Characterization of a new bacteriophage which infects bacteria of the genus *Acidiphilium*

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A novel bacteriophage, termed φAc1, that infects strains of the genus *Acidiphilium* (acidophilic, heterotrophic, aerobic, Gram-negative eubacteria) most commonly isolated from acidic mine drainage environments, has been discovered and several of its properties have been determined. This is the first report of a bacteriophage infecting such cells. The virion has a lambdoid morphology and is larger than λ, as shown by electron microscopy and sucrose gradient centrifugation. The sedimentation coefficient of the virion is approximately 615S. The nucleic acid of φAc1 is dsDNA, approximately 102 kb in length. Several experimental results show that φAc1 is a temperate phage. The plaques are turbid, and most cells isolated from plaques produced on sensitive cells by filter-sterilized phage preparations contain the phage and are resistant to further phage infection. Southern blot analysis shows that φAc1 prophage DNA is integrated into the bacterial genome during the temperate growth phase.

Introduction

Acidophilic bacteria are important in the leaching of metal sulphide ores and the removal of pyritic sulphur from coal, both of which result in production of acidic mine drainage (Harrison, 1984). Among the varieties of bacteria that can be recovered from such environments are members of the genus *Acidiphilium*. This genus contains acidophilic, aerobic, heterotrophic, Gram-negative eubacteria (Harrison, 1983, 1989; Wichlacz & Unz, 1981; Wichlacz et al., 1986). The genetic properties of these bacteria have not been extensively studied. We are investigating various techniques for transferring genetic information into such cells and conditions for transformation of *Acidiphilium* with plasmid DNA have recently been reported (Glenn et al., 1992). A transducing bacteriophage derived from an endogenous temperate phage would constitute an alternative mechanism for introducing DNA, which might result in higher efficiency. Since bacteriophages that infect Gram-negative, acidophilic bacteria had not been reported, a search was made for endogenous phage in these cells. Such a bacteriophage has been discovered and termed φAc1. This report describes the discovery and several properties of this phage.

Methods

Chemicals and reagents. All chemicals were reagent grade. Yeast extract was obtained from Becton Dickinson and agarose was obtained from Sigma. Enzymes were purchased from Boehringer Mannheim, New England Biolabs and Promega, and were used according to the manufacturers’ instructions. Deionized water was used to prepare all media.

Strains. *Acidiphilium* strains CM1, CM3, CM3A, CM4, CM4A, CM5, CM7, CM9 and CM9A were isolated from water collected at the Blackbird cobalt mine, 32 km southwest of Salmon, Idaho (Wichlacz & Thompson, 1988). The other *Acidiphilium* strains used were isolated from acidic mine drainage sites in central Pennsylvania and were supplied by P. L. Wichlacz (Wichlacz & Unz, 1981; Wichlacz et al., 1986). Strain PW2 is ATCC strain 35904. Bacteriophage λ was a clear-plaque mutant obtained from Dr Larry Farrell at Idaho State University. The *Escherichia coli* host used to propagate λ was ATCC strain 29055. Bacteriophages P1, T5 and T4 and their corresponding *E. coli* host strains 25404 (P1) and 11303 (T5, T4) were also obtained from ATCC. *Acidiphilium* strains CM9A and CM9 have been deposited with the ATCC under the accession numbers ATCC 55305 and ATCC 55306, respectively.

