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Temperate Bacteriophage Infectious for Asporogenic Variants of *Bacillus pumilus*

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Bacillus pumilus strain NRRL B-3275 is lysogenic for an inducible, nondefective temperate bacteriophage ϕ75. ϕ75 infects and lysogenizes several asporogenic mutants of *B. pumilus* strain NRS 576 but does not productively infect the spore$^+$ parent. ϕ75 DNA is a linear duplex with a mol wt of about 29×10^6 and a buoyant density of 1.701 g/cm^3. The location of the ϕ75 prophage attachment site on the chromosome of both host strains is adjacent to a lysine marker. The apparent order is *ϕ75 att lys trp*.

None of the commonly used strains of *Bacillus subtilis*, *B. licheniformis*, or *B. pumilus* is known to be naturally lysogenic for a nondefective temperate phage. Strains of these bacteria do, however, harbor inducible phages (4, 8, 16, 19). The inducible phage carried by *B. subtilis* 168, PBSX, is a defective virus (16). The defective nature of the other inducible phages has not been firmly established, although all share with PBSX the property of being unable to productively infect any bacterium tested.

Ito and co-workers (5, 6) demonstrated the existence of two virulent phages that productively infect asporogenic mutants of *B. subtilis* but not the spore$^+$ parent. The basis for the sensitivity of the asporogenic mutants is not known. Because of the possible general occurrence of this phenomenon in *Bacillus*, we tested strains known to carry inducible (noninfectious?) phages for plaque-forming activity on sporulation-deficient mutants. Such plaque-forming activity would have been heretofore undetected because of the common practice of using wild-type bacteria as plating host. In the present communication we report the isolation of a temperate phage, ϕ75, by this method. *B. pumilus* strain NRRL B-3275 is naturally lysogenic for ϕ75. The phage productively infects and lysogenizes certain asporogenic mutants of *B. pumilus* strain NRS 576, but does not productively infect the spore$^+$ parent.

MATERIALS AND METHODS

Bacteria. Two strains of *B. pumilus* were used. Strain NRRL B-3275 (13, 14) (Table 1) is naturally lysogenic for ϕ75; strain NRS 576 (Table 2) has been described (9, 11). The two strains are closely related: hybridization studies show extensive homology between their respective DNA types (11), and many auxotrophic markers can be transferred between the strains by PBP1 and PBS1 transduction (10; unpublished data). Strain NRRL B-3275 contains no detectable covalently closed, circular duplex DNA. Strain NRS 576 carries the 576 plasmid (9).

Growth media and conditions. Penassay broth and tryptose blood agar base (TBAB) were from Difco. The minimal medium was that described by Spizizin (20) supplemented with D-biotin (0.05 μg/ml). Liquid AK medium was prepared as described in the *BBL Manual of Products* (17), omitting the agar. Incubation was at 37 C; liquid cultures were grown with rotary shaking 250 rpm.

PBS1 transduction. PBS1 transduction was performed as previously described (14). Both NRS 576 and NRRL B-3275 are sensitive to PBS1. It was previously reported that NRS 576 does not support plaque formation by PBS1 (7); however, the phage produces very turbid plaques on NRS 576 at an efficiency nearly identical to that obtained on NRRL B-3275.

Isolation of ϕ75-sensitive variants of B. pumilus NRS 576. ϕ75 does not form plaques on strain NRS 576 (efficiency of plating $< 10^{-10}$). Phage-sensitive variants of NRS 576 have been obtained by two procedures. NRS 576 harbors about two copies per chromosome of a circular DNA element, the 576 plasmid (9, 11). Plasmid-negative variants, the W mutants, spontaneously appear in NRS 576 cultures at low frequency. W mutants are distinguished from the plasmid-carrying parent by their elevated sporulation frequency (9). ϕ75 does not form plaques on W mutants. Cultures of each of 10 W mutants examined contained a second spontaneous mutant type referred to as the G mutants. G mutants occur infrequently (frequency of 10^{-3} and lower). G mutants are all asporogenic (sporulation frequency below 10^{-9}), and spore$^+$ revertants of the G mutants have not been detected. All of 22 G mutants tested are sensitive to plaque formation by ϕ75. Selection of G mutants is based on the greenish hue of the mutant colonies on TBAB. Although we refer to these mutants collectively as G mutants, there is no evidence that they

represent a single mutant class other than their phenotypic similarity. G mutants have not been detected in cultures of NRS 576.

