Characterization of bacteriophage BFK20 from *Brevibacterium flavum*

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Bacteriophage BFK20 was isolated from a *Brevibacterium flavum* strain that had become contaminated during industrial fermentation. BFK20 has a polyhedral head 50 nm wide and a non-contractile tail 200 nm long and 10 nm in diameter. The genome of this bacteriophage consists of a linear double stranded DNA molecule of 44–45 kb with cohesive ends. The capsid of phage BFK20 contains nine polypeptides with molecular masses from 22·0–108·0 kDa. BFK20 DNA was used as a donor for fragments carrying promoters and transcription-terminators.

Introduction

Brevibacterium flavum and related bacteria ('brevibacteria') are Gram-positive, coryneform, glutamic-acid-producing bacteria that are very important for industrial production of various L-amino acids and flavour enhancing nucleotides (Yoshinaga & Nakamori, 1983).

The existence of bacteriophages in corynebacteria has been known for many years (Hongo *et al.*, 1972; Ozaki *et al.*, 1984; Pátek *et al.*, 1985; Trautwetter *et al.*, 1987a, b), and brevibacteria are susceptible to a number of these phages. Although bacteriophages are useful tools for studying the biology of their hosts only a few have been used for this purpose in brevibacteria. A cosmid vector that can be packaged *in vivo* has been developed (Miwa *et al.*, 1985), but phage cloning vectors have not yet been described and little is known about the life cycles of these bacteriophages.

We describe in this paper the characteristics of bacteriophage BFK20 and the isolation of fragments of phage DNA with promoter activity using the promoter probe vector pJUP05 (Barák *et al.*, 1990). We also describe the isolation of a transcription-terminator from phage BFK20 DNA.

Methods

Bacterial strains, bacteriophage and plasmid. The following bacterial strains were used: *Brevibacterium lactofermentum* BLOB (Santamaria *et al.*, 1984), *Brevibacterium flavum* ATCC 21474, *Brevibacterium flavum* ATCC 21127, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium glutamicum* RM3 (restriction modification mutant) and *Brevi-*

bacterium flavum CCM 251. Phage BFK20 was isolated from a *Brevibacterium flavum* strain that had become contaminated during industrial fermentation. The promoter probe vector pJUP05 (Barák *et al.*, 1990), *Escherichia coli* HB101 (Boyer & Roulland-Dussoix, 1969) and *C. glutamicum* RM3 were used in DNA cloning experiments.

Media and cultures. LB medium (Maniatis *et al.*, 1982) was used as a medium for *E. coli* and strains of brevibacteria. BHI medium (Yeh *et al.*, 1986) was used as a medium for protoplasts of brevibacteria.

Ampicillin (Ap, 50 mg l⁻¹), chloramphenicol (Cm, 10 mg l⁻¹) and kanamycin (Km, from 10 to 1000 mg l⁻¹) were used for *E. coli*; and Cm (8 mg l⁻¹) and Km (from 10 to 1000 mg l⁻¹) for strains of brevibacteria, which were grown under aerobic conditions at 37 °C and 30 °C, respectively. LB medium supplemented with 5 g NaCl l⁻¹, 2 g glucose l⁻¹ and 10 mM-MgSO₄ (LBS medium) (Trautwetter *et al.*, 1987a) was used as a liquid medium for preparation of phage lysates. Solid media were prepared by adding 1·5% (w/v) agar (Difco) to the liquid media; 0·6% agar was added to the liquid media for preparation of the soft overlay.

Phage titration and lytic spectrum. These were determined by the method described by Adams (1959). The precise titre was determined by mixing appropriate amounts of bacteria and phages in soft agar.

