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Characterization of *Serratia entomophila* Bacteriophages and the Phage-Resistant Mutant Strain BC4B

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Successful large-scale fermentations of the bacterium *Serratia entomophila* for use in biological control of the soil-dwelling insect *Costelytra zealandica* has required the development of a phage-resistant mutant, BC4B. We report our investigations into *S. entomophila* phages and the nature of the phage resistance mechanism of strain BC4B. The parental strain of BC4B, A1MO2, was found to contain two previously unidentified prophages, ϕ9A and ϕ9B, which were UV inducible and also released spontaneously in large numbers. BC4B was shown to be completely cured of ϕ9A. Single lysogens of ϕ9A and ϕ9B were not homoimmune to any other *S. entomophila* phages. However, on the basis of DNA-DNA homology, all *S. entomophila* phages except ϕCW3 were shown to have significant regions of homology and also packaged their DNA via *pac*-like mechanisms. The failure of phage particles to adsorb was identified as the basis of phage resistance in BC4B. In addition, it was demonstrated that all known *S. entomophila* phages are naturally temperature sensitive.

The bacterium *Serratia entomophila* (member of the family *Enterobacteriaceae*) (7) has been successfully developed as a biological control agent for a major New Zealand pasture pest, the native grass grub *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae) (10). Following the first report of bacteriophages specific for *S. entomophila* (23), the frequent collapse of large-scale fermentations due to phage infections was identified as a major obstacle in the development of *S. entomophila* as a biological control agent. Prior to this work, three distinct phages, ϕCW1, ϕCW3, and ϕCW4, had been identified on the basis of restriction maps and Southern hybridization analysis (20, 23). Concurrent with this work, a fourth distinct *S. entomophila* phage, ϕAgRP8, was identified (14). Isolates with restriction patterns identical to that of the clear plaque phage ϕCW4, which have altered plaque morphologies and host ranges, have been reported; these include, for example, the turbid plaque variants ϕBT and ϕCW5 (14).

To overcome phage-induced fermentation collapses, ethylmethane sulfonate mutagenesis of the wild-type strain A1MO2 was used to generate a mutant strain, BC4B, that was completely resistant to all phages yet retained full pathogenicity and normal growth characteristics (15). Preliminary investigations into the phage-resistant phenotype of BC4B (20) demonstrated that A1MO2 genomic DNA harbored extensive amounts of phage DNA homologous to ϕCW1, while BC4B contained significantly less homologous DNA. However, no phages had been detected even after UV induction of A1MO2 with the standard phage host strains and indicators. The strain BC4B was also classified as nonlysogenic as a result of the failure of UV and heat induction to produce any detectable phage particles (15).

In this study, we report the identification of two new phages isolated from strains A1MO2 and a derived strain BC4B, which, along with previously reported phages, were differentiated from one another on the basis of DNA-DNA homology. The primary mechanism of phage resistance in BC4B as well as a natural temperature sensitivity common to all phages of *S. entomophila* was identified.

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MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. Strains of *S. entomophila*, phages, and all plasmids used in this study are described in Table 1.

Media and growth conditions. *S. entomophila* strains and phages were grown at 30°C unless otherwise stated. Luria Broth (LB), LB agar (LA), and H-Top agar were prepared as described by Miller (13). The antibiotic concentrations (in micrograms per milliliter) used were as follows: ampicillin, 50; tetracycline, 30; and kanamycin, 100. Lysates of *S. entomophila* phages were prepared by adding a single plaque to an exponential-phase culture of 5 to 10 ml (optical density at 600 nm, 0.4) of the appropriate strain and shaking for 5 h. Phage lysates were harvested by adding 50 μl of chloroform, shaking for 10 min, and then centrifuging (10 min, 10,000 × *g*) to remove the cell debris. Titers were determined by preparing serial dilutions of the lysate in LB and spotting on an H-Top lawn of the indicator strain. Plates were incubated at 30°C overnight. A collection of wild-type strains was screened for an isolate sensitive to phages spontaneously produced by A1MO2 by spotting chloroformed A1MO2 supernatants on H-Top lawns of the potential indicator strains.

Spontaneous release of phages in overnight cultures was determined by inoculating a single colony into LB and growing the culture for 16 h before centrifuging (10 min, 10,000 × *g*) and collecting the supernatant. The supernatant was vortexed for 15 s with 20 μl of chloroform, and the phage titer was determined. To compare the phage production rates at 30 and 37°C, single colonies were streaked for two successive transfers at 37°C on prewarmed LA plates. Cultures in prewarmed LB at 30 and 37°C were then initiated from the same colony, and the phage titer was determined after overnight (16-h) incubation as described above.

Production of lysogens. A sterile needle was touched to the turbid center of a single plaque, and bacterial cells were streaked to give single colonies on LA plates. Single colony transfers were repeated three times to eliminate any free phage. Putative lysogens thus obtained were tested for spontaneous phage release, UV induction, and superimmunity to their respective prophages but sensitivity to the other phages.

