppressing A25 replication upon infection, thus acting as a protective mechanism against superinfection (Fig. 6). The *cI*-like repressor and entire operator site from high-homology prophage MGAS10270.2 was cloned into site-specific integration vector p7int (37), omitting the *cro*-like repressor and any of the other downstream genes (Fig. 7A). This plasmid, containing the bacterio-

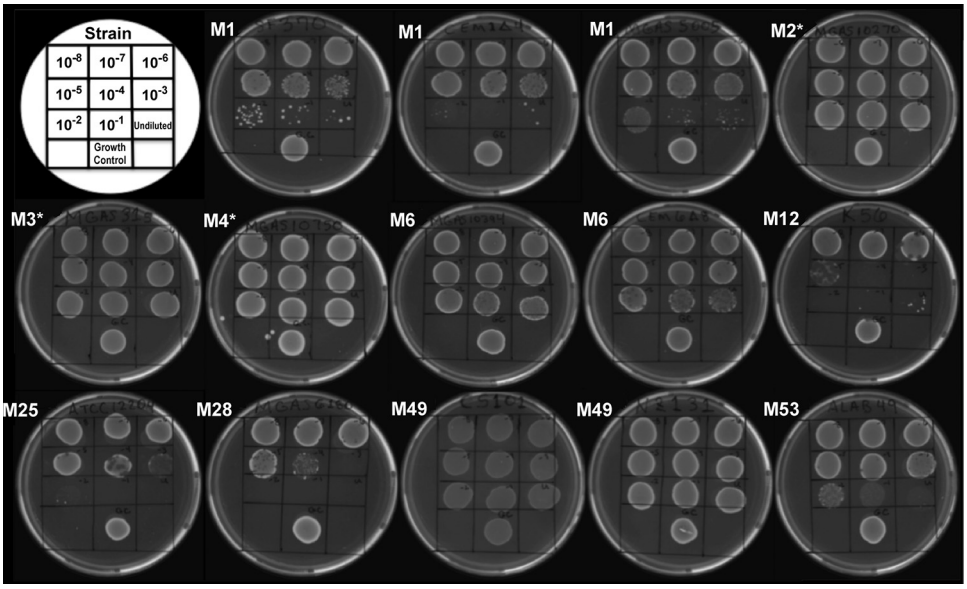

FIG 5 Group A streptococcal phage A25 host range. Overnight cultures of each strain were incubated with 2.8×10^8 PFU/ml undiluted or serially diluted phage A25 (strain M types are shown next to each plate). The plating scheme is shown in the upper left-hand corner of the image; a growth control with only diluent and bacteria was included for each strain tested. Strains were determined to be susceptible if plaques were visible. Strains in top row (from left to right): SF370 (M1), CEM1Δ4 (SF370 ΔSpyCIM1) (M1), MGAS5005 (M1), and MGAS10270 (M2). Strains in middle row (from left to right): MGAS315 (M3), MGAS10750 (M4), MGAS10394 (M6), CEM6Δ8 (MGAS10394 ΔSpyCIM6) (M6), and K56 (positive control; M12). Strains in bottom row (from left to right): ATCC 12204 (M25), MGAS6180 (M28), CS101 (M49), NZ131 (M49), and ALAB49 (M53). Strain K56 is the strain used for propagation of A25 and serves as a positive control. The majority of strains tested (9 of 14) were susceptible to A25 infection, with the level of susceptibility varying greatly. M types with an asterisk indicate the presence of a prophage containing near identity to the A25 genome within the remnant lysogeny regulatory module; all of these strains were completely resistant to A25 infection. Both M49 strains were resistant to A25. Strain NZ131 does not contain a prophage with homology to A25; the prophage carriage of strain CS101 is unknown, as its genome has not been sequenced to date. However, a screen by PCR for the A25 *cro*-like repressor and lysogenic operator region failed to amplify a product, suggesting that CS101 also lacks a high-homology prophage. Thus, A25 resistance in the M49 strains must employ another mechanism of protection instead of superinfection immunity. The image is representative of at least two biological replicates for each strain.

TABLE 3 CRISPR spacer sequences with homology to regions of the A25 genome

Organism	M type	RefSeq accession no.	Spacer sequence ID	Identity	E value
S. pyogenes strain SF370	1	NC_002737	NC_002737_1_5	1	9.00×10^{-7}
S. pyogenes strain MGAS5005	1	NC_007297	NC_007297_1_3	1	9.00×10^{-7}
S. pyogenes strain A20	1	NC_018936	NC_018936_1_3	1	9.00×10^{-7}
S. pyogenes strain MTB314	1	NZ_AP014585	NZ_AP014585_1_3	1	9.00×10^{-7}
S. pyogenes strain 5448	1	NZ_CP008776	NZ_CP008776_4_1	1	9.00×10^{-7}
S. pyogenes strain FDAARGOS_149	1	NZ_CP014027	NZ_CP014027_5_1	1	9.00×10^{-7}
S. pyogenes strain NGAS332	114	NZ_CP010449	NZ_CP010449_1_3	1	9.00×10^{-7}
Streptococcus dysgalactiae subsp. *equisimilis*		NC_012891	NC_012891_2_5	1	9.00×10^{-7}
S. dysgalactiae subsp. *equisimilis*		NC_012891	NC_012891_2_16	1	9.00×10^{-7}
S. dysgalactiae subsp. *equisimilis* strain ATCC 12394		NC_017567	NC_017567_2_19	1	1.00×10^{-4}
Streptococcus thermophilus strain ASCC 1275		NZ_CP006819	NZ_CP006819_7_6	0.96	1.90×10^{-2}
S. thermophilus strain MN-BM-A02		NZ_CP010999	NZ_CP010999_7_6	0.96	1.90×10^{-2}
S. thermophilus strain KLDS SM		NZ_CP016026	NZ_CP016026_3_6	0.96	1.90×10^{-2}
S. thermophilus strain ND07		NZ_CP016394	NZ_CP016394_2_7	0.96	1.90×10^{-2}
Dictyoglomus thermophilum strain H-6-12		NC_011297	NC_011297_3_67	0.93	6.60×10^{-2}
Clostridium difficile strain CD196		NC_013315	NC_013315_11_4	0.92	0.23
C. difficile strain R20291		NC_013316	NC_013316_11_4	0.92	0.23
C. difficile strain 2007855		NC_017178	NC_017178_11_4	0.92	0.23
C. difficile strain BI1		NC_017179	NC_017179_11_4	0.92	0.23
Leptolyngbya sp. strain O-77		NZ_AP017367	NZ_AP017367_26_110	1	0.8
Methanothermobacter sp. strain CaT2		NZ_AP011952	NZ_AP011952_15_28	0.95	0.8
Saprospira grandis strain Lewin		NC_016940	NC_016940_1_64	0.95	0.8
Brachyspira murdochii strain DSM 12563		NC_014150	NC_014150_2_44	0.89	0.8

phage T12 integrase and capable of site-specific integration at a highly conserved tmRNA site, was introduced into A25-susceptible strains SF370, SF370ΔSpyCIM1, and ATCC 12204. While the wild-type strains and vector-only controls were completely susceptible to A25 with complete lysis following exposure to undiluted A25, all three constructed strains containing the operator region and *cI*-like repressor from MGAS10270.2 became completely resistant to A25 infection, reversing susceptibility (Fig. 7B).

