Acta virologica 58: 152 – 159, 2014 doi:10.4149/av_2014_02_152

Immunodetection and N-terminal sequencing of DNA replication proteins of bacteriophage BFK20 – lytic phage of *Brevibacterium flavum*

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Received September 6, 2013; accepted May 19, 2014

Summary. – Phages are excellent models for studying the mechanism of DNA replication in prokaryotes. Identification of phage proteins involved in phage DNA replication is the first prerequisite for elucidation of the phage replication module. We focused on replication proteins gp41 (a putative helicase from SF2 superfamily), gp43 (a RepA-like protein), and gp44 (a putative DNA polymerase A) of phage BFK20 grown in *Brevibacterium flavum*. To identify them in the phage-host system, we prepared antibodies to these proteins which were cloned and expressed in *Escherichia coli* as his-tagged recombinant proteins. After purification to homogeneity the recombinant proteins served for raising specific polyclonal antibodies in mice. Using these antibodies in Western blot analysis the phage proteins gp41, gp43 and gp44 were detected during the phage growth cycle. The proteins gp41 and gp43, prepared from cell lysate by ammonium sulphate precipitation, were N-terminally sequenced and found to contain the sequences *N*-SVKPRELR-*C* and *N*-MLGSTML-*C*, respectively. This means that gp41 starts with serine but not with common methionine. We consider these findings an initial but important step towards more thorough characterization of replication proteins of phage BFK20.

Keywords: phage BFK20; Brevibacterium flavum; DNA replication; proteins; immunodetection; N-terminal sequence

Introduction

DNA replication is highly coordinated dynamic process where several protein complexes are linked together forming an active replisome (Roucourt and Lavigne, 2009). Bacteriophages use different mechanisms to replicate their genomes and most of them exploit several hosts' replication proteins for genome replication (Giraldo, 2003). The replication mechanism of particular bacteriophage depends on its own replication machinery and on its ability to recruit replication proteins from its bacterial host. Bacteriophage genomes can code for several families of initiator proteins, primases, helicase loaders, helicases, and DNA polymerases. Thorough report on the known replication proteins and mechanisms of bacteriophages has been provided by Weigel and Seitz (2006a,b).

The bacteriophage BFK20 is lytic phage of industrial strain *Brevibacterium flavum* CCM 251, L-lysine producer. BFK20 morphologically belongs to taxonomical group of unclassified *Siphoviridae* (Koptides *et al*., 1992) and it is the first corynephage with an entire sequenced and annotated genome. Fifty-four potential ORFs were identified on the phage genome (EMBL AJ278322, Bukovska *et al*., 2006) and the expression profile of individual ORFs was determined by using DNA microarray (Majtan *et al*., 2007). The genome organization of the phage has structure analogous to those of many phages of the *Siphoviridae* family (Brüssow and Desiere, 2001). It is composed of structural, lytic, DNA replication and transcriptional regulation modules. The research on BFK20 is now focused on the study of phage lytic and replication proteins and on elucidation of phage

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Abbreviations: gp = gene product; IMAC = immobilized-metal affinity chromatography; p.i.= post infection; WBA = Western blot analysis

host interactome. The biological function of endolysin gp24´ and its catalytic and binding domains was proved recently (Gerova *et al*., 2011). Three of the phage early proteins were previously identified by bioinformatic approach as putative helicase from superfamily SF2 (gp41), RepA-like protein with prim-pol domain and an SF4-type helicase domain (gp43) and putative DNA polymerase A (gp44). The genes ORF41, ORF43, and ORF44 were cloned and corresponding recombinant proteins have been studied and characterized. Gp43N is the deletion derivative of protein gp43 with primase and DNA polymerase activities and belongs to new archaeo-eukaryotic primase-polymerase group (Halgasova *et al*., 2012). Studies on biological functions of putative helicase gp41, the helicase domain of gp43 and exonuclease and polymerase domains of gp44 are on-going.

Characterization of replication proteins is important for elucidation of the BFK20 primosome and replisome structures and explanation of the phage DNA replication mechanism. The study of phage replication proteins, isolated from the host cell lysate, is difficult due to their limited amount. Moreover, the bacterial host replication proteins are present in lysate as well.

This study was aimed at preparation of specific antibodies against phage BK20 DNA replication proteins gp41, gp43 and gp44, which were cloned and expressed in *Escherichia coli*. Using these antibodies the replication proteins in cell lysate were identified and their N-terminal sequences were determined.

