

Molecular Characterization and Lytic Activities of *Streptococcus agalactiae* Bacteriophages and Determination of Lysogenic-Strain Features[∇]

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The application of mitomycin C induction to 114 genetically diverse *Streptococcus agalactiae* strains generated 36 phage suspensions. On electron microscopy of the phage suspensions, it was possible to assign the phages to the *Siphoviridae* family, with three different morphotypes (A, B, and C). Phage genetic diversity was evaluated by a PCR-based multilocus typing method targeting key modules located in the packaging, structural, host lysis, lysogeny, replication, and transcriptional regulation clusters and in the integrase genes and by DNA digestion with EcoRI, HindIII, and ClaI. Thirty-three phages clustering in six distantly related molecular phage groups (I to VI) were identified. Each molecular group was morphotype specific except for morphotype A phages, which were found in five of the six phage groups. The various phage groups defined on the basis of molecular group and morphotype had specific lytic activities, suggesting that each recognized particular host cell targets and had particular lytic mechanisms. Comparison of the characteristics of lysogenic and propagating strains showed no difference in the serotype or clonal complex (CC) identified by multilocus sequence typing. However, all the lysogenic CC17 and CC19 strains presented catabolic losses due to a lack of catabolic decay of DL-alpha-glycerol-phosphate substrates (CC17) and of alpha-D-glucose-1-phosphate (CC19). Moreover, the phages from CC17 lysogenic strains displayed lytic replication in bacterial hosts from all *S. agalactiae* phylogenetic lineages other than CC23, whereas phages obtained from non-CC17 lysogenic strains lysed bacteria of similar evolutionary origin. Our findings suggest that the adaptive evolution of *S. agalactiae* exposed the bacteria of this species to various phage-mediated horizontal gene transfers, which may have affected the fitness of the more virulent clones.

The Lancefield group B *Streptococcus*, *Streptococcus agalactiae*—a major cause of neonatal infections—has increasingly been reported as a common pathogen in nonpregnant adults since the 1970s (40). The proportion of neonatal infections caused by serotype III multilocus sequence type 17 (ST-17) strains is higher than would be expected on the basis of the proportion of women and infants colonized by ST-17 strains in control populations (3, 5, 24, 26, 30, 33). The major clonal complexes (CC) 1, 12, 17, 19, and 23 have been associated with infections in adults (4, 5, 14, 21). The presence in the *S. agalactiae* genome of particular insertion sequences, a group II intron, and prophage DNA fragments (17, 36, 49) suggests that horizontal genetic transfer may play an important role in genome diversification and the emergence of virulent clones in *S. agalactiae*.

Temperate phages affect bacterial fitness by modifying anchor points for genome rearrangements, by disrupting genes, by protecting against lytic infection, by lysing competing strains through prophage induction, and by introducing new fitness

factors (8, 18). Little is currently known about these phages. *S. agalactiae* phages were first isolated in 1969 (37). A phage-typing method has since been proposed for epidemiological investigations (1, 7, 45, 46). More recent analysis of sequenced *S. agalactiae* strains has revealed the presence of abundant regions resembling prophages (47, 48) although there is currently no evidence to suggest that these regions correspond to functional bacteriophages or remnants. We recently identified, cloned, and sequenced three prophage DNA fragments from *S. agalactiae* genomes that displayed significant alignment with previously identified prophage sequences from *S. agalactiae* and *Streptococcus pyogenes*. We found that these prophage DNA fragments were more frequent in the genomes of strains from a particular phylogenetic lineage isolated from cases of neonatal meningitis. Thus, genetic events coinciding with lysogeny may have increased the ability of strains to invade the brain endothelium (49).

We investigated lysogeny in *S. agalactiae* species by carrying out phage induction in 114 *S. agalactiae* strains representative of the genetic diversity of strains isolated from the genital tract and neonates. In accordance with the recommendations of the International Committee on Taxonomy of Viruses (11), the isolated phages were characterized by classical methods: electron microscopy analysis of virions, restriction endonuclease analysis (REA), and determination of the lytic spectrum of

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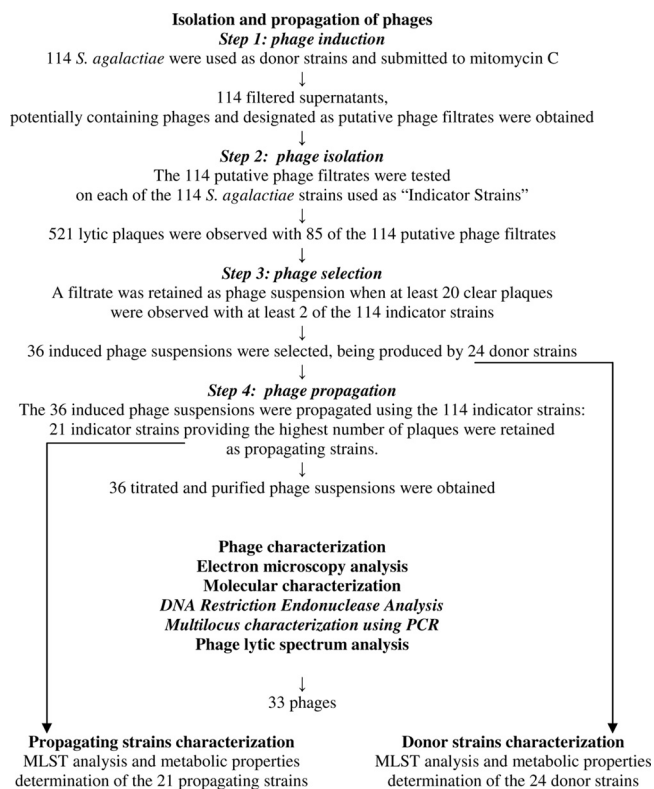


FIG. 1. The various steps in phage isolation.

phages. In addition, a PCR-based method targeting phage sequences located in the packaging cluster, the structural cluster, the host lysis cluster, the integrase genes, the lysogeny cluster, the replication cluster, and the transcriptional regulation cluster was developed for multilocus phage characterization. Molecular features were used to assess the genetic diversity of *S. agalactiae* bacteriophages. We characterized the phage donors and propagating strains by serotyping, multilocus sequence typing (MLST), and the assessment of carbon source oxidation to identify particular features of *S. agalactiae* lysogenic strains.