Phage A25 DNA packaging. Phage DNA packaging and transduction are linked to the discrimination of the terminase protein for its substrate. Both *pac*- and *cos*-type packaging mechanisms rely upon terminases in order to cleave concatemeric DNA to allow linear DNA packaging to occur. However, transducing phages typically employ *pac*-type substrate recognition, since the presence of suitable *pac* sequence homologs in the bacterial chromosome is more likely than that of *cos* sequence homologs, which must additionally be properly spaced for packaging to occur. The amino acid sequence of terminase large subunits is often conserved among tailed bacteriophages that

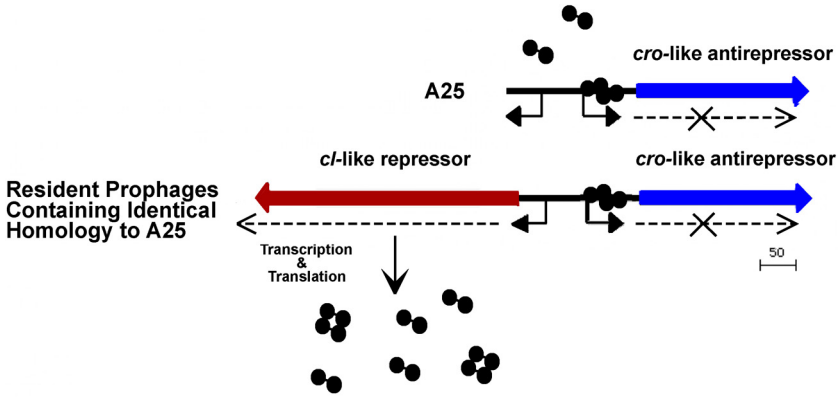

FIG 6 Proposed A25 resistance mechanism by prevention of superinfection by resident prophages: MGAS 315.5, MGAS10750, or MGAS10270. A25 and the above-listed resident prophages share nearly identical to identical homology within the residual lysogeny portion of the A25 genome. This includes the operator site containing the two promoters (designated by the closed arrows on the genome). As these sites are identical, the repressor from the resident prophages (shown in black as monomers and dimers) is likely to bind to the operator sites of not only the prophage but also A25 within these sites, inhibiting transcription (dotted lines) of the downstream genome. This event stops the replicative process, thus terminating early on an A25 infection.

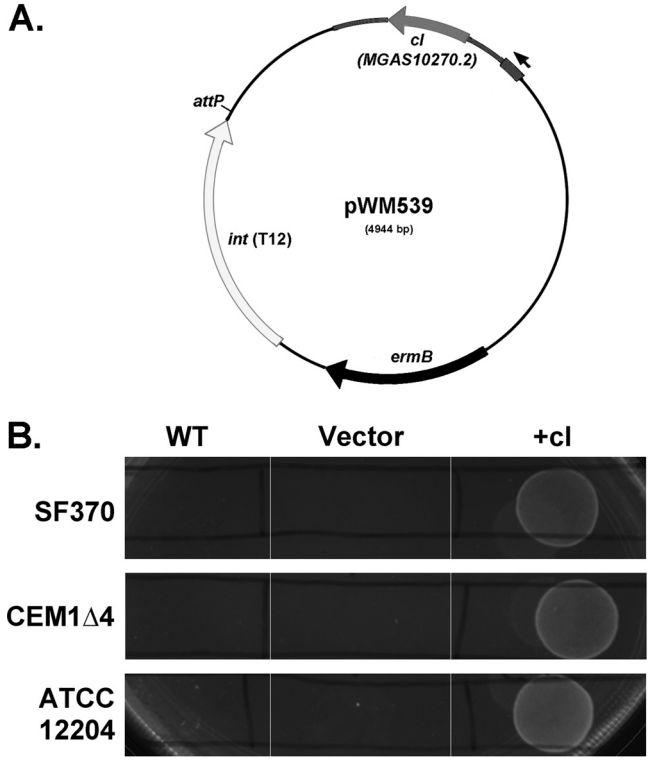

FIG 7 (A) Plasmid construct with *cI*-like repressor from MGAS10270.2. The *cI*-like repressor and operator site (labeled arrow and adjacent wider areas) from MGAS10270.2 was cloned into the vector plasmid p7int, a plasmid previously described containing the T12 integrase that undergoes site-specific integration at a highly conserved tmRNA site (32) to create pWM539. pWM539 was used to electroporate wild-type A25-susceptible strains: SF370, SF370 ΔspyCIM1, and ATCC 12204. (B) Complementation with the *cI*-like repressor and operator region from high-homology prophage MGAS10270.2 elicits A25 resistance. Wild-type (WT)-susceptible strains SF370, CEM1Δ4 (SF370ΔSpyCIM1), and ATCC 12204 were complemented with p7int vector only (Vector) or with p7int with the *cI*-like repressor from prophage MGAS10270.2 that was found to have high homology to the beginning portion of the A25 genome (+cI). Overnight cultures of each strain were coincubated with 20 μl of culture and 180 μl of undiluted ($\sim$2.8 $\times$ 10^8 PFU/ml) A25 for 15 min with 20 μl spotted onto the corresponding grid of the modified N6-Z6 agar medium. Once dried, plates were incubated overnight at 37°C with 5% CO_2 and imaged the following day. Resistance to A25 infection occurred only within the strains containing the *cI*-like repressor and operator region of MGAS10270.2 prophage, experimentally confirming immunity to superinfection as the resistance mechanism in *S. pyogenes* strains containing high-homology prophages to the beginning portion of the A25 genome.

employ either *cos*- or *pac*-type packaging mechanisms (38, 39), allowing for discrimination of packaging type by phylogenetic analysis of the terminase (large subunit). In order to better understand why A25 is a highly efficient transducing phage, we investigated the packaging method utilized by A25. Amino acid alignment of the A25 terminase large subunit to those from Gram-positive bacteriophages with known packaging mechanisms with subsequent phylogenetic tree construction revealed that A25 grouped with phages predicted to employ *pac*-type packaging mechanisms (Fig. 8A). More specifically, A25 was found to be most closely related to *S. pneumoniae* prophage MM1. Although the transduction efficiency of MM1 remains unknown, it is likely to be a high-efficiency transducing phage due to the relatedness to A25 within this region essential for packaging. Further confirming our phylogenetic prediction, we were able to detect a submolar *pac* sequence-containing fragment (Fig. 8B) via restriction endonuclease digestion using PvuII, which is a frequently occurring manifestation of the *pac*-type mechanism (40).