UV induction of lysogens. Exponential-phase cultures (optical density at 600 nm, 0.8) were centrifuged, and the supernatants were discarded. The cells were washed in 2 volumes of 10 mM $MgSO_4$ and resuspended in an additional 2 volumes of 10 mM $MgSO_4$. Five-milliliter aliquots were placed in sterile glass petri dishes (70-mm diameter) and exposed to 10 s of UV irradiation (germicidal UV lamp; Gelman Sciences biohazard hood model BH143, 254 nm), with constant swirling, 530 mm below the UV light source. Irradiated cells were added immediately to sterile, foil-covered flasks containing 550 μl of 10× LB and incubated for 2 h with vigorous shaking before collection of the supernatant and determination of the phage titer as described above.

Phage adsorption. Exponential-phase cultures (optical density at 600 nm, 0.7) were plated on LA to estimate the CFU per milliliter. One milliliter of a bacterial suspension (approximately 4×10^8 CFU) was added to an Eppendorf tube containing 4×10^5 PFU of phage ml^{-1} to give a multiplicity of infection of 0.001. The tubes were mixed by inversion and incubated for 15 min at 30°C before centrifuging (10 min, 10,000 × *g*) to remove the bacteria and any adsorbed

TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Genotype or description	Source or reference
Serratia entomophila		
A1MO2	Derivative of A1 wild-type Ap^r Path$^+$ ϕ9A+ϕ9B lysogen	LC[a]
BC4B	EMS induced phage-resistant derivative of A1MO2 Ap^r Path$^+$ ϕ9B lysogen	14
UC9	Clonal selection of A1MO2 Ap^r Path$^+$ ϕ9A+ϕ9B lysogen	LC
UC7	Clonal selection of A1MO2 Ap^r Km^r Path$^-$ ϕ9A+ϕ9B lysogen	LC
UC6	Clonal selection of a wild-type strain Ap^r Path$^+$ ϕ9A+ϕ9B lysogen	LC
UC53	Tn*phoA*::BC4B Path$^-$ mutant Ap^r Km^r ϕ9B lysogen	LC
UC58	Tn*phoA*::BC4B Path$^-$ mutant Ap^r Km^r ϕ9B lysogen	LC
562	Wild-type Ap^r Path$^+$	LC
A5	562 ϕ9A lysogen	This study
B2	562 ϕ9B lysogen	This study
222	Isolated from water in France	7
Serratia entomophila bacteriophages		
ϕCW1	Isolated from diseased larvae; turbid plaques	23
ϕCW3	Isolated from diseased larvae; large dull plaques	23
ϕCW4	Isolated from diseased larvae; clear plaques	23
ϕCW5	Isolated from diseased larvae; turbid plaques	23
ϕBT	Isolated from failed fermentation run; turbid plaques	15
ϕAgRP8	Laboratory isolate	14
ϕ9A	Isolated from A1MO2	This study
ϕ9B	Isolated from BC4B	This study
Plasmids		
pACYC177	Km^r Ap^r	5
pBR322	Ap^r Tet^r	1

[a] LC, Laboratory Collection, Department of Plant and Microbial Sciences, University of Canterbury.

phage. One hundred microliters of a 10^{-2} dilution of the supernatant (containing any unadsorbed phage) was added to 100 μl of indicator bacteria, mixed with 3 ml of molten H-Top agar (48°C), and spread on an LA plate. After overnight incubation, the number of plaques was counted to give an estimate of the residual titer. Control titers were estimated by incubating the phage with 1 ml of LB instead of the test bacteria and treating as described above, in duplicates. The percentage of phage adsorption was calculated by the following equation: percent adsorption = [(control titer − residual titer)/(control titer)] × 100.

Plasmid transduction. The generalized transducing phages ϕ9A and ϕ9B of *S. entomophila* (8) were used to transduce the plasmids pACYC177 and pBR322. Fresh overnight cultures of the *S. entomophila* recipient strains were pelleted and resuspended in equal volumes of 10 mM $MgSO_4$, centrifuged, and resuspended in equal volumes of LB. After 15 min of incubation with shaking, 1-ml aliquots of bacteria (approximately 3×10^9 CFU ml^{-1}) were mixed with 100 μl of phage lysate prepared from the donor strain (approximately 3×10^{10} PFU ml^{-1}) to give a multiplicity of infection of approximately 10:1 (phage/bacteria). Incubation at room temperature for 5 min was followed by incubation for 1 h at 30°C with shaking before plating of cells on LA plates containing the appropriate antibiotics.

DNA techniques. Phage DNA was prepared as described by Silhavy et al. (19), and bacterial genomic DNA was prepared by the method of Scott et al. (16). Restriction endonucleases were purchased from GIBCO BRL and used as specified by the manufacturer. Restriction fragments were analyzed in 0.8% horizontal agarose gels run in 1× Tris-acetate buffer, stained with ethidium bromide, and photographed under UV light.

