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Cloning and Characterization of Bacteriophage-Like DNA from *Haemophilus somnus* Homologous to Phages P2 and HP1†

RENO A. PONTAROLLO,[1]‡ CLEMENT R. RIOUX,[1]§ AND ANDREW A. POTTER[1,2]*

Veterinary Infectious Disease Organization,[1] and Canadian Bacterial Diseases Network,[2] University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E3

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In an attempt to identify and characterize components of a heme uptake system of *Haemophilus somnus*, an *Escherichia coli* cosmid library of *H. somnus* genomic DNA was screened for the ability to bind hemin (Hmb^+). The Hmb^+ phenotype was associated with a 7,814-bp *Hin*dIII fragment of *H. somnus* DNA that was subcloned and sequenced. Thirteen open reading frames (orfs) were identified, all transcribed in one direction, and transposon mutagenesis identified *orf7* as the gene associated with the Hmb^+ phenotype. Orf7 (178 amino acids) has extensive homology with the lysozymes of bacteriophages P-A2, P21, P22, PZA, ϕ-29, ϕ-vML3, T4, or HP1. The *orf7* gene complemented the lytic function of the *K* gene of phage P2 and the *R* gene of phage λ. A lysozyme assay using supernatants from whole-cell lysates of *E. coli* cultures harboring plasmid pRAP501 or pGCH2 (both of which express the *orf7* gene product) exhibited significant levels of lysozyme activity. The *orf6* gene upstream of *orf7* has the dual start motif common to the holins encoded by lambdoid *S* genes, and the *orf6* gene product has significant homology to the holins of phages HP1 and P21. When expressed from a *tac* promoter, the *orf6* gene product caused immediate cell death without lysis, while cultures expressing the *orf7* gene product grew at normal rates but lysed immediately after the addition of chloroform. Based on this data, we concluded that the Hmb^+ phenotype was an artifact resulting from the expression of cloned lysis genes which were detrimental to the *E. coli* host. The DNA flanking the cloned lysis genes contains orfs that are similar to structural and DNA packaging genes of phage P2. Polyclonal antiserum against Orf2, which is homologous to the major capsid precursor protein (gp*N*) of phage P2, detected a 40,000-M_r protein expressed from pRAP401 but did not detect Orf2 in *H. somnus* lysates. The phage-like DNA was detected in the serum-susceptible preputial strains HS-124P and HS-127P but was absent from the serum-resistant preputial strains HS-20P and HS-22P. Elucidation of a potential role for this cryptic prophage in the *H. somnus* life cycle requires more study.

Hemophilosis is a term used to describe a variety of bovine disease syndromes associated with *Haemophilus somnus*, a gram-negative opportunistic bacterial pathogen. *H. somnus* was first linked to infectious thrombotic meningoencephalitis in Colorado in 1956 (23). Since the mid-1960s, hemophilosis has been a major loss of income to high-density cattle operations (feedlot and dairy) due to reduced weight gains and mortality (15). The bacterium has now been associated with several septicemic, respiratory, and reproductive clinical manifestations (reviewed in references 27 and 30).

Several potential virulence factors of *H. somnus* have been identified, including the ability to acquire iron from bovine transferrin (11, 12, 16, 32, 35, 46, 63–66, 69). This is mediated by transferrin-binding outer membrane proteins that are expressed under conditions of iron deprivation, and it likely requires periplasmic and cytoplasmic membrane proteins to facilitate transport. Another potential source of iron for *H. somnus* is heme-associated iron which can be liberated from lysed erythrocytes (47). Several pathogens have the ability to utilize heme as an iron source, and specific heme-uptake systems have been identified in *Vibrio cholerae* (28) and *Yersinia enterocolitica* (50).

The original purpose of this study was to investigate the ability of *H. somnus* to acquire heme-associated iron and to characterize the mechanism(s) of heme uptake. This led to the discovery of *H. somnus* DNA similar to bacteriophage DNA. The existence of phage-encoded virulence factors in several bacterial species has been described (6). Phage conversion can occur due to the acquisition of a phage-encoded toxin or altered expression of bacterial toxins due to the lysogenic state (5, 31, 43, 45, 68). Increased serum resistance due to lysogenic conversion has been documented in *Salmonella choleraesuis* (44). Prophages and cryptic prophages have also been shown to enhance viability in bacterial cultures in an energy-limited environment (17). At present, there are no reports of lysogenic conversion in *H. somnus*. In this study, we suggest that the presence of the discovered phage-like DNA may alter the virulence of *H. somnus* by enhancing serum sensitivity.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and growth conditions. Bacterial strains, plasmids, and phages used in this study are described in Table 1. *H. somnus* HS25 was stored frozen in egg yolk at −80°C and cultured on Columbia blood agar plates (5% [vol/vol] sheep blood) and brain heart infusion broth supplemented with Trizma base (0.1% [wt/vol]) and thiamine monophosphate (0.001% [wt/vol]). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or Terrific Broth (56), and ampicillin was used at 100 μg/ml unless otherwise stated. Phage stocks were prepared at 10^6 to 10^8 PFU/ml as described previously (33).

For lysis kinetics, *E. coli* cultures containing cloned lysis genes were grown in LB broth with ampicillin to A_{600} = 0.5 and induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (5 mM). Optical density was monitored,

* Corresponding author. Mailing address: 120 Veterinary Road, Saskatoon, Saskatchewan, Canada S7N 5E3. Phone: (306) 966-7468. Fax: (306) 966-7478. E-mail: potter@sask.usask.ca.

† Veterinary Infectious Disease Organization journal series no. 195.

‡ Present address: Department of National Defence, Medical Countermeasures Section, Defence Research Establishment Suffield, Box 4000, Medicine Hat, Alberta, Canada T1A 8K6.

§ Present address: Unité de Recherche en Vaccinologie, Centre de Recherche en Infectiologie, Centre Hospitalier de Québec, Pavillon CHUL, Sainte-Foy, Québec, Canada G1V 4G2.

