

Bacteriophage HP2 of *Haemophilus influenzae*

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Temperate bacteriophages effect chromosomal evolution of their bacterial hosts, mediating rearrangements and the acquisition of novel genes from other taxa. Although the *Haemophilus influenzae* genome shows evidence of past phage-mediated lateral transfer, the phages presumed responsible have not been identified. To date, six different *H. influenzae* phages are known; of these, only the HP1/S2 group, which lysogenizes exclusively Rd strains (which were originally encapsulated serotype d), is well characterized. Phages in this group are genetically very similar, with a highly conserved set of genes. Because the majority of *H. influenzae* strains are nonencapsulated (nontypeable), it is important to characterize phages infecting this larger, genetically more diverse group of respiratory pathogens. We have identified and sequenced HP2, a bacteriophage of nontypeable *H. influenzae*. Although related to the fully sequenced HP1 (and even more so to the partially sequenced S2) and similar in genetic organization, HP2 has a few novel genes and differs in host range; HP2 will not infect or lysogenize Rd strains. Genomic comparisons between HP1/S2 and HP2 suggest recent divergence, with new genes completely replacing old ones at certain loci. Sequence comparisons suggest that *H. influenzae* phages evolve by recombinational exchange of genes with each other, with cryptic prophages, and with the host chromosome.

The host range of temperate bacteriophages is determined by multiple factors. Since phages require cellular components for replication, they become specialized to a compatible bacterial species. Within a host species, divergent restriction systems and surface receptors create barriers to interstrain transmission. Phages may overcome host range barriers by evolving DNA methylation systems (24) or by varying the structure of tail fiber proteins used for adsorption (31). The temperate bacteriophages of nonencapsulated (e.g., nontypeable) *Haemophilus influenzae* (NTHI) face adaptive challenges because of the unusually high genetic diversity of these bacteria (43). Here we describe a new temperate prophage, HP2, found in NTHI strains that are associated with unusual virulence.

Although six *H. influenzae* phages are described in the literature (HP1, S2A, B, C, N3, and ϕ flu), only HP1 and three types of S2 have been described in detail (4, 19, 21, 30, 43). Both HP1 and S2 infect *H. influenzae* Rd strains (4, 19), which were originally derived from an encapsulated serotype d (Sd) strain, but do not possess the genes for capsular biosynthesis (2, 15). We have discovered a new member of the HP1/S2 family that occurs as a prophage in the chromosome of strain R2866, a nontypeable invasive *H. influenzae* isolate (26).

DNA sequence analysis of HP1 and S2 types A, B, and C shows that these phages are closely related. Closer examination shows that type C is probably the original HP1 (37). Type A has many similarities to type C, but differences in the structures of the early promoter region suggest a different regulation of the lytic-versus-lysogeny decision. The type B variety

appears to be a chimera between types A and C. The original host of the HP1/S2 bacteriophages is unknown, but UV-induced mixed-culture filtrates lysogenized an Rd derivative. All HP1 and S2 type phages have similar morphologies when viewed with an electron microscope. The N3 bacteriophage has a similar head structure, but a longer tail. The N3 phage is found only in particular NTHI strains, and on restriction analysis, it has a pattern distinct from HP1 (43). No other information or sequence data on N3 are available. ϕ flu is an incomplete phage found in the Rd KW20 genome and has genes homologous to ones in HP1 (21).

HP1, with its 32-kb genome, belongs to the family of bacteriophages represented by *Escherichia coli* P2. Historically HP1 was used to elucidate the mechanism of natural transformation in *H. influenzae* (6, 27, 33, 41, 42). HP1 is a temperate phage capable of either a lytic infection or lysogeny of the host. The promoters controlling the lysis-versus-lysogeny decision are located near the 5' end of the genome (9): one leftward and two rightward promoters transcribe *cI* and *cox*, which have genetic and functional homology to transcriptional regulators in lambda. In vitro HP1 *cI*, *cox*, and *int* function similarly to their counterparts in lambda. In HP1, the majority of the genes downstream from these regulators appear to encode proteins that are part of phage structure and assembly apparatus. The function of these downstream genes is inferred on the basis of homology to genes in other phages.

The S2 phages also appear capable of a temperate life cycle in Rd hosts. The 5' 5.6 kb of this phage was sequenced for comparison to HP1 (36). Major sequence differences between S2 and HP1 are interspersed with regions of high homology.

While investigating a previously described invasive NTHI strain (26, 46), we found a prophage whose range was limited

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TABLE 1. Bacterial strains used in this work

Strain	Relevant characteristics	Source or reference
<i>E. coli</i> DH5 α	Cloning host	Gibco BRL
<i>H. influenzae</i>		
Rd	Derivative of Garf Sd	2
Rd KW20	Genome sequence	45
R3153	Rd strain for plaquing (BC200)	3
Rd McI	Minicell producing strain	32
R3152	HP1 lysogen	6
R2866	HP2 lysogen	26
R539	Type a (Pittman 610)	ATCC 9006
R538	Type b (Pittman 641)	ATCC 9795
R540	Type c (Pittman 624)	ATCC 9007
R541	Type d (Pittman 611)	ATCC 9008
R542	Type e (Pittman 595)	ATCC 8142
R543	Type f (Pittman 644)	ATCC 9833
Other <i>Haemophilus</i> sp. strains		
	<i>H. somnus</i>	ATCC 43625
R1966	<i>H. parainfluenzae</i>	ATCC 33392
R3358	<i>H. aegyptius</i>	ATCC 11116
R1968	<i>H. aphrophilus</i>	ATCC 33389
R1969	<i>H. paraphrophilus</i>	ATCC 29241
R1970	<i>H. haemolyticus</i>	ATCC 33390
R1985	<i>H. parahaemolyticus</i>	ATCC 29237
R1972	<i>H. haemoglobinophilus</i>	ATCC 19416
R1973	<i>H. segnis</i>	ATCC 33393
R1974	<i>H. parasuis</i>	ATCC 19417
R1976	<i>H. equigenitalis</i>	ATCC 35865
R1985	<i>H. parahaemolyticus</i>	ATCC 10014
R1986	<i>H. paracuniculus</i>	ATCC 29986
R1989	<i>H. paragallinarum</i>	ATCC 29545
R1990	<i>H. avium</i>	ATCC 29546
R1992	<i>H. ducreyi</i>	ATCC 33940
R1975	<i>H. pleuropneumoniae</i>	ATCC 27088
Other gram-negative spp.		
	<i>N. gonorrhoeae</i>	Joan Knapp, Centers for Disease Control and Prevention, Atlanta, Ga.
	<i>P. multocida</i>	ATCC 8369
	<i>P. aeruginosa</i>	ATCC 27853
<i>H. influenzae</i> strain constructs		
R3420	R2866 with HP2 deleted, Rib ^r	This study
R3422	R2866 with HP2 deleted, Cm ^r	This study
R3403	R3152 with first 5 kb of HP2 (HP1/HP2 ^P)	This study
R3404	R2866 with first 5 kb of HP1 (HP2/HP1 ^P)	This study
R3435	R2866 with TSTE insertion in HP2 <i>dam</i> , Rib ^r	This study

to this strain and a few other NTHI strains. To elucidate whether the phage provided clues to the unusual virulence of this strain, we sequenced its chromosome and found a close relationship to HP1 and S2. *H. influenzae* Sd strains and Rd derivatives are not lysogenic for HP2; however, HP2 can lysogenize a phage-deleted form of its original host.