Materials and Methods

Phage and bacteria. Bacteriophage BFK20 was propagated in the strain *Brevibacterium flavum* CCM 251 (hse^-, Aec^r) industrial producer of L-lysine according to Halgasova *et al*. (2005). Purification of phage DNA from phage particles was performed according to Sambrook and Russell (2001). *E. coli* XL1 Blue (Stratagene) was used for cloning experiments and *E. coli* BL21(DE3) (Novagen) for the expression of recombinant proteins. Bacterial strains were grown in LB medium, *B. flavum* CCM 251 at 30°C and *E. coli* at 37°C.

Plasmid constructs. The purified genomic DNA of the phage was used as template for PCR amplification of the gene sequences of gp41 and gp44, which were cloned into the expression vector pET28a+ (Novagen). Plasmid for expression of recombinant protein gp43N was prepared as deletion derivative according to Halgasova *et al*. (2012). The primers used for preparation of the phage genes, the expression plasmids and expected protein products are listed in Table 1. All constructs were verified by sequencing using an eight-column capillary ABI 3100-Avant Genetic Analyser (Applied Biosystems).

Expression and purification of recombinant proteins. The recombinant proteins gp41HN, gp43N and gp44N were expressed as his-tagged proteins in *E. coli*. Cells were induced at A600 = 0.5 with 0.5 mmol/l IPTG and each protein was expressed by incubation for 3 hr at 37°C. Protein gp43N was expressed mostly in soluble fraction of cell lysate and protein isolation was performed using IMAC according to Halgasova *et al*. (2012). The purified gp43N was dialysed against 20 mmol/l sodium phosphate (pH 7.0), 0.1 mmol/l EDTA, 40% (v/v) glycerol.

Proteins gp41HN and gp44N were expressed mostly in insoluble fraction of cell lysate and were isolated from inclusion bodies according to Singh and Panda (2005) with minor modifications. The overnight incubation after the second sonication was shortened to 1 hr and the last 30 min incubation in Tris-HCl buffer to 10 mins. Purified inclusion bodies of gp41HN or gp44N, were solubilised via incubation in 1.8 ml of 8 mol/l urea, 20 mmol/l Tris-HCl (pH 8.5), 1 mmol/l EDTA for 1hr at 37 °C and then proteins were renaturated by dialysis against 2 mol/l urea, 50 mmol/l Tris-HCl (pH 8.0), 0.5 mmol/l EDTA, 10% glycerol, 5% sucrose for 16 hr. The protein gp41HN was stored in renaturation buffer and gp44N in 20 mmol/l Tris-HCl pH 8.0, 150 mmol/l NaCl, 40% glycerol.

Table 1 Strategy for expression of recombinant phage proteins

ORF	PCR primers (5'–3')	Restr. site	Recombinant plasmid/ recombinant protein (M_r)	Phage protein (M_r)
41	F: TC**GAATTC**ATGAGTGTGAAGCC	*Eco*RI	pET28-41HN/	
	R: TTC**GTCGAC**ACATAGCGTTCATC	*Sal*I	**gp41HN (61.7 K)**	gp41 (57.9 K)
43	F: TC**GAATTC**ATGCTCACAACCTT	*Eco*RI	pET28-43-1/	
	R: TC**GTCGAC**GAACTAAGGTCGAG	*Sal*I	gp43-1 (111.3 K)	gp43 (108.0 K)
		*Xho*I	pET28-43N/	
			gp43N (45.5 K)	
44	F: CATATG**GCTAGC**ATGACCGACCCTAT	*Nhe*I	pET28-44N/	
	R: GCTC**GAATTC**TCAGCAATATTCCCA	*Eco*RI	**gp44N (72.7 K)**	gp44 (70.2 K)

Forward (F) and reverse (R) primers used for PCR amplification of DNA fragments containing ORF41, ORF43, and ORF44 are summarized. Restriction enzymes used for cloning of particular ORFs are listed in the table and their positions in primers are in bold. The *Xho*I restriction site indicates the digestion which was used for preparation of expression plasmid pET28-43N for expression of gp43-1 deletion derivative, the protein gp43N. The expressed recombinant proteins and their phage partners with corresponding relative molecular masses are listed in the table. Recombinant proteins isolated by immobilized metal affinity chromatography and used for preparation of antibodies are in bold.