TABLE 1. Bacterial strains, plasmids, and bacteriophages used in this study

Strain, plasmid, or bacteriophage	Relevant characteristics (reference)
Strains	
Haemophilus somnus	
HS25	Pneumonic isolate used in challenge model (26)
HS-20P and HS-22P	Preputial isolates; serum-resistant (13)
HS-124P and HS-127P	Preputial isolates; serum-sensitive (13)
Escherichia coli	
MC1061	K-12 strain; host for cosmid library (10)
JM105	K-12 strain; host for pTZ19 and derivatives (70)
C-1a	C strain; prototrophic (57)
C1757	C strain; *rpsL* (Sm^r) *supD* (61)
CC118	K-12 strain; *phoA* mutant, used to generate Tn*phoA* mutants (38)
Plasmids	
pHC79	Cosmid cloning vector (29)
pRAP117	Hmb^+ pHC79 derivative with a ~30-kb *Sau*3A1 fragment of HS25 genomic DNA (this study)
pTZ19R	Multipurpose cloning vector (40)
pRAP401	Hmb^+ pTZ19 derivative with 7,814-bp *Hin*dIII fragment of pRAP117 ligated to the *Hin*dIII site (this study)
pUEX1	Expression vector used to make Cro-LacZ fusion polypeptides with λ *c*I857 regulation (9)
pCAP109	pUEX1 derivative with 1,062-bp *Ase*I-*Xba*I fragment of pRAP401 (filled-in) ligated to the *Sma*I site and overexpresses Cro-LacZ-Orf2 fusion polypeptide (this study)
pRAP501	Hmb^+ pTZ19 derivative with 2,884-bp *Xba*I fragment of pRAP401 ligated to the *Xba*I site (this study)
pTn*phoA*101	Hmb^- and Pho^+ pRAP501 derivative with Tn*phoA* inserted in frame in Orf7 (this study)
pAA507	Expression vector with tac promoter and a synthetic leader polypeptide (66)
pGCH2	pAA507 derivative with the 1,210-bp *Xmn*I-*Sma*I fragment of pRAP501 ligated to the *Sma*I site and overexpresses Orf7 (this study)
pGH433	Expression vector with tac promoter (66)
pRAP505	pGH433 derivative with the 301-bp *Kpn*I-*Xmn*I fragment of pRAP501 ligated to the *Sma*I site and overexpresses Orf6 (this study)
pRAP506	pGH433 derivative with the 1,219-bp *Nco*I-*Sma*I fragment of pGCH2 ligated to the *Nco*I/*Sma*I site and overexpresses Orf7 (this study)
Bacteriophages	
P2 *vir*1	Does not lysogenize (3)
P2 *vir*1 *K*am218	Lysis deficient (61)
P2 *vir*1 *L*am79	DNA packaging defect (61)
P2 *vir*1 *M*am32	DNA packaging defect (61)
λ *c*I857	Temperate at 30°C and lytic at 42°C (62)
λ *c*I857 *R*am54am60	Lysis deficient (20)
λ::Tn*phoA*	Used to construct Tn*phoA* mutants (38)

and cell viability was verified by plating dilutions of the culture on LB agar with ampicillin.

Intracellular incubation of *H. somnus* in bovine blood monocytes was performed as described previously (35). Briefly, monocytes were isolated from a yearling heifer by standard techniques (16, 35). Phagocytosis was performed at a 1:1 bacterium:monocyte ratio in 1 ml of Hanks balanced salt solution with 10% (vol/vol) heat-inactivated anti-*H. somnus* hyperimmune serum (66). Monocytes containing bacteria were incubated 18 h, collected, and lysed with 1% (vol/vol) saponin for 5 min at 20°C. The liberated bacteria were pelleted by centrifugation and used to prepare whole-cell lysates for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting and total cellular RNA for Northern blotting.

Nucleic acid techniques and computer analysis. Restriction enzymes, calf intestinal phosphatase, Klenow fragment, T4 DNA ligase, random hexamers, the Nested Deletion Kit, and the T7 Sequencing Kit were used as recommended by the supplier (Pharmacia, Baie d'Urfe, Canada). Routine manipulation of plasmid DNA, Southern blotting, and Northern blotting were performed as described previously (56). The DNA probe used in the Southern and Northern blots was prepared by incorporating [α-^{32}P]ATP into the 7,814-bp *Hin*dIII fragment from pRAP401 with random hexamer primers and Klenow fragment. Bacterial genomic DNA was prepared by the rapid method as described previously (56), and total cellular RNA was isolated by the hot phenol method (41). Oligonucleotides were prepared on the Pharmacia LKB Gene Assembler Plus, and DNA sequence data was assembled by using PCGene 6.7 (IntelliGenetics). Protein and DNA sequence similarity searches in the GenBank and Swiss-PROT databases were done with BLAST version 1.4 (1). Local alignment of polypeptides was performed by using PAlign 1.05 with conservative amino acid changes permitted (42). MACAW 2.05 was used to align phage-encoded lysozymes based on primary and secondary structure (58).

Cosmid library and plasmid construction. The cosmid library was prepared as described previously (14) and screened for the ability to bind hemin (Hmb^+) as described previously (24). All plasmids are described in Table 1 and Fig. 1. Plasmid pCAP109 was constructed by filling in the *orf2*-containing 1,062-bp *Ase*I-*Xba*I fragment of pRAP401 with Klenow and blunt-end ligating into the *Sma*I site of vector pUEX1. The in-frame fusion was verified by sequencing with primer HSR6 (5′-TTAATGTGCCGATGCGT-3′). The *orf6*-containing 301-bp *Kpn*I-*Xmn*I fragment originating from pRAP501 was treated with Klenow and ligated into the *Sma*I site of vector pGH433 to create plasmid pRAP505. Plasmid pGCH2 was constructed by ligating the *orf7*-containing 1,210-bp *Xmn*I-*Sma*I fragment of pRAP501 into the *Sma*I site of pAA507.

