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Isolation and characterization of bacteriophage BCJA1, a novel temperate bacteriophage active against the alkaliphilic bacterium, *Bacillus clarkii*

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Abstract The isolation and characterization of a novel bacteriophage active against the obligately alkaliphilic bacterium *Bacillus clarkii* is described. The bacteriophage, designated BCJA1, is a member of the Siphoviridae family with a B1 morphology. It possesses an isometric head, which measures 65 nm between opposite apices, and a noncontractile tail of 195 nm length. It had a buoyant density of 1.518 g/ml and an estimated particle mass of 37×10^7 daltons. BCJA1 was stable over the pH range of 6–11. A one-step growth experiment conducted at pH 10 demonstrated a latent period of about 40 min and a burst size of approximately 40. The purified bacteriophage appeared to consist of 10 proteins with the major head and tail proteins likely to be of molecular weight 36500 and 28000, respectively. The genome size was estimated to be between 32.1 and 34.8 kb. The percent G + C content of purified bacteriophage DNA was 45.6. The wildtype bacteriophage is temperate but a clear plaque mutant was isolated.

Key words Alkaliphile · Bacteriophage · *Bacillus clarkii* · Temperate · Clear plaque mutant

Introduction

Microbial extremophiles are important organisms for study from ecological, applied, and basic research perspectives. Alkaliphilic bacteria, which have a pH optimum for growth between 9 and 11, are widespread in nature, being readily isolated from naturally occurring alkaline environments such as soda lakes and from man-made alkaline environments generated from commercial processes such as cement manufacturing, lye treatment of animal hides, and bauxite processing (Grant et al. 1990; Agnew et al. 1995). In addition, alkaliphiles can be found in soil, feces, and deep sea sediments (Horikoshi 1996). In highly saline, alkaline brines, alkaliphilic archaea belonging to the genera *Natronobacterium* and *Natronococcus* have been isolated (Grant et al. 1990) and an alkaliphilic anaerobic community located in an alkaline lake at pH 10 has recently been reported (Zhilina and Zavarzin 1994).

Alkaliphiles have already made a significant applied impact, especially in their role in biological detergents (Horikoshi 1996; Grant et al. 1990). Alkaliphilic bacteria have also proven to be important experimental organisms in which to study fundamental questions about pH regulation and bioenergetics. since these organisms possess a reverse pH gradient at external pH values above 7.5 but must still generate ATP at these submaximal electrochemical proton gradient (Δp) values (Krulwich 1995).

For many years, the only validly published obligately alkaliphilic *Bacillus* species was *Bacillus alcalophilus*, isolated from human feces (Vedder 1934). Recently, *Bacillus cohnii* isolated from horse meadow soil (Spanka and Fritze 1993), *Bacillus vedderi* isolated from bauxite-processing waste (Agnew et al. 1995), and several other soil isolates, including *Bacillus clarkii*, have been reported (Nielsen et al. 1995).

Reports of bacteriophages active against alkaliphilic bacteria are rare in the literature, with only a single bacteriophage, Al-K-I, being reported (Horikoshi and Yonezawa 1978). We have been studying the microbiology of alkaline red mud, derived from bauxite processing, a thus far poorly studied man-made alkaline environment which has yielded several new isolates (Agnew et al. 1995). In this communication, we report the isolation from alkaline red mud and characterization of a novel temperate bacteriophage BCJA1 and a clear plaque mutant, which are both active against the obligate alkaliphile *Bacillus clarkii* strain JaD.

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Materials and methods

Bacterial strains and growth media

Strain JaD is an obligate alkaliphile that was isolated from alkaline red mud. A detailed description of the isolate was published previously (Agnew et al. 1995). Comparison of the partial sequence of the 16S rRNA gene indicated at that time that strain JaD had 100% similarity to DSM 8720, an alkaliphile isolated from soil. The latter isolate had not been formally described (Nielsen et al. 1994) but was subsequently named *Bacillus clarkii* (Nielsen et al. 1995). Hence, we will refer to strain JaD as *Bacillus clarkii* strain JaD. Similarly, strain JaA was isolated from red mud and described by us previously (Agnew et al. 1995). It had a partial 16S rDNA similarity value of 99.7% compared to DSM 8715, another soil isolate which had not been formally described at that time (Nielsen et al. 1994) but which was subsequently described as a new species, *Bacillus pseudofirmus* (Nielsen et al. 1995). Hence, we will refer to strain JaA as *Bacillus pseudofirmus* strain JaA. *B. clarkii, B. pseudofirmus*, *Bacillus vedderi* strain JaH (DSM 9768; Agnew et al. 1995), *Bacillus alcalophilus* (ATCC 27647, obtained from the American Type Culture Collection), and *Bacillus cohnii* (DSM 6307, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were grown at 37°C in the medium recommended for *B. alcalophilus* (Slepecky and Hemphill 1992). This consisted of: Part A: KH_2PO_4, 10.0 g; peptone, 5.0 g; yeast extract, 5.0 g; glucose, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; distilled H_2O, 900 ml; Part B: Na_2CO_3, 20 g; distilled H_2O, 100 ml. Solutions A and B are mixed after sterilization to give a final pH of approximately 10.