The isolated proteins were quantified spectrophotometrically using calculated extinction coefficient.

Western blot analysis. SDS-PAGE was performed on 8 or 10% gel according to Laemmli (1970). His-tagged proteins were detected by Western blot analysis using His-tag monoclonal antibody (Novagen) and goat anti-mouse immunoglobulin G alkaline phosphatase conjugate (Novagen) as secondary antibody. For detection of phage proteins, the polyclonal antibodies against proteins gp41HN, gp43N, and gp44N prepared in this work were used as primary antibodies.

Preparation of specific antibodies to recombinant proteins. The isolated recombinant proteins gp41HN, gp43N, and gp44N were used for immunization of inbred mice C57BL/6. The protein gp41HN was quite unstable and prone to precipitate. Therefore, both the soluble and the insoluble corpuscular forms of gp41HNs and gp41HNc were used for immunization, respectively. The immunogens were prepared according to Thönes *et al*. (2008) using 500 μg of $Al(OH)_3$ gel (Sigma-Aldrich), 50 μg of Sigma Adjuvant System (Sigma-Aldrich) and 50 μg of protein. For immunization, four groups of three to four weeks old mice C57BL/6 (female) were formed with six to seven subjects in each group. Each group was immunized with one of the proteins (gp41HNs, gp41HNc, gp43N, gp44N) three times at ten-day intervals with 50 μg of protein per mouse weighing 20–25 g. Ten days after the last immunization, mice were anesthetized by pentobarbital, the sera were collected and by combining sera from individual subjects in each group four mixed sera with different types of antibodies were created. Plasma was stored at -20°C.

Dot blot analysis. Titer of antibodies against individual replication proteins was determined using dot blot analysis with HRP-conjugated secondary antibody according to protocol Version D 080101 28-0138 (Invitrogen), with some modifications. Two μl of protein samples gp41HNs, gp41HNc, and gp44N (all about 3 mg/ml) or gp43N (about 6 mg/ml) were spotted onto 10 nitrocellulose paper strips. One ml of serially diluted antibodies in blocking buffer was added to each strip. The negative controls represented strip without antibodies. As the secondary antibody, goat anti-mouse IgG conjugated to HRP (Sigma-Aldrich) diluted 1:500 was used.

Detection of phage proteins by Western blot analysis. The host strain *B. flavum* CCM 251 was infected with phage at MOI = 10. After infection, 50 ml of samples were taken after 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, and 220 min. Cell lysates from samples of the early period of infection (10–100 min) were prepared from pelleted cells by resuspending them in 0.5 ml of lysis buffer (20 mmol/l Tris-HCl pH 8.0, 150 mmol/l NaCl, lysosyme 5mg/ml), incubated 20 min at 30°C and then 5 μl of Protease inhibitor cocktail (Sigma-Aldrich) and 10 μg of DNase I from Bovine pancreas (Serva) were added. Cell lysates were prepared by sonication 7 times for 10 sec. As negative control, cell lysates prepared from non-infected cells by the same procedure was used. Cell lysates of samples from late period of infection (100–220 min) were prepared by sonication of taken aliquots 4 times for 10 sec and centrifugation to remove the cell debris. The proteins in the supernatants were precipitated by adding saturated solution of ammonium sulphate (called 100 % solution in terms of the saturation) to final 40% percent saturation in the sample. After 16 hr at 0°C, the precipitated proteins were pelleted by centrifugation (2,700 g, 30 min, 4°C) and dissolved in 70 μl of 20 mmol/l Tris-HCl (pH 7.5). The aliquot from non-infected culture was treated before sonication with lysosyme at final concentration of 0.5 mg/ml for 60 mins at 37°C. Phage proteins in cell lysates were detected by Western blot analysis.

Preparation of phage proteins for N-terminal sequencing. The 100 ml cell culture of *B. flavum* CCM 251 was harvested after 60 mins after phage infection. The pellet was resuspended in 1 ml of 20 mmol/l Tris-HCl (pH 7.5) supplemented with 10 μl of Protease inhibitor cocktail (Sigma-Aldrich) and 20 μg of DNase I from Bovine pancreas. After sonication 12 times for 20 sec, the cell debris was removed by centrifugation (20,000 g, 20 min, 4°C). Proteins in supernatant were selectively precipitated by adding saturated