MATERIALS AND METHODS

Bacterial strains. Phage induction was carried out in 114 *S. agalactiae* strains; 113 *S. agalactiae* strains were selected on the basis of previously established characteristics (33, 36) as representative of the diversity of strains colonizing the vagina or responsible for neonatal infections. The remaining isolate was strain 2603V/R, which has already been sequenced (48). Fifty-nine strains were colonizing strains isolated from the vaginas of asymptomatic pregnant women ($n = 37$) and asymptomatic neonates ($n = 22$), and 55 strains originated from the cerebrospinal fluid samples of neonates suffering from meningitis. Twenty-eight strains were of serotype I, 19 were of serotype II, 55 were of serotype III, 3 were of serotype IV, and 4 were of serotype V, including the 2603V/R strain.

Isolation and propagation of *S. agalactiae* phages. *S. agalactiae* phages were isolated in a four-step process (Fig. 1), as follows.

Step 1: phage induction. Each of the 114 *S. agalactiae* strains was assessed as a potential donor strain and subjected to mitomycin C induction (45). Each strain was plated on Trypticase soy agar containing 5% (vol/vol) sheep blood and incubated overnight at 37°C. A single colony was then added in 4.5 ml of modified Todd Hewitt broth (mTHB) consisting of 30 g of THB base (Becton Dickinson, le Pont de Claix, France), 2 g of yeast extract (Oxoid LTD, Basingstoke Hampshire, England), 12 mg of CaCl₂ (VWR Prolabo, Fontenay-sous-Bois, France), and 10 mg of L-tryptophan (Merck, Darmstadt, Germany) per

liter; the culture was then incubated for 2 h at 30°C. Mitomycin C (Sigma-Aldrich, St.-Quentin-Fallavier, France) was added to the mTHB at a final concentration of 1 µg per ml. Incubation was continued for 2 h. The culture was then centrifuged at 1,500 × *g* for 15 min. The supernatant was filtered through a 0.45-µm-pore-size filter (Millipore, Bedford, MA). The 114 filtered supernatants, corresponding to putative phage filtrates, were stored at −80°C.

Step 2: phage isolation. We assessed the lytic activity of each of the 114 putative phage filtrates against the 114 *S. agalactiae* strains used as indicator strains. Each phage filtrate was tested with each indicator strain, using a multiloop applicator: 10 µl of each filtrate was poured onto a base layer (20 ml) of mTHB containing 0.7% agar (wt/vol) and inoculated with an overnight culture of an indicator strain in mTHB. Plates were incubated overnight at 30°C and examined for the presence of lytic plaques.

Step 3: phage selection. A putative phage filtrate was considered to be a phage suspension if it provided at least 20 clear plaques with at least two different indicator strains. A clear plaque was then picked and dissolved in 1 ml of mTHB. The phage suspensions thus obtained were stored at −80°C.

Step 4: phage propagation. The indicator strain yielding the largest number of plaques for a given phage suspension was defined as the propagating strain for that phage suspension. Each phage was then propagated in its propagating strain. We added 1 ml of phage suspension to 10 ml of semisolid medium (mTHB plus 0.4% agar [wt/vol]) and 800 µl of an overnight culture of the propagating strain in mTHB. The mixture was then poured onto a base layer (20 ml) of mTHB containing 0.7% agar (wt/vol) and incubated for 24 h at 30°C. Phage propagation resulted in visible plaques, which were recovered in 10 ml of mTHB. The semisolid medium was then taken up and centrifuged at 1,500 × *g* for 15 min. The supernatant was passed through a filter with 0.45-µm pores and titrated by plating serial dilutions on a lawn of the propagating strain on modified Todd-Hewitt agar. The titrated induced phage preparations were kept at −80°C and named φn.

Phage characterization. (i) **Electron microscopy analysis of phages.** Based on the guidelines of the International Committee on Taxonomy of Viruses (19), the induced phage preparations were centrifuged on a sucrose density gradient. Phage particles were negatively stained with 2% uranyl acetate, examined in a JEOL 1230 transmission electron microscope at an accelerating voltage of 120 kV, and photographed. Morphological types were defined on the basis of phage tail length.

(ii) **Molecular characterization of isolated phages.** (a) **REA.** Phage DNA isolation and purification were carried out with a Lambda Minikit (Qiagen, Valencia, CA). The phage DNA was digested separately with EcoRI, HindIII, and ClaI (Roche Diagnostic, Meylan, France) by adding 15 µl of DNA to 15 IU of each restriction endonuclease. After 3 h of incubation at 37°C, the DNA fragments were separated by electrophoresis in 0.8% agarose gels using a voltage gradient of 50 V over 16 h. Digestion patterns were examined under UV transillumination after staining with ethidium bromide and manually compared.