Genetic complementation. Plasmids pTZ19R, pRAP501, pTn*phoA*101, and pRAP401 were transformed into *E. coli* nonsuppressing strain C-1a and suppressing strain C1757. Log-phase cultures were inoculated into molten top agar and were poured onto LB agar plates containing ampicillin and $CaCl_2$ (0.5 mM). After the agar solidified, plates were spotted with 10^6 PFU of the phage to be tested. The formation of a zone of lysis after 18 h at 37°C for phage P2 or 42°C for phage λ was scored as a positive complementation.

Lysozyme assay. Lysozyme activity was assayed in a procedure adapted from Tsui and Mark (67). Substrate cells (JM105) were grown in 100 ml of LB broth to an A_{600} of 1.0, pelleted by low-speed centrifugation, and resuspended in an equal volume of 0.1 M EDTA (pH 8). The cells were agitated gently for 5 min, repelleted, resuspended in 100 ml of 10 mM Tris-HCl (pH 7.4), and stored on ice.

Whole-cell lysates were prepared from 50 ml of LB broth-ampicillin cultures of JM105 harboring plasmids pTZ19, pRAP501, pTn*phoA*101, or pGCH2. JM105/pGCH2 was grown to an A_{600} of 0.6 and induced by the addition of IPTG to 5 mM (negative control was not induced) and incubated for 1 h. The cultures were sonicated for 90 s on ice (output = 5, duty cycle = 50%; Sonic Materials Vibracell). Cell debris was removed by centrifugation for 20 min at 12,000 × *g*, and the supernatants were concentrated to 1 ml with a Centriprep 10 (Amicon, Oakville, Canada). Then 50 μl of supernatant was added to 1 ml of substrate cells (A_{600} = 1.0) and the change in A_{600} was monitored for a 10-min interval.

Specific antiserum against Orf2. JM105/pCAP109 was grown to A_{600} = 0.6 and induced at 42°C for 2 h to express the Cro-β-gal-Orf2 fusion polypeptide. The fusion protein was isolated in the form of inclusion bodies (22) and solubilized in 0.5% (wt/vol) SDS. Rabbit 94-034 was inoculated at 20 subcutaneous dorsal