The second approach that has yielded ϕ75-sensitive variants involves overnight growth of strain NRS 576 (plasmid$^+$) in Penassay broth containing 0.1 μg of mitomycin C per ml (a growth inhibitory concentration). This procedure results in cultures of low cell number (approximately 10^7 to 10^8 cells per ml). More than 50% of the cells give rise to colonies that are about one-fourth the diameter of the parent colonies on rich medium (TBAB or nutrient agar). About 0.1% of the small-colony variants are sensitive to infection by ϕ75. One of these small-colony variants was used in the present study and is designated as C3. C3 is asporogenic (sporulation frequency of approximately 10^{-8}). Spore$^+$ revertants of C3 have been obtained. C3 carries the 576 plasmid, which cosediments with the 576 plasmid obtained from wild-type cells in neutral sucrose gradients (unpublished data). C3 and NRS 576 are equally sensitive to mitomycin C in disk tests.

TABLE 1. *Derivatives of B. pumilus strain NRRL B-3275*[a]

Strain[b]	Relevant properties	Origin or reference
NRRL B-3275	Wild type	14
BpB2	*lys-1*	14
BpB10	*trp-2*	14
BpB12	*gly-2*	15
BpB38	*serC4*	12
BpB10d11	*trp-2 def-11*[c]	NG[d] of BpB10
BpB12d7	*gly-2 def-7*	NG of BpB12
BpB38d2	*serC4 def-2*	NG of BpB38

[a] ϕ75 did not form plaques on any of the strains tested (efficiency of plating $<10^{-10}$).

[b] NRRL B-3275 and all mutant derivatives were lysogenic for ϕ75.

[c] "*def*" indicates defective lysogen.

[d] NG, *N'*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Bacteriophage techniques and purification. The plaque assay for ϕ75 was as reported for PBS1 (14) except that semisolid overlays were incubated 18 to 20 h at 37 C. Unless specifically noted, C3 was the indicator host. ϕ75 was routinely propagated by harvesting the semisolid overlays from confluently lysed plates. Isotopically labeled phage was prepared by including 100 μCi of carrier-free ^{32}P (as H_3PO_4; New England Nuclear Corp.) per ml of the semisolid agar overlays. ϕ75 was purified by differential centrifugation followed by equilibrium centrifugation in CsCl, both as reported for PBP1 purification (7). The visible phage band was removed with a syringe by puncturing the side of the gradient tube. The phage was mixed with an equal volume of TMA buffer (7) and dialyzed against TM buffer (7). Unless specifically noted, only purified ϕ75 was used in the present study.

ϕ75 DNA studies. Purified ϕ75 particles (labeled with ^{32}P) were suspended in TES buffer (0.02 M Tris-hydrochloride, 0.1 M NaCl, 0.005 M EDTA, pH 7.5) at about 10^{11} PFU/ml and gently shaken with an equal volume of TES-saturated phenol (Mallinckrodt Chemical Works) for 10 min at room temperature. After low-speed centrifugation, the aqueous layer was re-extracted once and then dialyzed exhaustively against TES at 4 C. The DNA was mixed with CsCl (average ρ = 1.70), and 2-ml portions were centrifuged for 36 h at 40,000 rpm in an SW50 rotor at 5 C. Each