(b) **Multilocus characterization of phages by a PCR-based method.** A PCR-based method was used to study the genetic diversity of phages and to assess the level of similarity between isolated phages. As *S. agalactiae* phages have yet to be sequenced, we performed an in silico analysis of the genomes of eight sequenced *S. agalactiae* strains: strains 515, A909, 18RS21, COH1, H36B, and CJB111 (47); strain 2603V/R (48); and strain NEM316 (16). We identified 15 target genes, numbered 1 to 15, encoding phage proteins (Fig. 2 and Table 1). These 15 target genes are located in four long sequences corresponding to four prophages—Sa1 (strains 2603V/R, 18RS21, and 515), Sa3 (strains A909, H36B, and 515), Sa4 (strains A909 and CJB111), and Sa5 (strains A909, 18RS21, COH1, H36B, and CJB111)—or in prophage remnants in the *S. agalactiae* genomes of strains A909, 515, H36B, and 18RS21. Using BLAST software, we aligned the genome sequences obtained, with a view to identifying sequences specific to each of the target genes. Primers for amplifying these specific sequences were designed with primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 1). PCR was performed on each of the isolated phages with the 15 primer pairs. The sequences amplified were located in the packaging cluster (PCR 1), the structural cluster (PCRs 2, 3, 4, and 5), the host lysis cluster (PCRs 6 and 7), the integrase genes (PCRs 8 and 9), the lysogeny cluster (PCRs 10, 11, and 12), the replication cluster (PCR 13), and the transcriptional regulation cluster (PCRs 14 and 15). The PCR assay was performed on a Chromo 4 System instrument (Bio-Rad, Hercules, CA) in a final volume of 25 µl containing 5 µl of extracted DNA, the necessary primers (each at a concentration of 0.5 µM), and 1× iQ SYBR Green Supermix (Qiagen SA, Courtabouef, France), including 3 mM MgCl₂. Amplification was performed over 40 cycles of 10 s at 94°C, 10 s at a hybridization temperature specific for each primer set (Table 1), and 30 s at 72°C. The reaction products were cooled to 35°C and subjected to a post-PCR melting cycle by increasing the temperature by 0.2°C for each 10-s cycle, up to

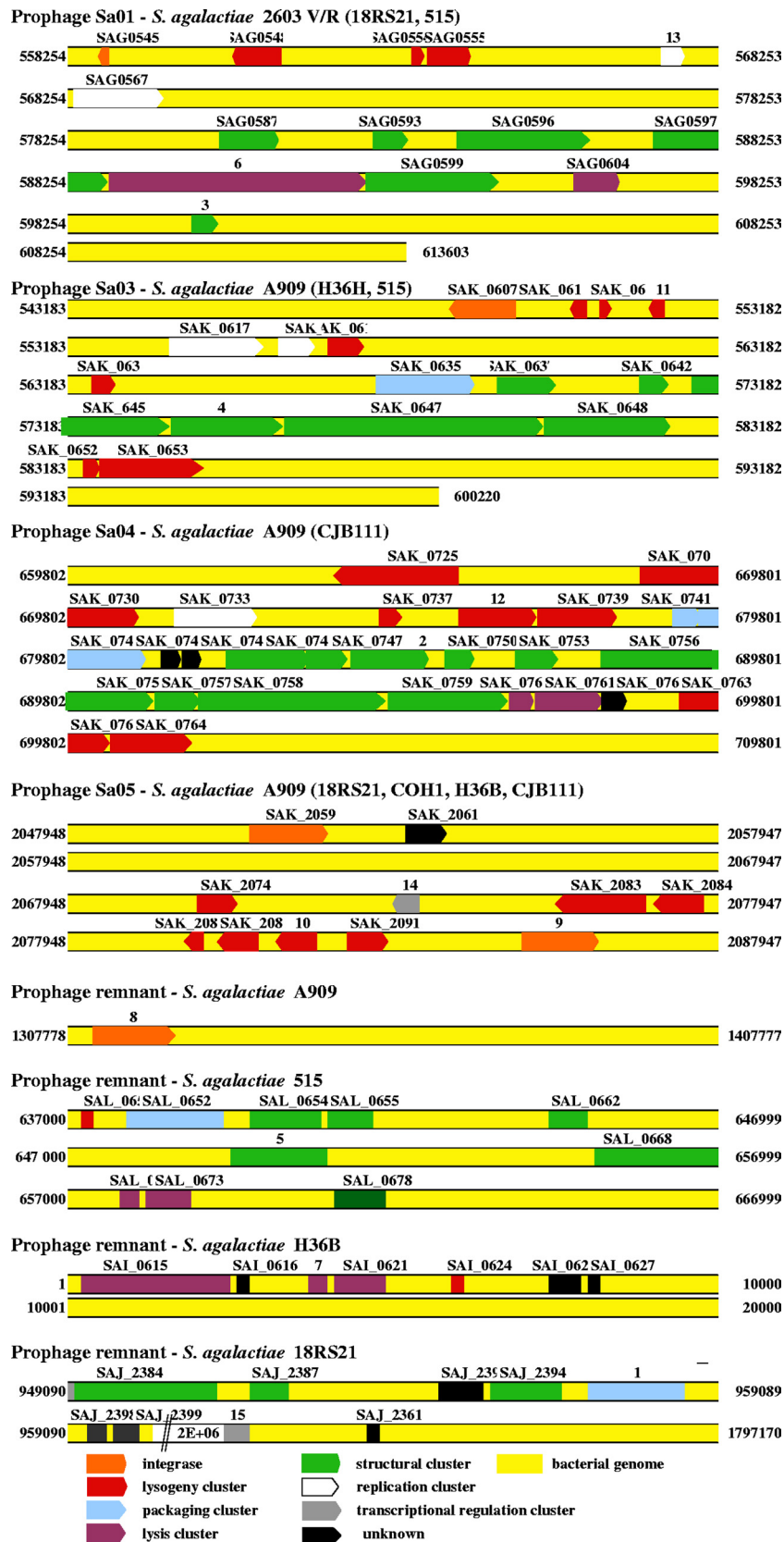


FIG. 2. Identification and localization of the 15 target genes used for multilocus characterization of phages by a PCR method and identified by an in silico analysis of eight sequenced *S. agalactiae* genomes. The target genes numbered 1 to 15 were located in the packaging cluster (1), the structural cluster (2, 3, 4, and 5), the host lysis cluster (6 and 7), the integrase genes (8 and 9), the lysogeny cluster (10, 11 and 12), the replication cluster (13), and the transcriptional regulation cluster (14 and 15).