MATERIALS AND METHODS

Bacteria and media. The bacteria used in this study are described in Table 1. Strain R2866, originally described as Int1, is a biotype V, nontypeable *H. influenzae* strain isolated from the blood of an immunocompetent child with signs of meningitis (26). This strain is serum resistant and harbors a 54-kb conjugal plasmid that encodes a β -lactamase. sBHI broth was made up of brain heart infusion (BHI) medium (Difco, Becton Dickinson, Sparks, Md.) supplemented with 10 μ g (each) of hemin-HCl (Sigma, St. Louis, Mo.), L-histidine (Sigma), and β -NAD (Sigma) per ml. The heme solution was prepared by mixing 100 mg of hemin-HCl and L-histidine in 100 ml of 50°C water, to which 0.4 ml of 10 N

NaOH (Sigma) is added. The solution was filter sterilized with a 0.22- μ m-pore-diameter filter and stored at 4°C in a lightproof container for no more than 3 weeks. β -NAD was dissolved in water to a concentration of 1 mg/ml, filter sterilized, and stored at 4°C. One volume of these solutions was aseptically added to 100 volumes of BHI broth prior to use. Chocolate agar was prepared as described by Difco with GC Media base. To avoid contamination with gram-positive organisms, bacitracin was added to all solid *H. influenzae* growth media at a final concentration of 500 U/liter (10 μ g/ml), and all incubations were done at 37°C in air. Luria-Bertani (LB) agar and broth (Difco) were used for *E. coli*.

Phage induction. To induce bacteriophage from the lysogens, a 100-ml sBHI culture was grown with shaking at 1,200 rpm at 37°C to an A_{600} of 0.15 to 0.2. Mitomycin C (Sigma) was added to a final concentration of 35 ng/ml, and the culture was shaken at 50 to 100 rpm. Bacterial replication continued to an A_{600} of 1.0, after which the optical density decreased, presumably due to phage-mediated lysis. When the optical density reached its minimum, generally 4 to 6 h after the addition of mitomycin C, the cells were pelleted by centrifugation at 20,000 \times g for 15 min at 4°C in a Beckman J21 centrifuge. The supernatant was removed, and the centrifugation step was repeated to remove residual intact cells. The resulting supernatant was passed through a 0.22- μ m-pore-diameter

TABLE 2. Oligonucleotide primers used in this work

Primer	Sequence ^a	Target/use
1	GAGACGGATCCGTTTGCACAACCTACGGGCTTA	Cloning of the 5' terminus of HP2 upstream of <i>attP</i> (<i>Bam</i> HI)
2	GAGACCGCTCGAGCGGATGGCTTGCAGGAAGTTTATG	Cloning of the 5' terminus of HP2 upstream of integrase (<i>Xho</i> I)
3	GAGAGGAAGATCTCCCGGTCAAATCTACCCGAAA	3' terminus of HP2 (<i>Bgl</i> II)
4	GAGACGGAATTCGCTTTAGTTTGTCCGCAACC	Cloning of the 3' terminus of HP2 at position 29,742 (<i>Eco</i> RI)
5	GCTGCTCTACCGACTGAGCTA	Creation of a PCR probe to the early genes of HP1 + HP2
6	AGACGGTGAGGCACGTTTAG	Creation of a PCR probe to the early genes of HP1 + HP2
7	AAGGGGGAAATAATGGCAAC	Cloning of HP2 genes in the pR promoter group
8	AAAGGATTGTTATTGCCCC	Cloning of HP2 genes in the pR promoter group

^a Sequences run 5' to 3', with restriction sites listed in target use underlined.

filter, after which chloroform was added (20 μ l/100 ml). Phage-containing supernatant was stored at 4°C until further use. To concentrate the bacteriophage particles, the supernatant was centrifuged in 33-ml ultracentrifuge tubes at 40,000 rpm in a Ti 50.2 rotor for 3 h at 15°C. The resulting pellet was resuspended in a minimal amount of phosphate-buffered saline (PBS) overnight at 4°C with gentle shaking.

Plaque assay. Bacteria were grown in sBHI broth to an A_{600} of 0.2 and then mixed with 1:5 to 1:100,000 dilutions (in PBS) of culture supernatant prepared as described above for phage induction. Soft agar consisted of 5 ml of 0.7% sBHI agar layered on a standard sBHI agar plate. The target strain was grown in sBHI broth to an A_{600} of 0.2 and diluted 1:100 in the same medium, an aliquot was added to the phage preparation, and 0.1 ml was spread over the surface of the soft agar. After overnight incubation at 37°C, clear plaques were counted (44).

Electron microscopy. Concentrated bacteriophage stocks were stained in uranyl acetate (5) and visualized by T.P. with a JEOL 1200 EX transmission electron microscope at the Electron Microscopy Core at the University of Missouri—Columbia.

DNA isolation. To purify phage for sequencing, 200 μ l of resuspended phage pellet in sBHI was treated with 0.1 U of DNase I (Gibco-BRL, Rockville, Md.) for 30 min at 37°C. After DNase treatment, the phage preparation was extracted with an equal volume of Tris-saturated phenol (pH 8.0)–chloroform–isoamyl alcohol in proportions of 25:24:1. The aqueous layer was removed, and the extraction was repeated with an equal volume of fresh phenol solution. The DNA was precipitated from the aqueous layer by addition of 1/10 volume of 3 M sodium acetate (pH 4.0) and 2.5 volumes of absolute ethanol at –20°C and concentrated by centrifugation, and the pellet was washed with 1 ml of 70% ethanol at room temperature. After centrifugation at 12,000 \times g for 15 min at 4°C, the ethanol solution was aspirated, and the pellet was allowed to air dry before resuspension in 50 μ l of water or PBS.

Sequencing. Sequencing was performed at the University of Washington Genome Center as described by Stover et al. (40). Phage DNA was cloned into pUC19, and the insert was sequenced with primers synthesized by that unit. The data set involved 462 dye-terminator and 123 dye-primer sequencing reads, sampled at random from the phage genome. The average number of q20 bases per read was 408. A q20 base is a base call with an estimated error rate of 1% as calculated by the PHRED base-calling software (11, 12). The redundancy of the data, in terms of q20 bases, was 7.6. Low-quality regions were resolved by a combination of manual and automated finishing procedures as described previously (17). An estimate of the number of remaining errors in the sequence based on quality scores was calculated with the phrap assembly software (16), which can be accessed at <http://www.phrap.org>. The expected number of residual errors in this 31.5-kb sequence was 0.16. In our experience, sequence with less than one predicted error usually has no errors. In addition, both strands of the first 10 kb of HP2 from *attP* to *orf10* were independently sequenced at the University of Missouri DNA Core by using the same vector and method, and no differences were observed.

DNA analysis. Open reading frames (ORFs) were identified using the ORF finder function in the OMIGA software program (Oxford Molecular). The ribosome binding sites in the HP2 ORFs were compared to the previously determined HP1 and S2 sequences to verify the most likely start codons. Similarity plots were obtained with the GCG software program available (Wisconsin Genome Center).

H. influenzae transformation. *H. influenzae* was transformed by the M-IV technique (39). Gel-purified PCR fragments or linearized plasmid DNA was added to competent *H. influenzae*, and dilutions were plated on chocolate agar plates containing either ribostamycin (Sigma) at 30 μ g/ml (for the TSTE cassette) or chloramphenicol at 5 μ g/ml (for the *cat* cassette).

Southern analysis. DNA was transferred from agarose gels to nylon membranes (Osmonics, Inc., Minnetonka, Minn.) by using a vacuum-assisted apparatus (Hoefer Scientific). Agarose gels were deproteinized in 0.25 M HCl for 1 h, followed by denaturation in 1.5 M NaCl containing 0.5 M NaOH for 30 min (29). Transfers were performed for \geq 3 h in 20 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate), after which the membrane was treated with UV light to cross-link the DNA. Chemiluminescent detection was performed with which digoxigenin-labeled oligonucleotide probes or double-stranded PCR products as recommended by the manufacturers (Roche, Indianapolis, Ind.).

