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Functional Genomic Analysis of Two *Staphylococcus aureus* Phages Isolated from the Dairy Environment▽

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The genomes of the two lytic mutant *Staphylococcus aureus* bacteriophages, vB_SauS-phiIPLA35 (phiIPLA35) and vB_SauS-phiIPLA88 (phiIPLA88), isolated from milk have been analyzed. Their genomes are 45,344 bp and 42,526 bp long, respectively, and contain 62 and 61 open reading frames (ORFS). Enzymatic analyses and sequencing revealed that the phiIPLA35 DNA molecule has 3′-protruding cohesive ends (*cos*) 10 bp long, whereas phiIPLA88 DNA is 4.5% terminally redundant and most likely is packaged by a headful mechanism. N-terminal amino acid sequencing, mass spectrometry, bioinformatic analyses, and functional analyses enabled the assignment of putative functions to 58 gene products, including DNA packaging proteins, morphogenetic proteins, lysis components, and proteins necessary for DNA recombination, modification, and replication. Point mutations in their lysogeny control-associated genes explain their strictly lytic behavior. Muralytic activity associated with other structural components has been detected in virions of both phages. Comparative analysis of phiIPLA35 and phiIPLA88 genome structures shows that they resemble those of ϕ12 and ϕ11, respectively, both representatives of large genomic groupings within the *S. aureus*-infecting phages.

Staphylococcus aureus is an important etiologic agent of food-borne diseases due to its ability to produce heat-resistant staphylococcal enterotoxins (SEs) when it grows in foods. In fact some *S. aureus* strains may produce up to 20 serologically distinct SEs, which could be responsible for food poisoning (30). SEs have been divided initially into serological types SEA through SEE, and recently the existence of new types of SEs has also been reported (5).

S. aureus strains harboring enterotoxin genes have been isolated from a variety of foods (38) including dairy products (9, 46, 56). Mastitis caused by this pathogen and poor hygienic processing conditions are the most important sources of dairy product contamination. Growth of enterotoxigenic *S. aureus* in both raw milk and dairy products poses a potential health hazard to consumers. In this context, new biocontrol strategies to prevent growth of *S. aureus*, suitable to be applied in the food industry, are being explored.

Currently, there is a renewed interest in exploiting the antimicrobial potential of bacterial viruses for bacterial-control applications in agriculture, aquaculture, and the food industry (11, 18, 23, 49). In fact, the use of phages for the treatment of infectious diseases (or phage therapy) has a long successful history in the countries of Eastern Europe (or former Soviet Union) (50). Specifically, *S. aureus* bacteriophages have been assayed in the treatment of venous leg ulcers and eye infections (22, 42).

Prior to any phage application, genome analysis is a prerequisite to examine the safety of the phages, specifically, traits which might enhance the virulence of the infected bacterium. In addition, genome analysis might uncover novel antibacterial targets or agents (33) with promising biotechnological applications (6). For example, various lytic phage proteins (endolysins) have shown great potential in veterinary and human medicine for the treatment and prophylaxis of infections (12) and have been applied as biocontrol agents in dairy products (36). Several technologies employing phages and endolysins for pathogen detection and decontamination have also been patented (7).

To date, genomes of over 47 *S. aureus* phages are available in public databases. The number of known, strictly lytic phages is limited to the close-knit *Myoviridae* genus of the SPO1-like viruses, containing phages K, Twort, and G1. Apart from this group, a large number of genomes from unclassified *Siphoviridae* in lysogenic *S. aureus* strains are available (26, 37). Some temperate bacteriophages may play an important role in the pathogenicity of *S. aureus* by carrying virulence factors, mediating lateral gene transfer, and even facilitating the adaptation of the pathogen during infection (1, 21, 52).

In previous work, we have characterized phiIPLA35 and phiIPLA88 *S. aureus* phages (17). These two lytic phages, previously named ϕ35 and ϕ88, were selected as mutants of the temperate phages ϕA72 and ϕH5, respectively, isolated from raw bovine milk. They belong to the *Siphoviridae* family of double-stranded DNA bacterial viruses in the order *Caudovirales*. Remarkably, these phages infect *S. aureus* of bovine and dairy origin while clinical isolates appear to be resistant. Both phiIPLA35 and phiIPLA88 are very well adapted to the dairy environment and effectively inhibit *S. aureus* growth in milk and curd-manufacturing processes (17, 20).

In this study, we have sequenced and annotated the genomes of both bacteriophages, elucidated their physical genome structures, and identified peptidoglycan hydrolytic activities. Comparative genome analysis also allowed us to put phiIPLA35 and phiIPLA88 into a phylogenetic context.

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MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *S. aureus* Sa9 was used as the host strain of phages phiIPLA35 and phiIPLA88 (17). *Escherichia coli* strain DH10B (Invitrogen) was used for recombinant DNA work. Both were grown in 2× TY broth (45) at 37°C with vigorous shaking. Plates of 2× TY broth contained 2% (wt/vol) bacteriological agar. For the selection of plasmid-bearing cells, ampicillin was added at 100 μg/ml. Bacterial stocks were stored at −80°C in 2× TY supplemented with 20% (final concentration) glycerol.

Bacteriophage propagation and DNA purification. Bacteriophages phiIPLA35 and phiIPLA88 were routinely propagated on *S. aureus* Sa9 as described previously (17). Phage enumeration was performed by the double-layer technique (45) using soft 2× TY medium (0.75% agar plus 10 mM $CaCl_2$ and 10 mM $MgSO_4$) in the upper layer. In order to prepare RNA-free DNA for sequence analysis, the phages were dialyzed against SM buffer (20 mM Tris-HCl, 10 mM $MgSO_4$, 10 mM $CaCl_2$, 100 mM NaCl, pH 7.5), and the DNA was extracted and purified as described previously (15).

One-step growth curve. One-step growth curves were performed in 2× TY medium using a multiplicity of infection of 1, as previously described (25).

Phage genome sequencing and analysis. Approximately 10 μg of both phiIPLA35 and phiIPLA88 DNA was sheared by sonication, size selected (2 to 3 kbp), and cloned into pUC18. Individual clones were sequenced and assembled. Trace assembly was done with the phredPhrap package (10). Each sequenced base had at least a fivefold coverage. Primer walking was used to close gaps in the sequence. Open reading frames (ORFs) were predicted with ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) software and by visual inspection. The primary nucleotide sequence was scanned in all reading frames for start codons with a threshold of 50 codons. BLASTX and BLASTP (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) were used to search for homologous proteins. Structural predictions and motif searches were performed with InterProScan (http://www.ebi.ac.uk/InterProScan/), Pfam (http://pfam.sanger.ac.uk/search?tab=search-SequenceBlock), and YASPIN (http://www.ibi.vu.nl/programs/yaspinwww/). σ^{70} promoter sequences were identified using Bprom (http://www.softberry.com) and PPP (Prokaryotic Promoter Prediction [http://bioinformatics.biol.rug.nl/websoftware/ppp/ppp_start.php]). Putative terminator sequences were detected with the terminator function of the GCG program (version 10.2). The search for putative tRNA-encoding genes was performed with tRNAscan-SE 1.21 (http://selab.janelia.org/tRNAscan-SE/). Prediction of +1 and −1 frameshifting was carried out with FSFinder 2 (http://wilab.inha.ac.kr/fsfinder2/). Cumulative GC skew (http://mips.gsf.de/services/analysis/genskew) was used to predict the *oriC*. Pseudoknot predictions were carried out by pknotsRG (41). Dlot plot analyses were carried out by Nucleic Acids Dot Plots (http://www.vivo.colostate.edu/molkit/dnadot/index.html).

Determination of phage genome physical structure. To test whether the phage genomes have cohesive ends (*cos*), purified DNA (0.5 μg) was incubated with T4 DNA ligase, followed by digestion with the restriction enzymes BamHI, EcoRI, HindIII, and PstI (Takara, Otsu, Shiga, Japan) and fragment separation by agarose gel electrophoresis. A nonligated DNA sample was equally treated but heated at 70°C for 10 min prior to the electrophoresis. To obtain the sequence of the single-stranded *cos* extensions, the ends of phiIPLA35 DNA were sequenced directly using primers A (5′-AGTTATACGACACAAGTACACGAG G-3′) and B (5′-TGAGTGACTTGCTCTGCATACCATG-3′) and aligned with the circular phiIPLA35 genome.

To determine if the phiIPLA88 DNA molecule has open ends, phage DNA (5 μg) and phage DNA heated at 70°C for 10 min were incubated with 20 units of the double-stranded DNA exonuclease Bal31 (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The DNA was ethanol precipitated, dissolved in Tris-EDTA buffer, digested with PvuII, and analyzed by agarose gel electrophoresis. As a control, we used lambda DNA (Quimigen, Madrid, Spain) known to have specific ends.