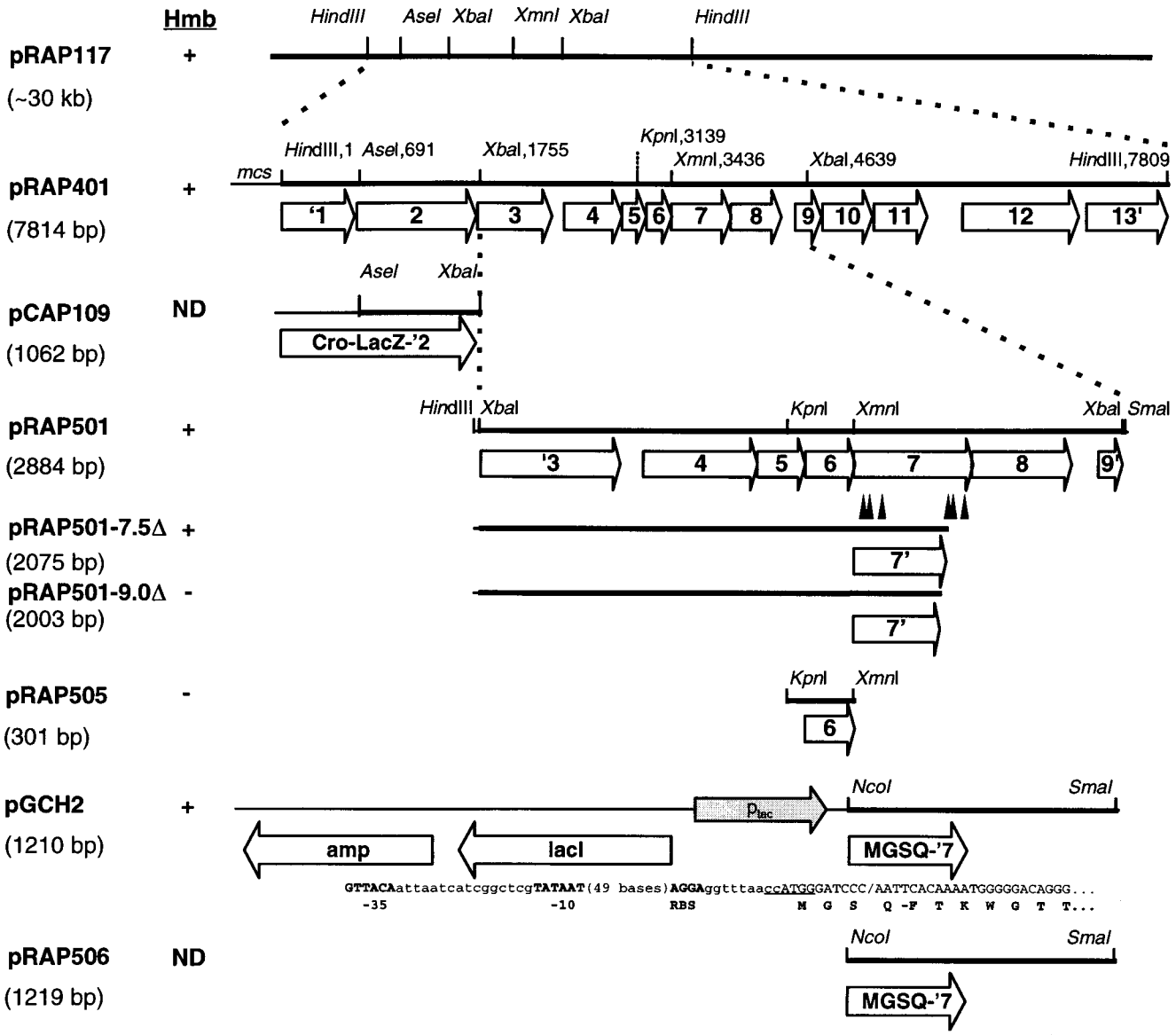

FIG. 1. Subcloning of Hmb$^+$ cosmid clone pRAP117. Relevant restriction enzymes sites are shown, and insert sizes are indicated in parentheses. Plasmids pRAP401, pRAP501, pRAP501-7.5Δ, and pRAP501-9.0Δ were constructed in vector pTZ19R. Arrows beneath pRAP401 indicate orfs deduced from DNA sequence data. Vertical arrowheads beneath Orf7 in pRAP501 represent insertion sites of Tn*phoA* that resulted in the loss of the Hmb$^+$ phenotype. Plasmid pRAP505 is a pGH433 derivative which expresses Orf6. Plasmids pGCH2 and pRAP506 were constructed in vectors pAA507 and pGH433, respectively, and express Orf7 with a MGSQ-leader peptide. The p_{tac} promoter region and the fusion joint of the leader peptide to Orf7 are detailed beneath pGCH2. Plasmid pCAP109 is a pUEX1 derivative and expresses the Cro-LacZ-Orf2 fusion protein. See text for subcloning details.

sites with a total of 100 μg of the fusion polypeptide in Freund's complete adjuvant (day 1) or in Freund's incomplete adjuvant (day 21) and bled out (day 31).

SDS-PAGE and immunoblotting. SDS-PAGE of proteins was performed in the Laemmli system (34). Whole-cell lysates were prepared for electrophoresis by resuspending cell pellets from a 1.5-ml culture in 100 μl of double-distilled H_2O and 100 μl of 2× SDS-PAGE sample buffer with β-mercaptoethanol. Transfer of proteins to nitrocellulose membranes was performed as recommended by the manufacturer (Biorad, Mississauga, Canada), and blots were treated by using standard techniques (65). The secondary antibodies were either goat anti-rabbit immunoglobulin G (IgG) (heavy and light chain) or goat anti-bovine IgG (heavy and light chain). Both were coupled to alkaline phosphatase.

Accession number. The sequence of the 7,814-bp *Hin*dIII fragment of *H. somnus* can be retrieved from GenBank by using accession number U28154.

RESULTS

Cloning, identification, and sequencing of the gene associated with the Hmb$^+$ phenotype. A plate bioassay was used to determine that *H. somnus* HS25 could use hemin as a sole source of iron (data not shown). Therefore, an *E. coli* MC1061 cosmid library of HS25 genomic DNA was screened for individual colonies with dark brown pigmentation (Hmb$^+$) on M9CA agar plates containing hemin. Restriction endonuclease digests of plasmids from 50 Hmb$^+$ colonies revealed five different clones, three of which had a common 7.8-kb *Hin*dIII fragment. The cosmid clone pRAP117 (Fig. 1) was selected for further studies.

Plasmid pRAP401, a pTZ19R derivative, contains the 7.8-kb *Hin*dIII fragment (Fig. 1 and Table 1) and confers the Hmb$^+$ phenotype on *E. coli* JM105. The DNA sequence of pRAP401 (7,814 bp) contains 13 potential open reading frames (orfs), all transcribed from the same DNA strand (Fig. 1) (19, 39). All orfs, excluding the truncated *orf1* and *orf13*, are preceded by a