TABLE 2. *Derivatives of B. pumilus strain NRS 576*

Strain	Relevant properties[a]	Origin or reference	ϕ75 sensitivity[b]
NRS 576	Wild type pl$^+$	9	0
CAT-1	*ade-100* pl$^+$	11	0
W1[c]	pl$^-$	11	0
W2	pl$^-$	11	0
W7	*ade-100* pl$^-$	11	0
W20	*ade-100* pl$^-$	11	0
GM1[c]	pl$^-$ Spo$^-$	Spontaneous mutant of W1	+
GM2	*trp-100* Spo$^-$ pl$^-$	NG of GM1	+
GM40	*ade-100* Spo$^-$ pl$^-$	Spontaneous mutant of W20	+
GM41	*ade-100 ilv-100* Spo$^-$ pl$^-$	NG[d] of GM40	+
GM45	*ade-100 ilv-100 lys-100* Spo$^-$ pl$^-$	NG of GM41	+
GM45d6	*ade-100 ilv-100 lys-100 def-6* Spo$^-$ pl$^-$	NG of GM45 (ϕ75)	0
GM47d6	*ade-100 trp-100 lys-100 def-6* Spo$^-$ pl$^-$	td[e]	0
C3	*ade-100* Spo$^-$ pl$^+$	Mitomycin C of CAT-1	+

[a] "pl$^+$" or "pl$^-$" indicate the presence or absence, respectively, of the 576 plasmid; Spo$^-$ indicates strain is asporogenic (sporulation frequency below 10^{-8}); *def* indicates strain is a defective lysogen.

[b] 0, ϕ75 did not form plaques on strain (efficiency of plating $<10^{-10}$); +, ϕ75 formed plaques on strain. The efficiency of plating of ϕ75 on all sensitive strains was the same ($\pm$10%).

[c] "W" in strain designation indicates a white mutant; "G" indicates a green mutant.

[d] NG, *N*-methyl-N'-nitro-*N*-nitrosoguanidine.

[e] Strain constructed by PBS1 transduction. Donor was GM2 and recipient was GM45d6. Selection was for Ilv$^+$.

gradient was collected in approximately 35 fractions through a hole pierced in the tube bottoms. Ten-microliter portions of each fraction were dried onto Whatman no. 1 filter paper disks (24 mm) and counted in toluene-Omnifluor (New England Nuclear Corp.). Only a single peak of ^{32}P-labeled material was detected in each gradient. The fractions comprising the peak were pooled and dialyzed exhaustively against TES buffer. The experiments reported in the present investigation were performed by using CsCl gradient-purified ϕ75 DNA. However, the sedimentation properties of ϕ75 DNA in neutral and alkaline sucrose gradients were the same before and after CsCl gradient purification of the phage DNA.

Sucrose gradient centrifugation was as previously reported (9), using [^{3}H]thymidine-labeled T7 DNA as reference. Buoyant density of ϕ75 DNA in CsCl was determined in a model E analytical ultracentrifuge (13), using *Escherichia coli* DNA (ρ = 1.710) as reference. Calculations were according to the equations of Schildkraut et al. (18).

ϕ75 antiserum. Purified ϕ75 particles (10^{10} PFU) were mixed with an equal volume (0.3 ml) of Freund complete adjuvant (Difco) and injected into a rabbit every 2nd or 3rd week for 6 months. Antiserum used in the present study had a K value of approximately 200.

RESULTS

Isolation and properties of temperate phage ϕ75. *B. pumilus* strains NRS 576 and NRRL B-3275 are closely related as evidenced by the large number of genetic markers that can be transferred between the strains by PBP1 and PBS1 transduction (10; unpublished data) and by the extensive homology exhibited by their DNA in hybridization studies (11). Treatment of strain NRRL B-3275 with mitomycin C resulted in the induction of a phage designated as ϕ75 (Fig. 1). The plaque-forming activity in induced lysates banded as a single peak in CsCl gradients (Fig. 2). The morphology of gradient-purified ϕ75 particles (Fig. 3) was distinct from that of two other phagelike particles known to be carried by strain NRRL B-3275 (8; unpublished data). ϕ75 formed turbid-centered plaques on all G mutants tested (22 were examined) and on C3. ϕ75 did not form plaques (efficiency of plating $< 10^{-10}$) on any of five spore$^+$ revertants of C3 or NRS 576 nor on any of 32 plasmid-negative derivatives of NRS 576 (the W mutants) (Table 2).

