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Research Article

T5-like phage BF23 evades host-mediated DNA restriction and methylation

Mikhail Skutel[1,†], Aleksandr Andriianov[1,†], Maria Zavialova[1,2], Maria Kirsanova[1], Oluwasefunmi Shodunke[1,3], Evgenii Zorin[1], Aleksandr Golovshchinskii[4], Konstantin Severinov[5], Artem Isaev[1,*]

[1]Skolkovo Institute of Science and Technology, Bolshoy Boulevard 30/1, 143028, Moscow, Russia
[2]Institute of Biomedical Chemistry (IBMC), Pogodinskaya 10/8, 119435, Moscow, Russia
[3]Moscow Institute of Physics and Technology, Institutskiy Pereulok 9, 141701, Dolgoprudny, Russia
[4]School of Pedagogical Skills Center, Olympiyskiy Prospect 11/1, 129272, Moscow, Russia
[5]Waksman Institute of Microbiology, 190 Frelinghuysen Rd, NJ 08854, Piscataway, United States
*Corresponding author. Skolkovo Institute of Science and Technology, Bolshoy Boulevard 30/1, 143028, Moscow, Russia. E-mail: Artem.Isaev@skoltech.ru
†Mikhail Skutel and Aleksandr Andriianov contributed equally to this work
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Abstract

Bacteriophage BF23 is a close relative of phage T5, a prototypical *Tequintavirus* that infects *Escherichia coli*. BF23 was isolated in the middle of the XXth century and was extensively studied as a model object. Like T5, BF23 carries long ~9.7 kb terminal repeats, injects its genome into infected cell in a two-stage process, and carries multiple specific nicks in its double-stranded genomic DNA. The two phages rely on different host secondary receptors—FhuA (T5) and BtuB (BF23). Only short fragments of the BF23 genome, including the region encoding receptor interacting proteins, have been determined. Here, we report the full genomic sequence of BF23 and describe the protein content of its virion. T5-like phages represent a unique group that resist restriction by most nuclease-based host immunity systems. We show that BF23, like other *Tequintavirus* phages, resist Types I/II/III restriction–modification host immunity systems if their recognition sites are located outside the terminal repeats. We also demonstrate that the BF23 avoids host-mediated methylation. We propose that inhibition of methylation is a common feature of *Tequintavirus* and *Epseptimavirus* genera phages, that is not, however, associated with their antirestriction activity.

Keywords: bacteriophage; phage BF23; *Escherichia* phage T5; *Escherichia coli*; genome; proteome; methylation; antirestriction

Introduction

The lytic bacteriophage BF23 was first described in 1949 by Pierre Fredericq (Fredericq 1949), and its similarity to *Escherichia coli* classical bacteriophage T5 was noted in 1968 (Nisioka and Ozeki 1968). In addition to similar genetic maps and efficient formation of hybrids (Beckman et al. 1973, Heller 1984), T5 and BF23 phages have common requirement for Ca^{2+} during infection (Nisioka and Ozeki 1968, Bonhivers and Letellier 1995), contain multiple nicks in the minus strand of the virion-packaged gDNA (Abelson and Thomas 1966, Kiyotaka and Yoshiro 1980), inject their DNA into infected cells via a two-stage mechanism (Lanni 1968, Davison 2020) and have similar gene expression strategies (Szabo et al. 1975, Kikuchi et al. 1988).

BF23 and T5 can infect both *E. coli* and *Salmonella* (Guterman et al. 1975, Mojica-a and Garcia 1976) but they utilize different strategies for adsorption. The BF23 receptor is the BtuB protein, a vitamin B_{12} (cobalamin) transporter (Buxton 1971). In contrast, T5 exploits the FhuA protein, which is involved in iron uptake (Braun 2009). While many T5-like phages carry L-shaped tail fibers (LTF) that facilitate binding to the host through interactions with lipopolysaccharide O antigen, the exact target of the BF23 LTF has not been identified (Heller and Braun 1979a, Heller 1984).

Recent cryoelectron microscopy studies shed light on the T5 host receptor recognition and injection initiation (van den Berg et al. 2022, Degroux et al. 2023). The T5 receptor binding protein (RBP) pb5, located at the tip of the tail, partially inserts into the cavity of FhuA. This triggers a large conformational change leading to bending of the initially straight tail fiber and opening of the tail tube, followed by release of the tape measure protein and formation of a channel in the outer membrane (Degroux et al. 2023). The RBP of BF23 is encoded by the *hrs* gene (Mondigler et al. 1996, 2006), which shares limited similarity with the T5 *oad* gene encoding pb5. However, BF23 Hrs can functionally replace T5 pb5 (Krauel and Heller 1991). Hrs of BF23 is homologous to the RBP of other BtuB-binding T5-like phages, such as DT57C, EPS7, and φR2-01 (Hong et al. 2008, Golomidova et al. 2016, Happonen et al. 2021).

Both T5 and BF23 orchestrate lytic conversion, i.e. block receptor accessibility after the primary infection has initiated (Decker et al. 1994). This is achieved by the binding of viral protein Llp to the cytoplasmic side of the receptor and locking it in an inactive conformation (van den Berg et al. 2022). While the Llp proteins of T5 and BF23 are nonhomologos, the modular structure of the receptor-binding and receptor-blocking loci appears to be preserved among these and other T5-like phages (Mondigler et al. 2006, Happonen et al. 2021).

Like T5, BF23 carries ~9.7 kb-long terminal direct repeats (Wiest and McCorquodale 1990), and after adsorption, only the

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5′ terminal repeat, which encodes the First-Step Transfer (FST) pre-early genes, is injected into the host cell. The FST genes have diverse functions, including shut-off of host transcription and translation, destruction of host DNA, and excretion of free nucleobases from the cell (McCorquodale et al. 1977, Davison 2015). How both phages avoid degradation of their own DNA is not clear. The FST genes of T5 and BF23 also provide protection against the RecBCD exonuclease of the host (Sakaki 1974) and other nuclease-based immunity systems. While T5 genomic DNA is subject to digestion by restriction endonucleases *in vitro*, the T5 infection is resistant to Types I, II, and III restriction–modification (R–M) defenses, as well as to Type I and II CRISPR-Cas and SspE/SspFGH systems, provided that their recognition sites are absent in the FST region (Brunel and Davison 1979, Davison and Brunel 1979a, b, Strotskaya et al. 2017, Xiong et al. 2020, Ramirez-Chamorro et al. 2021, Wang et al. 2021). This insensitivity to nuclease-based defenses of the host may be a common feature of all *Tequintavirus* phages, not characteristic for the phages from the close genus *Epseptimavirus* (Maffei et al. 2021).

The shut-off of expression of pre-early genes located in the FST region requires viral protein A1, although the exact mechanism of its function is unknown. The shut-off may be associated with the release of the injection brake and the transfer of the rest of the genome (Second Step Transfer) into the infected cell (McCorquodale et al. 1977, Davison 2015, 2020). Early phage genes are divided into two regulatory classes (Ea and Eb) and encode proteins involved in nucleotide metabolism and phage DNA replication. Late genes encode structural components of phage virions (Kikuchi et al. 1988, Wang et al. 2005). T5 and BF23 exploit host RNA polymerase for transcription of their genomes, and promoter switching between viral expression classes is regulated by phage-encoded proteins (Kikuchi et al. 1988, Klimuk et al. 2020).

In early research, phage BF23 attracted attention as a tool for typing of colicin resistant bacterial hosts (Fredericq 1949, Strobel and Nomura 1966). *Escherichia coli* strains insensitive to colicins E and Ib are also resistant to BF23; however, the source of this resistance is different. Colicins are proteinaceous toxins that are expressed by some *E. coli* strains and penetrate into target cells through membrane channel-forming proteins. Since BtuB serves as an entry point for colicin E (Kurisu et al. 2003) and is also a receptor for BF23, *btuB* mutants are resistant to both (Buxton 1971). In contrast, Col1b$^+$ hosts restrict T5 and BF23 through an abortive infection (Abi) mechanism without affecting phage adsorption (Mizobuchi and McCorquodale 1974). The Col1b genes are carried on a plasmid that contains a locus responsible for the abortive phenotype and denoted *ibf*, from inhibition of BF23 infection (Uemura and Mizobuchi 1982, Duckworth and Pinkerton 1988). The *ibf* locus (also called *abi*) displays features of a typical Abi system: it senses infection through a specific trigger, pre-early phage product A2 (the trigger was initially denoted as an independent protein A3) (Mizobuchi and McCorquodale 1974, Rose and McCorouodale 1990). The presence of A2 induces a toxic response leading to the loss of membrane potential and cell death (Cheung and Duckworth 1977). The products of the *ibf/abi* gene have not been identified and the exact defense mechanism is yet to be determined. It was also shown that the incompatibility group I1 (IncI1) plasmid R64 provides an Abi defense against BF23 infection. A gene responsible for this phenotype was also named *ibf* (Furuichi et al. 1984), though it is not related to the *ibf* genes of ColIb plasmids (Sampei et al. 2010). IncI1 plasmids are widespread in *Enterobacteriaceae*, and *ibf* (R64) was shown to affect phage types of *S. enterica* serovar Typhimurium isolates, showing an ecological impact of the Ibf (R64) system (Hiley et al. 2021).