Media and growth conditions. *Acidiphilium* strains were grown in glycerol salts medium [1 mM-(NH4)2SO4, 2 mM-KCl, 0.86 mM-K2HPO4, 13.6 mM-MgSO4, 8.7 mM-CaCl2, 3.5 mM-Al2(SO4)3, 0.7 mM-MnSO4 and 0.1% glycerol, pH 3.0] at 32 °C. For growth of phage-producing cells, 0.01% yeast extract was also added. Bottom agarose contained glycerol salts without Al2(SO4)3 and with 0.01% yeast extract and 1% agarose. Top agarose for titration and preparation of phage contained glycerol salts medium minus Al2(SO4)3, plus 0.01% yeast extract and 0.3% agarose. Phage were stored and diluted originally in phage buffer [20 mM-potassium acetate, pH 5.0, 1 mM-(NH4)2SO4, 2 mM-KCl, 20 mM-MgSO4, 20 mM-CaCl2, 0.7 mM-MnSO4 and 100 μg/ml BSA] and more recently in SM buffer (Sambrook et al., 1989).
Plate stocks of phage were prepared by mixing $1 \times 10^6$ to $2 \times 10^7$ lysogens or $5 \times 10^6$ to $7.5 \times 10^7$ plaque-forming units (p.f.u.) of phage with $0.3$ to $0.4$ ml of early- to mid-log (approximately $1 \times 10^7$ to $5 \times 10^7$/ml) sensitive cells, adding $3.2$ ml of molten top agarose ($45^\circ C$), vortexing, and spreading on $100$ mm bottom agarose plates. In some early experiments, a $10$ min incubation to allow phage attachment was included before addition of top agarose, but it was found that this did not increase the number of plaques, so this incubation was routinely omitted. Plates were incubated at $32^\circ C$ for $15$ to $24$ h to allow plaques to develop. The highest titres were obtained when the plaques did not overlap or barely touched each other. The top agarose was scraped into tubes, and each plate was rinsed with $1$ ml of phage buffer. The rinse was added to the tubes and the pH was adjusted to $5.0$ to $7.5$ with KOH. The mixture was incubated for $3$ to $6$ h at $4^\circ C$ and then centrifuged at $12000$ g at $4^\circ C$ for $20$ min to remove agarose and cell debris. The supernatant was passed through a $0.45 \mu m$ filter and stored at $4^\circ C$. These plate stocks generally had titres between $10^3$ and $10^6$ p.f.u./ml. No bacterial colonies were observed when these filtered preparations were plated on bottom agarose. Attempts to produce phage stocks using growth in liquid culture were not successful.

**Electron microscopy.** Filtered plate stocks of phage were fixed with $0.6$% glutaraldehyde, mounted on carbon-coated copper grids, and negatively stained with $1$% uranyl acetate. Observations were made using Philips EM420 and Zeiss EM900 electron microscopes.

**Sucrose gradient centrifugation.** Phage particles were centrifuged on linear $5$ to $20\%$ sucrose gradients in phage buffer ($\phi Acl$) or SM buffer ($\lambda$). $\lambda$ ($1.5 \times 10^6$ p.f.u.) and $\phi Acl$ ($1.8 \times 10^7$ p.f.u.) were layered on respective gradients. Centrifugation was in a Beckman SW40 rotor at $30000$ r.p.m. at $4^\circ C$ for $25$ min. Gradients were fractionated by puncturing the tube bottoms and collecting approximately $0.5$ ml fractions, which were then assayed for the respective phage.

**Isolation of phage DNA.** DNA from bacteriophages $\phi Acl$, $\lambda$, P1, T5 and T4 was isolated by SDS extraction of concentrated plate stocks of these phages. Phage (13 ml) were pelleted in a Beckman SW40 rotor at $20000$ r.p.m. at $4^\circ C$ for $60$ min. Frequently, additional $13$ ml aliquots of phage stock were added back to the tube, and pellets from two or three $13$ ml aliquots were built up on one another before extraction. The supernatant was discarded, and the pellet of phage was gently resuspended in $0.4$ ml of $50$ mM-NaCl, $10$ mM-Tris–HCl pH $7.5$, $1$ mM-EDTA, and dialyzed against the same solution. Solid SDS was added to a final concentration of $0.5\%$, followed by incubation at $65^\circ C$ for $15$ min. After cooling to room temperature, KCl was added to a final concentration of $0.5$ m to precipitate SDS and protein. Following incubation on ice for $15$ min, the precipitate was removed by centrifugation at $7500$ g at $4^\circ C$ for $5$ min. Finally, the supernatant was dialysed against $100$ mM-NaCl, $10$ mM-Tris–HCl pH $7.5$, $1$ mM-EDTA for $12$ h at $4^\circ C$ with three changes of buffer.

**Field-inversion gel electrophoresis of phage DNA.** Isolated phage DNA was electrophoresed in $0.7\%$ TEROse (Clontech) gels in $0.5 \times$ Tris–borate–EDTA (TBE) (Sambrook et al., 1989). An M.J. Research programmable power inverter (PPI-200) was used to control the applied voltage with a switching cycle of $0.15$ to $4.8$ s. After staining and photography as described above, the DNA was transferred to Biotrace RP nylon membranes (Gelman) according to Reed & Mann (1985). The blot was rinsed for $5$ min in $5 \times$ SSC (Sambrook et al., 1989) and baked for $1$ h at $80^\circ C$. Labelling of EcoRV-digested $\phi Acl$ DNA with digoxigenin using random primers, hybridization and colour development were performed according to manufacturer's instructions (Boehringer Mannheim 'Genius' non-radioactive DNA labelling and detection kit).