DISCUSSION

The conversion of a lysogen to a lytic phage through loss of the ability to integrate has been observed several times in the literature (41–46). In the case of A25, this

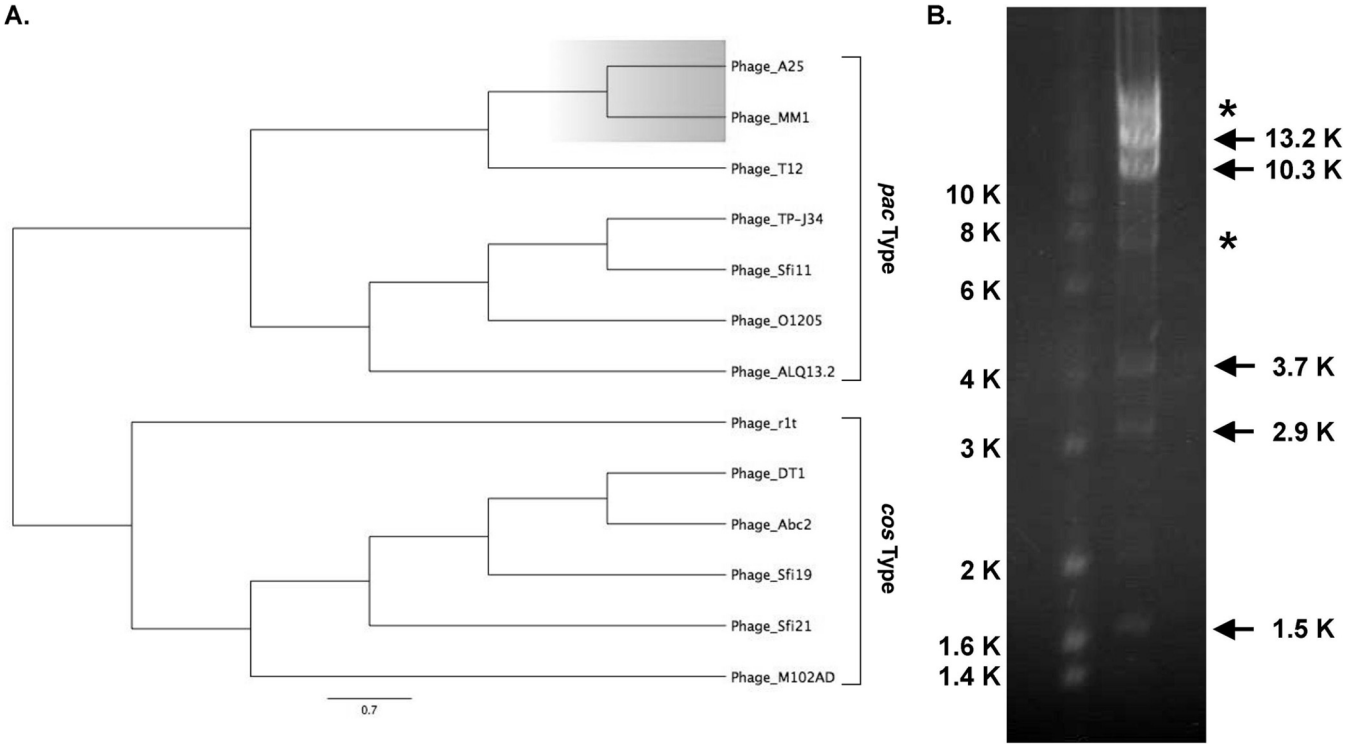

FIG 8 (A) Phage A25 is predicted to use *pac*-type DNA packaging. Phylogenetic tree construction of the terminase large subunit following Clustal alignment to bacteriophages with known packaging mechanisms and infecting Gram-positive bacteria predicts that A25 employs *pac*-type DNA packaging. The shaded region indicates the location of A25 within the group. A25 was found to group with *pac*-type packaging bacteriophages and was phylogenetically separate from *cos*-type packaging bacteriophages. (B) The presence of submolar digest fragments confirms *pac*-type packaging mechanism in A25. A frequent consequence of *pac*-type packaging is the presence of submolar restriction digestion fragments, which result from circularly permuted and terminally redundant sequences following genome replication (40). A25 genomic DNA was digested with PvuII, and the fragments were separated by reversed-field gel electrophoresis. The digestion products predicted by the genome sequence are indicated by the arrows on the right. Asterisks indicate the presence of submolar digestion fragments; these regions are associated with the region of the genome that would be expected to contain the *pac* sequence. Molecular weight markers in kilobases are shown on the left.

conversion probably was not a recent event since its genome is a mosaic of genetic material from phages of several streptococcal species, including *S. pneumoniae*. The presence of a unique integrase gene (acquired from a group D streptococcal prophage) was previously described within the closely related phage Str01, further supporting the theory of distant lysogeny escape, as the rescued lysogeny phenotype was acquired after the multiple rounds of recombination occurred. Further, the phage A25 genome revealed that lytic and lysogenic bacteriophages of *S. pyogenes* can share ancestry. This bacteriophage historically has been classified as a lytic phage capable of high-frequency generalized transduction. Obligatory lytic bacteriophages differ from lysogenic bacteriophages by lacking the genes necessary to facilitate site-specific integration and excision within the bacterial genome. Lysogenic phages also contain genes that regulate lysogenic or lytic cycling of the phage, known as the *cI*-like repressor and *cro*-like antirepressor in those phages employing typical lambdoid regulation. These helix-turn-helix proteins form homodimers, which bind with various levels of affinity to the overlapping promoter sites for each of the regulatory genes, thus controlling the expression of genes involved in lysogeny or the lytic cycle. Interestingly, sequencing revealed residual lysogeny regulatory elements, i.e., the *cro*-like antirepressor gene and operator site that included the promoter for the *cro*-like antirepressor and an orphan promoter for the now-lost *cI*-like repressor, indicating that lytic A25 had escaped lysogeny. This region was found to have high homology to prophages from *S. pyogenes*. Additionally, the A25 genome was found to have a modular configuration that was organized similarly to *S. pyogenes* prophages. Further phylogenetic studies revealed that A25 held nearly identical homology with prophages from M2, M3, and M4 strains,

including prophages MGAS315.1, MGAS10270.2, MGAS10750.2, and other resident prophages from genomic strains M3-b and STAB902. This area of high homology not only included the residual lysogeny module but also continued through the modules involved in regulation, DNA replication, and endonuclease activity. This region of homology provides the potential for recombination events to occur, thus facilitating homologous recombination between the lytic portions of the resident prophage and A25. As group A prophages commonly harbor virulence factors within the lytic portion of their genome, this event would lead to the transfer of virulence genes to the A25 genome and, upon successful replication, allow dissemination of the virulence genes. This scenario in theory could extend beyond group A streptococci, as A25 is known to have a wide host range, including groups C, G, L, and H, allowing both intra- and intertransduction events to occur.