In summary, BF23 is a classical coliphage and its infection cycle, host takeover mechanisms, receptor recognition, transcriptional strategy and interaction with the host immunity systems have been studied in some detail. However, the complete genome sequence of BF23 has never been published, and only short fragments of the genome encoding minor and major tail proteins (Nakayama et al. 1994), RBP and llp (Mondigler et al. 2006), A2–A3 (Wiest and McCorquodale 1990) and a tRNA genes region (NC_042564) have been deposited in the GenBank. To address this gap, we present here the complete genome sequence of the phage BF23 and describe the protein content of its virion. Additionally, we demonstrate that BF23, as well as phages T5 and Bas27, maintain their genomic DNA in a hypomethylated state, suggesting that they inhibit host methyltransferases.

Results and discussion

The BF23 virion morphology

Although BF23 has been extensively studied, the electron microscopy images of the phage virion have not been revealed (Nisioka and Ozeki 1968, Heller 1984). Negative uranyl acetate staining and imaging on Titan Themis Z demonstrated that BF23 virions possess a typical T5-like syphovirus morphology with a long noncontractile tail and icosahedral capsid (Fig. 1A). We confirmed the sheath-like multimeric structure of the BF23 tail and the presence of 3 LTF attached to the baseplate hub, which extends into the straight tail fiber that, based on cryo-EM studies of T5 virions, carries the RBP at the tip (Degroux et al. 2023). The diameter of the BF23 phage capsid (averaged at 82 nm from vertex to vertex, V_1–V_4) is comparable to a 90-nm capsid of T5 (Effantin et al. 2006) (Fig. 1B). The reported T5 tail lengths range from 160 to 250 nm (Effantin et al. 2006, Zivanovic et al. 2014). The BF23 tail lengths measured starting from the portal to the LTF attachment ring (as most clearly evident structures on images) were ∼175 nm (Fig. 1B). It should be noted that capsids sometimes overlay with the portal side of the tail, leading to ambiguity of such measurements. The estimated length of the LTF is ∼60 nm, while the central straight fiber together with baseplate hub average at ∼60 nm in length, though these structures are often not clearly visible on TEM images, and the size of RBP can not be accurately measured. Inclusion of the baseplate hub, straight tail fiber and RBP extends the length of the BF23 tail up to 250 nm.

General features of the BF23 genome

BF23 has a 114 544-bp linear double-stranded DNA genome with 9743-bp long direct terminal repeats, as detected by PhageTerm (Garneau et al. 2017). T5-like phages are known to carry nicks in the genomic DNA and this feature was also reported for BF23 (Abelson and Thomas 1966, Kiyotaka and Yoshiro 1980). Indeed, electrophoretic analysis of genomic DNA samples purified from T5 and BF23 virions sometimes demonstrated fragmentation patterns that can be explained by breaking of DNA at the positions of pre-existing DNA nicks (Figure S1A, Supporting Information). The average GC content of BF23 genome is ∼39.2%, which substantially differs from its *E. coli* host (∼50.78% for BW25113 strain) (Grenier et al. 2014). A total of 193 BF23 ORFs (including duplicated and completely identical 18 ORFs encoded in the terminal repeat regions) was predicted (Fig. 2; Table S1, Supporting Information). Out of 175 unique ORFs, 16 are absent from the T5 genome (Table S1, Supporting Information). T5-like phages encode an almost complete set of tRNAs, which were suggested to optimize translation of phage mRNAs in a host whose genomic GC content

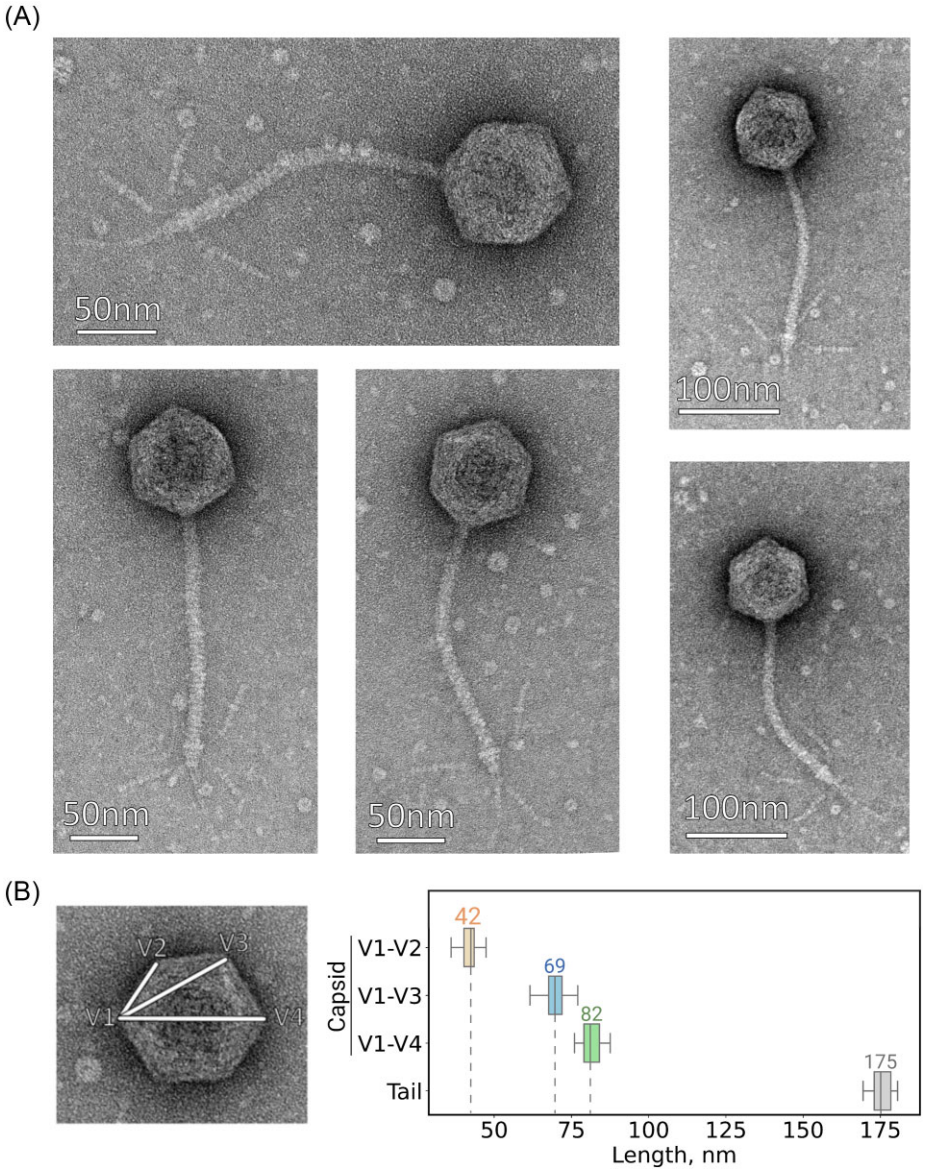

Figure 1. The BF23 virions. (A) Representative TEM images of BF23 virions. (B) Distributions of observed lengths of BF23 capsids diagonals (from vertices V_1–V_4), edges from (V_1–V_2), the V_1–V_3 distances, and tails.

is significantly different from that of the virus (Yang et al. 2021). Recently, it was proposed that phage-encoded tRNAs can also compensate the toxicity caused by host tRNA-cleaving abortive immunity nucleases (van den Berg et al. 2023). This function was confirmed by the demonstration that T5 $tRNA^{Tyr}$ can rescue toxicity of the Eco7 (Ec78) retron (Azam et al. 2023). The BF23 genome lacks 5 of 24 tRNA genes encoded by T5 but has 2 tRNA genes of its own, coding for $tRNA^{Gln}$—CUG and $tRNA^{Gly}$—GCC. Cumulatively, the BF23 genome encodes a set of 20 tRNAs specific for all but two (Arg and Trp) proteinogenic amino acids (Table S2, Supporting Information).

Prediction of promoters with the Sigma70Pred tool and manual comparisons with T5 allowed to delineate three classes of BF23 genes. Pre-early genes are located in the duplicated FST region and organized into two inversely oriented transcriptional units. While some early genes encode nucleic acids metabolism and replication-related proteins, as well as tRNAs, for most pre-early and early genes no function can be predicted and their products likely participate in host takeover and anti-immunity activities. Late genes encode structural components of the capsid. Similar to T5, a long noncoding region is evident at the border between FST and SST (8.6–10.3 kB). This region is enriched with direct and inverted repeat seqeunces (Figure S1B, Supporting Information), as well as with DnaA-binding boxes. While it was proposed that these sites are required for the orchestration of the two stage injection of T5 genome (Davison 2020), the experimental studies of their functional role are lacking.