TABLE 1. Multilocus PCR-based molecular characterization of phage: primers used and amplicon size

Prophage	PCR no. ^a	Target gene		Reference strain(s)	Primer		Hybridization temp (°C)	Amplicon size (bp)
		Locus	Description		Direction	Sequence (5' → 3')		
Sa01	13	SAG0566	Single-strand binding protein prophage lambda Sa1	2603 V/R, 18RS21	Forward	GTGCTTTGGTTGGAATTAC	54	132
	6	SAG0598	Prophage lambda Sa1; N-acetylmuramoyl-L-alanine amidase, family 4	18RS21, 2603V/R	Reverse	TCTGTGTTGGCTATTGC	54	289
	3	SAG0610	Hypothetical protein Sa1	2603V/R, H36B, COH1, 515, A909, CJB111	Forward	TTGATATACTCCACATTAGC	49	192
					Reverse	CCTTCCTTGTTCATACG		
Sa03	4	SAK_0646	Prophage lambda Sa03; tail component; putative	A909, 2603V/R	Forward	AATACAATACAATAGAAGATTAC	54	233
	11	SAK_0613	Prophage ps2; protein 07; excisionase	H36B, A909	Reverse	TCCGTCTTGAGTATAGTC	49	128
Sa04	12	SAK_0738	DNA methylase prophage lambda W4	CJB111, A909	Forward	GACTATGGCGATTATGTG	54	172
	2	SAK_0748	Phage major capsid protein; HK97 family	CJB111, A909	Reverse	CTATGCGTTGGATTATTG	52	136
Sa05	14	SAK_2079	Prophage Sa05; ArpU family transcriptional regulator	A909, COH1, CJB111, 18RS21	Forward	GGGATAAGAAAGCCAATC	47	67
	10	SAK_2090	BRO domain protein, prophage antirepressor prophage Sa05	A909, H36B, CJB111	Reverse	ACATAGATAGACGCATCG	54	102
	9	SAK_2094	Prophage Sa05 site-specific recombinase; phage integrase family	A909, H36B, CJB111, 18RS21, COH1	Forward	TGATTTCTCTACTACTGGATTG	49	526
	8	SAK_1326	Site-specific recombinase, phage integrase family	A909, H36B, CJB111	Reverse	CGCTTCTGGTAGAACGAG	50.5	261
	5	SAL_0666	Putative prophage lambda Sa1; minor structural protein	515, 18RS21	Forward	GCGTTACCCAGTTTGATATAG	50.5	289
	7	SAI_0620	Phage holing; LL-H family	H36B, 515, 2603V/R	Reverse	CATAGAGATACACGACATC	47	94
	1	SAJ_2395	Phage terminase-like protein, large subunit	18RS21, 515	Forward	TGATAGATAAGTATGTGAGATTG	50.5	251
	15	SAJ_2357	gp31	18RS21, 515	Reverse	TTGCTTTCCGAGTTTAGC	50.5	247
					Forward	ACTATTATATCATACGAGGAG		
					Reverse	ATTGCTTCTAATTCTTGTTTC		

^a PCRs were numbered as defined in Fig. 2.

95°C. PCR amplifications were validated by sequencing one of each of the amplicons obtained with each formulated primer pair. For all phages, PCR amplification was considered positive if an amplicon of the expected size was generated.

We investigated the genetic relationship between the genomes of isolated phages by carrying out a hierarchical analysis by the Jaccard dichotomy coefficient method with SYSTAT12 (LogiLabo, Paris, France). The genetic features used for analysis were the amplification by PCR of the 15 phage gene sequences studied and of each of the DNA fragments obtained by REA with the three enzymes used in this study. An absence of gene amplification was not considered to indicate similarity between the phages studied.

(iii) Determination of phage lytic spectrum. The lytic activity of the induced phage preparations was assessed against the 114 *S. agalactiae* strains. Each strain was used to inoculate 4.5 ml of mTHB, which was then incubated at 30°C for 2 h. For each strain, two petri dishes (90 mm) containing modified Todd-Hewitt agar were flooded with fresh cultures. Excess fluid was removed with a pipette, and the plates were dried. In accordance with the phage typing procedure (46), we used induced phage preparation dilutions corresponding to 10⁶ and 10⁷ phages per ml. The induced phage preparations were applied to the dried bacterial lawns with a multiloop applicator (Biddulf and Co., Manchester, United Kingdom). Plates were incubated overnight at 30°C and examined with oblique transmitted light against a dark background. Using a previously published method (27), we subjected the lytic reactions observed, characterized by >50 plaques or confluent lysis, to hierarchical analysis by the Jaccard dichotomy coefficient method, with SYSTAT12 (LogiLabo, Paris, France). A representation of the phages clustered as a function of their reactions with strains is shown as a dendrogram.

Characterization of donor and propagating strains. Donor and propagating strains were serotyped and analyzed by MLST, as described by Jones et al. (21). Using the unweighted-pair group method with arithmetic mean, a tree was generated from allelic profile data, using Phylodendron and the entire group B streptococcus MLST database (<http://pubmlst.org/sagalactiae/>). CCs were designated on the basis of reported findings (21). We also assessed the ability of these strains to oxidize carbon sources, which is known to be variable in this species,

with the Biolog system as previously described (13). Differences in catabolic functions between the donor and propagating strains were studied by chi square tests or Fisher-Yates tests. A *P* value of <0.05 was considered to indicate statistical significance.

RESULTS

Mitomycin C induction, according to the procedure summarized in Fig. 1, generated 36 induced phage suspensions from 24 different donor strains. We selected 21 indicator strains.

Electron microscopy analysis of phages isolated from the 36 *S. agalactiae* phage suspensions. Examination of the 36 induced phage preparations (Fig. 3) showed that the phages belonged to the *Siphoviridae* family, characterized by isometric, nonenveloped, 54-nm-diameter heads and filamentous cross-banded tails with short terminal fibers. Three morphotypes were defined on the basis of tail length (34): morphotypes A (108 nm ± 10 nm), B (145 nm ± 10 nm), and C (215 nm ± 10 nm). Eight of the 36 phages observed were of morphotype A (22%), 17 were of morphotype B (47%), and 11 were of morphotype C (31%) (Fig. 3).