For electron microscopy of phage DNA, phage were dialyzed on a 0.025-μm-pore-size Millipore filter against TBT buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$). The phage were spread with 50% formamide, carbonate buffer, and cytochrome *c* on a water surface as described earlier (48). Plasmid RSF1010 DNA (47) was added as an internal standard for length comparison with the released phage DNA.

Proteomic analysis of virion proteins. Phage structural proteins were extracted, purified as described previously (15), and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Miniprotean III (Bio-Rad, Richmond, CA). Proteins were stained with Coomassie R-250 blue or silver (PlusOne silver staining kit; GE Healthcare, Piscataway, NJ). The bands of interest were excised manually and digested with porcine trypsin (Promega, Madison, WI), and the resulting peptides were analyzed by matrix-assisted laser desorption ionization—time of flight mass spectrometry, essentially as previously described (16). N-terminal amino acid sequences were determined in bands excised from polyvinylidene difluoride membrane blots using an Applied Biosystems 477A automated protein sequencer.

Zymogram analysis. Purified phage suspensions were dialyzed against SM buffer, mixed with loading buffer (1% SDS, 6% sucrose, 100 mM dithiothreitol, 10 mM Tris, pH 6.8, 0.0625% bromophenol blue) and boiled for 5 min before loading onto 12% SDS-PAGE gels containing 0.2% *S. aureus* Sa9 autoclaved cells (31). Gels were cast according to Laemmli (28), except that only 0.01% SDS was used to allow protein renaturation. After electrophoresis, gels were washed for 30 min with water and then soaked for 1 day at room temperature in 150 mM sodium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and 10 mM $MgCl_2$. Zymograms were stained for 3 h with 0.1% methylene blue in 0.001% KOH and washed with water. Peptidoglycan hydrolase activity was detected as a clear zone in a dark blue background of stained peptidoglycan.

Nucleotide sequence accession numbers. The sequences of phiIPLA35 and phiIPLA88 have been deposited in the GenBank under accession numbers EU861004 and EU861005, respectively.

RESULTS AND DISCUSSION

One-step growth curve of phiIPLA35 and phiIPLA88. The stages of infection of the phages phiIPLA35 and phiIPLA88 have been followed using a one-step growth curve. We observed that phages behaved slightly differently when propagated on *S. aureus* Sa9 (Fig. 1). The eclipse phase of phage phiIPLA35, determined by the release of infecting particles after the treatment with chloroform, was longer than that of phiIPLA88 (30 versus 22.5 min). However, in both cases infecting particles were detected in the supernatant after 40 min postinfection. The burst sizes were estimated around 27 and 45 new virions per infecting particle of phiIPLA35 and phiIPLA88, respectively. These values are relatively lower than the burst size reported for ϕMR11 (34).

Physical structure of the phiIPLA35 and phiIPLA88 genomes. The phiIPLA35 *cos* sites were localized using restriction pattern analysis of ligated and unligated DNA samples, revealing typical fragments arising from the ligation of the *cos* sites (data not shown). Moreover, direct sequencing with appropriate oligonucleotides pointing outwards from the putative *cos* site and sequence alignment with the circular DNA show that phiIPLA35 DNA has 10-bp single-stranded 3′ extensions with the sequence 5′-CGGCGGGGGC-3′, located upstream from the terminase small subunit (position 19942 to 19951).

In contrast, no evidence could be found for the presence of *cos* ends in the phiIPLA88 DNA molecule. Ligation of its DNA did not alter the restriction patterns, which were compatible with a circular map (data not shown). Restriction endonuclease digestions of phiIPLA88 DNA also failed to reveal a "submolar" fragment, which would contain the *pac* site for initial recognition and subsequent cutting of sequentially packaged phage concatemers by the terminase enzyme (2). Another approach to determine genome ends involved a time-limited treatment of phiIPLA88 DNA with the exonuclease Bal31 (an exonuclease which degrades double-stranded linear DNA from both ends simultaneously), followed by complete digestion with a restriction enzyme (Fig. 2). In this situation, specific degradation of the restriction fragments overlapping the genome ends would be observed. To this end, the lambda phage DNA was used as a positive control. Bal31 treatment followed by digestion with the restriction enzyme EcoRI showed that the two expected EcoRI-specific fragments were degraded (Fig. 2A). Similarly, two specific phiIPLA88 DNA PvuII re-

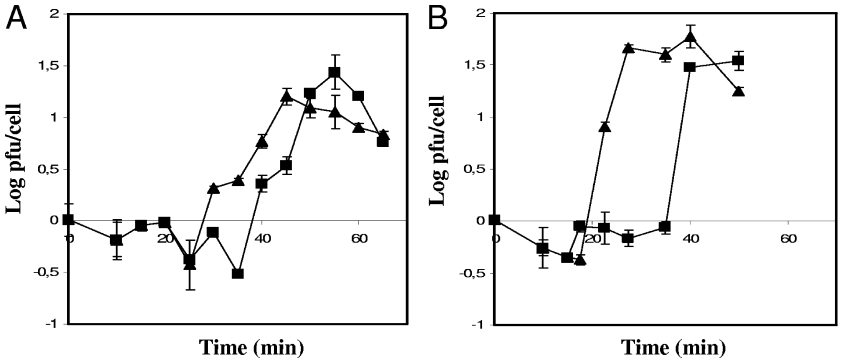


FIG. 1. One-step growth curves of phages phiIPLA35 (A) and phiIPLA88 (B) on exponential cultures of *S. aureus* Sa9 incubated in 2× YT medium at 37°C under agitation. Cells were chloroform treated (triangles) or left untreated (squares).

striction fragments of 21.5 and 10.7 kbp were simultaneously shortened (Fig. 2B). These fragments would bear the physical genome ends of phiIPLA88. In fact, they are located in the surrounding region of a possible *pac* site placed upstream of gene product 35 (gp35), the small terminase subunit. This position is where *pac* sites are usually found in headful packagers (3).

The phiIPLA88 DNA was measured on transmission electron micrographs. The size was 44,466 kb ± 1,025 kb (n = 36), which is significantly longer (+1,940 kb) than that derived from sequencing, and suggested that the phiIPLA88 DNA genome is about 4.5% terminally redundant.

Genome overview of phiIPLA35 and phiIPLA88 phages. The complete genome sequences of phiIPLA35 and phiIPLA88 were determined. The phiIPLA35 genome comprised 45,344 bp coding for 62 putative ORFs of more than 50 codons, while in the phiIPLA88 genome 42,526 bp and 61 putative ORFs were identified (Tables 1 and 2; Fig. 3 and 4). The G+C content of phiIPLA35 and phiIPLA88 was 33.2% and 34.9%, respectively, which is slightly higher than that of bovine *S. aureus* strains (32.8%) (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

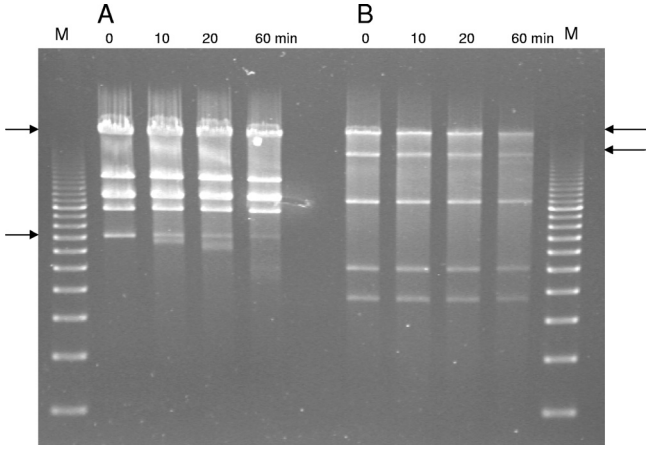


FIG. 2. Time-limited digestion with Bal31 exonuclease (0 to 60 min, as indicated) of control phage lambda DNA (A) and phiIPLA88 DNA (B), followed by restriction enzyme digestion (EcoRI in panel A and PvuII in panel B). The arrows point to the two sequentially degraded fragments of the linear control DNA and phiIPLA88 DNA. M, molecular size marker (0.5 to 10 kbp). Sampling times are indicated in minutes.

The overall genome organization of phiIPLA35 and phiIPLA88 is described below. As shown in Fig. 3 and 4, both genomes are apparently organized into three major gene clusters, containing (i) the "lysogeny control" region, which is probably transcribed mostly leftwards based on the gene orientation; (ii) the "early" region, encoding products for the replication, recombination, and modification of the phage DNA; and (iii) the "late genes," coding for structural and assembly proteins, DNA packaging proteins, and the lysis proteins.