Bacillus firmus (ATCC 14575, obtained from the ATCC) was grown in Tryptic Soy Broth (TSB; BBL, Becton-Dickinson Microbiology Systems, Cockeysville, MD, USA) at 37°C.

Escherichia coli was routinely grown in TSB at 37°C and used as the host for coliphage T7.

Bacteriophage BCJA1 and a clear plaque mutant BCJA1c were propagated on *B. clarkii* strain JaD using the overlay method (Adams 1959) with *B. alcalophilus* medium solidified with 1.5% (w/v, bottom agar) or 0.6% agar (w/v, top agar).

Bacteriophage BCJA1 (accession number HER 406) and BCJA1c (accession number HER 428), as well as *B. clarkii* strain JaD (accession number HER 1406) have been deposited with the Felix d'Herelle Reference Center for Bacterial Viruses, Department of Microbiology, Faculty of Medicine, Laval University, Quebec, Canada.

Bacteriophage isolation

Standard bacteriophage enrichments were set up using *B. clarkii, B. pseudofirmus, B. vedderi*, and *B. alcalophilus* as host and using alkaline red mud, garden soil, and sewage as potential sources of bacteriophage. Plaques were only observed on examination of enrichments using *B. clarkii* as host and alkaline red mud as bacteriophage source. A single, turbid plaque morphology was observed. A well-isolated plaque was stabbed with a sterile toothpick and used to inoculate log phase *B. clarkii* cells. Several rounds of plaque purification were performed to ensure a pure phage stock. The new bacteriophage has been designated BCJA1, with the first two letters of the name indicating the host genus and species name, in accordance with the recommendations of Ackermann et al. (1992).

Isolation of a clear plaque mutant

During our studies of the bacteriophage BCJA1, a spontaneous clear plaque variant was obtained. This clear plaque variant was plaque purified several times as just described. Since it routinely grew to significantly higher titers than the turbid plaque isolate, the clear plaque mutant, designated bacteriophage BCJA1c, was used for isolation of purified bacteriophage for protein composition and DNA analysis.

pH stability

Part A of the alkaline growth medium was adjusted with either 5N NaOH or with 2M HCl to obtain a pH range of approximately 4–12. Bacteriophage BCJA1 (100 µl) was added to 100 ml of the pH-adjusted media to attain a concentration of approximately 3×10^3 plaque-forming units (pfu)/ml. After a 30-min incubation at room temperature, 100-µl aliquots were plated, using *B. clarkii* as indicator, to determine the remaining viable bacteriophage. The pH of each solution was remeasured at the end of the 1-h incubation period.

Temperature stability

Bacteriophage BCJA1 (at approximately 3×10^3 pfu/ml) was incubated over a temperature range of 30°–90°C for 1 h before the viable remaining bacteriophage were determined using *B. clarkii* as indicator.

Chloroform sensitivity of bacteriophage BCJA1

One ml of bacteriophage BCJA1 (3×10^3 pfu/ml) was mixed with an equal volume of chloroform and left to incubate statically at room temperature. After 30 min, the remaining viable bacteriophage were titered with *B. clarkii* as host.

Buoyant density determination

The buoyant density of bacteriophage BCJA1 was determined by the method of Bachrach and Friedmann (1971) except that the centrifugation was for 2 h (Jarrell and Kropinski 1981). Coliphage T7 was used as an internal standard.

Bacteriophage particle mass

A mixture of bacteriophage BCJA1 and coliphage T7 was carefully layered over a preformed sucrose gradient (10%–50% (w/v) in 10 mM Tris-HCl, pH 7) and centrifuged at 77000 $\times g$/30 min. The $s°_{20,w}$ of bacteriophage BCJA1 was determined by its position in the gradient relative to that of T7 using 453 as the $s°_{20,w}$ of T7 (Dubin et al. 1970). Bacteriophage BCJA1 mass (m) was calculated from the equation $s_1/s_2 = [m_1/m_2]2/3$ (Dubin et al. 1970) using 49.9×10^6 as the molecular weight of T7 (Dubin et al. 1970; Bancroft and Freifelder 1970).

One-step growth experiment

The one-step growth experiment was performed essentially as described by Adams (1959). *B. clarkii* was grown to an A_{600} of 0.4 and bacteriophage BCJA1 was added to give a final concentration of approximately 2×10^3 pfu/ml. At timed intervals, 100-µl samples were removed and either plated directly or serially diluted and plated. Unadsorbed bacteriophage were estimated by removing an aliquot at 5 min to a tube at 4°C containing 900 µl of media and 100 µl of chloroform. After vortexing to kill the host cells, the viable (unadsorbed) bacteriophage remaining were determined.