This region of shared regulatory elements was found to influence strain susceptibility, as all three tested strains (MGAS315, MGAS1270, and MGAS10750) containing prophages with high homology to A25 within the lysogenic regulatory module were resistant to A25 infection. No A25-specific CRISPR sequences were found to exist within these genomes, suggesting that the mechanism of resistance stemmed from the *cI*-like repressor of the resident high-homology prophages. While maintaining lysogeny for the resident prophages through *cI*-like repressor expression, the homodimer *cI*-like protein was able to silence A25 infection, facilitating resistance by providing immunity to superinfection. All three strains tested (SF370, SF370ΔSpyCIM1 [CEM1Δ4], and ATCC 12204), wild-type susceptible to A25, were complemented with the *cI*-like repressor and lysogenic operator site. With this complementation, all three became completely resistant to A25 infection, experimentally confirming superinfection immunity as the mechanism of action conferring resistance to A25. There were two additional strains, both M49 strains, that did not contain prophages with high homology to A25. This was confirmed for NZ131 by blasting the sequenced genome to A25 and through searching the CRISPR database for A25 spacer-specific sequences. As the genome for CS101 is not sequenced, we are unable to rule out CRISPR-Cas-mediated resistance but through screening by PCR did determine that CS101 failed to contain prophages with high homology to A25 using primers specifically screening for the *cI*-like repressor and operator region. As both M49 types were resistant, we propose that specific cell surface moieties may form some barrier blocking A25 adsorption, as literature has indicated that the A25 receptor is peptidoglycan within the cell wall (47–49). Screening more M49 strains is needed, however, to further strengthen this hypothesis.

Another interesting find of this study was in comparing A25 susceptibility among isogenic pairs that were with and without the phagelike chromosomal island known as *Streptococcus pyogenes* chromosomal island (SpyCI). These isogenic pairs were M1 strains SF370 and SF370ΔSpyCIM1 and M6 strains MGAS10395 and MGAS10395ΔSpyCIM6. In SF370, our laboratory has demonstrated that SpyCIM1 is involved in altering global transcription (50). Genes upregulated in the presence of SpyCIM1 include *emm* and capsule gene *hasB*. Both of these genes can alter the surface of bacterial cells. When SpyCI was present, an increase in resistance (in the form of more-resistant colonies in SF370 and less plaque formation in MGAS10395) was observed. The global transcriptional changes conferred by the phagelike chromosomal island held implications for A25 infection, as literature has demonstrated that increase in M protein as well as overproduction of capsule leads to A25-resistant cells and that A25 infections of susceptible group A streptococcal strains can lead to selection of mucoid colonies that are resistant to A25 infection (2, 48). Additionally, these A25-resistant mucoid colonies were observed to be more virulent than A25-susceptible colonies in a murine infection model, leading to more-rapid killing (2, 51). Although the M protein and capsule have been demonstrated not to be receptors for A25, the increased production of M protein and capsule was found to be sufficient to block adsorption during A25 infection (2, 5, 48, 52). These observations argue that phage-bacterium interactions can be dynamic and have a multitude of implications (53).

One of the most important factors that may explain the transduction capabilities of

A25 is the packaging mechanism employed. There are two known packaging mechanisms that are utilized by bacteriophages: cohesive end (*cos*) and headful type packaging (*pac*) types. DNA cleavage, facilitated by terminases, is a necessary step to initiate and to terminate bacteriophage genomic packaging. For both *cos*- and *pac*-type mechanisms, the DNA cleavage that begins the packaging process is sequence specific (39); however, the termination of packaging differs between these mechanisms. For *cos*-type packaging, DNA cleavage that terminates packaging is also sequence specific, but for *pac*-type packaging it is not. Instead, *pac*-type mechanisms are based on volume size packaged within the capsid and less on site-specific termination sites (39). This facilitates a less stringent mechanism for packaging, which holds implications for transduction efficiencies. Not all bacteriophages that employ the *pac*-type method of genomic packaging are highly efficient transducing phages, as stringency can vary, but there is a correlation among high-efficiency transducing phages employing *pac*-type packaging mechanisms. A25 is historically known to be a highly efficient transducing bacteriophage. In this regard, our phylogenetic *in silico* packaging predictions were of little surprise. A25 was found to group with known *pac*-type packaging phages and was most closely related to *S. pneumoniae* bacteriophage MM1. Our previous phylogeny studies found MM1 to have homology with A25 within the region of DNA packaging and head morphogenesis. Although the transduction efficiency of MM1 has not been described, we predict, as with A25, that this bacteriophage is likely a high-efficiency transducing phage due to the large-subunit terminase homology among these two phages.

The results of this study underscore the value of group A streptococci bacteriophage genome sequencing to better understand the origins of, and the roles in horizontal gene transfer played by, these elements. A25's ability to infect a multitude of streptococcal species allows for genetic recombination with other streptococcal phages, facilitating genetic diversity within the A25 genome. As A25 was found to have high homology with resident group A prophages, which commonly contain virulence factors, homologous recombination followed by horizontal gene transfer is a plausible explanation for the movement of virulence factors and other genetic traits from group A to group C and G streptococci via phage transduction. This has clinical implications, as groups C and G are known human pathogens (54). Further research is necessary to determine whether similar themes exist among other clinically relevant bacteria or if this phenomenon occurs among group A streptococci in order to promote genetic diversity.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in this work are listed in Table 2. Modified P-broth medium (broth no. 1) for A25 propagation was prepared as previously described (5, 12) with the addition of 1% heat-inactivated horse serum (56°C for 30 min) and 1.36% (wt/vol) hyaluronidase extracted from bovine testes (Sigma-Aldrich, St. Louis, MO). Z6 medium for soft agar overlays was prepared as described previously (48) but with the omission of hyaluronidase. The solid agar medium used for the host range studies and for A25 lysate quantification was a modification of the Z6 recipe and N6 recipe previously described (48, 49). The recipe is as follows: 4% protease peptone number 3, 0.2% yeast extract, 0.88% agar, and 0.2% Tris. To this autoclaved mixture, sterile 0.2% glucose, 0.02% $CaCl_2$, and 6.8% hyaluronidase were added to the medium cooled to 50°C. Once mixed, 25 ml of medium was sterilely poured into plates and allowed to solidify. *S. pyogenes* strains were cultured in modified P broth and incubated at 37°C with 5% CO_2 unless otherwise stated.