Molecular characterization of phage DNA. (i) REA. DNA extracted from the 36 induced phage preparations was digested with EcoRI, HindIII, and ClaI. EcoRI restriction provided 12 profiles (E1 to E12), HindIII restriction provided 12 profiles (H1 to H12), and ClaI restriction provided 13 profiles (C1 to C13). Restriction profiles were not obtained in seven cases.

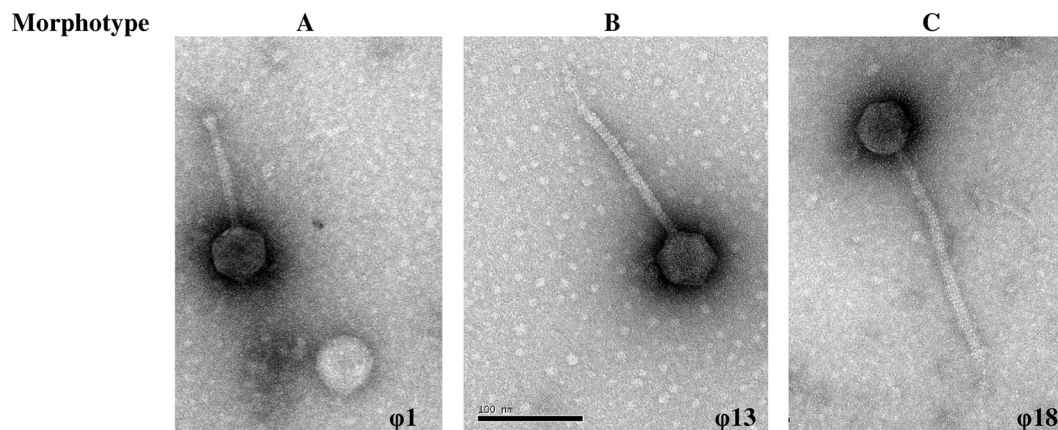


FIG. 3. Electron micrograph of *S. agalactiae* phages. Three morphotypes were found (A, B, and C).

Twenty-three REA types were recovered by combining the REA profiles obtained with the three enzymes. Five of these REA types contained more than one phage (Fig. 4).

(ii) **Multilocus phage characterization.** In cases of positive PCR amplification, the amplicons obtained with primer pairs targeting the 15 studied phage gene sequences were all of the expected sizes. The selected primers gave a high rate of amplification of phage gene sequences from the transcriptional regulation cluster (33/36, or 92%), the replication cluster (24/36, or 67%), the host lysis cluster (23/36, or 64%), and the structural cluster (32/36, or 89%) (Fig. 5). Amplification with the two primer pairs targeting gene sequences from the transcriptional regulation cluster resulted in the identification of four different genetic patterns. The two primer pairs targeting gene sequences in the host lysis cluster identified four genetic patterns, and the four primer pairs targeting gene sequences in the structural cluster identified six genetic patterns. By contrast, the selected primers gave low rates of amplification for sequences from the lysogeny cluster (10/36, or 28%; four genetic patterns), the integrase genes (4/36, or 11%; two genetic patterns), and the packaging cluster (7/36, or 19%) (Fig. 5).

Using the 15 PCR targeting phage genes, we obtained 25 different genetic patterns for the 36 phage suspensions studied.

(iii) **Genetic relationship between phages.** Hierarchical analysis using the molecular phage characteristics established by REA and multilocus characterization was used to estimate the genetic relationship between the isolated phages (Fig. 5, dendrogram). Among the 36 induced phage preparations, 33 phages displayed genetic dissimilarity ($\phi 1$ to $\phi 33$) (Fig. 5) but clustered in six distantly related molecular phage groups (I to VI), with a level of 42% dissimilarity (Fig. 5). Each molecular phage group was characterized by a specific phage morphotype (Fig. 5). Phage group I clustered two phages ($\phi 1$ and $\phi 2$) of morphotype A; phage group II clustered 11 phages ($\phi 3$ to $\phi 13$) of morphotype B. Phage groups III and IV contained mostly (five of seven phages) morphotype phages C ($\phi 15$ to $\phi 19$), and phage group V contained mostly (four of five phages) morphotype B phages ($\phi 21$ and $\phi 23$ to $\phi 25$). Phage group VI contained two major lineages, one of morphotype C ($\phi 27$ to $\phi 30$) and the other of morphotype A ($\phi 31$ to $\phi 33$). Thus, morphotype A phages were found in five of the six phage molecular groups and may therefore be considered the most diverse genetically.

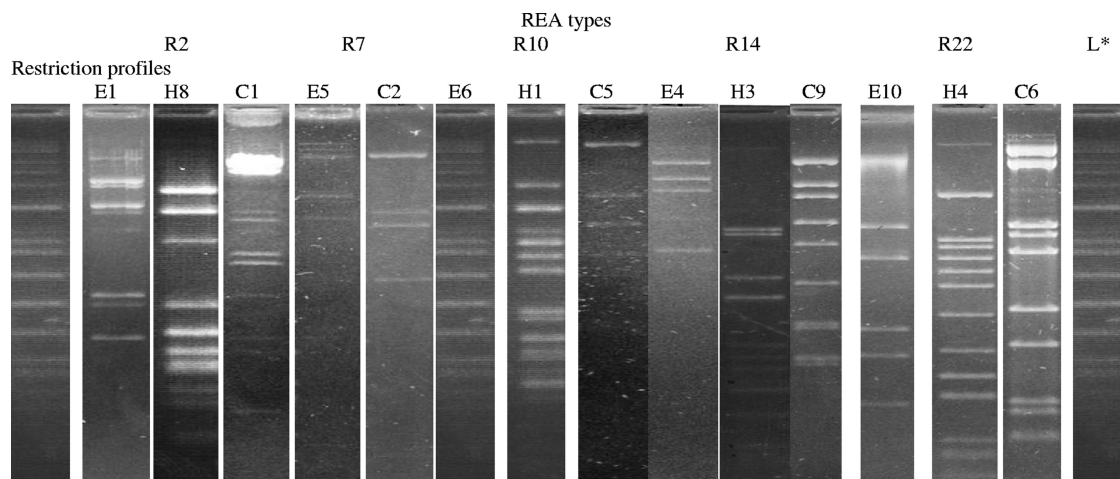


FIG. 4. EcoRI, HindIII, and ClaI profiles of the five major REA types observed for phages from 36 phages suspensions. L*, Raoul ladder (MWRAU300; Q-Biogene, Strasbourg, France).