Purification of bacteriophage

Large-scale purification of the new bacteriophage was undertaken for protein composition analysis and DNA isolation. For large-scale purification, the clear plaque mutant BCJA1c was used as it routinely grew to higher titers. By using various multiplicities of infection (m.o.i.) and various initial cell densities, it was determined that an m.o.i. of 0.01 at an initial cell density of *B. clarkii* of 2×10^8 colony-forming units (cfu)/ml resulted in the highest yield of bacteriophage. *B. clarkii* was grown overnight and subcultured at 1% (v/v) inoculum into ten 250-ml volumes of medium. When the optical density at 600 nm reached 0.3, the cells were diluted with an equal volume of medium and bacteriophage BCJA1c was added to give an m.o.i. of 0.01. After approximately 4 h, lysis was complete. The lysate was centrifuged for 15 min at 10000 $\times$ g to remove debris and unlysed cells. DNase and RNase were added to reduce the viscosity. NaCl was added to 0.5 M to the supernatant and then polyethylene glycol (Carbowax PEG 8000, Fisher Scientific, Ottawa, Canada), was added to 7% (w/v) (Yamamoto et al. 1970). The lysate was placed at 4°C overnight. The phage precipitate was collected by centrifugation (10000 $\times$ g/15 min) and the pellet resuspended in 40 ml of 100 mM Tris-HCl (pH 8.8). After resuspension, the sample was centrifuged at 10000 $\times$ g/10 min to remove debris and the supernatant containing the majority of the bacteriophage was centrifuged at 80000 $\times$ g/2 h. The bacteriophage pellet was resuspended in 100 mM Tris-HCl (pH 8.8) and layered over a CsCl step gradient (Bachrach and Friedmann 1971) which was centrifuged at 100000 $\times$ g/2 h. The bacteriophage band was removed, diluted with buffer, and the bacteriophage pelleted following centrifugation at 80000 $\times$ g/2 h. The bacteriophage were resuspended in 100 mM Tris-HCl pH 8.8 and treated with RNase and DNase for 1 h at room temperature and then rebanded in CsCl as before. The bacteriophage band was collected and the bacteriophage pelleted again at 80000 $\times$ g/2 h. This purified bacteriophage sample was used for protein and DNA analysis. Steps in the purification, including the location of the bacteriophage in the CsCl gradient, were followed by electron microscopy.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (*SDS-PAGE*)

SDS-PAGE of purified bacteriophage BCJA1c was performed by the method of Laemmli (1970), using a minigel system. Gels were stained using a Coomassie brilliant blue G250- perchloric acid solution (0.04% Coomassie brilliant blue G250 in 3.5% perchloric acid). The gel was microwaved in the stain for about 20 s (so that the stain became hot but did not boil). The gel was left in the hot stain with shaking for 2 min and then destained by microwaving the gel in water for 2 min . The protein bands were visible at this point but the gels were shaken in the hot water for an additional 15 min before photography (Faguy et al. 1996). The prestained protein molecular weight markers (Bio-Rad Laboratories. Mississauga, Canada) used were lysozyme (M_r 20900), soybean trypsin inhibitor (29100), carbonic anhydrase (35500), ovalbumin (50600), bovine serum albumin (83000), and phosphorylase B (101000).

Isolation of bacteriophage DNA

CsCl gradient purified bacteriophage were resuspended in 100 mM Tris-HCl, pH 6.8 (500 µl) and extracted three successive times with an equal volume of phenol:chloroform (1:1). The final aqueous phase was made 0.5 M with respect to NaCl and the DNA precipitated by the addition of 2 volumes of ice cold ethanol. The DNA was collected by centrifugation for 10 min in a microfuge, washed with 70% ethanol, and the DNA pellet resuspended directly into standard saline citrate (SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0).

Determination of G + C content

The mole percent guanine plus cytosine content of bacteriophage BCJA1c DNA was determined from its melting temperature (T_m) in 1$\times$ SSC using the equation of DeLey (1970) where mol% G + C = 2.44 (T_m − 69.4°C).

Estimation of genome size

Bacteriophage BCJA1c DNA was digested individually with a variety of restriction endonucleases and the fragments separated by electrophoresis on a 0.8% (w/v) agarose gel. A 1-kb ladder and λ DNA digested with *Sty*I served as

molecular weight markers (19329 bp, 7743 bp, 6223 bp, 4254 bp, 3472 bp, 2690 bp, 1882 bp, 1489 bp, 925 bp, 421 bp, 74 bp).

Electron microscopy

Bacteriophage BCJA1 was stained with 2% aqueous uranyl acetate (pH 4.0) and the grids examined with a Philips EM 300 electron microscope at 60 kV.