Preparation and purification of phage lysates. *B. flavum* CCM 251 was grown to an OD₅₇₀ of 0·3–0·5 in 100 ml LB and infected with phage BFK20 in the presence of 10 mM-MgSO₄. After incubating for 30 min at 30 °C without shaking, the infected culture was diluted in 900 ml LB supplemented with 10 mM-MgSO₄ and then incubated at 30 °C with shaking until complete lysis occurred, generally for 6 h. The phages were concentrated by the method of Yamamoto & Alberts (1970). Phages were then purified on CsCl gradients as described by Maniatis *et al.* (1982).

Electron microscopy. A drop of phage BFK20 purified as described above was placed on a copper grid coated with Formvar and stained with 2% (w/v) uranyl acetate for 30–40 s. The microscope used was a Tesla (Brno, CSFR) model BS540.

SDS - PAGE. The purified phage proteins were suspended in 60 mM-Tris/HCl (pH 6·8) containing 5% (v/v) 2-β-mercaptoethanol and 2% (w/v) SDS, boiled at 100 °C for 5 min, and applied to a 10% (w/v) polyacrylamide gel (Laemmli, 1970). After migration of proteins, the

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gels were stained either with Coomassie brilliant blue (Laemmli, 1970) or with silver (Oakley *et al.*, 1980).

Extraction of phage DNA. This was done as described for bacteriophage by Maniatis *et al.* (1982).

Plasmid DNA purification and DNA manipulation. Plasmid DNAs from *E. coli* were isolated by the method of Birnboim & Doly (1979). The reaction conditions for the enzymes used were described by Maniatis *et al.* (1982). For shotgun cloning procedures, 0.05 mg of vector pJUP05 linearized with *Bam*HI and treated with calf intestine phosphatase plus 0.5 mg of partially *Sau*3A-digested bacteriophage DNA were ligated for 16 h in 10 µl at 14 °C with 0.1 U of T4 DNA ligase (Maniatis *et al.*, 1982).

Protoplasts of *C. glutamicum* RM3 were transformed with pJUFt3 by the method of Yeh *et al.* (1986).

The number of copies of plasmid DNA per chromosome were determined as described by Taylor & Brose (1988).

DNA hybridization analysis. The *Xba*I-*Kpn*I DNA fragment from plasmid pJUF1 carrying promoter activity and the *Kpn*I-*Pvu*II fragment carrying the transcription-terminator were labelled with [α -³²P]dCTP by the nick-translation procedure of Rigby *et al.* (1977) and used as a probe for Southern hybridization analysis. Phage BFK20 DNA (0.5 mg), both digested with restriction nucleases and not digested, was separated in a 0.8% agarose gel. After electrophoresis, DNA was transferred onto a nitrocellulose membrane by the procedure of Southern (1975). Prehybridization, hybridization and stringent washing procedures were as described by Maniatis *et al.* (1982).

NPTII assay. Neomycin-phosphotransferase (NPTII) was assayed by the method of Cabanes-Bastos *et al.* (1989). *C. glutamicum* and *E. coli* were grown in 100 ml of LB medium to OD₅₇₀=0.8 and 0.6 respectively. The cells were harvested and the pellets were washed and suspended in 10 mM-Tris/HCl, pH 7.5, 10 mM-MgCl₂, 25 mM-NH₄Cl, 0.6 mM-mercaptoethanol. After sonication, the crude extract was obtained by centrifugation at 100000 g for 1 h. Crude enzyme was precipitated by addition of 60% (w/v) ammonium sulphate to the supernatant, collected by centrifugation, dissolved in the minimum volume of buffer as above, and dialysed against the same buffer. A 10 µl volume of this solution was used for assay of NPTII.

Results and Discussion

Morphology and host spectrum of phage BFK20

Bacteriophages isolated previously from *Brevibacterium* and *Corynebacterium* strains have belonged to Bradley's group B (Bradley, 1967): they have a polyhedral head 40–70 nm wide and a tail 100–300 nm long (Hongo *et al.*, 1984; Ozaki *et al.*, 1984; Pátek *et al.*, 1985; Trautwetter *et al.*, 1987a, b; Trautwetter & Blanco, 1988). Phage BFK20 looked, on electron micrographs (Fig. 1), like phages of Bradley's group B, since it has a 50-nm-wide polyhedral head and a non-contractile tail 200 nm long and 10 nm wide.