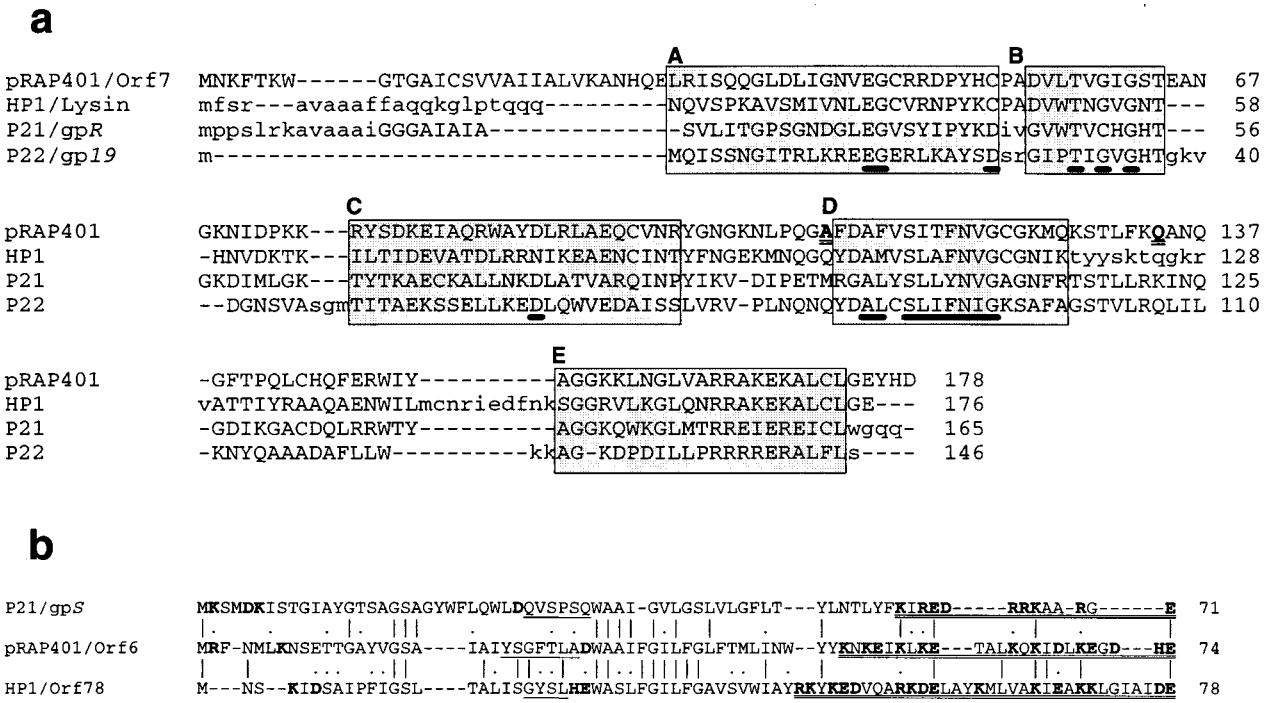

FIG. 2. Alignment of potential *H. somnus* lysis proteins with lysozymes (a) and holins (b) from other phages. The number of amino acid residues of each protein is indicated at the end of the sequence. Gaps (-) were introduced to optimize the alignments. (a) Orf7 alignment with known or presumed β(1-4) hydrolases (EC 3.2.1.17) of phages HP1, P21, and P22 determined by using MACAW 2.05 segment pair overlap with a minimum threshold of 20. Capital letters indicate regions similar to Orf7. Blocks of structurally conserved regions in shaded boxes (A to E) and residues critical to lysozyme function in P22 gp*19* are underlined. The Ala-110 and Gln-134 are in boldface and double underlined to indicate where Orf7 is truncated in plasmids pRAP501-9.0Δ and pRAP501-7.5Δ, respectively. (b) Orf6 alignment with the holins of phages HP1 and P21 performed with PAlign 1.05, allowing conservative replacements. |, identity; ·, conservative change. Charged residues are in boldface, potential β-turns are underlined, and the highly charged C termini are double underlined.

potential ribosomal binding site (60). The G+C% of the potential coding regions ranges from 35.9% (*orf13*) to 42.1% (*orf11*), which is typical for *H. somnus* (2). However, codon usage analysis indicated that 61.8% of codons prefer A or T at the wobble position, which is significantly lower than known *H. somnus* genes which average 77% A or T at the third position.

Further subcloning of pRAP401 localized the Hmb^+ phenotype to a 2,884-bp *Xba*I fragment contained in plasmid pRAP501 (Fig. 1 and Table 1). Transposon mutagenesis of pRAP501, with λTn*phoA*, was used to determine which orf was associated with the Hmb^+ phenotype. Six Hmb^- clones were isolated, and restriction endonuclease analysis and sequencing confirmed that Tn*phoA* had inserted in frame in *orf7* (Fig. 1). These Tn*phoA* constructs also had alkaline phosphatase activity (Pho^+), which suggested the *orf7* gene product (Orf7) was exported to the cell envelope. Nested deletions made in pRAP501 from the 3′ end of *orf7* lost the Hmb^+ phenotype when Orf7 was truncated after Ala-110 in pRAP501-9.0Δ (Fig. 1 and 2a). Deletion subclone pRAP501-7.5Δ retains the Hmb^+ phenotype and would express Orf7 truncated after Gln-134. These data eliminated the possibility of a polar effect caused by Tn*phoA* insertion and confirmed that *orf7* was associated with the Hmb^+ phenotype.

Liquid cultures of *E. coli* harboring pRAP401 or pRAP501 grew to low optical densities, would not form solid pellets after centrifugation, and had viscous supernatants. All procedures involving these clones were performed with overnight cultures of freshly transformed cells because the frozen stock cultures had poor viability.

Computer analysis of predicted polypeptides of pRAP401. A nonredundant database search revealed that Orf7 has 56% similarity with the putative lysozyme of bacteriophage HP1 (Fig. 2a) (18). HP1 is a temperate phage of *H. influenzae* Rd, and the lysozyme is involved in host cell lysis during the lytic cycle (71). Orf7 also has significant homology to the lysozymes expressed by phages P21 (gp*R*) (8), PA-2 (gp*R*) (7), PZA (gp*15*) (49), ϕ-29 (gp*15*) (21), P22 (gp*19*) (53), ϕ-vML3 (lysin) (59), and T4 (gp*e*) (48). An alignment of Orf7 with the lysozymes from HP1, P21, P22, and T4 confirmed that five structurally conserved regions of lysozymes (52) are present in Orf7 (Fig. 2a).

Bacteriophage lysozyme genes are often preceded by an overlapping lysis-associated gene which encodes the holin proteins. Holins form a lesion in the cytoplasmic membrane that allows the lysozyme to access and degrade the peptidoglycan (71). *orf6*, which overlaps *orf7*, has several holin-like features, including two in-frame methionine start codons and two potential Shine-Dalgarno sites which encode polypeptides of 71 and 74 amino acids. An alignment of Orf6 with the holins from phages HP1 (57% similarity, 32.4% identity) and P21 (40.8% similarity, 23.9% identity) predicts that Orf6 has other holin-like features including a highly charged C terminus and two hydrophobic membrane spanning helices separated by a β-turn (Fig. 2b) (71).

Orf1 to Orf5, Orf10, and Orf11 of pRAP401 have significant homology with DNA packaging, capsid, and tail synthesis proteins of phage P2 (Fig. 3) (36, 37). The truncated Orf1 is 56% similar to P2 gp*O*, the scaffolding protein, and is missing about 30 N-terminal residues based on its alignment with gp*O* (55). Orf2 has homology to gp*N*, the major capsid protein precursor. Orf3 is similar to gp*M* (terminase subunit), and Orf4 is similar to gp*L* (capsid completion protein). Orf5 is homologous to the

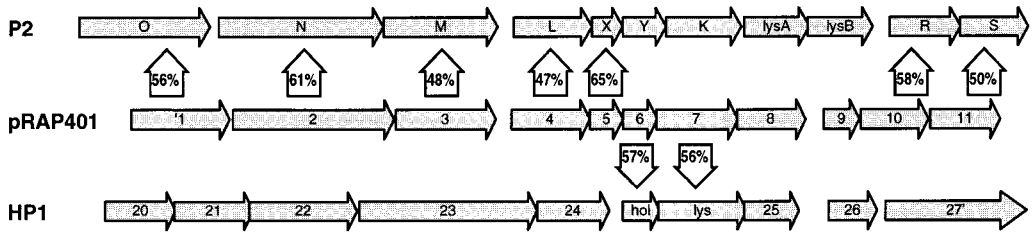

FIG. 3. Alignment of pRAP401 Orf1 to Orf11 (nucleotides 1 to 5700) with homologous proteins from phages P2 and HP1. The P2 orfs were deduced from nucleotides 3167 to 9367 from the left end of the P2 genome (36, 37, 73). The HP1 Orfs were deduced from nucleotides 16,588-22,888 of the complete HP1 genome (18). The hollow vertical arrows indicate the homology between polypeptides; e.g., Orf5 of pRAP401 is 65% homologous to gp*X* of phage P2. Polypeptide homology was determined with PAlign 1.05, allowing conservative replacements.

recently described gp*X* of P2 which is involved in phage tail synthesis (73). Also, *orf1* through *orf5* have the same transcriptional order as the P2 genes *ONMLX* (37). Orf10 and Orf11 are homologous to gp*R* and gp*S* of phage P2, two proteins involved in completion of phage tails (36). Orf8, Orf9, Orf12, and Orf13 had no significant homology with any known proteins. Therefore, the 7,814-bp *Hin*dIII fragment appears to have a phage HP1-like lysis cassette within the organization of phage P2-like DNA packaging and structural genes.