Isolation and analysis of lysogens

Bacterial growth from the center of the turbid plaques of BCJA1 was streaked onto fresh plates and a number of single colonies were picked and restreaked for purity. Spot tests of these lysogens onto lawns of *B. clarkii* strain JaD showed zones of lysis, indicating the presence of lysogeny. DNA was isolated from *B. clarkii* and several lysogens using the liquid nitrogen procedure (Jarrell et al. 1992). The chromosomal DNA of the wildtype and lysogen KJ55 were digested with *Kpn*I, and electrophoresed through a 0.8% agarose gel and used in Southern blot experiments with a DIG-labelled bacteriophage DNA fragment (a 3.3-kb *Kpn*I fragment, isolated from an agarose gel using Prep-A-Gene (BioRad, Mississauga, Canada) as probe. The probe was labelled using the random prime technique with digoxigenin-11-UTP (DIG-UTP; Boehringer-Mannheim, Germany). The membranes were prehybridized for 1 h at 50°C and hybridized with labelled probe at 50°C overnight. After washing (2 × 5 min in 2× SSC/0.1% SDS at room temperature followed by 2 × 15 min in 0.1× SSC/0.1% SDS at 50°C), the labelled membrane was developed using anti-digoxigenin antibody conjugated with alkaline phosphatase and the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo {3.3.1.1^{3,7}}decan}-4-yl) phenyl phosphate (CSPD, Boeheinger-Mannheim) according to the manufacturer's instructions. The treated membrane was then exposed to Kodak XAR film for detection.

Results

Reports of bacteriophages active against obligate alkaliphiles are rare. Since we have been studying a poorly analyzed man-made alkaline environment generated through the processing of bauxite, which yielded a variety of novel alkaliphilic *Bacillus* strains, this material was used in enrichments for bacteriophages. While we used several alkaliphilic *Bacillus* species as potential hosts and several potential sources of bacteriophages including soil, sewage, and alkaline red mud, it was only the combination of *B. clarkii* strain JaD as host and al-kaline red mud as enrichment source that yielded bacteriophage. A single turbid plaque morphology was observed and this plaque type was purified and analyzed in this study.

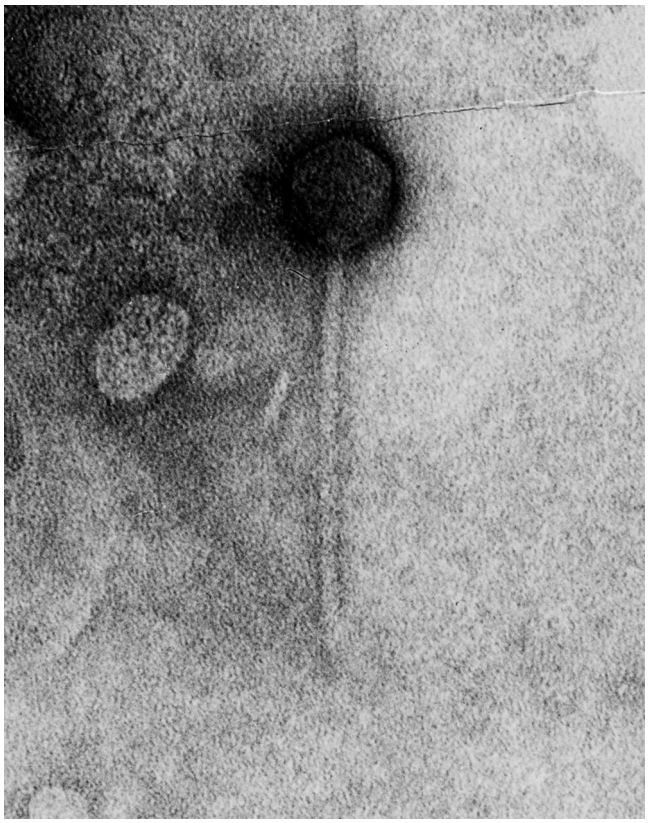

Fig. 1. Electron micrograph of bacteriophage BCJA1. The bacteriophage was stained with 2% uranyl acetate. Final magnification is 297000×

Bacteriophage morphology

Bacteriophage BCJA1 belongs to the Siphoviridae family and has the frequent B1 morphology (Ackermann 1992) with an isometric head and a long noncontractile tail (Fig. 1). The bacteriophage head measures 65 nm between opposite apices and the tail is 195 nm in length.

Host range

Bacteriophage BCJA1 (at 2 × 10^9 pfu/ml) was spot-tested onto lawns of a variety of alkaliphilic or alkalitolerant *Bacillus* species. Lysis was only detected against *B. clarkii* strain JaD.

One-step growth experiment

The infection cycle of bacteriophage BCJA1 was characterized by a one-step growth experiment (Fig. 2). Such experiments indicated that bacteriophage BCJA1 grown on *B. clarkii* strain JaD had a latent period of 40 min, a rise period of 30 min, and an average burst size of approximately 40. Similar results were obtained at both pH 10 and 9 (data not shown).