PCR amplification. Table 2 lists the primers used for PCR amplification of selected portions of the HP1 and HP2 prophages. The locations of these primers on the HP2 genome map are shown in Fig. 1. For PCR amplification of fragments shorter than 2 kb, standard *Taq* polymerase was used according to the manufacturer's instructions (Perkin-Elmer, Boston, Mass.). An Eppendorf thermocycler (model, Mastercycler) was set to run 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s in that order. Occasional primer sets required adjustment of the annealing temperature. For larger products of up to 18 kb, the long-range PCR kit from Roche (GeneAmp XL) was used.

Construction of an HP2 host. The plasmids used in the HP2 host construct are described in Table 3. Using PCR primers 1 and 2, a 1.7-kb fragment of HP2 DNA containing *int* and *attP* was amplified and ligated to pTcHisB restricted with *Bam*HI and *Xho*I (pBJ102). PCR primers 3 and 4 were used to amplify a 2.0-kb downstream portion of the HP2 prophage that was subsequently ligated into pBJ102 digested with *Bgl*II and *Eco*RI (pBJ102.2). A *Bam*HI-restricted TSTE cassette was ligated into *Bgl*II-digested pBJ102.2 to create pBJ102.3. The TSTE cassette contains the *aph(3')* gene flanked by *H. influenzae*-specific uptake (*hUS*) sequences (34). The TSTE cassette confers ribostamycin resistance to *H. influenzae* and kanamycin resistance to *E. coli*. Plasmid pBJ102.3 was digested with *Bam*HI and *Eco*RI and used to transform competent *H. influenzae* strain R2866 with selection for ribostamycin resistance. Of 12 ribostamycin-resistant transformants, 2 were shown to be devoid of most of the prophage genome by Southern blotting and to lack phage production after mitomycin C treatment, as assessed by electron microscope observation and infection assays (data not shown). One such mutant was designated R3420 (Fig. 2). R3422 is a derivative of R3420 with a chloramphenicol acetyltransferase cassette replacing the *aph(3')* gene, inserted between two *Hinc*II sites.

Construction of hybrid lysogens. Early experiments indicated that HP2 would not form plaques on any of the Rd derivatives or strain R3420, its original host, from which HP2 was isolated. To identify the genetic regions determining the host range of HP2, we created hybrid lysogens of HP1 and HP2 (Fig. 2). This was accomplished by first cloning a 7.5-kb *Hind*III prophage fragment containing the HP2 immunity genes from strain R2866 into the *Hind*III site of pUC18. This plasmid, designated pBJ100.1, contains a portion of a threonine synthetase gene and a *Bam*HI site in an intergenic region 5' to the prophage. After cloning TSTE into this *Bam*HI site, the plasmid (pBJ100.2) was linearized and transformed into competent R3152 selecting for ribostamycin resistance. One transformant (designated HP1/HP2^P [strain R3403]) of 12 examined acquired the HP2 immunity region as indicated by PCR. The chromosomal DNA of another transformant that retained the HP1 immunity region was digested and transformed into R2866. One transformant of the 12 that acquired HP1 immunity region was designated HP2/HP1^P (strain R3404) (Table 1). To verify the construction of the hybrid phages, we performed a Southern analysis of *Bgl*I-restricted DNA harvested from phage preparations of HP1, HP2, HP1/HP2^P (R3403), and HP2/HP1^P (R3404) by using a digoxigenin-labeled PCR product generated from primers 5 and 6 as a probe. HP2 contains a 2.0-kb fragment, while in HP1, the hybridizing fragment is smaller, as predicted. HP1/HP2^P has the 2-kb *Bgl*I fragment, while HP2/HP1^P has the smaller fragment.

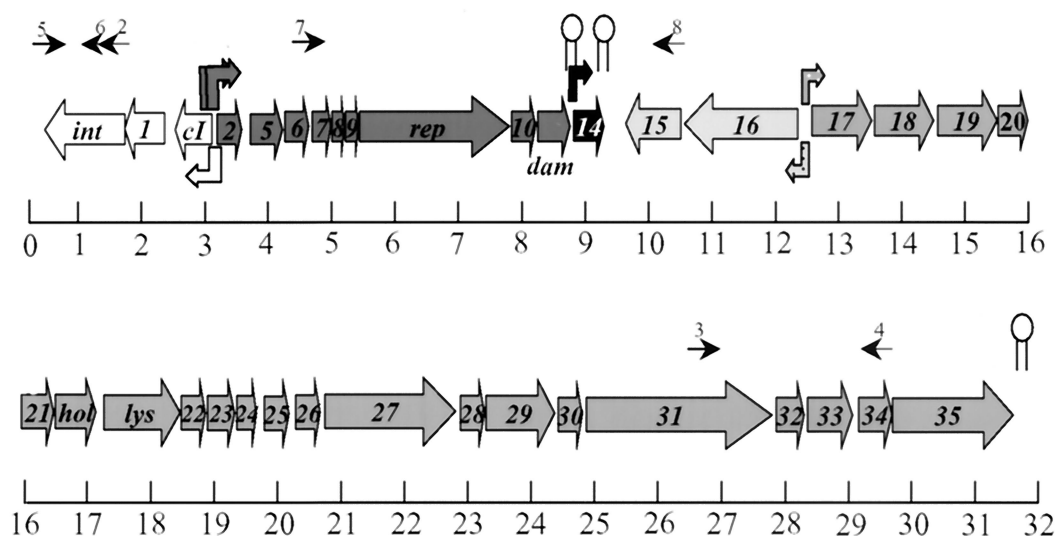


FIG. 1. Genetic map of HP2. Straight arrows indicate the approximate position of each ORF. The scale is in kilobases. Bent arrows indicate the locations of RNA polymerase promoter elements. Ball-and-stick figures indicate the locations of transcription terminators. Arrows above the map indicate locations of primers used in this work. The length of the HP2 chromosome is 31,508 bp. Primer 1 is homologous to coordinates 3088 to 3072 of the *H. influenzae* Rd KW20 genome (section 9 of 163).

Construction of marked HP2 derivative. To assess the ability of HP2 to lysogenize a host, we created a prophage mutant in R2866 with the TSTE cassette marking the phage. The insertion of TSTE into the phage *dam* gene resulted in no detectable phenotypic changes in growth rate or phage yields. This insertion was created by first cloning a portion of the prophage with primers 7 and 8 to amplify a 7-kb segment of the phage containing most of the genes driven by the pR promoters. This PCR product was digested with *Hind*III and *Eco*RI and ligated into pKS to create pBJ105. pBJ105 was digested with *Nco*I, which cuts this plasmid uniquely in the *dam* gene, and was treated with T4 polymerase. A *Bam*HI-digested, T4 polymerase-treated TSTE cassette was ligated to this plasmid to yield pBJ105.2. This plasmid was digested with *Hind*III and *Eco*RI and transformed into R2866 with subsequent selection of ribostamycin-resistant colonies. Southern analysis of chromosomal DNA and phage extract DNA from eight colonies revealed one mutant, R3435, which contained the TSTE cassette in the *dam* gene of the HP2 prophage (data not shown).

Nucleotide sequence accession number. The HP2 sequence has been deposited in GenBank under accession no. AY027935.

RESULTS AND DISCUSSION

The HP2 genome. The HP2 chromosome consists of 31,508 bp, similar to the size of S2 phage types A and B based on restriction mapping (28). The molar percentage of adenine and thymidine (A+T%) in the HP2 chromosome is 60.04%, a value similar to that in the Rd KW20 chromosome (61.86%) (15). The frequency of the triplet base combinations, coding and noncoding, in HP2 is also very similar to that in Rd KW20 (data not shown), which suggests that this bacteriophage was not recently introduced into *H. influenzae*.