A25 propagation. An overnight culture of *S. pyogenes* K56 (ATCC 14919) was diluted 1:10 into prewarmed modified P broth and incubated at 30°C until mid-log phase ($A_{600\ nm}$ = 0.35 to 0.45). A25 (ATCC 12204B1) phage lysate was added to achieve a final concentration of 1×10^8 PFU/ml. The mixture was incubated for 4 h at 30°C with gentle shaking at 125 rpm and subsequently spun for 15 min at 5,000 × *g* and 4°C to pellet bacterial debris. The supernatant was filtered using a 0.2-μm filter and stored at 4°C.

A25 DNA isolation and purification. To isolate A25 phage DNA, a lysate containing ~10^8 to 10^9 PFU/ml was treated with 7.5 μg/ml of DNase I (Sigma-Aldrich, St. Louis, MO) and RNase A (Sigma-Aldrich, St. Louis, MO) each for 30 min at 37°C to remove contaminating bacterial nucleic acid. The lysate was subsequently centrifuged at 40,000 × *g* for 2 h at 4°C, and the phage pellet was suspended in fresh P broth and allowed to resuspend completely by slanted storage at 4°C overnight. For phage capsid lysis, resuspended A25 was treated with 0.1 mg/ml of proteinase K (Amresco LLC, Solon, OH) and 0.5% (wt/vol) SDS, mixed, and incubated in a 56°C water bath for 1 h. Once cooled to room temperature, a standard

phenol-chloroform DNA extraction method was performed (55) followed by precipitation using 0.1 volume of 3 M sodium acetate, pH 5.2, and 0.7 volume of isopropanol for at least 30 min at −20°C (if necessary, samples were stored at −20°C until needed). DNA pellets were washed twice with 70% ethanol and then resuspended in nuclease-free water. DNA was further purified using the ZR-96 Genomic DNA Clean & Concentrator-5 purification kit (Zymo Research, Irvine, CA). Quantification and purity of DNA were assessed using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

A25 genome sequencing and annotation. Sequencing was performed by the University of Oklahoma Health Sciences Center Laboratory for Molecular Biology and Cytometry Research, using the Illumina MiSeq (Illumina Inc., San Diego, CA) sequencing platform with paired-end 250-bp sequencing at high coverage (~2,400-fold coverage; total number of reads, 389,342). Fifty nanograms of total A25 genomic DNA was used to generate the DNA library, observing the Nextera DNA library kit protocol (Illumina Inc., San Diego, CA). To ensure library quality and insert size, the library was run on the Agilent High Sensitivity DNA chip (Agilent Technologies Inc., Santa Clara, CA). To ensure sequencing quality, the samples were also spiked with 10% phiX library. Raw sequence data were aligned and analyzed using CLC Genomics Workbench (Qiagen Inc., Germantown, MD). Due to the relatively small size of the genome, no further sequencing was required, as no gaps in sequencing occurred. Genome annotation was performed using software packages NCBI BLASTX (56), PHAST (57), and Artemis (58). Promoter predictions were performed using the PePPER webserver (59). Circular genomic configuration was confirmed through sequencing of PCR using the junction primers A25.2-L, 5′-GGAAAACGGTTCTTTGG ACA, and A25.2-R, 5′-CTGGCAATTCCTTTGGAAAA, using the following PCR cycling parameters: an initial denaturing step of 95°C for 3 min followed by 35 cycles of 95°C for 15 s, 53°C for 30 s, and 72°C for 2 min. A final elongation step of 72°C for 5 min was included before holding at 10°C until use.

Phylogenetic analysis. A25 and group A streptococcal prophage homology studies using the Circoletto map construction were performed using the Circos software (60). Mauve and multiple bacteriophage genome alignments were conducted using Geneious version 6.0.6 (61) and Base by Base software (62). These were performed to assess the mosaicism of the A25 genome. For A25 DNA packaging prediction studies, Geneious 6.0.6 was used for ClustalW alignment and phylogenetic tree construction of the terminase large subunit. This packaging prediction strategy has been previously described (39). Briefly, bacteriophages used for terminase large-subunit comparison included bacteriophages of Gram-positive organisms with known packaging mechanisms. Diagrams of the A25 genome and the proposed novel A25 resistance mechanism were constructed using the Gene Construction kit 4.0 (63) and Graphic Converter 9 (Lemke Software GMBH, Peine, Germany). To determine the presence of A25-specific CRISPR spacer sequences within genomic strains of *S. pyogenes* utilized within this study (Table 2), the A25 genome was compared against the CRISPR database (http://crispr.i2bc.paris-saclay.fr) (36) using an E value threshold of one.

Detection of submolar restriction endonuclease digestion fragments. One microgram of purified A25 DNA was digested with excess restriction endonuclease PvuII (20 units; New England BioLabs Inc., Ipswich, MA) to ensure complete digestion. The digestion protocol was performed according to the manufacturer's instructions. For band separation, the following conditions of pulsed-field electrophoresis were used: forward pulse, 66 ms; reverse pulse, 22 ms; 3/h ramp with 150 V, using a 0.8% agarose gel in 0.5× Tris-borate-EDTA (TBE) for 5 h. The gel was stained for 1 h in ethidium bromide prior to imaging.

Titration and *S. pyogenes* host range screen to A25 infection. A25 titers were determined using the double agar overlay method as previously described (49). Briefly, using Z6 soft agar as an overlay, serial dilutions of 10^{-5} to 10^{-7} were performed using phosphate-buffered saline as diluent. Equal volumes of 0.1 ml of an overnight K56 culture and A25 lysate were combined, gently vortexed, and allowed to incubate at 37°C for 15 min. To this, 3 ml of 46°C molten sterile Z6 agar was added and mixed, and the mixture was poured onto the modified Z6-N6 agar plate. To determine the A25 host range within commonly used *S. pyogenes* strains, overnight cultures of strains listed in Table 2 were incubated for 15 min with serial dilutions ranging from 10^{-1} to 10^{-8} of A25 lysate; the starting undiluted A25 lysate contained on average 2.8×10^8 PFU/ml. These cultures were subsequently dripped onto the corresponding grid of Z6-N6 modified agar medium. The plates were incubated overnight and imaged the following day using Flash & Go imager (Neu-tec group Inc., Farmingdale, NY). A growth control with only diluent was included. This experiment consisted of two biological replicates. Susceptibility to A25 infection was defined by the presence of observable plaques.