Activities of Orf6 and Orf7. Functional studies were performed to support the lysis functions predicted by the primary structure analysis of Orf6 and Orf7. Plasmid pGCH2 was constructed to overexpress a fusion polypeptide that contains all but three N-terminal residues of Orf7 (Table 1 and Fig. 1). Concentrated whole-cell lysates from cultures of *E. coli* with plasmid pRAP501 or pGCH2 (IPTG induced) incubated with EDTA-killed JM105 substrate cells caused a drop in optical density over 10 min of 34.3 or 49.2%, respectively (Fig. 4). Lysates from cells with plasmids pTZ19R, pTn*phoA*101, and pGCH2 (uninduced) produced less than a 15% drop in optical density, which was equivalent to the natural sedimentation rate of the substrate cells. Concentrated HS25 lysates did not have lytic activity (data not shown).

The lysis activities of Orf6 and Orf7 were further delineated by cloning *orf6* and *orf7* into the expression vector pGH433 to construct plasmids pRAP505 and pRAP506, respectively (Table 1 and Fig. 1). *E. coli* JM105 harboring these plasmids were treated with IPTG and monitored for growth (Fig. 5). The addition of IPTG to the JM105/pRAP505 culture resulted in a cessation of growth and greatly reduced viability ($<10^3$ CFU/ml at A_{600} = 0.660), but no host cell lysis occurred. However, growth and viability were unaffected (8×10^8 CFU/ml at A_{600} = 0.860) in uninduced JM105/pRAP505. This result is consistent with the theory of holin activity, i.e., cell death as a result of lesions formed in the cell membrane (71). IPTG-induced JM105/pRAP506 cultures did not differ significantly from uninduced controls with respect to growth rates and viability. However, the addition of chloroform to the induced JM105/pRAP506 culture led to instantaneous host cell lysis (Fig. 5). This result is consistent with that for cells carrying the mutant λ S^- R^+ (holin deficient) prophage, which do not lyse upon induction but lyse immediately when chloroform is added (71).

Complementation of lysis-deficient phages by *orf7*. Table 2 summarizes an experiment designed to determine if *orf3*, *orf4*, or *orf7* could act in *trans* to complement the growth of DNA packaging-defective P2 phages or lysis-deficient P2 and λ phages. Complementation of amber mutations in the *M* and *L* genes of P2 was not possible by pRAP401 *orf3* or *orf4*, respectively. However, the nonsuppressed host, *E. coli* C-1a containing plasmid pRAP501 was capable of complementing amber mutations in the lysozyme (endolysin) genes of phages λ *c*I857 *R*am54am60 and P2 *vir-1 K*am218. Complementation of the lysozyme genes was not possible in strain C-1a containing plasmid pTZ19R or pTn*phoA*101. As expected, *E. coli* C1757 (amber suppressing *supD*) was lysed by all phages tested. This experiment, in conjunction with the lysozyme assay, the lysis activities of Orf6 and Orf7, and the protein homology data, constitutes substantial evidence to suggest that *orf6* and *orf7* encode a holin and lysozyme which we hypothesize are of bacteriophage origin.

Identification of Orf2. The *orf2* gene product was chosen for further study because it was homologous to the major capsid precursor protein (gp*N*) of phage P2. Therefore, it was the ideal candidate to identify phage particles associated with the phage-like DNA. Plasmid pCAP109 (Table 1 and Fig. 1) was constructed to express a Cro-β-gal-Orf2 fusion protein that was used to raise specific antiserum in rabbits. A Western blot of whole-cell lysates was probed with this antiserum to detect Orf2 (Fig. 6). The Orf2 protein (40,000 M_r) was detected in JM105/pRAP401 (lane 2), but the native protein was not detected in *H. somnus* (lane 6). An attempt was made to detect Orf2 and the *orf2* transcript in HS25 cultures following UV irradiation (35 μW/cm^2 for 20 s), mitomycin treatment (1 μg/ml), heat shock (42°C), ethanol shock (5% wt/vol), iron depri-

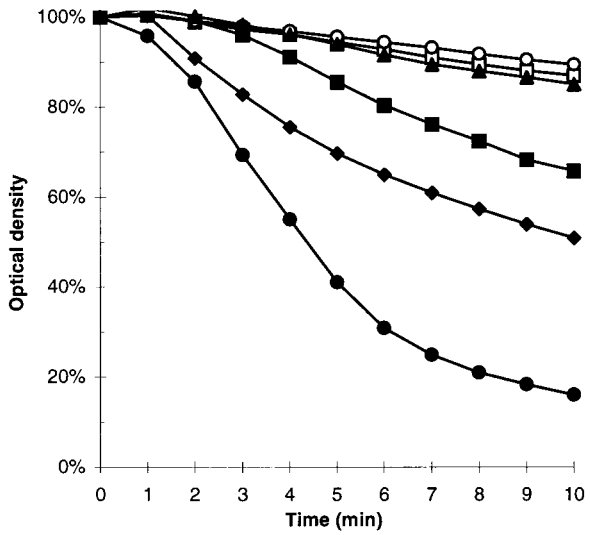

FIG. 4. Lysis activity in whole-cell lysates from *E. coli* JM105 expressing Orf7. Concentrated lysates from transformants containing the indicated plasmids were added to suspensions of EDTA-killed JM105 substrate cells. ○, pTZ19; ■, pRAP501; ▲, pGCH2 (uninduced); ◆, pGCH2 (IPTG induced); □, pTn*phoA*101; ●, hen egg white lysozyme (2.54 μg/ml; Sigma). The percentage reduction in optical density (A_{600}) of the substrate over 10 min is depicted. All curves are the average of five independent assays.