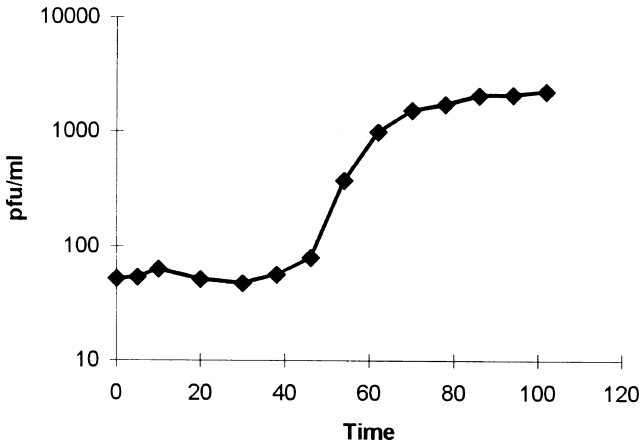

Fig. 2. One-step growth experiment of bacteriophage BCJA1 grown on *B. clarkii* strain JaD. *B. clarkii* was grown at pH 10 to an A_{600} of 0.4 and bacteriophage BCJA1 was added to give a final concentration of approximately 2×10^3 pfu/ml. At timed intervals, 100-µl samples were removed and either plated directly or serially diluted and plated. Unadsorbed bacteriophages were estimated by removing an aliquot at 5 min to a tube at 4°C containing 900 µl of media and 100 µl of chloroform. After vortexing to kill the host cells, the viable (unadsorbed) bacteriophages remaining were determined. *pfu*, plaque-forming units

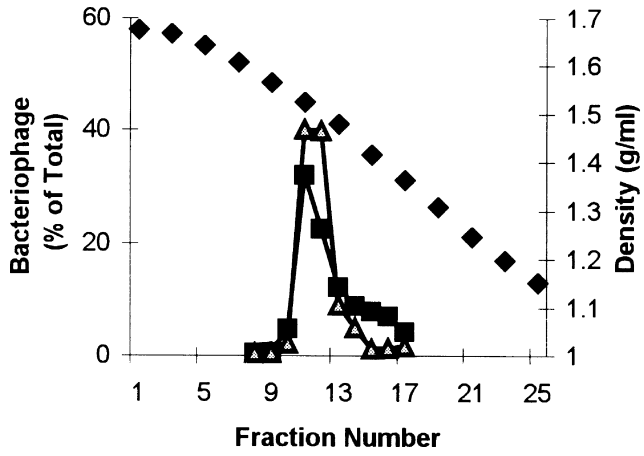

Fig. 3. Buoyant density of bacteriophage BCJA1. A mixture of coliphage T7 and bacteriophage BCJA1 (approximately 10^8 pfu/ml each) was layered over a CsCl step gradient and after centrifugation ($28\,500 \times g$/2 h), the gradient was fractionated and the location of the bacteriophages was determined. *Squares*, bacteriophage BCJA1; *triangles*, coliphage T7; *diamonds*, density

Stability of bacteriophage BCJA1

Bacteriophage BCJA1 was stable to a 1-h exposure to chloroform indicating that it probably does not contain lipid. For long-term storage of the bacteriophage, it was routinely kept in screw-capped tubes over chloroform. Bacteriophage BCJA1 was stable over the pH range 6–11 (data not shown). Almost complete inactivation of the bacteriophage occurred at pH 12 while about 25% of the bacteriophage survived exposure to pH 4 for 1 h. This seems to be a wider stability range than that reported for the alkaliphilic bacteriophage Al-K-I (Horikoshi and Yonezawa 1978). Bacteriophage BCJA1 was stable to temperatures below 45°C but was rapidly inactivated at temperatures above 50°C (data not shown).

Physical characteristics of bacteriophage BCJA1

Bacteriophage BCJA1 cobanded with coliphage T7, used as an internal standard in a CsCl gradient, demonstrating a buoyant density of approximately 1.518 g/ml (Fig. 3). This compares favorably with the literature value of 1.515 g/ml for the buoyant density of T7 (Bancroft and Freifelder 1970). The density of alkaliphilic bacteriophage Al-K-I was reported as 1.580 g/ml (Horikoshi and Yonezawa 1978).

The $s^\circ_{20,w}$ of bacteriophage BCJA1, as determined by centrifugation through a preformed sucrose gradient, with T7 as an internal standard, showed that bacteriophage BCJA1 did not migrate as far into the gradient as T7 (Fig. 4). From the data, the estimated particle mass of BCJA1 was 37×10^6 daltons, compared to 49.9×10^6 for T7 (Bancroft and Freifelder 1970; Dubin et al. 1970).