The organization of the HP2 genome is shown in Fig. 1; cohesive ends are similar to those in HP1 (data not shown). HP2 appears to contain five transcriptional units, with the control of each of these units directing or repressing bacterio-

TABLE 3. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pKS	Cloning vector	Stratagene
pUC18	Cloning vector	47
pTrcHisB	Vector for production of His-tagged fusion	Invitrogen
pUC4DEcat	<i>cat</i> gene used in <i>H. influenzae</i> cloning Chlor ^r	7
pTSTE	<i>apH</i> (3') <i>I</i> flanked by <i>H. influenzae</i> uptake sequences in pBR322; Rib ^r	34
pBJ100.1	<i>Hind</i> III fragment of R2866 chromosome from position 139332 (in HI0123) to bp 5194 of the prophage in pUC18	This study
pBJ100.2	pBJ100.1 with TSTE located in <i>Bam</i> HI site upstream of the prophage <i>attP</i> site	This study
pBJ102	TrcHisB containing a 1.7-kb fragment from the 5' end of the HP2 prophage	This study
pBJ102.2	pPB102 containing a 2.0-kb fragment of the 3' end of the HP2 prophage 3' to the early fragment	This study
pBJ102.3	<i>apH</i> (3') <i>I</i> inserted between the two prophage fragments in pBJ102.2	This study
pBJ102.4	<i>dCAT</i> inserted between the two prophage fragments in pBJ102.1	This study
pBJ105	pKS containing the <i>Hind</i> III- <i>Eco</i> RI fragment of the HP2 prophage located in the pR transcription frame	This study
pBJ105.2	pBJ105 with TSTE located in the <i>Nco</i> I site in <i>dam</i>	This study

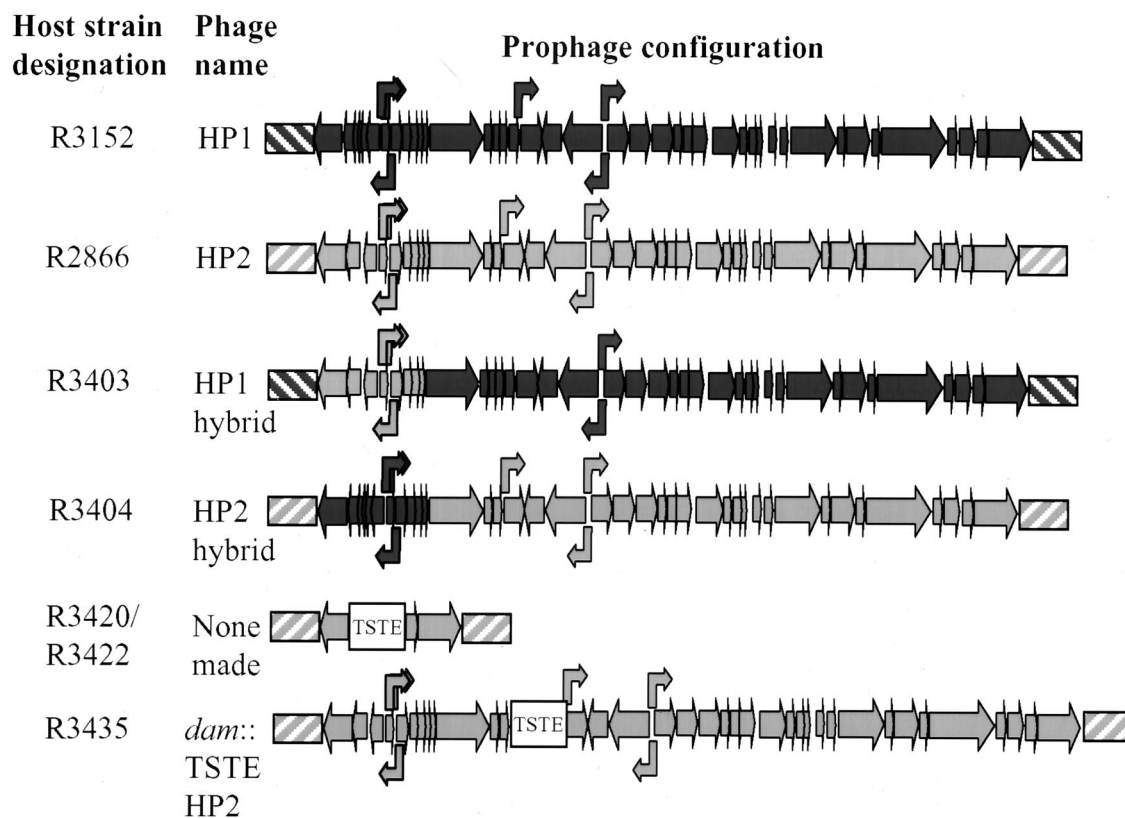


FIG. 2. Genetic maps of strains used in this work. Straight arrows indicate ORFs and their orientation. Bent arrows indicate the locations of the transcription promoters. Diagonal striped boxes indicate the boundaries of host DNA. DNA originating from the HP1 or HP2 host is shown in dark or light gray, respectively. The TSTE box indicates the location of the antibiotic cassette used for genetic manipulations. These maps are not to scale but approximate the total numbers of genes and their relative sizes.

phage replication. As in HP1, the pR_1 , pR_2 , and pL_1 promoters of HP2 adjoin the early regulatory elements. Flanking these promoters are elements believed to control the lysis-versus-lysogeny decision (13). If the products of the pL_1 promoter dominate, lysogeny is maintained, repressing all other bacteriophage gene expression. If the pR_1 and pR_2 promoters are activated, the lytic cycle will ensue. Products of the pR_1 - and pR_2 -activated transcript should control bacteriophage DNA replication and presumably activation of the downstream genes through hypothetical promoter elements between *orf16* and *orf17*. Genes responsible for bacteriophage particle production and host lysis reside in these diverging transcripts, one of which contains *orf15* and *orf16*, while the other contains *orf17* through *orf35*. Many of the ORFs in the latter transcript show homology to structural proteins of P2 and other phages. As in HP1, *orf14* appears to have its own promoter and terminator. The role of this gene in HP1 and HP2 is unknown. It is unique in being the only gene in these phages that appears capable of independent control.

HP2 regulatory elements. The pR and pL promoters controlling the lysis-versus-lysogeny decision differ among HP1, HP2, and S2 phages. Analysis of these regions indicates that both of the pR promoters are maintained in HP2, whereas the pR_1 promoter, and its corresponding *cI*-coded protein binding site, is missing from S2 (Fig. 3). The nucleotide sequences of these promoter regions differ at numerous sites: areas that are

conserved are the -10 , -35 , and *cI*- and *cox*-coded protein binding sites. This suggests HP2 has retained a functional control unit for phage induction and repression. As in S2, the *cox* homologue of HP1 is absent. Whereas the *orf2* genes of HP2 and S2 are similar to *cox* (see below), the finding of intact Cox protein binding sites suggests that a Cox-like protein performs this function. The spacing between the -10 and -35 sites of pR_1 in HP2 is 16 or 17 bp, depending on which thymidine residue is considered the start of the -10 site. The pR_1 promoter may be functionally redundant, since S2 lacks pR_1 , yet appears fully capable of controlling lysis versus lysogeny in *H. influenzae* Rd strains.

Outside of this promoter region, the sequence of HP2 is very similar to that of S2, while within the promoter region, HP2 is more homologous to HP1 (Fig. 4). All three phages have identical sequences at the -10 and -35 sites of pL , the leftward promoter, and the sequence between these promoters, suggesting a close relationship between S2 and HP2. It seems unlikely that HP2 is a simple recombinant of S2 and HP1, because certain regions in each phage have a different nucleotide sequence.