Of five strains of brevibacteria examined (*B. flavum* ATCC 21474, *B. flavum* CCM 251, *B. flavum* ATCC 21127, *B. lactofermentum* BLOB and *C. glutamicum* ATCC 13032), only *B. flavum* CCM 251 was sensitive to phage BFK20. Titres of about 8×10^8 p.f.u. ml⁻¹ were routinely obtained.

Fig. 1. Electron micrograph of phage BFK20. Bar, 200 nm.

Fig. 2. SDS-PAGE of the structural polypeptides of phage BFK20. The acrylamide gel (10%, w/v) was stained with silver. Lane 1, molecular mass markers: phosphorylase b (92.5 kDa), bovine albumin (67.0 kDa), egg albumin (45.0 kDa), carbonic anhydrase (29.0 kDa). Lane 2, molecular mass markers: carbonic anhydrase, (29.0 kDa), trypsin inhibitor (21.0 kDa), cytochrome c (12.5 kDa). Lane 3, purified BFK20.

Fig. 3. Restriction map of the phage BFK20 chromosome. Sizes of the restriction fragments are shown in Table 1: Pv, *Pvu*I; Bg, *Bgl*II; Ec, *Eco*RI; Xb, *Xba*I. f₁, Fragment carrying promoter activity.

Fig. 4. Mapping of the 'cos' sequence of phage BFK20. Lanes PP', BB' and PvPv' represent BFK20 DNA digested with *Pst*I, *Bam*HI and *Pvu*I, respectively. Samples in lanes P, B and Pv were loaded directly onto an agarose gel; samples in lanes P', B' and Pv' were loaded after heating for 15 min at 72 °C in 6% (w/v) sucrose and 44 mM-EDTA, and rapidly cooling on ice. In each case heating produced new fragments (A₁, A₂; B₁, B₂; C₁, C₂), while one restriction fragment (A, B, C) disappeared or was reduced in intensity.

Structural polypeptides of BFK20

The polypeptides of CsCl-purified phage BFK20 were analysed by SDS-PAGE (Fig. 2). The capsid of phage BFK20 contained polypeptides with molecular masses of 22.0, 25.1, 33.3, 50.1, 58.4, 68.1, 85.7, 92.5 and 108.0 kDa. The 50.1 kDa protein appeared in very high amounts, apparently being the constituent of a major structural phage component(s).

Genome organization of phage BFK20

The genome of bacteriophage BFK20 is a double stranded DNA molecule with cohesive ends. As determined by electron microscopy of the phage DNA (data not shown) and by summing the sizes of the restriction fragments produced by each of the restriction endo-

nucleases the genome size was 44–45 kb. The restriction map of bacteriophage BFK20 DNA was constructed according to the results of (i) the single, double and triple digestion analysis with restriction endonucleases, (ii) partial digestion analysis of BFK20 DNA and (iii) the determination of homology between various single or partially digested BFK20 DNAs and ³²P-labelled DNA fragments from the same phage. The restriction map of phage BFK20 DNA is shown in Fig. 3. The circularization of DNA is apparently a result of annealing of cohesive ends since the circular configuration is unstable and is suppressed by heating in buffered sucrose (Fig. 4). In each of the three restriction digests tested, heating provoked disappearance of one of the restriction fragments (A, B or C) and gave rise to two new fragments (A₁, A₂; or B₁, B₂; or C₁, C₂), which together accounted for the missing bands.