***cI*-like repressor complementation in wild-type A25-susceptible strains SF370, SF370 ΔSpyCIM1, and ATCC 12204.** The *cI*-like repressor and operator region was amplified by PCR from the beginning genome A25 high-homology prophage MGAS10270.2 using the following primers: cI-L, 5′-CCACATTGTGTCTGGGAACT, and cI-R, 5′-ACTTGCTCCTCTCTTAATTTGTTTT. The cycling parameters used included an initial 94°C denaturing step for 3 min followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s. A further 72°C 5-min step was included for elongation, and then the samples were maintained at 10°C until further use. This was subsequently cloned into the vector plasmid p7int (37) that was cut using SmaI restriction enzyme (New England BioLabs, Ipswich, MA). The *cI*-like repressor and operator PCR construct was ligated in using T4 DNA Ligase (Monserate, San Diego, CA). The above-mentioned strains were then electroporated either with intact p7int vector only or with MGAS10270.2 *cI*-like repressor region cloned p7int. Strains were confirmed by PCR and sequencing.

Accession number(s). The A25 genome data were deposited into GenBank and are available through the National Library of Medicine (accession number KT388093.1).

ACKNOWLEDGMENTS

We thank A. Gillaspy and the Laboratory for Molecular Biology and Cytometry Research at OUHSC for assistance in the sequencing of the A25 genome. We also thank C. W. Euler, Rockefeller University, for providing *S. pyogenes* strain CEM6Δ8.

REFERENCES

1. Maxted WR. 1952. Enhancement of streptococcal bacteriophage lysis by hyaluronidase. Nature 170:1020–1021.
2. Maxted WR. 1955. The influence of bacteriophage on Streptococcus pyogenes. J Gen Microbiol 12:484–495. https://doi.org/10.1099/00221287-12-3-484.
3. Read SE, Reed RW. 1972. Electron microscopy of the replicative events of A25 bacteriophages in group A streptococci. Can J Microbiol 18:93–96. https://doi.org/10.1139/m72-015.
4. Fischetti VA, Barron B, Zabriskie JB. 1968. Studies on streptococcal bacteriophages. I. Burst size and intracellular growth of group A and group C streptococcal bacteriophages. J Exp Med 127:475–488.
5. Friend PL, Slade MD. 1966. Characteristics of group A streptococcal bacteriophages. J Bacteriol 92:148–154.
6. Malke H. 1970. Characteristics of transducing group A streptococcal bacteriophages A 5 and A 25. Arch Gesamte Virusforsch 29:44–49. https://doi.org/10.1007/BF01253879.
7. Moynet DJ, Colon-Whitt AE, Calandra GB, Cole RM. 1985. Structure of eight streptococcal bacteriophages. Virology 142:263–269. https://doi.org/10.1016/0042-6822(85)90334-4.
8. Colon AE, Cole RM, Leonard CG. 1972. Intergroup lysis and transduction by streptococcal bacteriophages. J Virol 9:551–553.
9. Colon AE, Cole RM, Leonard CG. 1971. Lysis and lysogenization of groups A, C, and G streptococci by a transducing bacteriophage induced from a group G Streptococcus. J Virol 8:103–110.
10. Leonard CG, Colon AE, Cole RM. 1968. Transduction in group A streptococcus. Biochem Biophys Res Commun 30:130–135. https://doi.org/10.1016/0006-291X(68)90459-2.
11. Malke H, Kohler W. 1973. Transduction among group A streptococci: transducibility of strains representative of thirty different M types. Zentralbl Bakteriol Orig A 224:194–201.
12. Wannamaker LW, Almquist S, Skjold S. 1973. Intergroup phage reactions and transduction between group C and group A streptococci. J Exp Med 137:1338–1353. https://doi.org/10.1084/jem.137.6.1338.
13. Malke H. 1970. Resistance pattern and genetics of erythromycin-resistant mutants of Streptococcus pyogenes. J Gen Microbiol 64:353–363. https://doi.org/10.1099/00221287-64-3-353.
14. Skjold SA, Maxted WR, Wannamaker LW. 1982. Transduction of the genetic determinant for streptolysin S in group A streptococci. Infect Immun 38:183–188.
15. Malke H. 1973. Phage A25-mediated transfer induction of a prophage in Streptococcus pyogenes. Mol Gen Genet 125:251–264. https://doi.org/10.1007/BF00270747.
16. Kalia A, Spratt BG, Enright MC, Bessen DE. 2002. Influence of recombination and niche separation on the population genetic structure of the pathogen Streptococcus pyogenes. Infect Immun 70:1971–1983. https://doi.org/10.1128/IAI.70.4.1971-1983.2002.
17. Lefebure T, Stanhope MJ. 2007. Evolution of the core and pan-genome of Streptococcus: positive selection, recombination, and genome composition. Genome Biol 8:R71. https://doi.org/10.1186/gb-2007-8-5-r71.
18. Marks LR, Mashburn-Warren L, Federle MJ, Hakansson AP. 2014. Streptococcus pyogenes biofilm growth in vitro and in vivo and its role in colonization, virulence, and genetic exchange. J Infect Dis 210:25–34. https://doi.org/10.1093/infdis/jiu058.