**Results**

**Initial observation of phage $\phi Acl$.**

Our strategy for searching for an endogenous phage infecting *Acidiphilium* strains was based on the premise that among our collection of isolates at least one strain would harbour such a phage and a second strain would
be sensitive to that phage. A series of experiments was therefore performed in which cells of one strain were irradiated with u.v. light (in the hope of inducing lysogenic phage), and a small number of these cells were mixed with a large number of cells of a second strain, followed by plating in soft agar. The first experiment contained all pairwise combinations of six Acidiphilium strains (CM1, CM3, CM3A, CM4A, CM5 and CM9A). Circular zones of lysis with sharp edges were observed with some combinations of strains and not with others. Eventually, strains CM1, CM3A and CM9A were shown to produce zones of lysis, and strains CM3, CM5, CM9 and PW2 were shown to be sensitive to all producer strains. Several other strains were neutral in that they were neither sensitive nor lysis-producing.

For some time it was not clear whether this phenomenon was due to bacteriophage or to lytic, bacteriocin-type molecules originating from the producing cells. Zones of lysis appeared when producer cells were mixed with sensitive cells, but filtered (sterile) plate lysates produced no such zones or very few zones when mixed with sensitive cells. The lysates were being kept in the (pH 3) growth medium of the cells, as is done for E. coli phages. Eventually, it was determined that this phenomenon is due to a bacteriophage, but that the phage is unstable under these storage conditions. A phage stock was prepared using producer strain CM3A and sensitive strain CM3, and experiments were performed to test the effects of various components on phage stability. pH was found to have a significant effect. Phage particles are more stable at pH 5 (half-life of approximately 4.5 days) than at pH 3 (half-life of approximately 12 to 13 h). Phage particles have even greater stability between pH 7 to 7.5 (half-life approximately 40 to 45 days). Since our plate stocks are produced in cells growing at pH 3, a significant number of phage particles are probably inactivated before neutralization of the top agar layer. Attempts to produce phage stocks in cells growing at pH 4.0 and 5.0 were unsuccessful, presumably because the cells do not grow as well at these pHs.

In standard phage buffer, pH 5.0, 1 mM-DTT and high concentrations (1 g/ml) of CsCl were also found to decrease phage stability significantly (half-life of approximately 27 to 28 h and complete loss of plaque-forming ability in 2 h, respectively) compared to phage buffer alone. This latter result precludes the use of CsCl gradient centrifugation for purification of this phage. Other components tested (glycerol, yeast extract, KCl, sucrose) did not have a significant effect on phage stability. The stability of the phage is also increased by removing the aluminium from the storage buffer and the media used to produce phage stocks. Conditions that result in long-term stability of the phage have not yet been determined. The titre of phage plate stocks is also not very reproducible, indicating there are unknown factors affecting production of these stocks.

An experiment was performed to determine the relationship among the phages in the three producing strains. DNA was isolated from phage stocks produced on strain CM9 using these three strains, digested with restriction endonuclease RsaI, and analysed by agarose gel electrophoresis (Fig. 1). The band patterns of the three phage DNA preparations were indistinguishable, suggesting that the phages in all three producing strains are very closely related.

**Electron microscopy**

Electron microscopy was performed to investigate the morphology and size of the phage particle. The electron micrograph (Fig. 2) shows that Ac1 has a morphology similar to λ with a polyhedral head and a relatively long tail. This morphology puts it in Group B of Bradley (1967) or alternatively in the family Siphoviridae (Syphoviridae) (Ackermann & DuBow, 1987; Fraenkel-Conrat, 1985). Ac1 has a head diameter of approximately 78 nm compared to 54 to 55 nm for λ (Fraenkel-Conrat, 1985; Fraenkel-Conrat et al., 1988) and a tail length of approximately 213 nm.

**Sucrose gradient centrifugation**

Parallel sucrose gradients were prepared and run on Ac1 and λ to investigate the sedimentation properties of Ac1. The results (Fig. 3) show that Ac1 sediments
approximately 1.53-fold further than \( \lambda \), which has a sedimentation coefficient of 416S (Fraenkel-Conrat, 1985). Calculating by the method of Griffith (1986) yields a sedimentation coefficient of approximately 615S for \( \phi Acl \).