Southern hybridizations. Restriction enzyme-digested DNA that had been separated on an agarose gel was transferred to a nylon membrane (Hybond; Amersham) with the Vacugene system (Pharmacia). Southern hybridizations were carried out by the nonradioactive enhanced chemiluminescence technique (22) as specified by the manufacturer (Amersham). Blots were washed under low-stringency conditions with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Electron microscopy. Phages were purified and concentrated by ultracentrifugation through a glycerol step gradient (19). Phage particles (>10^{12} PFU ml^{-1}) were negatively stained by mixing with an equal volume of 2% phosphotungstic acid. Five-microliter aliquots were mounted directly onto Formvar-coated copper grids. Excess fluid was drawn off with absorbent filter paper, and grids were allowed to air dry. Grids were examined with a JEOL JEM-1200EX electron microscope at an accelerating voltage of 80 kV and a magnification of ×50,000. Electron micrographs were taken at a magnification of ×150,000.

RESULTS

Identification and characterization of prophages in A1MO2 and BC4B. Screening a collection of isolates of wild-type *S. entomophila* resulted in the identification of strain 562, which was sensitive to phages present in both UV-induced and noninduced lysates from A1MO2 and BC4B. The phage detected in A1MO2 supernatants was named ϕ9A and produced a plaque with a turbid center surrounded by a clear area. The phage from BC4B was called ϕ9B and gave a much duller plaque with easily distinguishable concentric rings. A1MO2 and BC4B were resistant to both phages. A closer inspection of plaques from A1MO2 lysates revealed a small number of ϕ9B type plaques, suggesting that A1MO2 was lysogenic for ϕ9A and ϕ9B, while BC4B appeared to contain only a functional ϕ9B prophage.

Separate lysogens of ϕ9A and ϕ9B were generated from strain 562 to produce strains A5 and B2, respectively. The strains spontaneously produced phage (Table 2) and were resistant to only their respective lysogenic phages. By using A5 and B2 as indicator strains, it was confirmed that A1MO2 also produced ϕ9B spontaneously (Table 2). As shown in Table 2, BC4B released no ϕ9A phage, while A1MO2 released 5.5 times as many ϕ9A as ϕ9B phages in overnight cultures. Examination of the A1MO2 derivatives UC9 and UC7 as well as UC6, a clonal selection of a wild-type *S. entomophila*, showed that they all produced phages that grew on A5 and B2 with the

TABLE 2. Numbers of ϕ9A and ϕ9B phages spontaneously released from overnight cultures of strains A1MO2, BC4B, A5, and B2[a]

Strain	PFU of phage/ml on indicator strain[b]:		
	562	A5 (for ϕ9B)	B2 (for ϕ9A)
A1MO2	ND[c]	1.2×10^7	6.6×10^7
BC4B	3.1×10^9	3.1×10^9	0
A5	1.2×10^9	0	1.2×10^9
B2	4.0×10^9	4.0×10^9	0

[a] Plaques on strains A5 and B2 are those of phages ϕ9B and ϕ9A, respectively.
[b] Each value is the mean of 10 independent experiments.
[c] ND, not determined.

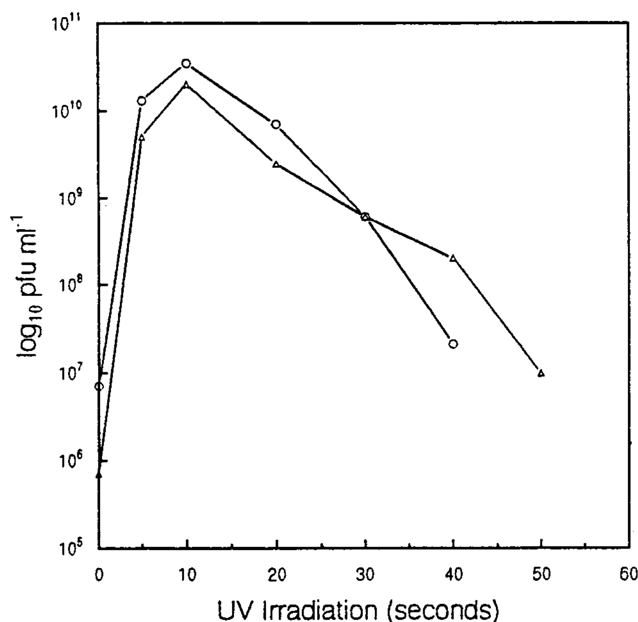

FIG. 1. Numbers of phages produced by *S. entomophila* A5 and B2 after induction by UV irradiation for various durations. Symbols: △, A5 (ϕ9A); ○, B2 (ϕ9B).