ϕ75 (10^6 PFU/ml) adsorbed equally efficiently (>90% adsorption in 10 min at 37 C in Penassay broth) to cells of NRS 576, W1, C3, and GM45 (each at 5 [$\pm$2] $\times$ 10^8/ml). Thus, the resistance of NRS 576 (and the W mutants) to ϕ75 plaque formation is not due to an inability of the virus to adsorb to the cells.

ϕ75 was carried by NRRL B-3275 and each of 10 mutant derivatives of this strain that were

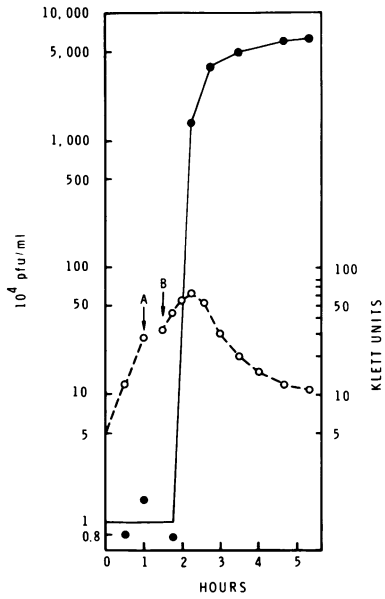

FIG. 1. *Induction of ϕ75 by treatment of strain NRRL B-3275 with mitomycin C. An auxotrophic derivative of NRRL B-3275 (BpB10) was grown to log phase in 20 ml of Penassay broth. Mitomycin C was added (at arrow A) to 2 μg/ml. Incubation was continued for 10 min. Cells were then washed twice at room temperature, resuspended to the original volume in broth, and returned to the 37-C shaker at arrow B. During the incubation, samples were periodically withdrawn, shaken with chloroform, and assayed for PFU on C3 cells. Turbidity was monitored in a Klett-Summerson colorimeter (filter 66). One Klett unit is equivalent to 1 ($\pm$0.5) $\times$ 10^7 cells/ml.*

tested. G mutants lysogenic for ϕ75 were isolated by repeated single-colony isolation of cells from the turbid centers of plaques. G-mutant lysogens liberated the phage spontaneously, and the phage could be induced by mitomycin C treatment essentially as shown in Fig. 1. Attempts to cure three G-mutant lysogens by repeated overnight growth in Penassay broth containing ϕ75 antiserum (K = 3) were unsuccessful.

Apparent lysogens of C3 were constructed. These were resistant to superinfection by ϕ75 and they spontaneously liberated the phage. However, C3 lysogens were observed to occasionally segregate nonlysogenic clones. Because of this apparent instability, C3 was used only as a plaquing indicator for ϕ75. Plaques produced by ϕ75 on C3 were generally less turbid than plaques produced on G mutants. The efficiency of plating of ϕ75 was the same ($\pm$10%) on C3 and the G mutants.