**Characterization of \( \phi Acl \) nucleic acid**

\( \phi Acl \) virion nucleic acid was treated with a number of enzymes, followed by agarose gel electrophoresis to determine the effect of those treatments. The nucleic acid was completely digested by DNase I, but was not affected by RNase A. Native \( \phi Acl \) DNA was not digested by nuclease S1, but heat-denatured \( \phi Acl \) DNA (95°C, 15 min) was completely digested by nuclease S1 (data not shown). Finally, \( \phi Acl \) DNA was digested to smaller fragments by the restriction endonucleases Rsal (Fig. 1), EcoRV (Fig. 5), and Sau3AI, HindIII and SinI (data not shown). Such enzymes generally do not digest ssDNA (Hofer et al., 1982). These results show that \( \phi Acl \) virion nucleic acid is dsDNA.

The size of \( \phi Acl \) DNA was analysed using field-inversion gel electrophoresis (Carle et al., 1986). The DNAs of bacteriophages \( \lambda \), P1, T5 and T4 were used as size markers. The switching programme described in Methods was found to give good resolution of these DNA molecules. From the results (Fig. 4), a size of 102 kb was calculated for \( \phi Acl \) DNA, based on the reported sizes of 99.7 kb for P1 DNA (Yun & Vapnek, 1977) and 121.3 kb for T5 DNA (Rhoades, 1982). This size is in good agreement with preliminary electron microscopic measurements of virion DNA molecules spread using the method of Griffith (1978) with pBR328 DNA as an internal standard (M. L. Shean & V. Winston, unpublished). [The faint, smaller band in the P1 DNA preparation represents DNA from ‘small-headed’ virions (P1S) which are present in preparations of P1 (Ikeda & Tomizawa, 1965; Walker et al., 1979).]

In an attempt to produce a restriction map, isolated \( \phi Acl \) DNA was treated with a series of restriction endonucleases. Several enzymes, including EcoRI, HindIII and BamHI, failed to cut this DNA. These enzymes were active on other DNA preparations at the time. The digestions were repeated with the addition of plasmid pBR328 DNA to the reaction mixtures. Again the \( \phi Acl \) DNA was not cut, whereas the pBR328 DNA in the reaction was digested (Fig. 5), demonstrating that there was no general inhibition of restriction enzyme activity by the phage DNA preparation. (The two additional faint bands in lane 7 are produced by EcoRI acting on pBR328 DNA, as can be seen from lane 9, and do not arise from \( \phi Acl \). The faint bands in lane 6 presumably arise through a similar mechanism during HindIII digestion of pBR328.) These results indicate either that the phage DNA is somehow specifically protected against digestion by these enzymes or that there are no recognition sequences for these enzymes in \( \phi Acl \) DNA. Preliminary results indicate that a segment of \( \phi Acl \) DNA cloned in \( E. coli \) (using Sau3AI partial digestion products), can be cleaved by both EcoRI and HindIII, indicating that recognition sequences for these enzymes are present in this DNA. This leads to the
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Fig. 5. Failure of restriction enzymes to digest φAc1 DNA. φAc1 DNA, pBR328 DNA (4.9 kb) and mixtures of the two were treated with various restriction enzymes, followed by agarose gel electrophoresis (0.7% agarose, 0.5 x TBE, 3.5 V/cm, PPI-200 programme no. 3, 8 h). Lane 1, uncut φAc1; lane 2, φAc1 plus pBR328, EcoRV-cut; lane 3, φAc1, EcoRV-cut; lane 4, uncut pBR328; lane 5, pBR328, EcoRV-cut; lane 6, φAc1 plus pBR328, HindIII-cut; lane 7, φAc1 plus pBR328, EcoRI-cut; lane 8, φAc1 plus pBR328, BamHI-cut; lane 9, pBR328, EcoRI-cut.

Conclusion that virion DNA is protected in some way against digestion by these enzymes. This protection could be due to some type of modification of the DNA, since the DNAs of a number of bacteriophages are modified (Huang et al., 1982; Warren, 1980), or it could be due to the presence of a protective protein or other agent that is specifically bound to the phage DNA, preventing its digestion. The phage DNA used for the experiment shown in Fig. 5 had been further purified using a NACS-52 column (BRL) before incubation with the restriction enzymes. Despite extensive analysis, we have not been able to detect any modified bases in φAc1 DNA using HPLC analysis of virion DNA hydrolysed either enzymatically to nucleosides or to free bases using acid. We thus have no satisfactory explanation for these observations. This inability to digest φAc1 DNA with several restriction enzymes has prevented us from constructing a restriction map of this phage. The protection is not effective for all enzymes since, as mentioned above, isolated virion DNA can be digested to smaller fragments by several restriction enzymes, including Rsal (Fig. 1), EcoRV (Fig. 5), and Ssp3AI, HindIII and SstI (data not shown). However, we have no way of knowing whether the DNA is being cleaved at every recognition sequence by these enzymes.