The majority of ORFs presented an AUG start codon, while two (*orf34* and *orf38*) initiated at GUG in phiIPLA35 and four (*orf31*, *orf32*, *orf50*, and *orf57*) in phiIPLA88. In addition, *orf2* from phiIPLA35 and *orf1* from phiIPLA88 have UUG as a start codon. The genomes are densely coded, having noncoding regions that comprise only 7.6% and 7.2% for phiIPLA35 and phiIPLA88, respectively. Furthermore, the phiIPLA35 and phiIPLA88 genomes encode 18 and 16 ORFs, respectively, in which the start codon overlaps with the stop codon of the preceding gene. Several putative promoters in phiIPLA35 and in phiIPLA88 were found by searching for the *S. aureus* σ^{70}-dependent promoter consensus motif (Fig. 3 and 4). Regions forming stem-loop structures, probably representing factor-independent transcriptional terminators (Fig. 3 and 4), were also determined.

The amino acid sequences of the predicted ORFs were screened for similarities with sequences from the available databases for preliminary functional assignments (Tables 1 and 2). Significant matches were obtained for 26 ORFs from phiIPLA35 and for 32 ORFs from phiIPLA88, and biological functions were assigned. No tRNA genes were found. No virulence genes were clearly identified. However, a VirE motif, found in bacterial virulence protein E, was detected in phiIPLA35 gp32. VirE motifs had also been described in several phage DNA replication proteins in the databases and might have a particular role in DNA metabolism (51). Nevertheless, a putative role in promoting host pathogenicity cannot be excluded.

Comparative genomics of phiIPLA35 and phiIPLA88 phages. From a comparative perspective, BLAST results and subsequent proteome-based clustering using CoreGenes/CoreExtractor (29) quickly reveal that ϕ12, ϕ47, ϕ3A, ϕ42E, and

TABLE 1. Features of bacteriophage phiIPLA35 ORFs, gene products, and functional assignments

ORF	Position (nt)		Length (nt)	No. of amino acids	Size (kDa [pI])	Predictive function	Closest hit (e value)	% Amino acid identity (% similarity)	Accession no.	Predicted domain (protein ID) (e value)[a]
	From	To								
1	1206	1	1,206	401	47.2 (10.24)	Integrase	*S. aureus* MSSA476 (0.0)	100 (100)	YP_043018.1	Phage integrase (PF00589) (1.3e−46)
2	1333	1956	624	207	24.3 (9.53)	Hypothetical protein	*Staphylococcus* ϕPVL108 (1e−34)	100 (100)	YP_918893.1	Signal peptide; transmembrane regions
3	2549	2154	396	131	15.3 (4.46)	Hypothetical protein	*Staphylococcus* ϕPV83 (3e−70)	99 (99)	NP_061593.1	
4	3011	2577	435	144	15.8 (9.54)	Putative lipoprotein	*S. aureus* MSSA476 (4e−73)	93 (97)	YP_043022.1	Signal peptide
5	3029	3400	372	123	14.7 (8.78)	Unknown conserved protein	*S. aureus* MSSA476 (3e−66)	99 (100)	YP_043023.1	DUF955 (PF06114) (2.9e−18)
6	3833	3579	255	84	9.9 (9.99)	Truncated repressor	*S. aureus* Mu50 (8e−15)	90 (92)	NP_371375.1	HTH_3 (PF01381) (1.1e−12)
7	3994	4185	192	63	7.2 (5.59)	Cro/CI family transcription regulator	*S. aureus* MSSA476 (1e−28)	100 (100)	YP_043025.1	HTH_3 (PF01381) (2.9e−10)
8	4273	4449	177	58	6.8 (4.63)	Hypothetical protein	*S. aureus* MSSA476 (2e−26)	98 (100)	YP_043026.1	
9	4676	4446	231	76	8.8 (6.21)	Hypothetical protein	*S. aureus* USA300 (1e−36)	100 (100)	YP_494616.1	
10	4732	4947	216	71	84 (9.93)	Hypothetical protein	*S. aureus* MSSA476 (5e−33)	100 (100)	YP_043028.1	
11	4972	5235	264	87	10.4 (9.83)	Hypothetical protein	*Staphylococcus* ϕ12 (4e−43)	97 (98)	NP_803315.1	
12	5247	5408	162	53	6.1 (4.16)	Hypothetical conserved protein	*Staphylococcus* ϕ47 (2e−21)	98 (100)	YP_240044.1	DUF1270 (PF06900) (3.4e−36); transmembrane region; signal peptide
13	5487	5810	324	107	12.5 (7.85)	Hypothetical protein	*Staphylococcus* ϕ47 (1e−55)	100 (100)	YP_240046.1	
14	5825	6187	363	120	13.6 (4.63)	Hypothetical protein	*S. aureus* MSSA476 (3e−54)	93 (95)	YP_043032.1	
15	6184	7350	1,167	388	44.3 (5.39)	Hypothetical protein	*S. aureus* MSSA476 (0.0)	98 (99)	YP_043033.1	
16	7376	7933	558	185	20.1 (4.65)	Hypothetical protein	*S. aureus* ϕ12 (1e−99)	99 (100)	NP_803317.1	
17	8001	9953	1,953	650	73.6 (6.59)	Phage DNA polymerase A	*S. aureus* MSSA476 (0.0)	98 (99)	YP_043035.1	DNA_pol_A (PF00476) (1.9e−06)
18	9966	10151	186	61	7.1 (4.19)	Hypothetical protein	*Staphylococcus* ϕ12 (9e−27)	100 (100)	NP_803319.1	
19	10148	10552	405	134	16.7 (10.35)	Primase	*S. aureus* MSSA476 (1e−72)	99 (100)	YP_043037.1	PVL_ORF50 (PF07768) (1.7e−90); HTH motif
20	10552	10809	258	85	9.7 (5.45)	Hypothetical protein	*Staphylococcus* ϕ12 (8e−43)	96 (98)	NP_803321.1	
21	10812	11012	201	66	7.5 (10.78)	Hypothetical protein	*Staphylococcus* ϕNPH82 (1e−16)	67 (83)	YP_950654.1	
22	11009	11698	690	229	27.1 (4.93)	Putative methyltransferase	*Listeria welshimeri* SLCC5334 (1e−53)	49 (68)	YP_849399.1	N6_Mtase (PF02384) (1.6e−05)
23	11712	11918	207	68	7.9 (4.68)	Hypothetical protein	*S. aureus* JH9 (2e−26)	82 (89)	YP_001245710.1	
24	11921	12349	429	142	16.3 (5.16)	Hypothetical protein	*Staphylococcus* ϕ71 (9e−24)	48 (57)	YP_240442.1	
25	12346	12540	195	64	7.5 (9.24)	Hypothetical protein	*Staphylococcus* ϕ187 (1e−24)	88 (95)	YP_239554.1	
26	12570	12821	252	83	10.1 (8.62)	Hypothetical protein	*Staphylococcus* ϕPV83 (1e−35)	93 (97)	NP_061617.1	
27	12814	13062	249	82	9.2 (4.00)	Hypothetical conserved protein	*Staphylococcus* ϕ71 (2e−38)	97 (100)	YP_240444.1	DUF1024 (PF06260) (2.5e−61)
28	13055	13615	561	186	22.1 (4.77)	dUTPase	*Staphylococcus* ϕX2 (5e−105)	99 (100)	AAX92028.1	dUTPase_2 (PF08761) (3.7e−08)
29	13852	14055	204	67	7.8 (9.80)	Hypothetical conserved protein	*Staphylococcus* ϕ42E (9e−30)	98 (98)	YP_239920.1	DUF1381 (PF07129) (7.4e−38)
30	14052	14204	153	50	5.9 (4.33)	Transcriptional activator RinB	*Staphylococcus* ϕtp310-2 (1e−20)	96 (98)	YP_001429932.1	RinB (PF06116) (6.3e−35)
31	14272	14472	201	66	7.8 (9.23)	Hypothetical conserved protein	*Staphylococcus* ϕ42E (2e−29)	100 (100)	YP_239924.1	DUF1514 (PF07438) (1.5e−48); signal peptide; transmembrane regions
32	14524	16971	2,448	815	94.3 (4.97)	Hypothetical protein	*S. aureus* MRSA252 (0.0)	99 (99)	YP_040926.1	VirE (PF05272) (4.1e−127)
33	17312	17602	291	96	11.2 (9.81)	Putative nuclease	*Staphylococcus* ϕ42E (4e−51)	100 (100)	YP_239929.1	VRR_NUC (PF08774) (1.1e−20)
34	17583	18950	1,368	455	52.9 (6.66)	Putative helicase	*Staphylococcus* ϕ42E (0.0)	100 (100)	YP_239930.1	SNF2_N (PF00176) (3.7e−05); DEAD-like helicase superfamily (SM00487) (1.6e−13)
35	18963	19400	438	145	17.0 (9.76)	Transcriptional regulator (RinA)	*Staphylococcus* prophage L54a (2e−78)	100 (100)	YP_185256.1	Phage_rinA; phage transcriptional regulator (IPR006523) (9.6e−16)
36	19557	19871	315	104	12.5 (8.58)	HNH endonuclease family protein	*Staphylococcus* ϕ42E (5e−53)	98 (100)	YP_239933.1	HNH (PF01844) (1.6e−10)
37	19980	20303	324	107	12.3 (8.98)	Terminase small subunit	*Staphylococcus* ϕ12 (3e−55)	100 (100)	NP_803335.1	Terminase_4 (PF05119) (9.4e−31)
38	20284	21984	1,701	566	65.4 (5.64)	Terminase large subunit	*S. aureus* MSSA476 (0.0)	100 (100)	YP_043059.1	Terminase_1 (PF03354) (8.1e−93)
39	21998	23227	1,230	409	47.3 (5.62)	Phage portal protein	*Staphylococcus* ϕ3A (0.0)	100 (100)	YP_239936.1	Phage portal (PF04860) (3.6e−116)
40	23211	23984	774	257	29.1 (4.77)	Clp protease	*Staphylococcus* ϕSLT (9e−142)	100 (100)	NP_075503.1	Clp_protease (PF00574)(1.5e−51); transmembrane regions
41	23996	25159	1,164	387	43.5 (4.95)	Major head protein	*Staphylococcus* ϕ12 (0)	100 (100)	NP_803339	Phage capsid (PF05065) (0.0012)
42	25228	25506	279	92	10.8 (4.93)	Possible DNA packaging protein	*Staphylococcus* ϕSLT (0)	100 (100)	NP_075505	Put_DNA_pack: uncharacterized phage protein (2.7e−09)
43	25518	25850	333	110	12.9 (5.77)	Hypothetical protein	*Staphylococcus* ϕ12 (3e−58)	100 (100)	NP_803340.1	
44	25847	26248	402	133	15.1 (10.08)	Hypothetical protein	*Staphylococcus* ϕ12 (1e−68)	99 (99)	NP_803341.	
45	26249	26644	396	131	15.5 (8.76)	Hypothetical protein	*Staphylococcus* ϕSLT (1e−68)	100 (100)	NP_075508	