19. Feil EJ, Holmes EC, Bessen DE, Chan MS, Day NP, Enright MC, Goldstein R, Hood DW, Kalia A, Moore CE, Zhou J, Spratt BG. 2001. Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. Proc Natl Acad Sci U S A 98:182–187. https://doi.org/10.1073/pnas.98.1.182.
20. Shousha A, Awaiwanont N, Sofka D, Smulders FJ, Paulsen P, Szostak MP, Humphrey T, Hilbert F. 2015. Bacteriophages isolated from chicken meat and the horizontal transfer of antimicrobial resistance genes. Appl Environ Microbiol 81:4600–4606. https://doi.org/10.1128/AEM.00872-15.
21. Ross J, Topp E. 2015. Abundance of antibiotic resistance genes in bacteriophage following soil fertilization with dairy manure or municipal biosolids, and evidence for potential transduction. Appl Environ Microbiol 81:7905–7913. https://doi.org/10.1128/AEM.02363-15.
22. Calero-Caceres W, Muniesa M. 2016. Persistence of naturally occurring antibiotic resistance genes in the bacteria and bacteriophage fractions of wastewater. Water Res 95:11–18. https://doi.org/10.1016/j.watres.2016.03.006.
23. Quiros P, Colomer-Lluch M, Martinez-Castillo A, Miro E, Argente M, Jofre J, Navarro F, Muniesa M. 2014. Antibiotic resistance genes in the bacteriophage DNA fraction of human fecal samples. Antimicrob Agents Chemother 58:606–609. https://doi.org/10.1128/AAC.01684-13.
24. McShan WM, Nguyen SV. 2016. The bacteriophages of Streptococcus pyogenes. *In* Ferretti JJ, Stevens DL, Fischetti VA (ed), Streptococcus pyogenes: basic biology to clinical manifestations. University of Oklahoma Health Sciences Center, Oklahoma City, OK.
25. Pomrenke ME, Ferretti JJ. 1989. Physical maps of the streptococcal bacteriophage A25 and C1 genomes. J Basic Microbiol 29:395–398. https://doi.org/10.1002/jobm.3620290621.
26. Benchetrit LC, Gray ED, Wannamaker LW. 1977. Hyaluronidase activity of bacteriophages of group A streptococci. Infect Immun 15:527–532.
27. Arendt EK, Daly C, Fitzgerald GF, van de Guchte M. 1994. Molecular characterization of lactococcal bacteriophage Tuc2009 and identification and analysis of genes encoding lysin, a putative holin, and two structural proteins. Appl Environ Microbiol 60:1875–1883.
28. Loessner MJ, Gaeng S, Wendlinger G, Maier SK, Scherer S. 1998. The two-component lysis system of Staphylococcus aureus bacteriophage Twort: a large TTG-start holin and an associated amidase endolysin. FEMS Microbiol Lett 162:265–274. https://doi.org/10.1111/j.1574-6968.1998.tb13008.x.
29. Aunpad R, Panbangred W. 2012. Evidence for two putative holin-like peptides encoding genes of Bacillus pumilus strain WAPB4. Curr Microbiol 64:343–348. https://doi.org/10.1007/s00284-011-0074-3.
30. Fernandes S, Sao-Jose C. 2017. Probing the function of the two holin-like proteins of bacteriophage SPP1. Virology 500:184–189. https://doi.org/10.1016/j.virol.2016.10.030.
31. Saier MH, Jr, Reddy BL. 2015. Holins in bacteria, eukaryotes, and archaea: multifunctional xenologues with potential biotechnological and biomedical applications. J Bacteriol 197:7–17. https://doi.org/10.1128/JB.02046-14.
32. Nelson D, Schuch R, Zhu S, Tscherne DM, Fischetti VA. 2003. Genomic sequence of C1, the first streptococcal phage. J Bacteriol 185:3325–3332. https://doi.org/10.1128/JB.185.11.3325-3332.2003.
33. Madsen SM, Mills D, Djordjevic G, Israelsen H, Klaenhammer TR. 2001. Analysis of the genetic switch and replication region of a P335-type bacteriophage with an obligate lytic lifestyle on Lactococcus lactis. Appl Environ Microbiol 67:1128–1139. https://doi.org/10.1128/AEM.67.3.1128-1139.2001.
34. Bruttin A, Brussow H. 1996. Site-specific spontaneous deletions in three genome regions of a temperate Streptococcus thermophilus phage. Virology 219:96–104. https://doi.org/10.1006/viro.1996.0226.
35. Bruttin A, Foley S, Brussow H. 1997. The site-specific integration system of the temperate Streptococcus thermophilus bacteriophage phiSfi21. Virology 237:148–158. https://doi.org/10.1006/viro.1997.8769.
36. Grissa I, Vergnaud G, Pourcel C. 2007. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinformatics 8:172. https://doi.org/10.1186/1471-2105-8-172.
37. McShan WM, McLaughlin RE, Nordstrand A, Ferretti JJ. 1998. Vectors containing streptococcal bacteriophage integrases for site-specific gene insertion. Methods Cell Sci 20:51–57. https://doi.org/10.1023/A:1009773309163.
38. Casjens S. 2003. Prophages and bacterial genomics: what have we learned so far? Mol Microbiol 49:277–300. https://doi.org/10.1046/j.1365-2958.2003.03580.x.
39. Casjens SR, Gilcrease EB. 2009. Determining DNA packaging strategy by analysis of the termini of the chromosomes in tailed-bacteriophage