Isolation of a phage BFK20 DNA fragment carrying a promoter

Using a promoter probe vector we isolated DNA fragments carrying promoter activity. *Sau*3A fragments from phage BFK20 DNA were cloned into plasmid pJUP05 digested with *Bam*HI and dephosphorylated. *E. coli* HB101 cells were transformed with the ligation mixture. From the positive clones which grew on LB media containing kanamycin (100 mg l⁻¹) we isolated plasmid pJUF1. This plasmid was a derivative of the vector pJUP05 carrying a 0.3 kb inserted fragment. The strength of fragment f₁ and the original promoter of Km^R was determined semi-quantitatively. The relative strength of the promoter was calculated from the minimal inhibitory concentration (MIC) of Km and the number of copies of the plasmids in cells of *E. coli* HB 101. The results are shown in Table 1. The Km gene from transposon Tn5 with its own promoter is situated on plasmid pJU2. Each of the two promoters, the original promoter of the Km^R gene and the promoter from fragment f₁ worked effectively in *E. coli*. The highest expression in *E. coli* was achieved by the promoter from fragment f₁. The localization of fragment f₁ on the DNA of bacteriophage BFK20 (Fig. 3) was determined using Southern hybridization.

C. glutamicum strain RM3 was transformed with plasmid pJUF1. Only deleted derivatives, bearing a deletion in the *Sal*I fragment of plasmid pJUF1, could be obtained from the transformed clones. These results show structural instability of plasmid pJUF1, probably caused by the influence of the strong promoter situated in front of the Km^R gene.

Introducing the F1 promoter into *C. glutamicum*

It is known from previous work that cloning of strong promoters can be made possible by downstream replacement of a RNA-termination signal (Gentz *et al.*, 1981). Cadenas *et al.* (1991) and Bartone & Blanco (1991) have shown that transcription-terminators which work efficiently in *E. coli* do not do so in corynebacteria. For this reason we used as a transcription-terminator donor phage BFK20 DNA. We introduced *Sau*3A fragments into the *Bgl*III site upstream of the Km^R gene. Transformants of *C. glutamicum* RM3 were selected on BHI plates supplemented with Cm (8 mg l⁻¹). From clones which grew on LB plates supplemented with Km (70 mg l⁻¹) but failed to growth on LB plates supplemented with Km (80 mg l⁻¹), we isolated plasmid pJUFT3, which is a derivative of pJUF1 with a 0.2 kb inserted fragment. Plasmid pJUFT3 was introduced into *E. coli* HB101. The MIC of Km for *E. coli* HB101 clones harbouring plasmid pJUFT3 decreased from 1000 mg l⁻¹ to 70 mg l⁻¹.

Table 1. Relative strength of promoters in *E. coli* HB101

N, copies of plasmids per chromosome; *S*, relative strength of promoter (MIC/*N*).

<i>E. coli</i> HB101 with plasmid:	MIC (mg l ⁻¹) of kanamycin									<i>N</i>	<i>S</i>
	50	100	200	300	400	600	700	800	1000		
pJUP05	—	—	—	—	—	—	—	—	—	40	0
pJU2	+	+	+	+	+	+	+	+	—	47	17
pJUF1	+	+	+	+	+	+	+	+	+	38	>26

Table 2. Expression of NPTII (neomycin-phosphotransferase) in *E. coli* and *C. glutamicum*

—, No clones harbouring plasmid pJUF1 were detected.

Plasmid	NPTII specific activity (pmol min ⁻¹ mg ⁻¹)	
	<i>E. coli</i>	<i>C. glutamicum</i>
None	0	0
pJUF1	210	—
pJUFT3	14	4.5

The specific activity of neomycin-phosphotransferase in *E. coli* HB101(pJUF1, pJUFT3) and *C. glutamicum* (pJUFT3) is shown in Table 2.

Hybridization experiments located terminator T3 on a 9.1 kb *Xba*I fragment of the phage restriction map.

Our data show that sequences recognized by the RNA polymerase of corynebacteria as promoters and transcription-terminators work in both corynebacteria and *E. coli*. However, promoter F1 seems to be stronger in *C. glutamicum* than in *E. coli*, indicating certain differences in the recognition specificity of *E. coli* and corynebacterial RNA polymerase.

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