Continued on following page

TABLE 1—*Continued*

ORF	Position (nt)		Length (nt)	No. of amino acids	Size (kDa [pI])	Predictive function	Closest hit (e value)	% Amino acid identity (% similarity)	Accession no.	Predicted domain (protein ID) (e value)[a]
	From	To								
46	26679	27320	642	213	23.4 (4.75)	Major tail protein	*Staphylococcus* ϕ12 (2e−120)	100 (100)	NP_803343	Phage_tail (PF04630) (2.2e−99)
47	27412	27867	456	151	16.0 (4.39)	Tail protein	*Staphylococcus* ϕ12 (6e−81)	99 (99)	NP_803344	Big_2 (PF02368) (1.1e—07)
48	27925	28275	351	116	13.6 (4.35)	Hypothetical protein	*Staphylococcus* p ϕSLT (4e−60)	100 (100)	NP_075511	
49	28317	28475	159	52	6.1 (4.65)	Hypothetical protein	*Staphylococcus* ϕ42E (3e−21)	100 (100)	YP_239870	
50	28489	34689	6,201	2,066	226.05 (10.23)	Tail tape measure protein	*Staphylococcus* ϕSLT	99 (99)	YP_494090.1	Lytic transglycosylase-like SLT (PF01464) (0.0013); Tape_meas_TP901 (1.4e−99); Peptidase_M23 (PF01551) (5.5e−36)
51	34689	35513	825	274	31.2 (4.96)	Hypothetical protein	*Staphylococcus* ϕ12 (2e−158)	99 (99)	NP_803347.1	DUF1306 (PF06997) (1.4e−157)
52	35522	37102	1,581	526	60.9 (5.66)	Conserved hypothetical protein	*Staphylococcus* ϕ47 (0.0)	98 (99)	AAX91197.1	DUF1142 (PF06605) (0)
53	37102	37392	291	96	10.5 (5.04)	Unknown conserved protein	*Staphylococcus* ϕ42E (3e−49)	100 (100)	YP_239876.1	
54	37408	39318	1,911	636	73.1 (5.80)	Minor structural protein	*Staphylococcus* ϕ12 (0.0)	99 (100)	NP_803350.1	
55	39318	40784	1,467	488	54.1 (5.42)	Hypothetical protein	*S. aureus* MSSA476 (0.0)	98 (99)	CAG42725.1	
56	40784	41173	390	129	14.7 (4.56)	Hypothetical protein	*Staphylococcus* ϕ12 (1e−65)	100 (100)	NP_803352.1	
57	41166	41330	165	54	6.5 (4.06)	Hypothetical protein	*Staphylococcus* ϕ12 (1e−24)	100 (100)	AAL82328.1	Phage_XkdX: phage (TIGR01669) (7.5e−15)
58	41376	41675	300	99	12.0 (7.63)	Hypothetical protein	*Staphylococcus* ϕSLT (9e−51)	100 (100)	NP_075520.1	Transmembrane regions
59	41811	42113	303	100	11.1 (9.58)	Holin	*Staphylococcus* ϕ12 (3e−50)	100 (100)	NP_803354.1	Signal peptide; Phage_holin (PF04688) (1.1e−20)
60	42124	43578	1,455	484	53.7 (9.62)	Amidase	*Staphylococcus* ϕtp310-1 (0.0)	98 (99)	YP_001429893.1	Amidase_3 (PF01520) (2.6e−09); CHAP (PF05257) (9.9e−43); SH3_5 (PF08460) (7.3e−08)
61	43969	44319	351	116	13.3 (9.82)	Hypothetical protein	*Staphylococcus* p ϕ71 (2e−44)	92 (93)	YP_240409.1	Transmembrane regions
62	44552	45007	456	151	17.1 (9.88)	Hypothetical protein	*Staphylococcus* p ϕ71 (4e−78)	100 (100)	YP_240410.1	Signal peptide; transmembrane regions

[a] ID, identifier; HTH, helix-turn-helix; PVL, Panton-Valentine leukocidin.

ϕSLT are the closest relatives of phiIPLA35. The phiIPLA88 genome is practically identical to a prophage found in *S. aureus* RF122 and strongly related to ϕ11, ϕETA2, ϕ69, ϕ53, ϕNM, ϕ85, and ϕ187. Comparison of the phiIPLA35 sequence with the related ϕ12 sequence by the dot plot method (Fig. 5A) revealed colinearity throughout almost the entire genomic region. The extensive similarity of phiIPLA35 with these phages is underlined by the close relationship of some of them previously observed. The 3A, 42e, and 47 phages have been ascribed to class II, clade B (26), and display a similar B2 morphotype with a long, noncontractile tail and elongated head as described for phiIPLA35 (17). Detailed analysis between phiIPLA88 and ϕ11 by dot plot, as shown in Fig. 5B, revealed that the late gene cluster extending from the DNA packaging genes to the host lysis genes is more closely related than the early cluster comprising the lysis-lysogeny and replication clusters. However, no official taxonomic status can be given to phiIPLA35 and phiIPLA88 since both phages are artificially selected mutants (17).

The deficient lysogeny module. phiIPLA35 and phiIPLA88 are lytic derivatives selected after sodium pyrophosphate treatment of their parental temperate phages ϕA72 and ϕH5, respectively (17). Hence, the presence of a lysogeny module was expected. In both phages, gp1 was identified as the phage integrase (Tables 1 and 2), which would catalyze site-specific integration as it displays the C-terminal catalytic domain of DNA breaking-rejoining enzymes and of Cre integrases. The other module that is commonly present in temperate *Siphoviridae* is the lysis/lysogeny switch. The phiIPLA35 gp6 and phiIPLA88 gp4 proteins share extended similarity with repressors of the CI type. However, phiIPLA35 gp6 lacks 25 amino acids in the carboxy-terminal sequence (Fig. 3B), and the start codon was lost in phiIPLA88 gp4 (Fig. 4B). After the corresponding DNA regions of the parental temperate phages were sequenced and compared, two point mutations were mapped: (i) a 1-base insertion in phiIPLA35 at position 3614 and (ii) a 1-base replacement in phiIPLA88 that shifted the ATG codon to TTG. The presence (or absence) of a truncated CI repressor would explain why the lytic derivative phages are unable to lysogenize (17). It is worth mentioning that, in spite of the use of pyrophosphate that destabilizes the phage capsid and, consequently, selects for smaller genomes, no differences were observed between the restriction patterns of the temperate phages versus the lytic variants (data not shown), precluding the presence of relatively large DNA deletions. Therefore, it is likely that the lytic phages are spontaneous mutants, and the method used to obtain them would have