The position of BF23 within the group of T5-like phages

The BF23 genome is 96.61% identical to that of T5 (assembly AY587007.1) with 85% query coverage, according to blastn alignment, while the overall intergenomic similarity between BF23 and T5 calculated by VIRIDIC (Moraru et al. 2020) is 79.664%. The closest relatives of BF23 among the currently sequenced phages are *Escherichia* phages vB_EcoS_EASG3 (MK373799.1) and vB_EcoS_HASG4 (MK373797.1), *Salmonella* phage NBSal005

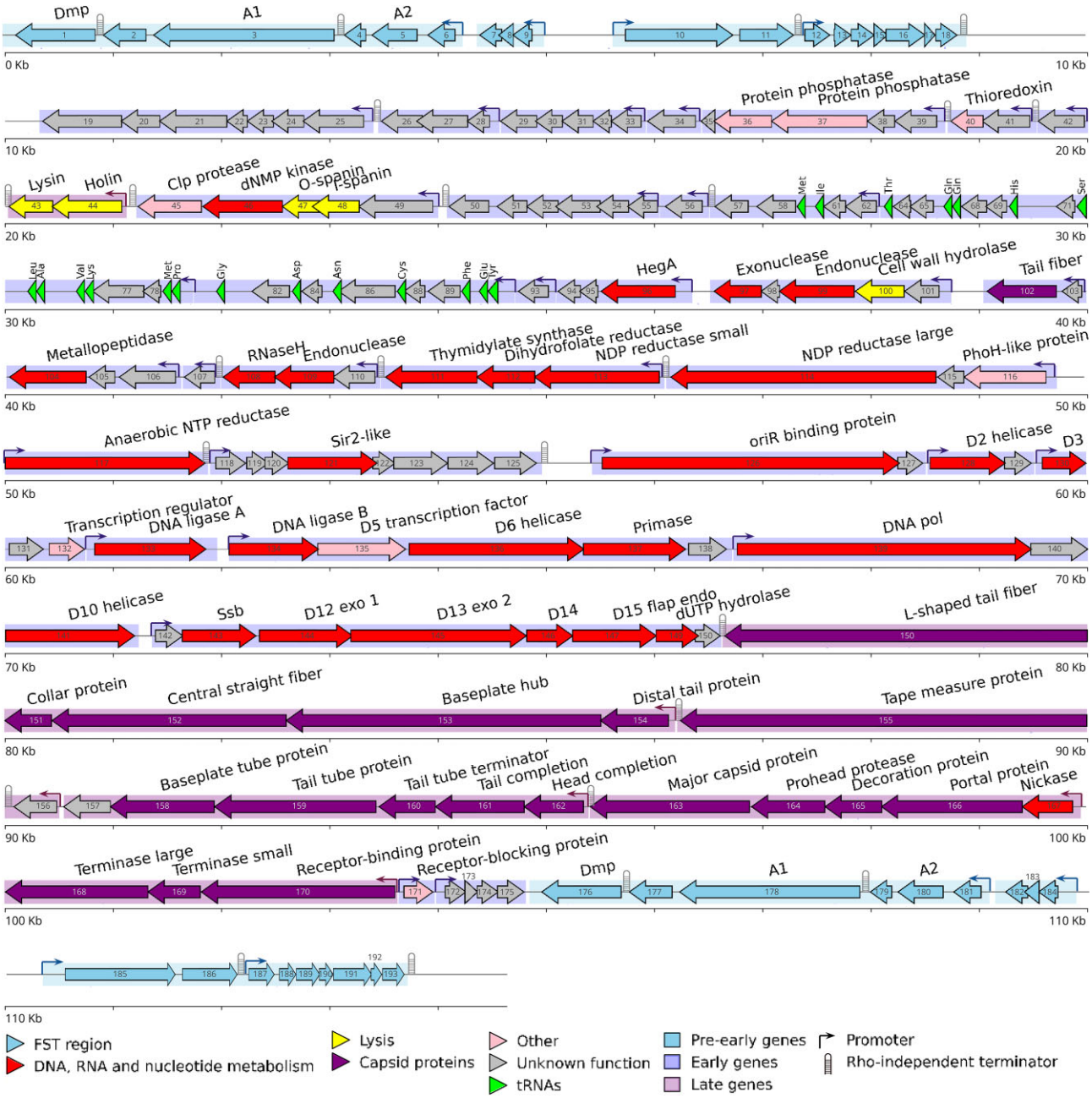

Figure 2. The BF23 genome annotation. Functional gene groups are indicated by different colors of the arrows. Gene classes [pre-early (FST region), early, and late] are identified by colored backgrounds. Predicted promoters and rho-dependent terminators are shown by arrows indicating the direction of transcription and stem-loop like structures, respectively. The figure was prepared with pyGenomeViz.

(NC_048857.1) (Llanos et al. 2020), and *Klebsiella* phage KPP2018 (OQ031075.1) (Figure S2, Supporting Information). Phylogenetic reconstruction of the BF23 position within the group of T5-like phages carried with VICTOR (Meier-Kolthoff and Göker 2017) places it within the T5 branch of the *Tequintavirus* genus, distinct from the clade of Gostya9 (Golomidova et al. 2019) and DT57C (Golomidova et al. 2015) phages, although the tree is not supported with high GBDP pseudo-bootstrap values, reflecting proximity between genomes (Fig. 3). Full genome alignment demonstrated complete synteny between BF23 and selected members from the *Tequintavirus* and the closely related *Epseptimavirus* genera (Fig. 3). On the background of this synteny, local genome rearrangements were evident. The most significant contribution to observed differences is due to genes encoding homing endonucleases such as F-Tfl H-N-H endonucleases of T5 (Akulenko et al. 2004). Homing nucleases are highly mobile egoistic elements that often "parasitize" on phage genomes resulting in mosaicism (Hafez and Hausner 2012, Barth et al. 2023). Compared to T5, BF23 has a ~4 kb deletion in the early genes region that removes five genes (ORF071-075 according to T5 assembly AY587007.1), including ORF071, which was previously annotated as primase due to the presence of the TOPRIM domain, but was later established as Rad—an inhibitor of the retron immunity systems (Azam et al. 2023). This suggests the loss of the antidefense locus, due to the lack of pressure from host immunity systems and highlights mobility of such "anti-defense" islands. The deletion is flanked by two different nuclease genes, whose products (ORF96–HegA and ORF97; Table S1, Supporting Information) could have been

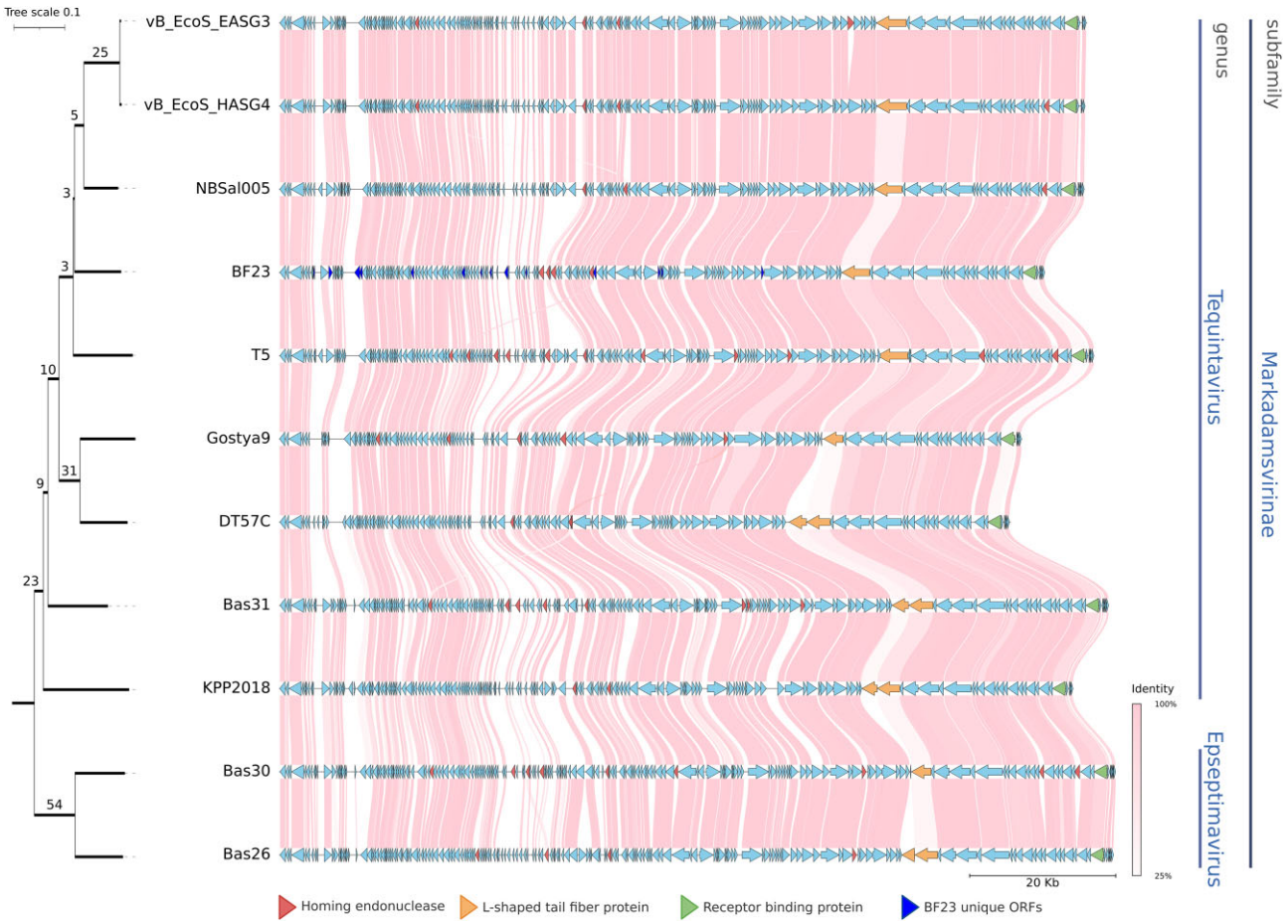

Figure 3. Left—phylogenetic tree of BF23 and selected members of the *Tequintavirus* and *Epseptimavirus* genera calculated by Victor using the λ phage genome as an outgroup. Numbers above the branches indicate GBDP pseudo-bootstrap values (100 replicas). D6 formula was used to build the tree. Right—whole-genome alignments were performed with MMseqs and visualized with pyGenomeViz. Genes that vary the most between compared phages are color-coded (red—homing nucleases; yellow—LTF proteins; green—RBPs; and blue—ORFs found in BF23, but not in the T5 genome). Genomic start positions were reassigned for some phages, while a ~20-kb duplication was removed from the KPP2018 genome.