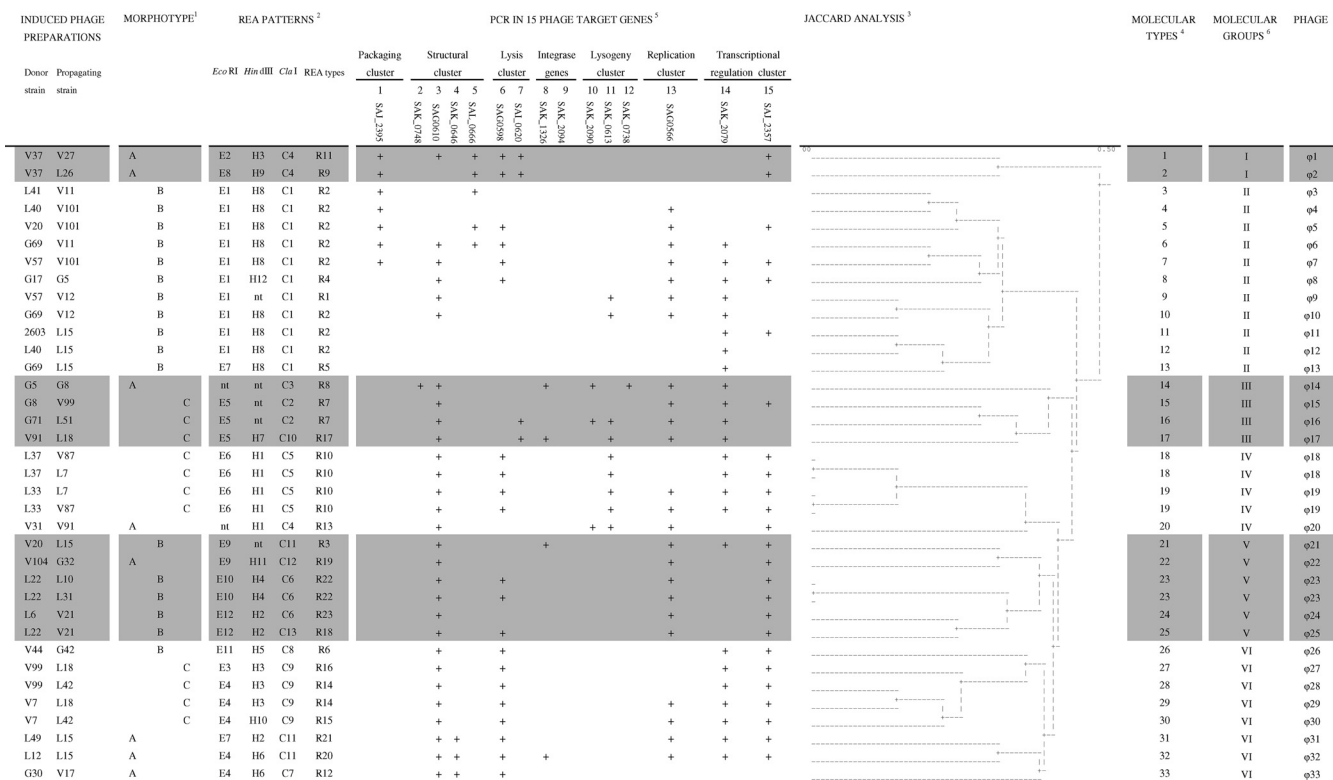


FIG. 5. Characteristics of 36 *S. agalactiae* phages. Morphotypes were defined as a function of tail length (1). REA types were obtained by combining the restriction profiles obtained with the three restriction enzymes (2). Jaccard analysis shows a dendrogram of similarity values for the molecular characteristics of *S. agalactiae* phages, combining REA type and results for the 15 prophage sequences (3). Molecular types were obtained by combining REA type and results for the 15 prophage sequences (4). PCR results with screening for the 15 prophage sequences found in published *S. agalactiae* genome sequences are shown (5). Molecular groups were defined at a level of 42% dissimilarity (6).

Lytic activities of *S. agalactiae* phages. We observed 340 lytic reactions in tests of the lytic activity of the 36 induced phage preparations against the collection of 114 *S. agalactiae* strains. Hierarchical analysis of the lytic reactions observed for each phage distinguished 31 different lytic types (Fig. 6, dendrogram); 30 of these 31 lytic types were distributed in two major branches of the dendrogram, including 12 in branch A and 18 in branch B (Fig. 6). The diversity of phage groups, as defined by their genetic characteristics and morphotypes, was therefore correlated with their lytic activity. The 11 molecular group II/morphotype B phages had diverse lytic types (L1 to L12) (Fig. 6), but all these lytic types were related and clustered in major branch A of the dendrogram, suggesting similarities in terms of the host cell targets recognized or lytic mechanisms involved. The molecular group IV/morphotype C phages (ϕ 18 and 19) had lytic activities L18 and L19, displaying 87% similarity (Fig. 6, branch C), and the four molecular group VI/morphotype C phages (ϕ 27 to ϕ 30) had similar activities of the L23 type (Fig. 6, branch D), indicating strong similarities in terms of the host cell targets recognized or the lytic mechanisms involved. By contrast, the four molecular group V/morphotype B phages (ϕ 21, the pair ϕ 23 and ϕ 24, and 25) and the three molecular group III/morphotype C phages (ϕ 15 to ϕ 17) had diverse lytic activities. The group of phages of morphotype A (ϕ 1, ϕ 2, ϕ 14, ϕ 20, ϕ 22, and ϕ 31 to 33), which was the most genetically diverse, also displayed the highest degree of diversity in lytic activities. Nevertheless, the lytic types generated by

these morphotype A phages were all related and located in branch B of the dendrogram (Fig. 6).