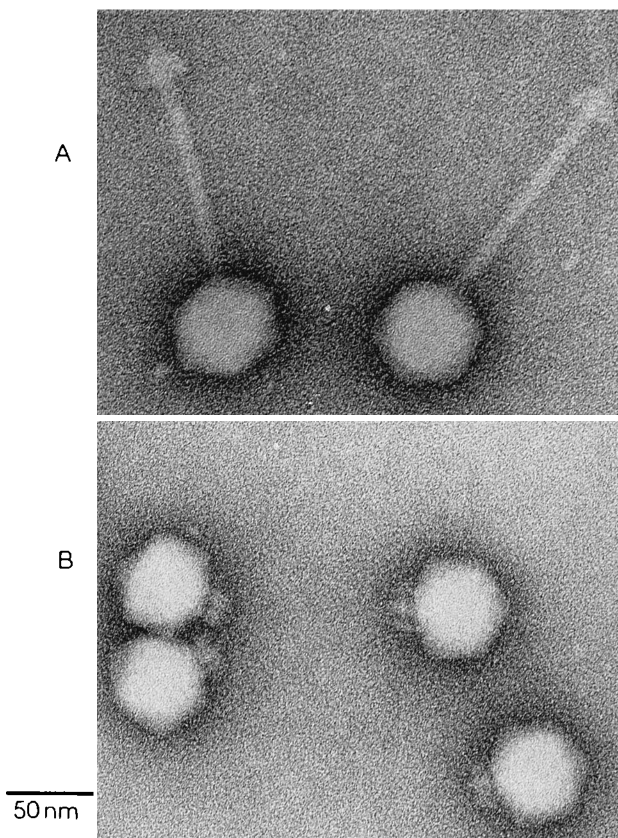

FIG. 2. Electron micrographs of *S. entomophila* phages. (A) ϕ9A; (B) ϕ9B. Bar, 50 nm.

same plaque morphologies as ϕ9B and ϕ9A, respectively. Phages from the strains described above failed to form plaques on the double lysogen A1MO2 (data not shown). The unpublished results simply confirmed that all three strains were lysogenic for both ϕ9A and ϕ9B like the parental strain A1MO2.

UV induction. Preliminary experiments showed that both phages ϕ9A and ϕ9B were UV inducible, with 10 s of exposure being the optimum (Fig. 1). Ten seconds of exposure was used for all further UV inductions. Strains A1MO2 and BC4B were also UV induced, and the numbers of ϕ9A or ϕ9B were determined (Table 3). UV treatment failed to induce any ϕ9A phages from BC4B.

Electron microscopy. Examination of purified ϕ9A and ϕ9B particles by electron microscopy showed morphologies typical of the double-stranded DNA bacteriophages (2). Figure 2A shows ϕ9A with a head measuring 60 by 60 nm and a long noncontractile tail (150 by 10 nm) with a base plate attached. ϕ9A was classified as a group B phage belonging to the *Styloviridae* family in the classifications of Bradley and Matthews, respectively (2, 12). ϕ9B (Fig. 2B) had a short noncontractile tail, 17 nm in length, with a head of 60 by 60 nm, and was classified as a group C phage (2), belonging to the *Podoviridae* family (12). The supernatant from a UV-induced culture of strain 562 was also concentrated by a glycerol step gradient. Examination under the electron microscope failed to detect any phages.

Phage DNA analysis. Restriction analysis of ϕ9A and ϕ9B DNA with *Bam*HI, *Hin*dIII, and *Eco*RI provided confirmation that ϕ9A and ϕ9B were different from all previously described *S. entomophila* phages. No bands in common with those of the previously identified phages were observed when DNA was cleaved with *Bam*HI (data not shown). Restriction with *Eco*RI showed two bands (2.2 and 6.3 kb) in common with those of ϕ9A and ϕ9B (Fig. 3A) but no bands in common with those of the other phages. *Hin*dIII produced two fragments of 0.6 and 0.7 kb present in ϕ9A, ϕ9B, ϕCW1, ϕCW4, and ϕBT. Additional bands of common size were present at 0.8, 1.1, and 3.9 kb only in ϕ9A and ϕ9B after restriction with *Hin*dIII. These results suggested ϕ9A and ϕ9B may be related to ϕCW1, ϕCW4, and ϕBT but have more homology with each other

TABLE 3. Numbers of phages released from *S. entomophila* A1MO2 and BC4B after UV induction

Strain	PFU of phage/ml on[a]:					
	Indicator strain B2 (for ϕ9A)			Indicator strain A5 (for ϕ9B)		
	After 0 s of UV induction	After 10 s of UV induction	Increase	After 0 s of UV induction	After 10 s of UV induction	Increase
BC4B	0	0		5×10^7	1.4×10^{11}	2.8×10^3
A1MO2	1.3×10^6	9.7×10^{10}	7.5×10^4	2×10^6	5.3×10^9	2.7×10^3

[a] Each value is the mean of three independent experiments.

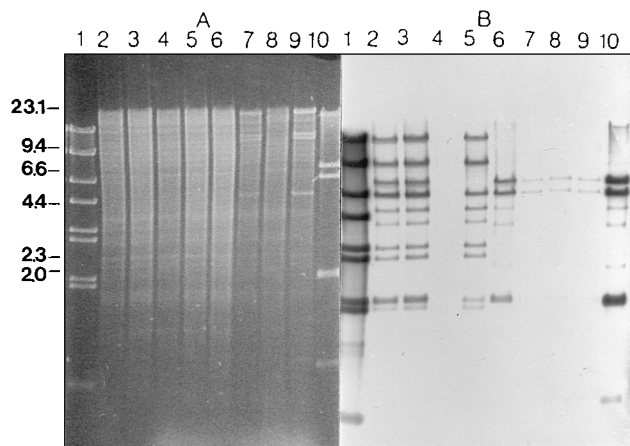

FIG. 3. (A) Gel electrophoresis of *Eco*RI digests of DNA from phages ϕ9A and ϕ9B and lysogenic and nonlysogenic hosts. (B) Autoradiogram prepared after hybridization of labeled ϕ9A DNA with DNA fragments. Lanes: 1, ϕ9A; 2 and 3, A1MO2; 4, 562; 5, A5; 6, B2; 7, UC53; 8, BC4B; 9, UC58; 10, ϕ9B. Numbers on the left indicate molecular weight markers (in thousands).