responsible for this rearrangement. The BF23 region encoding receptor-binding and receptor-blocking proteins is different from that of T5. The BF23 RBP clusters with those of vB_EcoS_EASG3, vB_EcoS_HASG4, and NBSal005 (Fig. 4A), suggesting that these phages also utilize host BtuB protein for adsorption. Finally, two variants of loci encoding the LTF proteins can be distinguished among the analyzed phages. Similar to DT57C, phages KPP2018, Bas31, and Bas26 encode two LTF proteins (LtfA and LtfB), while BF23 and T5 encode one distinct variant (Fig. 3). It was proposed that LtfA forms a fully functional LTF, attached to the phage tail, while a shorter LtfB represents an additional LTF "branch," attached to LtfA (Golomidova et al. 2016). The *ltfA* and *ltfB* genes form two distinct clades, *ltfA* clustering with *ltf* of T5, while *ltfB* clustering with *ltf* of BF23 (Fig. 4B). The LTF proteins of BF23 and T5 share 82.5% identity, and although BW25113 lacks O-antigen, the LTFs of T5-like phages are required for reversible adsorption to O-antigens of other *E. coli* strains (Golomidova et al. 2016), and targets of BF23 and T5 were proposed to be different (Heller and Braun 1979b). Thus, it can be expected that LtfA and LtfB also recognize distinct O-antigen receptors and where both *ltfB* and *ltfA* genes are present, they must have emerged not through duplication but through recombination events that likely expanded the host range of resulting phages. In summary, the BF23 genome is very close to that of T5 and other *Tequintavirus* and *Epseptimavirus* members with major differences reflecting different host recognition and repositioning of mobile genes.

Proteomic characterization of BF23 virion

We performed mass-spectrometric analysis of BF23 virions proteome. One impetus for this analysis was almost complete restriction-insensitivity of BF23 and T5 *in vivo*, the mechanism of which remains unknown (Davison and Brunel 1979, Davison 2015). Since some phages package antirestriction proteins into their capsids and inject them together with genomic DNA (Iida et al. 1987), we wanted to determine whether BF23 incorporates nonstructural components in its virions. Based on SDS-PAGE, the pattern of protein bands observed in BF23 and T5 lysates was highly similar (Fig. 5A). The most intense bands corresponded to the major capsid and tail tube proteins; electrophoretic mobility of major capsid proteins band corresponded to proteolytically processed form (Zivanovic et al. 2014). Unexpectedly, we found that the predicted transcription factor D5 (ORF135) was also very abundant in lysates of both phages. PEG-precipitated BF23 virions were subjected to SDS-PAGE (Fig. 5B) and tandem MS/MS analysis. The abundance of specific proteins was estimated by peptides intensity and only confident hits with a minimum of two unique peptides were considered (Table 1). Consistent with previous studies of T5 (Zivanovic et al. 2014, Vernhes et al. 2017) we

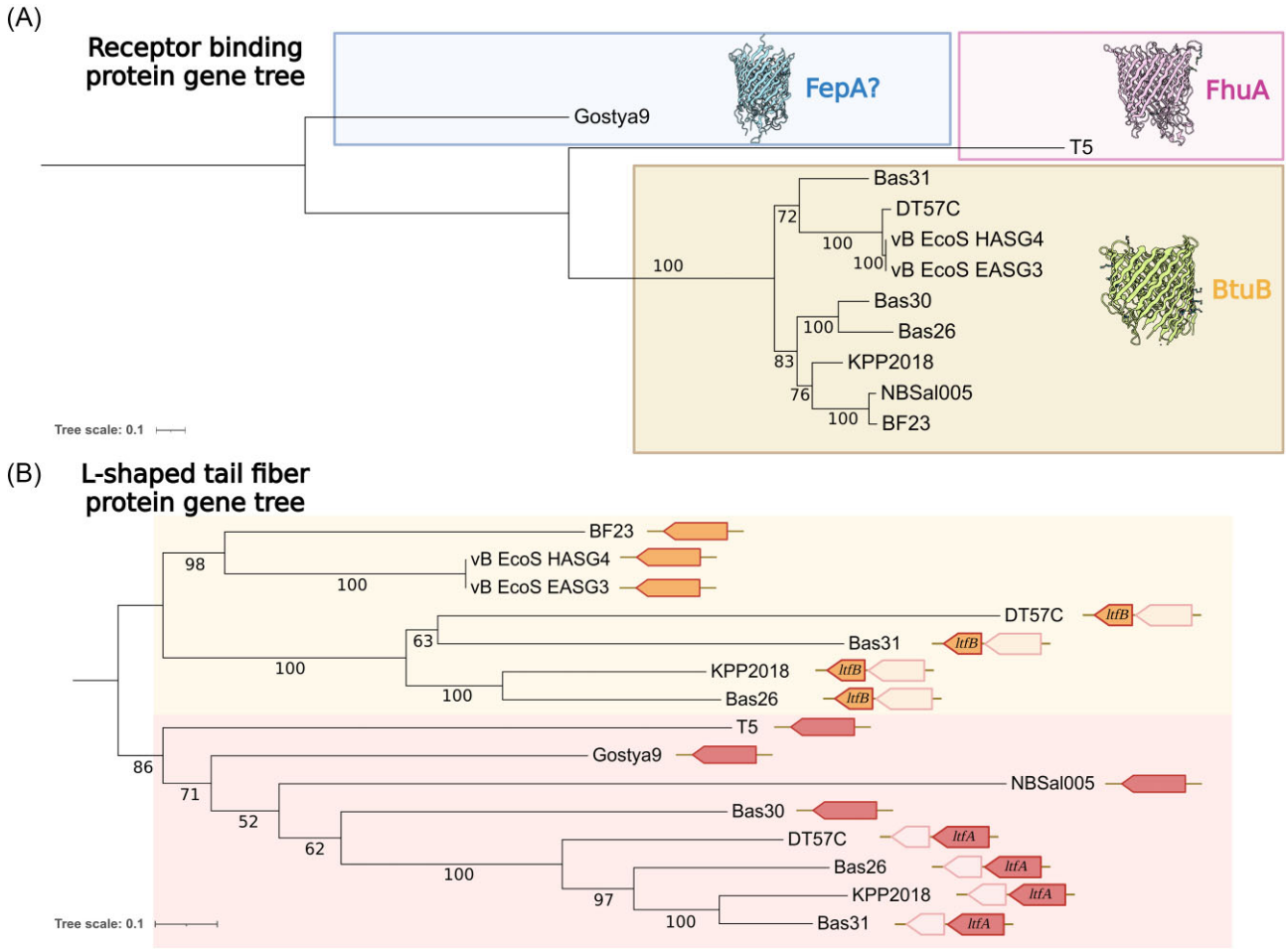

Figure 4. (A) A phylogenetic tree of RBP genes of BF23 and its relatives. Known or predicted targets of phage RBPs (host receptor proteins) are shown. (B) A phylogenetic tree and organization of LTF protein genes of BF23 and its relatives. For phages encoding two homologous LTF proteins (A and B), both genes were used for tree construction. Alignments were performed in MAFFTv7.490 and trees rooted at midpoint were visualized with IQ-TREE v1.6.12, bootstrap values are indicated above the branches (100 replicas). Alignments can be found in the Supplementary files.

Table 1. MS/MS identification of proteins detected in BF23 virion.

Protein	ORF number*	Mass, kDa**	Peptide counts	Intensity
Major capsid	163	50.775	30	39 940 000 000
Tail tube	159	50.302	20	4 563 500 000
Decoration	165	17.09	11	2 330 800 000
Portal	166	45.417	21	1 485 300 000
LTF	150	137.17	28	976 030 000
Tape measure	155	132.6	43	974 790 000
Prohead protease	164	23.427	5	694 440 000
Baseplate hub	153	107.02	19	241 810 000
Central straight fiber	152	76.357	15	231 670 000
Head completion	162	19.165	9	209 210 000
Collar protein	151	15.023	5	185 570 000
Dihydrofolate reductase	112	19.711	6	170 010 000
Baseplate tube	158	34.31	8	106 180 000
Receptor binding	170	63.711	10	60 834 000
Distal tail	154	22.79	2	39 760 000
Clp protease	45	22.927	3	27 828 000
Tail tube terminator	160	18.36	2	23 464 000
Tail completion	161	27.856	3	15 892 000

*—ORFs description can be found in Table S1 (Supporting Information).
**—Predicted mass calculated based on the unprocessed protein sequence of corresponding ORFs.