Characteristics of donor and propagating strains. Determination of the serotypes and phylogenetic origins of the 24 donor and 21 propagating strains by MLST indicated that the distribution of strains within serotypes and within the *S. agalactiae* lineages of the studied population—CC10, CC17, CC19, and CC23—did not differ between donor and propagating strains (Fig. 7 and 8). By contrast, studies of catabolic functions showed a loss of catabolic function in the lysogenic strains of CC17 and CC19. Indeed, all five CC19 donor strains (100%) and only one of the six CC19 propagating strains (17%) showed no decay of alpha-D-glucose-1 phosphate substrate (H10) ($P = 0.013$). All six CC17 donor strains (100%) and only one of the three CC17 propagating strains (33%) showed no decay of DL-alpha-glycerol phosphate substrate (H12) ($P = 0.083$) (Fig. 7 and 8). All nine CC17 strains (100%) showed no decay of the D-ribose substrate (D7), whereas this was the case for only a few of the strains in the other CCs (3/27, or 11%; $P < 0.001$).

Characteristics of phages as a function of the phylogenetic position of donor and propagating strains. Isolated phages were phylogenetic lineage specific within *S. agalactiae* (Fig. 7). Indeed, the lysogenic CC23 donor strains provided phages only of the genetically diverse morphotype A group. CC17 strains produced phages of molecular group II/morphotype B or of molecular group IV/morphotype C. In addition, phages of

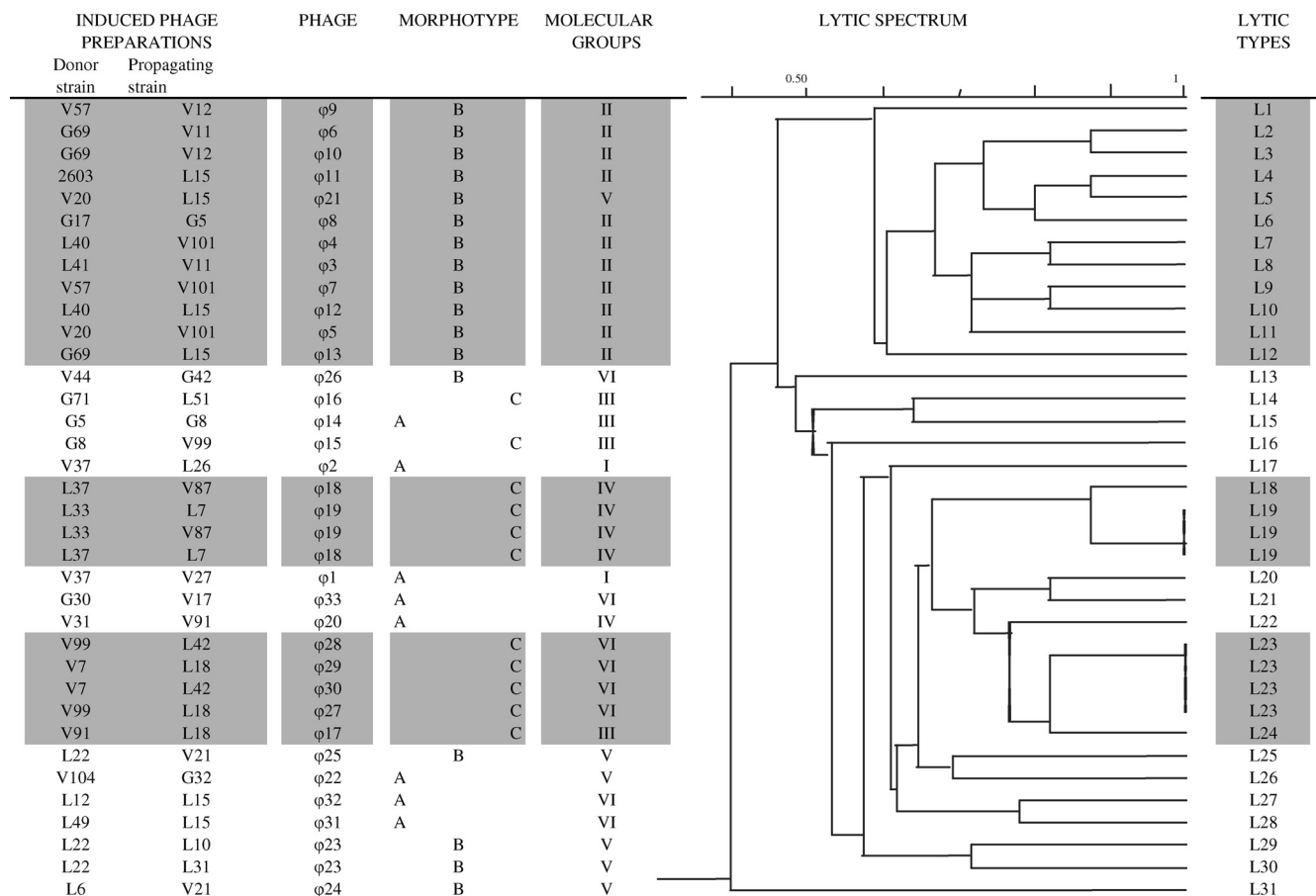


FIG. 6. Lytic activities of *S. agalactiae* phages. The dendrogram representing the lytic spectrum was generated by the Jaccard dichotomy coefficient method using the lytic reactions against the 114 *S. agalactiae* strains. Phages were defined by their morphotypes and molecular characteristics (molecular groups). Thirty-three lytic types were found.

these two groups were rarely supplied by strains of phylogenetic lineages other than CC17: only 2 of the 11 molecular group II/morphotype B phages and none of the molecular group IV/morphotype C phages were produced by strains of other lineages. The phages of molecular group V appeared to be more specifically responsible for the lysogeny of CC19 strains, given that only one of the five phages (φ21) of this molecular group was generated by a non-CC19 strain. Similarly, the phages of molecular group III and of molecular group VI/morphotype C appear to be more specifically able to integrate into CC10 strain genomes as all the phages displaying these characteristics were produced by strains of this phylogenetic lineage.