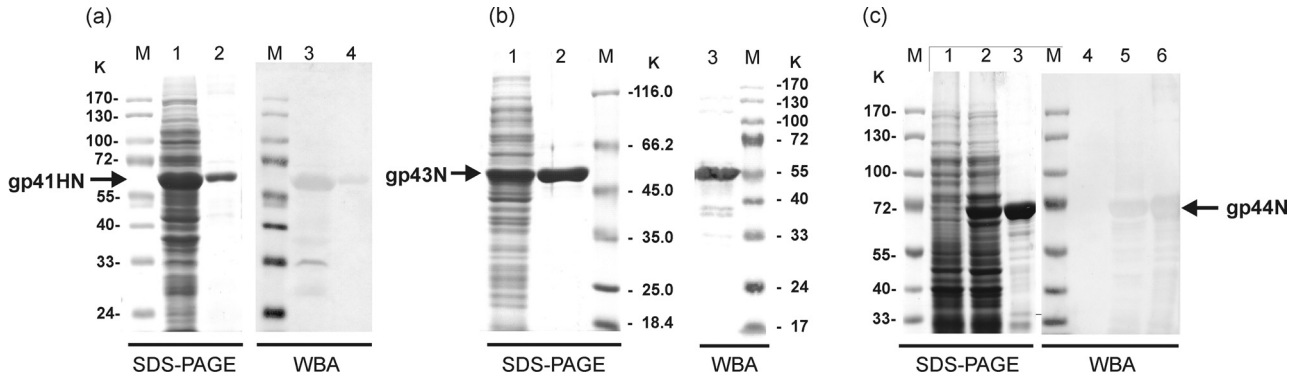

Fig. 1
Purification of recombinant phage proteins
Proteins were separated by SDS-PAGE (left lanes) and detected by Western blot analysis (right lanes). (a) gp41HN in cell lysate (lanes 1 and 3) and after purification (lanes 2 and 4). (b) gp43N in cell lysate (lane 1) and after purification (lanes 2 and 3). (c) gp44N in cell lysate of non-induced (lanes 1 and 4) and induced cells (lanes 2 and 5), and after purification (lanes 3 and 6). Protein size marker (lanes M).

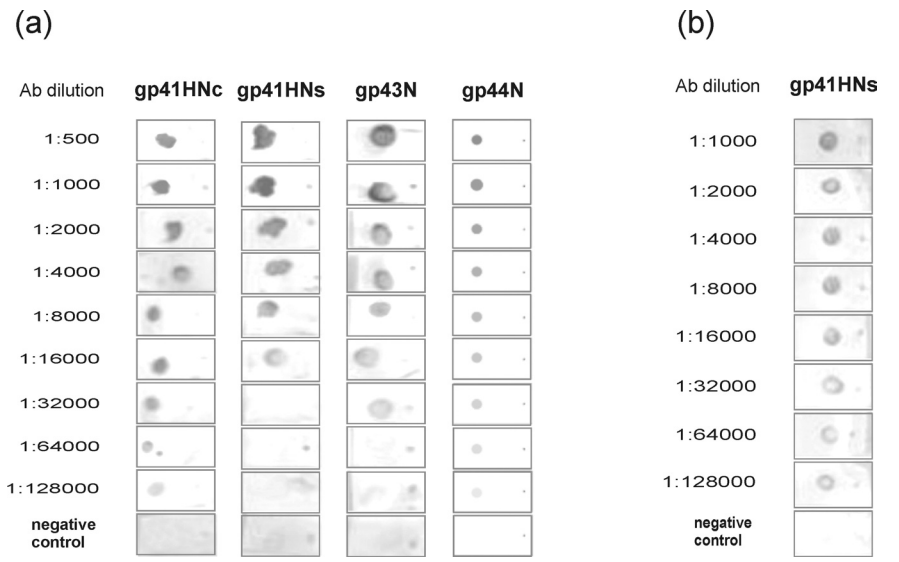

Fig. 2
Titration of antibodies to recombinant phage proteins
Titers were determined using the dot-blot method and homologous (a) and heterologous (b) antibodies. Heterologous antibody to gp41HNc was used for detection of gp41HNs.