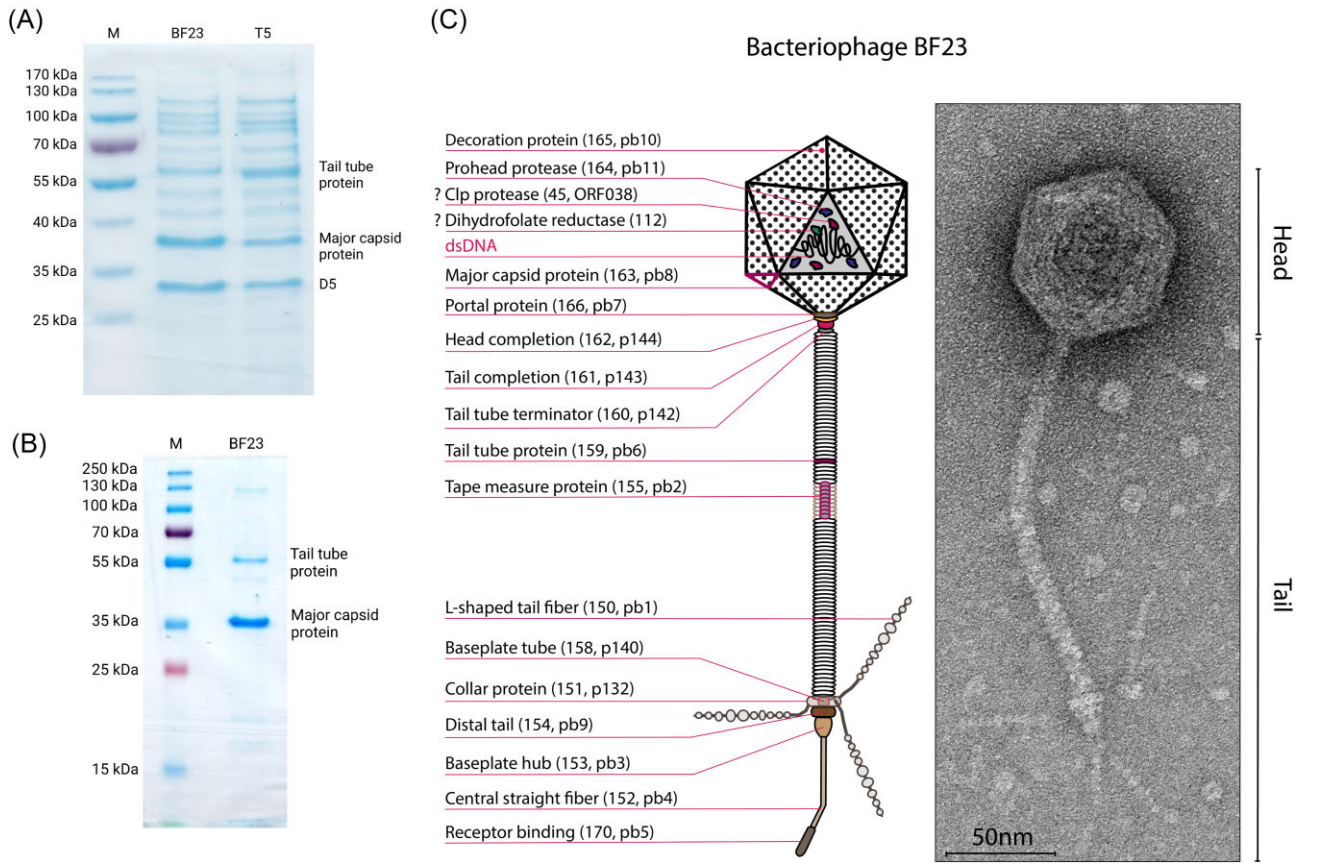

Figure 5. (A) 12% SDS-PAGE analysis of proteins in T5 and BF23 phage lysates. (B) 12% SDS-PAGE analysis of PEG-precipitated BF23 virions. (C) A model of the BF23 virion with depicted positions of proteins identified by MS/MS analysis of phage virion. For each protein, the number of the corresponding BF23 ORF and the name of the T5 homolog is indicated in the brackets. Positions of proteins are based on previously established T5 proteins positions (Zivanovic et al. 2014).

identified all expected capsid, tail, and baseplate proteins as well as the abundant capsid decoration protein (Table 1, Fig. 5C). The D5 (ORF135) protein that was abundant in the lysates was absent from purified virions. In addition to structural proteins, the prohead protease or maturase (ORF164), required for processing of the major capsid protein (Huet et al. 2016) and the Clp protease (ORF45), the function of which is currently unknown, were found. We also detected intense signals from dihydrofolate reductase (DFR—ORF112; Table 1). Localization of this protein within the T5 virion was not reported before. Early works suggested the structural role of DFR in the virion of an unrelated phage T4, though these findings were not confirmed by subsequent immunoblotting (Mosher and Mathews 1979, Chen et al. 1995). No BF23 components that could perform an antirestriction function were detected.

BF23 and other tequintavirus phages overcome diverse R–M systems

To gain further insights into restriction insensitivity of BF23 and other T5-like phages and to understand the specificity of this phenomenon, we carried a comprehensive EOP screen of a panel of phages from the Epseptimavirus (Bas26-29) and Tequintavirus (BF23, T5, DT57C, Gostya9, and Bas31–34) genera against hosts equipped with Types I/II/III R–M and BREX defenses. Infection with phage λ was used to validate the activity of each defense system. The results, presented as a heatmap of the efficiency of defense provided by each system, are shown in Fig. 6, and phage titers obtained on each host are provided in Figure S3 (Supporting Information). T5 phage was shown to be resistant to Type II R–M defenses, except when restriction sites were located in the FST region (Davison and Brunel 1979). Thus, for each defense system, we specifically labeled the recognition sites when present in phage FST regions.

The results of the screen confirmed T5 resistance to EcoRV and EcoRI systems (Davison and Brunel 1979, Chernov and Kaliman 1987) and the sensitivity/resistance pattern of BASEL (Bas) phages against EcoKI, EcoRI, EcoRV, and EcoPI systems (Maffei et al. 2021). The Epseptimavirus phages were generally more sensitive to R–M systems than Tequintaviruses, irrespective of the presence of recognition sites in their FST regions. Yet, Type II R–M Eco29kI and Esp1396I systems were not efficient against Epseptimaviruses despite the presence of multiple recognition sites, implying the existence of additional dedicated viral antirestriction mechanism(s).

BF23 and other Tequintavirus phages were highly resistant to most Type I and II R–M systems tested. When observed, sensitivity to an R–M defense was correlated with the presence of recognition sites in phage FST region. The Type I R–M systems require two recognition sites, at any orientation, for cleavage of DNA (Murray 2000, Isaev et al. 2021). Consistently, the Type I system EcoR124II provided the highest defense against phages Bas31 and Bas33 that carry two recognition sites in the FST, while other phages lacked an active sites configuration in the FST. The recognition site of EcoR124II is GAA$(N)_7$RTCG. Deletion of four amino acids from the HsdS subunit linker determining the distance between