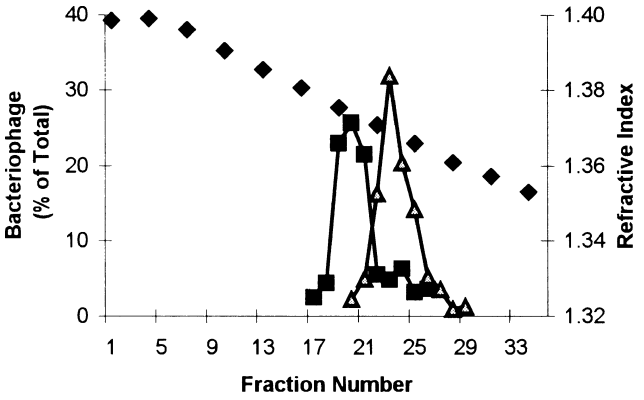

Fig. 4. Sedimentation of bacteriophage BCJA1 relative to coliphage T7. A mixture of coliphage T7 and bacteriophage BCJA1 (approximately 10^8 pfu/ml each) was layered over a 10%–50% (w/v) sucrose gradient. After centrifugation ($77\,000 \times g$/30 min), the gradient was fractionated and the location of the bacteriophage was determined. *Triangles* bacteriophage BCJA1; *squares*, coliphage T7; *diamonds*, refractive index

Composition of bacteriophage BCJA1c

A spontaneous clear plaque mutant of bacteriophage BCJA1, designated BCJA1c, was isolated (Fig. 5). Since it routinely grew to higher titers than the wildtype bacteriophage, it was used to purify bacteriophage for protein composition and DNA analyses. Purified bacteriophage BCJA1c, the clear plaque mutant, was subjected to SDS-PAGE analysis for examination of its protein composition. Examination of the stained gel (Fig. 6) indicated the bacte-

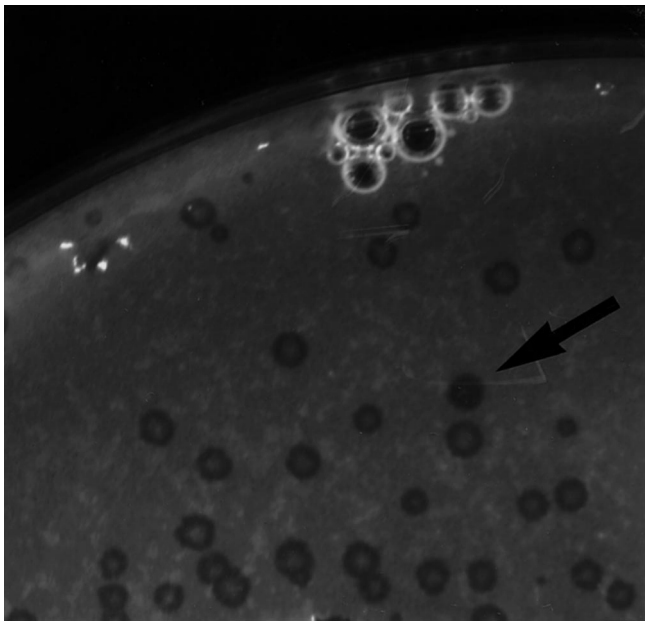

Fig. 5. Plaque morphology produced by the clear plaque mutant of bacteriophage BCJA1. The *arrow* points to the one clear plaque among a number of the typical turbid plaques

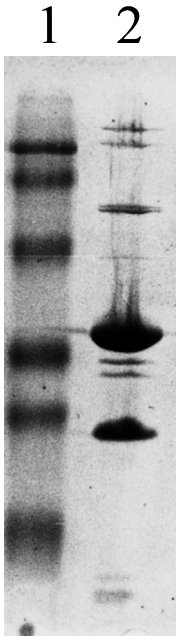

Fig. 6. Electrophoretic analysis of the total protein composition of purified bacteriophage BCJA1c. Proteins were analyzed by electrophoresis through a 12.5% acrylamide minigel and stained with Coomassie Blue G250. *Lane 1*, prestained molecular weight markers; *lane 2*, purified bacteriophage BCJA1c. The prestained protein molecular weight markers used were lysozyme (M_r 20900), soybean trypsin inhibitor (29100), carbonic anhydrase (35500), ovalbumin (50600), bovine serum albumin (83000), and phosphorylase B (101000)

riophage was composed of at least 10 proteins with apparent molecular masses of 120kDa, 106kDa, 70kDa, 50kDa, 36.5kDa, 34.5kDa, 33kDa, 28kDa, 18kDa, and 17kDa. The two major protein species were observed at 36.5 and 28kDa, and these are likely to represent the major head and tail proteins, respectively.

DNA isolated from the purified bacteriophage had a T_m in standard saline citrate of 88.1°C, which corresponds to a mol% G + C content of 45.6. The DNA was digested with a variety of restriction endonucleases (Fig. 7). Several restriction enzymes either cut the bacteriophage DNA not at all (*Xba*I) or at only 1–3 sites (*Eco*RV, *Sac*II, *Kpn*I, *Xho*I). However, digestions of bacteriophage DNA with *Bam*HI , *Hind*III, or *Pst*I resulted in multiple fragments of sizes that could be used to estimate the total genome size. Such estimates ranged from 32.1kb to 34.8kb.