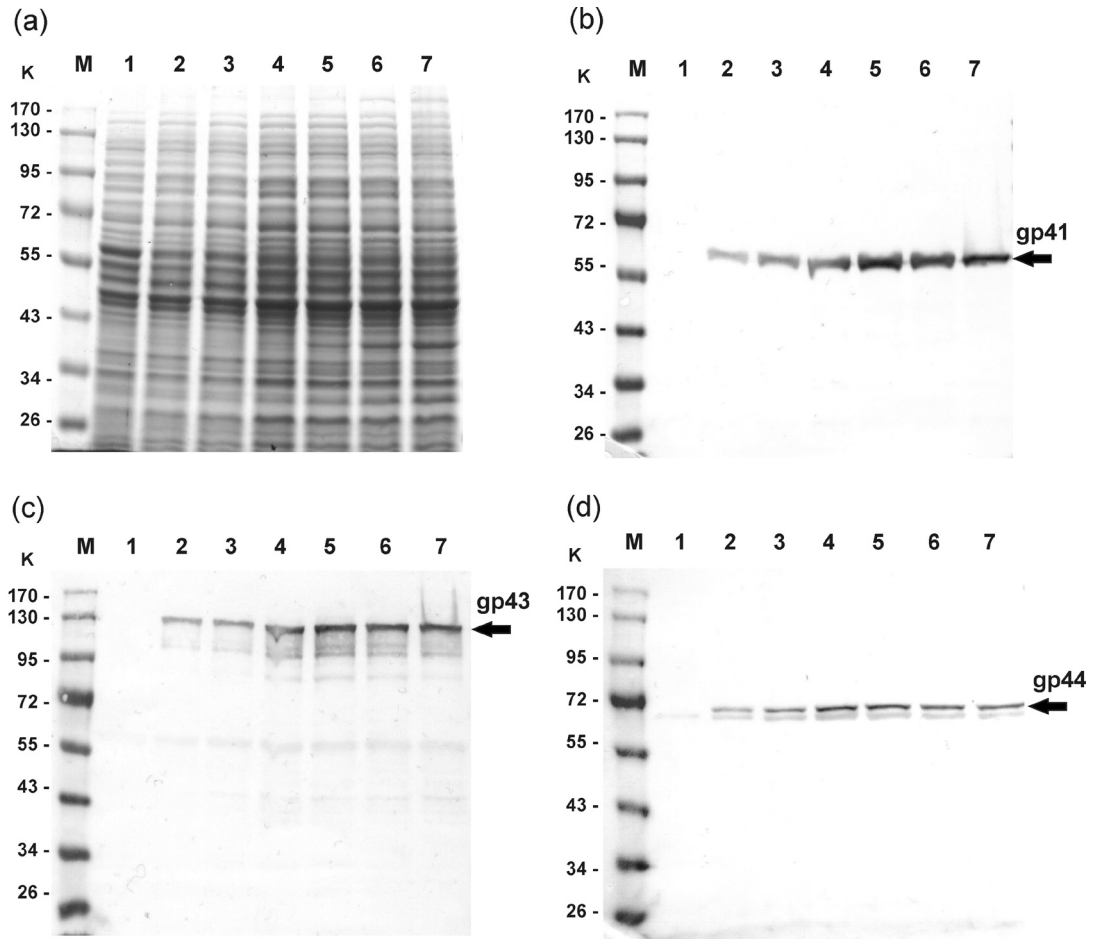

Fig. 3
Detection of phage proteins in early stages of infection
SDS-PAGE of cell lysates prepared from non-infected culture (lane 1) and from 10, 20, 40, 60, 80, and 100 min p.i. (lanes 2–7) (a). Detection of gp41 (b), gp43 (c) and gp44 (d) by Western blot analysis. Protein size marker (lanes M).

solution of ammonium sulphate up to final concentration of 40% percent in the sample. After incubation for 20 min at 0°C, the precipitated proteins were pelleted by centrifugation (20,000 g, 20 min, 4°C) and then resuspended in 150 µl of 20 mmol/l Tris-HCl (pH 7.5). Aliquots were probed by Western blot analysis using antibodies against gp41HN and gp43N. The proteins were separated by SDS-PAGE, transferred to PVDF membrane and the marked proteins were subjected to an automated Edman degradation sequencing (Department of Protein Biochemistry, IOCB CAS).

Results

Preparation of recombinant phage proteins

The recombinant protein gp43N with His-tag sequence on both the N- and C-termini was purified from soluble fraction of cell lysate using metal affinity chromatography. Gp41HN and gp44N are N-termini His-tagged proteins. Both proteins were expressed mostly in insoluble fraction of cell lysate and isolated from inclusion bodies. The presence of the His-tag sequence on the expressed proteins was confirmed by Western blot analysis (Fig. 1a–c).