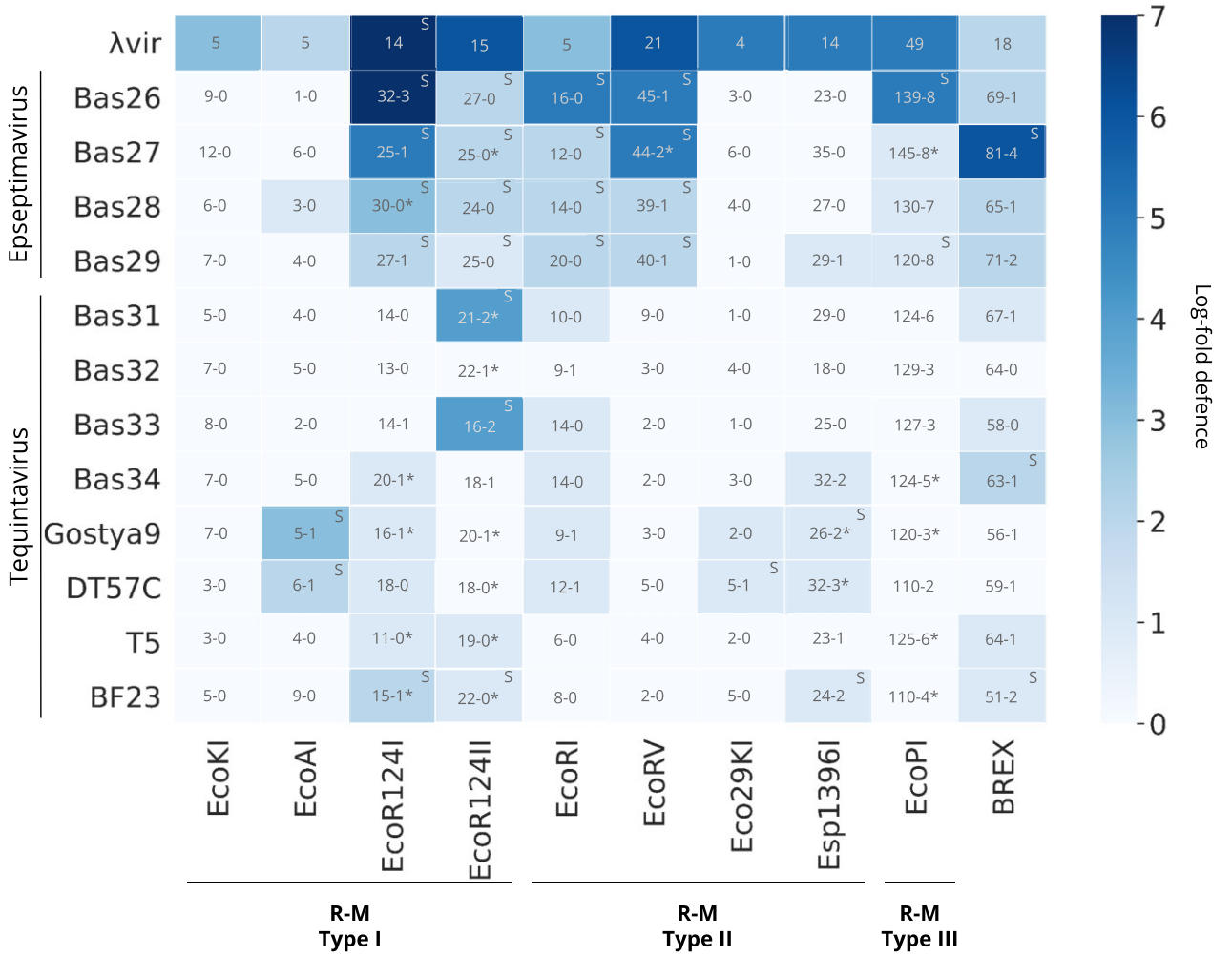

Figure 6. Efficiency of host defense systems against a panel of T5-like phages from Tequintavirus and Epseptimavirus genera. For each cell of the heatmap, the first number reflects the amount of defense system recognition sites in the tested phage genomes, the second number (after a hyphen) shows the number of sites in the FST region (not accounting for FST duplication). Since the exact position of the injection stop signal is not determined for the majority of phages, asterisk indicates cases where at least one additional restriction site is present in the noncoding region between FST operons and early genes, and it is not known whether this site is available during the FST step. "S" in the corner indicates reduced plaque size. EcoKI site—AACNNNNNNGTGC; EcoA site—GAGNNNNNNNGTCA; EcoR124I site—GAANNNNNNRTCG; EcoR124II site—GAANNNNNNNRTCG; EcoRI site—GAATTC; EcoRV site—GATATC; Eco29kI site—CCGCGG; Esp1396I site—CCANNNNNTGG; EcoPI site—AGACC; and BREX HS site—GGTAAG.

the bipartite recognition site changes the EcoR124II specificity to GAA$(N)_6$RTCG (Price et al. 1989). The resulting system is referred to as EcoR124I. We converted plasmid-borne EcoR124II–EcoR124I and found that it no longer protected cells from Bas31 and Bas33 infection, consistent with the lack of recognition sites in the FST. At the same time, EcoR124II–EcoR124I conversion increased defense against phages Bas26 and Bas27 that contain EcoR124I sites in the FST. The result strongly supports a causal relationship between the efficiency of defense and location of active sites in the FST. However, some phage/R–M pairs (Gostya9/Eco29kI or DT57C-T5/EcoR124I) did not follow this rule, suggesting a more complex dynamics of the R–M system interaction with phage DNA during infection.

BF23 and other Tequintaviruses were also resistant to the Type III R–M system EcoPI, despite the presence of multiple recognition sites in their FSTs. Type III R–M systems require two inversely oriented nonpalindromic sites for cleavage and some phages are known to evade this defense through a strand bias in recognition sites localization in their DNA (Krüger et al. 1995). However, most phages tested in our screen contained at least one "active" restriction site (comprised of two AGACC sequences located on the opposing DNA strands) in their FSTs, suggesting that Type III R–M resistance is mediated by a yet undetermined mechanism.

The BREX system weakly restricted BF23 and some other Tequintavirus and Epseptimavirus phages. Although no direct correlation between efficiency of defense and the number of BREX recognition sites in phage genomes was observed, phage Bas27, with the highest BREX sensitivity, carried 4 recognition sites in the FST region, which is more than any other phage tested.

In summary, the EOP screen revealed that BF23, like other Tequintavirus phages, is broadly insensitive to Type I/II/III R–M defenses. The Type I and II R–M systems can target these phages only if active restriction sites are located in the FST region. How recognition of sites located in the SST region is avoided remains to be determined. Phages from the closely related Epseptimavirus genus are sensitive to R–M defenses, which should help determine Tequintavirus proteins responsible for antirestriction. We surmise that (at least) two factors influence the efficiency of R–M defense against T5-like phages. First, as is the case for other phages, the presence of restriction sites close to the end of the genome first in-

jected into the cell generally renders a phage more sensitive to the defense. This general consideration explains the R–M sensitivity pattern of Epseptimaviruses. At the same time, Tequintaviruses should encode additional antirestriction components that might be expressed during the FST stage to protect their genomes at later stages, thus only the presence of restriction sites in the FST allows cleavage.

Tequintavirus and epseptimavirus phages avoid host-mediated DNA methylation

Some phages protect their genomes from cleavage by host nucleases through incorporation of modified bases in their genomic DNA (Weigele and Raleigh 2016). While the T5 and BF23 genomes are efficiently digested by type II restriction enzymes *in vitro* (Figure S1A, Supporting Information), we investigated the nucleoside composition of T5 and BF23 genomic DNA to rule out modification-dependent protection. Phage DNA was digested to nucleosides and subjected to HPLC-MS/MS analysis (Lee and Weigele 2021). Genomic DNA of phage λ and BF23 relative Bas26 (an *Epseptimavirus*, also named vB_EcoS_TrudiGerster) were used as negative controls, since these phages are sensitive to type II R–M systems *in vivo* (Fig. 6) (Maffei et al. 2021). The T4 phage DNA, taken as a positive control, revealed the expected substitution of cytosines with α- and β-glycosylated hydroxymethylcytosines. Noncanonical bases were not detected in T5, BF23, and Bas26 DNA samples (Figure S4, Supporting Information). Interestingly, the intensity of N6-methyl-adenine (m6A) (m/z=266.125) in extracted ion chromatograms (EIC) of T5, BF23, and Bas26 DNA was significantly lower compared to that of T4 and λ (Fig. 7A). When the N6-methyl-adenine intensity was normalized to the adenine signal, a ∼20–60x under-representation of the modified base in the DNA of T5-like phages, compared to DNA of T4 and λ was revealed (Table S3, Supporting Information).

The N6-methyl modification of adenines can be installed by a host orphan methyltransferase Dam, which methylates the GATC sites and plays an important role in host metabolism. Dam methylation can also affect phage infection, for example, through methylation of promoters (Løbner-Olesen et al. 2005). T4 encodes its own Dam methyltransferase (Kossykh et al. 1995), while λ DNA is modified by the host Dam at ∼50% of GATC sites, since phage replication outcompetes the modification reaction (Szyf et al. 1984). Considering the abundance of the GATC sites in the genomes of T5, BF23, and Bas26, the observed lack of m6A suggested the presence of an active Dam suppression mechanism. To validate this finding, we performed *in vitro* digestion of T5, BF23, and Bas26 genomes with a set of Dam-sensitive and insensitive restriction nucleases using nonmethylated or methylated λ DNA and methylated plasmid DNA as controls (Fig. 7B; Figure S5A, Supporting Information). We used DpnI and DpnII enzymes, that cleave nonmethylated or methylated GATC sites, respectively, and Sau3AI that has the same recognition site and is not affected by methylation. To discriminate between intact and cleaved genomic DNA we also applied capillary electrophoresis, which allowed to separate fragments of higher molecular weights (Figure S5A, Supporting Information). T4 DNA was completely resistant to cleavage, while nonmethylated λ DNA and plasmid purified from a *dam*$^+$ host demonstrated expected cleavage patterns. λ DNA produced on a *dam*$^+$ host was only partially cleaved by both DpnI and DpnII, consistent with its intermediate methylation state (Szyf et al. 1984). Importantly, the BF23, T5, and Bas26 DNA was not cleaved by DpnI, and was fully cleaved by DpnII, confirming the absence of m6A-modified GATC sites.

Another host-mediated modification, C5-methylation of cytosines (m5C) at CCWGG sites is due to the action of the Dcm methyltransferase (Palmer and Marinus 1994). 5mC was detected in λ but not in other tested phages DNA by HPLC-MS (Fig. 7A). In the case of T4 cytosines are converted to 5-hydroxymethylcytocines prior to glycosylation, and thus are not available for C5 methylation. The Dcm methylation status in other phage DNA was determined in a restriction-sensitivity assay using SexAI, an enzyme that is sensitive to Dcm methylation and cleaves only nonmethylated sites, and Dcm-insensitive BstNI (Fig. 7C; Figure S5B, Supporting Information). The obtained restriction patterns matched expectations and the results obtained in the Dam-sensitivity assay: no signs of cytosine methylation at CCWGG sites in DNA of BF23, T5, and Bas26 were detected, demonstrating that these phages also avoid Dcm modification.

The ability of T5 to suppress host methylation in lysates of phage-infected cells was reported in the 60-s (Hausmann and Gold 1966) and our work demonstrates hypomethylated status of T5 genome and extends these observations for the BF23 and Bas26 phages, belonging to distinct genera. Methylation requires a methyl groups donor S-adenosyl-methionine (SAM), and some phages are known to affect its intracellular concentration. But unlike the case of T3, a phage that encodes SAM lyase and actively depletes SAM through its cleavage and inhibition of SAM synthase MetK (Simon-Baram et al. 2021, Andriianov et al. 2023), T5 infection does not result in SAM depletion (Gefter et al. 1966). While methylation inhibitors were not detected in the lysates of T5-infected cells, the inhibition of Dam, Dcm, and R–M specific methylation during infection (Chernov et al. 1985) suggests a nonspecific mechanism of methylation sppression. Further research is required to identify the basis for this broad antimodification activity.