TABLE 2. Features of bacteriophage phiIPLA88 ORFs, gene products, and functional assignments

ORF	Position (nt)		Length (nt)	No. of amino acids	Size (kDa [pI])	Predictive function	Closest hit (e value)	% Amino acid identity (% similarity)	Accession no.	Predicted domain (protein ID) (e value)[a]
	From	To								
1	1065	1	1,065	354	41.0 (9.63)	Integrase	*S. aureus* RF122 (0.0)	99 (99)	YP_417222.1	Phage integrase (PF00589) (2.2e−14)
2	1639	1124	516	171	19.2 (8.98)	Hypothetical protein	*S. aureus* RF122 (6e−93)	100 (100)	YP_417221.1	Prokar_Lipoprotein (PS51257) (0.0)
3	2143	1643	501	166	19.2 (9.58)	Putative excisionase	*S. aureus* RF122 (9e−89)	100 (100)	YP_417220.1	
4	2834	2196	639	212	24.2 (5.67)	Mutated repressor	*S. aureus* phiNM (3e−107)	98 (99)	YP_873953.1	HTH (PF01381) (1.7e−14); Peptidase_S24 (PF00717) (0.00011)
5	3025	3279	255	84	10 (9.03)	Cro	*S. aureus* phiNM (4e−37)	97 (100)	YP_873954.1	HTH (PF01381) (1.8e−13)
6	4050	3460	591	196	22.3 (6.23)	Hypothetical protein	*S. aureus* RF122 (2e−107)	100 (100)	YP_417216.1	Transmembrane region
7	4107	4856	750	249	28.5 (9.79)	Antirepressor	*S. aureus* RF122 (4e−144)	100 (100)	YP_417215.1	ANT (PF03374) (2.5e−102); AntA (PF08346) (3.8e−31)
8	4872	5087	216	71	8.7 (9.70)	Hypothetical protein	*S. aureus* RF122 (4e−34)	100 (100)	YP_417214.1	DUF771 (PF05595) (3.3e−6)
9	5102	5272	171	56	6.4 (8.60)	Hypothetical protein	*S. aureus* RF122 (5e−24)	100 (100)	YP_417213.1	DUF1270 (PF06900) (5.0e−34)
10	5277	5591	315	104	12.4 (9.60)	Hypothetical protein	*S. aureus* RF122 (6e−53)	100 (100)	YP_417212.1	
11	5656	5958	303	100	11.1 (4.48)	Hypothetical protein	*S. aureus* RF122 (8e−49)	100 (100)	YP_417211.1	
12	5963	6223	261	86	10.1 (4.40)	Hypothetical protein	*S. aureus* ϕPVL108 (4e−46)	94 (96)	YP_918906.1	DUF1108 (PF06531) (6.2e−60)
13	6233	6454	222	73	8.6 (5.42)	Hypothetical protein	*S. aureus* RF122 (4e−35)	100 (100)	YP_417209.1	
14	6447	7067	621	206	23.5 (5.65)	Topoisomerase	*S. aureus* RF122 (6e−119)	100 (100)	YP_417208.1	DUF1071 (PF06378) (2.7e−12)
15	7070	7495	426	141	15.8 (5.24)	ssDNA binding protein	*S. aureus* RF122 (9e−76)	100 (100)	YP_417207.1	SSB (PF00436) (1.6e−29)
16	7509	8180	672	223	26 (6.60)	Hypothetical protein	*S. aureus* RF122 (8e−131)	100 (100)	YP_417206.1	DUF968 (PF06147) (0.0)
17	8177	8932	756	251	29.4 (8.37)	DNA replication protein	*S. aureus* RF122 (1e−145)	100 (100)	YP_417205.1	DnaD and phage-associated region (IPR006343) (3.6e−10)
18	8932	9288	357	118	13.7 (5.74)	Hypothetical protein	*S. aureus* RF122 (1e−64)	100 (100)	YP_417204.1	
19	9285	10526	1,242	413	47.4 (5.14)	DNA helicase	*S. aureus* RF122 (0.0)	100 (100)	YP_417203.1	DnaB_C (PF03796) (7.2e−51)
20	10523	10738	216	71	8.5 (5.13)	Hypothetical protein	*S. aureus* RF122 (1e−34)	100 (100)	YP_417202.1	
21	10741	10962	222	73	8.5 (4.83)	Hypothetical protein	*S. aureus* RF122 (2e−35)	100 (100)	YP_417201.1	
22	10973	11377	405	134	16.1 (9.79)	Hypothetical protein	*S. aureus* RF122 (1e−72)	100 (100)	YP_417200.1	DUF1064 (PF06356) (2.7e−86)
23	11382	11567	186	61	7.2 (4.06)	Hypothetical protein	*S. aureus* ϕ29 (5e−25)	93 (96)	YP_240593.1	
24	11568	11930	363	120	14.5 (10.42)	Hypothetical protein	*S. aureus* RF122 (2e−65)	100 (100)	YP_417199.1	PVL_ORF50 (PF07768) (1.5e–65)
25	11930	12184	255	84	9.6 (4.99)	Hypothetical protein	*S. aureus* RF122 (8e−44)	100 (100)	YP_417198.1	
26	12184	12432	249	82	9.8 (5.90)	Hypothetical protein	*S. aureus* RF122 (9e−42)	100 (100)	YP_417197.1	Phage_Orf51 (PF06194) (3.1e−59)
27	12441	12677	237	78	9.6 (10.17)	Hypothetical protein	*S. aureus* RF122 (3e−39)	100 (100)	YP_417196.1	
28	12737	12991	255	84	9.5 (3.95)	Hypothetical protein	*S. aureus* RF122 (3e−40)	100 (100)	YP_417195.1	DUF1024 (PF06260) (1.3e−51)
29	12978	13139	162	53	6.1 (3.30)	Hypothetical protein	*S. aureus* phage 71 (1e−19)	98 (98)	YP_240445.1	
30	13139	13675	537	178	20.6 (4.36)	dUTPase	*S. aureus* RF122 (6e−100)	100 (100)	YP_417194.1	dUTPase_2 (PF08761) (6.5e−6)
31	13712	13957	246	81	9.5 (10.08)	Hypothetical protein	*S. aureus* RF122 (1e−35)	100 (100)	YP_417193.1	Transmembrane regions
32	13954	14160	207	68	7.7 (7.31)	Hypothetical protein	*S. aureus* RF122 (6e−32)	98 (100)	YP_417192.1	DUF1381 (PF07129) (2.2e−43)
33	14157	14330	174	57	6.5 (4.05)	Transcriptional activator	*S. aureus* RF122 (4e−25)	100 (100)	YP_417191.1	RinB (PF06116) (4.5e−38)
34	14492	14911	420	139	16.1 (9.52)	Transcriptional activator	*S. aureus* RF122 (5e−72)	100 (100)	YP_417190.1	Phage transcriptional activator RinA (IPR006523) (2.1e−32)
35	15099	15593	495	164	18.9 (6.24)	Terminase small subunit	*S. aureus* RF122 (3e−93)	100 (100)	YP_417189.1	Terminase_2 (PF03592) (1.3e−39)
36	15596	16885	1,290	429	50.2 (9.33)	Terminase large subunit	*S. aureus* RF122 (0.0)	100 (100)	YP_417188.1	Terminase_3 (PF04466) (2.2e−11)
37	16896	18437	1,542	513	59.6 (4.60)	Portal protein	*S. aureus* RF122 (0.0)	100 (100)	YP_417187.1	Phage_prot_Gp6 (PF05133) (2.8e−100)
38	18319	18504	186	61	7.1 (11.05)	Hypothetical protein	*Staphylococcus* ϕ80α (2e−24)	98 (98)	YP_001285357.1	
39	18549	19433	885	294	33.9 (9.38)	Head morphogenesis protein	*S. aureus* RF122 (1e−173)	100 (100)	YP_417186.1	Phage_Mu_F (PF04233) (5.1e−06)
40	19506	19676	171	56	6.5 (9.70)	Hypothetical protein	*S. aureus* ϕ53 (5e−22)	92 (94)	YP_239646.1	
41	19811	20425	615	204	23.6 (4.92)	Scaffold protein	*S. aureus* RF122 (4e−109)	99 (100)	YP_417185.1	
42	20439	21413	975	324	36.6 (4.93)	Major head protein	*S. aureus* RF122 (3e−171)	100 (100)	YP_417184.1	Major capsid protein gp5 superfamily (SSF56563) (2.8e–26)
43	21435	21722	288	95	10.9 (5.21)	Hypothetical protein	*S. aureus* RF122 (9e−47)	100 (100)	YP_417183.1	
44	21731	22063	333	110	12.8 (5.08)	DNA packaging protein	*S. aureus* RF122 (8e−56)	100 (100)	YP_417182.1	
45	22362	22709	348	115	13.4 (5.60)	Head to tail joining protein	*S. aureus* RF122 (3e−62)	100 (100)	YP_417180.1	TP901-1_ORF40 (PF07772) (1.1e−38)
46	22721	23104	384	127	14.7 (4.21)	Hypothetical protein	*S. aureus* RF122 (8e−70)	100 (100)	YP_417179.1	
47	23122	23703	582	193	21.2 (4.55)	Major tail protein	*S. aureus* RF122 (4e−109)	100 (100)	YP_417178.1	Phage_tail_2 (PF06199) (4.1e−84)
48	23765	24130	366	121	13.1 (4.30)	Hypothetical protein	*S. aureus* RF122 (4e−60)	100 (100)	YP_417177.1	
49	24160	24504	345	114	13.5 (4.41)	Minor structural protein	ϕ11 (7e−60)	100 (100)	NP_803294.1	
50	24521	27988	3,468	1,155	125.6 (10.37)	Tape measure protein	*S. aureus* ϕNM (0.0)	98 (99)	YP_874001.1	TMP (PF05017) (0.089); WD_REPEATS_1 (PS00678); transmembrane regions
51	28001	28948	948	315	37 (7.28)	Minor tail protein	*S. aureus* RF122 (0.0)	100 (100)	NP_803296.1	WD_REPEATS_1 (PS00678)
52	28957	30858	1,902	633	71.1 (6.96)	Putative anti-receptor protein	*S. aureus* RF122 (0.0)	99 (100)	YP_417173.1	Phage minor structural protein, N-terminal (IPR007119) (0.024); SGNH hydrolase IPR13830 (1.1e−24)
53	30873	32783	1,911	636	73.3 (6.64)	Minor structural protein	*S. aureus* RF122 (0.0)	98 (99)	YP_417172.1	
54	32783	34606	1,824	607	66.5 (4.62)	Minor tail protein	*S. aureus* RF122 (0.0)	99 (99)	YP_417171.1	
55	34606	34983	378	125	14.1 (4.30)	Hypothetical protein	*S. aureus* ϕ85 (2e−59)	92 (95)	YP_239743.1	
56	34984	35166	183	60	7.2 (6.69)	Hypothetical protein	*S. aureus* ϕ29 (9e−29)	98 (98)	YP_240554.1	Phage uncharacterized protein XkdX (IPR010022) (3.3e-22)