Preparation of specific antibodies to recombinant phage proteins

The purified proteins were used as immunogens for s.c. immunization of C57BL/6 mice and specific antibodies were isolated from mouse plasma. Antibody titers against individual proteins (gp41HNc, gp41HNs, gp43N, and gp44N) were determined by dot-blot analysis. The titers against corpuscular (c) and soluble (s) forms of gp41HN were 1:128,000 and 1: 16,000, respectively. The titer against gp43N was 1:32,000 and against gp44N reached 1:128,000. Results of dot blot analysis matching the reactions of used replication proteins with serially diluted partner antibodies are shown in Fig. 2a. The cross-reactions among the replication proteins and heterologous antibodies, which were isolated from mice immunized by other proteins, gave negative results. Only the reaction of soluble gp41HNs with antibodies against corpuscular gp41HNc was positive with the titer of 1:128,000 (Fig. 2b). This is explained by the fact that gp41HNc and gp41HNs are two forms of the same protein.

Detection of phage proteins using specific antibodies

The BFK20 growth cycle reached the end after 220 min as determined previously by Majtan *et al.* (2007), so the cell lysates were prepared from aliquots of samples taken periodically from 10 to 220 min post infection (p.i.) (Fig. 3a, Fig 4a). The presence of phage proteins in cell lysate during the phage growth cycle was determined by Western blot analysis using specific antibodies. The phage proteins gp41, gp43, and gp44 were already detected 10 min p.i., which is the immediate early stage of the phage growth cycle (Fig. 3b–d, lane 2). The most intensive bands for gp44 appeared 40 and 60 min p.i. (Fig. 3d, lane 4 and 5) and for gp41 (Fig. 3b, lane 5) and gp43 (Fig. 3c, lane 5) 60 min p.i. Interestingly, the immunopositive bands were detected 80 to 220 min p.i., which is late stage of phage development (Fig. 4). The sizes of the immunodetected proteins corresponded to the predicted sizes of proteins gp41: 57.9 K, gp43: 108.0 K, and gp44: 70.2 K, respectively.

Preparation of phage proteins gp41 and gp43 for N-terminal sequencing

The most intensive bands of immunodetected proteins gp41 and gp43 were revealed at 60 min of the phage growth cycle (Fig. 3). Therefore, for preparation of proteins for N-terminal sequencing, the cell lysate of *B. flavum* from 60 min p.i. of BFK20 cycle was used. Samples after ammonium sulphate precipitation were separated by SDS-PAGE and probed by Western blot analysis using antibodies against gp41 and gp43 (Fig. 5a,b). The proteins precipitated at concentration of 40% ammonium sulphate were separated in 8 and 10% polyacrylamide gel and transferred to PVDF membrane. The proteins gp41 and gp43 were submitted to N-terminal amino acid sequencing and found to contain the sequences *N*-SVKPRELR-*C* and *N*-MLGSTML-*C*, respectively. This means that gp41 starts not with common methionine but with serine.

Discussion

Bacteriophage proteome is composed of specified proteins - structural, lytic, replication and regulatory. The level of phage proteins in the host cells varies during the bacteriophage growth cycle and isolation of individual phage proteins can be difficult. In this study, we prepared specific antibodies against the recombinant phage replication proteins and used them for detection of the individual phage proteins. Using the pET expression system, we expressed and isolated three replication proteins of phage BFK20: gp41 – putative helicase from superfamily SF2, gp43 – RepA-like protein with prim/pol domain (gp43N) and SF4-type helicase domain and gp44 – DNA polymerase A. The recombinant proteins gp41HN and gp44N were expressed in *E. coli* cells mostly in insoluble form and both proteins were isolated from inclusion bodies. On the other hand, the protein gp43N was expressed in soluble form and we isolated this protein by IMAC to homogeneity (Halgasova *et al.*, 2012). The purified recombinant proteins gp41HN, gp43N, and gp44N were used as immunogens for s.c. immunization of C57BL/6