Isolation of lysogens

The turbid nature of the plaques of wild type bacteriophage BCJA1 suggested that the bacteriophage was temperate and that lysogens could be isolated from the center of these turbid plaques. Many independent potential lysogens were isolated this way. All lysogens gave a zone of clearing after overnight incubation when spot tested back onto the wild type *B. clarkii* strain JaD, indicating that the lysogens were, in fact, carrying the bacteriophage which was released and subsequently infecting and lysing the wild type *B. clarkii*. Overnight cultures of wild type *B. clarkii* spotted onto itself did not result in any zone of clearing. As further evidence of this, DNA was isolated from wild type *B. clarkii* and one of the lysogens (KJ55), digested with *Kpn*I, electrophoresed and used in a Southern blot to detect the presence of

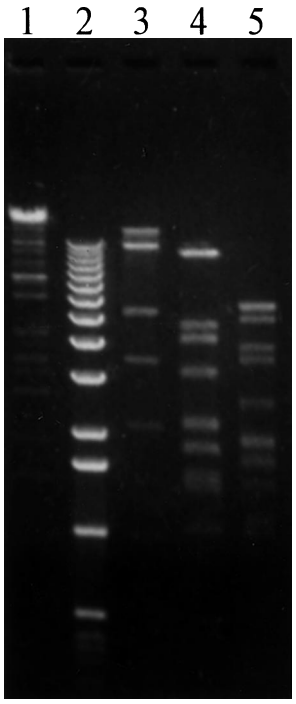

Fig. 7. Agarose gel electrophoresis of bacteriophage BCJA1c DNA digested with restriction enzymes. *Lane 1*, λ DNA digested with *Sty*I; *lane 2*, 1-kb ladder; *lane 3*, BCJA1c DNA digested with *Bam*HI; *lane 4*, BCJA1c DNA digested with *Hin*dIII; *lane 5*, BCJA1c DNA digested with *Pst*I. λ DNA digested with *Sty*I served as molecular weight markers (19329bp, 7743bp, 6223bp, 4254bp, 3472bp, 2690bp, 1882bp, 1489bp, 925bp, 421bp, 74bp)

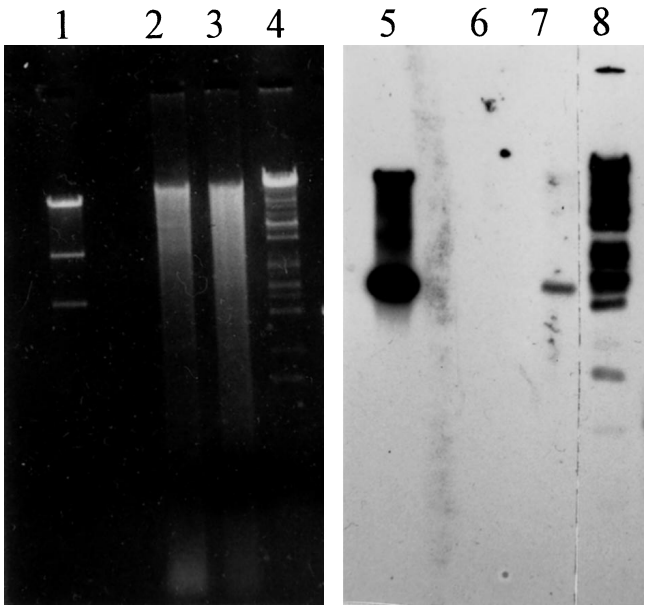

Fig. 8. Detection of BCJA1c DNA sequences in lysogens. Wildtype *B. clarkii* strain JaD and lysogen KJ55 DNA were digested with *Kpn*I, separated by agarose gel electrophoresis, and used in a Southern blot probed with a 3.2-kb *Kpn*I DIG-labeled BCJA1c fragment. *Lanes 1, 5*, bacteriophage BCJA1c DNA digested with *Kpn*I; *lanes 2, 6*, wildtype *B. clarkii* DNA digested with *Kpn*I; *lanes 3, 7*, *B. clarkii* lysogen KJ55 DNA digested with *Kpn*I; *lanes 4, 8*, λ DNA digested with *Sty*I. λ DNA digested with *Sty*I served as molecular weight markers (19329 bp, 7743 bp, 6223 bp, 4254 bp, 3472 bp, 2690 bp, 1882 bp, 1489 bp, 925 bp, 421 bp, 74 bp). *Lanes 1–4* show ethidium bromide stained gel. *Lanes 5–8* show chemiluminescent detection of D16-labelled probe

bacteriophage BCJA1 DNA. The probe used in these experiments was a 3.2-kb *Kpn*I fragment of bacteriophage. The results of the Southern blot (Fig. 8) indicated a 3.2-kb fragment hybridized with the probe in the lane of gel containing lysogen DNA whereas no binding was apparent in the gel lane containing wild type *B. clarkii* DNA. Furthermore, the *Kpn*I fragment hybridized in the lysogen DNA was the same size as the *Kpn*I fragment hybridized in the lane containing only bacteriophage BCJA1c DNA.