Continued on following page

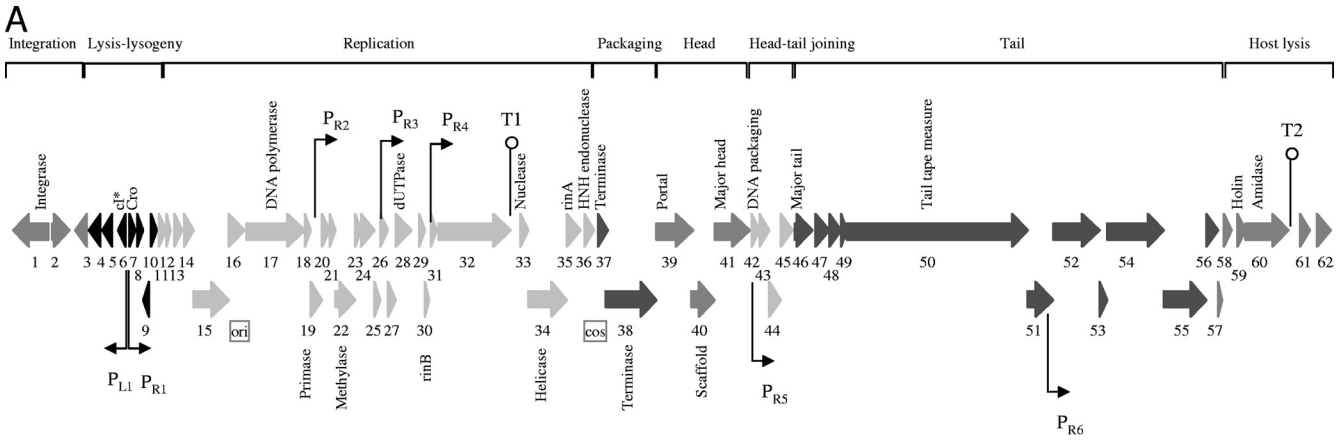
TABLE 2—*Continued*

ORF	Position (nt)		Length (nt)	No. of amino acids	Size (kDa [pI])	Predictive function	Closest hit (e value)	% Amino acid identity (% similarity)	Accession no.	Predicted domain (protein ID) (e value)[a]
	From	To								
57	35207	35506	300	99	12.0 (10.23)	Hypothetical protein	*S. aureus* ϕNM (2e−35)	89 (94)	YP_874008.1	Transmembrane region
58	35643	37547	1,905	634	72.5 (9.78)	Hydrolase	*S. aureus* RF122 (0.0)	99 (99)	YP_417168.1	CHAP (PF05257) (1.5e−30); LYZ2 (SM00047) (1e−51)
59	37660	39573	1,914	637	71.8 (6.48)	Tail fiber protein	*S. aureus* RF122 (0.0)	99 (99)	YP_417167.1	Collagen alpha chain (PTHR10499) (0.00037)
60	39624	40061	438	145	15.6 (4.52)	Holin	*S. aureus* RF122 (7e−79)	100 (100)	YP_417166.1	Transmembrane regions Phage_holin_1 (PF04531) (9.1e−50)
61	40042	41487	1,446	481	53.7 (8.71)	Amidase	*S. aureus* RF122 (0.0)	100 (100)	YP_417165.1	Amidase_2 (PF01510) (2.5e−38); SH3b (SM00287) (1.4e−12); CHAP (PF05257) (4.7e−47)

[a] ID, identifier; HTH, helix-turn-helix; PVL, Panton-Valentine leukocidin; SSB, single-stranded DNA binding protein.

been irrelevant. The second putative repressor-encoding gene (phiIPLA35 gp7 and phiIPLA88 gp5) specifies a polypeptide which resembles several phage-related transcription repressors and may represent a Cro analogue. Analysis of the phiIPLA88 *orf7* reveals a strong sequence similarity to designated antirepressors from a number of very different phages and could be responsible for inactivation/bypass of the CI transcription repressor.

The DNA replication, recombination, and modification module. Both phages have replication modules with genes in-



B

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3833  atggagaata ataaagtcag aaaaatttta tctgaaaacc ttcaagaact tatgaatgat aaaaatattg atcagagaga
 gp6    m  e  n    n  k  v    r  k  i  l    s  e  n    l  q  e    l  m  n  d    k  n  i    d  q  r
 wt     m  e  n    n  k  v    r  k  i  l    s  e  n    l  q  e    l  m  n  d    k  n  i    d  q  r

3753  acttgctgaa gctattggag tttctcaacc tacagtctcc aattggattc aacaaactaa atatccacga attaaaagaa
      e  l  a  e    a  i  g    v  s  q    p  t  v  s    n  w  i    q  q  t    k  y  p  r    i  k  r
      e  l  a  e    a  i  g    v  s  q    p  t  v  s    n  w  i    q  q  t    k  y  p  r    i  k  r

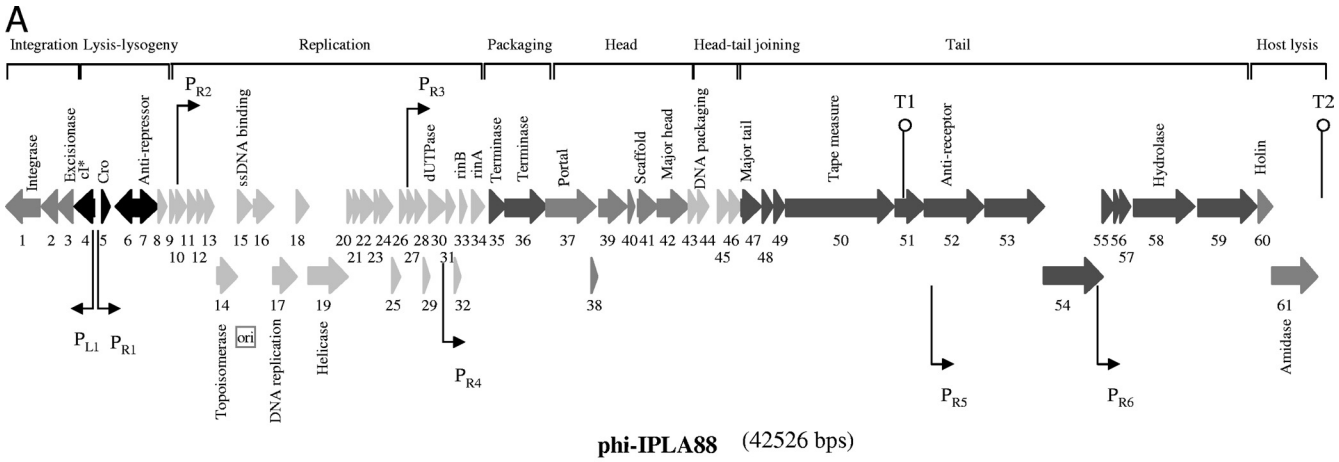
3706  ttcaacaact tgcagattac ttcaatgtac cgaaatcaag aattactgaa tcaaaaaaaa gatatacatc aagaaacaat
       i  q  q    l  a  d  y    f  n  v    p  k  s    r  i  t  e    s  k  k    r  y  t    s  r  n  n
       i  q  q    l  a  d  y    f  n  v    p  k  s    r  i  t  e    s  k  k    d  i  h    q  e  t