Infection of an AK broth culture of C3 with

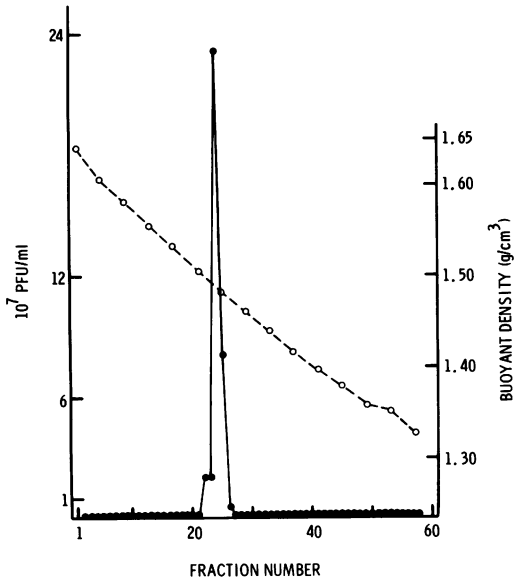

FIG. 2. *Equilibrium centrifugation of ϕ75 in cesium chloride. A mitomycin C-induced lysate (20 ml; 10^9 PFU/ml) was concentrated by centrifugation (2 h at 40,000 × g). The pellet was overlaid with 2 ml of TMA buffer and held at 4 C for 12 h. The resuspended pellet was centrifuged (15 min at 10,000 × g), and the supernatant fraction was mixed with CsCl to achieve a final buoyant density of 1.5 g/cm^3. A 5-ml portion was centrifuged 36 h at 40,000 rpm in an SW50.1 rotor at 4 C. Fractions were collected through the bottom of the tube. Each fraction was assayed for PFU on C3 cells, and the buoyant density of selected fractions was determined by refractometry (22).*

wild-type ϕ75 (or with a clear-plaquing spontaneous variant of the phage) resulted in a one-step growth curve, of which that shown in Fig. 4 is typical. By 35 to 40 min after infection, an approximate doubling of the initial number of infected cells occurred. The initial phage bursts were detected 10 to 15 min later. The burst size for the phage was about 70. Since the doubling time for C3 cells in AK was about 40 min, it seems that infected cells continue growth throughout the early stages of the infection. Liquid cultures of several G mutants (grown in Penassay or AK broth) were unable to support phage replication, although G mutants lysogenic for ϕ75 can be induced with mitomycin C when grown in either of these media.

Properties of ϕ75 DNA. The buoyant density of ϕ75 DNA was 1.701 g/cm^3 in CsCl gradients (Fig. 5). This is identical to the buoyant density of the bulk DNA extracted from the host strains NRRL B-3275 and NRS 576 (9, 13).

In neutral sucrose gradients, ϕ75 DNA sedimented as a single peak with a sedimentation velocity of 33.6*S* (±1%) (Fig. 6). This *S* value corresponds to a mol wt of 28.8 × 10^6 (21). In alkaline sucrose gradients, ϕ75 DNA sediments as a single component (38.8*S* ± 1%) with a single-stranded mol wt of 14.5 × 10^6 (21). Accordingly, ϕ75 DNA appears to be a linear duplex with a mol wt of about 29 × 10^6.

Genetic mapping of the ϕ75 prophage attachment site. Strain NRRL B-3275 and three G mutants lysogenic for ϕ75 did not contain detectable covalently closed, circular duplex DNA when their DNA was subjected to dye-buoyant density centrifugation. It therefore seemed probable that ϕ75 had a chromosomal attachment site.

Location of the ϕ75 attachment site was approached by mapping mutations that result in defective lysogens. Auxotrophic mutants of strain NRRL B-3275 were mutagenized with nitrosoguanidine (14). Survivors were stabbed into TBAB plates prespread with phage-sensitive cells (C3 cells), and the plates were incubated overnight at 37 C. About 0.1% of the mutagenized clones did not produce a zone of killing in the area surrounding the stab. After repeated single-colony isolations, these presumed defective lysogens were found to be resistent to superinfection by ϕ75. They could be induced to lyse with mitomycin C, but no infectious ϕ75 particles were detected in the lysates. The genetic lesion in three such independently isolated mutants (d2, d7, d11) was linked by PBS1 transduction to the *trp-2* marker (about 20% linkage) and to the *lys-1* marker (35 to 40% linkage). The results of a three-factor cross using one of these defective lysogens is shown in Table 3. From these data the most probable order is *ϕ75 att lys-1 trp-2*.