Regulation of lysis. Since the structures of the promoter elements and repressors controlling the lysis-versus-lysogeny decision differ between HP1 and HP2 (see above), we sought to determine if the HP2 immunity region could mediate mitomycin C induction in an Rd host. We generated a hybrid

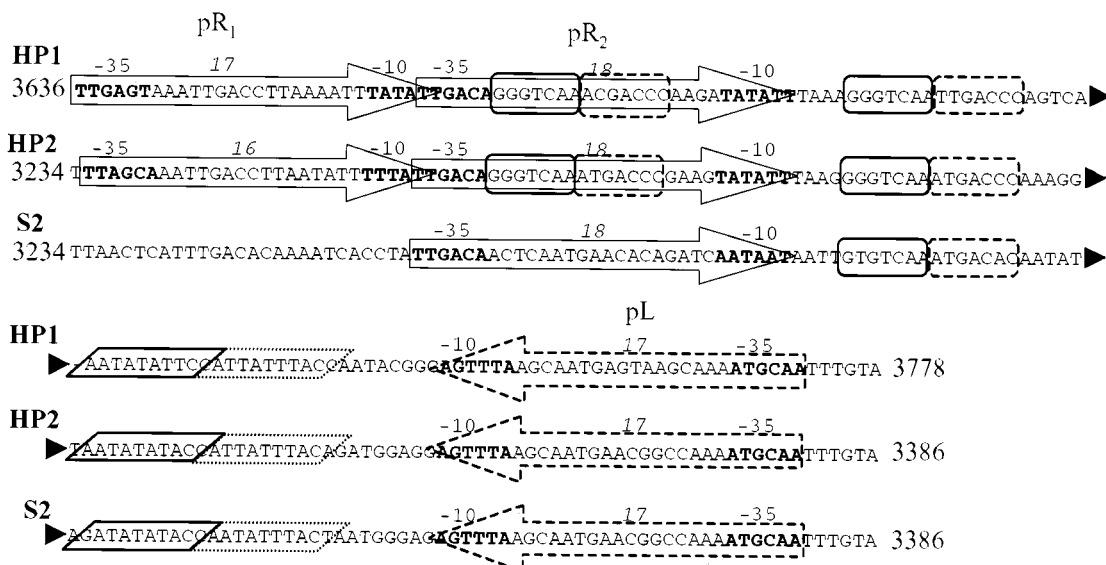


FIG. 3. Early promoter comparison among HP1, HP2, and S2 type A. The numbers on either side of the sequences denote the nucleotide positions of these loci in their respective phages. Large arrows delineate the promoter elements with the -35 and -10 sites in boldface and labeled. Italic numbers denote the spacing between the -35 and -10 sites. Dashed lines indicate elements contained on the opposite strand. Rounded boxes identify sequences consistent with the *cI* repressor binding sites, and the parallelograms indicate the *cox* repressor binding sites.

phage in which the first 5 kb of HP2 from *attP* to *orf7* was replaced with the homologous region of HP1 (HP2/HP1^P [strain R3404]). Conversely, we constructed an HP1 lysogen in which the first 5 kb was replaced with the homologous region of HP2 (HP1/HP2^P [strain R3403]). After mitomycin C induction, the A_{600} of the R3403 culture decreased in a manner similar to that of strain R3152 (Fig. 5). This indicates that the promoter region of HP2 is compatible with mitomycin induction and is capable of inducing phage replication and lysis in an *H. influenzae* Rd derivative. Strain R3404 grew only on solid media, precluding examination of the effect of mitomycin C.

Plaque formation. Strain R3152 typically yielded between 3.6×10^4 and 4.2×10^5 PFU/ml of culture supernatant with mitomycin C induction when it formed plaques on strain BC200. Similar titers were obtained when HP1 formed plaques on strains Rd and Rd Mc1. We did not observe HP1 plaques with any of the encapsulated *H. influenzae* strains, any of the other *Haemophilus* species, or *Pasteurella multocida*, *Neisseria gonorrhoeae*, or *Pseudomonas aeruginosa*. Similarly HP2 would not form plaques on any *Haemophilus* species listed in Table 1 or on *P. multocida*, *N. gonorrhoeae*, or *P. aeruginosa*.

At high phage concentrations, both HP1 and HP2 completely cleared lawns of strains Rd and R3422, respectively (Table 4). Plaques on a lawn of Rd became visible when HP1 was diluted 10,000-fold. The plaques produced by HP1 ranged from 1.5 to 2.5 mm in size and were usually turbid. Gradual dilution and infection of R3422 with HP2 resulted in lawns that gradually became more turbid as the phage concentration was decreased, but plaques were never observed. HP1 would not produce plaques in strain R3422. Thus, HP2 is restricted to its original host, while HP1 will only infect Rd derivatives. Lysogens of either phage, as well as the hybrid lysogens, were immune to infection by their own phage. Furthermore, the hybrid phage induced from strain R3403 had the same host

range, even though it contained the HP2 early promoter region and immunity genes. Thus, the differences in plaquing between HP1 and HP2 do not lie within the early control region. It is possible that R2866 and its derivative, R3422, are inherently resistant to lysis, including plaquing. Preliminary experiments indicate that R2866 is more resistant to polymyxin-induced lysis than strain Rd KW20 (data not shown).

Evidence for lysogenic conversion by HP2. While HP2 appears to infect the prophage-deleted mutant R3422, it was not clear if it could lysogenize infections. To determine whether HP2 was capable of lysogenic conversion of the strain with the phage deleted, strain R3435 (the TSTE antibiotic cassette located in the *dam* gene of the HP2 prophage) was created. The *dam* gene encodes an adenine methylase that does not appear necessary for growth, because this mutant prophage is still methylated by the host's methylase (data not shown). Phage induced from R3435 was mixed with strains Rd, R3422, and R2866 (Table 1) and plated on ribostamycin-containing chocolate agar. One hundred microliters of this supernatant contained ~16,000 ribostamycin-conferring units when monitored with strain R3422. Treatment of strain Rd with R3435 phage did not generate any ribostamycin-resistant colonies; however, treatment of strain R2866 generated ~160 ribostamycin-resistant colonies. The same phenomenon similarly occurred when marked HP1 or HP2 was mixed with ribostamycin-susceptible lysogens. When the phage preparation was treated with DNase, ribostamycin-resistant transformants were not obtained, indicating that transformation was the most likely mechanism of gene transfer. Transfer of ribostamycin resistance via phage from R3435 to R3422 was relatively DNase resistant compared to transfer to an HP2 lysogen. Furthermore, the transfer efficiency from R3435 was approximately 100-fold higher with transfer into strain R3422, which does not have large regions of homology for the phage recombination.