3593  tgctgctcat tttgataagg agggattaac tgaagaagag attgaagaag taaatagatt cattgaatgg gttagaaata
        c  c  s    f  -
      i  a  a  h    f  d  k    e  g  l    t  e  e  e    i  e  e    v  n  r    f  i  e  w    v  r  n

3513  gagacaaata a

       r  d  k   -
```

FIG. 3. Physical and genetic map of the bacteriophage phiIPLA35. (A) The ORFs are sequentially numbered, indicated by arrows proportional to their lengths and pointing toward their direction of transcription (L = left, R = right). Some ORFs have been placed below for clarity. The functional modules are indicated on top of the scheme, and the names of several putatively or experimentally identified genes are shown below. Promoter (P) and terminator (T) sequences are also indicated. (B) DNA sequence of gp6 showing the insertion mutation (in bold and underlined) and alignment of the putative CI amino acid sequence of the mutant and the wild-type (wt) phages, respectively. Numbers to the left of the DNA sequence are the genome coordinates.



B

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2834  acggctttca aaaattcaat aaaagaaatc agattgaaca atagattgtc taaagttgag atggctagaa aattagatgt
  gp4   t a f  k n s  i k e i  r l n  n r l  s k v e  m a r  k l d
  wt    m a f  k n s  i k e i  r l n  n r l  s k v e  m a r  k l d

2754  ttccgaaggt actataagaa tgtgggaaag tggaagaact gaacctagaa tgggtatggt cgaaaaaatt tcaagtttgt
      v s e g  t i r  m w e  s g r t  e p r  m g m  v e k i  s s l
      v s e g  t i r  m w e  s g r t  e p r  m g m  v e k i  s s l

2674  tcaatgtttc taaaggttat ctcttaggag aaattgaaga aattgtttta ccagaatttg atagcgaaat cgaggttcca
       f n v  s k g y  l l g  e i e  e i v l  p e f  d s e  i e v p
       f n v  s k g y  l l g  e i e  e i v l  p e f  d s e  i e v p

2874  tatttcggta aagtttctgc tggaaatttc gaggaagttg caattgataa tgaaaaatta aaagttccac catttgcttt
        y f g  k v s  a g n f  e e v  a i d  n e k l  k v p  p f a
        y f g  k v s  a g n f  e e v  a i d  n e k l  k v p  p f a

2974  taacggtcgt aaacctagcg aatgtatagc actaaaaata aacggagata gcatgaataa aatactcgct aacggttctt
      f n g r  k p s  e c i  a l k i  n g d  s m n  k i l a  n g s
      f n g r  k p s  e c i  a l k i  n g d  s m n  k i l a  n g s
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FIG. 4. Physical and genetic map of the bacteriophage phiIPLA88. (A) The ORFs are sequentially numbered, indicated by arrows proportional to their lengths and pointing toward their directions of transcription. Some ORFs have been placed below for clarity. The functional modules are indicated on top of the scheme, and the names of several putatively or experimentally identified genes are shown below. Promoter (P) and terminator (T) sequences are also indicated. (B) Partial DNA sequence of gp4 showing the replacement mutation (in bold and underlined) and alignment of the putative CI amino acid sequence of the mutant and the wild-type (wt) phages, respectively. Numbers to the left of the DNA sequence are the genome coordinates.

volved in DNA metabolism and replication. Gene products predicted to be DNA polymerases are found in both phages, as well as primases, single-stranded DNA binding proteins, helicases, and topoisomerases (Tables 1 and 2). In phiIPLA35, *orf22* encodes an N6-adenine methyltransferase, which probably specifically modifies newly synthesized viral DNA. phiIPLA35 gp28 and phiIPLA88 gp30 are predicted as dUTPases, the most conserved gene products among P335 phage species from *Lactococcus lactis* (27) and also present in many *S. aureus* phages. A region overlapping phiIPLA35 *orf16* and phiIPLA88 *orf15* also contains the putative *oriC*, as predicted from a cumulative GC skew analysis (Fig. 3 and 4). Within the phiIPLA35 replication module, gp36 is a zinc-dependent HNH homing endonuclease. It has been proposed that these endonuclease genes in phage genomes can be considered analogous to insertion or transposon elements in bacterial genomes (4, 43). phiIPLA35 *orf33* encodes a nuclease that may be involved in host DNA degradation. In both phages, homologs to the transcriptional regulators *rinA* and *rinB* were detected (Tables 1 and 2). These genes have been shown to be required for the activation of the staphylococcal ϕ11 integrase gene expression (55).

The structural and lysis module. The structural module can be divided into five submodules, ordered as packaging, head morphogenesis, head-tail connection, tail morphogenesis, and lysis (Fig. 3 and 4). Genes included specify proteins homologous to (or that bear domains typical of) small and large terminase subunits, portal proteins, tail tape measure, and head and tail structural proteins.

A putative translational frameshifting was observed in both phages in the two genes (*orf48* and *orf49*) just upstream from the tail tape measure gene. We have found a canonical −1 slippery site (G GGA AAG) located 6 and 20 codons upstream from the termination codon of *orf48* of phiIPLA35 and phiIPLA88, respectively. Putative pseudoknots downstream of each slippery sequence have also been detected (data not shown). This position is analogous to the phage lambda fusion protein gpG-T essential for tail assembly, even though it does not become part of the mature virion (32). In addition, in the case of tailed bacteriophages, the frameshifted products contained immunoglobulin-like domains which may be important to phage infection (14).

The lysis cassette would comprise the holin and lysin genes and was predicted in both phages (Fig. 3 and 4) and experi-

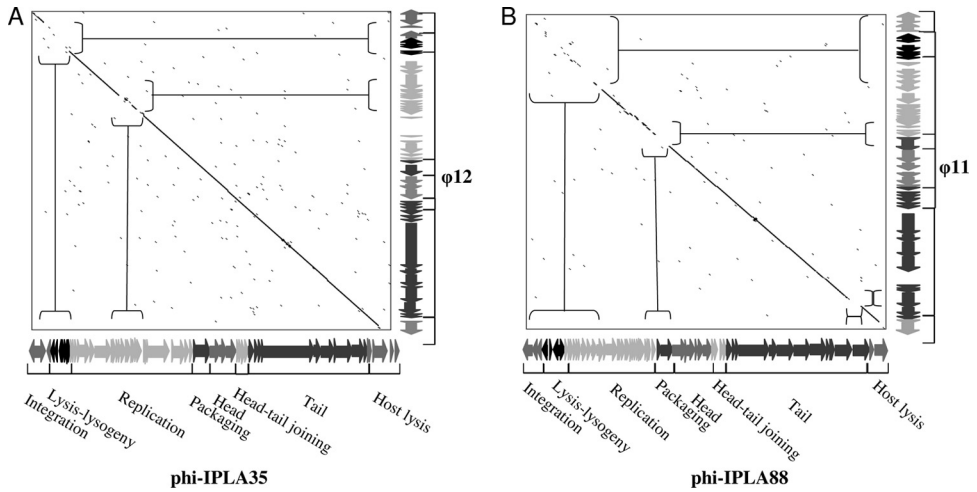

FIG. 5. Dot plot alignment of phiIPLA35 (A) and phiIPLA88 (B) genomes and related ϕ12 and ϕ11, respectively. The genome sequence is represented by the corresponding ORFs in each phage genome.

mentally verified previously for recombinant phiIPLA88 gp61 (36). The lysins show typical domains of amidase and endopeptidase activities.