It was previously proposed that restriction-insensitivity of T5 might stem from activity of a DNA-binding protein protecting the T5 genome from interaction with host components (Davison 2015). However, our results show that BF23/T5 avoidance of DNA methylation could not be directly associated with this mechanism, as gDNA of restriction-sensitive phage Bas26 is also m6A/m5C-depleted (Fig. 7B and C). Thus, T5-like phages represent a unique group that exploits two independent strategies to protect its genome against the action of the host-mediated restriction and methylation activities.

Materials and methods

Phage propagation and purification, bacterial strains

Bacteriophage BF23 was a kind gift of Dr Andrei Letarov and Dr Vladimir Ksenzenko, while the source of the stock phage can be traced back to the works of Dr K.J. Heller. The identity of our BF23 stock can be verified by the fact that assembled BF23 genome perfectly aligns with previously published genomic fragments deposited to GenBank (accession id: NC_042564, M37095, DQ097178, D12824, and X54455). Phage T5 was a kind gift of Dr David Bikard, while Dr Alexander Harms shared phages from BASEL collection (Maffei et al. 2021). BF23, T5, and Bas27 were routinely propagated on *E. coli* BW25113 host in LB medium supplemented with 2 mM CaCl2, 0.2% maltose and 5 mM MgSO4 was added for the infection with phage λ. BW25113 [F$^-$ Δ(araD–araB)567 ΔlacZ4787(:: rrnB-3) λ$^-$ rph^{-1} Δ(rhaD–rhaB)568 hsdR514] is an exemplary derivative of *E. coli* K-12, and a parental strain for the KEIO collection, which lacks active restriction–modification systems (Grenier et

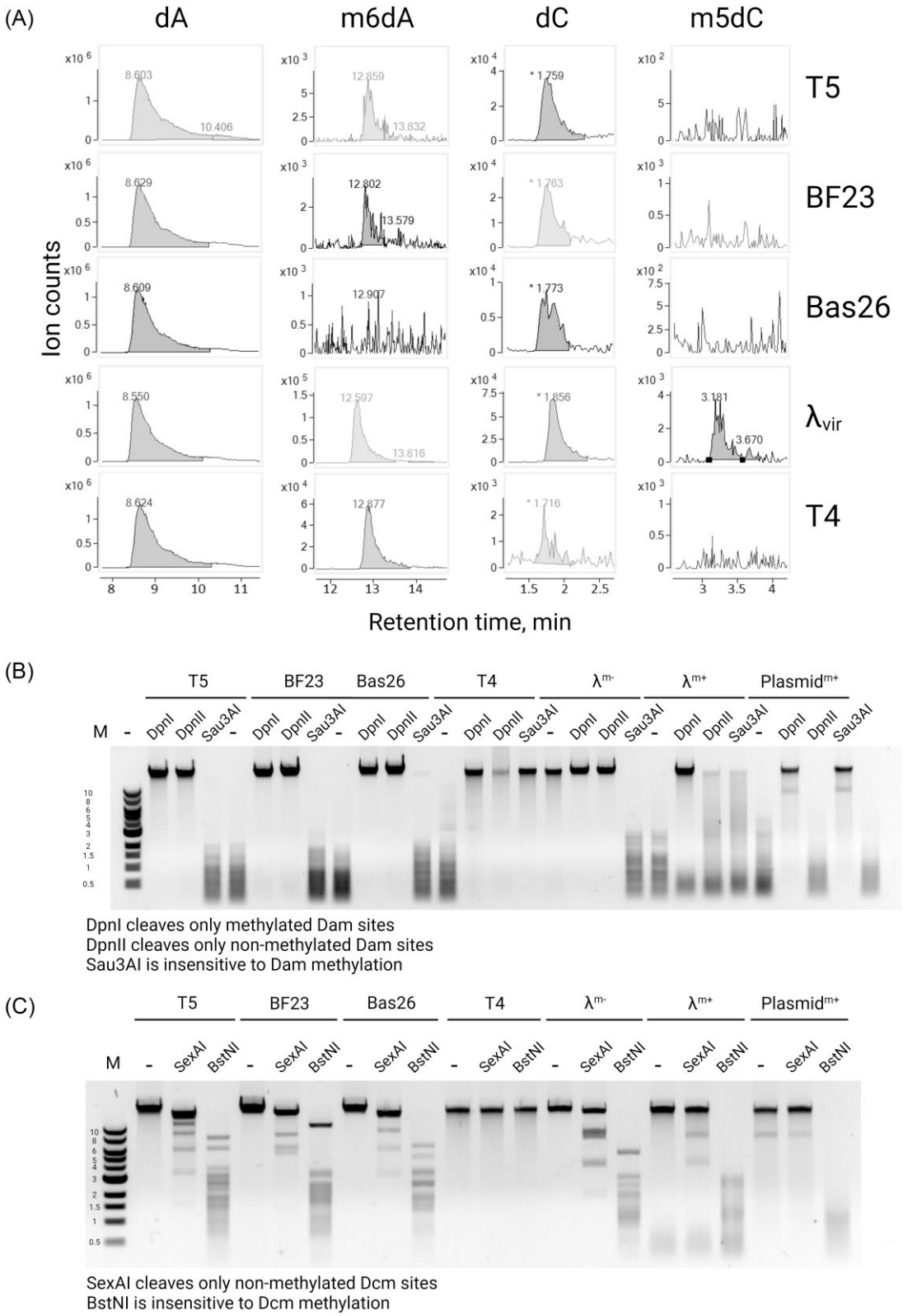

Figure 7. T5, BF23, and Bas26 DNA lacks the m6A and m5C modifications. (A) EICs for the adenine, N6-methyl-adenine, cytosine, and C5-methyl-cytocine deoxynucleosides in DNA of indicated phages. (B) and (C) *In vitro* restriction sensitivity assay performed with Dam-methylation sensitive (B) and Dcm-methylation sensitive (C) enzymes. λ^{m-} is a control nonmethylated phage λ DNA. λ^{m+} was produced on a *dam*$^{+}$*dcm*$^{+}$ *E. coli* host BW25113. The pBREX AL plasmid (Plasmid^{m+}) is fully methylated at Dam and Dcm sites. All tested DNA molecules contain sites for each of the restriction enzymes used. M–1 Kb+ DNA ladder (Thermo Scientific).

al. 2014). BF23 lysate was obtained from 10 ml of bacterial culture infected at OD_{600}~0.6 with low phage MOI (multiplicity of infection) and infection proceeded overnight. Cell lysate was spun-down by centrifugation (10 min at 6000 *g*) and treated with 50 μl of chloroform. For downstream genomic DNA extraction and proteomic analyses phage was precipitated with PEG. In short, 8 ml of lysate with titer ~10^{10} pfu/ml was treated with 2 μl DNAse I at 37°C for 30 min, to remove fragments of host DNA and mixed with 2 g of PEG 8000, NaCl was adjusted in solution to 1 M. Phage particles were precipitated at +4°C overnight with rotation and then collected by centrifugation (10 min at ~3600 *g* in bucket rotor). Precipitate was resuspended in 500 μl of STM buffer (NaCl—100 mM, $MgSO_4$—10 mM, Tris-HCl, and pH = 7.5–50 mM). To remove PEG, 500 μl of chloroform was added and mixture was rigorously vortexted for 1 min. Following centrifugation (5 min at 6000 *g*) the supernatant was collected and stored at 4°C. DNA of nonmethylated λ phage was from a commercial stock (SibEnzyme). To obtain methylated pBREX AL plasmid and λ_{vir} DNA we used dam^+dcm^+ *E. coli* BW25113 host. Plasmid was purified from 2 ml of overnight culture with GeneJET Plasmid MiniPrep (Thermo Scientific). λ_{vir} was propagated on LB agar plates with 0.6% top agar, concentration of phage particles was adjusted to achieve uniform lysis of bacterial lawns. Top agar layer was collected and liquid containing phage particles was separated from solid phase by centrifugation (10 min at 8000 *g*). All defense systems were expressed from plasmids and were investigated in a BW25113 *E. coli* background. List of plasmids used in the study is provided in Table S4 (Supporting Information).

Transmission electron microscopy

For negative staining transmission electron microscopy (TEM), the formvar/carbon Cu-supported TEM grid (Ted Pella, catalaog number 01801) was cleaned in Ar: O_2 plasma for 40 s (1070 Nanoclean, Fischione). A volume of 20 μl of phage lysate was dropcasted onto the carbon side of the grid and left for 1 min. The residual sample was blotted by touching the grid with the blot paper followed by two rinses in droplets of distilled H_2O. After that, the grid was immediately floated on top of the drop of uranyl acetate (UA, 1 wt.% solution, 9 μl) and was held in touch with UA droplet with tweezers for 45 s. The excess negative stain was blotted by gently sliding the side of the grid along the piece of blotting paper. The grid with stained sample was left in the air until complete dry.

Bright-field TEM images were acquired on a Titan Themis Z transmission electron microscope (Thermo Fisher Scientific) operated at 200 kV using a BM-Ceta 4 K × 4 K CMOS camera with 4 pixel binning.