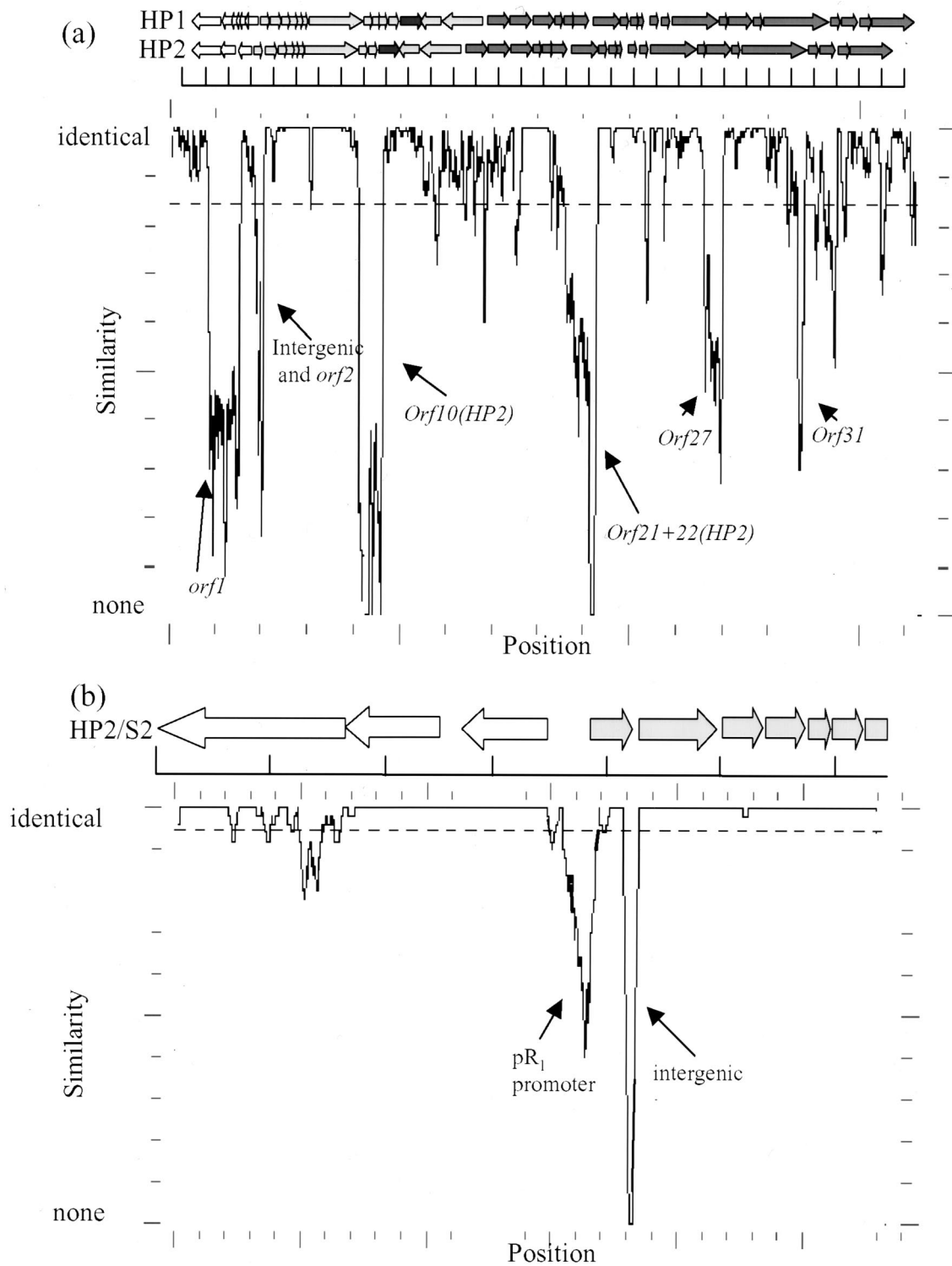


FIG. 4. Sequence comparison of HP2 to HP1 and S2 type A. Similarity plots are measured as percent difference from the designated window size. The dashed line represents the overall average similarity. Large peaks demonstrate the largest differences that are labeled with the HP2 gene or region showing this difference. The scale under the genetic maps is in kilobases. (a) Complete chromosome comparison between HP1 and HP2. Genetic maps are aligned to show differences in gene arrangement. A similarity plot scores the similarity over a 100-bp window. (b) Comparison of the first 5.6 kb of S2 type A with HP2. The genetic maps of HP2 and S2 are identical in this region. This similarity plot was calculated over a 50-bp window.

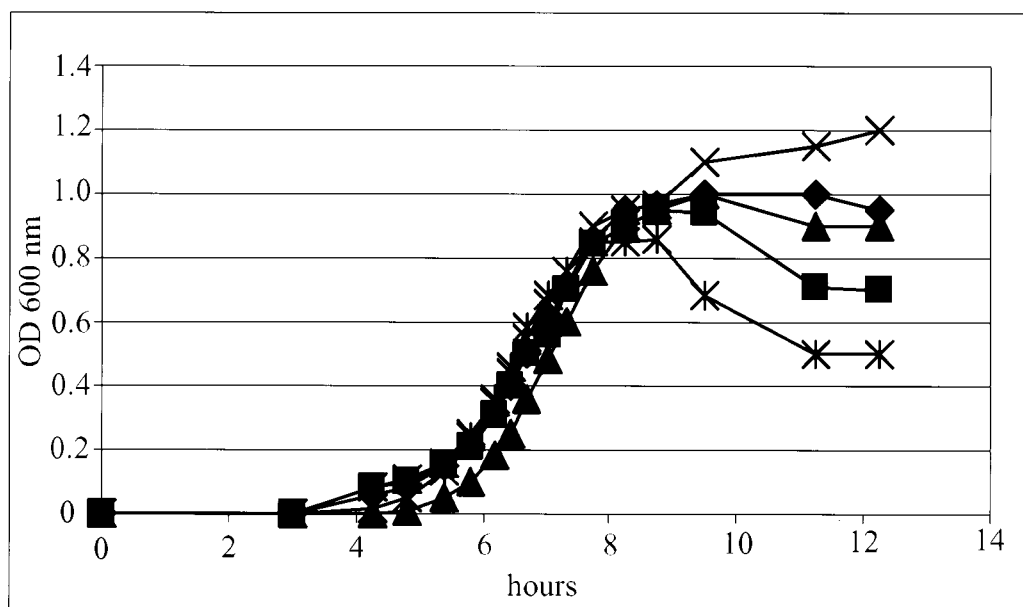


FIG. 5. Change in A_{600} (OD 600) after addition of mitomycin C. Mitomycin C was added when the culture reached an A_{600} of 0.15. \blacktriangle , R2866; \blacklozenge , R3422; \blacksquare , R3152; X, Rd; *, R3403.

This suggests that HP2 is capable of lysogeny in addition to lysis in strain R3422.

We would suspect phage infection to be much more efficient than transformation. However, we have observed high transformation rates (up to 10^4 CFU/ μ g of DNA) with TSTE-marked homologous DNA fragments into strain R2866. ScoCCA has reported that the HP1 phage is extremely fragile (personal

communication), and we suspect this contributes to a large amount of unpackaged phage DNA in these preparations that is available for transformation.

Electron microscopy studies. Using a concentrated HP2 solution, we used electron microscopy to determine the morphology of HP2 (Fig. 6). As predicted from the sequence similarities, HP2 is identical to HP1 by all visible measures. Its head

TABLE 4. Plaquing assays with HP1, HP2 and mutant phages^a

Strain producing phage	Phage pretreatment	Strain to be plaqued	Plaquing strain pretreatment	Result
R2866 (HP2)		R2866		Turbid lawn
R2866/R3404		R3422		Complete lysis, no growth
R2866/R3404	Diluted 1:100	R3422		Uniform incomplete lysis
R2866/R3404	Diluted 1:10,000	R3422		Turbid lawn
R2866/R3404		Rd		Turbid lawn
R3152 (HP1)		R3152		Turbid lawn
R3152/R3403		Rd		Complete lysis, no growth
R3152/R3403	Diluted 1:100	Rd		Uniform incomplete lysis
R3152/R3403	Diluted 1:10,000	Rd		1,000 plaques
R3152/R3403		R2866		Turbid lawn
R3152	Diluted 1:10,000, absorbed to Rd	Rd		Turbid lawn
R3152	Diluted 1:10,000, absorbed to R3422	Rd		Turbid lawn
R2866	Absorbed to Rd	R3422		Uniform incomplete lysis
R3152		Rd	Preincubated with HP2	Turbid lawn
R2866		R3422	Preincubated with HP1	Turbid lawn
R3152	Diluted 1:10,000	Rd	Preincubated with HP2	Turbid lawn
R3152	Diluted 1:10,000	Rd	Preincubated with <i>dam::TSTE</i> HP2	Turbid lawn without antibiotic selection, no growth with ribostamycin
R3152	Diluted 1:10,000	Rd	Preincubated with HP2 diluted 1:100	1,000 turbid plaques
R3152	Diluted 1:10,000	Rd	Preincubated with HP2 diluted 1:10,000	1,000 plaques
R3152	Diluted 1:10,000, absorbed to HP2 preabsorbed Rd culture	Rd		Turbid lawn
R2866	Absorbed to HP1 preabsorbed R3422 culture	R3422		Turbid lawn

^a The first column describes the host of the phage used in the plaquing assay. When more than one strain is listed, phages from either strain gave the same result. The second column describes any treatment the phage received prior to exposing it to the strain to be plaqued against in column 3. The fourth column describes any treatment the strain to be plaqued received prior to exposure with the test phage in column 1.

where 3' to the *orf2/cox* gene in the pR-driven transcript. Conservation of regulatory elements and long transcripts is a common theme in bacteriophages (1).