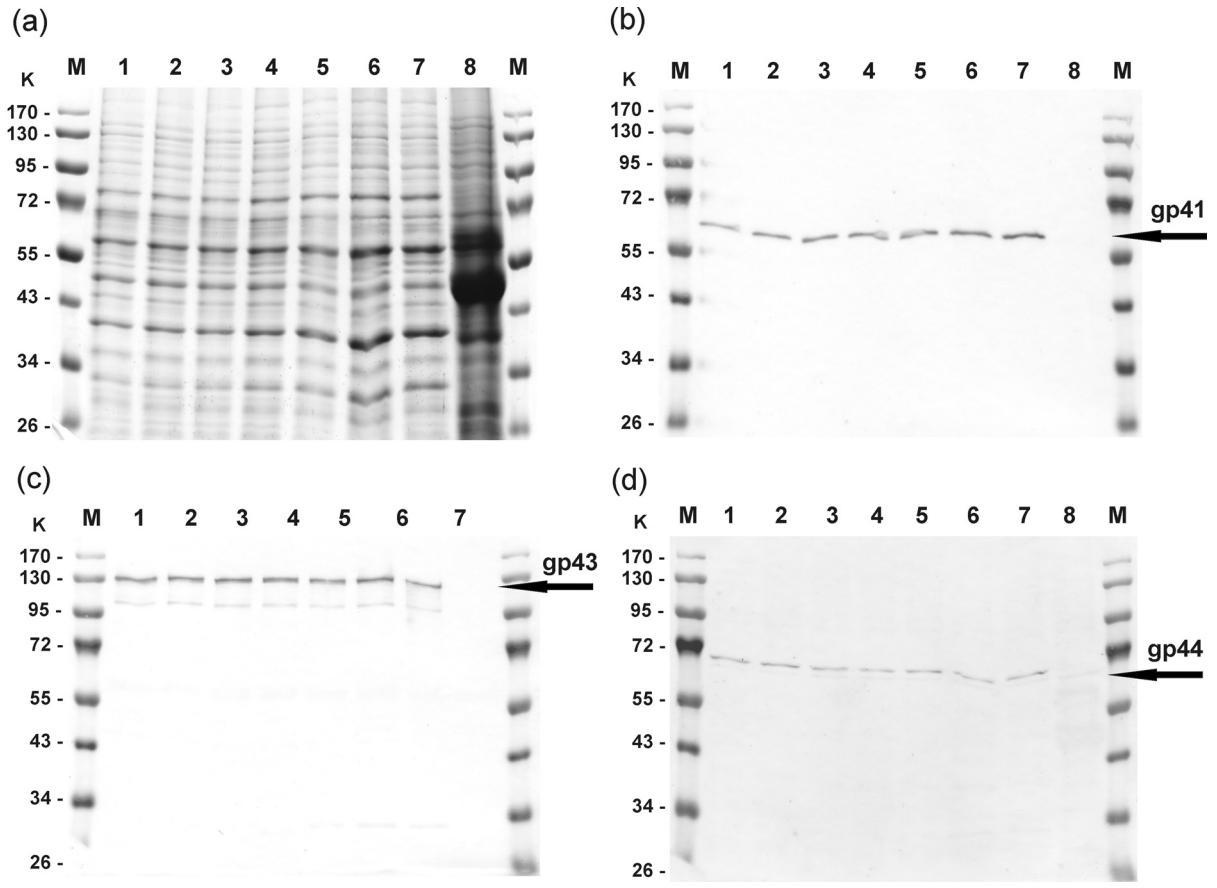

Fig. 4
Detection of phage proteins in late stages of infection
SDS-PAGE of cell lysates prepared from 100, 120, 140, 160, 180, 200 and 220 min p.i. (lanes 1–7) and from non-infected culture (lane 8) by precipitation at concentration of 40% ammonium sulphate (a). Detection of gp41 (b), gp43 (c) and gp44 (d) by Western blot analysis. Protein size marker (lanes M).

mice and the polyclonal antibodies were prepared according the method of Thönes *et al*. (2008). Authors compensated the lower immunogenicity of capsomeres of virus like particles with an adjuvant system containing 3-O-desacyl-4´-monophosphoryl-lipid *A* adsorbed to aluminium hydroxide. In our experiment we tried to increase immunogenicity of recombinant phage proteins also by using the similar Adjuvant System®. Moreover, the corpuscular proteins are more immunogenic than soluble ones and such proteins induce stronger antibody response (James, 1989). The higher immunogenicity of the corpuscular form of protein gp41HNc compared to the soluble form gp41HNs was confirmed by our results. The antibody prepared against gp41HNc reacted with both forms of protein gp41HNc and gp41HNs with the titer of 1:128,000, whereas the titer of antibody prepared against the soluble antigen gp41HNs was 1:16,000. To summarize, the polyclonal antibodies prepared against recombinant replication proteins gp41HN, gp43N, and