Genetic mapping of the ϕ75 prophage in a G mutant was accomplished as above. A G mutant (GM45; *ade-100 lys-100 ilv-100*) was lysogenized with ϕ75 yielding GM45 (ϕ75). GM45 (ϕ75) was mutagenized with nitrosoguanidine, and a defective lysogen, GM45d6, was isolated. The results of two two-factor crosses using this mutant as recipient and GM40 (ϕ75) as donor are shown in Table 4. These data suggest the order *ϕ75 att lys-100 ilv-100*. When the donor in this cross was nonlysogenic, none of 300 transductants selected for Lys$^+$ or Ilv$^+$ carried the wild-type phage marker.

GM45d6 was made tryptophan requiring by the introduction of *trp-100* (from GM2) through PBS1 transduction with selection for Ilv$^+$. A resulting transductant GM47d6 (*ade-100 lys-100 trp-100 def-6*) was isolated, cloned, and

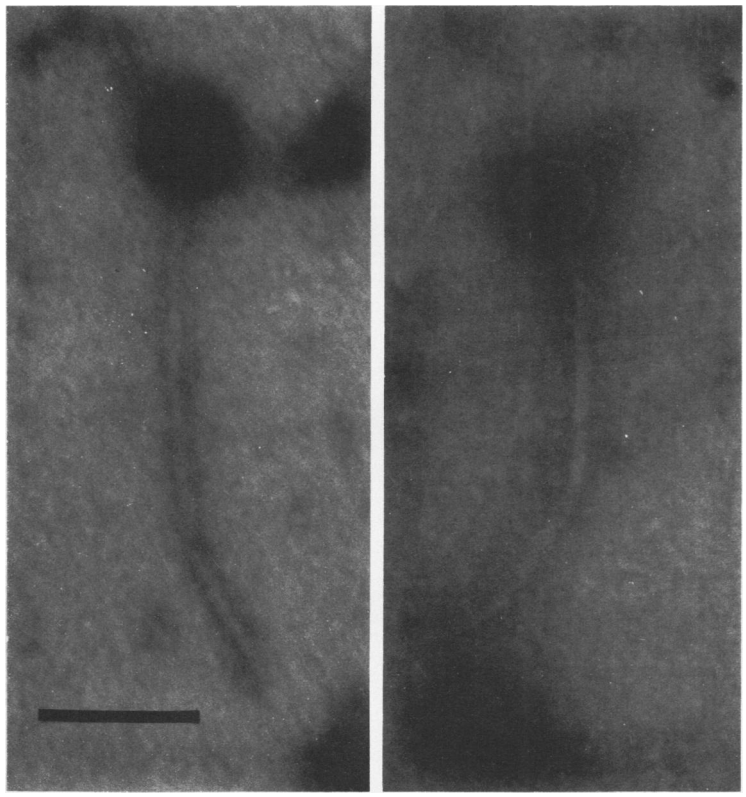

FIG. 3. *Electron micrograph of two ϕ75 particles stained with uranyl acetate. Bar represents 0.1 μm. Magnification, $\times$210,000.*

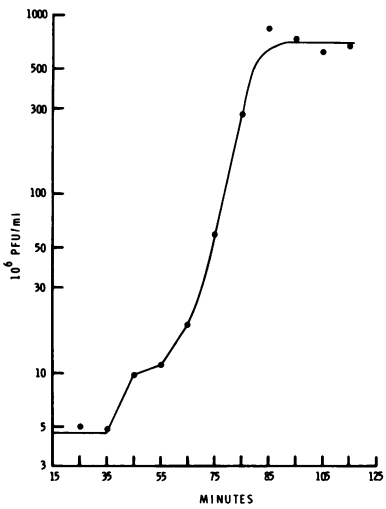

FIG. 4. *One-step growth curve of ϕ75 in C3 cells. The procedure followed is described by Adams (1), except that the cells were grown and infected in AK broth.*