Phages obtained from lysogenic strains of each of the *S. agalactiae* phylogenetic lineages other than CC17 were able to infect and induce a viral lytic cycle more specifically in bacteria of a similar evolutionary origin (Fig. 8). Indeed, 7 of the 10 phages isolated from CC10 donor strains (70%) were propagated by CC10 strains. All of the phages isolated from CC19 donor strains (100%) were propagated by CC19 strains, and three of the four phages isolated from CC23 donor strains (75%) were propagated by CC23 strains. By contrast, the phages isolated from CC17 donor strains (eight molecular group II/morphotype B phages and two molecular group IV/

morphotype C) were able to mediate lytic replication in bacterial hosts of each of the phylogenetic lineages of *S. agalactiae* other than CC23.

DISCUSSION

Phages have made an important contribution to bacterial evolution but have been little studied in the *S. agalactiae* species. *S. agalactiae* phages of bovine origin, assigned to the *Siphoviridae* family, were first isolated in 1969 (37), and a phage-typing method was proposed for investigations related to *S. agalactiae* infections in the early 1980s (45). Prophage sequences are abundant in the sequenced genome of *S. agalactiae* (47, 48), but little is known about the functionality of *S. agalactiae* bacteriophages. The mitomycin C prophage induction observed in *S. agalactiae* strains of four major lineages (CC10, CC17, CC19, and CC23) responsible for genital and neonatal colonization and infections indicates that *S. agalactiae* serves as a host for several temperate phages. Serotype V strains of CC1, which emerged in adults in the 1990s and in neonates more recently (5, 26), were not included in our collection, and their study is required. Although most of the donor strains (23/24) (Fig. 7) provided phages of the same molecular group and morphotype, polylysogeny could not be

phage features and lytic activities, each with its own ability to infect, replicate within, or integrate into host cells from well-defined intraspecies phylogenetic lineages (Fig. 7 and 8). Over the last 60 years, a marked change has occurred in the habitat of *S. agalactiae*, from predominantly bovine infections to several ecological niches in the environment, animals, and humans, with each colonizing population displaying specific features with respect to the various *S. agalactiae* lineages (23, 29, 39, 41, 50). As shown for other species (38), such changes in habitat expose bacteria to a wide range of environmental and nutritive constraints, imposing stressful conditions that lead to the induction of mutation and contribute to adaptive evolution. Our data suggest that these stress-induced conditions concomitantly expose the bacteria, which share their new habitat with specifically adapted bacteriophages, to different phage-mediated horizontal gene transfers.

Phages originating from the genetically homogeneous CC17 (ST-17) presented a wide spectrum of lytic activities against strains of all lineages (except CC23), whereas—as shown previously for a phage isolated from a CC19 strain (32)—phages originating from CC19, CC10, and CC23 presented a narrow range of lytic activity, principally against strains of the same CC (Fig. 8) (32). In recent years, *S. agalactiae* ST-17 strains have emerged as a major cause of *S. agalactiae* early-onset diseases (24). Our findings raise questions concerning the role of the lysogenic features observed here for ST-17 strains in the emergence of this pathogen. As suggested by experiments with cocultures of *Salmonella* strains carrying or lacking specific prophages (6), prophages may modify the competitive fitness of host strains through their ability to grow and, for a small proportion of the population, to annihilate rival bacteria through cell lysis. Phages active against isolates other than the strain causing disease were released from most of the bacterial isolates from septicemic patients, suggesting that the prophages present in sepsis-causing bacterial clones play a role in clonal selection during bacterial invasion (15). Given the particular ability of the phages of ST-17 strains to destroy extraclonal strains, such phenomena may confer a selective advantage for vaginal colonization or for invasive disease in neonates.

We found that lysogenic *S. agalactiae* strains of CC17 and CC19 had lost some catabolic functions (Fig. 7 and 8). Several studies in other species have demonstrated that genetic events causing a loss of catabolic function frequently induce changes in the expression of virulence (8, 12, 13, 28, 31, 43). In many species, lysogenic phages affect virulence because they carry genes encoding toxins, such as diphtheria toxin in *Corynebacterium diphtheriae* (10), Shiga toxin and type III secretion effectors in enterohemorrhagic *Escherichia coli* (42), staphylococcal enterotoxin A and Panton-Valentine leukocidin in *Staphylococcus aureus* (2, 22), streptococcal pyrogenic exotoxins (20), and cholera toxin in *Vibrio cholerae* (51). No such virulence factor associated with phages has been found in *S. agalactiae* CC17 and CC19, but lysogeny may affect the virulence of these two highly virulent clones by modifying bacterial fitness. For example, in vitro lysogenization of pneumococcal strains enhances bacterial adhesion to inert surfaces and pharyngeal cells (25). Localization of insertion sites of phages in the *S. agalactiae* genome and a greater understanding of temperate phage morons, particularly in phages isolated from

ST-17 strains, may provide insight into why these strains are more likely than others to invade the central nervous system of neonates.

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N.V.D.M.-M., A.S.D., and R.Q. conceived and designed the experiments. A.S.D. and N.V.D.M.-M. performed isolation, propagation, and characterization of phages; A.S.D. and P.Y.S. performed electron microscopy analysis; and G.H.A., M.F.L., L.M. (MLST), A.S.D., N.V.D.M.-M., and R.Q. performed experiments for serotyping and metabolic properties of strains. A.S.D., N.V.D.M.-M., and R.Q. analyzed the data and wrote the paper.

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