Evidence for the temperate nature of φAc1

Several experimental results show that φAc1 is a temperate bacteriophage. A majority of cells isolated from plaques produced on sensitive cells by filter-sterilized phage preparations contained the phage, since they were resistant to phage infection, and they produced plaques when plated with sensitive cells, similar to the original producing strains. To determine whether prophage DNA is integrated into the genome during the temperate growth phase, a Southern blotting experiment was performed on undigested whole cell DNA from a sensitive strain and two lysogenic strains. The results (Fig. 6) showed that labelled virion DNA hybridized to large size chromosomal DNA, and not to a separate,
extrachromosomal element, demonstrating that the phage is integrated into the genome during the temperate growth phase. As expected, prophage DNA was not detected in the sensitive strain.

Discussion

A bacteriophage termed ϕAc1, which infects strains of the genus Acidiphilium, has been discovered. This is the first report of a bacteriophage infecting obligately acidophilic, Gram-negative bacteria, and the first report of a temperate bacteriophage in any obligately acidophilic bacterium. Some strains of Acidiphilium contain the phage and are resistant to superinfection, whereas other strains, including isolates from different locales, are sensitive to the phage. In addition, some strains are resistant but do not appear to carry a phage. Under normal conditions, cultures of the lysogens show no evidence that they contain a phage.

We speculate that the increased stability of the phage at pHs nearer neutrality, compared to pH 3, may be due to the fact that the virions are assembled inside Acidiphilium cells, where the pH is near 6.0 (Goulbourne et al., 1986). However, severe ecological limitations would exist for a phage that is not stable in the external environment of the cells it infects. Phage liberated from a lysing cell would have a limited time to find new host cells before being inactivated. There may be unidentified stabilizing factors present in the cells' natural environment. Preliminary experiments do suggest that a high percentage of infections result in lysogeny, which is consistent with the low numbers of p.f.u. in phage preparations. Thus the long-term survival of the phage depends on its maintenance in the lysogenic bacterium.

The stimulus for phage induction is unknown. In the absence of u.v. treatment, the ratio of p.f.u.:c.f.u. in a lysogen population varies between 0.04 and 0.34, depending on the strain. Ultraviolet treatment (25 s, 60 cm from a GE G15T8 germicidal lamp) increases this ratio by up to a factor of two by reducing cell survival (c.f.u.), while the number of p.f.u. remains relatively constant. Thus this phage is not inducible by u.v. light in a manner similar to λ. There are significant numbers of free phage in lysogenic cultures [up to 10^5/ml in early log (3 x 10^6 c.f.u./ml) phase], as determined by assay of filtered culture supernatants. This indicates that lysogens produce phage spontaneously throughout much of their life cycle. The number of free phage declines significantly as the culture ages.

There is strong evidence that ϕAc1 is a temperate bacteriophages. It was isolated by performing a series of pairwise incubations with different Acidiphilium strains, none of which individually showed any evidence for the presence of bacteriophage. The majority of cells isolated from plaques produced on sensitive cells by filter-sterilized phage preparations contain the phage, since they produce plaques when plated with sensitive cells and are resistant to phage infection. These laboratory-produced lysogens thus behave similarly to the original producer strains.

The recent isolation of what appears to be a lytic (clear) mutant or variant of ϕAc1 (D. F. Bruhn, unpublished) is also consistent with the conclusion that ϕAc1 is a temperate bacteriophage. Finally, Southern blot analysis of undigested ϕAc1 virion DNA and whole cell DNA from both 'natural' and laboratory-produced lysogens shows that prophage DNA is present in the chromosomal DNA of the lysogens and not as an extrachromosomal element (Fig. 6). Therefore the phage is integrated into the bacterial genome during lysogenic growth. We do not know whether this phage integrates into a unique site or different sites in the bacterial chromosome, nor how many copies of the phage are integrated in any given cell.

There has been one previous series of reports on a bacteriophage infecting acidophilic bacteria. That phage (ϕNS11) contains lipid and infects the thermophilic, acidophilic, Gram-positive bacterium Bacillus acidocaldarius (Sakaki & Oshima, 1976; Sakaki et al., 1977a, b, 1979). It is stable in the acidic environment of its host cells and does not appear to be temperate.

Further study of ϕAc1 should provide insights into various aspects of the genetics of the genus Acidiphilium, since bacteriophages have been extensively used to investigate genetic mechanisms in a variety of bacteria. It is also possible that this phage could be modified for use as a genetic engineering vector for these cells, as numerous other bacteriophages have been exploited for genetic manipulation of their host cells.

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