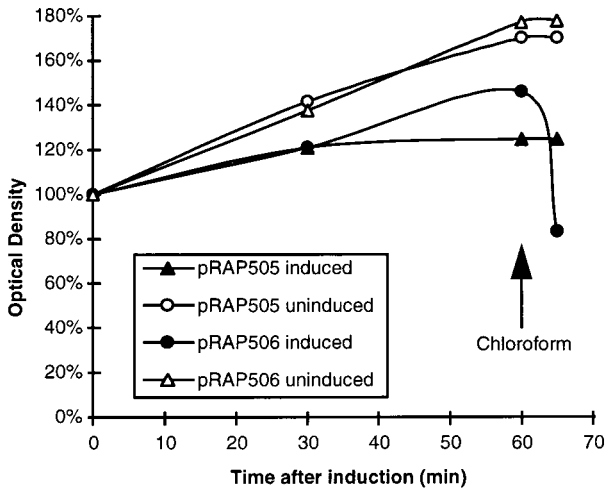

FIG. 5. Lysis activity of cloned *orf6* and *orf7* genes. JM105 cultures (5 ml) with the indicated plasmids were grown to mid-log phase (which is defined as 100%) at 37°C, induced by adding IPTG to 5 mM, and monitored for growth (A_{600}) for an additional hour. Chloroform (150 μl) was added to the pRAP506 cultures 60 min after induction.

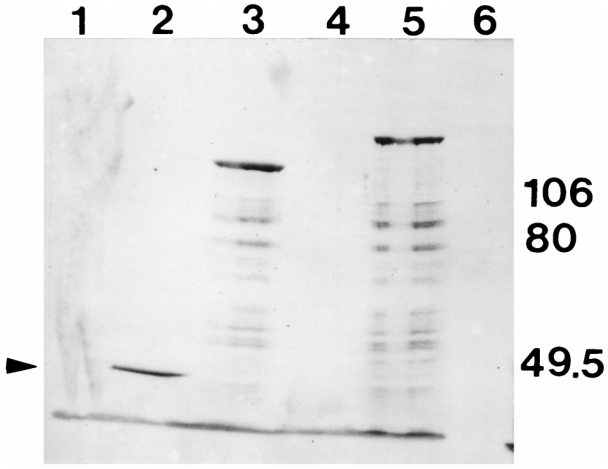

FIG. 6. Identification of Orf2 in Western blots of whole-cell lysates. Each lane contains approximately 15 μg of protein. Lane 1, JM105/pTZ19R; lane 2, JM105/pRAP401; lane 3, JM105/pUEX1 (induced); lane 4, JM105/pCAP109 (uninduced); lane 5, JM105/pCAP109 (induced); lane 6, HS25. Biorad prestained low molecular size markers (in kilodaltons) are shown on the right. Orf2 is indicated (➤) in lane 2.

vation [200 μM ethylamine di-*o*-(hydroxyphenylacetic acid)], and intracellular growth in bovine monocytes. However, neither the protein nor the transcript was detected by Western or Northern blotting, respectively (data not shown).

Detection of phage-like DNA in other *H. somnus* strains. Serum-sensitive and serum-resistant preputial strains of *H. somnus* were tested for the presence of the phage-like DNA from strain HS25 (Fig. 7). DNA homologous to the 7,814-bp *Hin*dIII fragment of HS25 was present in the two serum-sensitive strains tested, while the two serum-resistant strains did not contain the phage-like DNA.

DISCUSSION

Some pathogens, including *H. somnus*, are able to utilize heme-associated iron, which may be an important virulence factor (47, 51). Therefore, an *E. coli* cosmid library of *H. somnus* genomic DNA was screened for colonies with dark brown pigmentation (Hmb^+) on agar plates containing hemin. This screening procedure was previously used to isolate a gene from *H. influenzae* type b that encodes for a lipoprotein, HbpA (hemin binding protein), that may be a periplasmic binding protein for a heme uptake system (25). In our study, a 7,814-bp *Hin*dIII fragment of *H. somnus* DNA conferred the Hmb^+ phenotype on the host strain. Further subcloning and transposon (Tn*phoA*) mutagenesis identified *orf7*, which encodes a 178-amino-acid (19.8-kDa) polypeptide, to be associated with the Hmb^+ phenotype. However, a comparison to the GenBank nonredundant database indicated that Orf7 is similar to several phage-encoded β-(1-4) hydrolases (Fig. 2a) (71). Most importantly, five of eight Orf7 amino acids are identical to five of the eight critical amino acids that form the active site cleft of the phage T4 lysozyme. This includes the Orf7 Glu-43 (Glu-11 of T4), Gly-44 (Gly-12), Thr-58 (Thr-26), Gly-60 (Gly-28), and Gly-62 (Gly-30). The three nonidentical residues in Orf7 are acceptable substitutions that had little effect on lysozyme activity when tested in T4 gp*e* (52).

A series of experiments support the Orf7-lysozyme homology data. First, Orf7-containing lysates exhibited lytic activity (Fig. 4). Although this activity may appear weak, the substrate cells were not treated with chloroform to damage the outer membrane, as was done in other laboratories (73). This would inhibit access of the phage-encoded lysozyme to the cell wall

TABLE 2. Complementation[a] of bacteriophage P2 and λ mutations by *H. somnus* phage-like genes

Phage	Result for *E. coli* strain/plasmid				
	C1757/ pTZ19	C-1a/ pTZ19	C-1a/ pRAP501	C-1a/ Tn*phoA*101	C-1a/ pRAP401
P2 *vir*1	+	+	+	+	+
P2 *vir*1 *K*am218	+	−	+	−	+
P2 *vir*1 *L*am79	+	−	−	−	−
P2 *vir*1 *M*am32	+	−	−	−	−
λ*c*I857	+	+	+	+	+
λ*c*I857 *R*am54am60	+	−	+	−	+

[a] 10^6 phage were spotted onto a confluent top agar plate of *E. coli*/plasmid. The formation of a zone of lysis after incubating 18 h was scored as a positive (+) complementation (−, negative).