In HP2, the promoter controlling expression of *orf14* is identical to the one in HP1. In fact, *orf14* shows 100% identity at the protein level between the two phages. As in HP1, there do not appear to be any *cox*- or *cI*-coded protein binding sites near the *orf14* promoter presumably putting it under control of an alternative regulator: *orf14* may be capable of transcription, independent of the usual phage regulators.

Comparison to HP1 and S2. The HP2 phage appears to be closely related to the HP1 phage (Fig. 4a). Its chromosome is 31.5 kb (1 kb smaller than that of HP1), and it does not contain as many ORFs as HP1: 36 in HP2 compared to 41 in HP1. Of the 41 HP1 ORFs, 35 are hypothetical based on the ORF encoding a protein of >7 kDa and the presence of a potential ribosomal binding site (9). According to the same criteria, the HP2 chromosome contains 36 ORFs. The organization of the first four ORFs suggests that HP2 is very closely related to the S2 type A phage (Fig. 4b). As in S2, *orf2*, *orf3*, and *orf4* of HP1 are missing in HP2: *orf11* and *orf12* of HP1 are also missing from HP2. All of these small genes were contained in the early regulatory region. The downstream sequence, believed to contain the genes encoding phage structural elements, appears to be highly conserved. The promoters and terminators of the downstream transcripts are identical, as is the number of genes compared to HP1. This mosaic pattern is typical when comparing closely related phages and suggests that divergence occurs by recombination with each other, host DNA, and probably cryptic phages such as ϕ flu (25, 31, 36).

One area of the chromosome is unique to HP2: a small portion of noncoding DNA, labeled as "intergenic" in Fig. 4a and b. This 37-bp sequence is 92% identical to the DNA encoding a portion of the gs60 antigen of *Pasteurella haemolytica* (A. Mellors and R. C. Lo, unpublished observations [GenBank accession no. U42028]). While this DNA does not code for a product, it does suggest a possible lateral genetic exchange. Lateral DNA transfer occurs from *H. influenzae* to *N. gonorrhoeae* and *Neisseria meningitidis* (8, 23), and it seems likely to occur between closely related genera like *Haemophilus* and *Pasteurella*.

Restriction map of HP1, HP2, and S2. While the complete sequence of the S2 phage is unknown, a restriction map of S2 types A, B, and C has been reported (28). Since the first 5.6 kb of each of the S2 and HP2 sequences shows a great deal of homology, it might be concluded that they are the same phage in different hosts. A comparison of the limited restriction map of HP1 and the S2 phages with that of HP2 is shown in Table 5: HP2 has a number of differences in comparison to HP1 and to the three S2 subtypes. Since the restriction map of HP2 was based on sequence and that of S2 was based on restriction digests, some differences may be artifactual. Secondary structure may also conceal some restriction sites, and host modification of the phage DNA may account for some differences, because the restriction systems in R3152 and R2866 are likely different.

Protein differences between HP1 and HP2. Table 6 compares the levels of homology of the predicted protein products between HP1 and HP2. The names of the ORFs of the *H. influenzae* bacteriophages were derived from the original

TABLE 5. Restriction fragment sizes from the HP1/S2 family of bacteriophages

Restriction fragment	Fragment size (kb) in ^a :				
	HP1	HP2	S2A	S2B	S2C
<i>Bam</i> HI	26.3	31.5	26.3	26.3	26.3
	6.0		5.3	5.3	6.1
<i>Bgl</i> I	17.6	17.9	10.1	17.6	17.6
	6.2	6.6	7.5	4.8	6.1
	5.0	3.2	4.8	3.0	4.8
	2.4	2.6	3.0	2.6	2.35
	0.89	0.94	2.6	2.4	0.85
	0.28	0.28	2.4	0.85	0.35
			0.85	0.35	
<i>Bgl</i> II	10.5	9.6	10.0	10.0	10.8
	9.6	9.3	9.6	9.6	9.6
	6.9	6.2	6.2	7.1	7.1
	5.2	6.1	5.9	5.0	5.0
	0.11	0.11	0.12	0.12	0.12
<i>Eco</i> RI	17.8	12.9	18.6	17.8	17.8
	12.9	10.5	12.7	12.2	12.9
	1.6	8.1		1.7	1.7
<i>Hae</i> III	6.7	8.3	6.75	6.75	6.75
	5.5	6.2	6.0	5.3	5.6
	3.9	3.9	5.3	3.8	3.8
	3.8	3.8	3.8	3.7	3.75
	3.5	3.0	3.7	3.45	2.45
	2.5	2.8	1.8	2.45	2.45
	2.4	1.0	1.1	1.8	2.35
	1.0	1.0	1.0	1.1	1.1
	1.0	0.86	0.95	1.0	1.0
	0.97	0.32	0.90	0.95	0.95
	0.86	0.07	0.2	0.90	0.9
	0.18			0.2	0.2
0.05					
<i>Msp</i> I	19.0	14.7	29.0	29.0	32.4
	13.4	11.3	2.45	2.45	
		2.29			
		0.40			
		0.34			

^a The fragment sizes of HP1 and HP2 were calculated with sites determined by sequence rather than actual restriction cuts. We have verified the locations of the *Bam*HI, *Bgl*II, and *Eco*RI sites in HP2 (data not shown).

HP1 designation by Esposito et al. (9). When the first 5.6 kb of the S2 phage was sequenced, the ORFs were assigned numbers that matched the HP1 designation, although they were not consecutive. While most of the proteins show a large degree of similarity, there are several striking differences: the *orf10*(HP2), *orf21*(HP2) and *orf22*(HP2) proteins are encoded by genes with no homology with any known DNA sequence in the National Center for Biotechnology Information database. While there are differences in sequence, the amino acid similarity scores of these proteins in HP1 in comparison to HP2 suggest conservation of function.

(i) *orf10*(HP2). Bacteriophage S2 has *orf1* and -2, which are unique to S2, but the next hypothetical ORF is *orf5*, since it is identical to *orf5* of HP1. S2 lacks the third and fourth ORFs found in HP1; hence there is no *orf3* or *orf4* in S2. A similar situation arises in HP2. The gene following *rep* in HP2 is not