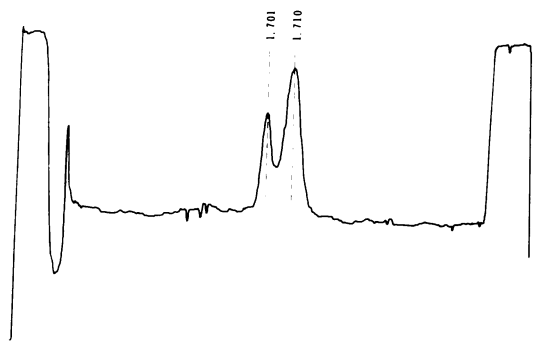

FIG. 5. *Microdensitometer tracing of a CsCl solution containing ϕ75 DNA and E. coli K-12 DNA after equilibrium centrifugation in an analytical ultracentrifuge. E. coli DNA (2.5 μg) was assumed to have a buoyant density of 1.710. ϕ75 DNA (1 μg) has a calculated buoyant density of 1.701 (18).*

used as recipient in the three-factor cross shown in Table 5. The results of this cross suggest that the marker order is *ϕ75 att lys-100 trp-100.*

Confirmation of the location of the ϕ75 at-

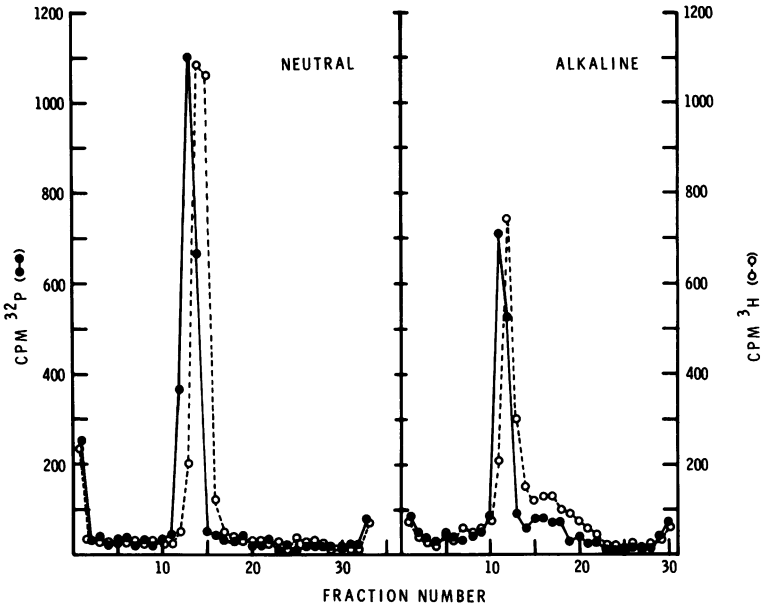

FIG. 6. *Centrifugation of φ75 DNA and T7 DNA through 5 to 20% neutral and alkaline sucrose gradients. φ75 DNA was labeled with ^{32}P (●); T7 DNA was labeled with [^{3}H]thymidine (○). T7 was assumed to have a sedimentation velocity of 32S in neutral gradients and 37S in alkaline gradients (21).*

TABLE 3. *Location of def-11 on the chromosome of B. pumilus NRRL B-3275 by a three-factor PBS1-mediated cross*[a]

Phenotype of transductants	No.	% of total
Lys$^+$ φ75Def[b]	193	48.5
Lys$^-$ φ75Def	121	30.6
Lys$^+$ φ75[b]	11	2.8
Lys$^-$ φ75	72	18.2

[a] Donor was BpB2 (*lys-1* φ75); recipient was BpB10d11 (*trp-2 def-11*); selection was for Trp$^+$. Transductants were streaked twice on the medium used for initial selection prior to scoring the non-selected markers. The phage marker was scored by stabbing transductants into TBAB plates seeded with C3 cells.