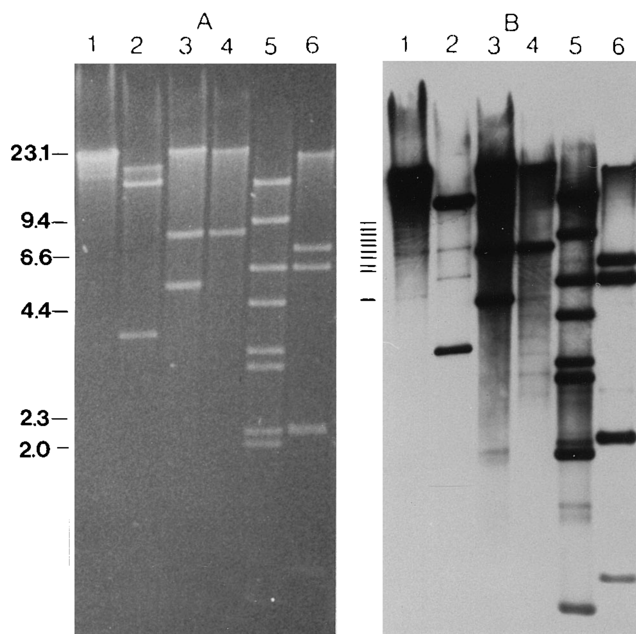

FIG. 4. (A) Gel electrophoresis of phages ϕ9A and ϕ9B DNA after digestion with various enzymes. (B) Autoradiogram detecting subfragments in ϕ9A and ϕ9B DNA after hybridization of labeled ϕ9A DNA with DNA fragments. Lanes: 1, 3, and 5, ϕ9A; 2, 4, and 6, ϕ9B. Lanes 1 and 2 were digested with *Bam*HI, lanes 3 and 4 were digested with *Kpn*I, and lanes 5 and 6 were digested with *Eco*RI. The bars to the left of panel B indicate the positions of the subfragments detected in *Bam*HI digests of ϕ9A. Numbers to the left of panel A indicate molecular weight markers (in thousands).

than with the phages described above. The ϕ9A and ϕ9B genome sizes were estimated to be 53 and 44 kb, respectively. On the basis of restriction patterns, phages ϕ9A and ϕ9B are also very distinct from ϕAgRP8, having no similarities after digestion with the restriction enzymes used (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, and *Sal*I) (data not shown).

BC4B no longer contains a ϕ9A prophage. *Eco*RI-cut genomic DNA from strains A1MO2, A5, B2, 562, BC4B, and the nonpathogenic (Path$^-$) mutants UC53 and UC58 that were derived from BC4B was probed with an *Eco*RI-cut ϕ9A phage probe (Fig. 3B). A comparison of strains A5 (Fig. 3B, lane 5), B2 (lane 6), UC53 (lane 7), UC58 (lane 9), and BC4B (lane 8) showed that BC4B and its derivatives contain no ϕ9A (lane 1) prophage DNA. The same three bands that the ϕ9A probe hybridized strongly to in the B2 (ϕ9B lysogen) bacterial and ϕ9B phage genomes were detected in UC53, UC58, and BC4B genomic DNA, indicating that the only sequences detected with homology to sequences of ϕ9A that existed in BC4B were due to the ϕ9B prophage. In comparison, A1MO2 genomic DNA (lanes 2 and 3) can be seen to contain all of the *Eco*RI fragments to which the ϕ9A probe hybridizes in the genomes of the separate ϕ9A and ϕ9B lysogens.

Phage-packaging mechanisms. Restriction of ϕ9A and ϕ9B DNA with *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, and *Sal*I followed by heating to 70°C for 0, 5, 10, or 15 min and then rapidly cooling the DNA on ice before electrophoresis failed to detect any evidence of *cos* sites for either phage (data not shown).