virions. Methods Mol Biol 502:91–111. https://doi.org/10.1007/978-1-60327-565-1_7.
40. Casjens SR, Gilcrease EB. 2009. Determining DNA packaging strategy by analysis of the termini of the chromosomes in tailed-bacteriophage virions, p 91–111. *In* Clokie MRJ, Kropinski AM (ed), Bacteriophages: methods and protocols, vol 2. Molecular and applied aspects. Humana Press, Totowa, NJ.
41. Clore AJ, Stedman KM. 2007. The SSV1 viral integrase is not essential. Virology 361:103–111. https://doi.org/10.1016/j.virol.2006.11.003.
42. Mikkonen M, Dupont L, Alatossava T, Ritzenthaler P. 1996. Defective site-specific integration elements are present in the genome of virulent bacteriophage LL-H of Lactobacillus delbrueckii. Appl Environ Microbiol 62:1847–1851.
43. Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VA, Fouts DE, Gill SR, Pop M, Holmes M, Brinkac L, Beanan M, DeBoy RT, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Chris Lee P, Holtzapple E, Scanlan D, Tran K, Moazzez A, Utterback T, Rizzo M, Lee K, Kosack D, Moestl D, Wedler H, Lauber J, Stjepandic D, Hoheisel J, Straetz M, Heim S, Kiewitz C, Eisen JA, Timmis KN, Dusterhoft A, Tummler B, Fraser CM. 2002. Complete genome sequence and comparative analysis of the metabolically versatile Pseudomonas putida KT2440. Environ Microbiol 4:799–808. https://doi.org/10.1046/j.1462-2920.2002.00366.x.
44. Trotter M, McAuliffe O, Callanan M, Edwards R, Fitzgerald GF, Coffey A, Ross RP. 2006. Genome analysis of the obligately lytic bacteriophage 4268 of Lactococcus lactis provides insight into its adaptable nature. Gene 366:189–199. https://doi.org/10.1016/j.gene.2005.09.022.
45. Labrie SJ, Josephsen J, Neve H, Vogensen FK, Moineau S. 2008. Morphology, genome sequence, and structural proteome of type phage P335 from Lactococcus lactis. Appl Environ Microbiol 74:4636–4644. https://doi.org/10.1128/AEM.00118-08.
46. Lieb M. 1953. The establishment of lysogenicity in Escherichia coli. J Bacteriol 65:642–651.
47. Fischetti VA, Zabriskie JB. 1968. Studies on streptococcal bacteriophages. II. Adsorption studies on group A and group C streptococcal bacteriophages. J Exp Med 127:489–505.
48. Cleary PP, Johnson Z. 1977. Possible dual function of M protein: resistance to bacteriophage A25 and resistance to phagocytosis by human leukocytes. Infect Immun 16:280–292.
49. Cleary PP, Wannamaker LW, Fisher M, Laible N. 1977. Studies of the receptor for phage A25 in group A streptococci: the role of peptidoglycan in reversible adsorption. J Exp Med 145:578–593. https://doi.org/10.1084/jem.145.3.578.
50. Hendrickson C, Euler CW, Nguyen SV, Rahman M, McCullor KA, King CJ, Fischetti VA, McShan WM. 2015. Elimination of chromosomal island SpyCIM1 from Streptococcus pyogenes strain SF370 reverses the mutator phenotype and alters global transcription. PLoS One 10:e0145884. https://doi.org/10.1371/journal.pone.0145884.
51. Wessels MR, Moses AE, Goldberg JB, DiCesare TJ. 1991. Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. Proc Natl Acad Sci U S A 88:8317–8321.
52. Krause RM. 1957. Studies on bacteriophages of hemolytic streptococci. I. Factors influencing the interaction of phage and susceptible host cell. J Exp Med 106:365–384.
53. Leon M, Bastias R. 2015. Virulence reduction in bacteriophage resistant bacteria. Front Microbiol 6:343. https://doi.org/10.3389/fmicb.2015.00343.
54. Zaoutis T, Attia M, Gross R, Klein J. 2004. The role of group C and group G streptococci in acute pharyngitis in children. Clin Microbiol Infect 10:37–40. https://doi.org/10.1111/j.1469-0691.2004.00732.x.
55. Sambrook J, Fritsch EF, Maniatis T. 1987. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
56. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. https://doi.org/10.1093/nar/25.17.3389.
57. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. 2011. PHAST: a fast phage search tool. Nucleic Acids Res 39:W347–W352. https://doi.org/10.1093/nar/gkr485.
58. Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B. 2000. Artemis: sequence visualization and annotation. Bioinformatics 16:944–945. https://doi.org/10.1093/bioinformatics/16.10.944.
59. de Jong A, Pietersma H, Cordes M, Kuipers OP, Kok J. 2012. PePPER: a webserver for prediction of prokaryote promoter elements and regulons. BMC Genomics 13:299. https://doi.org/10.1186/1471-2164-13-299.
60. Darzentas N. 2010. Circoletto: visualizing sequence similarity with Circos. Bioinformatics 26:2620–2621. https://doi.org/10.1093/bioinformatics/btq484.
61. Drummond AJ, Buxton S, Cheung M, Cooper A, Duran C, et al. 2011. Geneious version 6.0.6. Biomatters, Inc., Newark, NJ.
62. Brodie R, Smith AJ, Roper RL, Tcherepanov V, Upton C. 2004. Base-By-Base: single nucleotide-level analysis of whole viral genome alignments. BMC Bioinformatics 5:96. https://doi.org/10.1186/1471-2105-5-96.
63. Troen BR. 1997. The gene construction kit. DNA sequence analysis and presentation. Methods Mol Biol 70:257–272.
64. Ferretti JJ, McShan WM, Ajdic D, Savic DJ, Savic G, Lyon K, Primeaux C, Sezate S, Suvorov AN, Kenton S, Lai HS, Lin SP, Qian Y, Jia HG, Najar FZ, Ren Q, Zhu H, Song L, White J, Yuan X, Clifton SW, Roe BA, McLaughlin R. 2001. Complete genome sequence of an M1 strain of Streptococcus pyogenes. Proc Natl Acad Sci U S A 98:4658–4663. https://doi.org/10.1073/pnas.071559398.
65. Sumby P, Porcella SF, Madrigal AG, Barbian KD, Virtaneva K, Ricklefs SM, Sturdevant DE, Graham MR, Vuopio-Varkila J, Hoe NP, Musser JM. 2005. Evolutionary origin and emergence of a highly successful clone of serotype M1 group a Streptococcus involved multiple horizontal gene transfer events. J Infect Dis 192:771–782. https://doi.org/10.1086/432514.
66. Beres SB, Richter EW, Nagiec MJ, Sumby P, Porcella SF, DeLeo FR, Musser JM. 2006. Molecular genetic anatomy of inter- and intraserotype variation in the human bacterial pathogen group A Streptococcus. Proc Natl Acad Sci U S A 103:7059–7064. https://doi.org/10.1073/pnas.0510279103.
67. Beres SB, Sylva GL, Barbian KD, Lei B, Hoff JS, Mammarella ND, Liu MY, Smoot JC, Porcella SF, Parkins LD, Campbell DS, Smith TM, McCormick JK, Leung DY, Schlievert PM, Musser JM. 2002. Genome sequence of a serotype M3 strain of group A Streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence. Proc Natl Acad Sci U S A 99:10078–10083. https://doi.org/10.1073/pnas.152298499.
68. Banks DJ, Porcella SF, Barbian KD, Beres SB, Philips LE, Voyich JM, DeLeo FR, Martin JM, Somerville GA, Musser JM. 2004. Progress toward characterization of the group A Streptococcus metagenome: complete genome sequence of a macrolide-resistant serotype M6 strain. J Infect Dis 190:727–738. https://doi.org/10.1086/422697.
69. Kjems E. 1958. Studies on streptococcal bacteriophages. 2. Adsorption, lysogenization, and one-step growth experiments. Acta Pathol Microbiol Scand 42:56–66.
70. Green NM, Zhang S, Porcella SF, Nagiec MJ, Barbian KD, Beres SB, LeFebvre RB, Musser JM. 2005. Genome sequence of a serotype M28 strain of group a streptococcus: potential new insights into puerperal sepsis and bacterial disease specificity. J Infect Dis 192:760–770. https://doi.org/10.1086/430618.
71. Jacks-Weis J, Kim Y, Cleary PP. 1982. Restricted deposition of C3 on M+ group A streptococci: correlation with resistance to phagocytosis. J Immunol 128:1897–1902.
72. McShan WM, Ferretti JJ, Karasawa T, Suvorov AN, Lin S, Qin B, Jia H, Kenton S, Najar F, Wu H, Scott J, Roe BA, Savic DJ. 2008. Genome sequence of a nephritogenic and highly transformable M49 strain of Streptococcus pyogenes. J Bacteriol 190:7773–7785. https://doi.org/10.1128/JB.00672-08.
73. Bessen DE, Kumar N, Hall GS, Riley DR, Luo F, Lizano S, Ford CN, McShan WM, Nguyen SV, Dunning Hotopp JC, Tettelin H. 2011. Whole-genome association study on tissue tropism phenotypes in group A Streptococcus. J Bacteriol 193:6651–6663. https://doi.org/10.1128/JB.05263-11.