Phage genomic DNA purification and sequencing

Phage genomic DNA was extracted from PEG-precipitated particles. Phage in STM buffer was treated with Proteinase K (100 μg/ml), SDS (0.5%), and EDTA (20 mM) at 50°C for 1 h. To remove proteinaceoues components, 1 V of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the solution and mixed, followed by centrifugation (10 min at 6000 *g* at 4°C). Supernatant was collected and treatment was repeated if required. After this, DNA was precipitated in ethanol (2 V) with sodium acetate (0.3 M) at −20°C for 2 h or overnight. To collect DNA precipitate, solution was centrifuged (12 000 *g*, at least 30 min at 4°C) and pellet was washed twice in 70% ethanol and once in 96% ethanol. Air dried DNA pellet was resuspended in 100 μl TE buffer (Tris-HCl, pH = 7.5–10 mM, and EDTA—1 mM) and concentration was measured with Qubit 3 Fluorometer. λ_{vir} DNA was additionally gel-purified with QIAquick Gel Extraction Kit (Qiagen) to get read of small copurified RNA and DNA fragments. BF23 DNA libraries were prepared by a standard procedure using NEBNext® Ultra™ II DNA Library Prep Kit (NEB) and sequenced on Illumina MiniSeq platform with paired-end 150 cycles (75 + 75).

Phage genome assembly and annotation

Raw reads quality was assessed with FastQC, followed by trimming with trimmomatic v0.39 (Bolger et al. 2014). Genome was assembled with SPAdes implemented in Unicycler (Wick et al. 2017) and terminal repeats were determined with PhageTerm (Garneau et al. 2017). Initial annotation was carried with MultiPhATE2 (Ecale Zhou et al. 2021), followed by manual curation. Open reading frames were predicted in parallel by Glimmer version 3, Phanotate, and Prodigal and only predictions made at least by two of these methods were retained. Functional annotation was performed using blastp search against pVOGs, PHANTOME, NCBI Swissprot, NCBI Refseq Protein, NCBI Virus databases, and using hmmscan search against pVOG database of viral proteins HMM profiles (Grazziotin et al. 2016). All genes were manually compared with their homologs in T5 phage genome. tRNAs were predicted using Aragorn (Laslett and Canback 2004). Since T5-like phages exploit host RNA-polymerase for transcription, σ70 subunit binding sites were searched with Sigma70Pred to determine promoters in intergenic regions and at positions aligned with reported T5 phage promoters (Wang et al. 2005, Patiyal et al. 2022). Rho-independent terminator sites were predicted using ARNold (Naville et al. 2011). The results were visualized using the pygenomeviz 0.3.2 python package (https://github.com/moshi4/pyGenomeViz).

Phylogenetic analysis

To determine the relative taxonomic position of BF23, we reconstructed phylogenetic tree with selected members of the Tequintavirus and Epseptimavirus genera using VICTOR, a tool specifically developed for the classification of prokaryotic viruses (Meier-Kolthoff and Göker 2017). All pairwise comparisons of the nucleotide sequences were conducted using the Genome-BLAST Distance Phylogeny (GBDP) method (Meier-Kolthoff et al. 2013). The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME including SPR postprocessing (Lefort et al. 2015). To validate the resulting tree, 100 pseudobootstrap replicates were carried out. Obtained tree was visualized using iTOL web server (Letunic and Bork 2021) and rooted at midpoint. Full genome alignment between BF23 and relative phages was prepared with MMseqs (Steinegger and Söding 2017) at 0.5 minimum identity level and visualized with pyGenomeViz 0.3.2 (https://github.com/moshi4/pyGenomeViz).

To construct a phylogenetic tree of RPF and LTF genes, nucleotide sequences were aligned using MAFFT v7.490 (Katoh et al. 2009) with FFT-NS-2 strategy. Molecular phylogenetic sequence distances were determined using maximum likelihood approach in IQ-TREE 1.6.12 using the HKY substitution model (Nguyen et al. 2015). The branches were supported with standard nonparametric bootstrapping procedure with 100 replicates. The resulting tree was rooted at the midpoint and visualized with iTOL (Letunic and Bork 2021).

Proteomic characterization of BF23 virion

First, lysates of BF23 and T5 cultures, prepared as described above, were subjected to 12% SDS-PAGE. Identity of proteins in gel bands was verified by MALDI-TOF mass-spectrometric analysis on a Rapiflex system (Bruker). Proteins were tryptic digested in gel with Trypsin Gold (Promega) according to manufacturer's instructions. Peptides mixture after trypsinolysis was analyzed by MS using 2,5-DHB matrix, Peptides mass fingerprint search was performed against SwissProt database with 100 ppm error, using Mascot server. Next, to carry full characterization of the virion protein content, PEG-precipitated phages were loaded on 5% SDS-PAGE after boiling in standard SDS-loading dye. Proteins were allowed to enter the gel and concentrate for ~10 min, after which the current was stopped. The region of gel containing proteins was sliced and digested with Trypsin Gold (Promega). To elute peptides, gel was incubated in a microtube shaker with 50 μl of 50% acetonitrile/5% formic acid for 45 min at room temperature. Liquid was removed and extraction was repeated. Final extraction step was carried with 50 μl of 90% acetonitrile/5% formic acid for 5 min. Collected peptides were vacuum dried and solubilized in 5 μl of 0.1% formic acid. Peptides were subjected to LC-MS/MS analysis with Q Exactive HF-X mass spectrometer (Thermo Scientific) as described before (Laptev et al. 2020). Raw data was processed with MaxQuant software and peptides were searched against BF23 proteome.

HPLC-MS analysis of nucleosides

From 1 to 5 μg of purified phage genomic DNA was digested with Nucleoside Digestion Mix (NEB) at 37°C overnight. Nucleosides were loaded onto Agilent Poroshell 120 SB-C18 column (4.6 x 100 mm, 2.7 μm) and were analyzed on Agilent 1200 HPLC-MS system with ESI source and Q-TOF detector (Agilent). Gradient conditions were as following: solution A—5 mM ammonium acetate, pH = 5.3; solution B—90% acetonitrile; LC run was carried at 40°C, 0.3 ml/min speed and 1 μl of sample was loaded. The column was washed for 5 min with 2% B, followed by linear increase to 30% B till 30 min, linear increase to 100% B till 36 min, and linear decrease to 2% B till 40 min. UV detection was carried at 260 nM. LC-MS/MS data were analyzed in MassHunter, nucleosides and their modified variants were searched in EICs of expected m/z values taken from the table of common phage gDNA modifications (Lee and Weigele 2021).

In vitro digestion of bacteriophage genomic DNA

A total of 100 ng of phage genomic or control plasmid DNA was incubated with indicated enzymes (DpnI, DpnII, Sau3AI, SexAI, and BstNI from NEB) in commercial reaction buffer at 37°C for 60 min (except for BstNI—60°C for 60 min), followed by 0.5% agarose gel electrophoresis with ethidium bromide staining. To discriminate between higher molecular weight DNA fragments, 1 μl of reaction products was subjected to capillary electrophoresis on Agilent TapeStation 4150 (Agilent Technologies) with Genomic DNA ScreenTape System. Genomic DNA of T5 and BF23 was also investigated using pulse field gel electrophoresis with CHEF-DR III system (Bio-Rad). DNA was run for 14 h in 1% agarose 0.5x TBE gel using the following conditions: switch time—50 (initial)—90 (final) s; angle—120°; voltage gradient—6 V/cm; and temperature—14°C. Gel was stained after run, through incubation in 0.5x TBE supplied with 0.25 μg/μl ethidium bromide.

Efficiency of plating (EOP) assay

To determine the titer of active phage particles in cell lysates and the level of defense provided by the host R–M systems, the double agar overlay method was used. Overnight cultures of bacteria (100 μl) were mixed with 10 ml of 0.6% top LB agar supplemented with appropriate antibiotics and poured on the surface of precast 1.2% bottom LB agar plates. A volume of 10 μl drops of serial 10-fold phage lysate dilutions were spotted on the top agar, allowed to dry and plates were incubated at 37°C overnight. Efficiency of R–M systems defense was calculated as the phage titer obtained on the nonrestrictive host (BW25113) divided by the titer of phage obtained on the host with defense system. All experiments were performed in biological triplicates.

Construction of EcoR124I variant

The specificity of the EcoR124II system was changed by modifying hsdS gene in the pKF650 (Table S4, Supporting Information). One of three regions encoding linker sequence (TAEL) determining the distance between bipartite recognition sites (Price et al. 1989) has been removed through KLD mutagenesis with Q5 Site-Directed Mutagenesis Kit (NEB). Primers HsdS_delta_repeat_F (5′accgctgagcttaacatgcg) and HsdS_delta_repeat_R (5′ ctgcacttacgctgagctt) were used in the PCR reaction.

Authors' contributions

Conceptualization, A.I.; methodology, A.I., M.S., and A.A.; TEM analysis, M.K.; bioinformatic analysis, A.I. and M.S.; proteomic analysis, A.I. and A.A.; construction of EcoR124I plasmid, O.S.; modification-sensitivity assay M.S., M.Z., E.Z., and A.G.; writing—original draft preparation, A.I.; writing—review and editing, A.I. and K.S.; visualization, A.I., M.S., and A.A. All authors have read and agreed to the published version of the manuscript.

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Supplementary data

Supplementary data is available at *FEMSML Journal* online.

Conflict of interest: The authors declare no conflict of interest.

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Data availability

Complete genome of BF23 has been deposited to the GenBank under accession number OR083247. Phage BF23 and other materials used in this work are available upon request from the lead contact, Dr Artem Isaev (Artem.Isaev@skoltech.ru). Alternatively, phage BF23 can be also requested from Dr Konstantin Severinov, Rutgers University, USA (severik@waksman.rutgers.edu).

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