However, a pattern of subfragments characteristic of phages with *pac* sites (4) was detected in a *Bam*HI digest of ϕ9A DNA. Three faint bands were also detected in *Bam*HI digests of ϕ9B DNA. Digestion with 15 other restriction enzymes failed to detect any further evidence of *pac* sites. Confirmation of the presence of *pac* sites was achieved by probing a Southern blot of *Bam*HI-, *Kpn*I-, and *Eco*RI-digested ϕ9A and ϕ9B DNA with a ϕ9A *Eco*RI-cut probe (Fig. 4B). *Kpn*I and *Eco*RI restriction of ϕ9A and ϕ9B DNA produced a confusing pattern of faint fragments (Fig. 4B). This is to be expected for enzymes with sites close to *pac*. In Fig. 4B, at least 10 separate faint bands were detected in the *Bam*HI digests of ϕ9A DNA, suggesting at least five successive rounds of packaging from a single concatemer. The terminal redundancy for ϕ9A was estimated to be 1.6 kb. At least eight faint bands, not previously observed, were detected in the *Kpn*I digests of ϕ9B (Fig. 4B), which therefore has a minimum of four successive rounds of packaging per linear concatemer. The two faint bands at 9.1 and 6.9 kb detected in the *Bam*HI digests of ϕ9B may represent successive packaging events from the same *Bam*HI fragment, suggesting a terminal redundancy of 2.2 kb.

Homology between phage DNAs and host genomic DNA. An *Eco*RI-cut ϕ9A DNA probe hybridized strongly to many of the *Eco*RI fragments from all of the other phages except ϕCW3 (Fig. 5B, lane 3). ϕ9A had the highest degree of homology with ϕ9B, then with ϕCW1, followed by ϕBT and ϕCW4.

*Eco*RI-cut ϕ9B DNA was used to probe the same blot as that described above. ϕ9B hybridized most strongly to ϕ9A; again, no hybridization was observed with ϕCW3 DNA (Fig. 5C). Compared with the ϕ9A probe, ϕ9B had only slightly reduced homology to ϕCW4 and ϕBT but had significantly less homology with ϕCW1. Probing the same blot with ϕCW1 DNA showed that it had extensive homology with ϕBT, ϕCW4, and ϕ9A DNA and also with ϕ9B to a lesser extent (data not shown). The ϕCW1 probe also failed to hybridize to ϕCW3 or strain 562 DNA.

The ϕ9A, ϕ9B, and ϕCW1 probes all detected faint bands in the ϕCW1, ϕCW4, and ϕBT lanes that had not been observed in agarose gels. This suggests that these three phages also use a *pac* mechanism for DNA packaging.

A comparison was made of *Eco*RI-digested ϕ9A DNA (Fig. 5A) and *Eco*RI-digested genomic DNA from the lysogenic strain A5 hybridized with labeled ϕ9A DNA (Fig. 5B). Results indicated that the ϕ9A *att* site is contained in the 2.1-kb *Eco*RI fragment of the phage genome, since this band could not be detected in the lysogen. This observation also explains the appearance of the 5.2-kb *Eco*RI fragment in A5, which is due