TABLE 6. Comparison of the HP2 ORFs to those of HP1 and S2

Gene ^a	Position ^b		Protein identity/similarity ^c	Predicted function
	HP2	HP1		
Integrase	702–1712	698–1711	97/97, 98/98	Integrates phage into host chromosome to establish lysogeny
<i>orf1</i> (S)	1715–2476	1698–2315	0/0, 100/100	?
<i>cI</i>	2671–3243	3061–3636	96/97, 95/97	Maintains lysogeny by repressing pR promoters
<i>orf2</i> (S) <i>cox</i>	3311–3571	3574–3993	39/59, 100/100	Inhibits action of <i>cI</i> and competes with integrase (acts as excisionase)
<i>orf5</i>	3710–4210	4050–4553	93/94, 100/100	?
<i>orf6</i>	4232–4597	4572–4940	98/98, 99/99	?
<i>orf7</i>	4600–4782	4940–5125	100/100, 100/100	?
<i>orf8</i>	4797–5075	5137–5418	100/100, 100/100	?
<i>orf9</i>	5129–5386	5469–5729	100/100, 100/100	?
<i>rep</i>	5338–7716	5732–8059	98/98	Phage DNA polymerase
<i>orf10</i> (HP2)	7731–8031	8071–8370	37/61	?
<i>dam</i>	8052–8567	9169–9687	98/98	Dam methylase
<i>orf14</i>	8871–9269	9989–10390	100/100	?
<i>orf15</i>	96411–10675	10575–11794	97/98	Portal
<i>orf16</i>	10668–12488	11784–13607	96/97	Terminase
<i>orf17</i>	12692–13582	13826–14722	91/93	Scaffold
<i>orf18</i>	13588–14595	14726–15736	96/97	Capsid
<i>orf19</i>	14618–15454	15750–16595	96/97	Packaging
<i>orf20</i>	15450–15899	16588–17040	90/94	Packaging
<i>orf21</i> (HP2)	15890–16372	17028–17528	67/79	?
<i>orf22</i> (HP2)	16323–17057	17506–18189	42/61	?
<i>orf23</i>	17332–18459	18204–19334	99/99	Tail sheath
<i>orf24</i>	18489–18915	19338–19790	100/100	Tail tube
<i>hol</i>	18987–19238	19877–20113	98/98	Holin
<i>lys</i>	19255–19791	20106–20666	95/96	Lysis
<i>orf25</i>	19799–20123	20651–20998	98/99	?
<i>orf26</i>	20319–20624	21185–21493	100/100	?
<i>orf27</i>	20816–22942	21682–23751	73/81	?
<i>orf28</i>	22949–23281	23755–24090	98/99	?
<i>orf29</i>	23398–24443	24083–25264	98/99	?
<i>orf30</i>	24456–24980	25261–25785	100/100	?
<i>orf31</i>	25007–27736	25815–28592	78/86	Tail fibers
<i>orf32</i>	27751–28380	28604–29206	91/96	Tail collar
<i>orf33</i>	28396–29160	29239–30015	97/98	?
<i>orf34</i>	29150–29707	30002–30565	94/96	?
<i>orf35</i>	29711–31309	30562–32163	96/98	?

^a ORFs with names not beginning in “*orf*” have experimental functions. Gene names that include “(S)” designate a gene novel to S2 which is also found in HP2. Gene names that include “(HP2)” designate a gene novel to HP2 and that has not been described in either HP1 or S2.

^b The position numbers refer to the nucleotides composing the start-to-stop codons beginning with the first nucleotide of the phage chromosome as it is packaged in the bacteriophage head.

^c Identity/similarity scores refer to the score of amino acid identity or similarity of the given ORF with scores of HP2 versus HP and HP2 versus S2 listed in italics.

identical to *orf10* of HP1 (Fig. 4a). Although the inferred gene product shares the same N terminus, a distinct sequence of 270 bp makes up the rest of the 309-bp ORF. Thus, the gene in HP2 is identified as *orf10*(HP2) to distinguish it from the same region in HP1. Since we were unable to obtain an S2 lysogen, it is unclear whether this gene is unique to HP2. *orf10*(HP2) is also found in invasive lysogens closely related to R2866 (unpublished observations), so it is not likely to be a spurious finding. Since this phage appears to function without the HP1 equivalents of *orf11* and *orf12*, these genes may not be necessary for phage function. This suggests that HP2 may have lost these nonessential genes in its evolution from HP1. The protein encoded by *orf10*(HP2) has weak homology to that coded for by *orf10* of HP1 (37% identity, 61% similarity) and may serve a similar purpose.

(ii) **Lytic transcript differences.** Downstream of *orf10*(HP2), the nucleotide sequence of HP2 is similar to that of HP1. One

large difference occurs between bases 15992 and 17307, including *orf21* and *orf22* (Fig. 4a). This region has no known match at the nucleotide level in GenBank. However, the putative products of these genes each share 64% similarity and 42% identity (respectively) in spite of the large difference in nucleotide sequence. This suggests that the function of these gene products is conserved. As with most of the genes in this transcript, a function is not known or suggested by motif searches. *orf22* was implicated as the location of the *ts2* mutation in HP1 that produces a tailless phage (14). If *orf22* is involved in some aspect of tail biosynthesis, our data suggest that the tail structures may be different between HP1 and HP2.

HP2 *orf27* also differs from HP1 (Fig. 4a). A change in the nucleotide sequence yields a mosaic with a nearly identical N terminus and a divergent C terminus compared to those of HP1. The last 50 amino acids of the *orf27*(HP2) gene product are identical to those of *orf27* of HP1. This suggests a con-

served function for the N terminus of this protein. As in *orf21* and *orf22*, it could also be hypothesized that differences in *orf27* account for some of the phenotypic differences with regard to plaquing and lysogenization.

There are two changes in the sequence of HP2 *orf31* in comparison to that of HP1 (Fig. 7). Based on homology to P2 and phage 186, *orf31* is thought to encode the tail fibers of the bacteriophage (9). While the protein product of *orf31*(HP2) is nearly identical to that of *orf31* in HP1 for the N-terminal 360 amino acids, the sequence of amino acids 361 to 440 is unique and not highly conserved. This region is followed by near-complete identity until HP2 position 606, where there are some conserved changes until residue 638. There is only modest conservation of the 79 amino acids at the C terminus. If this ORF encodes the tail fibers, it is highly suggestive of an altered binding motif at the C terminus that may allow HP2 to distinguish its host from other *H. influenzae* strains. The variation across the middle of this ORF may alter the three-dimensional structure of the tail fiber or allow it to be presented to the host surface in a slightly different orientation.

Our data suggest that HP2 represents a variant of HP1 that diverged before S2 had evolved to its current structure. In this scenario, HP2 acquired features of S2 before it evolved to its current state of differentiation from HP1. This evolution is supported by a decrease in the number of genes in HP2 and S2 in comparison to the number in HP1. Bacteriophages evolve to efficiency, and losing unnecessary genes is more likely than gaining extra small genes. The loss of the pR₁ promoter supports this contention. Evidence from Esposito et al. suggests that pR₂ functions as well as pR₁, thus negating a need for two promoters at such close proximity (10). *cI* and *cox* binding experiments suggest that both pR₁ and pR₂ are regulated by the same elements. The S2 promoter lacks pR₁, suggesting it is not necessary. HP2 contains pR₁, but in a configuration that may be less active than the HP1 version (16 bp between the -10 and -35 regions rather than the ideal 17-bp configuration). This comparison places HP2 between HP1 and S2 in the evolving loss of pR₁.

Uptake elements. hUSs consist of a 9-bp core sequence and occur in the Rd KW20 genome, on average, once every 1,249 bp (38). The ability of the *H. influenzae* phage DNA to be introduced by transformation suggests that the phage genomes would have many hUSs. As an alternative to transfection, transformation could serve as a means for phage DNA dissemination in *H. influenzae*, and transformation bypasses restriction-modification surveillance, unlike bacteriophage infection (42). The HP1 genome contains only 17 hUSs, an average density lower (0.53/kb) than that of the Rd KW20 genome in general (0.81/kb) (9). HP2 also has 17 hUSs, although at different locations from HP1. We have found that bacteriophage DNA containing a kanamycin resistance cassette transforms with frequencies equivalent to those of chromosomal DNA (containing antibiotic resistance markers) into competent strain Rd KW20 (unpublished observation).

Attachment sites. The integration site for HP1 and S2 is the stem-loop of the gene encoding tRNA^{Leu} (18, 20, 35). The *attP* target for HP2 is the same (data not shown). The anticodon stem-loop of the tRNA^{Leu} is in the middle of an operon encoding tRNA^{Lys}, tRNA^{Leu}, and tRNA^{Gly}. As in HP1 and S2, the *attP* site in HP2 carries duplication of these genes to main-

tain transcription into functional tRNA molecules. More than one chromosomal attachment site has been described in strain Rd KW20 based on the presence of phage DNA in different *SmaI* fragments of chromosomal DNA (22). Since the *SmaI* restriction fragment patterns determined by pulsed-field gel electrophoresis differ between Rd KW20 and strain R2866, comparing the usage of these alternate attachment sites by HP2 is not possible.

We conclude that HP2 is closely related to HP1 but has a different host: unencapsulated *H. influenzae*.

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