Proteomic analysis of virion particles. The bioinformatics analysis of the phiIPLA35 and phiIPLA88 genomes suggests that the regions from *orf39* to *orf57* in phiIPLA35 and from *orf37* to *orf59* in phiIPLA88 harbor the genes encoding the proteins that build up the mature virions. Combining both N-terminal sequencing and mass spectrometry analysis after trypsin digestion of Coomassie blue-stained and silver-stained gels, respectively, six proteins of phiIPLA35 and eight of phiIPLA88 were identified, and their structural roles predicted by genome analysis were confirmed (Fig. 6). The additional faint bands on the gel could not be identified or represented mixtures of degradation products from the structural proteins.

Several examples of posttranslational processing were detected in both phages. N-terminal methionine processing takes place in each of the major tail proteins gp46 and gp47 (Fig. 6A and B). In phiIPLA88 virions, 14 amino acids were removed from the predicted major head protein gp42.

The N-terminal amino acid sequence obtained for the major head protein gp41 of phiIPLA35 is encoded upstream of the

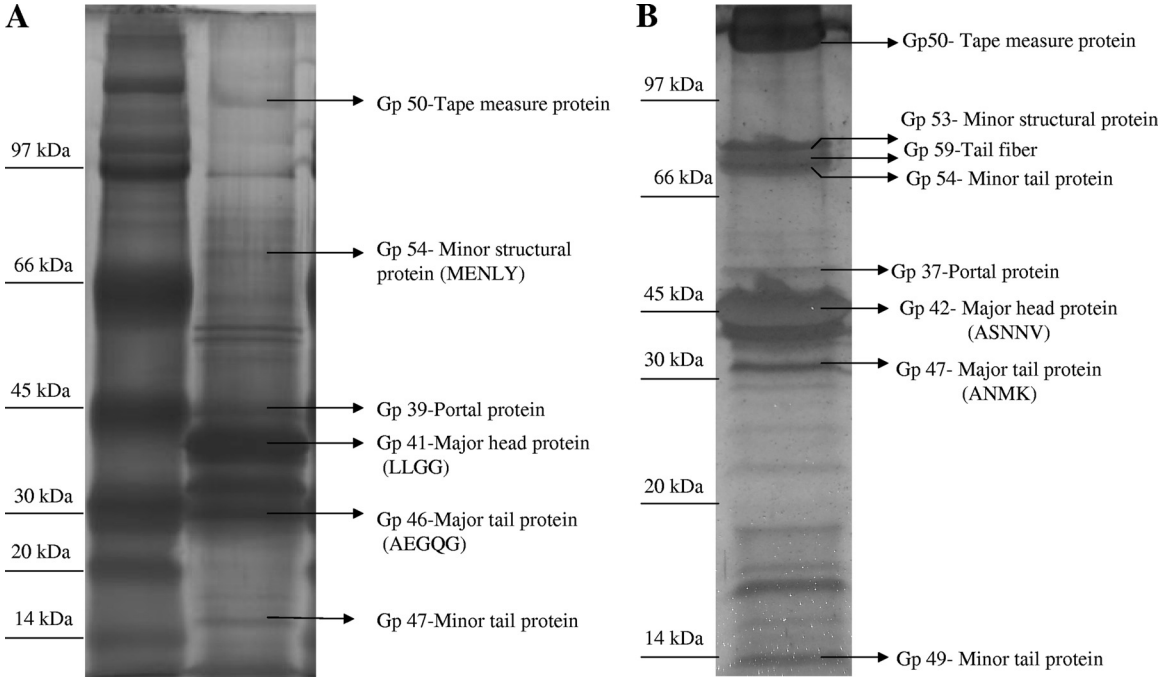

FIG. 6. Silver-stained SDS-PAGE analysis of the phiIPLA35 (A) and phiIPLA88 (B) virion proteins. Proteins identified by mass spectrometry are indicated on the right. Unassigned bands contained mixes of two or more structural proteins. If available, the N-terminal sequence is shown in parentheses. The migration of the molecular mass markers is shown at the left.

predicted ATG codon. This strongly suggests that gp41 might be synthesized as a fusion protein with gp40 and further processed. Indeed, closer inspection of the region between *orf40* and *orf41* reveals a potential −1 translational frameshifting element. Specifically, a slippery region, U UUA AAA (0 frame), and a pseudoknot that starts at position 23970 (3 nucleotides after the slippery sequence) and expands to position 24170 are observed at the 3′ end of *orf40*. The −1 frameshift would result in a 649-amino-acid protein combining both the scaffolding protein gp40 and the major head protein gp41. Bacteriophage frameshift occurrence is considered a strategy to compress as much information as possible into a very small genome or an alternative to obtain definite proportions of two proteins (16, 44). The fusion protein must be further processed to constitute the mature head protein detected in the virions. Interestingly, gp40 matches with proteases in its amino-terminal part (up to residue 186) and with scaffolding proteins in its carboxy-terminal part which, in addition, is predicted to be structured almost exclusively as a row of α-helices. It is tempting to speculate that the fusion protein could be autoprocessed by its own protease domain. The major capsid protein of *Pseudomonas aeruginosa* bacteriophage PAJU2 is thought to be synthesized as a protein fused to a prohead protease and is autocatalytically cleaved (53). Similar domain architecture is found in the capsid protein from *Stenotrophomonas* bacteriophage S1 (19). This protein matches three types of proteins in the databases: proteases, scaffold protein, and major head proteins. Matrix-assisted laser desorption ionization–time of flight analysis revealed that the major capsid protein is possibly autoprocessed by its protease domain, giving rise to the scaffold and the major capsid polypeptides.

Virion proteins with peptidoglycan hydrolytic activity. Mature virions are often endowed with peptidoglycan hydrolases involved in host cell wall degradation prior to injecting their genetic material during infection (35, 40). The genomes of the two *S. aureus* phages were screened for the presence of putative peptidoglycan hydrolytic domains, different from those found within the lysis cassette. A lytic transglycosylase-like domain (nucleotides [nt] 1838 to 1964) and a peptidase domain (nt 1705 to 1806) were identified in the tail tape measure protein, gp50, of phiIPLA35. Recently, it has been demonstrated that some tail tape measure proteins have structural domains similar to peptidoglycan hydrolases, enabling the entry of phage DNA through the thick peptidoglycan layer of host bacteria such as *Mycobacterium* (8, 39). In phiIPLA88, gp58 could also be an infection-related hydrolase as it bears two domains for peptidoglycan hydrolysis (LYZ2 and CHAP).

To confirm the presence of structural components with peptidoglycan hydrolytic activity in phiIPLA35 and phiIPLA88 virions, zymogram assays were performed with autoclaved *S. aureus* Sa9 cells. Upon electrophoretic separation of the virion proteins, the zymogram revealed a single 70-kDa band with muralytic activity in each phage (Fig. 7). The size is consistent with the virion protein gp58 (72.5 kDa) of phiIPLA88, which contains the predicted peptidoglycan hydrolytic domains. In contrast, for phiIPLA35 the size corresponding to this activity is smaller than the estimated size for gp50 (226 kDa). However, proteolytic processing of this protein might explain this result.

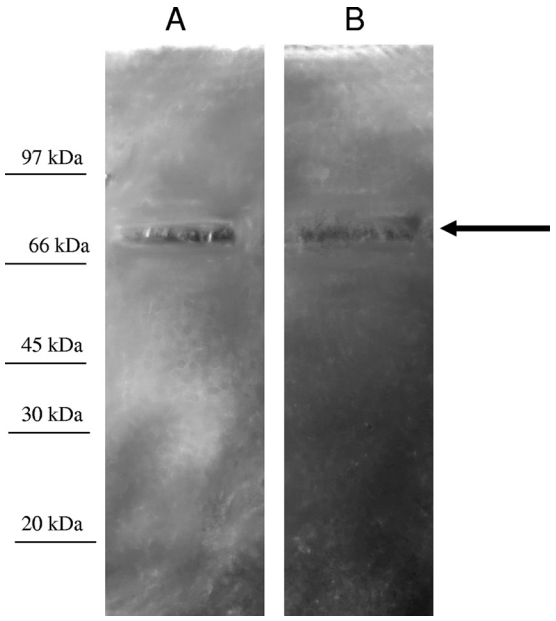

FIG. 7. Zymogram gels of phiIPLA35 (A) and phiIPLA88 (B) phages. About 10^{11} particles were loaded on each lane for the zymogram analysis. Molecular mass markers are indicated at the left.

Conclusions. In this study, we have presented a detailed genomic and molecular characterization of two *S. aureus* lytic derivative phages, phiIPLA35 and phiIPLA88, isolated from the dairy environment. The analysis revealed the mutations responsible for the loss of their ability to lysogenize. In addition, these genomes do not clearly encode known virulence factors, whereas new peptidoglycan lytic activities have been identified. All these elements suggest that these phages could be useful as natural antimicrobials against *S. aureus* in dairy products.

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