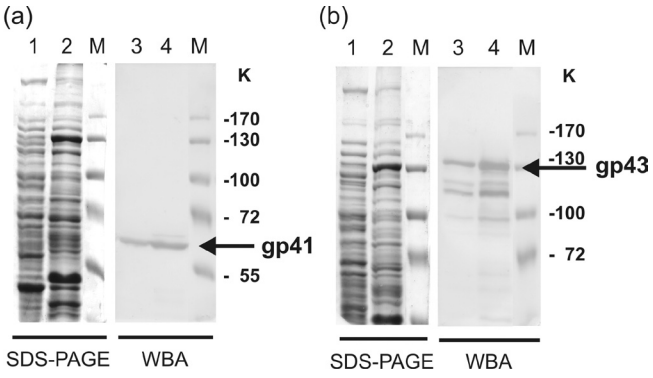

Fig. 5
Preparation of phage proteins gp41 and gp43 for N-terminal sequencing
Proteins were separated by SDS-PAGE (left lanes) and detected by Western blot analysis (right lanes). Soluble gp41 (lanes 1 and 3) and gp41 precipitated at concentration of 40% ammonium sulphate (lanes 2 and 4) (a). Soluble gp43 (lanes 1 and 3) and gp43 precipitated at concentration of 40% ammonium sulphate (lanes 2 and 4) (b). Protein size marker (lanes M).

gp44N were specific, with high titer and active against phage proteins.

By using the specific polyclonal antibodies, we detected the presence of phage proteins in cells of *B. flavum* during the phage growth cycle. Proteins gp41, gp43, and gp44 were present not only during the early stage of phage development, but also at the beginning of the late stage. According to our previous results (Majtan *et al.*, 2007) the transcripts of early genes of ORF40–ORF46 first appeared between 3 and 7 min p.i. of the phage growth cycle and expression of ORF41 and ORF42 lasted until 120 min. The gene expression patterns of ORF40–ORF42 showed the presence of two peaks as well, 15 and 40 min p.i. of the phage growth cycle. Obviously, the expression of immediate early gene transcripts is not strictly terminated after the switch to early and late gene transcription. In our experiments, the phage proteins gp41, gp43, and gp44 were detected by Western blot analysis not only in the immediate early stage at 10 min of the phage growth cycle, but also in samples from 220 min p.i. which is the late stage of phage cycle. Probably the replication proteins, mainly gp41, could be involved not only in phage DNA replication processes, but also in others such as phage recombination, repair or regulation of replication origin. Similar properties were also shown by phage T4 helicase UvsW (Carles-Kinch *et al.*, 1997).

The estimated sizes of the immunodetected proteins corresponded to the predicted ones. However, by using the antibody against gp43, we also observed minor bands on immunoblot. This degradation could be due to presence of proteolytic enzymes in the sample or by autoproteolytic activity. Similar degradation appeared in samples of the recombinant variants of protein gp43. Moreover, the pattern of minor bands was similar in several samples so we assumed that autoproteolytic degradation of this protein occurred.

We determined N-terminal amino acid sequence of two proteins gp41 and gp43. The first amino acid of gp41 is serine, not methionine. This is probably the result of the host methionine aminopeptidase activity (Lowther and Matthews, 2000). Also structural protein gp12 of BFK20 starts with serine as was determined previously (Bukovska *et al.*, 2006). During the phage growth cycle, the expression of the phage protein gp44 was lower compared to those of gp41 and gp43. Therefore, its identification was difficult. Moreover, in all samples of cell lysates including the non-infected negative control, protein band overlapping the gp44 was present. Probably this protein originated from the host *B. flavum* and blocked us from identifying the gp44 and determination of the N-terminal sequence.

In conclusion, we expressed and isolated three BFK20 replication proteins using the pET expression system. Recombinant proteins were used as vaccines for immunization of inbred mice and polyclonal specific antibodies against these proteins were isolated from mouse plasma. These antibodies served for detection of phage replication proteins during the phage growth cycle. Characterization of phage replication proteins in their original form is the first step in elucidation of phage replisome composition.

Acknowledgements. The authors thank Dr. V. Kery (Agensys, Inc., CA, USA) for helpful comments. This study was supported by the research grants VEGA 2/0110/11 and APVV-0354-07.

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