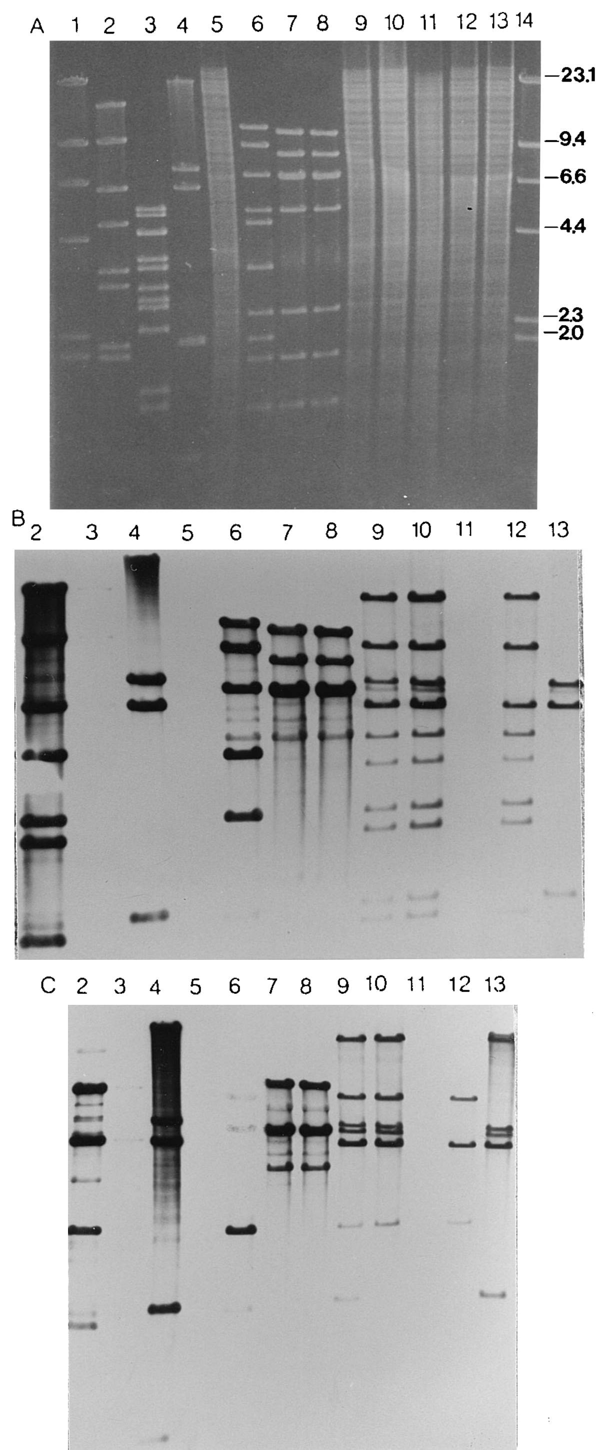

FIG. 5. (A) Gel electrophoresis of *S. entomophila* phages, lysogens, and host DNA after digestion with *Eco*RI. (B) Autoradiogram prepared after hybridization of labeled ϕ9A DNA with DNA fragments in panel A. (C) Autoradiogram prepared after hybridization of labeled ϕ9B DNA with DNA fragments in panel A. Lanes: 1 and 14, λ *Hin*dIII standard; 2, ϕ9A; 3, ϕCW3; 4, ϕ9B; 5, 222; 6, ϕCW1; 7, ϕBT; 8, ϕCW4; 9 and 10, A1MO2; 11, 562; 12, A5; 13, B2. Numbers to the right of panel A indicate molecular weight markers (in thousands).

TABLE 4. Ability of phages to adsorb to *S. entomophila* BC4B, A1MO2, and 562

Phage[a]	% Phage adsorption to[b]:			Indicator strain
	BC4B	A1MO2	562	
CW1 (3)	0	98.3	ND[c]	UC9
CW3 (6)	0	93.4	ND	UC9
CW4 (2)	0	80.0	ND	UC9
CW5 (5)	0	94.4	ND	UC9
9A (6)	13.9	ND	98.2	B2
BT (2)	0	96.9	ND	UC9

[a] The number of independent experiments is shown in parentheses.
[b] Percent phage adsorbtion calculated by [(control titer − residual titer)/(control titer)] × 100.
[c] ND, not determined.

to the inclusion of bacterial DNA adjacent to the bacterial *att* site in one of the *Eco*RI fragments. Alternatively, the 5.2-kb *Eco*RI fragment may not be visible in agarose gels of phage DNA because it is close to the *pac* site. Detection of the same banding patterns of phage DNA in A1MO2 and BC4B as in A5 and B2 also suggests that ϕ9A and ϕ9B insert into a specific site in the bacterial chromosome. The detection of only the single, large, low-copy-number plasmid in A1MO2 and BC4B (6) confirms that lysogeny for these phages does not involve a plasmid state as it does for some temperate phages such as P1 of *E. coli* (24).

Nature of BC4B phage resistance. The ability of BC4B to adsorb various phages was compared with that of A1MO2 and 562 (Table 4). Only phage 9A showed any detectable adsorption to BC4B, but this occurred at a much reduced rate which did not result in the formation of any plaques. Exploitation of the generalized transducing abilities of ϕ9A and ϕ9B demonstrated that both of these phages failed to inject DNA into BC4B. The plasmids pBR322 and pACYC177 were readily transduced between 562 and A1MO2 donors and recipients, but repeated attempts with BC4B as the recipient strain failed to produce any transductants despite the ability of both plasmids to replicate in BC4B (8).

Temperature sensitivity of *S. entomophila* phages. *S. entomophila* and its phages are normally grown at 30°C, although the bacterium grows well at 37°C. When H-Top lawns of host strains and phages were incubated at 37°C, no plaques were observed for any of the *S. entomophila* phages, including ϕAgRP8 (data not shown).

The effect of overnight growth at 37°C on both the spontaneous production of phages and the number of viable cells in A1MO2 and BC4B cultures was determined (Table 5). Growth at 37°C resulted in an approximately 10-fold decrease in CFU of A1MO2 and BC4B per milliliter when compared with growth at 30°C (Table 5). However, there was a dramatic reduction in viable phages detected in the supernatants. For A1MO2, the proportion of phages produced per viable cell at 37°C was just 0.02% of that at 30°C (Table 5). For BC4B, only 3.7% of the PFU per CFU were produced at 37°C when compared with growth at 30°C.

DISCUSSION

Prior to this work, it had not been appreciated that all of the common laboratory strains of *S. entomophila* used for molecular analysis contained prophages. In certain genera, most species are polylysogenic for several distinct phages, and it has been suggested that lysogeny is the norm for bacteria in nature rather than the exception (9). Strains A1MO2 and BC4B have

TABLE 5. Growth and spontaneous release of phages from *S. entomophila* BC4B and A1MO2 after 16 h of growth at 37 or 30°C[a]

Strain	30°C			37°C			% PFU at 30°C/CFU[b] at 37°C[c]
	Cell yield (CFU/ml)	Phage production (PFU/ml)	PFU/CFU	Cell yield (CFU/ml)	Phage production (PFU/ml)	PFU/CFU	
A1MO2	4.93×10^9	4.77×10^8[d]	9.7×10^{-2}	3.25×10^8	5.23×10^3	1.6×10^{-5}	0.02
BC4B	5.71×10^9	1.27×10^9	2.2×10^{-1}	3.66×10^8	3.00×10^6	8.2×10^{-3}	3.70