Discussion

This manuscript documents a rare occurrence: the isolation of a bacteriophage active against an obligate alkaliphile, *Bacillus clarkii*, using alkaline red mud as source material for the enrichment. According to Ackermann et al. (1992), this bacteriophage, designated BCJA1, must represent a new species since no bacteriophages have been reported against the host bacterium, *B. clarkii*, itself a very recent isolate (Nielsen et al. 1995; Agnew et al; 1995). Enrichments using other obligate or facultative alkaliphiles (see Materials and Methods) as hosts did not yield bacteriophage.

Following the recommendations of Ackermann et al. (1992) for the characterization of novel bacteriophages, BCJA1 morphology was studied by electron microscopic techniques, its DNA was isolated and characterized with regards to its G + C content, genome size, and restriction digest patterns, and the bacteriophage particle weight, protein profiles, and stability to heat and chloroform were determined.

Bacteriophage BCJA1 possesses an icosahedral head approximately 65 nm in diameter and a long noncontractile tail of about 195 nm. This places the bacteriophage into the family Siphoviridae and the frequent B1 morphotype (Reanney and Ackermann 1981). This compares with the only other bacteriophage active against an alkaliphilic *Bacillus*, namely phage Al-K-I, which is also a tailed bacteriophage with a head diameter of 100 nm and a tail 210 nm in length (Horikoshi and Yonezawa 1978). A survey completed in 1992 (Ackermann 1992) found, of over 4000 phages reported in the literature, greater than 96% were tailed and more than 60% belonged to the *Siphoviridae*. As of 1994, there were 33 *Bacillus* bacteriophage species (Ackermann et al. 1994).

BCJA1 was stable to long-term chloroform contact, indicating that it did not contain lipid in its capsid. In addition, BCJA1 was stable over a wide pH range especially on the alkaline side where it was completely stable at pH 11 for 1 h. The phage was stable to incubation at 50°C for 1 h but was rapidly killed above this temperature.

Analysis of the protein profile of purified BCJA1 indicated the presence of at least 10 protein bands ranging in molecular mass from 120 KDa to 17 kDa, with the two major proteins being 36.5 kDa and 28 kDa. These proteins are likely to represent the major head and major tail protein, respectively. This compares to other well studied bacteriophages of similar morphology. For example, T5 (head 65 nm diameter and tail 180 nm long) has one major head protein of 32 kDa and one major tail protein of 51 kDa, which account for 65% and 17% of the total protein, respectively (Zweig and Cummings 1973). Bacteriophage lambda has one major head protein (37.5 kDa), and one major tail protein (32.5 kDa), accounting for 57% and 19% of the total protein, respectively (Buchwald et al. 1970).

The G + C content of the bacteriophage DNA was estimated to be 45.6%, similar to the G + C content of the host bacterium, *B. clarkii* strain JaD, which has a G + C content of 41.8% (Agnew et al. 1995).

Analysis of restriction digests of the DNA of BCJA1 by agarose gel electrophoresis allowed an estimate of the total genome size from the summation of the resulting DNA fragments. A marked parallelism between capsid size and DNA content has been noted (Ackermann et al. 1994). There are several *Bacillus* phages of the *Siphoviridae* family which have similar dimensions to that of BCJA1 and similar sized genomes compared to the genome size of 32–35 kb estimated for BCJA1. For example, phage TP-15 has a head of 57 nm and a genome of 35 kb, and phage γ has a head of 59 nm and a genome of 40 kb (Ackermann et al. 1994).

The current state of genetic transfer systems in obligately alkaliphilic bacteria is poor. The lack of phages and hence transduction systems has already been alluded to. The dearth of plasmids found in alkaliphilic bacteria has made the development of conjugation systems and plasmid based

vectors difficult (Horikoshi 1986). Recently, protoplast transformation protocols have been successfully used for the facultative alkaliphiles *Bacillus* strain C-125 (Aono et al. 1994), a strain similar to *Bacillus subtilis* (Kudo et al. 1990), and *Bacillus firmus* (Gilmour and Krulwich 1997; Ito et al. 1997). While many genes from alkaliphilic *Bacillus* strains have been cloned and expressed in heterologous hosts (Horikoshi 1986), the reintroduction of alkaliphile DNA back into alkaliphiles is vital to address many questions unique to alkaliphiles, such as the bioenergetic and biotechnology ones (Krulwich and Guffanti 1989). Perhaps bacteriophage BCJA1, shown here to be a temperate bacteriophage, can be used as the basis of a transduction system for obligate alkaliphiles.

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