[b] "φ75Def" indicates that clone did not produce a halo of lysis when stabbed into C3 seeded plates; "φ75" indicates that clone did produce a halo of lysis when stabbed into C3 seeded plates.

tachment site was obtained by curing GM45d6 of the prophage by transduction. PBS1 grown on a nonlysogenic donor (GM40) was used to transduce GM45 (φ75d6). Of 306 transductants selected for Lys$^+$, six were sensitive to plaque formation by φ75. These six were presumably cured of the prophage. Transductants selected for Ilv$^+$ (total of 302) all remained immune to plaque formation by φ75.

DISCUSSION

The location of the φ75 prophage attachment site is in a region of the *B. pumilus* chromosome

TABLE 4. *Location of def-6 on the chromosome of B. pumilus NRS 576 by two two-factor PBS1-mediated crosses*[a]

Phenotype of transductants	No.	% of total
Selection for Lys$^+$		
Ilv$^+$ φ75Def	83	32.1
Ilv$^-$ φ75Def	118	45.7
Ilv$^+$ φ75	2	0.8
Ilv$^-$ φ75	55	21.3
Selection for Ilv$^+$		
Lys$^+$ φ75Def	59	22.7
Lys$^-$ φ75Def	201	77.3
Lys$^+$ φ75	0	
Lys$^-$ φ75	0	

[a] Donor was GM40 (*ade-100* φ75); recipient was GM45d6 (*ade-100 ilv-100 lys-100 def-6*). For other details, see footnotes to Table 3.

TABLE 5. *Location of def-6 on the chromosome of B. pumilus NRS 576 by a three-factor PBS1-mediated cross*[a]

Phenotype of transductants	No.	% of total
Lys$^+$ φ75Def	221	53.3
Lys$^-$ φ75Def	176	42.5
Lys$^+$ φ75	16	3.8
Lys$^-$ φ75	1	0.2

[a] Donor was GM40 (*ade-100* φ75); recipient was GM47d6 (*ade-100 trp-100 lys-100 def-6*); selection was for Trp$^+$. For other details, see footnotes to Table 3.

where several mutations resulting in asporogenesis have been mapped (P. S. Lovett, unpublished data). An apparently analogous region of the *B. subtilis* chromosome harbors the SpoA mutations (3). Efforts are in progress to determine whether ϕ75 can mediate transduction of genetic markers adjacent to its attachment site.

The approach used in the isolation of ϕ75 depended on the sensitivity of several asporogenic mutants to the virus. It is not possible to suggest a direct relationship between asporogenesis and phage sensitivity since little is known of the properties of the phage-sensitive mutants. However, the fact that spore$^+$ revertants of C3 and spore$^+$ transductants of three G mutants (unpublished data) are resistant to plaque formation by ϕ75 indicates a correlation between asporogenesis and phage sensitivity.

We have considered that the apparent resistance of NRS 576 (and the W-mutant derivatives) could result if the bacterium harbored a temperate phage related to ϕ75 with respect to repressor immunity. The G mutants might then be explained as spontaneous cured derivatives. Cultures of NRS 576 and W1, and of W4 and W7, contained no activity before or after mitomycin C induction that formed discernible plaques on GM45 or C3. Therefore, if such a temperate phage is carried by NRS 576, it is noninfectious for GM45 and C3. If the sporulation defect in G mutants were the consequence of excision of a phage genome from the chromosome (i.e., curing), then the mutation in independent G-mutant isolates would presumably map at a similar chromosome location. The limited number of genetic markers presently available in NRS 576 has precluded the mapping of these mutations. However, spore$^+$ transductants have been obtained in crosses between pairs of G mutants (e.g., GM1 and GM45), indicating the nonidentity of the mutations in these G mutants. Further genetic study of the G mutants is clearly required to explain the basis of this variant type.

During our limited screening for a temperate phage that plaques apparently exclusively on sporulation-deficient variants, we did not examine *Bacillus* species other than *pumilus*. The wider application of such an approach might prove fruitful considering the relatively common occurrence of inducible, apparently "noninfectious" viruses in spore-forming bacteria.

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