[a] Each value is the average of three independent experiments.
[b] Number of plaques formed at 37°C as percentage of the number formed at 30°C.
[c] Obtained by determining the following: [(PFU/CFU at 37°C)/(PFU/CFU at 30°C)] × 100.
[d] The indicator strain was 562.

been shown to be truly lysogenic for their respective phages by (i) the failure of repeated single-colony purifications to dilute out the phage, (ii) the UV-inducible nature of the association, and (iii) in the case of A1MO2, superimmunity to ϕ9A and ϕ9B. The separate lysogens isolated for these phages, A5 and B2, had the expected properties for lysogens of the individual phages. The ability of all other phages to infect A5 and B2 indicated that the repressors of ϕ9A and ϕ9B did not recognize the operator sites of any other phage. Although the spontaneous release of phages from the lysogens A5 and B2 did not differ widely relative to one another, wide variations can occur between different lysogenic strains of the same species, being a function of the type of prophage rather than the host (11). In polylysogenic strains, each phage is produced at its own characteristic rate, independent of the other phage, although this is often not the case for double lysogens of related phages as demonstrated for A1MO2 (Table 2).

The large numbers of ϕ9A and ϕ9B produced spontaneously by A1MO2 could have an influence on the phage typing scheme developed by O'Callaghan et al. (14) to monitor the persistence of applied *S. entomophila* strains in the field. They used strain A1MO2 to propagate all phages except ϕAgRP8, which would result in lysates containing significant numbers of ϕ9A and ϕ9B. The lack of any UV-inducible prophage or an uninducible prophage with homology to ϕ9A, ϕ9B, or ϕCW1 in strain 562 would make this a more suitable strain for phage propagation. The isolation of the new phage types could also have applications in refining the phage typing scheme described above.

Restriction analysis of phage DNA confirmed ϕ9A and ϕ9B to be two distinct phage types that had not been identified previously. Also demonstrated was the possession of *pac* sites by ϕ9A and ϕ9B and all the previously described phages, with the exception of ϕCW3. The generalized transducing capabilities of ϕ9A, ϕ9B, ϕCW1, and ϕBT (8) confirm that these phages use a *pac*-like mechanism to package their genomes, although previously, Wilson et al. (23) had postulated that ϕCW1 contained a *cos* site. Electron microscopy of ϕ9A and ϕ9B particles showed their heads to be indistinguishable from one another and of a similar size to all previously described *S. entomophila* phages except the smaller ϕAgRP8. The long noncontractile tail of ϕ9A with an obvious base plate was also similar to the tails of the other phages with the exception of ϕAgRP8, for which a tail has not been observed (14). ϕ9B had a short noncontractile tail of a type not seen before in an *S. entomophila* phage.

Despite the different tail morphologies of ϕ9A and ϕ9B, the Southern hybridization data showed them to be more closely related to each other than to any other phage. It has been postulated that the *Escherichia coli* phage lambda has evolved not as a single organism but as a family of continually interbreeding phages (3). The extensive regions of homology among all of the *S. entomophila* phages analyzed in this study, with the exception of ϕCW3, suggests that they belong to a similar family of phages.

The demonstration that BC4B and derivative strains no longer contain a ϕ9A prophage does not appear to be related to the phage-resistant phenotype of these strains. However, the remaining prophage, ϕ9B, could be a problem, as there are documented instances of virulent phages arising from lysogenic starter strains used in industrial fermentations (17).

The inability of phages to adsorb was identified as the primary phage resistance mechanism of strain BC4B. The only phage that demonstrated any ability to adsorb to BC4B, ϕ9A, did so at a much reduced rate and did not result in any observable plaque-forming ability. Failure of ϕ9A and ϕ9B to inject their DNA into BC4B was demonstrated by their inability to transduce plasmids into this strain, which serves as an indication that even the resident ϕ9B prophage is incapable of adsorption to BC4B. The ability of the ϕ9B prophage to be induced and successfully enter the lytic cycle established that once the cell wall was bypassed, phage development occurred normally in BC4B. The possession by BC4B of single or multiple mutations that affect the receptor(s) of all known *S. entomophila* phages, resulting in total resistance, makes this strain an interesting candidate for further study.

The temperature-sensitive nature of all isolated *S. entomophila* phages could have a future application in the control of phage infections. The failure of some phages to multiply at a temperature at which their host bacteria grow actively has been observed in phages for several species, including *E. coli*, *Bacillus subtilus*, and *Streptococcus cremoris* (18, 21). Sozzi et al. (21) found that only a small proportion of phages tested for the lactic acid bacteria were affected by temperatures less than the maximum of their hosts and concluded that temperature control had little potential application in the reduction of phage infections in the dairy industry. This contrasts with *S. entomophila*, in which an inhibition of growth of all phages occurred at 37°C, accompanied by only a 10-fold reduction in bacterial numbers after overnight growth.

Further investigation of *S. entomophila* phages and their temperature sensitivity as well as the phage-resistant phenotype of strain BC4B are warranted in view of the long-term challenges that phages may pose in the industrial fermentation of this bacterium.

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