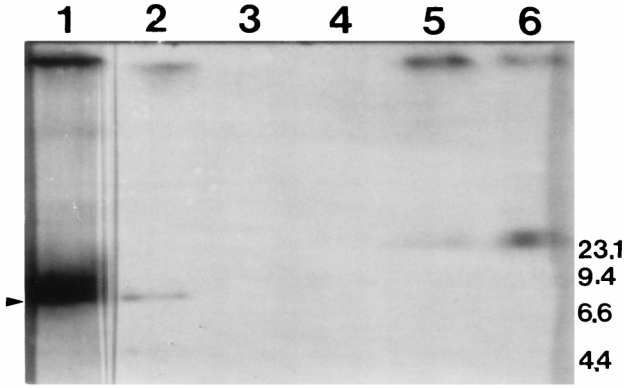

FIG. 7. Southern blot of *H. somnus* strains with pRAP401 7,814-bp *Hin*dIII fragment. All samples were digested with *Hin*dIII. Lane 1, pRAP401 control; lane 2, HS25 control; lane 3, HS-20P; lane 4, HS-22P; lane 5, HS-124P; lane 6, HS-127P. The band at the top in lanes 1, 2, 5, and 6 is the well. The probe hybridized with a ~25-kb *Hin*dIII fragment in strains HS-124P and HS-127P.

and thus reduce the activity. Lysozyme extracted from egg whites does not appear to be inhibited by outer membranes. Second, *orf7* was able to complement lysis-deficient P2 and λ phages (Table 2). The P2 *K* gene and λ *R* gene encode transglycosylases which are not true lysozymes. However, this distinction is irrelevant to the lysis mechanism and this assay since λ *R* gene defects can also be complemented by the true lysozymes of phages P22 and T4 (71). The third experiment utilized separately cloned *orf7* and *orf6* genes to delineate the function of Orf7 from Orf6 (Fig. 5). Orf7 expression was not lethal to the host but caused instantaneous lysis when chloroform was added to the culture. Orf6 expression did not cause lysis but was lethal to the host cell. These results parallel the activities of holins and lysozymes from other phages (71) and confirm that Orf6 and Orf7 are indeed lysis proteins with bacteriophage origins. Lysis genes usually include a λ *Rz*-like gene located downstream of the endolysin (72). However, analysis of the gps of *orf8* and *orf9* revealed no homology to any known proteins.

The Hmb^+ phenotype appears to be an artifact due to expression of cloned lysis proteins. This problem has been described for other situations in which other lysis genes were cloned into high-copy-number plasmids, where low background levels of expression are unavoidable (71). Background expression of the lysis genes would explain the poor viability of *E. coli* hosts expressing both the holin and lysozyme. It is possible that the hydrophobic heme molecule partitioned into the lipid bilayer of the outer membrane of the dead (or dying) cells, where its accumulation resulted in the phenotype. The PhoA activity resulting from the Tn*phoA* insertions into *orf7* also suggests low-level holin function in these clones.

The orfs upstream and downstream of the lysis genes of pRAP401 have extensive homology with the DNA packaging, capsid, and tail synthesis genes of phage P2 (Fig. 3). Orf3 is similar to the terminase subunit (gp*M*) of phage P2 (Fig. 5), which promotes circularization of the P2 genome before packaging. Two low-affinity DNA-binding signatures, KPK and GRP, are thought to aid in this process (37), but Orf3 does not have these signatures, which could explain why *orf3* did not complement the gene *M* mutation (Table 2).

The cloned *orf2* gene product was identified by Western blotting of whole-cell lysates of JM105/pRAP401; however, the native Orf2 was not identified in *H. somnus* lysates (Fig. 6). All attempts to induce expression of Orf2 by *H. somnus* by several environmental stresses were unsuccessful, as were attempts to express Orf7 (51). It should be noted that lysogenic phage P2, to which the phage-like DNA is most homologous, is uninducible by UV light (4).

The codon bias of the cloned region showed a 61.8% preference for A or T at the third position. This is similar to that in phage HP1 (61.4%) but significantly lower than that in most *H. somnus* genes, which average 77% A or T at the wobble position. Only 32% of the phage P2 DNA packaging and capsid gene codons end with A or T (37). The fact that the codon bias of the region is between that of phage P2 and *H. somnus* suggests that the phage-like DNA may have been acquired by *H. somnus* from a P2-like ancestor.

Defective, incomplete, and cryptic prophages are thought to be ubiquitous in all species of bacteria. Phage genes can be beneficial to the host by enhancing its survival in some limiting environment (17) and in some cases can be considered virulence factors (6). To test if the phage-like genes were involved in pathogenesis, we screened four preputial *H. somnus* strains that have been described as serum sensitive or serum resistant (13) for the presence of the phage-like DNA. Interestingly, the phage-like DNA was present only in the serum-sensitive strains HS-20P and HS-22P (Fig. 7). Due to the limited sample population, a general correlation cannot be drawn between phage-like DNA and serum sensitivity from this experiment. However, it would be worthwhile to screen a panel of geographic, clinical, and carrier isolates of *H. somnus* for the prevalence of the phage-like DNA. This data could then be correlated with the pathogenicity of *H. somnus.*

Although bacteriophages are likely to be associated with *H. somnus* field isolates, to date, none have been described. No phage particles or native proteins relating to the phage-like DNA have been detected in *H. somnus* cultures (51), suggesting that the genes from the cloned fragment are not expressed in *H. somnus*. Therefore, data presented here suggests that the cloned DNA is part of a cryptic or defective prophage that may have a significant role in *H. somnus* pathogenesis.

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